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NK cell modifications to advance their anti-tumor activities

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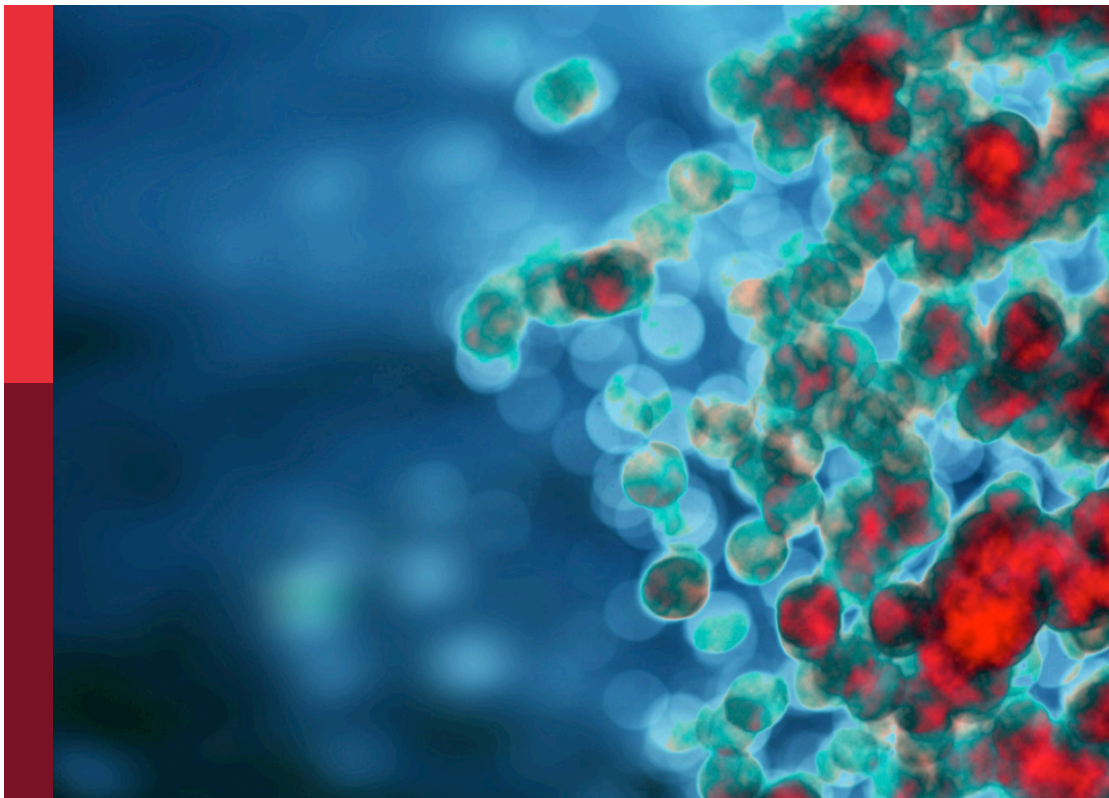

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NK cell modifications to advance their anti-tumor activities

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Natural Killer Cells and Regulatory T Cells Cross Talk in Hepatocellular Carcinoma: Exploring Therapeutic Options for the Next Decade

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Despite major advances in immunotherapy, hepatocellular carcinoma (HCC) remains a challenging target. Natural Killer (NK) cells are crucial components of the anti-HCC immune response, which can be manipulated for immunotherapeutic benefit as primary targets, modulators of the tumour microenvironment and in synchronising with tumour antigen specific effector CD8 cells for tumour clearance. Regulatory T cells shape the anti-tumour response from effector T cells *via* multiple suppressive mechanisms. Future research is needed to address the development of novel NK cell-targeted immunotherapy and on restraining Treg frequency and function in HCC. We have now entered a new era of anti-cancer treatment using checkpoint inhibitor (CPI)-based strategies. Combining GMP-NK cell immunotherapy to enhance the frequency of NK cells with CPI targeting both NK and CD8 T cells to release co-inhibitory receptors and enhance the cells anti-tumour immunity of HCC would be an attractive therapeutic option in the treatment of HCC. These therapeutic approaches should now be complemented by the application of genomic, proteomic and metabolomic approaches to understanding the microenvironment of HCC which, together with deep immune profiling of peripheral blood and HCC tissue before and during treatment, will provide the much-needed personalised medicine approach required to improve clinical outcomes for patients with HCC.

Keywords: liver, NK cells, regulatory T cells, hepatocellular carcinoma, tumour microenvironment, GMP cell therapy

THE LIVER AS AN ORGAN OF IMMUNOTOLERANCE

The liver is a unique lymphoid organ which plays a key role in the immunological function of the human body. Embryologically, the human liver is derived from the endoderm layer and resides between two venous circulatory systems; the portal vein, receiving venous flow from the gastrointestinal tract and the systemic venous circulation. The liver has a unique immunological

environment containing both professional antigen presenting cells, dendritic cells and Kupffer cells (resident hepatic macrophages) as well as non-professional antigen presenting cells (sinusoidal endothelial cells and biliary epithelium) (1). There is also an abundance of natural killer (NK) cells, innate lymphoid cells (ILCs) and innate mucosa associate invariant T (MAIT) cells, all of which play integral roles in the innate immune response of the liver.

The liver is constantly filtering harmful and harmless antigens from the gut acting as an immunological firewall (1). Hepatic tolerance to gut antigens is achieved by a combination of both immune cells and parenchymal cells. The constant exposure to gut-derived bacteria triggers a downregulation of Toll-like receptor 4 (TLR4) on the hepatic sinusoidal endothelial cells (HSEC). Liver-resident dendritic cells (DC) have distinct properties that promote tolerance rather than an immune response. These tolerogenic DCs secrete anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β) to dampen the immune response and can promote T-cell "hyporesponsiveness" (2, 3). Kupffer cells (KCs), known as sinusoidal firewalls, also contribute to hepatic tolerance by continuously phagocytosing the microbial products from the portal vein (4). Hepatic regulatory T cells (Treg) also play a crucial role in maintaining the tolerogenic environment by continuously controlling the cytokine production and proliferation of intrahepatic auto-reactive effector CD4 and CD8 T cells (5).

The liver is known as a "graveyard" of immune cells due to apoptosis of activated lymphocyte populations (6). It can mount an effective immune response to invading pathogens and cancer cells or when there is insult or loss of peripheral self-tolerance in immune-mediated liver injury. The balance between immunity and tolerance is established by competition for primary activation of effector T cells between the liver and its draining lymphoid tissues. For example, naive $CD8^+$ T cells, activated within liver-draining portal lymph nodes are capable of mediating hepatitis, while cells undergoing primary activation within the liver exhibit defective cytotoxic function and do not induce hepatocellular injury (7). The hepatic immune response will depend on the nature of the injury. In acute hepatic injury due to, for example, viral infection or drug-induced liver injury, an innate immune cells infiltrate appears important, with eosinophil or neutrophils and natural killer (NK) cells being the predominant immune cells. Adaptive immune cells are dominant in chronic injury resulting from, for example, chronic hepatitis B and C infection, alcoholic and non-alcoholic steatohepatitis, or autoimmune hepatitis. In the context of HCC, both innate cells such as NK cells and adaptive T cells are involved in auto-tumour immunity. In general, the balance of effector and regulatory T cells determines the outcome of inflammation, either resolution or chronic active hepatitis (8).

HEPATOCELLULAR CARCINOMA

In 2018, HCC was the sixth most common neoplasm diagnosed globally and was the fourth leading cause of cancer related death. In the vast majority of cases, primary hepatocellular carcinoma (HCC) arises on a background of cirrhosis, driven by chronic inflammation from a number of causes. These include viral (hepatitis B and hepatitis C) and non-viral (non-alcoholic fatty liver disease and alcoholic liver disease). Improved treatments for chronic viral hepatitis, coupled with the global epidemic of obesity imply that in the coming years the global epidemiology of HCC may shift from infectious to non-infectious causes.

The recent observation that hepatocytes within cirrhotic nodules have a higher mutational load than normal liver characterizes cirrhosis as a truly pre-malignant condition (9). Coexistent with this is the immune dysfunction associated with cirrhosis. This is characterized by evidence of peripheral activation of circulating immunocytes exposed to higher than normal levels of bacterial antigens, but a more profound central tolerance. A healthy liver is a tolerogenic organ, however this state is exacerbated by changes in immune sub-populations and their dysfunction in cirrhosis. The combination of an increased hepatic mutational burden together with decreased immune surveillance underpins the development of HCC.

IMMUNE DYSFUNCTION IN CIRRHOSIS

As described above, in a healthy liver, immune system homeostasis is achieved through immunosurveillance of its dual blood supply (portal vein and hepatic artery), protecting the host from microbe-associated molecular patterns and damage-associated molecular patterns (MAMPs and DAMPs respectively) from the gut (10). Concurrently the liver displays features of local immune tolerance to non-pathogenic material and helps mediate the appropriate immune response through the synthesis of pro-inflammatory and anti-inflammatory cytokines (11). The immune system thus plays a decisive role in both the pathogenesis of cirrhosis and subsequent immune dysfunction. Chronic factors including infection, alcohol and obesity, inflict persistent hepatocyte damage leading to fibrosis *via* hepatic stellate cell (HSC) activation (12). Disease specific alterations may compound these factors and augment the rate of fibrosis progression. Once cirrhosis becomes established, the liver loses its ability to appropriately protect the body from pathogens as a consequence of disordered immune cell activation termed "cirrhosis-associated immune dysfunction".

In early cirrhosis, changes in the intrahepatic immune compartment are often due to persistent innate immune cell stimulation. As disease progression ensues, ultimately leading to decompensated cirrhosis, immune hyporesponsiveness and increased tolerance develops (13). This is driven by the innate immune system in which long-term exposure of toll-like receptors to bacterial products such as lipopolysaccharide, can lead to a dampened innate immune response (14). Cirrhosis is associated with a multitude of abnormalities in the innate and adaptive arms of the immune system leading to a generalised immune hyporesponsiveness. The reticulo-endothelial system becomes compromised in the context of cirrhosis as a result of fibrotic damage to the sinusoidal vascular space. This leads to capillarisation, porto-systemic shunts and loss of the KC

population, which are also dysfunctional, with impaired phagocytosis (10, 15). Consequently, the capacity for clearance of endotoxin and microbes is attenuated, lowering the threshold for bacterial infection. This dysfunction may be exacerbated by a combination of changes in the gut microbiota and increased intestinal permeability (16). Evidence for this persistent immune stimulation is supported by both human disease studies and mouse models (17). Cirrhotic livers possess a reduced ability to synthesise innate immune proteins, such as complement and pattern recognition receptors thus reducing its bactericidal capacity. This is further exacerbated by reduced numbers of MAIT cells which have the capacity to respond directly to bacterial metabolites (18, 19).

Compromised immune function not only occurs at a local level in cirrhosis, but also systemically, and affects many different immune cell sub-populations. Neutrophils are reduced, with impaired chemotaxis and subsequent phagocytosis of bacteria as illustrated by a lower response to peptidoglycan recognition proteins (20). Monocytosis is observed in cirrhotic patients with evidence of cellular dysfunction, such as impaired function of the Fc-γ receptors, which are responsible for the clearance of bacteria (21). In alcohol and hepatitis C virus (HCV) related cirrhosis, a reduced frequency of $CD27^+$ memory B cell function suggests defective antibody function (22). T cell defects have also been noted including T cell lymphopenia affecting both CD4 and CD8 T cells. There is depletion of naïve and memory T cells, although naïve T cells appear to be more profoundly affected putatively related to splenic pooling, and there is also impaired proliferation of peripheral T lymphocytes (23). NK cells are shown to be lower in number in the periphery but also less responsive to cytokine stimuli which hampers their cytotoxic and anti-fibrotic roles (24). Within the intrahepatic compartment, innate lymphoid cells type 2 (ILC-2) cells appear to be the dominant ILC population (25, 26). The observed changes in immune cell function alters the equilibrium of immunosurveillance and immunotolerance within the liver, promoting the latter through relative immune deficiency. Due to the observed microenvironmental shift towards immunotolerance, there is an increase in host vulnerability to tumorigenesis and the occurrence of HCC.

NATURAL KILLER CELLS

Natural Killer (NK) cells are a key part of the innate immune response against viruses and tumours, and more recently, have been shown to participate in the adaptive immune response through cross-talk with dendritic cells and T cells. They make up between 5-20% of circulating lymphocytes, but a much larger fraction (~50%) of the intrahepatic lymphocyte compartment. NK cells are usually characterised as $CD3^-CD56^+$ lymphocytes and in general do not require priming to initiate anti-viral or anti-tumoral cytotoxic or cytokine secretory effects (27). Recent work has identified that they also have adaptive properties (28), which can be both receptor or cytokine driven. Particular interest has been generated in the utility of cytokine-induced memory NK cells as agents for immunotherapy (29).

Surveillance of diseased cells can be mediated through the 'missing self' model (loss of inhibition) or through recognition of stress-induced molecules (gain of activation) (30). The net effects of both of these mechanisms is a change in the balance between activating and inhibitory signals transduced by the NK cell such that activation is favoured. Both these mechanisms may operate in cancer. In the missing-self model, healthy cells which express major histocompatibility complex (MHC) class I are spared from lysis through the engagement of inhibitory receptors on the NK cell surface, such as killer cell immunoglobulin-like receptors (KIR) or CD94:NKG2A. Thus if MHC class I is downregulated, the tonic inhibitory signal to the NK cell is lost and the cell becomes activated (31). Conversely NK cells can be activated by augmenting activating signals. Key activating receptors include the natural cytotoxicity receptors (NKp30, NKp44 and NKp46) and C-type lectin-like receptors, especially NKG2D. In stressed, transformed or infected cells MHC class I is often downregulated and ligands for NKG2D are up-regulated, shifting the NK cell balance towards activation and tumour lysis (31, 32).

The KIR are MHC class I-specific receptors that perform a fundamental role in self-recognition and in functional "licensing" of NK cells. The tuning of the activity of NK cells may be a more dynamic process than previously considered, which is relevant for NK cells within immunosuppressive tumour microenvironments (33, 34), resulting in induced-hyporesponsiveness. The KIR exhibit an extraordinarily high level of diversity at the gene content and allelic levels. In combination with the diversity of MHC class I ligands, the KIR form a complex immunogenetic network, which has been associated with development and outcomes of cancer. The KIR gene family is found on chromosome 19 and comprises 13 functional KIR genes and 2 pseudogenes (35). Two haplotypes KIR-A and KIR-B have been identified with the former having a fixed gene content but substantial allelic diversity, and the latter haplotype displaying variation in gene content and also allelic diversity. There also is substantial diversity in the frequencies of KIR-A and KIR-B haplotypes amongst different human populations (36). This has been proposed to account for some of the diversity noted in anti-cancer responses. For instance, the activating KIR, KIR2DS2, has been associated with protective responses against acute myeloid leukaemia and other solid tumours including HCC (37, 38). Interestingly, $KIR2DS2^+$ NK cells appear to express higher amounts of FcγRIII (CD16), a medium-low affinity IgG receptor essential for antibody-dependent cellular cytotoxicity, providing a potential molecular basis for enhanced protection (39). Improved HCC outcomes have been observed in individuals with different KIR : HLA genotypes including HLA-C group 1, KIR2DS5 and the compound genotypes KIR2DL2: HLA-C group 1, KIR3DS1:HLA-$Bw4^{80T}$ and KIR3DS1: HLA-$BBw4^{80I}$ (38, 40).

HEPATOCELLULAR CARCINOMA AND NATURAL KILLER CELLS

In comparison to other cancers, HCC is relatively cold immunologically with only about 25% having an immune

reactive phenotype (41, 42). Individuals with HCC have reduced numbers of NK cells within the periphery and these have lower levels of functionality (43). Within the tumour they are present at low frequencies in contrast to myeloid and other lymphoid cells (44). In HCC accumulation of NK cells in intratumoral tissue, as compared to peritumoural tissue, has increased expression of the activation marker CD49a and an increased $CD56^{bright}$:$CD56^{dim}$ ratio, demonstrating localised differences in these two subtly distinct microenvironments (45). Individuals with higher frequencies of NK cells and enhanced cytotoxic and cytokine secretory functions have improved overall survival in HCC following liver resection (46–48). Patient survival also positively correlates with the frequencies of both circulating and intratumoral NK cells in HCC (49), and in two separate studies the response to sorafenib was better if a higher frequency of intratumoral NK cells was present (48, 50). Conversely, overall survival is worse in individuals with fewer intratumoral NK cells and a higher proportion of $CD56^{bright}$ to $CD56^{dim}$ NK cells. The $CD56^{bright}$ sub-population are considered less mature and have lower levels of cytotoxicity than the $CD56^{dim}$ subpopulation, indicating that NK cell functionality is important in determining the outcome of HCC.

NK cells express multiple activating receptors and therefore can engage many different molecules expressed by tumours. Changes in the balance of expression of activating and inhibitory receptors can determine NK cell function in a "rheostat" model. Thus, in advanced HCC there may be upregulation of the inhibitory receptor NKG2A and this, combined with a reduction in the effector molecules perforin and granzyme B, contributes to a hypofunctionality of NK cells (43, 46). Down-regulation of granzyme B is a feature of intra-tumoral, as opposed to peri-tumoral, NK cells and correlates with expression of IL-10-positive tumour-associated macrophages (51). This immunosuppressive gradient also correlates with expression of exhaustion-associated markers such as PD-1, Tim-3, and Lag-3. In addition to modulating T cell functions these molecules may act as checkpoints for NK cells and are often co-expressed on activated or exhausted NK cells (52). CD96 (TACTILE) is another immunological checkpoint associated with HCC, that in combination with its ligand negatively associates with the outcome of HCC (53). Thus, together this group of checkpoints form potential therapeutic targets for HCC.

A number of activating NK cell receptors have been associated with HCC. Differential splicing of NKp30 leads to preferential generation of an inhibitory isoform that predominates in advanced HCC (54). However, most attention has focussed on NKG2D which engages multiple ligands including MIC-A/B and the ULBP proteins. These stress-induced proteins are express on tumours and may be released into the circulation following proteolytic cleavage, through molecules including ADAM9, which is upregulated in HCC (55). Soluble receptors bind and down-regulate NKG2D on the surface of NK cells thus rendering cells less active. In HCC high levels of soluble ULBP1 is associated with poor survival (56). The observation that in mouse models of HCC, NKG2D can drive tumorigenesis, probably through promotion of the chronic inflammation that leads to mutagenesis, indicates the complexity of the molecular pathology of this disease (57, 58). Nevertheless, in general down-regulation of the NKG2D: NKG2D ligand axis is associated with poorer outcomes (59).

REGULATORY T CELLS

Sakaguchi and colleagues first described regulatory T cells (Tregs) in the late 1990s (60). Tregs are generated in the thymus. They are a subset of CD4 T cells, expressing high levels of CD25 (the α-chain of the IL-2 receptor) and low levels of IL-7 receptor (CD127). Thus, Tregs are defined by surface receptors as CD4, $CD25^{high}$, $CD127^{low}$ (61). Tregs are crucial in maintaining peripheral immune tolerance (62). Their phenotype and function are controlled by the transcription factor Foxp3 (63). Tregs represent 2–5% of CD4 T cells in humans and about 10% in rodents.

Tregs are present in the human liver. Our group has previously demonstrated that Tregs reside together with effector CD4 and CD8 T cells, CD11c dendritic cells in both interface and lobular hepatitis areas to control liver inflammation (5). The suppressive function of Treg is reduced in the inflamed microenvironment (64). The main function of Tregs is to control autoreactive effector T cells, thereby maintaining hepatic immune tolerance. We have shown that Treg recruitment *via* hepatic sinusoids to inflamed human liver is mediated by the chemokine receptor CXCR3 and the integrin VLA-4 on Tregs and the chemokine ligands CXCL9, 10, 11 and VCAM expression on inflamed sinusoids. Following recruitment, post endothelial migration through the fibrous stromal framework occurs *via* LFA-1 and VLA-4 integrin's on Treg and cell adhesion molecules ICAM and VCAM on the stroma cells, and subsequently Tregs reside around the area of hepatitis to control liver inflammation (5). Their suppressive function in the human liver is mainly executed *via* CTLA-4, CD39 or IL-10 dependent mechanisms and low dose IL-2 can upregulate functional CTLA-4 on the surface of Tregs surface *via* STAT-5 (65, 66).

Tregs have the potential to be plastic towards effector Th1 or Th17 lineages especially in the inflamed human liver and tumour microenvironment (8). The immune response mediated by T lymphocytes plays an important role in anti-tumour immunity. Tregs secrete immunosuppressive cytokines such as IL-10 and IL-35 to suppress the aberrant immune response, while Th1/Treg and Th17/Treg cells can release not only anti-inflammatory cytokines but also proinflammatory Th1 cytokines such as IFNγ, TNFα, and Th17 cytokines IL-17, IL-22 (67, 68). The ratio of Treg to Th17 cells is closely associated with the outcome of many immune mediated diseases and cancers (69).

HEPATOCELLULAR CARCINOMA (HCC) AND TREGS

Multiple studies have reported that the frequency of $CD4^{+}CD25^{++}$Treg cells in the peripheral blood of HCC

patients is significantly higher than in the blood of healthy individuals (70, 71). $CD4^+CD25^{++}$Tregs in advanced (stage III-IV) HCC patients is significantly increased compared to early (stage I-II) HCC patients, implying that the presence of $CD4^+CD25^{++}$Tregs is closely related to tumour progression and enhances the invasiveness and metastasis of HCC (71). In addition, accumulation of Tregs correlates with reduced infiltration of CD8 T cells in HCC tumour regions, and the expression of granzymes and perforin functional molecules is less in tumour-infiltrating CD8 T cells (72). Furthermore, in this study an increased quantity of circulating Treg was associated with a high mortality and reduced survival time of HCC patients.

$CD4^+CD25^{++}$Tregs suppress the anti-tumour immune response either in the draining lymph nodes or in tumour tissue. $CD4^+CD25^{++}$Tregs in the tumour-draining regional lymph node inhibit the proliferation of effector T cells, and Tregs prevent effector T cells from killing tumour cells in the primary tumour tissue (73). It has been shown that the level of Treg cells in cancer tissue was significantly higher than that in adjacent tissues (74). It is thought that Treg cells may decrease the proliferation of effector CD4 and CD8 T lymphocytes in the tumour microenvironment by contact inhibition, subsequently reducing the anti-tumour immune response and resulting in the potential for tumour cells to escape immune surveillance. HCC is one of the most common and aggressive human malignancies and $CD4^+CD25^{++}$Treg promote hepatocellular carcinoma invasion *via* TGF-β1 dependent mechanisms (75). Furthermore, $ICOS^+$ $FOXP3^+$ Tregs contribute to the immunosuppressive HCC microenvironment and lead to an unfavourable prognosis for HCC patients (76). Thus, removal or reduction of the Treg cell population, or inhibition of $CD4^+CD25^{++}$Treg function in the HCC microenvironment may facilitate the efficacy of tumour immunotherapy (77). In addition, the Th17/Treg ratio is a risk factor for HCC (78). The majority of expanded Tregs do not express CD45RA suggesting that Tregs have a memory phenotype and exponential expansion of these tumour antigen exposed memory Tregs has been a distinct finding in HCC (79).

TREG AND NK CELL INTERACTIONS IN HEPATOCELLULAR CARCINOMA

The balance of NK cells which provide tumour clearance and Tregs which inhibit tumour immunity may determine the outcome of HCC (**Figure 1**). A potential role for Tregs in dampening NK cell functions was first suggested in a murine leukaemia model. In this model, the depletion of Tregs by administration of anti-CD25 mAb before tumour inoculation abolished tumour growth and promoted the generation of cytotoxic cells characterized as NK cells (80). In HCC, the NK cell frequency within the tumour-infiltrating lymphocyte compartment is less than in the non-tumour tissue, and these intratumoral cells demonstrate impaired cytotoxicity and IFN-γ production (43, 81), We have also previously demonstrated that there is a parallel increase in NK cells and Treg in hepatic inflammation (82) suggesting that taming the Treg population will allow NK cells to function more efficiently.

Freshly isolated human Tregs can directly inhibit human NK cell cytotoxicity against K562 (83). TGF-β maintains the inhibitory functions of Treg and plays a suppressive role by inhibiting the expansion of NK cells and their cytotoxic functions (84, 85). TGF-β can also have a negative effect by facilitating the onset of tumours due to a reduction of immunosurveillance and anti-cancer responses. Resting NK cells harbour surface expression of TGF-β receptors, rendering them susceptible to soluble TGF-β (86). Resting human Tregs express membrane-bound TGF-β that is associated with the protein, latency-associated protein (LAP) (87). When associated with LAP, TGF-β remains inactive. Membrane-bound TGF-β is involved in the inhibitory function of Tregs on NK cells since anti-TGF-β blocking antibodies can restore the cytotoxicity IL-12-induced IFN-γ secretion of human NK cells (88). When exposed to TGF-β or on Treg encounter, Smad signalling in NK cells blunts expression of cytotoxic molecules such as granzyme B and perforin (89). In addition, high levels of TGF-β have been associated with impaired NK cell function and NKG2D expression (90). Therefore, Tregs bearing TGF-β on their membrane can present it directly to NK cells resulting in a reduction in NKG2D expression (88). Activated Tregs can also suppress NK cells responses *via* an IL-2 mediated mechanism that is crucial for NK cell survival (91). This reduces the capacity of NK cells to secrete IFN-γ on stimulation with IL-12 but not on activation by IL-2 and IL-15 suggesting the regulation of NK cell control by Tregs is critically dependent on the cytokine milieu in the HCC microenvironment.

THE INFLUENCE OF THE TUMOUR MICROENVIRONMENT ON NK CELL FUNCTION

The intrahepatic microenvironment is crucial for both NK and Treg phenotypic stability, functionality, survival and proliferation (8). The HCC microenvironment is an active component of the tumour rather than merely a passive structural support for tumour growth, which changes dynamically and consequently affects HCC behaviour. These immunosuppressive features of HCC are a challenging barrier to clinicians to design effective immunotherapies. The HCC microenvironment is composed of not only growth factors, cytokines, metabolites and chemokines generated by stroma and tumour cells, but also tumour-infiltrating macrophages, myeloid-derived suppressor cells, neutrophils, cancer-associated fibroblasts and regulatory T cells. They all play key roles in the clinical outcome of HCC and success or failure of immunotherapies.

Hepatic stellate cells (HSCs) are the major framework of HCC and can directly promote tumour cell proliferation. Conditioned medium collected from HSCs induce not only proliferation and migration of HCC cells but also promote HCC growth through the activation of NF kappa B and extracellular-regulated kinase

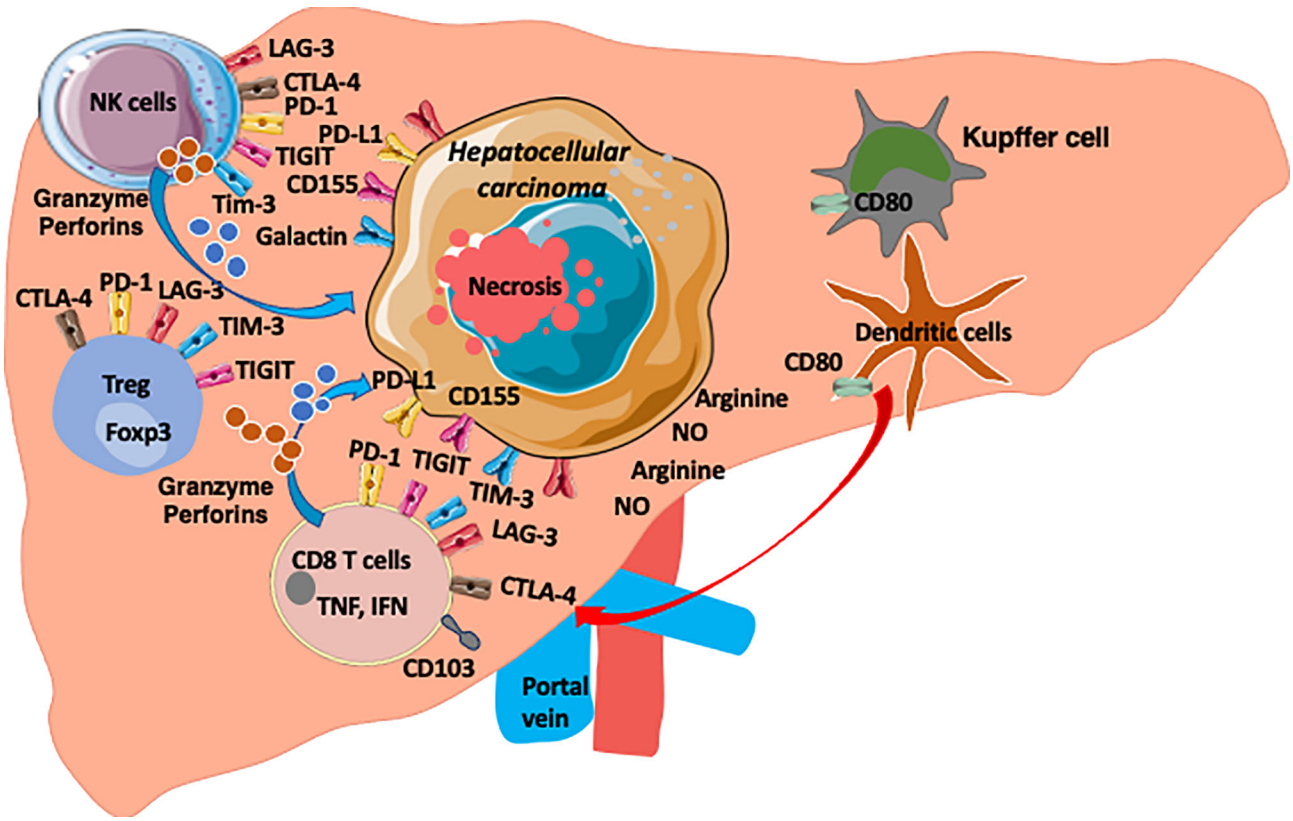

FIGURE 1 | The HCC microenvironment is enriched with anti-tumour CD8 T cells, NK cells and Treg cells. Both T cells and NK cells express checkpoint molecules (PD1, TIGIT, TIM3, LAG3 and CTLA-4) as common targets. HCC express ligands for these checkpoints (PDL1, CD155, Galactin). Arginine and Nitrous oxide are also enriched in HCC environment and the microenvironment is hypoxic environment and enriched with chemokines, cytokines, metabolites and microbial products (8).

(ERK) pathways (92). Cancer-associated fibroblasts are the major cell type within the tumour stroma and play a critical role in tumour-stromal interactions (93). They are activated by TGF-β and are responsible for the synthesis, deposition and remodelling of excessive extracellular matrix thus modulating the biological activities of HCC. HCC cell growth, extravasation and metastatic spread are dependent upon the presence of these fibroblasts. HCC cells can reciprocally stimulate proliferation of tumour associated fibroblasts, suggesting their key role in tumour-stromal interaction (94). Stroma from HCC express several growth factors, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and Wnt family members, stromal-derived factor (SDF)-1α and IL-6 (95).

Additionally, myeloid derived suppressor cells (MDSC) exert multiple mechanisms of immunosuppressive activity in the tumour microenvironment. MDSCs induce differentiation and expansion of Tregs during tumorigenesis; inhibit DCs and NK cells *via* TGF-β; deprive T cells of essential amino acids such as L-arginine and L-cysteine; and generate the oxidative stress that is associated with HCC progression (96). MDSCs co-cultured with autologous T cells induce an increased number of Tregs, PD-1^{+}-exhausted T cells, and an increase in immunosuppressive cytokine levels in HCC patients (97). MDSCs can also impair NK cell function. In HCC, MDSCs inhibit NK cell cytotoxicity and cytokine release mediated by the NKp30 receptor (98). MDSCs also inhibit TLR-ligand-induced IL-12 production and inhibit the T-cell stimulating activity of DCs in HCC (99). Tumour-associated neutrophils can also recruit macrophages and Treg cells into HCCs to promote their growth, progression, and resistance to sorafenib therapy (100, 101).

Furthermore, immunosuppressive cytokines, extracellular matrix and inflammatory cytokines in the HCC microenvironment can define HCC biology and prognosis. Global gene expression profiling of human HCC indicates that TGF-β gene signatures cluster HCC into two homogeneous groups with early or late TGF-β signatures (102). Importantly the late TGF-β signature is associated with an invasive HCC phenotype and an increased risk of tumour recurrence. MMP1 and TIMP1 were also signature genes in the immature hepatoblast subtypes of HCC that is associated with a poor prognosis (103). Inflammation-associated pathways, gene expression signatures, NF-κB, TNF-α, and IL-6 from the adjacent benign tissue can also predict late recurrence of HCC (104). IL-6, a major pro-inflammatory cytokine, is one of the signature genes in the hepatoblast phenotype signature (103).

Osteopontin, secreted from Kupffer or stellate cells in response to inflammatory cytokines, is also associated with metastasis of HCC (105).

The tumour microenvironment also shapes NK cell metabolism and effector functions. Understanding immunometabolic suppression is critical in engineering a new generation of effective natural killer cell-based immunotherapies targeting solid tumours such as HCC. Multiple factors can modulate NK cell metabolism in the tumour microenvironment. HCC exert immunosuppressive effects through a number of mechanisms, a key driver of which is hypoxia. High oxygen consumption by tumour cells can generate hypoxic regions. Hypoxia impairs NK cell effector functions, but also sustains HIF1α, which promotes glycolytic metabolism. Hypoxia fuels the generation of adenosine from the cancer-associated ectoenzymes CD39, expressed on Treg (106), and CD73 on antigen presenting cells. Tumour cells also generate extracellular adenosine through the CD39 and CD73 ectonucleotidases, thus compromising NK cell function through competition for nutrients.

Thus, the interaction between the tumour and stroma interaction generates the microenvironment within HCC tissue and can suppress the effect of surrounding tissues or cell types that stimulate hepatocarcinogenesis, tumour progression, invasion, and metastasis. Sorafenib, an oral multi-kinase inhibitor, which is the most widely used HCC medication, inhibits VEGFR-2/-3 and PDGFR as well as Raf kinase, disrupting tumour-stromal interactions and resulting in decreased cell proliferation and angiogenesis. The efficacy and safety of sorafenib have been demonstrated in Phase III clinical trials, and it is currently the standard of care for patients with advanced stage HCC (104, 107).

IMMUNOTHERAPEUTIC INTERVENTIONS AND HOW THEY MAY ENHANCE NK FUNCTION AND TAME TREG SUPPRESSION

Currently the treatment for HCC is challenging, often due to the stage at which many patients present. Therapeutic options include surgery, transplantation and locoregional therapies can be curative, and systemic therapies with tyrosine kinase inhibitors including sorafenib and lenvantinib result in a modest prolongation of survival. As a result there is much interest in novel therapeutics and therapeutic combinations which have been recently reviewed by Llovet et al. (108).

In terms of immunotherapy, trials of checkpoint inhibitors have been the best studied. The PD-1 checkpoint inhibitors nivolumab and pembrolizumab have been used for the treatment of patients with HCC (109), but only leads to clinical responses of 10-20%. Several factors including the expression of programmed cell death-Ligand 1 (PD-L1), tumour mutational loads, and tumour-infiltration immune cells correlate with patient responses using these medications (110). This relative lack of efficacy implies that combination therapy should be considered and applied to each patient as personalized treatment approaches in HCC. Consistent with this combining a PDL1 inhibitor (Atezolizumab) with an anti-VEGF antibody (Bevacizumab) increased survival for unresectable HCC by approximately six months compared to sorafenib monotherapy (111). As checkpoint inhibitors predominantly target T cells, one attractive approach is to simultaneously target NK cells thus effectively mobilizing two arms of the immune system.

Enhancing NK Cell Frequency and Function

The field of NK cell therapeutics is rapidly growing. The observations of Ruggeri et al. demonstrating that NK cell alloreactivity was beneficial in refractory leukaemia acted as the cornerstone for the development of NK cell-based approaches (112). Initial work focussed on culturing NK cells *in vitro* and then infusing them, met with success mainly for haematological malignancies rather than solid tumours (113). However, this may be augmented by combining NK cells with monoclonal antibody therapy thus targeting the ADCC function of NK cells, or by genetically modifying NK cells to express chimeric antigen receptors (CAR-NK cells). Both these strategies have reached clinical trials (114, 115). Encouragingly the use of CD19-transduced CAR-NK cells did not result in the increase in cytokine levels associated with the systemic toxicity of CAR-T cell therapies. An alternative strategy is to target inhibitory receptors. As NK cells are held in check by dominant inhibitory signals then they are excellent candidates for having their activity unleashed by blocking these inhibitory receptors using monoclonal antibody therapeutics. These have targeted both the KIR receptors and NKG2A. Importantly, the clinical effects of the anti-NKG2A monoclonal antibody Monalizumab may be related to unleashing both T and NK cells for its anti-tumour effects (116).

In current clinical trials of adoptive tumour immunotherapy, large dosages of NK cells have been used ranging between 5×10^6 to 5×10^7/kg body weight (117). An approach to achieve these large numbers of NK cells is *via* the enrichment of NK cells from donor-derived leukapheresis products. Multiple protocols have been successfully developed to generate GMP NK cell products through immunomagnetic depletion of T and B cells and positive selection of $CD56^+$ cells (118). The necessity of the NK cell products to be of a high-purity, which requires not only a long manufacturing process and compromises the viability and potency of the NK cell product, combined with the limited availability of autologous leukapheresis products makes the task of obtaining sufficient GMP NK cells from a single leukapheresis challenging. Therefore, an alternative method is to make NK cell products *via* the expansion of NK cells from PBMCs using feeder cells. An example of this are K562 cells modified with membrane-bound molecules such as IL-15 and 4-1BB ligand which can rapidly expand NK cells from PBMCs by 21.6-fold in 7 days (119). This method also produces NK cell products with purities in the range of 60-70%. To achieve purities needed for allogenic use a further expansion up to 21 days or

enrichment of NK cells is still required (117, 119). NK cells can also be derived from induced pluripotent stem cells (IPSCs) and this has the advantage of hugely increasing their availability, as well as the prospect of selecting NK cell populations based on their alloreactivity potential, which may enhance their anti-cancer response (120).

IL-2 can also be used for short term activation of NK cells without feeder cells. IL-2 activation of NK cells can be coupled with $CD3^+$ T-cell and $CD19^+$ B cells depletion to increase the purity of the cell product (121). GMP NK cells have high IFNγ expression upon cultivation with K562 tumour cells and are highly cytotoxicity toward tumour cell lines *in vitro* (122). These data confirm that NK cells can have high clinical potency and potential for a significant role in tumour immunotherapy, including HCC.

Many companies have developed GMP cell isolation equipment and GMP reagents and have a manufacturing platform which has been utilised for multiple clinical trials utilising NK cell therapy (**Figure 2**). There are currently only a few MHRA and HTA approved GMP facilities in the UK focusing on GMP T cells and NK cell therapy for patients with both autoimmune diseases and cancer. GMP grade magnetic isolation or GMP cell sorting of clinical grade CD56-positive NK cells and applying these cells as immunotherapy in HCC could be one of the future treatment for these patients.

Suppressing Treg Frequency and Function in HCC

Restraining Tregs in HCC is important for many reasons. Anti-tumour T cell responses are severely compromised in advanced HCC patients through multiple immunosuppressive pathways comprising Tregs, PD-1^+ T effector cells, and inhibitory cytokines (72, 97, 123). In HCC, expression of PD-1 is increased on CD8 memory T effector cells and interaction with its ligand PD-L1 on HCC cells blocks signalling, proliferation, and cytokine secretion of anti-tumour CD8 T cells (124, 125). The association between the infiltration of CD8 T cells in HCC and patient survival has been well recognised (126, 127). Granzyme B, perforin and IFNγ secretion by CD8 T cells generates potent anti-tumour activity but high expression of PD-1 on exhausted T cells contributes to ineffective effector T cell function, and selective *in vitro* depletion of these immunosuppressive cells results in improvement of T effector cell function in HCC patients (128, 129).

Activated Tregs also have the ability to inhibit effector T cells *via* contact-dependent interactions between checkpoint molecules and their ligands including PD-1 with PD-L1, Tim-3 with galectin-9, CTLA-4 and GITR. Tregs also contribute to the strongly immunosuppressive HCC microenvironment by releasing the inhibitory cytokines TGF-β and IL-10 (130, 131). The mechanism of inhibition of anti-tumour effector T cells by Treg involves several molecular pathways: 1) Tregs may inhibit proliferation and cytokine secretion of T effector cells by IL-10, adenosine production from CD39 on its surface and IL-35, which can be reversed by adding neutralising antibodies (132, 133); 2) *via* the PD-1/PD-L1 pathway to suppress anti-tumoral immunity in HCC (134); and also *via* 3) the co-inhibitory molecule CTLA-4 (135).

Suppressing Tregs and Boosting NK Cells in HCC

Combination of these approaches by inhibiting Tregs and enhancing NK cells is an exciting option which has not been tried before (**Figure 3**). One of the potential approaches would be sequential manipulation by administering Basilizumab (anti-CD25) to deplete $CD4^{pos}$ $CD25^{high}$ Treg cells followed by GMP NK cells either *via* a peripheral route or direct administration along with transarterial chemoembolization (TACE) as GMP-NK TACE therapy. This would prevent systemic depletion of Treg thus reducing the potential for inducing autoimmunity. With the remarkable success of chimeric antigen receptor (CAR)-engineered technology, developing CAR-engineered NK (CAR-NK) cells for cancer therapy could offer some significant advantages, including better safety by a lack or minimal cytokine release syndrome, multiple different mechanisms for inducing cytotoxic activity including checkpoint inhibition, and off-the-shelf manufacturing. CAR-NK cells could also have better infiltration into solid tumour such as HCC and overcome the resistant tumour microenvironment.

Targeting intratumoral Treg cells may offer a therapeutic direction to modulate the tumour microenvironment. The combination of nivolumab with the Treg-depleting anti-CCR4 antibody, mogamulizumab has been explored by Doi et al. (136). In their proof of concept study this combination provided anti-tumour activity and can thus be a potentially effective option in cancer immunotherapy. A recent study found that CD36 was selectively upregulated in intratumoral Treg cells as a central metabolic modulator. They genetically ablated *CD36* in Treg cells resulting in suppressed tumour growth, a decrease in intratumoral Treg cells and enhanced antitumour activity (137). In addition, a recent report identified widespread *HLA-E* expression in tumour samples, with levels correlating to those of *NKG2A*. This is of importance as one mechanism of tumour resistance to immune cells is mediated by the expression of peptide-loaded HLA class I molecule (HLA-E) in tumour cells. HLA-E suppresses NK cell activity *via* ligation of inhibitory receptor, NKG2A (138). Furthermore, blockade of NKG2A results in the enhancement of tumour immunity by promoting both NK and CD8+ T cell effector functions in mice and humans. As described above, monalizumab is a humanised anti-NKG2A antibody which has been shown to enhance the activity of NK cells against various tumour cells and rescue CD8+ T cell function in combination with blockade of the PD-x axis (116).

THE POTENTIAL FOR FUTURE COMBINATION IMMUNOTHERAPY IN HCC

Checkpoint Therapy to Enhance T and NK Cell Function

Both T cells and NK cells express co-inhibitory molecules or checkpoint inhibitors, which can be targeted using CPI to

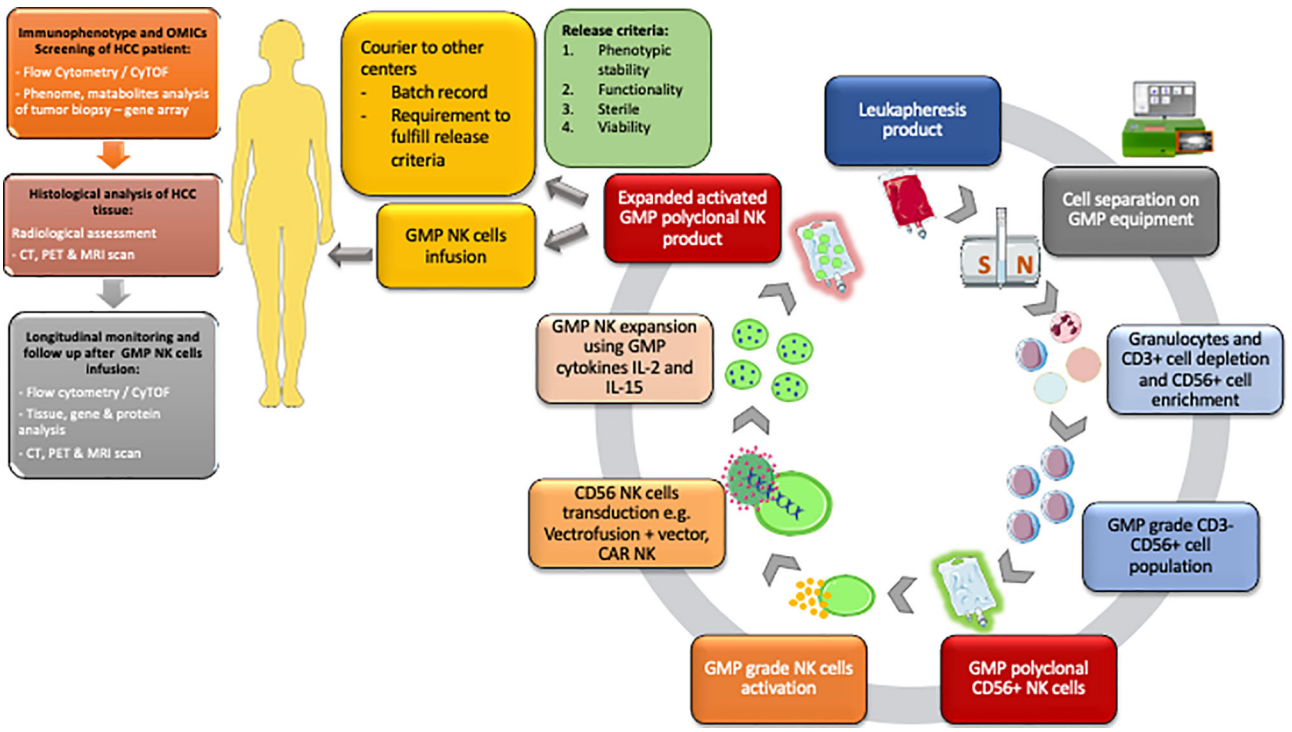

FIGURE 2 | Workflow for the isolation of GMP polyclonal NK product and subsequent pathways from the NK cell isolation to distribution to other centres. GMP NK cell product must fulfil the release criteria according to MHRA criteria. Flow cytometry/CyTOF and histological analysis, CT, PET and MRI scans are performed before cell infusion to help identify individuals for therapy. Following GMP NK cell infusion the patient immune system is monitored using a combination of flow cytometry/ CyTOF, tissue analysis and cross-sectional radiological imaging. HCC, Hepatocellular carcinoma; OMICs, genomic proteomic metabolomic; CyTOF, cytometry time of flight.

unleash potent anti-tumour immunity by recovering both NK and T cell function (116, 139). These include PD-1, CTLA-4, TIGIT and LAG-3. The mechanism of action of PD-1 involves the engagement of its ligands PD-L1 and PD-L2 to deliver inhibitory signals that regulate the balance between T cell exhaustion, tolerance and immunopathology (140). Tumour cells expressing PD-1 ligands on their surface use the PD-1 pathway to attenuate tumour immunity and facilitate tumour progression (141). PD-1/PD-L1 has been recognised to play a role of critical importance in immune escape in HCC after the successful treatment of nivolumab in patients with advanced HCC, achieving an objective response rate of 15-20% (142). Furthermore, elevated PD-L1 expression in HCC significantly correlates to poor survival and tumour aggressiveness (143–146). Blockade of the PD-1 and PD-L1 interaction using monoclonal antibodies produces durable clinical responses in patients with diverse advanced tumour types (147).

CTLA-4 outcompetes the co-stimulatory molecule CD28 for binding to B7-1/CD80 or B7-2/CD86 expressed on the surface of antigen presenting cells, including tumour infiltrating dendritic cells. This is due to its higher affinity for CD80/CD86 as compared to CD28. As a result of this, CTLA-4 negatively regulates T-cell activation, inactivating T lymphocytes in the G1 phase. CD8 T lymphocytes are able to exert their anti-tumour cytotoxic effects *via* the secretion of TNFα and IFNγ leading to the apoptosis of tumour cells (148), when the T-cell receptor (TCR) binds its cognate peptide:MHC antigen expressed on tumour cells (149–153). CTLA-4 blockade can then provide long-lasting tumour remission due to its impact on memory T cells response to cancer (154). After blocking CTLA-4, an increase in the number and breadth of protective T cells can be seen in the blood as evidenced by TCR V-β analysis (154). However, its role in the anti-tumour NK cell response requires further study.

TIGIT (T cell immunoglobulin and ITM domain) is an inhibitory receptor and is expressed on activated T cells and can also be found on NK cells as well as memory T cells, a subset of Treg cells as well as follicular T helper (Tfh) cells (155–160). TIGIT is recognised for its protective role in autoimmune diseases as well as cancer. To date, tumour associated lymphocytes expressing TIGIT have been shown to exist in acute myeloid leukaemia, non-small cell lung cancer, colo-rectal carcinoma and melanoma (161–163). TIGIT is a key checkpoint inhibitor in anti-tumour responses and thus presents a promising target for future immunotherapies (161). TIGIT binds to its ligand PVR or CD155 on the tumour cells with a much higher affinity than its activating counterpart CD226 (DNAM-1), thereby inhibiting the interaction between CD226 and CD155 which is widely expressed

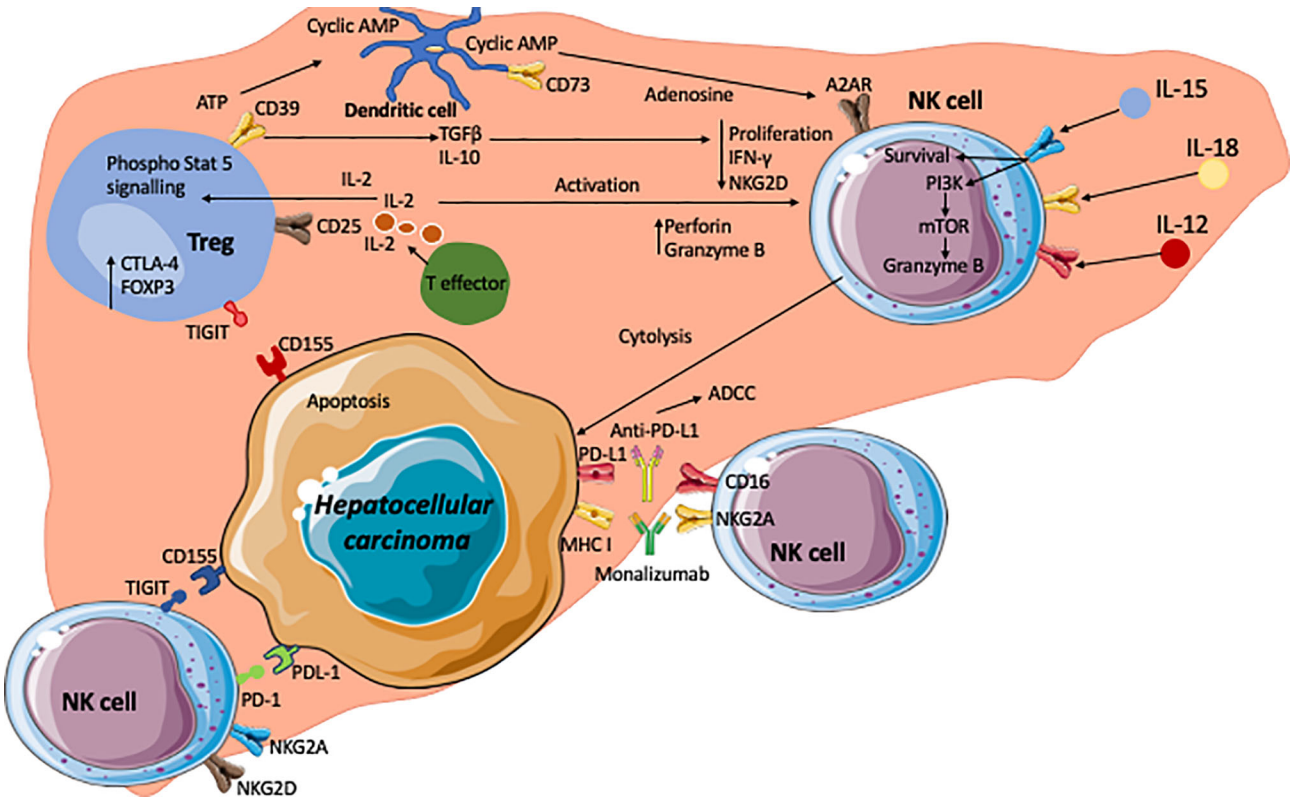

FIGURE 3 | The hepatocellular carcinoma microenvironment and crosstalk of NK, Treg and HCC with the key signalling cascades. Effector T cells are the source of interleukin-2 (IL-2) which acts on the IL-2α receptor in CD25 on regulatory T cells. This leads to phosphorylation of STAT5 which subsequently upregulates Treg functional molecule CTLA-4 and the transcription factor Foxp3. The tumour microenvironment is enriched with adenosine triphosphate (ATP). CD39 on Tregs generates cyclic AMP from ATP. CD73 expressed on intrahepatic Tregs subsequently generate immunosuppressive adenosine from the cyclic AMP. Adenosine act on A2AR on NK cells which leads to the suppression of NK cell function. In addition, immunosuppressive cytokines TGFβ and IL-10 are secreted by Tregs and these cytokines lead to reductions in proliferation, IFNγ production and expression of NKG2D. Conversely, high concentrations of IL-2 lead to an increase in perforin and granzyme B expression on NK cells. IL-12, IL-15 and IL-18 act on their corresponding receptors on NK cells and leading to enhanced cell survival, and granzyme B production *via* PI3Kinase and mTOR pathways. NK cells express inhibitory receptors such as TIGIT and PD-1, which interact with corresponding ligands CD155 (PVR) and PDL-1 expressed on tumour cells. Inhibition of these receptors with check point inhibitors could lead to unleashing of NK cell cytotoxic activity to tumour cells *via* secretion of granzymes, perforin, IFNγ and TNFα. Monoclonal antibodies against inhibitory receptors such as immune checkpoint inhibitor monalizumab are developed to block MHC-I ligands and NKG2A interactions and enhance NK cell cytotoxicity towards cancer cells. CD16 receptors on NK cells allows them to carry out ADCC targeting such molecules as PD-L1.

on tumour cells (158–160, 164). CD226 promotes cytotoxicity and enhances anti-tumour responses (165, 166), whereas TIGIT, which outcompetes CD226, negatively regulates anti-tumour responses (167). After TIGIT/CD155 ligation, TIGIT's immunoglobulin tail tyrosine-like motif becomes phosphorylated at Tyr225 and binds to cytosolic adapter Grb2. This in turn leads to NK cell immunosuppression and dysfunction, including downregulation of IFN-γ production (168). It is now recognised that TIGIT expression on tumour-infiltrating NK cells is associated with tumour progression and was also linked to functional immune exhaustion (139). The role of TIGIT in liver cancer is still under investigation. Recent research has shown that survival and prognosis of HCC patients is positively correlated with NK cell numbers in blood and tumour tissue (169). Tumour progression of these HCC patients was associated with dysfunction of the tumour-infiltrating NK cells (170), and exhausted tumour-infiltrating NK cells correlate with poor clinical outcome for HCC patients. Importantly, this NK cell exhaustion was reversed by manipulating the TIGIT pathway (171). Thus, the clinical application of anti-TIGIT CPI immunotherapy will enhance the NK cell anti-tumour immune response and is a promising new approach towards treating HCC.

Lymphocyte activation gene-3 (LAG-3) belongs to the immunoglobulin superfamily and is expressed on tumour infiltrating lymphocytes (TILs) (172), NK cells (173), B cells (174) and DCs (175). LAG-3 has a high binding capacity to MHC II (173). Current data suggests that modulating LAG-3 can impact autoimmunity, cancer and chronic viral infection (164). Fibrinogen-like protein 1 (FGL1) is a new major ligand for LAG-3 and it has recently been demonstrated that blocking the FGL1-

LAG-3 pathways results in the stimulation of tumour immunity and inhibits tumour growth (176). Furthermore, co-expression of LAG-3 and PD-1 on TILs has been observed in several mouse tumour models. One of the first pre-clinical cancer models using anti-LAG-3 demonstrated enhanced activation of tumour-specific T cells at the tumour site and disruption of tumour growth, especially when used in combination with anti-PD-1 (177). Currently human studies involving LAG-3 as a target are usually performed in combination with PD-x axis blockade, providing a wider checkpoint blockade than monotherapy.

The use of monoclonal antibodies targeting CTLA-4, PD-1 and PD-L1 has seen excellent success, especially in settings such as melanoma and non-small-cell lung cancer. However, most HCC patients have underlying cirrhosis, and there is a concern about the risk of decompensation related to CPI-induced immune mediated hepatitis. This appears relatively rare occurrence during checkpoint inhibitor therapy for HCC with grade ≥3 side effects affected under 5% of patients (142, 178, 179). Nivolumab and pembrolizumab have now received approval from the FDA as second-line treatments for advanced HCC based on both the Checkmate 040 (142) and Keynote 224 (178) clinical trials. Although subsequent phase III trials have failed to show statistically significant data for survival improvement in either first-line (nivolumab vs. sorafenib) or second-line (pembrolizumab vs. placebo) setting (179, 180). These therapeutics now have potential to be combined with anti-VEGF therapies (111), however there remains an unmet clinical need for investigating combinatorial blockade targeting the novel inhibitory receptors, such as TIGIT and LAG-3.

Combination of GMP NK Infusion and Check Point Inhibitors in HCC

NK-cell therapy in cancer has made significant progress in the past decade with many milestones. It has been shown that NK-cell alloreactivity can eliminate the risk of leukaemia relapse and graft rejection as well as protecting against graft-versus-host disease in transplant patients (112). High-risk myelodysplastic syndrome (MDS) patients have been shown to be responsive to NK-cell therapy due to the infused donor NK cells causing a reduction in high-risk clones and a less pronounced host immune activation (181). The infusion of NK cells has also been successful in patients with refractory acute myeloid leukaemia achieving remission in one third of patients (113). A recent phase I trial investigating the use of NK-cell therapy in combination with trastuzumab in HER2-positive cancer patients demonstrated that the therapy was well tolerated and that target engagement and anti-tumour activity was seen in the patients (115). CAR-NK cell infusion is also an exciting and promising therapy for cancer patients with a recent phase I and II trial showing that the majority (8 out of 11) patients responded to the treatment, of which 7 patients had complete disease remission (114). Recent developments suggested that NK cells derived from induced pluripotent stem cells (iPSCs) produce inflammatory cytokines and exert strong cytotoxicity against a variety of hematologic and solid tumours. iPSC-derived NK cells were also found to recruit T cells and cooperate with T cells and anti-PD-1 antibody, subsequently enhancing inflammatory cytokine production and promoting tumour lysis (120). Additionally, NK-CAR-iPSC-NK cells have been shown to significantly inhibit tumour growth resulting in prolonged survival in an ovarian cancer xenograft model (182).

Anti-PD-1, anti-PD-L1 and anti-CTLA-4 monoclonal antibodies also enhance NK cell tumour trafficking and release cytokines against tumours whilst simultaneously supressing Treg function (183–186). Anti-CTLA-4 monoclonal antibodies have also been shown to induce the release of TNFα against tumour cells *via* CD16 binding to antibody-bound tumour cells, and simultaneously to induce Treg inactivation (187–189). Blocking TIGIT and its ability to exploit both T cell and NK cell responses is also a strategy that is currently being explored by multiple pharmaceutical companies undergoing phase I/II clinical trials. Experimental drugs such as tiragolumab (anti-TIGIT) is currently undergoing phase I trials in various cancers (https://clinicaltrials.gov/ct2/show/NCT02913313) in combination with atezolizumab and nivolumab. Specific clinical trials looking at patients with HCC include looking at the use of SRF388 which is a fully human IgG1 antibody against IL-27 that decreases the expression of inhibitory immune checkpoint receptors (https://clinicaltrials.gov/ct2/show/NCT04374877?term=TIGIT+HCC&draw=2&rank=).

Thus, combining the massive increase in CPI therapy with the growth in adoptive NK cell therapeutics indicates that there is now a great potential to generate novel therapeutic combinations that target both CD8 T cells, NK cells and Tregs generating a holistic approach to cancer immunotherapy. Such an approach could simultaneously positively modulate the tumour microenvironment and directly cytotoxicity against tumours. In particular, the combination of augmenting both CD8 T cells and NK cells means that tumours that down-regulate MHC class I to avoid CD8 T cell mediated lysis, lose an important inhibitory signal for NK cells, and thus render themselves susceptible to NK cell killing. However, new therapeutic combinations have the potential to exacerbate toxicity, especially those related to autoimmunity and so these approaches need caution. In general, though NK cell therapeutics have proven relatively safe with little systemic toxicity observed, so these combinations may be more advantageous than those that target solely T cells. Nevertheless as with all therapeutic combinations it is important that future studies are carefully monitored for signs of unexpected toxicity.

CONCLUSION

HCC remains a challenge for clinicians and researchers to approach in partnership. The recent development of multiple new immunotherapies including monoclonal antibodies and cell products present new opportunities for the clinician to treat HCC. However, understanding the immunological micro environment in which HCC occurs will be key to successfully combining these. Fundamental research is required to unpick the different intrahepatic immunological microenvironments on which HCC occurs. Understanding these on a personalized basis will be the key to selecting the optimal combination of immunotherapies for each patient.

AUTHOR CONTRIBUTIONS

AB and FW contributed equally to this work. All authors contributed to the article and approved the submitted version.

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REVIEW
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Current Perspectives on "Off-The-Shelf" Allogeneic NK and CAR-NK Cell Therapies

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Natural killer cells (NK cells) are the first line of the innate immune defense system, primarily located in peripheral circulation and lymphoid tissues. They kill virally infected and malignant cells through a balancing play of inhibitory and stimulatory receptors. In pre-clinical investigational studies, NK cells show promising anti-tumor effects and are used in adoptive transfer of activated and expanded cells, *ex-vivo*. NK cells express co-stimulatory molecules that are attractive targets for the immunotherapy of cancers. Recent clinical trials are investigating the use of CAR-NK for different cancers to determine the efficiency. Herein, we review NK cell therapy approaches (NK cell preparation from tissue sources, ways of expansion *ex-vivo* for "off-the-shelf" allogeneic cell-doses for therapies, and how different vector delivery systems are used to engineer NK cells with CARs) for cancer immunotherapy.

Keywords: natural killer cells, CAR-NK cells, immunotherapy, NK cell expansion, lentiviral delivery, AAV delivery, killer immune receptors, GMP manufacturing

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NATURAL KILLER CELL BIOLOGY

Human natural killer (NK) cells are innate cytotoxic lymphoid cells derived from CD34+ precursors originating from hematopoietic stem cells (1, 2) and play an essential role in tumor surveillance. Unlike T cells, NK cells can kill malignant cells in an antigen-independent manner and have shown promise in a number of clinical trials involving both solid and hematological cancers (3). NK cells do not require HLA matching. Their ability to act in an antigen-independent manner makes them a viable option for an "off the shelf" therapy that can be manufactured on a large scale and easily distributed to cancer patients.

NK cells are subdivided into two populations based on their relative expression of CD56 (neural cell adhesion molecule; NCAM) and CD16: immature $CD56^{bright}$ $CD16^{neg}$ NK cells, and mature $CD56^{dim}$ $CD16^{pos}$ NK cells (4). The $CD56^{bright}$ population accounts for 10% of NK cells circulating in the blood and are located primarily in lymph nodes. Immature $CD56^{bright}$ NK cells have an immunoregulatory function and produce cytokines, such as interferon-gamma (IFN-γ), TNFα, TNF-β, IL-10, and GM-CSF (5). In contrast, the mature $CD56^{dim}$ $CD16^{pos}$ population accounts for up to 90% of the circulating NK cells (6). The key function of mature $CD56^{dim}$ $CD16^{pos}$ NK cells is natural and Ab-mediated cell cytotoxicity. Mature $CD56^{dim}$ NK cells express high amounts of killer cell immunoglobulin receptors (KIRs) (7).

The mechanism for the transition from $CD56^{bright}$ to $CD56^{dim}$ is still widely unknown, but the change in surface markers is a major indicator for transitioning to maturity (2, 7). CD16, CD27, CD56, CD57, and perforin are all markers for NK cell maturation (7, 8). CD27 is a marker of immature NK cells, associated with the TNFα receptor group and found on three times as many immature $CD56^{bright}$ as mature $CD56^{dim}$ (7). Inversely CD57 and perforin are markers for terminal maturity and are highly expressed on mature NK cells (7, 9).

Located throughout the body, NK cells represent 5-20% of all lymphocytes in the blood and organs with high concentrations in the bone marrow, spleen, liver, lungs, skin, kidneys, uterus, and secondary lymphoid tissue (8, 10). The tissue-specific location has been shown to have a significant impact on NK cell functionality and cytokine production. Mature NK cells in the lung are shown to produce higher amounts of granzyme B, a serine protease associated with cytotoxicity, than those NK cells found in the lymph nodes or gut (8).

NK cells secrete a number of pro-inflammatory cytokines, such as TNF and IFN-γ that stimulate an adaptive immune response and prevent tumor angiogenesis (5). The production levels of IFN-γ and TNFα from NK cells can be stimulated through various cytokines such as IL-12, IL-15, and IL-18 (8).

NK cells function by killing virally infected, stressed, and cancerous cells in an antigen-independent manner (1, 2, 8). Additionally, NK cells work to activate other immune cells using co-stimulatory signals (2). NK cell's cytolytic function is based on an array of activation and inhibitory signals (**Figure 1**) as well as self-major histocompatibility complexes (MHC) class I molecules (1, 2). NK cells recognize target cell MHC class I molecules which bind to the NK cell KIRs allowing the NK to identify "self." This self-identification inhibits the cytotoxic activity against normal cells (1, 2, 7). In addition to preventing cytotoxic function, this binding also prevents inflammation and helps with the "licensing" of the immature NK cells (7). Tumor cells often downregulate MHC class I expression to avoid lysis by cytotoxic T cells. Additionally, DNA damage and cellular stress upregulates tumor ligands' expression on malignant cells, which are recognized by NK cell-activating receptors (**Figure 2**). NK cells will trigger cell-mediated lysis (1) if a cell down-regulates its MHC class I molecules and upregulates activation ligands.

Once a cell is designated as infected, stressed, or cancerous, NK cells work to kill it through a direct release of cytolytic granules containing perforin and granzyme B. The contents of cytolytic granules are released from the cell *via* degranulation (**Figure 2**). The granules from the NK cell form a synapse with the target cell, releasing the cytolytic contents. Perforin and granzyme B are key components of cytolytic granules and trigger apoptosis through caspase-dependent and independent mechanisms. Perforin aids in the entry of the granzyme B into the target cell, which ultimately leads to target cell death (11).

In addition to direct lysis of malignant or virally infected cells, $CD56^{dim}$ $CD16^{pos}$ NK cells mediate antibody-dependent cellular cytotoxicity (ADCC). ADCC is triggered when NK cells recognize an antibody opsonized target cell. The binding of CD16 with the Fc portion of IgG antibodies trigger the release of perforin and granzyme B which lyse the target cell (11). ADCC is provoked by several therapeutic monoclonal antibodies (mAbs) and may enhance the homing and efficacy of NK cell therapy (12).

NK CELL-BASED STRATEGIES IN CLINICAL TRIALS TARGETING DIFFERENT INDICATIONS

Autologous and allogeneic NK cell therapies have shown great potential in preclinical studies and clinical trials. Different strategies are considered in clinical trials using NK cells for cancer therapies, including utilizing an agonist to NK cell activation receptors (mABs; transtuzumab, rituximab, etc., + IL-2 and anti PD1) or by blocking NK cells inhibitory receptor signals with mABs to KIR (NKG2A-CD94 or with CTLA-4 and PD-1 checkpoint inhibitor) (13). Recent findings demonstrate the potential of allogeneic NK cells for hematological malignancies and solid tumors (14). Unlike T cells, NK cells do not induce graft-*versus*-host-disease (GVHD) and their alloreactivity is enhanced under KIR mismatch with HLA ligands on cancer cells (15). Several clinical trials have highlighted the safety of the allogeneic transfer of NK cells (16). Allogeneic NK cells were used to target different cancers including hematological malignancies, lymphoma, leukemia, and solid tumors such as melanoma, neuroblastoma, gastric cancers, ovarian and breast tumors (**Table 1**).

An "off the shelf" NK cell therapy solves the one-donor, one-patient limitation that makes -autologous cell therapy processes labor-intensive. A critical step to enable allogeneic NK cell-based therapies would require a healthy donor source for NK cells and expanding to clinically relevant doses. Most clinical trials of NK cells require large numbers of cells for infusion, ranging from 5×10^6 to 1×10^8 $CD3^{neg}CD56^{pos}$ NK cells per kilogram body weight (5).

SOURCES OF NATURAL KILLER CELLS FOR IMMUNOTHERAPY

NK cells for therapy can be acquired from various sources such as umbilical cord blood (UCB) (17), peripheral blood (PB) (18, 19), human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) (20) as well as cells lines such as NK-92 (21). To date, most of the NK cell clinical trials are based on UCB-NK cells, PB-NK cells, and the lymphoma-derived NK cell line NK-92. There are critical challenges in the manufacturing process of the final therapeutic cell doses. For example, isolation and expansion of PB-NK cells and UCB-NK cells result in a mixed composition (22). The cell line NK-92 is derived from a cancer patient with non-Hodgkin lymphoma; thus, the cells need to be irradiated before infusion, limiting the

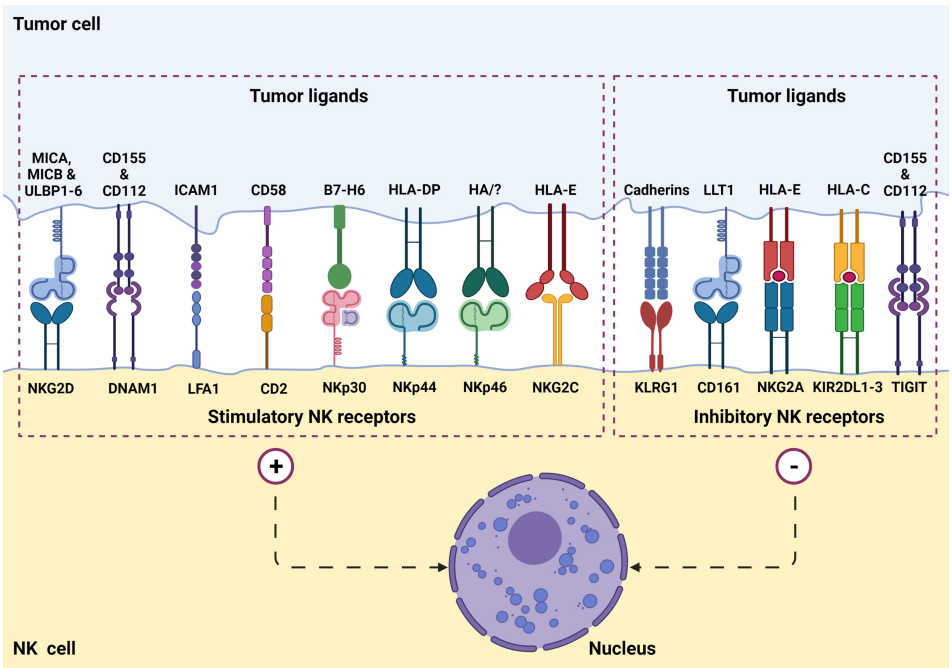

FIGURE 1 | NK cell surface receptors and ligands on tumor cells are involved in tumor recognition. NK cells express a set of stimulatory (or activation) receptors as well as inhibitory receptors to recognize healthy cells and aberrant cells such as virus-infected or a potential tumorigenic cell through MHC-1 receptor appearance.

NK cell persistence (23). In contrast to these limitations, hESC-NK cells and iPSC-NK cells are more homogenous and can be generated in sufficient cell numbers for allogeneic clinical use (24). Pluripotent (hESC/iPSC) derived NK cells can result in allogeneic therapy providing a standard cell-based treatment option for different diseases (24–26). Processing workflow of NK cell isolation from different donor sources through expansion for adaptive transfer is described (**Figure 3**).

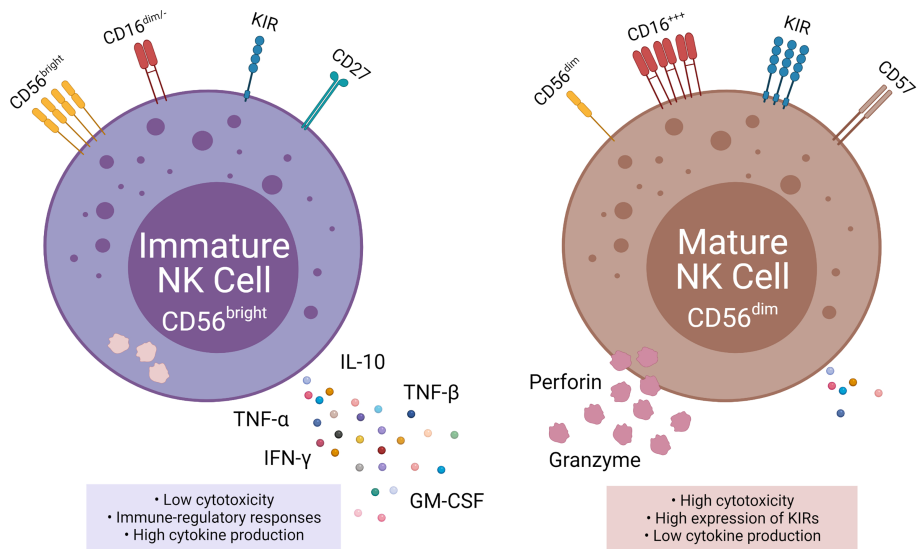

FIGURE 2 | Phenotypic and functional properties of immature (left) and mature (right) NK cells. Immature NK cells express $CD56^{bright}$, absent, or $CD16^{dim}$, low KIR, and CD27 and are also known as $NK^{regulatory}$ that exhibit low cytotoxicity, but high cytokine production. Mature NK cells, in contrast, express $CD56^{dim}$, high CD16, high KIRs, and CD57 and are also knows as $NK^{cytotoxic}$ that exhibit high cytotoxicity and low cytokine production.

TABLE 1 | Completed allogeneic NK cell clinical trials.

	NCT Number	Title	NK Cell source	Status	Conditions	Interventions	Clinical trial phase	Population	Sponsor/ Collaborators	Dates	Locations / Outcome
1	NCT03358849	Phase 1 Clinical Trial to Evaluate the Safety of Allogeneic NK Cell ("SMT-NK") Cell Therapy in Advanced Biliary Tract Cancer	Not available	Completed: No results posted	• Advanced Biliary Tract Cancer	• Biological: Natural killer cell	Study Type: Interventional Phase: Phase 1	Enrollment: 9 Age: 18 Years to 75 Years (Adult, Older Adult) Sex: All	• Yonsei University	Study Start: October 17, 2017 rimary Completion: September 27, 2018 Last Update Posted: January 16, 2019	• Division of Gastroenterology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea, Republic of
2	NCT02008929	to Evaluate the Efficacy and Safety of MG4101(Ex Vivo Expanded Allogeneic NK Cell)	Allogeneic expanded NK Cells	Completed: No results posted	• Hepatocellular Carcinoma	• Biological: MG4101	Study Type: Interventional Phase: Phase 2	Enrollment: 5 Age: 20 Years to 69 Years (Adult, Older Adult) Sex: All	• Samsung Medical Center	Study Start: August 2014 Last Update Posted: December 3, 2015	• Samsung Medical Center, Seoul, Korea, Republic of
3	NCT01212341	Allogeneic Natural Killer (NK) Cell Therapy in Patients With Lymphoma or Solid Tumor	Allogeneic NK Cells	Completed - No results posted	• Malignant Lymphomas • Solid Tumors	• Biological: Allogeneic NK cells	Study Type: Interventional Phase: Phase 1	Enrollment: 18 Age: 18 Years and older (Adult, Older Adult) Sex: All	• Seoul National University Hospital • Green Cross Corporation	Study Start: September 2010 Primary Completion: August 2012 Last Update Posted: August 19, 2013	• Seoul National University Hospital, Seoul, Korea, Republic of
4	NCT00383994	Immunotherapy With NK Cell, Rituximab and Rhu-GMCSF in Non-Myeloablative Allogeneic Stem Cell Transplantation	Blood derived NK Cells	Completed No resultrs posted	• Lymphoma • Leukemia • Transplantation, Stem Cell • Lymphoid Malignancies • Disorder Related to Transplantation	• Drug: GM-CSF • Drug: Rituximab • Biological: NK Cell Infusion	Study Type: Interventional Phase: Phase 1	Enrollment: 6 Age: Child, Adult, Older Adult Sex: All	• M.D. Anderson Cancer Center • Bayer Healthcare Pharmaceuticals, Inc./Bayer Schering Pharma	Study Start: September 2006 Primary Completion: July 22, 2019 Last Update Posted: July 31, 2019	• University of Texas MD Anderson Cancer Center, Houston, Texas, United States
5	NCT00402558	Alloreactive NK Cells for Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS)	Completed - no results posted	Completed No results posted	• Myelodysplastic Syndrome • Leukemia	• Drug: Thymoglobulin • Drug: Busulfan • Drug: Fludarabine • Procedure: Alloreactive NK Infusion	Study Type: Interventional Phase: Phase 1	Enrollment: 15 Age: up to 70 Years (Child, Adult, Older Adult)	• M.D. Anderson Cancer Center	Study Start: May 2006 Primary Completion: April 2014 Last Update Posted: May 8, 2015	• UT MD Anderson Cancer Center, Houston, Texas, United States

(Continued)

TABLE 1 | Continued

	NCT Number	Title	NK Cell source	Status	Conditions	Interventions	Clinical trial phase	Population	Sponsor/ Collaborators	Dates	Locations / Outcome
						• Drug: G-CSF • Drug: Tacrolimus • Drug: Methotrexate • Drug: Interleukin-2		Sex: All			
6	NCT01853358	Phase I of Infusion of Selected Donor NK Cells After Allogeneic Stem Cell Transplantation	HLA identical allogeneic NK Cells	Completed-Phase 1-No results posted	• Hematological Malignancy	• Biological: NK Cell infusion	Study Type: Interventional Phase: Phase 1	Enrollment: 17 Age: 18 Years to 70 Years (Adult, Older Adult) Sex: All	• Institut Paoli-Calmettes	Study Start: April 2013 Primary Completion: March 15, 2018 Last Update Posted: July 12, 2018	• Institut Paoli-Calmettes, Marseille, France
7	NCT01287104	A Phase I Study of NK Cell Infusion Following Allogeneic Peripheral Blood Stem Cell Transplantation From Related or Matched Unrelated Donors in Pediatric Patients With Solid Tumors and Leukemias	Allogeneic Bone marrow NK Cells	Completed - Has results	• Leukemia • Lymphoma	• Biological: Natural Killer (NK) Cell Infusion • Biological: Stem Cell Infusion - Pag	Study Type: Interventional Phase: Phase 1	Age: 4 Years to 35 Years (Child, Adult) Sex: All	• National Cancer Institute (NCI) • National Institutes of Health Clinical Center (CC)	Study Start: January 29, 2011 Primary Completion: June 28, 2018 Last Update Posted: August 22, 2019	• National Institutes of Health Clinical Center, Five of 9 transplant recipients experienced acute graft-versus-host disease (GVHD) following aNK-DLI, with grade 4 GVHD observed in 3 subjects.
8	NCT02716571	Recruiting Blood Donor With Allogeneic Natural Killer Cell	Allogeneic natural killer cell	Completed: No results posted	• Healthy Volunteers	• Other: Leukapheresis or Plasmapheresis	Study Type: Interventional Phase: Not Applicable	Enrollment: 90 Age: 20 Years to 60 Years (Adult) Sex: All	• Seoul National University Hospital	Study Start: March 28, 2016 Primary Completion: June 2, 2017 Last Update Posted: July 31, 2017	• Seoul National University Hospital, Seoul, Korea, Republic of
9	NCT00877110	Anti-GD2 3F8 Antibody and Allogeneic Natural Killer Cells for High-Risk Neuroblastoma	Allogeneic NK Cells from a family member who shares half of the HLA proteins	Completed: No results posted	• Neuroblastoma • Bone Marrow, Sympathetic Nervous System	• Drug: cyclophosphamide, vincristine, topotecan ,allogenei NK cells & 3F8	Study Type: Interventional Phase: Phase 1	Enrollment: 71 Age: Child, Adult, Older Adult Sex: All	• Memorial Sloan Kettering Cancer Center	Study Start: April 2, 2009 Primary Completion: January 7, 2019 Last Update Posted: January 10, 2019	• Memorial Sloan Kettering Cancer Center, New York, New York, United States

(Continued)

TABLE 1 | Continued

	NCT Number	Title	NK Cell source	Status	Conditions	Interventions	Clinical trial phase	Population	Sponsor/ Collaborators	Dates	Locations / Outcome
10	NCT02301065	Analysis of T Cell and Natural Killer (NK) Cell in Relation to Viral Infections in Pediatric Stem Cell Transplant Patients and Donors	Blood derived FcRg-CD56 +CD3- NK cells in pediatric allogeneic HSCT patients and healthy donors	Completed: No results posted	• Hematologic Malignancies		Study Type: Observational Phase:	Enrollment: 35 Age: up to 21 Years (Child, Adult) Sex: All	• St. Jude Children's Research Hospital • Michigan State University	Study Start: October 13, 2016 Primary Completion: February 6, 2017 Last Update Posted: July 17, 2017	• St. Jude Children's Research Hospital, Memphis, Tennessee, United States
11	NCT02845999	Allogenic Immunotherapy Based on Natural Killer (NK) Cell Adoptive Transfer in Metastatic Gastrointestinal Carcinoma Treated With Cetuximab	Haploidentical Natural Killer (NK) cells	Completed-No Results posted	• Gastrointestinal Metastatic Cancer	• Biological: allogenic immunotherapy based on Natural Killer cells adoptive transfer • Biological: cetuximab • Drug: Cyclophosphamide • Drug: fludarabine • Drug: interleukin-2	Study Type: Interventional Phase: Phase 1	Enrollment: 9 Age: 18 Years to 65 Years (Adult, Older Adult) Sex: All	• Centre Hospitalier Universitaire de Besancon • National Cancer Institute, France	Study Start: November 2009 Primary Completion: January 2013 Last Update Posted: July 27, 2016	• University hospital of Besançon, Besançon, France
13	NCT01181258	Penostatin, Rituximab and Ontak and Allogeneic Natural Killer (NK) Cells for Refractory Lymphoid Malignancies	Interleukin 2-activated Allogeneic Natural Killer Cells	Completed-Has results	• Non-Hodgkin Lymphoma • Chronic Lymphocytic Leukemia	• Drug: Rituximab • Biological: Interleukin-2 • Biological: Natural killer cells • Drug: Cyclophosphamide • Drug: Methylprednisolone • Drug: Fludarabine	Study Type: Interventional Phase: Phase 2	Enrollment: 16 Age: Child, Adult, Older Adult Sex: All	• Masonic Cancer Center, University of Minnesota	Study Start: August 2010 Primary Completion: September 2015Study Completion: July 2016 First Posted: August 13, 2010 Results First Posted: May 18, 2017 Last Update Posted: February 6, 2018	• Masonic Cancer Center, University of Minnesota, Observations support development of donor NK cellular therapies for advanced NHL as a strategy to overcome chemoresistance
14	NCT01105650	Allogeneic Natural Killer (NK) Cells for Ovarian, Fallopian Tube, Peritoneal and Metastatic Breast Cancer	Allogeneic donor cells	Completed-Has results	• Ovarian Cancer • Fallopian Tube Cancer • Primary Peritoneal Cancer • Breast Cancer	• Drug: Fludarabine • Drug: Cyclophosphamide • Drug: Cyclosporine	Study Type: Interventional Phase: Phase 2	Enrollment: 13 Age: 18 Years and older (Adult,	• Masonic Cancer Center, University of Minnesota	Study Start: July 2010 Primary Completion: April 2014 Last Update	• Masonic Cancer Center, University of Minnesota, Some adverse events reported - not published

(Continued)

TABLE 1 | Continued

	NCT Number	Title	NK Cell source	Status	Conditions	Interventions	Clinical trial phase	Population	Sponsor/ Collaborators	Dates	Locations / Outcome
						• Biological: Natural killer cells • Drug: IL-2 • Drug: Methylprednisolone • Drug: Interleukin-2		Older Adult) Sex: Female		Posted: December 28, 2017	
15	NCT00586703	Safety Trial of NK Cell DLI 3-5/6 Family Member Following Nonmyeloablative ASCT	CD56-NK cells from mismatched donors	Completed-Has results	• Lymphoma	• Device: NK-CD56	Study Type: Interventional Phase: Phase 1	Enrollment: 21 Age: 18 Years and older (Adult, Older Adult) Sex: All	• David Rizzieri, MD • Duke University	Study Start: April 2005 Primary Completion: April 2013 Last Update Posted: June 12, 2014	• Duke University Health Systems" A 1-step, high-yield process is feasible, and results in high doses of NK cells infused with little toxicity. NK cell-enriched DLIs result in improved immune recovery and outcomes for some
16	NCT02118285	Intraperitoneal Natural Killer Cells and INCB024360 for Recurrent Ovarian, Fallopian Tube, and Primary Peritoneal Cancer	haploidentical donor NK cells and IL-2	Completed-No results posted	• Ovarian Cancer • Fallopian Tube Carcinoma • Primary Peritoneal Carcinoma	• Drug: Fludarabine • Drug: Cyclophosphamide • Biological: NK cells • Biological: IL-2 • Drug: INCB024360	Study Type: Interventional Phase: Phase 1	Enrollment: 2 Age: 8 Years and older (Adult, Older Adult) Sex: Female	• Masonic Cancer Center, University of Minnesota • Incyte Corporation	Study Start: July 28, 2014 Last Update Posted: December 5, 2017	• University of Minnesota Masonic Cancer Center, Minneapolis, Minnesota, United States
18	NCT00526292	Chemotherapy and a Donor Natural Killer Cell Infusion in Treating Patients With Relapsed or Persistent Leukemia or Myelodysplastic Syndrome After a Donor Stem Cell Transplant	Allogeneic NK Cells from a family member who shares half of the HLA proteins	Completed:-Has results	• Leukemia • Myelodysplastic Syndromes	• Biological: natural killer cell therapy • Drug: cyclophosphamide • Drug: fludarabine	Study Type: Interventional Phase: Phase 2	Enrollment: 12 Age: up to 120 Years (Child, Adult, Older Adult) Sex: All	• Memorial Sloan Kettering Cancer Center • National Cancer Institute (NCI)	Study Start: August 2007 Primary Completion: July 2015 Last Update Posted: February 12, 2016	• Memorial Sloan Kettering Cancer Center, New Yor: Results not conclusive as 4/6 patients showed some adverse events
19	NCT02854839	A Study of MG4101 (Allogeneic Natural Killer Cell) for Intermediate-stage of Hepatocellular Carcinoma	allogeneic Natural killer cells	Completed No results posted	• Hepatocellular Carcinoma	• Biological: MG4101	Study Type: Interventional Phase: Phase 2	Enrollment: 78 Age: 18 Years to 80 Years (Adult, Older Adult) Sex: All	• Green Cross LabCell Corporation	Study Start: November 28, 2016 Primary Completion: September 27, 2018 Last Update Posted: September 26, 2019	• Seoul National University Hospital, Seoul, Korea, Republic of • Seoul Asan Medical center, Seoul, Korea, Republic of • Samsung Medical Center, Seoul, Korea, Republic of and others

(Continued)

TABLE 1 | Continued

	NCT Number	Title	NK Cell source	Status	Conditions	Interventions	Clinical trial phase	Population	Sponsor/ Collaborators	Dates	Locations / Outcome
20	NCT01386619	NK DLI in Patients After Human Leukocyte Antigen (HLA)- Haploidentical Hematopoietic Stem Cell Transplantation (HSCT)	HLA haploidentical -CD3-depleted/ CD56+ selected natural killer cells collected from apheresis products	Completed No results posted	• Leukemia, Myeloid, Acute • Precursor Cell Lymphoblastic Leukemia- Lymphoma • Myelodysplastic Syndromes • Lymphoma • Neuroblastoma • Rhabdomyosarcoma	• Biological: CD3-depleted/CD56+ selected natural killer cells collected from apheresis products	Study Type: Interventional Phase: • Phase 1 • Phase 2	Enrollment: 15 Age: Child, Adult, Older Adult Sex: All	• University Hospital, Basel, Switzerland	Study Start: January 2004 Primary Completion: March 2011 Last Update Posted: September 15, 2015	• Universitätsklinikum, Frankfurt, Germany • University Hospital, Basel, Switzerland
21	NCT00274846	Donor Peripheral Stem Cell Transplant in Treating Patients With Relapsed Acute Myeloid Leukemia	Peripheral Blood derived NK cells and also stem cells from the same allogeneic donor	Completed Has Results	• Leukemia	• Biological: aldesleukin • Biological: therapeutic allogeneic lymphocytes • Drug: cyclophosphamide • Drug: fludarabine phosphate • Procedure: in vitro treated peripheral blood stem cell transplantation	Study Type: Interventional Phase: Phase 2	Enrollment: 21 Age: 2 Years and older (Child, Adult, Older Adult) Sex: All	• Masonic Cancer Center, University of Minnesota	Study Start: March 2005 Primary Completion: June 2008 Last Update Posted: December 28, 2017	• Masonic Cancer Center, Minneapolis, : Supports the need to optimize the in vivo cytokine milieu where adoptively transferred NK cells compete with other lymphocytes to improve clinical efficacy in patients with refractory AML

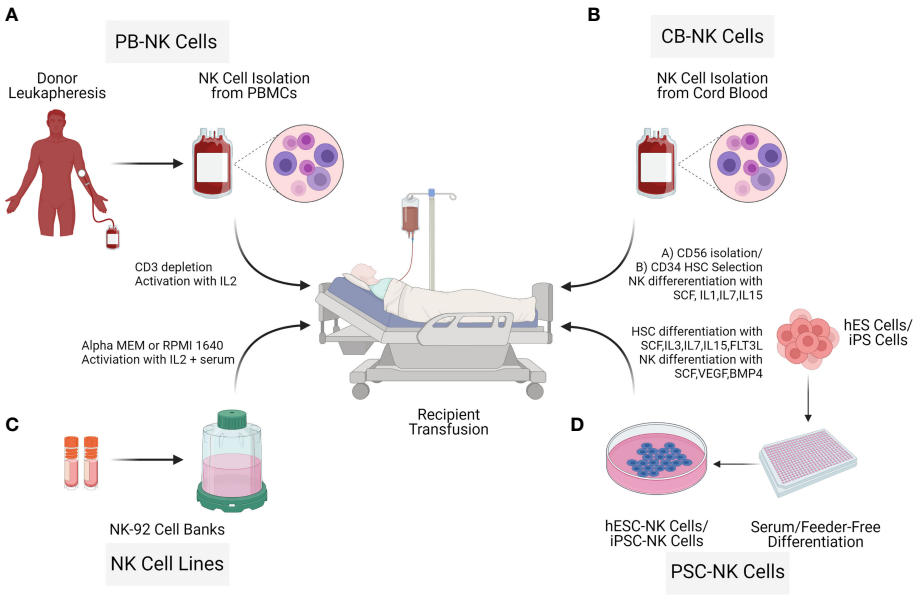

FIGURE 3 | Sources of Natural killer cells for immunotherapy. NK cells for cell therapy applications can originate from different sources: Peripheral blood NK cells (PB NK cells) **(A)**, allogeneic umbilical cord blood NK cells (CB-NK Cells) **(B)**, NK cell cancer cell lines (NK-92) **(C)**, human embryonic stem cells (hESC) and inducible pluripotent stem cells (iPSCs) **(D)**. Advantages and limitations with the different NK cell sources vary as described in the NK cell isolation section.

Umbilical Cord Blood

The umbilical cord is an abundant source of cytotoxic $CD56^{pos}CD16^{pos}$ NK cells, with high lytic potential of cancer cells (27). UCB-NK cells are isolated from cord blood after birth, *via* venipuncture of the umbilical cord, and purification by density gradient centrifugation (28). Alternatively, CD34 hematopoietic stem cells can be isolated from UCB and differentiated to NK cells (19, 29). NK cells generated from CD34 cells from HLA matched umbilical cord blood units showed good tolerance, no GVHD or toxicity (30). UCB is a readily available source with the potential to manufacture multiple doses from a single frozen vial of NK cells isolated from a healthy donor (21, 31). In addition, UCB NK cells are of a younger and more proliferative phenotype relative to PB NK cells (32, 33).

Peripheral Blood

Peripheral blood contains NK cells and is a reliable source of CD34 progenitor cells from individuals undergoing GCS-F mobilization (34). Isolating large numbers of PB NK cells and hematopoietic stem cells is difficult as the percentage derived from leukapheresis can be low and highly variable (22, 35, 36). Further, cryopreservation of PB NK cells lowers the cytotoxic ability (35, 37). Allogeneic NK cells can be isolated from PBMCs by either CD3/CD19 depletion (38) or CD3 depletion and subsequent CD56 enrichment (39). The second round of purification based on CD3 depletion can also be implemented post-expansion (39) to increase NK cell purity. An evaluation of 94 samples with CD3/CD19 depletion and 13 samples with CD3 depletion/CD56 enrichments for NK cell isolations in support of 8 clinical trials demonstrated limitations and benefits with NK cell isolation strategies (34). CD3/CD19 depletion resulted in a mean NK cell recovery of 74% and viability of 96%. However, CD3 depletion/CD56 enrichment resulted in a high NK cell purity (90%), with 5% CD14 monocytes (38).

iPSC or hESC Derived NK Cells

Pluripotent stem cells (iPSC or hESC) are an unlimited source for the derivation of human NK cells for therapy. NK cells derived from iPSC/hESC result in a homogenous population, which can be expanded on a large scale and can be genetically modified (40). NK cells are generated from different iPSC cell lines (41–43) on stromal feeders using IL-3, IL-7, IL-5, Stem cell factor (SCF), fms-like tyrosine kinase receptor-3 ligand (FLT3L) (24). NK cells derived this way are homogenous and express CD56, KIR, CD16, NKp44, NKp46, and are capable of killing tumor cells (24). Similar to iPSCs, hESCs can also be differentiated to NK cells based on stromal cell-mediated differentiation, involving CD34+CD45+ cell sorting and NK cell differentiation with IL-3, IL-5, IL-7, fms-like tyrosine kinase receptor 3 ligand (FLT3L), and Stem cell factor (SCF) (26).

Recently a stromal-free process for iPSC NK cell generation has been established based on embryoid bodies (EB) as self-stromal cells are formed inside the EB (40). Feeder-dependent hESC/iPSC was adapted to a feeder independent system before EB generation (44). To generate EBs, hESC/iPSC are seeded in APEL media containing SCF, BMP4, VEGF, Rocki (rho kinase inhibitor). In the second and final step, NK cells are generated by transferring EBs to gelatin-coated wells containing NK cell differentiation media with IL-3, SCF, IL-7, and IL-15. After

four weeks of culture, differentiated NK cells stained positive for CD45 and CD56 markers were harvested (40).

NK Cancer Cell Lines

Among the available NK cancer cell lines, only NK-92 cell line has shown antitumor activity in a variety of tumors and has worked well in pre-clinical studies (21, 45). Furthermore, NK-92 cancer cell line has received FDA approval for clinical phase patient trials (46, 47). The NK-92 cancer cell line is well characterized and robust clinical protocols are available for cGMP manufacturing (48). These cells can be genetically engineered, but with a variable efficiency of 4% - 95% (49) and expanded to substantial numbers. However, NK-92 cancer cell line requires irradiation prior to infusion, as it is cytogenetically abnormal. Select advantages and disadvantages with NK cells derived from different tissue sources are shown in **Table 2**.

NK CELL EXPANSION FOR THE CREATION OF ALLOGENEIC DOSES

Regardless of how the NK cells are sourced, every method of NK cell expansion can be classified as either a feeder-cell-based system or a feeder-free system. A multitude of cells and cell lines are used as feeders to stimulate allogeneic NK cell expansion. K562 leukemia cells have been successfully used in this regard for several decades (50) and are the most used and well-characterized example. Other examples including EBV transformed lymphoblastoid (EBV-LCL) (51), HEK293 (52), autologous irradiated PBMCs (53), Jurkat cells (54), the Wilms tumor cell line, HFWT (la5), RPMI1866 (55), MM170 (56), and Daudi (57) have also been applied with varying degrees of success. Strategies to prime and propagate NK cells using EBV-transformed lymphoblastoid cells and irradiated PBMCs continue to show promise, but protocols employing K562 cells remain superior in terms of both the magnitude and speed of expansion. Still, many groups attempt to improve the outcome even more by supplementing the culture with antibodies, such as OKT-3 (58, 59) and other cyto-stimulants, such as PHA, ionomycin (53), and concanavalin A (60). One group has even claimed an extremely robust average of 50,000-fold expansion in 21 days (about 3 weeks) using a modified K562 line that expresses membrane-bound IL-21 (61). A potential pitfall of employing feeder cells is that they are associated with a multitude of regulatory concerns. These cells must be stringently qualified using cumbersome assays and viral testing to ensure that they are free of microbial contaminants, such as mycoplasma (62). Moreover, additional actions need to be taken to ensure that the final product is free from the feeder cells. This has encouraged researchers to develop and employ several feeder-free systems in the cultivation of NK cells.

To date, there has been a clear trade-off in that feeder-free systems alleviate many regulatory concerns but result in much lower yields. Several cell-free methods can be explored to activate and stimulate NK cells, including cytokines, and antibodies. Cytokines represent the most widely studied and earliest feeder-free method for activating NK cells. IL-2 is the most potent NK cell stimulant and elicits immunostimulatory signaling, increases cytokine release (63), promotes cell motility (63), and enhances cytotoxicity (64). More recently, many alternative immunogenic cytokines have garnered attention for NK stimulation, including interleukins-15, -21, -12, -18, and -27. Much like IL-2, IL-15 stimulates NK cell proliferation, immunostimulatory receptor expression, and cytotoxicity (65, 66), which makes it a great candidate to be used as an NK stimulant in a stand-alone fashion. In addition, it boasts several benefits over IL-2. Marks-Konczalik and colleagues reported that IL-15 inhibited activation-induced cell death that results from continuous IL-2 stimulation (67)and unlike IL-2, IL-15 does not induce activation and proliferation of Tregs (68), which results in peripheral tolerance and potentially leads to a more robust anti-tumor response. However, there is a tradeoff, research conducted by Felices et al. recently demonstrated that sustained IL-15 signaling results in exhausted NK cells and a loss of *in vitro* and *in vivo* efficacy (69). Several groups have tried to stimulate NK cells with lower doses of IL-2 or IL-15 in combination with some of the other cytokines or they have developed cytokine schedules to alleviate some of the drawbacks associated with persistent stimulation with the one cytokine over the entire expansion protocol (70). IL-21 alone is not sufficient to stimulate significant NK-cell expansion (71, 72), however, there is a synergistic proliferative effect when IL-21 is

TABLE 2 | Advantages and drawbacks of NK Cells from different sources.

The Source of NK Cells	Advantages	Drawbacks
Peripheral Blood derived NKs (PB-NKs)	High expression of CD16+ Highly cytotoxic Expression of CD57, a marker of terminal differentiation of NK Cells	Low number of NK Cells in PB Lower or no expression of CXCR4
NK-92 cancer cell line	Cell line product -easy to obtain Clinically approved CD16 negative	Need for irradiation before injection Tumorigenesis potential Safety concerns
Umbilical Cord Blood derived NKs (UCB-NKs)	High expression of CXCR4 Minimize GvHD Ready reconstitution after transplant	Reduced cytotoxicity (against K562 tumor cell line) Low numbers Immaturity of NK cells
Placental blood derived NKs (p-NKs)	Placenta rich source for NK cells Easily, readily available	Low cytolytic activity
iPSC derived NKs (iNKs)	Resource to generate unlimited numbers relevant for therapy Minimal immune rejection	Complex differentiation steps Clinical effectiveness still to be proven Safety issues

combined with other immunostimulatory cytokines like IL-2 and IL-15 (71, 72). Furthermore, the addition of IL-21 to NK cell culture has been associated with increased immunostimulatory cytokine production (73) and upregulation of perforin and granzyme A and B (74), leading to enhanced NK cell cytotoxicity (75, 76). IL-12, IL-18, and IL-27 are slightly less characterized but have also displayed the ability to positively contribute to NK cell expansion, especially when used in conjunction with the IL-2 or IL-15. Research demonstrates that IL-12 can have a synergistic effect with IL-2, which results in enhanced NK cell cytokine secretion, proliferation, and cytotoxic capacity (77, 78). IL-18 and IL-27 have recently been combined with IL-15 to boost NK cell fold expansion (79). Another advantage of combining the cytokines can result in a lower dose of the individual cytokines, which can lead to a higher percentage of memory NK cells (19). The combination of IL-12, IL-15, and IL-18 drives preferential expansion of memory-like NK cells, which exhibit heightened responses when they encounter tumor cells (79–81) and longer lifespans following engraftment (79–81). An additional benefit of these cells is an increased capacity to produce immunostimulatory cytokines upon secondary challenge. This memory response is an intrinsic quality that is passed on to all cellular progeny (79–81).

Apart from these most common feeder-cell and feeder-free cytokine systems, several groups have moved towards strategies that are a hybrid of the two. Several groups have engineered feeder cells that express immunostimulatory signaling molecules, such as 41BB, IL-15 (82, 83), and IL-21 (63, 84–86) on their cell surfaces. These strategies have resulted in highly cytotoxic NK cells that display both extremely high proliferative capacities (up 50,000-fold expansion) (61), extended survival, and the ability to secrete immunogenic cytokines, leading many groups to adopt these methods into their clinical protocols. This approach has recently been taken one step further to avoid safety concerns by stimulating NK cells with K562-mb21-41BBL cell lysates (87).

Most of the experiments and trials discussed in this review have utilized small-scale, open methods for NK cell activation and expansion, such as flasks and G-Rex vessels. However, these methods are hampered by logistical hurdles, inconsistencies, and safety concerns. To reach the desired cell numbers for allogeneic manufacturing and clear all regulatory and safety hurdles associated with drug approval, it will be necessary to develop closed, and automated systems with large-scale capabilities. Hence, clinical scale NK cell manufacturing development suitable for effective allogeneic therapy production is a priority. Several options have been explored, including a G-Rex-based method that was developed under good manufacturing practice (GMP) conditions and required little to no manual intervention for the 8- to 10-day expansion and yielded 19 billion functional NK cells (88). Another example of static culture is the use of large, gas-permeable culture bags, which were successfully applied in combination with feeder cells, antibodies, and cytokines to yield an NK cell fold expansion of 15,000 (89). A more recent trend for achieving clinical scale NK cell expansion has been the use of bioreactors. In addition to large cell capacity, these devices are highly adaptable for closed and automated manufacturing processes (**Figure 4**). Robust NK cell expansion with the Xuri Cell Expansion System W25 (Cytiva) has been demonstrated by several groups (90–92). The most common approach is to expand the isolated NK cells in static culture before transferring them to rocking bioreactors, which effectively nourish high cell densities (90–92). These workflows were able to generate 50 billion highly cytotoxic NK cells (91). Stirred tanks are another type of dynamic culture bioreactor that has gained favor in the NK cell therapy community. Pierson and colleagues first demonstrated that the cultivation of NK cells in a 750ml-stirred tank significantly outperforms that in a comparable static vessel (93). Moreover, it was recently shown that NK cell propagation in 2L stirred tanks scaled up exceptionally well to 50L stir tanks (94) making this platform an excellent fit for allogeneic manufacturing workflows. Aside from the well-known wave motion reactors and stirred tank reactors, there has also been success using lesser-characterized reactors, such as the ZRP Bioreactor 50M, which was able to grow massive amounts of highly pure and functional NK cells (95).

NK CELL THERAPY PACKAGING AND RELEASE TESTING

Once the desired expansion is achieved, a major challenge is the downstream processing of these cells and preparing the allogeneic doses. Manufacturing and storing these "off-the-shelf" doses remotely, requires cryopreservation, which is often problematic in the case of NK cells. In addition to a loss in cell viability, it is common to see a significant drop in cytotoxicity after thawing. This functional loss routinely corresponds to a reduction in the expression of CD16 on NK cell surfaces (63). However, many groups are attempting to mitigate these issues with different strategies. A few more promising examples are to expose thawed cells to IL-2 immediately, thereby restoring their cytotoxic capacity (60), using twice as many cells in the dose to compensate for the reduced function per cell (96), and inoculating the NK cells immediately after thawing them (37, 96). A separate, but related concern, is a 6-fold decrease in motile NK cells following cryopreservation (37). Efforts to develop effective cryopreservation solutions that preserve NK cell numbers and functionality are currently a priority to carry this field forward.

Beyond viability and cytotoxicity issues following the cryopreservation and recovery cycle, there is a multitude of other criteria that should be considered before confidently releasing the NK cells for administration as a therapeutic dose. Safety is the overarching theme for most of these considerations. Several of these requirements are focused on confirming that there are no undesirable trespassers in the dose, such as endotoxins, mycoplasma, bacteria, or feeder cells if they were used for expansion. Confirmation that the dose consists of the desired cellular population is also highly important in preventing the onset of adverse effects that these cellular contaminants can cause. This can be done by setting a minimum requirement for the percent of $CD56^{pos}/CD3^{neg}$ cells and a maximum allowed

FIGURE 4 | Bioreactors offer several advantages to the clinical manufacturing of cell therapies. The shift from static vessels on the left toward dynamic bioreactors on the right allows for several process improvements, such as scalability, automated and closed operation, digital integration, and intimate control of liquids. These capabilities result in increased safety and consistency, reduced labor requirement and cost, and improved quality of cellular output.

amount of $CD3^{pos}$ T cells, $CD19^{pos}$ B cells, and $CD14^{pos}$ monocytes that can safely be released in a dose. These are the key regulatory principles that agencies across different geographical locations will require for cell therapies. Several additional ideas could be incorporated to further ensure therapeutic efficacy. An example of this could be flow cytometric characterization of activating receptors, such as NCRs, NKG2D, NKG2C, NKG2E, 2B4 and the inhibitory receptors NKG2A and KIRs. In addition, indicators of cytotoxic capabilities, CD16 and CD25, markers of differentiation status, CD62L, CD45, HLA-DR, CD69, and CD57, and functional analysis of IFNγ or TGF-β can be included (20) (**Figure 5**). It may also be beneficial to modify the cellular requirement based on the characteristics of the disease state. The tumors and surrounding microenvironments pose significant obstacles that are directly opposed to the proper function of adoptive cell therapies, such as NK cell therapies (97). While many of the escape mechanisms are identified, there is often no way to identify which ones a particular tumor is employing. Thus, understanding the individual challenges associated with each tumor through a standardized molecular imprint could go a long way in cultivating the most effective cell therapy or combination therapy.

CAR-NK ENGINEERING

When NK cells are engineered with a tumor-specific chimeric antigen receptor (CAR), superior NK cell elicited cytotoxicity and improved cell infiltration into the tumor microenvironment are noticed. Genetic modification of NK cells by transducing with CAR receptors directed against tumor specific antigens may enhance both NK cell tumor specificity and NK cell persistence. CARs are engineered receptor proteins that recognize a target antigen on tumor cells and are successfully used in T cell therapy for lymphoid leukemias. Most of the CAR-T trials are restricted to autologous therapies, which are cumbersome, although strikingly efficient in targeted tumor cell killing (98). The development of allogeneic CAR-T cells is challenging, as these treatments must be specifically tailored to avoid graft *versus* host diseases (GVHD) and elimination by the host immune system (99). In contrast, several advantages are recognized with CAR-NK cell therapy over CAR-T cell therapy clinical approaches. First, there are less side effects such as low/no GVHD (100), cytokine release syndrome (101) and neurotoxicity (102). Second, CAR-NK cells can also eliminate tumor cells efficiently in a CAR-independent manner through their stimulatory and inhibitory receptors and CD16-mediated ADCC (103). Therefore, several researchers are exploring different approaches to genetically engineer NK cells with CARs to augment the efficiency of NK cells to kill tumors (104).

NK cells are successfully engineered to express CARs against several tumor-specific antigen targets and are shown to be efficient for *in vitro* and *in vivo* killing of tumor cells in experimental investigational studies. Human iPSC-derived NK cells engineered with specific CAR constructs demonstrated significantly enhanced targeted anti-tumor activity in an ovarian cancer xenograft model (105). Although autologous NK cells can be generated *in vitro*, they have limited efficiency against own patient's tumor cells. There are currently 72 clinical trials using CAR-NK cell lines and 35 primary CAR-NK preclinical studies based on PubMed and Global data (www.carnkreview.com) targeting different tumors (106). However, only 5 studies are ongoing in phase I & II clinical trials at www.clincaltrials.gov (**Table 3**). CAR constructs for NK cells consist of three domains: an extracellular antigen recognition domain, a transmembrane domain, and an intracellular cytoplasmic signaling domain (**Figure 6**). The ectodomain contains a single-chain variable fragment (scFv) derived from an antibody recognizing the tumor antigen. The transmembrane domain anchors the CAR structure to the effector cell membrane. CAR recognition of specific antigen triggers intracellular activation domain that results in the killing of the target cells. From the limited number of CAR-NK trials so far, no significant adverse events are noted, and the CAR-NKs showed robust

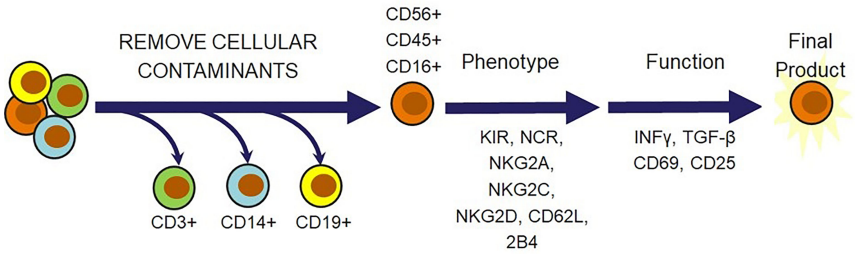

FIGURE 5 | Natural killer cell-specific strategies for NK cell therapy release criteria. In addition to verifying that cell therapies are free from endotoxins, mycoplasma, bacteria, and feeder cells, there is a multitude of cell markers that can be selected to ensure that the therapeutic population possesses desired phenotypic and functional qualities. T cells, monocytes, and B cells must be removed for safety. Receptors and cytokines can then be evaluated to confirm that the outgoing cell population is responsive, cytotoxic, and safe.

cytolytic activity. In the CAR-NK trials that fit the allogeneic and off-the-shelf approach, CAR-NK cells from a single healthy donor were expanded in cell culture for appropriate dosing. The infused CAR-NKs persisted and expanded at a low level, based on PCR results, for a year within the tumor microenvironment in an ablative conditioning regimen. Most engineered CAR-NK cells are directed against blood-related malignancies, such as CD19 for B cell lymphomas, CD22 for refractory B-cell lymphomas and solid tumors, NKG2D-ligand for pancreatic cancers, and CD33 and ROBO1 specific BiCAR-NK/T for malignant and metastatic solid tumors (107). Barriers to a successful implementation of CAR-NK in solid tumors are recently reviewed, including off-tumor effects, impaired antigen recognition, poor cell trafficking, harsh tumor environment, and immune evasion (108).

DELIVERY SYSTEMS TO ENGINEER NK CELLS

A critical aspect of CAR-NK generation is the introduction of genetic elements into NK cells, referred to as CAR-NK engineering. Once the genetic element is introduced into NK cells, the subsequent expansion of the CAR-NK cells with the cytotoxic killing of the target tumor cell will be another important consideration. The introduction of genetic material into NK cells is carried out using either viral vectors (retrovirus, lentivirus, and Adeno associated virus) or non-viral methods (mRNA and DNA). Examples of the viral and non-viral vector delivery systems with select pros and cons are shown in **Table 4** and **Figure 7**.

Retroviral Vector Systems

Recent studies from the Rezvani laboratory at MDACC used retroviral vectors to deliver anti-CD19 CAR into NK cells along with IL-15 and inducible caspase 9. The CAR NK cells were used to treat CD19 positive tumors; 7 of 11 patients (64%) had a complete response (4 of 5 patients with CLL and 4 of 6 with non-Hodgkin's lymphoma). All 8 patients had an objective response (73%) at 13.8 month follow-up (109). Gene transfer did not change the function or phenotype of NK cells, nor did it change the proliferative or cytotoxic ability post engineering. Another recent study used retroviral vector systems that ectopically expressed iC9/CAR.19/IL15 to generate CAR-CD19-NK cells from cord blood that persisted for a long time in the tumor microenvironment (110, 111). In both studies, the retroviral vector ectopically produced IL15 that is crucial for NK cell survival and conditionally expressed a caspase 9 (iC9)- an inducible suicide gene that could be activated to shut off the system by eliminating transduced cells when needed. Though retroviral vector systems have high transfection efficiency, the cDNA can integrate into the NK cell genome causing insertional mutagenesis and sometimes an induction of immune response (112).

Lentiviral Vector Systems

Lentiviral transduction is the preferred choice to modify NK cells. The lentiviral method allows transduction of primary and non-activated NK cells, and unlike the retroviral vector system, does not require dividing cells (113). Single lentiviral transduction usually results in lower transduction efficiencies, PB NK (<10%) or CB NK (<30%) (114). In Japan, a study led by Dr. Ueda used a lentiviral system to express CAR-NK-GPC3 for solid tumors of hepatocellular carcinoma (HCC) and ovarian cancers that are treated in *in vivo* animal models with good success (115). Recent studies have improved the efficiencies of lentiviral delivery, by using statins to upregulate the low-density lipoprotein (LDL) receptor on NK cells enhancing the transduction efficiency by 30-50% (116).

Pseudotyped lentiviral particles are glycoproteins derived from other enveloped viruses that enable the tropism of the lentiviral. The ability to generate CAR-NK cells depends on the envelope protein lentivirals express. Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotype particles showed the highest transduction efficiency of primary NK cells compared to retroviral vectors (117). Feline endogenous retrovirus envelope protein RD114-TR was similar to VSV-G pseudotype particles for primary human NK cells (118). Further, a Baboon envelope pseudotyped lentiviral vector BaEV-LV was significantly better than both the RD-114-TR and VSV-G pseudotyped lentiviral vector (119). Choosing which LV pseudotypes VSV-G *versus* RD114-TR *versus* BaEV-LV has the best transduction efficiency

TABLE 3 | On-going CAR-NK Clinical Trials.

	NCT Number	Title	Status	Conditions	Source of NK Cells	Interventions	Clinical trial phase	Population	Sponsor/ Collaborators	Locations
1	NCT04324996	A Phase I/II Study of Universal Off-the-shelf NKG2D-ACE2 CAR-NK Cells for Therapy of COVID-19	Recruiting	• COVID-19	Cord blood :NKG2D CAR- NK cells,ACE2 CAR-NK cells, NKG2D-ACE2 CAR-NK cells	• Biological: NK cells, IL15-NK cells,NKG2D CAR- NK cells,ACE2 CAR-NK cells,NKG2D-ACE2 CAR-NK cells	Study Type: Interventional Phase: • Phase 1 • Phase 2	Enrollment: 90 Age: 18 Years and older (Adult, Older Adult) Sex: ll	• Chongqing Public Health Medical Center • Chongqing Sidemu Biotech • Zhejiang Qixin Biotech	• Chongqing Public Health Medical Center, Chongqing, China
2	NCT03940833	Clinical Research of Adoptive BCMA CAR-NK Cells on Relapse/Refractory MM Study Documents:	Recruiting	• Multiple Myeloma	Engineered NK-92 Cells	• Biological: BCMA CAR-NK 92 cells	Study Type: Interventional Phase: • Phase 1 • Phase 2	Enrollment: 20 Age: 18 Years to 80 Years (Adult, Older Adult) Sex: All	• Asclepius Technology Company Group (Suzhou) Co., Ltd.	• Department of Hematology, Wuxi People's Hospital, Nanjing Medical University, Wuxi, Jiangsu, China
3	NCT03940820	Clinical Research of ROBO1 Specific CAR-NK Cells on Patients With Solid Tumors Study Documents:	Recruiting	• Solid Tumor	ROBO1 Specific CAR-NK Cells	• Biological: ROBO1 CAR-NK cells	Study Type: Interventional Phase: • Phase 1 • Phase 2	Enrollment: 20 Age: 18 Years to 75 Years (Adult, Older Adult) Sex: All	• Asclepius Technology Company Group (Suzhou) Co., Ltd.	• Radiation Therapy Department, Suzhou Cancer Center, Suzhou Hospital Affiliated to Nanjing Medical University, Suzhou, Jiangsu, China
4	NCT04887012	Clinical Study of HLA Haploidentical CAR-NK Cells Targeting CD19 in the Treatment of Refractory/ Relapsed B-cell NHL Study Documents:	Recruiting	• B-cell Non Hodgkin Lymphoma	HLA haploidentical CAR-NK cells targeting CD19	• Biological: anti- CD19 CAR-NK	Study Type: Interventional Phase: Phase 1	Enrollment: 25 Age: 18 Years to 75 Years (Adult, Older Adult) Sex: All	• Second Affiliated Hospital, School of Medicine, Zhejiang University	• 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China
5	NCT05020678	NKX019, Intravenous Allogeneic Chimeric Antigen Receptor Natural Killer Cells (CAR NK), in Adults With B-cell Cancers Study Documents:	Recruiting	• Lymphoma, Non- Hodgkin • B-cell Acute Lymphoblastic Leukemia • Large B-cell Lymphoma • Mantle Cell Lymphoma	allogeneic CAR NK cells targeting CD19	• Biological: NKX019	Study Type: Interventional Phase: Phase 1	Enrollment: 60 Age: 18 Years and older (Adult, Older Adult) Sex: All	• Nkarta Inc.	• Colorado Blood Cancer Institute, Denver, Colorado, United States • Peter MacCallum Cancer Center, Melbourne, Victoria, Australia

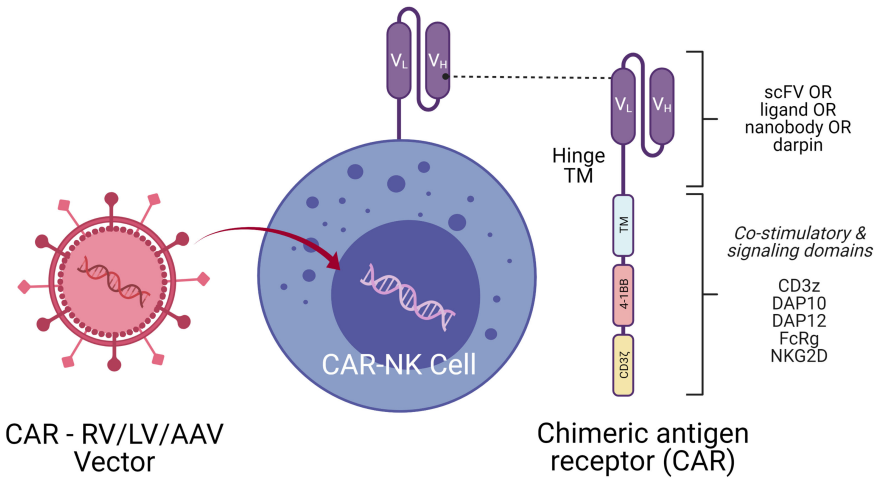

FIGURE 6 | CAR-NK Molecule Delivery of genetic cargo into NK cells with CAR encoding retro (RV), lenti (LV), or adeno-associated (AAV) vectors. CAR molecule is shown on the right side with single-chain variable fragment (scFv including V_H and V_L chains), hinge, transmembrane (TM), and signaling domain. Co-stimulatory signaling domains are indicated in different colors.

should be an essential consideration for CAR-NK generation. The advantages and disadvantages of different LV pseudotypes have been recently reviewed (120, 121).

Adeno Associated Viral Delivery

Alternative viral vectors with a better safety profile are adenovirus-associated virus (AAV) vectors. One way to improve the efficiency of NK cell cytotoxicity is by blocking their inhibitory receptors. Using CRISPR/cas9 driven delivery by recombinant adeno-associated virus serotype6 (rAAV6), highly efficient knockout of A Disintegrin and Metalloproteinase-17 (ADAM17) and programmed cell death 1 (PDCD1) genes in NK cells was accomplished (121). KO of ADAM17 and PDCD1 improved NK activity, cytokine production and cancer cell cytotoxicity. These approaches demonstrate an easy-to-use strategy for efficient gene editing and delivery with AAV vector systems for NK cell therapies (121). However, one limitation with AAV is its packaging capacity (~5kb) that limits a large gene transfer. NK cells in general have a low propensity for viral transduction, and higher cell death. Hence, commercially available viral transduction enhancers such as LentiBOOST, PGE2, PS, Vectofusin-1, ViraDuctin, Retronectin, Stauro and 7-hydroxy stauro are sometimes employed to improve vector transduction.

TABLE 4 | Advantages and disadvantages with different gene delivery vectors.

Vector	Advantages	Drawbacks
Viral Vectors		
Adenovirus	• Deliver large dsDNA (~8kb)	• Transient expression • Elicit immune response
Adeno-associated virus	• Deliver to dividing and nondividing cells • ssDNA (~4kb)	• Difficulty producing vectors • Limited transgene • Elicit immune response
Retrovirus	• Deliver to dividing cells • Sustained vector expression • ssRNA (~8kb)	• cannot transfect non-dividing cells • Low transfection rate *in vivo* • Elicit immune response • Risk of insertion
Lentivirus	• Deliver to non-dividing cells • Genome integration into host • ssRNA (~8kb)	• Possibility for insertional mutagenesis
Non-Viral Vectors		
	• Less/No insertional mutagenesis • Low/No immunogenicity • Can scale-up • Can be chemically modified • Relatively less expensive	• Less effective • Transient expression

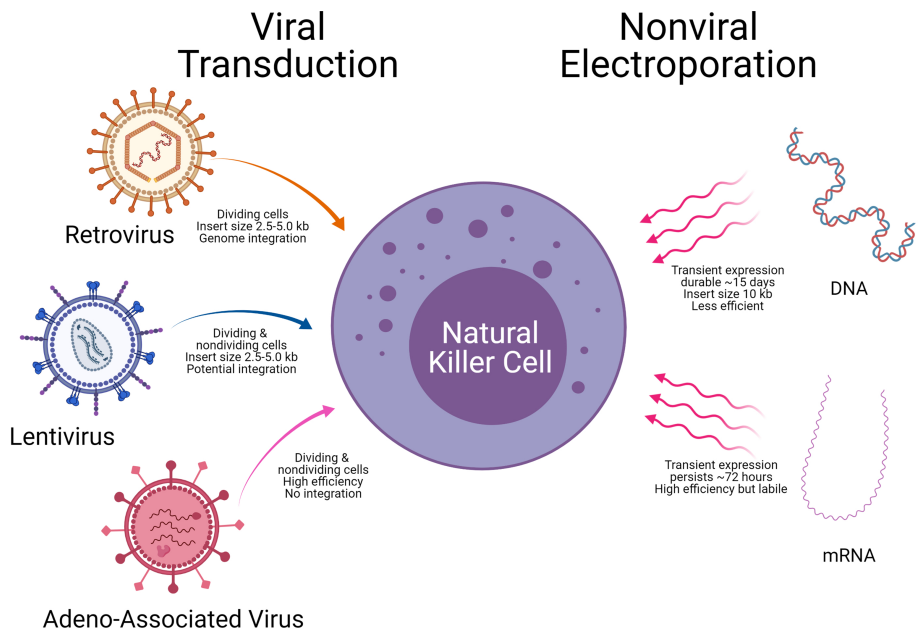

FIGURE 7 | Delivery technologies to engineer Natural killer cells. Modes of genetic cargo delivery into NK Cells by viral transduction and non-viral electroporation for gene engineering of NK Cells. Specific advantages and limitations are noted below the arrows.

Non-Viral DNA Transfection and mRNA-Electroporation

Successful electroporation of DNA into the NK-92 cancer cell line was shown, but not in primary NK cells from PBMCs or cord blood (33). Recently, an improved method with NK cells expanded with IL-2 was reported with 40% efficiency of DNA plasmid transfer (122). Following DNA transfer by electroporation, the viability of NK cells was lower, due to harsh electroporation conditions, and the DNA transfection efficiency was less compared to resting NK cells. The real advantage of this approach is complex constructs can also be transferred efficiently into the cells. Plasmid DNA of small (~3.5Kb) and large sizes (~12.5Kb) are transferred with a substantial increase of transfection up to 5-fold compared to the standard electroporation approach (123).

Some researchers are exploring electroporation to express CAR molecules on NK cells (124, 125). Unlike DNA electroporation, mRNA electroporation of NK cells may be an efficient alternative, but it induces only transient expression of the transferred gene. mRNA electroporation efficiencies are usually high (80-90%) for PBMCs or cord blood cells and require cytokine stimulation such as IL-2 for post-transduction expansion or the use of feeder cells that are engineered to secrete IL2 for better viability of cells (126). Transfection efficiency with mRNA by electroporation depends on the dose of mRNA (25-200 ug/ml) (127). High dose of mRNA results in poor viability of cells following transfection. In general, post electroporation expansion is contraindicated with mRNA approaches as it leads to dilution of the mRNA.

Recently another charge-based chemical method has been tried successfully to deliver CAR mRNA into non-dividing NK cells using a nucleofection approach that showed high efficiency (128). A specific advantage of using mRNA delivery system is the transient expression of protein by mRNA, thus avoids the risk of genome integration, least expensive to manufacture and savings of time (129). Another strategy that has been less frequently used for stable non-viral gene delivery is employing DNA transposons to transduce NK cells which is cost effective, has large cargo (ex: CAR in combination with activating receptors or cytokines) deliver capacity with stable integration. Their disadvantages include potential insertional mutagenesis and the transposon must be delivered as DNA (130, 131). Despite the limitations described above, the most successful non-viral gene delivery for primary NK cells is still rapid transient expression by electroporation.

Engineering NK Cell Receptors

For CAR-engineered cells to act, identifying specific tumor antigens as targets is a challenge, whether for T cells or NK cells. Human NK cells have innate inhibitory receptors such as KIRs and NKG2A molecules that recognize MHC class I and evoke response through immunoreceptors tyrosine-based inhibitory motif (ITIM). T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is an inhibitory receptor expressed on T and NK cells and is a promising emerging target for cancer immunotherapy. TIGIT interacts with ligands CD115 and CD112 expressed on tumor cells. There is evidence that TIGIT blockade can help tumor regression (132).

In contrast, activating receptors on NK cells such as NKG2D and DNAX accessory molecule 1 (DNAM-1) play a crucial role in tumor surveillance since this receptor has a wide range of ligands that provide target specificity (133). Preclinical study data using CAR-NKG2D is promising in colorectal cancer patients (124) and multiple myeloma patients (134). Natural cytotoxicity receptors (NCRs) like NKp30, NKp44, and NKp46, can recognize multiple stress ligands in infection and oncogenic transformation. Harnessing these receptors on NK cells and their ligands on tumor cells is another new strategy to create CAR-NK cells that can induce anti-tumor immunity.

With the advances made in viral and non-viral gene delivery approaches to generate better CAR-NK molecules, there will be a heightened focus on how these cells perform in clinical trials over the next few years. These results will help determine whether CAR-NKs can effectively target and kill tumor cells (135). The viability of CAR-NK cells in the tumor microenvironment is central to the success of therapy, in addition to the repeated dosing of the cells. CAR engineering of NK cells primarily resides between two choices of stable high expression by viral vectors or rapid transient expression of non-viral delivery systems using electroporation.

CONCLUSIONS AND PERSPECTIVES

NK cells are critical in immune surveillance of invading viruses and kill tumor cells without the need of tumor specific antigen presentation. Pre-clinical data from early phase clinical trials has significantly increased our knowledge for the use of allogeneic donor NK cells across a wide range of hematological malignancies and solid tumors. Recent advances include developing NK cell expansion protocols without the use of feeders, serum, activation technologies, validation of NK cells from different tissue sources, ability to selectively use donor NK cells with minimal HLA matching, genetic modification to create CAR-NK constructs, and transfer of genetic material using viral and nonviral delivery technologies. These advances point towards a true "off-the-shelf" NK cell therapy. Despite impressive advances, there are multiple outstanding challenges with NK cell therapies. Methods following good manufacturing practices to generate large clinical doses from a single healthy donor and selective expansion appropriate NK cell subsets with best predictive KIR/HLA are needed. Additionally, tumor immune evasion remains a large barrier. Once the NK or CAR-NK cells are infused into the patient, the long-term persistence of these cells *in-vivo* in the tumor microenvironment needs to be ensured and monitored. Another limitation with NK and CAR-NK cells is the memory property *in vivo*, which is not fully understood as in the case of memory T cells in adaptive immunity. Identifying novel CAR targets and generation of NK specific CAR constructs will enable CAR-NK cell homing and persistence in solid tumors contributing to breakthrough approaches driving allogeneic NK therapies towards the next frontier of cancer immunotherapy.

AUTHOR CONTRIBUTIONS

EH, EZ, TS, AH, NK and MCV wrote the manuscript and, SB prepared figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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CAR-NK Cells: From Natural Basis to Design for Kill

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Chimeric antigen receptors (CARs) are fusion proteins with an extracellular antigen recognition domain and numerous intracellular signaling domains that have been genetically modified. CAR-engineered T lymphocyte-based therapies have shown great success against blood cancers; however, potential fatal toxicity, such as in cytokine release syndrome, and high costs are some shortcomings that limit the clinical application of CAR-engineered T lymphocytes and remain to overcome. Natural killer (NK) cells are the focal point of current immunological research owing to their receptors that prove to be promising immunotherapeutic candidates for treating cancer. However, to date, manipulation of NK cells to treat malignancies has been moderately successful. Recent progress in the biology of NK cell receptors has greatly transformed our understanding of how NK cells recognize and kill tumor and infected cells. CAR-NK cells may serve as an alternative candidate for retargeting cancer because of their unique recognition mechanisms, powerful cytotoxic effects especially on cancer cells in both CAR-dependent and CAR-independent manners and clinical safety. Moreover, NK cells can serve as an 'off-the-shelf product' because NK cells from allogeneic sources can also be used in immunotherapies owing to their reduced risk of alloreactivity. Although ongoing fundamental research is in the beginning stages, this review provides an overview of recent developments implemented to design CAR constructs to stimulate NK activation and manipulate NK receptors for improving the efficiency of immunotherapy against cancer, summarizes the preclinical and clinical advances of CAR-NK cells against both hematological malignancies and solid tumors and confronts current challenges and obstacles of their applications. In addition, this review provides insights into prospective novel approaches that further enhance the efficiency of CAR-NK therapies and highlights potential questions that require to be addressed in the future.

Keywords: CAR-NK, CAR-T, clinical trials, HLA, KIR, cancer immunotherapy, receptors, tumor

INTRODUCTION

Natural killer (NK) cells are typical peripheral blood (PB) lymphocytes (5–10%) that were first identified in mice approximately 45 years ago (1). NK cell distribution varies among healthy tissues owing to the presence of unique chemokine receptors. NK cells are mainly found in the bone marrow, liver, spleen and PB; however, a few of them are also found in the lymph nodes (2). NK cells were initially described to exert cytolytic activity and directly kill tumor or virus-infected cells without any specific immunization, hence the name. Subsequently, NK cells were found to produce a large number of cytokines, especially interferon-gamma (IFN-γ), in several physiological and pathological conditions. They secrete various pro-inflammatory and immunosuppressive cytokines including tumor necrosis factor-alpha (TNF-α); interleukin-10 (IL-10) and growth factors such as granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IL-3. Similarly, several chemokines, such as CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), XCL1 (lymphotactin) and CXCL8 (IL-8), are also produced by NK cells (3). The physiological role of the growth factors produced by NK cells remains unclear. The colocalization of NK cells in the inflamed area with other hematopoietic cells such as dendritic cells (DCs) is caused by their production of chemokines (4). Moreover, T cell responses in the lymph nodes are governed by NK cells. Stimulated NK cells release granzymes and perforin to mediate the killing of targeted cells (**Figure 1**) (5). Perforin increases permeability by creating a transmembrane (perforation) channel on the target cell, allowing granzymes to enter more easily and causing osmotic lysis. Granzymes at the cleavage site help in the lysis and killing of target cells (**Figure 1**). NK cells affect DCs indirectly, probably by secreting IFN-γ. The interaction of naïve T cells and NK cells entering the secondary lymphoid compartments from the site of inflammation plays a significant role in mediating T cell responses (6). As the first line of defense, NK cells prevent pathogen invasion and tumorigenesis. After viral infection, NK cells are activated quickly, without prior sensitization, to avoid anomalous cells and infection (7). Compared with autologous NK cells, allogeneic NK cells are usually more potent and highly toxic against tumors (8). NK cells are categorized into subpopulations based on their functional attributes and maturation levels. Advancements in the profound understanding of tumor immunology have made NK cell biology, especially its clinical applications, an interesting focus area for research in recent years. A few features that make NK cells unique for their use in future immunotherapies are described below.

Receptors and Their Mechanisms for Regulation of NK Cells

Several cytoplasmic membrane receptors, including activating, inhibitory, cytokine and chemokine receptors, are expressed on NK cells (**Figure 2**) (5). The classical and non-classical major histocompatibility complex (MHC) class I molecules present on normal cells are recognized by inhibitory or activating receptors expressed on NK cells. A balance between the signaling of inhibitory and activating receptors regulates the activation and role of NK cells (9).

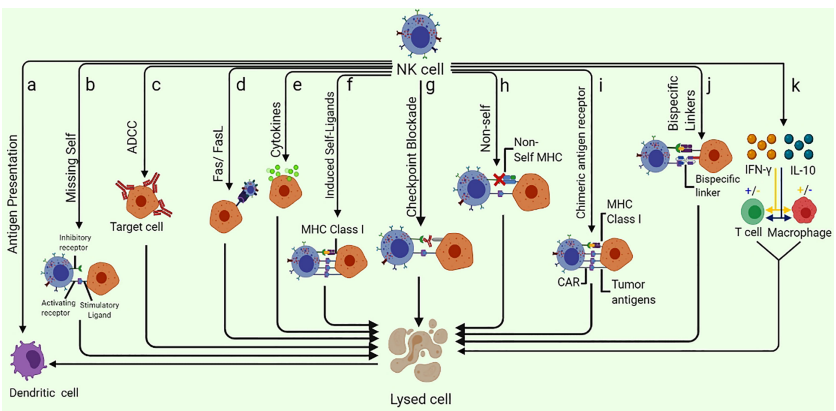

FIGURE 1 | Different ways of NK-cell mediated tumor killing and immune system modulation: **(A)** NK cells are capable of enhancing the antigen presentation to T cells by killing the immature DC while promoting the IFN-γ and TNF-α mediated maturation of DC. **(B)** NK cells can specifically recognize the cells that lack the expression of self-MHC class I molecules (Missing-self). **(C)** ADCC can kill the target cell. **(D)** Fas/FasL pathway is a very effective NK cell-mediated cell killing as the binding of FasL to Fas results in delivering a "death signal" to the target cell that undergoes apoptosis shortly. **(E)** Cytokine pathway can exert anti-tumor potential as Cytokines such as NK cells secret several cytokines such as TNF-α. **(F)** NK cell receptors NKG2D are capable of recognizing the "induced-self" ligands that are express at a very high rate in response to the activation of tumor-associated pathways. **(G)** Checkpoint blockade may inhibit NK cell suppression by preventing the interaction of NK cell inhibitory receptors with their ligands. **(H)** As a result of adoptive NK cells transfer, the mismatch between donor and recipient, inhibitory KIRs, NK cells eliminate the allogeneic tumor cells that lack self-MHC. **(I)** CAR-NK cells designed specifically to target overexpressed tumor antigens are also useful in eliminating the specific tumor cells. **(J)** Specifically designed bispecific molecules are also being utilized to specifically eliminate tumor cells as these special molecules bind to activating NK cell receptors on one arm and tumor antigens on the other. **(K)** NK cells can enhance or diminish macrophage and T cell activities *via* IFN-γ and IL-10 production.

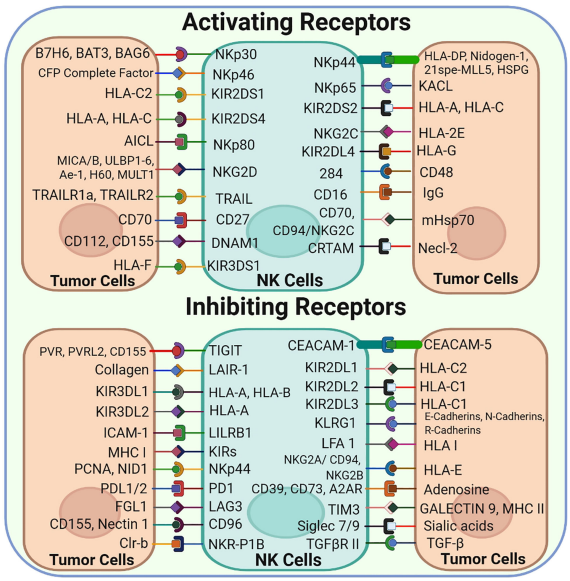

FIGURE 2 | NK-Cell Surface Receptors and their corresponding ligands: NK cells express a vast array of both Activating and inhibitory cell surface receptors that interact with their corresponding ligands found on the tumor cell surface.

MHC molecules or other inhibitory receptors that recognize the corresponding ligands are downregulated in tumor cells; however, NK cells are activated and subsequently kill tumor cells *via* the so-called 'missing-self' mechanism (**Figure 1**) (10). Normally, MHC molecules present on the surface of healthy cells act as ligands for inhibitory receptors and help in establishing the self-tolerance of NK cells. However, cells may lose these molecules owing to tumor development, resulting in reduced inhibitory signals to NK cells. Another major mechanism based on receptor–ligand interaction that triggers NK cell activation is called 'induced-self' mechanism. Several activating receptors, including NKG2D and activating killer immunoglobulin-like receptors (KIRs), are capable of recognizing their corresponding interactive 'induced-self' ligands that either lack or express merely on healthy cells but highly express on cancer cells in response to the activation of tumor-associated pathways (11, 12). Consequently, these 'missing-self' and 'induced-self' changes are characterized by significant cellular stress in the form of cellular ageing, DNA damage response and tumor suppressor genes that stimulate the robust expression of ligands for activating receptors. NK cells are activated under the influence of these activating receptors and eliminate target cells either directly *via* NK cell-mediated cytotoxicity or indirectly *via* pro-inflammatory cytokine-mediated killing (13).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is another way to target tumor cells. NK cells are characterized by the abundance of CD16 (FcγRIIIA) that serves as a receptor for IgG1 and IgG3 and is indispensable for NK cell-mediated ADCC. As a prototype NK cell-activating receptor, CD16 can trigger the cytotoxicity and secretion of cytokines and chemokines, thereby imparting antitumor activity to NK cells (14–16). A few circulating monocytes and macrophages also express CD16 (17), which is comprised of two extracellular Ig domains, a cytoplasmic tail and a transmembrane domain. The transmembrane domain helps CD16 to bind with the CD3ζ and FcεRIγ chains, resulting in the formation of immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits that associate these subunits with the intracellular signal transduction pathways to regulate the activation of numerous transcription factors and reorganization of cytoskeletal elements (13, 15). In NK cells, such pathways mediate ADCC characterized by target-oriented secretion of cytotoxic granules (perforin and granzymes) and Fas ligands and the involvement of TNF-related apoptosis-inducing ligand (TRAIL) death receptors (18, 19). Furthermore, CD16 engagement supports the survival and proliferation of NK cells (20, 21) and stimulates cytokine and chemokine secretion, leading to the recruitment and activation of tumor-infiltrating immune cells (22, 23).

A major pathway involved in NK cell-mediated cytotoxicity is known as the Fas/FasL pathway (24). Fas (Apo-1 or CD95) and Fas ligands (FasL or CD95L) are type-I and type-II transmembrane proteins, respectively, and belong to the TNF family. When FasL binds to Fas, it sends a 'death signal' to the target cell, causing it to undergo apoptosis. The secretion of several cytokines (e.g. TNF-α) from NK cells is referred to as the cytokine pathway (5), which kills target/tumor cells.

The modulation of macrophages and T cell responses can also kill target cells. NK cells can enhance or diminish macrophage and T cell activities *via* IFN-γ and IL-10 production (25). These cytokines lead to the release of hydrolases from the lysosomes of target cells by altering the phospholipid metabolism of the cell membrane. This altered metabolism activates endonucleases that degrade the genomic DNA of target cells (26). NK cells mediate immune responses *via* cross-talk between NK cells and DCs. NK cells can enhance antigen presentation to T cells by killing immature DCs and promoting maturation of DCs mediated by IFN-γ and TNF-α (25). Similarly, another potential mechanism to kill target cells is checkpoint blockade that manifests by preventing the interaction of inhibitory receptors with their respective ligands. Consequently, a mismatch between the KIRs and MHC class I molecules of donor cells and those of recipient NK cells may initiate the elimination of target cells. In addition, genetically expressed activating CARs on NK cells can specifically bind to tumor antigens. Interestingly, bispecific molecules are also being exploited to bind with the activating receptors on NK cells and tumor antigens simultaneously to stimulate NK cell-mediated tumor lysis. Usually, various signals against inhibitory receptors, instead of activating receptors, maintain homeostasis in the host (27). Inhibitory receptors act as checkpoints, such as those in T cells, to control NK cell activation (28). Overexpression of PD-1, TIM-3, inhibitory KIRs, NKG2A and T cell immunoreceptors with Ig and immunoreceptor tyrosine-based inhibition motif domains (TIGIT) on NK cell surface has been reported in the tumor microenvironment (TME) and viral infections (29). The

interaction of inhibitory receptors with the respective ligands on DCs, regulatory T cells (Tregs), tumor cells and infected cells generates signals that regulate the activation, effector function and even the subsequent functional exhaustion of NK cells (30). NK cells can, therefore, be activated *via* checkpoint receptor inhibition. For instance, anti-TIGIT and anti-NKG2A mAbs have shown a superior anticancer activity because they can restore the antitumor activities of both NK and T cells. Recently, a combination of cetuximab (anti-EGFR) and monalizumab (anti-NKG2A) demonstrated an objective response rate of 31% against head and neck squamous cell carcinoma (31).

Identification of HLA class I-specific inhibitory receptors, especially KIRs, reveals the mechanisms underlying the killing of tumor cells by NK cells. The next section provides a quick overview of KIRs.

Killer Immunoglobulin-Like Receptors

KIRs specifically expressed on human NK cells, which are encoded by LRC and found on human chromosome 19. They can bind to MHC class I molecules present on target cells. Both inhibitory and activating receptors are found in the KIR family. Inhibitory KIRs use the immunoreceptor tyrosine-based inhibition motifs (ITIMs) located in the extended cytoplasmic tails to transmit inhibitory signals. However, activating KIRs possess short tails and use DAP12 or FcγR as an adaptor molecule to transduce activating signals to NK cells (32). There are two (KIR2DL) or three (KIR3DL) extracellular Ig domains in inhibitory KIRs that equip them with their functional specificity for HLA molecules. Therefore, KIRs are highly sensitive to any change in HLA molecules during cancer and viral infections, as recently reviewed in detail (33). Normally, KIRs are expressed constitutively; however, inhibitory signals naturally predominate and suppress the activating signals and fine-tune NK cells to protect healthy cells, also known as NK cell self-tolerance. The phenomenon of self-tolerance was recognized to be regulated by the binding of some powerful inhibitory receptors (such as KIRs and NKG2A) to MHC class I molecules. Reduced expression of MHC class I molecules in malignancies (missing self) was presumed to prevent this inhibition and permit self-reactivity (34). KIR–HLA mismatch is also a significant factor that should be considered for cancer immunotherapy. Each KIR recognizes a particular HLA allotype as an inhibitory ligand; KIR2DL1, KIR2DL2/3 and KIR3DL1 specifically bind to group 2 HLA-C, group 1 HLA-C and HLA-Bw4, respectively. Therefore, a superior and more potent antitumor activity can be exhibited by recipients lacking specific HLA allotypes for inhibiting NK cells. Several previous studies have reported that KIR–HLA mismatch between a donor and a recipient results in a higher antitumor potential (35–37). Furthermore, to attain the best antitumor effects, the actual expression of KIRs should be considered because they are usually expressed in stochastic combinations (38). Interestingly, single-KIR$^+$ allogeneic NK cells that do not encounter any HLA-inhibitory signal mediate the antitumor activity (39). NK cells recover first from allogeneic hematopoietic stem cell transplantation (HSCT); however, it takes approximately 3 months for the NK receptor repertoires to reconstitute (40, 41), and single-KIR$^+$ NK cells are not completely functional until then (39). However, KIR-mismatched allogeneic NK cells might be rejected due to MHC mismatch. For instance, acute kidney transplant rejection resulting from KIR/HLA polymorphism was found in a KIR/HLA genotype study (42). Similarly, in a phase II clinical trial (NCT00703820), KIR–HLA-mismatched allogeneic NK cells were found ineffective against medium-high-risk acute myeloid leukemia (AML), possibly owing to their insufficient number and poor persistence (43).

NK cells are educated and licensed by inhibitory receptors that recognize MHC molecules to respond to MHC-I-deficient cells. The inhibitory receptors are expressed during NK cell development and ensure their functional competence. NK cells that lack these inhibitory receptors cannot undergo education and licensing and remain hyporesponsive. For instance, KIR3DL$^+$ NK cells of an individual having HLA-Bw6 are more hyporesponsive than those of an individual having HLA-Bw4 because Bw4 serves as a ligand for KIR3DL1. Therefore, the strength of inhibitory signals may influence NK cells (44, 45). Moreover, only a few NK cells that lack self-reactive receptors are anergic because NK cell 'licensing' or 'education' during maturation imparts this property (46). Inhibitory KIRs are downregulated whereas activating KIRs are upregulated in several cancer types, such as skin cancer, lymphoma, leukemia, breast cancer and biliary cancer (47). Changes in the expression patterns of these KIRs may aid tumor escape by inhibiting NK cell activation and subsequent anticancer activity.

Inhibitory KIRs were the first to be considered the checkpoint receptors in NK cells (48). Because mAbs can recognize inhibitory KIRs, they enhance the anticancer activity of NK cells by blocking their signal transduction. Therefore, mAbs are potential therapeutic candidates that are safer and have fewer side effects as compared with other therapeutic approaches, which have been employed in several clinical trials against several tumor types (49).

The outcomes of earlier studies employing autologous NK cells were disappointing owing to self-HLA molecules mediated repression (50–52). With the establishment of KIR–ligand mismatch in transplantation, there came a boom in the application of allogeneic NK cells in both HSCT and non-HSCT (53–55). Compared with autologous NK cells, allogeneic NK cells does not face a repression from self-MHC molecules. Furthermore, multiple studies have indicated that infusing haploidentical NK cells into patients with AML to investigate the missing-self mechanism is safe and can induce substantial clinical activity (53–55). Adoptive transfer of haploidentical NK cells to patients with AML was found to be safe because it did not result in graft-versus-host disease (GVHD), and IL-2 administration increased their persistence by approximately 4 weeks (54).

NK Cell Sources and Expansion

NK cells are commonly used in cell-based immunotherapies for hematological malignancies (56); however, they have also shown some hope for the treatment of solid tumors in preclinical studies (discussed in detail in later sections of the manuscript).

Previously, IL-2-stimulated autologous NK cells were used in NK cell immunotherapies; however, the binding of KIRs on NK cells to the ligands of endogenous HLAs present on cancer cells leads to suppression, limiting the clinical outcomes. This suppression can be prevented by transferring allogeneic or haploidentical NK cells having KIR–ligand mismatch owing to missing-self recognition of tumor cells (35). Consequently, allogeneic NK cell mixtures are considered safer and may help to eradicate leukemia regression and prevent GVHD, especially when HLA class I molecules are downregulated by cancer cells. Therefore, using allogeneic NK cells as specific unified sources of therapeutics may facilitate large-scale GMP-based 'drug' production, leading to overwhelming treatment effects.

Various NK cells obtained from different sources are under investigation in clinical settings (**Figure 3**) (57). Induced pluripotent stem cells (iPSCs) can be transformed into all cell types, including immune cells. Several differentiation methods are now available for producing immunological effector cells from iPSCs. Moreover, a quick, powerful and well-defined procedure for the differentiation of T and NK cells has been recently developed (58). The hematopoietic stem cell (HSC) stage is one of the most important intermediate phases in the development of iPSCs to fully differentiated immune cells. Adult HSCs produce all types of blood cells. Adult bone marrow and the umbilical cord of neonates are the two common sources of HSCs. The currently available protocols are not completely optimized to minimize HSC fatigue and differentiation; therefore, it is necessary to develop protocols to increase HSC multiplication and pluripotency for improved immunotherapeutic applications. It is encouraging that researchers are working towards developing a more targeted and efficient HSC growth medium (59). Owing to their adaptability of growth conditions, iPSCs are an alternative or complementary strategy to achieve a large number of HSCs (60, 61).

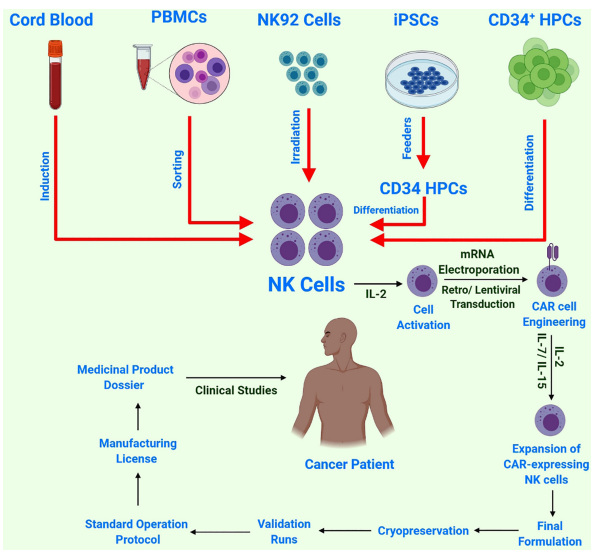

FIGURE 3 | Schematic of NK cell sources and CAR-NK cell/immune therapy workflow: NK cells harvested from multiple sources, followed by NK-cell activation by IL-2 and subsequent transduction with a construct encoding CAR. Next, these reprogrammed CAR-NK cells are expanded *ex-vivo* and passed through strict quality control testing before cryopreservation and subsequent validation and approval for final clinical administration.

Moreover, to overcome the critical problems encountered thus far, iPSCs have been used to create 'off-the-shelf' CAR-NK cells by taking advantage of iPSCs as a renewable source for NK cells, thus allowing for constant, precisely defined immunotherapy and genome editing in different anticancer modes of action. These CAR-iNK cells may be cryopreserved and supplied on demand to each patient, significantly decreasing the manufacturing costs. Moreover, NK cells from PB were found more advantageous in terms of safety and cytotoxic potential against tumors (62).

Cytokines have been reported to induce NK cells into a memory-like phenotype having a prolonged life expectancy under *in vivo* conditions (63). Memory-like NK cells undergo differentiation and result in an amplified IFN-γ yield and enhanced cytotoxicity against leukemia after brief preactivation with IL-12, IL-15 and IL-18, both *in vitro* and *in vivo*. The aforementioned memory-like NK cells have significantly depicted efficacy in AML, wherein 5 out of 9 patients showed complete remission (CR) (64). Briefly, 13 patients underwent several treatments that included memory-like NK cells at three distinct dosages, i.e. levels 1, 2 and 3. Of the 13 patients, 4 achieved CR/CR with incomplete blood count recovery (CRi) and 1 achieved morphologic leukemia-free state (MLFS), with an overall response rate of approximately 55% and a CR/CRi rate of 45%. Interestingly, IL-15-stimulated NK cells exhibited significant therapeutic efficacy against solid tumors. Of the 6 patients with refractory solid tumors, 4 exhibited a positive clinical response after receiving haploidentical stem cell transplants, 1 exhibited high partial remission, whereas 2 exhibited only partial remission (65). Unfortunately, the poor expansion of NK cells in patients with tumors makes their clinical application limited, and owing to strict evaluation of donors, those contributing allogeneic NK cells are quite difficult to find. Fortunately, the results of adoptive NK cell therapy have proved both autologous and allogeneic NK cells considerably safer and more tolerable (66).

NK cell expansion is stimulated by proliferative cytokines (IL-2 and IL-7/IL-15) (67). Similarly, the use of artificial antigen-presenting feeder cells (aAPC) in a culture system that facilitates gas permeability has been proposed as a new approach for growing NK cells from cryopreserved cord blood (CB) units. CB-NK cells expanded 1848-fold from fresh blood and 2389-fold from cryopreserved CB after 14 days, with >95% purity for NK cells ($CD56^+/CD3$) and <1% for $CD3^+$ cells (68). The co-culturing of NK cells using irradiated feeder cells in media supplemented with IL-2 and IL-15 is used to harvest a large number of NK cells, although an expansion system without the use of feeder cells is already available that used anti-CD3 antibodies (OKT-3) for NK cell expansion. RPMI8866, the Epstein–Barr lymphoblastoid cell line (EBV-LCL) and K562 are the most common feeder cell lines exploited for NK cell expansion (69). Activation by NK-sensitive K562 cells has been

found to enhance NK cell proliferation with IL-2, IL-15 and IL-21 (67). K562 are good activators of NK cells owing to the lack of HLA expression and loss of inhibitory signals. Recently, significantly high NK cell expansion rates were observed with genetically engineered (GE) feeder cells. The co-expression of IL-15 and 4-1BBL *via* membrane-bound K562 was found to act collaboratively to strengthen the activation capability of K562-specific NK cells and resulted in remarkable expansion of PB CD56+CD3−NK cells devoid of any satellite T lymphocyte build-up (70). Ojo et al. (71) recently developed an NK cell feeder cell line named 'NKF' by overexpressing membrane-bound IL-21. This cell line could induce robust and sustained proliferation (>10,000-fold expansion at 5 weeks) of highly cytotoxic NK cells. Compared with IL-2-activated non-expanded NK cells, the expanded NK cells were highly cytotoxic against several types of malignancies. Expanded NK cells were also effective in mouse models of human sarcoma and T cell leukemia (71). Copik et al. reported a unique method for efficient expansion of NK cells with the help of plasma membrane (PM) vesicles. Briefly, they cultured K562-mb15-41BBL cells and optimized the formation of PM vesicles with a higher level of 41BBL using the nitrogen cavitation method. The optimized PM vesicles (PM 15) resulted in a 293-fold expansion of NK cells after 12–13 days as compared with 173-fold expansion achieved with live feeder cells. NK cells not only expanded better after stimulation with PM-mb15 41BBL vesicles but also exhibited phenotypes, surface receptors and superior cytotoxicity comparable with those of NK cells expanded with live feeder cells (72). Furthermore, PM21 particles were prepared to employ K562-mb21-41BBL cells. Briefly, PBMCs were cultured for 28 days with PM21 particles, resulting in >90% NK cell expansion by day 14 and an exponential expansion of 100,000-fold by day 28 (73). Similarly, a method for better expansion of memory NK cells was described recently. Briefly, NK cells are preactivated using stimulatory cytokines, and these preactivated NK cells were then expanded using a vesicle having NK cell effector agents such as PM21 particles, EX21 exosomes or FC21 feeder cells (74).

Remarkably, iPSC-NK cells were reported to produce a large number of homogenous NK cells; therefore, they can be banked and stored (75). aAPC-expanded PB-NK and iPSC-NK cells exhibited a higher antitumor potential *in vivo* when compared with PB-NK cells that had undergone overnight activation (76). NK cell lines, especially NK-92, have demonstrated better antitumor efficacy and offered more advantages as an 'off-the-shelf' approach because they have reduced toxicity and lack most inhibitory KIRs, except a very mild expression of KIR2DL4 (77), as compared with other NK cell sources and have successfully entered multiple clinical trials (78). Moreover, a high dose (10^{10} cells) was found to be safer for effective results in patients with melanoma and lung cancer (78).

CHIMERIC ANTIGEN RECEPTOR

CAR is a fusion protein created intentionally and is found on T cell receptors (TCRs). It contains an extracellular antigen recognition domain and various intracellular signaling domains. Cross-reactivity of TCRs has only been observed in receptors with a supra-physiological affinity for cognate antigens. Moreover, CARs are capable of overcoming resistance observed in several malignancies because they, unlike TCRs, engage molecules independent of MHC recognition and antigen presentation by target cells. CAR constitutes an extracellular antibody-like region known as the single-chain variable fragment (scFv) which is intended to bind to a specific antigen and a hinge region of flexible lengths depending on the vicinity of the perceived epitope situated on the exterior of the target cell. It also contains a transmembrane domain along with one or more co-stimulatory domains and a signaling domain capable of persuading cytotoxicity as a consequence of antigen binding (79) (**Figure 4**). scFvs targeting a particular molecule can be derived from several sources such as murine or humanized antibodies. Furthermore, phage display libraries can be screened for the identification and synthesis of scFvs (80). The specificity of CARs is usually determined by the antibody scFv region; however, NK cell receptors have also been used for this purpose. The ligand-binding domains of scFv region are coupled with extracellular, transmembrane and signaling domains of other cell proteins using recombinant DNA technology. NK cells may identify distinct types of tumor cells through numerous cell surface receptors. The ligands recognized by NK cell receptors are present on various tumor cell types, making them promising targets for tumor-targeting therapies. The NK cell receptor NKG2D and its ligands have attracted significant attention for being involved in a possible tumor-killing approach. Various tumor cells express NKG2D ligands, exhibiting comparative selection between ligand expression on tumor and healthy cells (81). Previous studies have reported several tumor antigen-binding domains as CAR extracellular domains. In addition to the antigen-binding domain, a hinge region is located extracellularly along with the transmembrane domains of CD8 or IgG4. The intracellular signaling domain is the most important section of CARs because it decides the functionality of CAR. The most common intracellular domains are CD3, CD28, 4-1BB or OX40, which are premeditated to increase T cell activation. CARs that can directly identify CAR-targeted antigens are involved in T cell activation, proliferation, cytokine production and cytotoxicity against tumor cells that express CAR-specific antigens and are synthesized by genetically engineered T cells (82).

CAR GENERATIONS AND CAR-T THERAPY

CAR 'generations' generally denote the number and composition of the intracellular signaling domains. Currently, the fourth generation of CARs is under technical development because its use in clinical practice has not yet been approved (83). The first-generation CARs contained a domain equipped with scFv to recognize tumor antigens and an activation motif (ITAM, generally CD3ζ) (84). Unfortunately, these CARs could not

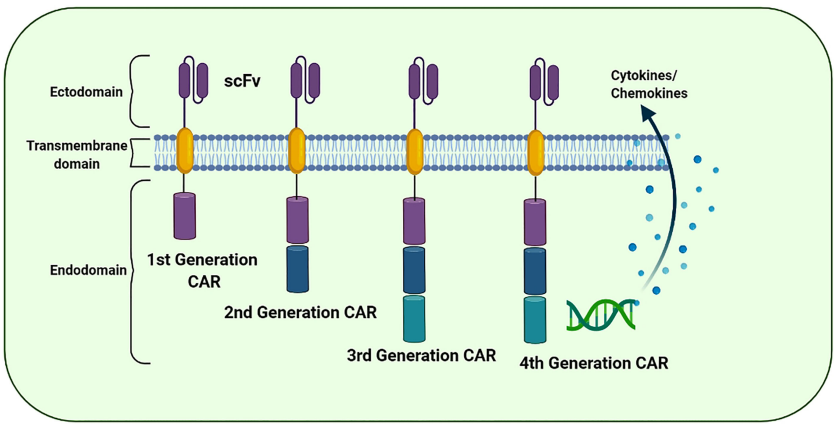

FIGURE 4 | The evolution of the chimeric antigen receptor (CAR) structure over time: The structural components of 1st, 2nd, 3rd, and 4th generation of CAR. The CAR "generations" denote the number and composition of the intracellular signaling domains. The 1st generation's CARs failed to deliver cell proliferation signals for the retention of anti-cancer potential. However, 2nd and 3rd generation CARs have CD28, CD134 (OX40), and CD137 (4-1BB) to promote the anti-tumor potential. The 4th CAR generation is designed to secrete cytokines to further improvise the therapeutic activity of the CAR-based immunotherapies.

support persisting cell proliferation indicators for resuming the anticancer activity. Furthermore, the second- and third-generation CARs were equipped with CD134 (OX40), CD28 and CD137 (4-1BB) to enhance their propagation and cytotoxic potential (85). In the subsequent (fourth) generation, CARs are designed to secrete cytokines and are usually provided with more than one co-stimulatory molecule such as CD134, CD28 or CD137 to enhance antitumor potential by stimulating the innate immune system (86). In addition, several next-generation CARs have also been developed and are in an experimental phase (**Figure 5**).

The first-ever CAR-T cell therapy (Kymriah; Novartis) was authorized in 2017 by the United States Food and Drug Administration (FDA) to treat B-cell acute lymphoblastic leukemia (ALL) (87, 88). Subsequently, another CAR-T cell therapy (Yescarta; Kite Pharma) was developed and approved to treat non-Hodgkin's lymphomas (89). Several hematological malignancies, including lymphoma, chronic lymphocytic leukemia (CLL) and ALL, are treated using CAR-modified T cell therapy, which has shown extraordinary results. Notably, feedback rates of 70–90% have been achieved in patients with ALL treated with CD19-targeting CAR-T cell therapy (90). Furthermore, the four CAR-T therapies approved by the FDA include Breyanzi, the first cell therapy product of Bristol Myers Squibb (BMS); Kymriah (tisagenlecleucel) by Novartis and Yescarta (axicabtagene ciloleucel) and Tecartus (brexucabtagene autoleucel) by Gilead/Kite. Tecartus, which was licensed in the United States and Europe in 2020, is currently only used to treat mantle cell lymphoma

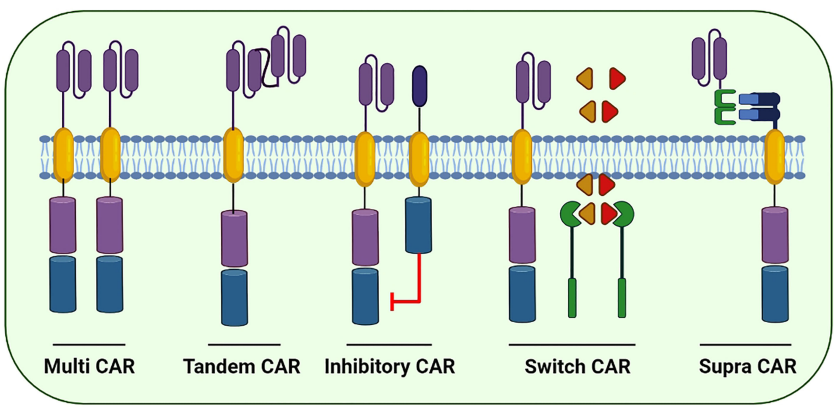

FIGURE 5 | Few of the next generation CARs to better cope with the immune escape and improve the cytotoxic potential of CAR-based immunotherapies: Multi CARs are equipped with two or more separate CARs expressing various ScFvs to target the cancer cells. Tandem CARs are equipped with two different scFvs in a single CAR molecule. Upon antigen recognition in healthy cells, Inhibitory CARs tend to inhibit immune cell activation. In switch CARs, certain chemicals capable of dimerization with the iCasp9 are conditionally administered to activates the downstream caspase molecules leading to the apoptosis of CAR-expressing cells. Supra CARs are equipped with two split structures; the antigen-binding domain (zipFV) and function domain (zipCAR) that upon binding activates the CAR-expressing cells.

(MCL), which is not among approved indications of other CAR-T therapies. The FDA has authorized Abecma (idecabtagene vicleucel; ide-cel) for treating adult patients with relapsed or refractory multiple myeloma.

The prime focus of the formulation of fourth-generation CARs is to address the prevailing challenges and constraints in its clinical applications. Next-generation CAR constructs can be further divided into subgroups including tandem, combinatorial, ON-switch, inhibitory, universal and T cells reprogrammed as global cytokine killing (TRUCK) CARs based on their function. Small molecules are required for the assembly of ON-switch CARs that promote controlled CAR activation *via* drug administration (**Figure 5**) (91). Similarly, another fragmented CAR design is of universal CARs that can target numerous cancer types by exchanging antigen-specific regions with the same TM and an intracellular signaling construct (92). Furthermore, OR-gate CARs present a novel strategy to prevent tumor escape by attaching two scFv domains to distinct targets adhered either to a single TM along with an intracellular domain (tandem CAR) or a complete dual CAR construct build on a single cell (dual CAR) (93), and T cells can be activated by signals transduced by either scFv. Similarly, AND-gate CARs also involve two scFvs; however, they require both antigens to be present on the same cell before signal propagation. This approach is unique in targeting non-tumor-specific antigens because the binary execution of two antigens, namely, combinatorial CAR and synNotch receptor, helps in achieving tumor specificity (94). Furthermore, a novel CAR design, known as TRUCK-CAR, carries a transgenic 'payload' to target solid tumors. These CARs modulate TME because they can induce cytokine transgene products such as IL-12 and mediate the release of 'payload' at the tumor site (86). In addition, an extracellular inhibitory domain is fused with an activating intracellular CAR domain to convert an immunosuppressive signal to an activating signal in inhibitory CARs (**Figure 5**) (95). Moreover, to ensure the safety of CAR-T therapy, a suicide switch has been developed that activates in case of any adverse effects; however, this safety measure is found in only 10% of the current clinical trials (96).

The development of CAR-T technology has revolutionised cancer therapy. However, currently available CAR-T technology presents significant barriers to its widespread acceptance. Several issues include high cost, patient-oriented manufacturing, inconsistency in CAR-T production and function because the immune system of patients is intrinsically weakened and potential side effects. However, NK cell treatment has the potential to address some of these problems. Multiple anticancer receptors are used by NK cells that do not induce GVHD. However, their decreased *in vivo* lifespan requires numerous doses, enhancing the chances of rejection. Currently available CAR therapies are susceptible to checkpoint blockades and other immunosuppression strategies that reduce their ability to kill *in vivo*.

NK CELLS: ALTERNATIVE HOST CELLS FOR CAR THERAPY

The success of CAR-T therapy in clinical trials has led to the development of CAR-NK cells. Extracellular, transmembrane and intracellular signaling domains are present in CAR-NK cells as they are in CAR-T cells. CAR-NK cells often have CD3 as their initial signaling domain and CD28 or CD137 (4-1BB) as a costimulatory domain to form an intracellular signaling motif. NK cells increase their cytotoxic capability and cytokine production through two more costimulatory molecules, namely, NKG2D and CD244 (2B4) (97). Owing to more enhanced tumor-specific targeting and cytotoxicity than those of CAR-T cells, CAR-modified NK cells have been used to target cancer cells (98). Notably, NK cells can serve as another candidate along with T cells in CAR-targeted immunotherapies.

CAR-NK cell therapy may serve as an alternative to CAR-T therapy in the future because CAR-NK cells possess several unique features detailed below (**Figure 6**).

First, allogeneic haploidentical NK cells are considerably safe for adoptive cell therapy (ACT) because they usually do not mediate and may diminish GVHD (99). Earlier

Properties	CAR-T	CAR-NK
Low risk of GVHD		✓
High tumor-killing potential	✓	✓✓
Low risk of Cytokine release syndrome		✓
High graft-versus-tumor (GVT) potential		✓
Low cost off-the-shelf cancer immunotherapy	✓	✓✓
Sources of harvestation	✓	✓✓

FIGURE 6 | A comparison of CAR-T and CAR-NK immunotherapy: CAR-NK cell therapies are becoming increasingly popular due to several advantageous features such as low safety concerns, low costs, and higher tumor potential.

investigations have revealed that NK cells are involved in the induction or aggravation of GVHD. Subsequently, it was observed in both patients and mouse models that NK cells regulate GVHD by suppressing alloreactive T cell responses. NK cells interact with other immune cell subsets during GVHD and suppress GVHD naturally by inhibiting T cell activation *via* their cytotoxic ability unless exogenous hyperactivation causes them to produce proinflammatory cytokines that can sustain T cell-mediated GVHD induction (100). Similarly, CAR-NK cells have considerably fewer safety concerns such as on-target/off-tumor effects, CRS and tumor lysis syndrome (101). For instance, a study that used HSCT to treat AML revealed that NK cells were the main factors in inducing the graft-versus-tumor (GVT) response (35). Moreover, NK cells only secrete a small number of IFN-γ and GM-CSF and do not produce IL-1 and IL-6 that initiate CRS. Second, tumor cells may not be detected by CAR-T cells owing to tumor escape because of a loss of either MHC class I expression or tumor-specific antigens (102). CAR-NK cells lack a self-antigen and can detect MHC class I-negative tumor cells because they retain innate cytotoxic potential against germline-encoded tumor/stress ligands (103). In addition, both HLA-A and HLA-B bind to KIR3D receptors, whereas HLA-C only binds to KIR2D receptors. CD94-NKG2A, which detects HLA-E, LILRB1 and all MHC class I molecules, is another inhibitory receptor that identifies MHC class I molecules expressed by NK cells (104). Normal MHC class I-sufficient cells are ignored by NK cells because their inhibitory receptors can detect MHC class I molecules; however, they are not inhibited after interacting with abnormal MHC class I low cells. Third, it is believed that low levels of MHC class I expression in cancer stem cells (CSCs) and the presence of NKp30, NKp44 and NKG2D (activating receptors) cause cytokine-activated NK cell-mediated death of CSCs (105). CSCs usually use two different mechanisms to escape NK cell detection: shedding the NKG2D ligands MICA and MICB in case of breast CSCs (106) and lacking NKG2D ligands in case of leukemia stem cells (107). Although NK cells can perform serial killing and have a limited life span, cytomegalovirus (CMV)-induced memory-like adaptive NK cells had a prolonged life span and an enhanced cytotoxic potential (108). For instance, after reactivation, $NKG2C^+$ NK cells from CMV naïve UCB grafts were found to expand preferentially in recipients, indicating a primary NK cell response after HSCT (109). The effects of donor/recipient CMV serostatus on the expression and activity of $NKG2C^+$ NK cells were then evaluated in donor HSCT recipients to identify responses to secondary CMV occurrences. After clinical CMV reactivation, $NKG2C^+$ NK cells increased in number. When both the donor and recipient were CMV-seropositive, the cells expanded in the absence of detectable CMV viraemia. CMV-positive recipients who received grafts from CMV-seropositive or -seronegative donors had higher levels of $NKG2C^+$ NK cells. These *in vivo*-expanded $NKG2C^+$ NK cells had a greater capacity for target cell-induced cytokine release, generated an inhibitory killer Ig-like receptor for self-HLA and acquired CD57 more quickly. Compared with seronegative donors with $NKG2C^+$ NK cells, seropositive donors with $NKG2C^+$ NK cells responded better to a subsequent CMV infection (110). Fourth, CAR-NK cells can regulate their activating receptors, including NKp30, NKp44, NKp46, NKG2D, KIR-2DS, KIR-3DS, 2B4, CD226, CD94/NKG2C and DNAM-1; therefore, the chances of relapse owing to the loss of CAR-targeting antigens is reduced. Moreover, T lymphocytes only kill their targets through a CAR-specific mechanism, whereas NK cells exhibit spontaneous cytotoxic activity and can kill target cells regardless of the presence of tumor-specific antigens. Tumor cells downregulate antigens to escape immune detection; however, NK cells are still effective against them. Furthermore, cytokines such as IFN-γ, IL-3 and GM-CSF produced by primary human NK cells are different from proinflammatory cytokines released by T cells, which induce CRS. Individual NK cells can survive after interacting with and destroying several target cells, potentially decreasing the number of cells that are adoptively transferred. Fifth, the availability of an off-the-shelf CAR-NK therapy enhances the pace of administration remarkably and first dosing to 1 day by minimizing the lag time from the decision to treat. Sixth, CAR-NK therapy is expected to decrease huge indirect costs because CAR-NK infusions can be administered with outpatient follow-up monitoring and do not require lengthy post-treatment hospitalization because they are safer and have no potential toxicity (111). Although iPSC-derived CAR-T cells can also serve as an off-the-shelf product, they require more rigorous efforts and extra genetic modifications to obtain a universal product that does not require HLA-matching and is devoid of any endogenous TCR. Therefore, iPSC-derived CAR-T cells require post-treatment hospitalization and hence cannot abolish the potential possible side effects associated with CAR-T cell therapy. In addition, NK cells can be harvested from multiple sources including iPSCs, PB, UCB, human embryonic stem cells and NK cell lines (112). Therefore, CAR-NK cells may be a reliable therapeutic candidate to mitigate these limitations and safety concerns.

Recently, a meta-analysis has reported that of the 520 active trials investigating a total of 64 different CARs worldwide, 96.4% of trials are investigating CAR-T cells (96). Therefore, the research on CAR-NK therapy is currently in its beginning stages owing to very few translations of laboratory investigations to clinical settings. Further investigation and clinical trials are required to ensure the safety profile of CAR-NK cells because they have a few side effects and a low incidence of CRS. It is quite delightful to mention that several ongoing clinical trials are investigating the safety and efficacy of CAR-NK cell therapy for both hematological and solid tumors, which are registered on ClinicalTrials.gov (**Table 1**) (113).

PRECLINICAL APPLICATIONS OF CAR-NK THERAPY

Allogeneic stem cell transplantation of NK cells has shown significant success for the treatment of AML in preclinical trials (114). However, a few limitations at the clinical level

TABLE 1 | Clinical trials of CAR-NK cell therapies against hematological malignancies and solid tumors.

Serial No.	Tumor Type	Specific Target	Source of NK-cells	Status	Phase	References
1.	Leukemia and lymphoma	CD7	NK-92	Unknown	I/II	NCT02742727
2.	Leukemia and lymphoma	CD19	NK-92	Recruiting	I/II	NCT02892695
3.	Leukemia and lymphoma	CD19	Umbilical cord blood	Recruiting	I/II	NCT03056339
4.	Leukemia and lymphoma	CD19	Umbilical cord blood	Withdrawn	I/II	NCT03579927
5.	Acute myeloid leukemia	CD33	NK-92	Unknown	I/II	NCT02944162
6.	Acute myeloid leukemia	CD19	Expanded donor NK cells	Completed	I	NCT00995137
7.	Acute myeloid leukemia	CD19	Haploidentical donor NK cells	Suspended	II	NCT01974479
8.	Relapsed & Refractory Acute myeloid leukemias	CD33	NK-92	Unknown	I/II	NCT02944162
9.	Relapsed & Refractory B Cell Lymphoma	CD19	Unknown	Not Yet Recruiting	Early phase I	NCT03690310
10.	Relapsed & Refractory B Cell Lymphoma	CD22	Unknown	Not Yet Recruiting	Early phase I	NCT03692767
11.	Relapsed & Refractory B Cell Lymphoma	CD19/CD22	Unknown	Not Yet Recruiting	Early phase I	NCT03824964
12.	Multiple Myeloma	BCMA	NK 92	Recruiting	I/II	NCT03940833
13.	Pancreatic Cancer	ROBO1	Unknown	Recruiting	I/II	NCT03941457
14.	Epithelial ovarian cancer	Mesothelin	Unknown	Not Yet Recruiting	Early phase I	NCT03692637
15.	Castration-resistant Prostate Cancer	PSMA	Unknown	Not Yet Recruiting	Early phase I	NCT03692663
16.	Non-small cell lung cancer	Unknown	CCCR-NK-92	Recruiting	I	NCT03656705
17.	Glioblastoma	HER2	NK-92	Recruiting	I	NCT03383978
18.	Solid tumors	MUC1	Unknown	Unknown	I/II	NCT02839954
19.	Solid tumors	NKG2D ligands	Autologous or allogeneic NK cells	Recruiting	I	NCT03415100
20.	Solid Tumors	ROBO1	Unknown	Recruiting	I/II	NCT03940820

need to be overcome, including the moderate activity of NK cells and tumor escape from immune surveillance (115). Therefore, CAR was proposed for reprogramming NK cells to enhance their efficacy and cytotoxicity against tumors. Currently, a large number of ongoing studies use CAR-modified NK cells and NK-92 cell lines against various types of tumors (116).

Preclinical Successes in Hematological Tumors

Recently, Romanski et al. (117) successfully improved the sensitivity and cytotoxic potential of NK cells against B-lineage malignant cells by constructing NK92-CD19-CD3ζ cells (117). Shimasaki et al. (118) successfully expressed CD19-41BB-ζ on NK cells employing electroporation technology for transfecting its mRNA (118). Subsequently, CAR-NK cell therapy was found very effective, *via* optical *in vivo* imaging, in reducing the growth of leukemia xenografts. Moreover, to improve the cytotoxicity of NK cells against rituximab-resistant Burkitt lymphoma, Chu et al. (119) successfully transfected CD20-BB-ζ mRNA in NK cells through nuclear transfection and co-cultivated them with K562-mb-IL15-41BBL for activating CAR-NK cells (119). Furthermore, CD138-CAR-NK-92 has also been reported to be very effective in improving the survival rate of mice with MM (120). Notably, the effective elimination of EBNA3C-expressing Epstein–Barr virus-positive T cells by CAR-NK92 cells indicates a remarkable cytotoxic potential of CAR-expressing NK cells (121). Similarly, Liu et al. (122) recently used CB-derived HLA-mismatched anti-CD19 CAR-NK cells to treat relapsed or refractory CD19-positive cancers. They found that CAR-NK cell administration was well tolerated and did not lead to CRS, neurotoxicity, GVHD and elevated inflammatory cytokines such as IL-6. Of the 11 patients, 8 exhibited a response to the therapy, 7 had complete remission, whereas 1 had remission of the Richter's transformation component but had persistent chronic lymphocytic leukemia (122). Recent preclinical studies using CAR-NK cells against various hematological cancers are summarized in **Table 2**.

Preclinical Success in Solid Tumors

The success of CS1-CD28/CD3ζ-NK92 cells in restricting the growth of MM, enhancing IFN-γ production and improving the survival rate (132) has encouraged oncologists to design CAR-NK immunotherapies against solid tumors. Consequently, CAR-NK-92 cells expressing EGFR-CD28-CD3ζ exhibited remarkable cytotoxicity and killing potential in glioblastoma (GBM) cells (136). Similarly, GD2-specific NK-92 cells were found to exert cytolysis and effectively eliminate neuroblastoma (137). NKG2D-DAP10-CD3ζ-expressing NK-cells were found to exhibit a remarkably strong antitumor potential in several different cancers including breast cancer, hepatocellular carcinoma (HCC), osteosarcoma and pancreatic cancer (97). Prostate stem cell antigen (PSCA)-DAP12 CAR-expressing PB-NK and YTS-NK cells were found highly beneficial against $PSCA^+$ tumors (138). A significantly high expression of human epidermal growth factor receptor 2 (HER2) in GBM (139), renal cell (140) and breast cancer (141) makes it an ideal candidate to develop immunotherapy using HER2-CAR-modified NK cells. CAR-NK92 cells designed to target epithelial cell adhesion molecules (EpCAMs) were highly efficient in killing breast cancer cells (142). The growth of ovarian cancer xenografts was remarkably reduced in mice after treatment with CAR-iPSC-NK cells (143). It is noteworthy that following a standard protocol, iPSC-derived NK cells can be synthesized on a larger scale (144), leading to feasible administration of multiple doses to treat refractory solid tumors more efficiently. Recent preclinical studies using CAR-NK cells against various solid tumors are summarized in **Table 3**.

TABLE 2 | Recent preclinical studies employing CAR-NK cells against various hematological cancers.

Serial No.	Tumor Type	Specific Target	Source of NK-cells	CAR composition	Clinical Outcomes	References
1.	T cell malignancies	CD5	NK-92	CD28+4-1BB+CD3ζ	Inhibition and control of disease progression	(123)
2.	B-cell malignancies	CD19	PB-NK	CD28+4-1BB+CD3ζ	Complete elimination of leukemia after 48 h	(124)
3.	B-cell malignancies	CD19	PB-NK	4-1BB+CD3ζ	Augmented cytotoxicity of NK cells	(118)
4.	B-ALL	CD19	PB-NK	CD28+CD3ζ	Complete and durable molecular remissions of pre-B-ALL	(125)
5.	B-cell malignancies	CD19	NK-92	CD3ζ	Successful inhibition of disease progression	(126)
6.	B-cell malignancies	CD19	NK-92	CD3ζ	Overcame NK resistance and markedly enhanced NK-cell-mediated killing	(98)
7.	B-cell malignancies	CD19	PB-NK	CD28+CD3ζ+IL15	Superior cytotoxicity with up to 90% specific killing activity	(127)
8.	B-cell malignancies	CD19	NK-92	41BB-CD3ζ,	Specific and efficient lysis of leukemia cell lines and lymphoblasts	(117)
9.	B-ALL	FLT3	NK-92	CD28+CD3ζ+iCasp9	Remarkable inhibition of disease progression and high antileukemic activity	(128)
10.	B-cell malignancies	CD19	UCB-NK	4-1BB+CD3ζ+iCasp9+IL15	Efficient killing of CD19-expressing cell lines and primary leukemia cells with marked prolongation of survival	(129)
11.	B-cell malignancies	CD20	PB-NK	4-1BB+CD3ζ	Significantly enhanced cytotoxicity and IFNγ production, extended survival time and reduced tumor size	(130)
12.	Burkitt lymphoma	CD20	PB-NK	4-1BB+CD3ζ+IL15	Significant anti-tumor effects and enhanced *in vitro* cytotoxicity	(119)
13.	MM	CD-38	NK-92	CD28-41BB-CD3ζ	Specific lysis of CD38-expressing tumor cell lines and effective depletion of MM	(131)
14.	MM	CD138	NK-92MI	CD3ζ	Remarkable cytotoxicity against human MM cell lines and elevated secretion of granzyme B, interferon-γ and CD107a proportion	(120)
15.	MM	CS-1	NK-92	CD28+CD3ζ	Enhanced MM cytolysis and IFN-γ production, efficient suppression of human IM9 MM cells and significant survival of mice	(132)
16.	Peripheral T cell lymphoma	CD-4	NK-92	CD28-41BBCD3ζ	Specific elimination of T-cell leukemia, lymphoma cell lines, and patient samples *ex vivo*	(133)
17.	T cell malignancies	CD-5	NK-92	2B4-CD3ζ	Specific cytotoxicity against $CD5^+$ malignant cells and prolonged survival of T-ALL xenograft mice	(134)
18.	T-ALL	CD-7	NK-92	CD28-41BBCD3ζ	Potent anti-tumor activity, elevated Granzyme B and IFNγ secretion, and significant inhibition of disease progression	(135)
19.	EBV+ T cell	EBNA3C	NK-92	4-1BB+CD3ζ	Exquisite specificity, potent cytotoxicity, and induction of ADCC toward the targeted T-cell epitope (TCE)	(121)
20.	Non-Hodgkin's lymphoma or chronic lymphocytic leukemia	CD-19	UCB-NK	CD28+CD3ζ+ IL15+iCasp9	complete remission in 7/11 (4 with lymphoma and 3 with CLL)	(122)

CLINICAL APPLICATIONS OF CAR-NK THERAPY

A large number of studies have been conducted on CAR-T cells; however, only a few clinical trials on CAR-NK cells (**Table 1**) have been registered on ClinicalTrials.gov. In addition to CD19 (NCT02742727), CD7 (NCT02742727) and CD33 (NCT02944162) are also the prime targets for CAR-NK cell therapy in clinical studies on lymphoma and leukemia. Furthermore, HER2-targeted GBM (NCT03383978) and costimulating conversion receptors are under investigation clinically to treat non-small-cell lung carcinoma (NSCLC) (NCT03656705). CAR-NK cell therapy against multiple refractory solid tumors targeting mucin 1 (MUC1), including pancreatic tumors, HCC, NSCLC and triple-negative invasive breast tumors, is also under investigation in clinical trials (NCT02839954). The details of ongoing clinical trials of CAR-NK cell therapies against hematological malignancies and solid tumors are summarized in **Table 1**.

CURRENT LIMITATIONS OF CAR-NK CELL THERAPY

Low Persistence

The lack of *in vivo* durability of infused cells in the absence of cytokine support is one of the key drawbacks of adoptive NK cell treatment. Although it may be safe, it may limit the efficacy of NK cell immunotherapy. Exogenous cytokines have been demonstrated to increase the proliferation and durability of adoptively infused NK cells; however, they can also cause undesired side effects (160), including the growth of inhibitory immune subsets such as Tregs (161). Multiple studies have reported promising results by engineering NK cells with transgenes encoding for cytokines that are either expressed on the membrane or released constitutively. In one such study, tumor-harboring mice with NK-92 cells or primary NK cells transduced with retroviral vectors producing IL-2 or IL-15 had increased proliferation and persistence (162). It has also been demonstrated that integrating IL-15 transgene into a CAR

TABLE 3 | Recent preclinical studies employing CAR-NK cells against various solid tumors.

Serial No.	Tumor Type	Specific Target	Source of NK-cells	CAR composition	Clinical Outcomes	References
1.	Osteosarcoma/ Prostate/HCC/ Breast cancer	NKG2D	PB-NK	NKG2D+DAP10+CD3ζ	Enhanced cytotoxicity and secretion of IFN-γ, GM-CSF, IL-13, MIP-1α, MIP-1β, CCL5, and TNF-α, and cytotoxic granules which persisted after 48h	(97)
2.	Multiple solid tumors	NKG2D	NK-92	DAP10+CD3ζ	Enhanced anti-tumor cytotoxicity both *in vitro* and *in vivo*	(145)
3.	Ovarian cancer	NKG2D	PBMCs	CD8α+ CD3ζ	Augmented tumor infiltration and significant antitumor responses	(146)
4.	Bladder carcinoma	PSCA	YTS/PB-NK	DAP12	Improved cytotoxicity, delayed tumor growth and complete tumor eradication	(138)
5.	Breast carcinoma	EpCAM	NK-92/ NKL	CD28+CD3ζ+IL-15	Predominantly intracellular expression of the cytokine, and STAT5 activation, high and selective cell-killing activity	(142)
6.	Colorectal cancer	EGFRvIII	NK-92	CD8α+ CD28+ CD3ζ	Development of a sensitive *in vitro* platform to evaluate CAR efficacy	(147)
7.	Breast cancer	EGFR	PB-NK/ NK-92	CD28+CD3ζ+oHSV	Enhanced cytotoxicity and IFN-γ production, efficient killing and significantly longer survival	(148)
8.	Renal cell carcinoma	EGFR	NK-92	CD28+4-1BB+CD3ζ	Potent antitumor activity and long-lasting immunological memory	(77)
9.	GBM	EGFRvIII	YTS	DAP12	Specific cytotoxicity, significantly delayed tumor growth, increased survival, and complete tumor remission	(149)
10.	GBM	EGFR/EGFRvIII	NK-92/ NKL	CD28+CD3ζ	Enhanced cytolytic capability, IFN-γ production, efficient suppression of tumor, and significantly prolonged the survival	(136)
11.	GBM	EGFR/EGFRvIII	NK-92	CD28+CD3ζ	High and specific cytotoxicity and tumor lysis, and marked extension of survival	(150)
12.	Melanoma	GPA7	NK-92	HLA-A2TM+CD3ζ	Enhanced tumor killing and suppression of the growth of human melanoma	(151)
13.	GBM/breast cancer	HER2	NK-92	CD8α+CD3ζ	Specific and efficient tumor lysis	(152)
14.	GBM/breast cancer	HER2	NK-92	CD28+CD3ζ	Potent *in vivo* antitumor activity, marked extension of survival	(139)
15.	Breast cancer/renal cell carcinoma	HER2	NK-92	CD28+4-1BB+CD3ζ	Efficient *in vitro* lysis, serial target cell killing, and reduction of metastasis	(140)
16.	Neuroblastoma	GD2	NK-92	CD3ζ	Remarkable cell killing activity	(137)
17.	Ewing sarcomas	GD2	PB-NK	CD28+4-1BB+CD3ζ	Enhanced *in vitro* responses and overcame resistance to NK cell lysis	(116)
18.	hepatocellular cancer	Glypican-3 (GPC3)	NK-92	CD28+41BB+CD3ζ CD3ζ CD28+CD3ζ DNAM1+CD3ζ DNAM1+2B4+CD3ζ	More quick expansion, more persistence, and higher cytotoxicity	(153)
19.	Ovarian cancer	Glypican-3 (GPC3)	iPSC	CD8α+CD28+CD137 +CD3ζ,	Enhanced cytotoxicity, IFN-γ production, and prolonged the survival	(154)
20.	Ovarian cancer	Mesothelin	iPSC	2B4+CD3z	Superior anti-tumor potential, significant inhibition of tumor growth and prolonged survival	(143)
21.	Ovarian cancer	Mesothelin	NK-92	CD28+41BB+CD3ζ	Specific *in vitro* killing, enhanced cytokine production, efficient tumor elimination, and prolonged survival	(11)
22.	Prostate Cancer	Prostate Stem Cell Ag (PSCA)	YST cell line, primary NK	DAP12	Improved cytotoxicity, delayed tumor growth and complete tumor eradication	(138)
23.	Colorectal Cancer	Carcinoembryonic antigen (CEA)	NK-92	CD3ζ	Improved recognition and lysis of the tumor cell lines	(155)
24.	ovarian cancer	CD133	NK-92	CD28-41BBCD3ζ	Enhanced cytotoxicity and IFN-γ production	(156)
25.	Liver cancer	c-MET	Peripheral blood	41BB+DAP12	Improved specific cytotoxic potential	(157)
26.	PD-L1$^+$Solid tumors	PD-L1	NK-92	41BB	Improved antitumor potential and significant inhibition of tumor growth	(158)
27.	Triple-negative breast Cancer	Tissue Factor (TF)	NK-92	CD28+41BB+CD3ζ	Superior tumor killing	(159)

construct improves NK cell proliferation, *in vivo* persistence and antitumor activity in patients with high-risk lymphoid malignancies, without increasing systemic levels of IL-15 or causing toxicity (122, 129). Other armored CAR-NK cells with cytokine transgenes are under development; however, there are no published reports yet. Another way to increase NK cell persistence is by inducing a memory-like phenotype, such as by preactivating them with a cytokine cocktail (IL-12, IL-15 and

IL-18) for a brief period to induce differentiation into cytokine-induced memory-like NK cells (64, 163). Memory-like NK cells were recently modified to express a CAR directed against CD19 and showed improved responses *in vitro* and *in vivo* against NK-resistant B-cell lymphoma (164).

Transport to the Required Tumor Site

Rapid homing to tumor beds is essential for adoptive cellular treatment efficacy and is governed by complicated interactions between chemokines released by NK cells and tumor cells (165). The efficiency of NK cell homing to tumor sites has been controversial, thus prompting efforts to improve it (166). The chemokine receptor CCR7 was transferred from K562 feeder cells to NK cells through trogocytosis, which resulted in improved homing of NK cells to the lymph nodes (167). In a xenograft-harboring mouse model of CXCL10-transfected melanoma, overexpression of CXCR3 on NK cells after *ex vivo* growth with irradiated EBV-LCL feeder cells and IL-2 resulted in better trafficking and antitumor activity (168). Several researchers have since investigated various engineering methods to improve NK cell homing. For example, NK cells were electroporated with mRNA coding for the chemokine receptor CCR7 to increase movement toward the lymph nodes that express the chemokine CCL19 (169). NK cells transduced with a viral vector encoding CXCR2 demonstrated better motility to renal cell carcinoma tumors expressing cognate ligands such CXCL1, CXCL2, CXCL5, CXCL6 and CXCL8 (170). The NK cell-recruiting protein-conjugated antibody (NRP-body) with a cleavable CXCL16 molecule was used in another study to increase NK cell trafficking and penetration into pancreatic tumors (171). Furin, an endoprotease expressed on the surface of pancreatic cancer cells, cleaves CXCL16, thus promoting NK cell infiltration *via* the ERK signaling cascade. In a mouse model of pancreatic cancer, this method was demonstrated to improve NK cell-mediated tumor suppression (171). CAR-NK cells have also been modified to improve their ability to travel to the tumor sites. Müller et al demonstrated that anti-EGFRvIII CAR-NK cells modified to produce CXCR4 conferred selective chemotaxis to CXCL12/SDF1-secreting glioblastoma cells in a mouse model of glioblastoma, leading to better tumor regression and survival (149). Furthermore, in mice with established peritoneal ovarian cancer xenografts, NKG2D CAR-NK cells modified to express CXCR1 significantly increased antitumor responses (146). To improve the success of NK cell immunotherapy in patients with solid tumors, several novel techniques to promote NK cell trafficking to tumor sites have been investigated in mice models; however, the efficacy of these approaches should be validated in clinical trials.

Immunosuppressive Tumor Microenvironment

TME, which includes immunosuppressive soluble chemicals, immunosuppressive cells and an unfavorable environment for optimal immune cell function, is a major barrier to successful CAR-NK cell therapy. TGF-β; adenosine; indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) are immunosuppressive cytokines and metabolites found in TME that can impair NK cell activity (23). Treg cells; regulatory B cells; myeloid-derived suppressor cells (MDSCs); tumor-associated macrophages (TAM); platelets; fibroblasts and several unfavorable metabolic factors such as hypoxia, acidity and nutrient deprivation induce immunosuppression in the malignant milieu (172, 173). Therefore, researchers are working towards developing CAR-NK cells that can prevent some of these immunosuppressive effects. Engineering NK cells to make them resistant to TGF-β has shown to be a promising strategy. The TGF-β receptor 2 (TGFβR2) gene was deleted using CRISPR/Cas9 technology in primary human NK cells, rendering them immune to this immunosuppressive growth factor without losing their efficacy against AML (174). Similarly, NK cells that were genetically engineered to express a dominant-negative TGF-β receptor, a high-affinity non-signal transducing receptor generated from TGFβR2, were able to counteract the suppressive effects of TGF-β on NK cells and restore their cytotoxicity (175). An anti-miRNA against miR-27a-5p, an miRNA that is elevated by TGF-β in NK cells, improved NK cell effector function both *in vitro* and *in vivo* (176). Furthermore, adenosine, an important immunosuppressive metabolite generated from ATP by the ectonucleotidases CD73 and CD39 in response to hypoxia and stress (177), has been targeted by blocking the high-affinity A2A adenosine receptor on NK cells, resulting in more potent antitumor activity in mouse models of breast cancer, melanoma and fibrosarcoma (178, 179). Another important method by which TME causes NK cell failure is checkpoint molecule interaction (30). To overcome this problem, genome editing is employed to eliminate checkpoint components from NK cells to improve their function. In tumor-harboring mice, TIGIT deletion was demonstrated to protect against NK cell depletion and enhance prognosis (180). Some researchers have investigated NKG2A and found that NKG2A^{null} NK cells exhibit increased cytotoxicity against HLA-E-expressing malignancies (181). Recent studies have suggested that combining CAR engineering with checkpoint deletion is effective in enhancing NK cell antitumor activity (by targeting CIS, a negative regulator of cytokine signaling) (182–184). The classic T cell checkpoints PD-1 and CTLA4 are two other inhibitory molecules that are under investigation in NK cells (185, 186). The use of checkpoint blockade to improve NK cell effector activity has recently been discussed in detail (187). Literature review reveals that creative engineering techniques and genome editing technologies may overcome the biological limitations of NK cells and hurdles caused by TME. Adoptive therapy with NK cells is likely to transform from a safe treatment with only moderate efficacy to a serious contender as a first-line treatment in cancer immunotherapy if strategies are developed to improve NK cell persistence, trafficking to tumor sites, and effector function in a hostile and malignant milieu.

Low Lentivirus Transduction Efficiency

Lentivirus-based transduction system is one of the most common approaches for gene modification and delivery in cells. However, owing to native characteristics, NK cells are

resistant to lentivirus, which makes lentivirus-based transduction a challenge. To improve viral transduction, various chemicals have been used. For instance, the electrical charge on cell membranes can be removed using protamine sulfate or polymers (dextran or polybrene) (188). Similarly, in HSCs and progenitor cells, cyclosporine A (189) and rapamycin (190) may help in removing different lentiviral restriction barriers. Interestingly, inhibition of intracellular antiviral defense mechanisms was reported to enhance the efficiency of lentiviral transduction in human NK cells (191). Furthermore, vectofusin-1 (192), prostaglandin E2 (188) and dextran (190) were found to promote transduction rates in human HSCs, T lymphocytes and primary NK cells, respectively (193). Furthermore, rosuvastatin has been discovered to increase the effectiveness of VSV-G lentiviral transduction of NK cells by upregulating LDLR levels (194). In addition, Colamartino ABL et al. (195) have revealed an effective and a resilient approach for NK cell transduction using baboon envelope pseudotyped lentivectors (BaEV-LVs) (195). They observed a transduction rate of 23.0 ± 6.6% and 83.4 ± 10.1% (mean ± SD) in freshly isolated human NK cells and those from the NK cell activation and expansion system (NKAES), respectively. Furthermore, CAR-CD22 transduced with BaEV-LVs exhibited robust CAR expression on 38.3 ± 23.8% (mean ± SD) of NKAES cells and particularly destroyed the NK-resistant pre-B-ALL-RS4;11 cell line. A larger vector encoding a dual CD19/CD22-CAR and a low viral titre were used to accomplish successful transduction and re-expansion of dual-CAR-expressing NKAES for efficient killing of both $CD19^{KO}$- and $CD22^{KO}$-RS4;11 cells (195). In addition, Bari R et al. (196) found that lentiviral vectors pseudotyped with a modified baboon envelope glycoprotein had a 20-fold or higher transduction rate than that of a VSV-G pseudotyped lentiviral vector (196). Moreover, using CD19-CAR, they achieved efficient and specific killing of CD19-expressing cell lines.

FUTURE STRATEGIES TO OVERCOME THESE LIMITATIONS/FUTURE PERSPECTIVES

Although CAR-NK cells are optional, potentially competitive cancer immunotherapeutic candidates along with CAR-T cells, numerous obstacles including heterogeneity, low persistence, trafficking to the tumor site, hostile TME and loss of tumor antigens remain to be overcome.

The most critical step in designing CARs is to identify highly and uniformly expressed target tumor antigens. Most tumor-associated antigens (TAAs) are also expressed by several healthy cells; therefore, achieving 'on-target, off-tumor' effects is inevitable (197). Moreover, huge differences may be observed in the expression of these TAAs among single-cell clones from the same tumor owing to two major strategies to evade immune surveillance—clonal evolution and decreased TAA expression. To overcome this problem, bispecific CARs were designed to target two different antigens simultaneously in prostate cancer, and very encouraging results were obtained (198).

Similarly, to overcome difficulties in the accessibility or trafficking of CARs in solid tumors, several approaches are used, including local administration, intraperitoneal administration and focused ultrasound-guided delivery. For instance, pleural injection was found very effective in an orthotopic model mimicking human pleural malignancies with even longer functional persistence than that obtained with intravenous injection (199). Regional administration of CAR-based immune cells may also help in reducing the therapeutic dose. Moreover, anti-HER2 CAR-NK-92 cells have been administered, using focused ultrasound, into the brain of mice with metastatic breast cancer (200). To minimize significant tissue damage caused by CAR-NK cells, ultrasound bursts along with intravenous injection of microbubbles were passed through the intact skull, allowing the temporary passage of NK-92 cells through the blood–brain barrier.

Tumors are equipped with several immunosuppressive factors such as TGF-β, IL-10, PD-1 or arginase. There are several ways to reduce the inhibitory effects of TGF-β. For instance, the combination of TGF-β kinase inhibitors and NK cells has been found to retain the cytotoxicity and expression of NKG2D and CD16 (201). Similarly, the use of either fresolimumab (TGF-β neutralizing antibody) or galunisertib (TGF-βRI inhibitor) has shown very encouraging results owing to their safety and tolerability in solid tumors (29). Moreover, the use of hybrid CARs equipped with an extracellular TGF-β receptor domain has been found quite successful in improving the antitumor potential of NK-92 cells (202). In addition, the cytotoxic activity of NK cells has been enhanced successfully by knocking down SMAD3 (downstream mediator of TGF-β) in solid tumors (145). Similarly, the expression of a dominant-negative TGF-β receptor has been found quite effective in retaining the ability of UCB-NK cells to produce IFN-γ and kill glioblastoma cells. Entinostat (a histone deacetylase inhibitor) has been reported to enhance MICA expression on cancer cells and NKG2D in primary NK cells, resulting in improved tumor cell recognition, and induce NK cytotoxicity despite the hypoxic conditions of tumors (203).

TME is further characterized by the scarcity of nutrients and significant hypoxia that lead to acidosis, eventually suppressing the immune responses (204). Hypoxia helps in tumor development by disturbing metabolism and enhancing the expression of several tumor growth factors and angiogenesis. Moreover, hypoxia promotes tumor growth and metastasis by decreasing the expression of several NK cell-activating receptors including NKG2D, NKp30, NKp44 and NKp46 (205). In addition, CD73 has been found to induce arginase (an immunosuppressive metabolite) in the hypoxic state to block the NK cell activity. Researchers have demonstrated an improved antitumor activity by enhancing the homing of NKG2D-CAR-NK cells to tumor sites in lung cancer (206).

Several immune checkpoints regulate and inhibit NK cell activity. These immune checkpoints act as 'a natural brake' to prevent autoimmune diseases or immuno-pathological

conditions caused by overactivation. Cancer cells can evade immune surveillance by expressing several checkpoint proteins that inhibit or block immune cell activation. Genetic deletion or blockage of these checkpoints can help CAR-NK cells to remain hyperactive and get rid of cancer and metastases more quickly. For instance, TIGIT has been found to prevent the cytotoxicity of NK cells by opposing CD226 (207). Moreover, decreased proliferation and effector potential was observed in PD-1^+ NK cells, whereas an improved effector activity was observed in PD-$L1^+$ NK cells (208). Subsequently, the reactivation of exhausted immune cells and long-lasting clinical outcomes have successfully been achieved by inhibiting PD-1 or PD-L1 *via* checkpoint blocking agents (209). Furthermore, persistent therapeutic benefits have been observed with a combination of CAR and checkpoint proteins (PD-1, CTLA-4, LAG3 and TIGIT) blockers (198). NK-92 cells expressing CD16, IL-2 and PD-L1-specific CARs have been found to destroy several human cancer cells, such as breast, lung and gastric cancer cells, by secreting a large number of perforin and granzymes (210). Interestingly, the use of antibodies as checkpoint inhibitors is under development in several clinical trials. For instance, two mAbs, namely, lirilumab (IPH2101 or 1-7F9) and IPH4102, have recently been developed to specifically target KIRs and KIR3DL2, respectively. Lirilumab has been engineered to target the common epitope shared by KIR2D that renders alloreactivity to NK cells to kill cancer cells by disrupting the inhibitory KIR-L/HLA interactions. The use of this antibody for NK stimulation in combination with lenalidomide has been found quite safe, tolerable and clinically effective against MM in a phase I trial (211). In addition, IPH4102 has been used for the treatment of cutaneous T cell lymphomas because these malignancies have a higher expression of KIR3Dl2 (212). This treatment has been found well tolerated and clinically effective in phase I trials, and the results are very encouraging and will prompt further large-scale clinical investigations (213).

Another important strategy to enhance the activity of CAR-NK cells that has not received the required attention is the modulation of tumor metabolism. Under hypoxic conditions, adenosine is produced by metabolizing ATP *via* CD39 and CD73, which are involved in immune evasion, blocking NK cell transportation to tumor sites, and preventing NK cell maturation (206). Moreover, the use of anti-CD39 and anti-CD73 antibodies to inhibit adenosine has been found quite successful in enhancing the effects of targeted therapy for ovarian cancer (214). CD73 may be an important target to treat several solid tumors such as glioblastoma, prostate cancer and lung cancer because it is highly expressed in these tumors. NKG2D-engineered CAR-NK cells have shown promising effects in treating lung cancer after anti-CD73 antibody-based inhibition (206).

To overcome the antigen loss after CAR therapy, more than one antigen can be targeted simultaneously. This can be achieved in many ways; for instance, different CARs targeting different antigens can be injected simultaneously (215); another approach may involve the use of vectors for two CARs that can be combined and used during the cell production step to obtain a mixture of cells equipped with single CARs and for both CARs as well. However, high costs involved in making multiple vectors and heterogeneity of CARs resulting in poor clinical analysis remain the major drawbacks of this strategy. Another important approach is to design a CAR that can recognize multiple antigens. This goal can be achieved *via* 'tandem CAR', where two binders are attached to a single molecule to improve the efficiency of immune synapse. In addition, the ribosomal skip sequences of internal ribosomal entry sites can help in generating multiple CARs on the same immune cell using a single vector, which is called 'bicistronic CAR'. Bielamowicz et al. recently targeted three different antigens on glioblastoma using a trivalent vector that encoded three independent CARs (216). It is quite promising that, recently, the number of trials using CARs that target multiple antigens simultaneously has increased. We anticipate that more trials will investigate CARs capable of simultaneously targeting two or more antigens in the future.

Another important strategy is to increase CAR-NK cell activation. A prime target for NK cell activation is CD16 that can induce the killing effect upon engagement. Identification of more such proteins/receptors for CAR-NK cells may enhance the efficacy of CAR-NK therapies. Other significant approaches to increase the safety profile of CAR-based NK cell therapies may involve the modification of CAR constructs by incorporating suicide genes (217) or developing bispecific CAR molecules to better target the tumor-specific antigens (150). Interestingly, CAR-NK cells can equally target tumors in CAR-dependent and CAR-independent manners; therefore, this property of NK cells should be conceivably used to exert an enhanced tumor-killing effect and develop non-signaling CARs. These non-signaling CARs lack direct killing signals but can enhance the legitimate killing technique of NK cells by promoting dwelling and adherence of these cells to targets (218). Another interesting strategy is to design CARs that can modulate or reprogramme the local TME via either immunosuppression or immunoactivation. One such CAR-based NK cell has already been developed and named either 'armored' CAR-NK cells or 'NK cell pharmacies'. These very special CAR-NK cells express several exogenous genes that can modulate the local TME to prevent any harmful effects (219). In line with it, a coalescence of CAR-based NK cell therapy and several alternative therapies might be a very effective option for efficiently eradicating the tumors. For instance, several chemicals can be used for immunosuppression before CAR-NK cell infusion to prevent or lagging the rejection of NK-cell by the host's defense system. Similarly, CAR-NK cell therapies could be made more effective in combination with radiotherapy. It has been previously proved that radiotherapy, especially stereotactic body radiotherapy (SBRT), helps in boosting the efficacy of immunotherapy (220). Radiations result in DNA damage that induces NKG2D expression on cancer cells and paves the path for NK cell activation and consequently killing effect. Hence, a combination of CAR-NK cell therapy and radiotherapy could be a sound option in lieu of targeting excrescences.

A large number of clinical investigations have been conducted using adoptive cell transfer of autologous NK cells for the eradication of several tumor types such as lymphoma, breast

cancer, colon cancer, and lung cancer (221). Nevertheless, the results were not satisfactory with poor antitumor activities due to the interplay of inhibitory receptors that are found on NK cells and self MHC class I that are found on cancer cells. This self-recognition prevented the stimulation of NK cells (222). For instance, mature NK cells bear a short lifespan at the point of malignancy, thus no long-term adverse effects were seen. Nonetheless, other kinds of NK cells, for instance, that are generated through cord blood or HSCs, have a longer lifespan and may cause long-term damage (223). To overcome this problem, researchers are investigating a new way for integrating caspase-controlled suicide vectors keen on CAR-NK cells, which might swiftly eliminate those cells that are transduced. In this regard, a contemporary investigation has shown that the addition of the matching small molecule dimerizer to the persuadable caspase 9 (iCAS-9) suicide schemes in CD19-CAR+IL15 NK cells caused apoptosis in 4 hours (129). HLA-mismatch donor haematopoietic transplantation in AML patients might prevent relapse and graft rejection without GVHD due to the donor-recipient NK cell alloreactivity that comes from KIR-ligand incompatibility (35). As several clinical investigations have highlighted the significance of KIR-HLA interactions in HSCT (224), KIR genotyping in the near future can serve as an important factor in donor selection. The cost of KIR genotyping is quite competitive and easy to perform; hence, can be done for donor screening together with HLA genotyping. Indeed, several trials that employ KIR genotyping for donor selection are in progress. Therefore, this approach can also opt for future target-oriented CAR-based immunotherapies.

Furthermore, KIR2DSs and KIR3DSs use ITAM (DAP-12) for the phosphorylation of tyrosine residue and recruitment of ZAP-70 or Syk that enhances NK cell activation and NK cell recognition of the tumor cells (225). Therefore, this potential of KIRs could be exploited for improved and highly efficient CAR-NK-based immunotherapies.

Adoptive immunotherapies are usually accompanied by certain side effects. One approach to minimize the risk associated with the adoptive immunotherapies is to endow the immune cells to target tumor-specific neoantigens. As the antigen screening technologies are progressing, more ways to identify the tumor neoantigens are being employed including inventory-shared neoantigen peptide library, whole-exome sequencing in combination with mass spectrometry, and neoantigen detection *via* trogocytosis. Hence, the future CAR-NK immunotherapies can be improved by employing this approach, to better treat the tumors resistant to conventional anti-cancer therapies.

Collectively, progress and advancement in the NK cell immunobiology field have led down the base of better and novel immune therapies. Excellent antitumor bloodlines of the NK cells have made them the center of focus of cell-based immunotherapies. Especially, the HLA phenotype independent NK cell recognition can be exploited to develop NK cell banks instead of modified CAR-NK cells. Subsequent CAR-NK cells are promising as novel anti-cancer therapies that could serve as "off-the-shelf" products. Advancements in the field of gene manipulation, antigen-screening technologies, and KIR-typing have allowed the development of novel, more powerful, and target-oriented CAR-NK cells with strong anti-tumor potential. Similarly, the use of bispecific CARs and Tandem CARs, genetic deletion/blocking checkpoint inhibition, and modulating tumor microenvironment are few other strategies that can better treat several tumor types. With an enhanced safety profile and promising success of CAR-NK immunotherapies in preclinical studies and clinical investigations, together with impressive efforts to overcome the existing challenges, we will witness progress and improvements in cancer treatment in the near future.

AUTHOR CONTRIBUTIONS

MK collected the data, draw figures, and wrote the manuscript. HS proposed the idea, modified, supervised, and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Natural Killer Cells in the Malignant Niche of Multiple Myeloma

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Natural killer (NK) cells represent a subset of CD3- CD7+ CD56+/dim lymphocytes with cytotoxic and suppressor activity against virus-infected cells and cancer cells. The overall potential of NK cells has brought them to the spotlight of targeted immunotherapy in solid and hematological malignancies, including multiple myeloma (MM). Nonetheless, NK cells are subjected to a variety of cancer defense mechanisms, leading to impaired maturation, chemotaxis, target recognition, and killing. This review aims to summarize the available and most current knowledge about cancer-related impairment of NK cell function occurring in MM.

Keywords: NK cells, multiple myeloma, inhibitory receptors, activating receptors, immunotherapy, microenvironment, niche

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INTRODUCTION

Multiple myeloma (MM) is a malignant disorder of plasma cells (PCs) with a median age of 65 years at diagnosis. MM evolves from monoclonal gammopathy of undetermined significance (MGUS) present in >3% of the population aged >50 years (1). The disease's clinical manifestations are mostly elevated serum calcium, renal failure, anemia, and bone involvement (the acronym CRAB) (2). Eventually, in up to 20% of cases, MM can progress into extramedullary disease (EMD), a soft tissue plasmacytoma, which represents a highly aggressive and treatment-resistant stage of MM (3–6). The mechanisms and biology of EMD are poorly understood, though PCs accumulate more chromosomal aberrations during EMD transformation (7).

Due to their natural tumor suppressor potential, natural killer (NK) cells became a subject of intensive research in cancer immunotherapy in both solid tumors and hematological malignancies (8, 9). Restoring or enhancing the effector abilities of NK cells for the treatment of MM has been one of the key topics in recent years (10–12). NK cell therapy is advantageous for several reasons: (1) NK cells are easy to isolate and expand *in vitro* using well-established methodologies; (2) these cells are capable of both direct killing and secretion of cytokines that can either potentiate other immune cells or suppress tumor cells; (3) overall biological features of NK cells are reducing the possibility of undesired side effects such as the ones observed with CAR-T cells; (4) NK cells are not antigen specific and there is no need for a specific target, although this can enhance the effectiveness of the therapy (13–15); and (5) the infusion of allogeneic NK cells is safe and does not cause the unwanted and deleterious graft vs. host disease (GvHD), thus opening the possibility of a more affordable off-the-shelf cancer cell-based immunotherapy (16).

Novel NK cell-based therapy possibilities include infusion of allogeneic/autologous NK cells, administration of *in vitro* expanded and genetically modified NK cells (including CAR-NK cells),

cytokine-stimulated NK therapy, and monoclonal antibody (mAb)-based NK therapy (13, 15, 17). Modification of inhibitory or activating surface molecules represent a promising option to potentiate efficacy of NK cells (18, 19). Another promising approach is priming of NK cells with certain interleukins (ILs). IL-2 and IL-15 supplementation *in vitro* was confirmed to enhance the NK cells' killing abilities, increasing the expression of activating NK cell receptors (20–22). Although the NK therapy seems to hold a huge potential for cancer therapy, a recent study showed that haploidentical NK cell transplantation in relapsed/refractory (RR) MM patients did not report significant therapeutic outcomes. The study had to be halted after all 12 patients relapsed within 90 days (23). Also, it is important to understand that mAb therapies for the treatment of MM act through (amongst others) NK-cell activities like antibody-dependent cell cytotoxicity (ADCC) mediated *via* either the mAb or mAb–drug conjugate (24–26). Anti-CD38 daratumumab (approved in 2015), anti-SLAMF7 elotuzumab (2015), and anti-CD38 isatuximab (2020) are mAbs used for the treatment of MM (25, 27, 28). The novel anti-CD38 MOR202 is now in the clinical trial phase in MM patients (29). Likewise, proteasome inhibitors and immunomodulating agents such as thalidomide, lenalidomide, and bortezomib have been proved to potentiate NK cell activity against MM (28).

Understanding the NK cell biology and mechanisms affecting the function of NK cells in MM is crucial for further progress in the field of targeted and NK cell therapy. This review summarizes the most recent and available data providing a necessary insight into the origin and development of NK cell subsets, their biology, antitumor abilities, and, mainly, impairment of function occurring in the MM microenvironment.

NK CELL DEVELOPMENT AND SUBSETS

NK cells represent 2–31% of peripheral blood (PB) lymphocytes (30). Although the organ and tissue distribution and circulation of NK cells are not fully understood, they are also present in the bone marrow (BM), liver, spleen, lungs, uterus, thymus, and secondary lymphoid tissues (31, 32). Maturation and differentiation of early NK subsets occurs in BM and secondary lymphoid organs. Even though NK cell development in humans is understood less than in mice, stages 1 to 6 were identified (8 overall with substages) in humans, each having a distinct immunophenotypic profile (**Figure 1**) (33, 34). Several ILs are crucial for the development of the NK lineage, mainly IL-2, IL-7, and IL-15, but also pro-inflammatory IL-12, IL-18, IL-27, and IL-35 (35, 36).

Similar to all other hematopoietic lineages, the NK lineage is derived from bone marrow-residing hematopoietic stem cells (HSCs), which transition into CD34+ CD45RA- CD133+ multipotent progenitor (MPP) cells and subsequently to CD34+ CD133+ CD45RA+ lymphoid-primed multipotent progenitors (LMPPs), determining the lymphoid line potential. Direct NK cell lineage precursors seem to be derived from the CD34+ CD133+ CD45RA+ CD10+ fraction, known as common lymphoid progenitors (CLPs) in the conservative model of hematopoiesis, or multi-lymphoid progenitors (MLPs) according to the proposed hematopoietic tree revision. The revision does not distinguish NK lineage potential and further NK cell development since the study aimed at early CD34+ progenitors and also mainly on the revision of the general myeloid and lymphoid progenitor potential (35, 37, 38). The earliest NK precursors with acknowledged NK lineage potential were identified as CD127+ in mice (39). This correlates with CD34+ CD133+ CD45RA+ CD7+ CD117+ CD127+ stage 2a phenotype in humans. CD7 (from the stage 2a) and CD122 (from the stage 2b) are subsequently expressed throughout the whole NK cell lineage. Stage 3 represents a transitional stage between NK precursors and mature NK cells with a complete loss of CD34, CD133, and CD127 but with prevailed high levels of CD117 (35, 40–42). Mature subtypes of NK cells (stages 4a, 4b, 5 and 6), all with the characteristic CD3- CD7+ CD45RA+ CD56+/dim immunophenotype are characterized by the progressive loss of CD117 from high to low levels in stage 4 to stage 6 (the final stage) being completely CD117-, and with the gain of CD56 (33, 43, 44).

CD56 and CD16 represent two of the most common and relevant markers used to identify NK cells (32, 45). For a proper flow cytometric detection of all mature NK cell subsets, both CD56 and CD16 should be included in the panel since CD16 is expressed only in stages 5 and 6, and CD56 alone is not sufficiently specific (32). CD3 should also be mandatory for correct NK cell evaluation to exclude CD3+ CD7+ CD56+ NK-like T cells (46). Based on the expression of CD56 and CD16, two main mature functional subsets are often described: CD56+/bright CD16-/dim and CD56dim CD16+/bright (47). CD56+ CD16- cells (accounting for 5-10% of circulating NK cells) are agranular with low cytotoxic activity and are considered mainly to be cytokine and chemokine producers. These cells co-express CD94/NKG2A in a high manner, meaning the CD56+ CD16- subset consist of both stages 4a and 4b. Contrary to this, CD56dim CD16+ cells (90%–95% of circulating NK cells) are designated as true killer cells with a high cytolytic potential against infected, tumor-transformed, or otherwise immunocompromised cells due to the expression of CD16 (Fcγ receptor III), which acts as a cell lysis signal transducer. A typical feature is the diminished expression of CD94/NKG2A compared to high levels of this antigen on the surface of CD56+ CD16- cells (45, 48–50).

CD57+ is a terminal marker of CD8+ T cells and also NK cells (51). The CD56dim CD16+ subset consists of developmental stages 5 and 6. The main difference in these two stages is in the expression of CD57 and regulatory surface molecules known as the Killer cell Ig-like Receptors (KIRs); stage 5 lacks CD57 and maintain only low levels of KIRs (NK stage 5 immunophenotype: CD56dim CD16+ CD57- KIRdim), whereas the terminal stage 6 expresses both CD57 and KIRs in a high manner (NK stage 6 immunophenotype: CD56dim CD16+ CD57+ KIR+) (32, 35).

The CD56- CD16+ subset was also identified in high numbers in individuals with chronic infections (HIV and HCV).

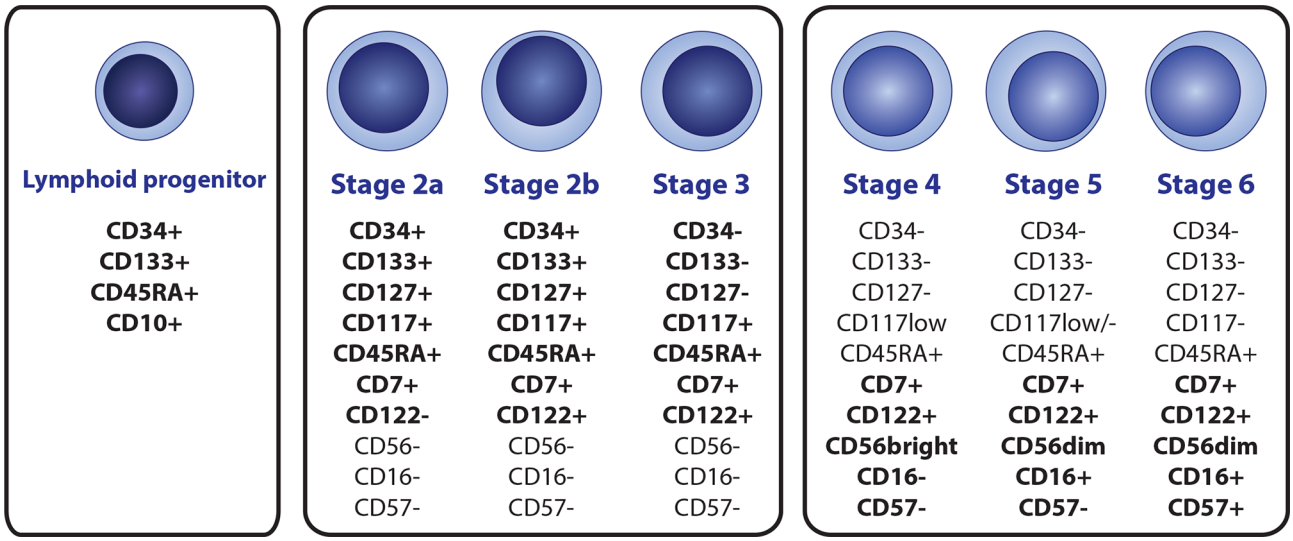

FIGURE 1 | A scheme of the NK cell development, with immunophenotypic profile of the most relevant markers that are suitable for identification of individual subsets by flow cytometry. NK cell lineage is derived from the CD34+ CD133+CD45RTA+CD10+ lymphoid progenitor (terned also as a stage 1), Immature stages 2a, 2b and 3 can be distinguished by the differential expression of CD34, CD122 and CD127, while high levels of CD117 are preserved. Mature stages 4 (4a + 4b), 5, and 6 can be distinguished by diverse expression of CD16, CD56 and CD57.

The subset is described as dysfunctional, with higher expression of NK inhibitory receptors, lower levels of NK cytotoxic molecules, and both limited cytotoxic function and secretion of anti-inflammatory cytokines (52, 53). NK subsets can also be described by different levels of CD27 and CD11b in both mice and humans (54, 55).

Although there are several known and accurately described subsets of NK cells, it seems that diversity in the expression of different NK surface molecules pushes the variability of NK cells beyond the limits of standard flow cytometry. Between 6,000 and 30,000 different NK phenotypes can be detected in one individual and up to 100,000 in a group of individuals using the mass cytometry approach (56).

NK CELL BIOLOGY IN ANTICANCER IMMUNITY

The role of NK cells in anticancer surveillance is unquestionable in the modern era. Many studies have highlighted the significance of NK cells in the elimination of malignant cells or in cancer progression regulation, a topic that has been heavily reviewed in recent years (49, 57, 58). NK cells were originally categorized as part of the innate immunity; however, memory and education abilities have been a matter of discussion lately (59). These cells lack specific antigen receptors compared to other lymphocyte subsets. The anti-cancer potential of NK cells is mediated either directly in a contact-dependent manner through their ability to induce programmed cell death, or indirectly in a contact-independent manner through the secretion of various cytokines, or in both manners by cooperation with other cells of the immune system (60). A broad spectrum of surface regulatory molecules is involved in NK regulatory actions (61).

Anticancer Mechanisms of NK Cells

The release of cytokines and chemokines, which are soluble, omnipresent, and crucial immune system regulators, is one of the key antitumor abilities of NK cells (48). The CD56+ CD16- NK cell subset is considered a major cytokine producer with low killing abilities (47). Nonetheless, CD56dim CD16+ cells, otherwise with a strong cytolytic potential and present in the majority in peripheral blood, also act as cytokine producers mainly in the initial immune response, which helps in mobilizing other immune cells (62). Tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) are among the most potent antitumor cytokines, but the NK cell cytokine repertoire also includes immunoregulatory IL-5, IL-10, and IL-13; chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES); and GM-CSF as well (63).

The ability to induce apoptosis of the target cell is a primary and well-known regulatory mechanism of NK cells. Apoptosis induced by NK cells can be mediated by degranulation, death receptors, or mAb-CD16 binding (60). The degranulation ability of NK cells was proved to be crucial in tumor and metastatic regulation (64). A specialized organelle called secretory lysosome, mainly containing perforin and granzyme granules, is involved in the highly coordinated and regulated process (65, 66). Death receptors are TNF superfamily receptors expressed on the surface of many cells (67). Death receptor ligands expressed by NK cells (such as Fas ligand, TNF, and TRAIL) bind specifically to the death receptor domains on the surface of target cells, resulting in a conformational change of the receptor,

recruitment of the adaptor protein, and apoptosis (65, 68). ADCC represents one of the cancer immunotherapy-related killing mechanisms of NK cells. ADCC is facilitated after the binding of an IgG mAb Fab fragment to the target surface antigen on one side and Fc fragment to the Fcγ receptor III (CD16) of the effector cell on the other side, creating the effector cell–mAb–target cell link with subsequent engagement of cytotoxic pathways (69).

Surface Effector Receptors

NK cell surface activating and inhibitory molecules play a crucial role in the regulation of NK cell killing abilities, cytokine production, and all actions, in general. These receptors are able to detect specific stress signals and changes in expression patterns of surface molecules on cells and consequently regulate NK cell activity, which is a deeply balanced process (61, 70). During differentiation, NK cells undergo a complex series of educational interactions between major histocompatibility complex type I (MHC-I) molecules and NK surface inhibitory receptors. Thus, they are educated to self-tolerate other healthy cells in the body (71, 72). Also, interactions between non-classical MHC and non-MHC molecules were described (73). The concept of induced tolerance and inhibition of NK cell activity by recognizing MHC is fundamental for the regulation of the anti-cancer response. During malignant transformation, a series of changes in gene expression occur in transforming cells, leading to the downregulation or upregulation in the expression of surface molecules (74). In this context, the most important is the diminution of surface MHC-I molecules, which tags transformed cells as a potential target for eliminating NK cell-regulatory mechanisms (75, 76).

NKG2A/CD94 heterodimer (CD159a), LAG-3, and a fraction of the killer Ig-like receptor (KIR/CD158) family (inhibitory KIRs [KIR2DL, KIR3DL subgroups]) are categorized as specific MHC-I/HLA-I recognizing inhibitory NK cell receptors (61, 77, 78). However, this does not necessarily mean that any cells lacking MHC-I are the target of NK cells. There are several other inhibitory and co-inhibitory NK cell molecules like the Siglec family (e.g., Siglec 7 and Siglec 9), Tactile (CD96), PD-1, TIGIT, CD112R, IL-1R8 and TIM-3 (**Figure 2**) (77, 79, 80).

Nevertheless, a "missing-self" signal is not enough for the activation of NK cells. Expression of stress-induced signals which stimulate the NK cell-activating receptors is crucial for activating NK cell response (81). Cellular stress activates a variety of DNA-damage response, senescence, and tumor-suppressor signaling pathways, which consequently lead to the expression of activating ligands that are recognized by NK cell activating receptors (82). Also, the synergistic action of multiple activating molecules is required for the activation of NK cells, except for CD16 and NKG2C, which are able to activate cell response on their own without any other co-stimulation (19, 83, 84). Several MHC-dependent and MHC-independent molecules are categorized as NK cell activating receptors, including activating KIRs (KIR2DS and KIR3DS subgroups), NKG2D, NKG2C, natural cytotoxicity receptors (NCRs [NKp30, NKp44, and NKp46]), Nkp80 (not clearly categorized as NCR), ICOS, DNAM-1 (CD226), CRTAM, and signaling lymphocyte activation molecule (SLAM) family members like 2B4 (CD244), CD48, Ly9 (CD229), NTB-A (CD352), and SLAMF7 (CD319) (**Figure 3**) (19, 85–92).

Originally, functional receptors of NK cells were categorized either as inhibitory or activating, but there are hints that the function of some molecules might be much more complex with a dual inhibitory and activating potential or at least a costimulatory function (13). For example, both the inhibitory and activating potential of 2B4 (CD244) was proved (93). There seems to be evidence that the activating molecule NKG2D also has broad costimulatory abilities of other activating receptors (94).

Other molecules are of course present on the surface of NK cells, but they are not clearly categorized among the activating or inhibitory receptors. Nevertheless, CD38, which is an important signal transducing, activating, and adhesion molecule, was also proved to activate NK cell effector response (95, 96). CD27, a T-cell co-stimulatory molecule, is not mentioned similarly in this context, but CD27 was connected with the enhanced cytotoxic activity of NK cells (97).

MM MICROENVIRONMENT

The BM niche, in general, is a deeply complex environment, which consists of cellular and noncellular components. The cellular compartment is represented either by hematopoietic cells, or nonhematopoietic cells such as mesenchymal stromal cells (MSCs), osteolineage cells, adipocytes, and endothelial cells. Cytokines, chemokines, growth factors, reactive species, extracellular matrix (ECM) proteins, and other molecules form the noncellular compartment (98, 99). BM function is negatively affected in hematological malignancies due to the tumor microenvironment (TME), which creates advantageous conditions for clonal cells and suppressive conditions for normal cells. In MM, disease manifestation, progression, and treatment resistance are often reflected with TME and its individual components (100, 101). Single-cell transcriptomics data revealed that alteration in the immune setup of the BM niche can be observed early from the MGUS stage, including increased frequency of NK cells, T cells, and monocytes. T cells exhibit accumulation of Treg and γδ T-cell subsets at MGUS, accompanied by decrease of CD8+ memory subsets at the stage of SMM. Importantly, the patterns of immune dysregulation are heterogeneous in MM patients and might represent a possible indicator for the risk stratification (102).

Non-Cellular Compartment

The role of cytokines, growth factors, extracellular vesicles, and other molecules was described in the process of TME transformation and MM progression (103). Furthermore, the presence of tumorigenic molecules plays a critical role in the concept of pre-metastatic niche describing slow and remote TME orchestration, connected to the disease dissemination (104, 105). Malignant BM is highly inflammatory and hostile to non-malignant cells (including NK cells), a fact that is reflected by

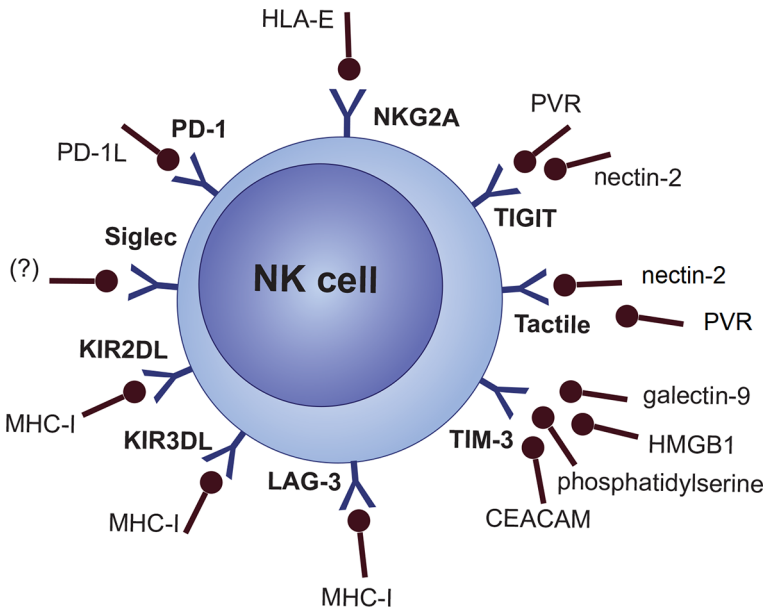

FIGURE 2 | NK cell inhibitory receptors will their cognate ligands.

elevated levels or altered expression of pro-inflammatory factors such as IL-1, IL-6, IL-10, IL-17, IL-18, IL-21, IFN-γ, TGF-β, TNF-α, HGF, EGF, and HIF-1α; chemotactic factors recruiting other pro-inflammatory cells such as CCL2, CCL3, CCL4, CXCL12 (SDF-1), CSF-1, GM-CSF, and MSP; and proangiogenic factors supporting neovascularization such as VEGF, IGF-1, FGF, PDGF, reactive oxygen species (ROS), and reactive nitrogen species (RNS). The overall profile of soluble factors is a deeply complex topic itself (106–110).

Cellular Compartment

Normal cells present in the BM stroma can be clearly reprogrammed to support the disease manifestation and progression. This is reflected by the fact that immature BM MSCs have an abnormal genomic profile compared to their normal counterparts and provide advantageous environment for the expansion of MM cells (111, 112). Hence, the role of MSCs in disease persistence was suggested. Single-cell transcriptomic analysis revealed that MSCs in MM are highly pro-inflammatory, their transcriptomic profile can be tracked even post-treatment, and unfortunately, therapy is not effective in normalizing the BM niche. The study also revealed that MM MSCs are stimulated by pro-inflammatory cytokines that are most likely produced by immune cells such as IFN-responsive T cells and CD8+ memory T-cell subsets (113). MSCs development is also disrupted in MM, and aberrancies were described in more mature osteolineage cells and adipocytes (114, 115). The differentiation of MSCs is shifted preferentially towards the adipocyte lineage in MM, and, if the high secretory potential of adipocytes is taken into consideration, this may favor further disease progression as well (116). *EPHB1, FBLN5, RELL1, ADAMTS17* are among the impaired genes in MM-affected MSCs. Downregulation of *BMP10*, the bone morphogenic protein 10 gene, in MM MSCs reflects the impaired osteoblastic differentiation, and it seems that BMP signaling is involved in MM bone disease progression. Therefore, inhibition of the BMP axis, as well as others such as TGFβ, Notch, Wnt, or Runx2/Cbfa1 signaling, represents a possible option for therapy improvement in MM (112, 117, 118). Interestingly, interactions between MM cells and BM MSCs trigger the production of IL-6 and a number of cytokines and chemokines, including TNF-α, VEGF, IGF-1, CXCL12, IL-1β, TGF-β, CCL-3, and CCL4 with immunomodulatory activity (119, 120).

Over-angiogenic potential of endothelial cells (ECs) was linked with neovascularization and disease progression in MM (121). ECs of MM patients also have a distinctive genetic profile that strongly supports their neoangiogenic potential. Genes involved in neovascularization, such as *bFGF, FGF-7, VEGF-A, VEGF-B, VEGF-C, VEGF-D*, and *GROα*, together with ETS-1, HIF-1α, ID3, and osteospontin transcription factors, are overexpressed in MM ECs (122). Also, filamin A, vimentin, and α-crystallin B proteins are overexpressed by MM ECs, though anti-MM drugs such as bortezomib and lenalidomide affect these proteins during treatment (123). The hypoxic niche and HIF-1α overexpression are key factors in MM neovascularization as well (124).

The most relevant hematopoietic cells contributing to the MM TME are without a doubt malignant PCs, macrophages, myeloid-derived suppressor cells (MDSCs), and T-regulatory lymphocytes (Tregs) (105). Overall impact of malignant PCs can be seen throughout the whole chapter, but briefly, the role of PCs in the organism is much more complex than just antibody production. They are able to produce many soluble factors, including IL-1, IL-10, IL-12, IL-17, IL-35, TNF-α, TGF-β, and GM-CSF, which indicate their role in immune and

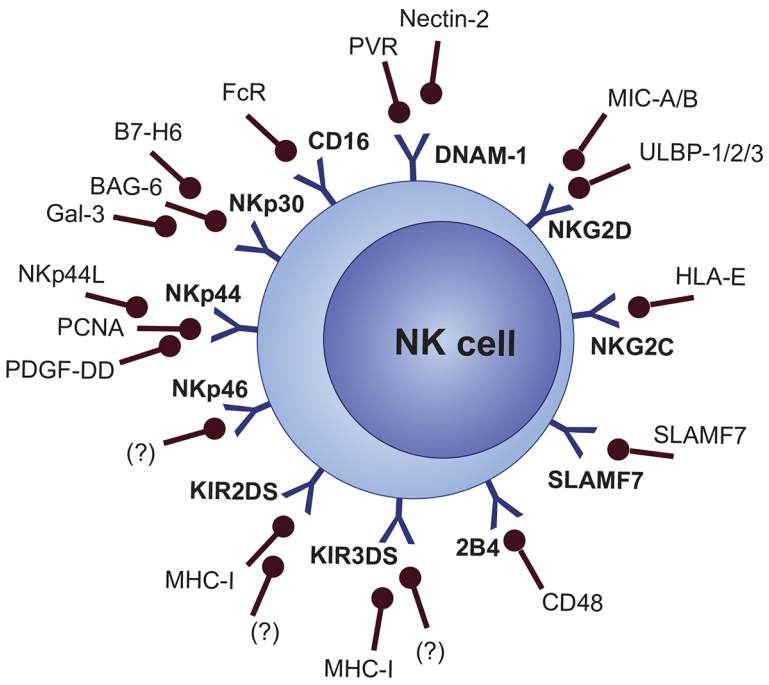

FIGURE 3 | NK cell activating receptors and their ligands.

hematopoietic modulation (125–127). Over 400 genes are deregulated in MM PCs compared to normal PCs, which could reflect their ability to alter the niche to favor myeloma progression (128).

Macrophages, in the context of the malignant niche, are divided into two groups: classically activated M1 macrophages, and alternatively activated M2 macrophages. The function and phenotype of macrophages depends on their microenvironment, and the general term "tumor associated macrophages" (TAMs) is used to distinguish these cells from normal macrophages (though M2 cells are sometimes classified as TAMs) (129). M1 cells are pro-inflammatory, anti-tumor macrophages activated by bacterial lipopolysaccharides or cytokines produced by Th-1 lymphocytes and secrete IL−1β, IL-6, IL-12, IL-23, TNF-α, ROS, and RNS. M2 macrophages are activated in response to factors produced by Th2 lymphocytes, such as IL-4, IL-10, IL-14, and glucocorticoids. These cells are considered tumorigenic, immunosuppressive and, among others, they produce IL-10, TGF-β, VEGF, matrix metalloproteinases (MMPs), and ARG-1 (109). A high frequency of TAMs was associated with worse prognosis and treatment resistance in MM (130). It was also proved that TAMs cooperate with other cells in the niche. They can mimic ECs in MM and promote neovascularization through VEGF and FGF−2 stimulation (131). Macrophage chemotaxis towards MM BM niche and shift to the tumorigenic M2 phenotype is mediated *via* CCL2, CCL3, CCL14, CXCL12, CSF-1, GM-CSF, MSP, PDGF, and TGF−β,which are produced by MM-associated MSCs (109).

MDSCs were confirmed as immune system inhibitors in cancer patients. These cells express typical CD33+ CD11b+ HLA-DR-/low immunophenotype, with further subdivision into CD15+ granulocytic (G-MDSCs) or CD14+ monocytic (M-MDSCs) subsets (132). It was proposed that G-MDSCs differentiate into tumor-associated neutrophils (TANs) and, similarly, that M-MDSCs are precursors of TAMs. However, possible polarization of normal neutrophils into TANs in the TME is also discussed (133). Increased frequency of MDSCs was found in MM patients, which was also correlated with disease progression and therapy outcome (134, 135). MDSCs produce ROS, RNS, and ARG1, which in detail impair the function of the CD3 T-cell co-receptor participating in the activation of both CD4+ and CD8+ T-cells. Also, MDSCs downregulate the expression of L-selectin (CD62L), thus decreasing T−cell trafficking to the malignant niche (133, 136, 137). Overall, an inhibitory role of MDSCs on the function of NK cells was proved by the co-culture of NK cells with MDSCs, which resulted in the downregulation of activating receptors, decreased secretion of IFN−γ, and decreased degranulation (138). Furthermore, data suggesting a pro-angiogenic potential of MDSCs in MM were published (139). RNS and membrane-bound TGF-β are among the MDSC-derived factors inhibiting NK cell function (140, 141). Overall cooperation of cells present in the malignant niche is reflected by a confirmed ability of MM MSCs to induce the upregulation of TNFα, ARG1, and pro-angiogenic PROK2 in MDSCs (135).

CD3+CD4+CD25+ Tregs are important modulators of normal immune response. The role of Tregs in MM progression seems to be a matter of discussion due to contradictory data. Both decreased and increased Treg frequency can be detected in MM. Increased Tregs were

associated with the disease progression, but contradictory data are published too. Nevertheless, these cells clearly contribute to dysfunctional immunity in MM, though the role seems to be heterogeneous (142–146). IL-10 and TGFβ are probably the most discussed cytokines produced by Tregs that may contribute to pathological features of MM BM (144). One of the key Treg-related aspects to maintain functional immunity in MM and tumors in general is a balance in the Treg vs. CD4+ T-helper 17 (Th17) cell ratio. Th17 cells contribute to the development and progression of chronic immune diseases, and cancer, by overall immune regulation and production of IFN-γ, TNF-α, IL-10, IL-17, IL-21, IL-22, and IL-26. It seems that the Treg/Th17 differentiation axis is skewed in MM by elevated levels of IL-6 and TGFβ. In the presence of TGFβ alone, naive T cells that express Foxp3 and differentiate into Tregs Th17 cells are generated in the combination of TGF-β and IL-6, or IL-21 (146). Again, contradictory data have been published on the topic of the Treg/Th17 cell relationship to MM prognosis, and further clarification is needed. Nonetheless, Th17 cells produce high levels of IL-17, which was proved to promote growth of MM cells *in vitro* and *in vivo (*147, 148).

Without a doubt, MM niche is a deeply complex environment contributing to disease progression and persistence through modulation of the immune response. Nevertheless, only limited data are published about how individual components affect the function of NK cells, which will be discussed in the next chapter.

NK CELLS IN THE MYELOMA NICHE

NK cells act as important regulators in the development and progression of hematological malignancies and their suppressor activity particularly against MM cells was confirmed in many studies (149–152). Nonetheless, significant changes in the distribution of NK subsets and dysfunctions of NK cells were described in MM patients (153, 154). The functional activity of NK cells was also correlated with disease staging (155). Recent studies provided an insight into mechanisms involved in the NK cell –mediated killing of malignant PCs and highlighted the role of interactions between surface effector receptors on the surface of NK cells and specific ligands (156, 157). The recognition of MM cells with activating receptors, including NKG2D, NKp46, and DNAM-1, has been proved (158). Also, a low expression of HLA-1 molecules on malignant PCs and the role of NK inhibitory receptor suppression was demonstrated in MM (150). Downregulation or upregulation of these surface molecules was associated with severe dysfunctions of NK cells in MM. However, details about involved mechanisms between NK cells and individual TME components remain poorly described (**Figure 4**) (159, 160) Data describing the NK cell distribution or functional capabilities in EMD lesions are missing completely, even though NK cell infiltration was connected with better overall survival in solid tumors (161).

Impairment of NK Cell Development

Since malignant populations are considered to be competitive to non-malignant cells, bone marrow brings a unique insight into the effect of myeloma on the development of healthy immune cells (100). In hematological malignancies, cancer niche disrupts normal hematopoiesis and results in a favorable environment for clonal cells (162). Several publications describe that overall lower percentage of circulating NK cells can be detected in the peripheral blood of MM patients in advanced disease stages with poor prognosis compared to controls, MGUS, and MM with good prognosis (163, 164). However, Pazina et al. recently published that frequencies of NK cells in PB of ND MM and smoldering multiple myeloma (SMM) patients are not significantly decreased compared to healthy donors (HD). Furthermore, overall numbers of PB NK cells in RR MM and post-stem cell transplant (post-SCT) patients were increased in this study, with CD56bright CD16- CD57- stage 4 subset prevailing. This was argued as a possible effect of NK lineage reconstitution after the disease and therapy depletion; hence, it might not reflect the actual disease impact. Frequencies of total NK cells in BM reflected the frequencies in PB, except post-SCT where the frequency was significantly lower in BM. Also, numbers of CD56dim CD57+ cells (representing the terminal and highly active stage 6) are lower in BM compared to PB of ND, RR, and post-SCT MM patients (165). To point out the importance of the terminal stage NK cells, MM patients with higher absolute numbers of CD57+ NK cells were associated with better prognosis compared to patients with higher numbers of more immature CD56bright CD16- CD57- cells (159). Similar to what was published by Pazina et al., overall NK cell numbers and cytotoxic abilities are reduced in B/T-ALL patients as a result of CD56bright CD16- cytokine-producing stage 4 accumulation. In this study, high numbers of cytokine CD56bright CD16- cells were also associated with poor prognosis (166). The accumulation of CD56bright CD16- subset and lower total frequencies of NK cells in the BM reflect that NK cell lineage differentiation is impaired during the progression of hematological malignancies. Nevertheless, scarce information is available in the context of altered NK cell maturation in the environment of malignant BM in general. No study so far has provided detailed data about NK progenitor subset distribution in leukemic or myeloma marrow (167, 168).

In general, early CD34+ CD38- HSCs are not depleted in leukemic marrow since they enter a self-protective quiescence. Nonetheless, leukemic niche affects hematopoietic differentiation leading to reduced levels of CD34+ CD38+ progenitors and subsequent cytopenias (162, 169). There is also evidence that NK maturation in BM is blocked in solid tumors, even though no direct contact is needed between tumor cells and NK cells. One of the reasons is most likely a remotely orchestrated IL-15R downregulation in cancer-altered BM stroma (170). The IL-15/IL-15R axis is indeed an important NK cell development regulator and proliferation inducer, acting *via* IL-2Rβ (CD122), and JAK/STAT, Ras/MEK/MAPK, or PI3K/AKT pathways (171, 172). Mutations in *GATA2* (absent CD56bright cells), *MCM4* (absent CD56dim cells), *IL2-R, JAK3, STAT5*, and *IL-15R* were associated with impaired NK maturation (44, 173, 174).

One of the possible suppressors of IL-15 signaling is prostaglandin E2 (PGE2), which downregulates the γ-chain of

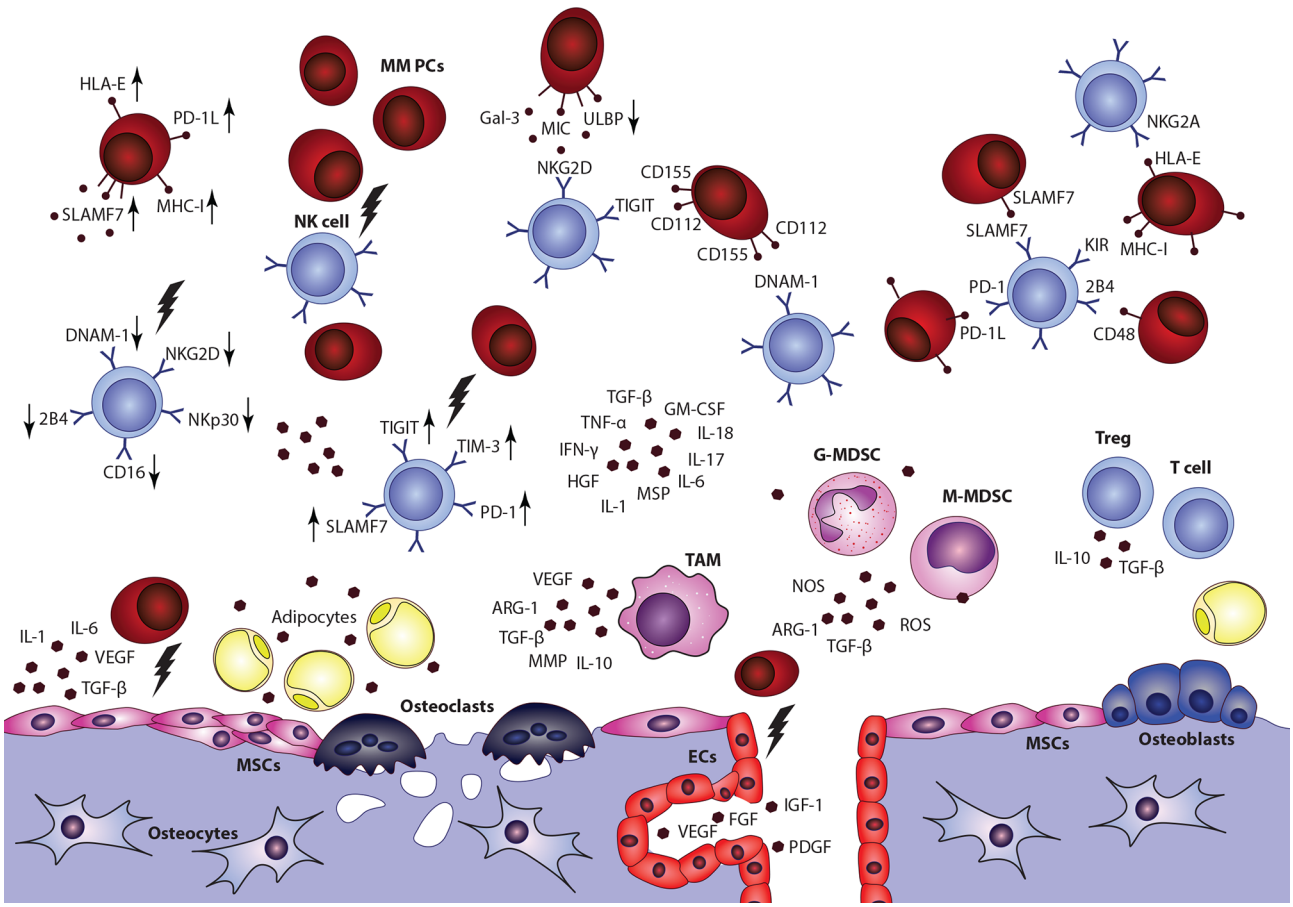

FIGURE 4 | Impact of multiple myeloma bone marrow microenvironment on the overall NK cell function is a complex topic, with only limited data available. Overall decrease of NK cells frequency, accumulation of CD56bright CD16-cytokine producing subset, impaired overall fuctional properties, and alterations of surface effector receptors were connected with the disease and its progression. Nonetheless, detailed data describing interactions between NK cells and individual components in the niche are incomplete. MSCs, mesenchymal stromal cells; ECs, endothelial cells; G-MDSC, granulocytic myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; TAM, tumor associated macrophage.

the IL-15R complex and subsequently inhibits NK cell function (175). Another candidate is ADAM17, which is activated through the IL-15 axis and reduces NK cell proliferation. Blockade of this metalloproteinase results in increased levels of L-selectin (CD62L) on NK cells, thus supporting the homing of these cells (176). However, not only downregulation of the IL-15R function, but also chronic exposure to IL-15 leads to the NK cell exhaustion (177). Without a doubt, impaired IL−15R/IL−2R signaling and distorted NK cell maturation contributes to the disease progression. Levels of soluble IL-2R in serum and surface expression of IL-2R on malignant PCs or mononuclear cells are significantly increased in MM, which also correlates with the active state of the disease (178, 179). Quite unexpectedly, defects related to the IL-21 axis affect NK cell lytic abilities but not the maturation, even though IL-21 promotes NK cell differentiation (180–182). The maturation capacity of NK cells, together with their ability to respond to the presence of malignant cells, is also reduced with age as the BM stroma deteriorates with time, thus the age of the patients may play a crucial role in this context (183).

The inhibitory role of Tregs in the NK cell differentiation was also confirmed both *in vitro* and *in vivo*. Presence of activated Tregs in the culture of HSCs, which were expanded with NK cell lineage differentiation protocol, led to 90% reduction in NK numbers compared to the control. Similar inhibitory role of Tregs was observed also in mice (184). This phenomenon seems to be caused by increased levels of membrane-bound TGF-β and active TGF-β signaling (184, 185).

Finally, the low numbers of NK cells represent a major issue not only from the view of disease control and progression, but it should also be noted that cancer patients are more susceptible to infections, which are one of the main causes of mortality in MM (186, 187). Understanding the maturation distortion and recovering the generation of mature NK subsets could be crucial for the therapy outcome and patient survival improvement.

Impairment of NK Cell Localization and Chemotaxis

TME-related downregulation or upregulation of chemotactic factors or their receptors is deeply beneficial for tumor growth, either to attract inflammatory or tumorigenic cells like MDSCs and TAMs, or to repel immunosuppressive cells. As already mentioned, it was well described that tumor infiltration by NK cells contributes to better prognosis. Thus, in hematological malignancies, it is only logical to expect the impairment of NK cell BM localization with consequent efflux to PB (161, 188). In general, several chemotactic receptors are expressed by NK cells, including CCR1, CCR2, CCR5, CCR7, CXCR1, CXCR3, CXCR4, CXCR6, CX3CR1, S1P5, CCRL2, and ChemR23. Indeed, aberrancies in these receptors were connected to lower NK cells' recruitment to the tumor (189).

CXCR4 is one of the key regulators of NK cell BM localization, and it is expressed in high levels by NK progenitors. With decreasing CXCR4 expression in mature stages, levels of CXCR3, CCR1, and CX3CR1 increase, whereas reduced CXCR4 expression, together with S1P5 activation, is necessary for NK cells to exit to the periphery and vice versa (190–192). In general, dysregulation in the CXCR3 and CXCR4 axes was connected to defective BM localization of NK cells in BM. Both of these pathways are closely connected. CXCR3 triggering possibly counteracts CXCR4-mediated BM retention by limiting the CXCR4 responsiveness. Only limited data are available about NK cell disrupted chemotaxis, BM localization and retention in MM, or in other hematological malignancies. In MM BM, several chemokine ligands engaging in NK cell BM localization show a disbalance, including increased levels of CXCL9 and CXCL10 (CXCR3 ligands) and decreased levels of CXCL12 (CXCR4 ligand) (**Figure 5**). Levels of CCL3, CCL5, and CX3CL1 ligands are most likely not subjected to any changes (193).

In other cancers, CXCL12 was confirmed to be downregulated, together with CXCR2 reduction on the surface of NK cells, though data suggest that these changes occur on the post-translational level (194). Another study revealed that tumor tissues tend to overexpress CXCL3 and CXCL5, while expression of CXCL1, CXCL2, and CXCL7 decreases (195). There are also hints, that deregulated CXCR3 signaling in malignant PCs could play a role in MM to EMD progression, although this needs to be confirmed (196). Also, IFN-γ-mediated CXCR3 activation was associated with lower overall survival, and it was proposed as an independent prognostic factor in MM (197). Indeed, inhibition of the CXCR3 axis resulted in better efficacy of IL-15 activated NK cells against malignant PCs (198). CXCR4 was proved to be downregulated in metastatic cells, which also demonstrates its role in malignancy dissemination (199).

Besides, it seems that obstructions in NK cell chemokine signaling and BM/PB localization are connected to the altered NK cell development and the prognosis-related CD56bright CD16- subset accumulation (as discussed previously). About 10%–20% of BM NK cells are localized in proximity to CXCL12 producing osteoblasts and reticular cells that are also able to express IL-15 and IL-15R. This localization is also dependent on the integrin chain α4 (200). Moreover, it was proved that the CXCR4/CXCL12 axis is essential for NK cell development in mice (201).

Chemokine signaling also actively participates in the recruitment of immune suppressor cells. CCR2 and CCR5 contribute to the migration of TAMs and MDSCs into the TME, whereas Tregs with higher expression of CXCR4 are attracted to the TME by their ligands CCL17 and CCL22, which can be produced by TAMs and cancer cells themselves (202). Further research is necessary to understand the chemokine ligand/receptor interactions between NK cells and TME. For example, studies evaluating chemokine/ligand expression profiles on MM cells and NK cell subsets in both BM and PB would probably uncover striking details regarding the role of chemokines in NK cell development and functional impairment, as well as MM-to-EMD progression.

NK Cell Inhibitory Receptors in MM

Blockade of the checkpoint axis PD-1/PD-1 ligand (PD-1/PD-1L) involved in the inhibition of the immune response has been discussed lately, although this therapy alone seems to be ineffective in MM and combination with other treatment approaches is necessary (203–205). In cancer, expression of PD–1L1 by tumor cells is considered an evasion mechanism promoting the suppression of immune cells (206). Malignant PCs in MM were shown to express higher levels of PD-1L compared to HD or MGUS patients, and significant upregulation can be observed in RR MM patients. Also, PD-1L expression on malignant PCs was connected with resistance to anti–myeloma agents, and the expression of this ligand on PCs was proposed as a marker of poor prognosis in combination with other factors such as age and cytogenetics (205, 207, 208).

Expression of PD-1 was confirmed on the surface of NK cells in MM while undetectable on healthy NK cells (209, 210). Indeed, it was proved that PD-1/PD-1L negatively regulates NK function. However, in this study, at least low levels of PD-1 were also detected on normal circulating or resting NK cells (211). To highlight the therapeutic potential, one study showed that inhibition of PD1/PD-1L signaling in NK cells can increase the degranulation or cytokine-producing ability *in vitro (*212). Of note, the data reflect the importance of a cautious approach during the flow cytometric detection of PD-1 and subsequent data evaluation since studies reported variable (low or none) levels of PD-1 on healthy NK cells. Furthermore, Pazina et al. encountered difficulties in the detection of any levels of PD-1 even in myeloma samples (165). A recent study proved that PD-1 mRNA and cytoplasmic PD-1 protein can be detected in NK cells, which suggests that surface PD-1 expression is inducible; hence, flow cytometry may provide variable data (213). There are data indicating the ominous role of the TME cellular compartment in PD-1/PD-L axis-related impairment of NK cells. PD-1L-positive MDSCs are present in higher frequency in cancer patients (214, 215). Furthermore, PD-1L expression in MM cells can be also induced by BM MSCs-derived IL-6, with subsequent engagement of JAK/TAT and MEK signaling (208, 216). Use of the JAK inhibitors (ruxolitinib) in MM truly

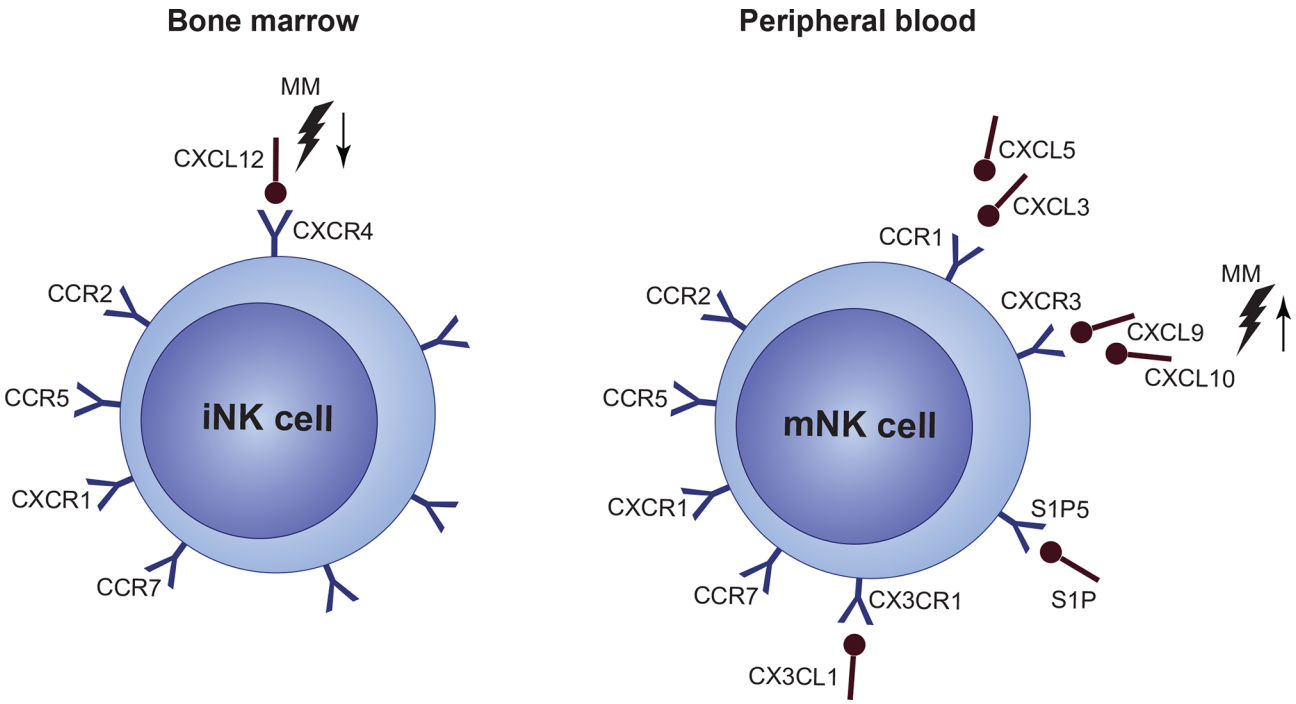

FIGURE 5 | Under normal conditions, immature NK cell (iNK cell) retention in the bone marrow (BM) is mediated via a high expression of CXCR4/CXCL12. Mature NK cells (mNK cell) express only low levels of CXCR4 and high levels of CCR1, CXCR3, CX3CL1 and S1P5 which mediate migration to the periphery. However, expression of individual chemokine receptors, or their ligands, as well as overall function of a crucial CXCR4/CXCR3 signaling, is impaired in multiple myeloma (MM). Occurring CXCL12 upregulation, together with CXCL9 and CXCL10 upregulation during the disease progression suggest, that NK cells are forced out of the BM by factors present in the MM niche. Furthermore, CXCR4/CXCR3 axes seems to be also crucial for the NK cell lineage development.

downregulates PD-1L expression in malignant PCs and makes them more susceptible to lenalidomide and steroids (217). Quite curiously, TGF-β, which is abundantly present in the MM niche, seems to have no effect on the expression of PD1-L1 and PD-L2 (218). However, other factors like IL-2, IL-7, and IL-15 were proved to upregulate levels of PD-1L (219). IL-2, IL-7, IL-15, IL-18, and IL-21 are able to upregulate the expression of surface PD−1 too, while IFN-α promotes the transcription of PD-1 (220, 221). HIF-1α is also directly involved in the PD-1L upregulation, which, together with the inhibitory role of HIF-1α in NK cells, propose a multilevel role of hypoxia in MM progression (222, 223).

CD94/NKG2A is an HLA-E-binding molecule recognized as an immune checkpoint like PD-1. Expression of this inhibitory receptor is increased in cancer-associated NK cells, which contributes to their exhaustion (224). Interestingly, HLA-E overexpression in tumors was connected to both poor and good prognosis (225, 226) Although there are data suggesting that the expression of NKG2A is not detrimental for the anti-MM activity of *in vitro* activated NK cells, NKG2A is still a valid target for consideration in immunotherapy. High levels of HLA-E in high-risk MM were proposed as a potential therapeutic candidate, and the experimental blocking of NKG2A by antibodies resulted in restored antitumor activity of NK cells (227–230). Among the MM niche factors, IFN-γ was proved to be involved in cancer-related HLA-E overexpression (231). Furthermore, HLA-E may serve to protect TAMs from CD94/NKG2A-mediated cell lysis (232). From the receptor point of view, TGF-β and IL-10 are among factors inducing the expression of NKG2A (224, 233). Also, IL-2 and IL-15 were shown to upregulate the expression of NKG2A, as well as NCRs, NKG2D, DNAM-1, and KIR2DL4 (234).

KIRs are crucial inhibitory regulators of NK cell response acting through interactions with MHC-I molecules, as already described. Tumor cells can temporarily upregulate their surface MHC-I expression to evade NK cell lysis. On the other hand, MHC-I downregulation is also a common mechanism to avoid immune response (235, 236). Masking the pathological origin (i.e., hiding the "missing-self" signal) by upregulating MHC-I levels is also a feature of MM PCs (237, 238). Moreover, increased levels of KIR molecules KIR2DL1 and KIR2DL2 were described on the PB NK cells of MM patients; however, no further details are available about involved mechanisms (61). Two strategies were proposed to exploit the KIR signaling for the therapy (239). The first is represented by HLA/KIR ligand-mismatched transplantation that showed promising results. Healthy donor NK cells provided a better response than NK cells of the patient, which are corrupted by the tumor inhibitory niche (240, 241). The second option is to directly block the KIR receptor with an antibody to inhibit its interaction with HLA ligand. However, in the MM clinical trial of the anti-KIR antibody IPH2101, this approach was ineffective due to the

monocytic trogocytosis of KIR molecules, eventually leading to the lack of education and hyporesponsiveness of NK cells (242). In addition, KIR downregulation leading to enhanced NK cell killing can be achieved also by the stimulation with IL-12, IL-15, and IL-18, while the expression can be restored back after 3 days of culture with IL-2, suggesting an interesting possibility of KIR exploitation with cytokine stimulation (243).

Recently, the upregulation of other possible novel therapeutic target molecules TIGIT, TIM-3, ICOS, and GITR on NK cells was proved in both the PB and BM of MM patients, which probably reflects on the additional immune evasion mechanism (165, 244). TIGIT is a newly identified NK cell immune checkpoint binding to PVR (CD155) and nectin-2 (CD112), which are shared ligands with activating molecule DNAM-1 (245). Nectin-2 was found to be overexpressed on MM PCs, and both PVR and nectin-2 expression were associated with poor prognosis in cancer (246, 247). TIGIT inhibition was proved to restore T-cell response in MM (248). Moreover, TIGIT ligands are highly expressed on cells residing in the BM, which also proposes a role of TIGIT signaling in the MM niche-mediated suppression of NK cell function (246). This is supported by the study showing that BM MSCs upregulate PVR on the surface of MM cells by IL-8 secretion (249). Also, a specific role of MDSCs in the TIGIT/CD155 axis was found. Co-culture of MDSCs with NK cells inhibited their cytotoxic abilities; however, this effect can be reversed either by the inhibition of ROS production (which led to upregulation of PVR in MDSCs) or by the blockade of TIGIT (250). Upregulation of TIM-3, a molecule associated with both inhibitory and activating functions, was also linked with cancer progression as well as CD8+ T cell exhaustion (251–254). In NK cells, it was proved that interactions of TIM-3 with its ligands HMGB1, CEACAM, phosphatidylserine, and galectin-9 inhibit the cytokine production and killing abilities (255). CEACAM ligand overexpression was described in MM, and the expression of HMGB1 was connected to therapy resistance and poor prognosis (256, 257). However, CEACAM downregulation was also correlated with cancer progression (258). These findings may only reflect a heterogeneous role of TIM-3 in the regulation of NK cells in cancer. Further investigation of inhibitory receptors is definitely necessary not only in the context of MM.

NK Cell Activating Receptors in MM

Both increased and decreased expressions of activating receptors were described in MM. These complex phenotypic changes are attributed to chronic ligand exposure and subsequent NK cell exhaustion (165, 259, 260). Ligands of these receptors were confirmed to be upregulated by MM PCs (238).

Downregulation of NKG2D, as well as 2B4/CD244 and NKp30, can be detected on BM NK cells but not in the PB of MGUS/MM patients (261). However, reduced levels of NKG2D (together with DNAM-1 and CD16) can be observed in both PB and BM of RR and post-SCT MM patients (165). These results reflect the fact that NK cell functional alteration is initiated in the MM BM and later, as the disease progresses, functional impairment is reflected even in circulating NK cells. NKG2D activation is induced by MHC-I-related ligands, which are upregulated as a signal of stress or malignant transformation (262). MIC-A, MIC-B, ULBP-1, ULBP-2, and ULBP-3 are well-known ligands for NKG2D. However, cancer cells are able to downregulate and shed these molecules from their surface. It was shown that high volumes of soluble NKG2D ligands, together with exosomes, are released from tumor cells to chronically exhaust T and NK cells (263–265). However, contradictory data were also published showing that another soluble NKG2D ligand, MULT-1, promotes NK cell function and tumor killing in mice. Nonetheless, the question is whether long-term exposure would not lead to effector cell exhaustion as well (266). One of the mechanisms behind the downregulation and shedding of NKG2D ligands is most likely TGF-β-induced expression of MMP2 (218). MIF was also proved to contribute to the transcriptional downregulation of NGD2D in NK cells (267). Moreover, the expression of NKG2D and NKG2D ligands is downregulated by IDO (indoleamine-2,3-dioxygenase) (268). Recently, CAR-NK cells transduced to express NKG2D-CAR showed a very good anti–myeloma efficacy *in vivo*, with minimal activity against healthy cells. Considering the greater efficacy and lesser toxicity compared to CAR-T cells, these are promising results reflecting the possible use of autologous-engineered CAR-NK cells in the treatment of MM (269).

SLAMF7 (CS1, CRACC, and CD319) is a surface signaling lymphocytic activation molecule (SLAM family) expressed on NK cells and PCs (both normal and malignant), while undetectable in other cells, which makes it a valid target for MM therapy (270, 271). Increased levels of surface SLAMF7 on NK cells were correlated with a worse prognosis in MM (165). Moreover, malignant PCs were proved to cleave SLAMF7 from their surface, leading to increased levels of soluble SLAMF7, which can be detected in MM patients, but not in MGUS. Thus, levels of SLAMF7 can be associated with disease progression. Data also confirmed that soluble SLAMF7 promotes MM cell growth *via* interaction with surface SLAMF7 on MM cells, with subsequent activation of ERK and SHP-2 signaling (272). Furthermore, it was predicted that soluble SLAMF7 could potentially interfere with the novel targeted therapy (273). Anti-SLAMF7 elotuzumab was FDA-approved in 2015, and since then, it has shown promising results in clinical studies. Elotuzumab in combination with lenalidomide and dexamethasone or bortezomib showed increased effectiveness and sustained benefit in progression free survival (274–276). In NK cells, elotuzumab binds to the CD16, which mediates ADCC against the anti-SLAMF7 antibody coupled with SLAMF7 on MM cells. Also, other mechanisms of action include NK cell co-stimulation through NKp30 and NKG2D, stimulation of IFN-γ and granzyme B secretion, as well as macrophage–mediated antibody dependent cell phagocytosis (276, 277). Very intriguing are findings indicating that anti-SLAMF7 antibodies disrupt adhesion of MM PCs to MSCs in the BM. This indicates that elotuzumab might be one of the pioneering agents with a multiple-hit strategy, both against malignant PCs as well as MM niche components (267).

As already mentioned, other activating receptors were proved to be downregulated in MM, including DNAM-1, 2B4 (CD244), and CD16 (165, 261). TGF-β was confirmed as one of the factors causing the downregulation of 2B4 and 2B4 adaptor proteins (DAP10 and SAP) (278, 279). Early studies on 2B4 showed an activating function of this receptor leading to increased killing and IFN-γ production; nonetheless, further research also proved an inhibitory role of this molecule (280, 281). Upregulation of 2B4 and downregulation of the associated adaptor protein SAP were related with inhibitory signaling, while downregulation of 2B4 and normal levels of SAP were associated with activating signaling (282). Among others, downregulation of 2B4 results in defective interactions with its ligand CD48. This also affects the co−stimulation of NCRs mediated through the 2B4-CD48 signaling (259). Lately, 2B4, which is also a member of SLAM family, was proposed as a target for immunotherapy, which could potentially have a double-hit impact affecting MM niche similar to SLAMF7. In particular, 2B4 expression was confirmed on MDSCs (283). Downregulation of CD16 logically results in impaired ADCC (259). However, counterintuitively, it was published that shedding of CD16 from the surface of NK cells leads to the positive stimulation of the immune response by engagement of other immune cells (284). Indeed, levels of soluble CD16 in the serum are significantly decreased in patients with MM compared to MGUS or healthy donors. This was also correlated with the disease staging (285).

The NCR family consists of 3 receptors: NKp30 (NCR3), NKp44 (NCR2), and NKp46 (NCR1). These molecules were originally categorized as activating receptors; nonetheless, it seems that different isoforms of NCRs may exist based on the environment, which then deliver either activating or inhibitory response. Blocking of individual NCRs with mAbs is rather ineffective, while effective inhibition caused by a combination of mAbs against multiple NCR receptors suggest a cooperative mechanism in the process of NK cell activation (286, 287). A positive role of NCRs in cancer control was proved by several studies. NKp46 was connected to metastatic prevention and the potentiation of NK cell antitumor activity by increased IFN-γ production (288, 289). Nevertheless, the activity and function of NCRs can be downregulated in cancer, which, particularly in NKp46, is associated with the progression of malignancy too (290, 291). In MM, NKp30 was proved to be downregulated on BM NK cells, but not in the PB (261). Also, increased expression of NKp30 (on CD56dim CD16+ subset) and NKp44 (CD56bright CD16- and CD56dim CD16+) and decreased expression of NKp44 (CD56bright CD16-) can be observed on PB NK cells in RR MM, but it is not possible to detect any similar changes in ND MM. Again, these data support the fact that functional NK cell properties are impaired preferentially in the site of disease manifestation, and that the impairment is minor (or the function is restored) in circulating NK cells during early states of MM. However, as the disease progresses, further dysfunctional evolution is reflected even in PB NK cells (165). NCRs can be activated by different viral and bacterial ligands, growth factors, ECM−derived and membrane-derived components, or stress related ligands (286). In cancer, several molecules were proved to play a role in NCR activation and cancer cell elimination, though data related to NCR ligands in cancer cells is scarce. B7−H6, BAG-6, and Galectin-3 were confirmed as NKp30 ligands. PCNA, NKp44L (a MLL5-variant protein), and PDGF-DD (platelet-derived growth factor isoform) are among the ligands of NKp44 that might play a role in cancer cell elimination (292–294). However, similarly to what was described in NKG2D ligand shedding, cleavage of B7-H6 ligands by the ADAM-10 and ADAM-17 MMP-related mechanism was showed to chronically exhaust NK cell actions mediated through the NKp30 receptor (295, 296). Galectin-3 can also be released in soluble form by cancer cells to inhibit the NKp30 function (297). Furthermore, tumor-derived TGF-β is one of the factors involved in NKp30 downmodulation (298). In hypoxic conditions, NK cells upregulate HIF−1α, and, curiously, maintain the killing abilities mediated *via* CD16. Nonetheless, the function of activating receptors, including NKp30, NKp44, and NKp46, is impaired (299). NCR-related therapeutical options are not clearly elucidated, though NKp30 was proposed as a target for immunotherapy. However, only CAR-T cells targeting this receptor has been explored, and data relevant to anti-NKp30 mAbs are still missing. Further research is needed regarding the NCR impairment, ligand identification and expression, as well as possible therapeutic options (300).

Impact of Anti-Myeloma Therapy on NK Cells

As already mentioned, NK cells are important cells mediating the anti-tumor effect of novel mAbs used in the treatment of MM, such as daratumumab, isatuximab, or elotuzumab, and induction of ADCC represents one of several important mechanisms of action induced by these antibodies. Moreover, additional effects of daratumumab mediated *via* NK cells were described, including monocyte activation, phagocytosis, and increased T-cell costimulatory abilities. Hence, any disruption of NK cell immune function might be of great concern and the overall impact of anti-myeloma therapy, including the above-mentioned mAbs, immunomodulatory drugs (IMiDs), or proteasome inhibitors, on NK cells needs to be studied thoroughly (301).

Since CD38 is expressed on the surface of NK cells as well, the question of whether anti-CD38 agents negatively affect or even possibly kill NK cells was raised. Indeed, it was described that daratumumab depletes CD38+ MDSCs, B cells, and Tregs (302). Data published 2 years later confirmed that CD38+ NK cells are also subjected to ADCC induced by daratumumab bound on their surface, which suggested an alarming issue of anti-CD38 therapy (303). A significant reduction of NK cell numbers can be detected in PB and BM of MM patients after initiation of the daratumumab-containing therapy, with the persistence of low NK cell counts during the whole course of the treatment. Nonetheless, no adverse effects on the overall efficacy of the therapy or function of NK cells were discovered. Furthermore, additional immunomodulatory mechanisms of daratumumab participating in the overall therapy efficacy were shown, including increased frequency of CD8+ T cells with

preferential generation of effector memory subset (304, 305). Isatuximab was described to mediate even stronger efficacy in the killing of target cells compared to daratumumab, and also the drug was confirmed to induce apoptosis of Tregs with higher CD38 expression than other T cells (28). Similar to daratumumab, reduction of NK cells can be observed after isatuximab application as well, together with the depletion of CD38high B-lymphoid progenitors. Isatuximab-treated NK cells exhibit deregulation of 70 genes, mostly connected to chemotaxis, cytolysis, and immune defense response (28, 95, 306). Anti-SLAMF7 mAb elotuzumab also strongly stimulates NK cell activation, induction of ADCC, and degranulation *via* engagement of CD16. Calcium signaling costimulation triggered by engagement of NKp46 and NKG2D in CD16-independent manner is also activated by this antibody. Regimens containing elotuzumab plus lenalidomide or bortezomib showed promising results, while no adverse effects of elotuzumab on the overall function or frequency of NK cells were observed (307).

Furthermore, proteasome inhibitors such as bortezomib or carfilzomib were described to potentiate NK cell cytotoxicity against MM cells, while no considerable adverse effects on NK cells were reported. Sensitization by downregulation of HLA-I molecules on the surface of malignant PCs is one of the involved mechanisms. Other mechanisms were revealed in studies involving other types of cancer, including bortezomib-induced upregulation of NK cell activating receptor ligands (MIC-A/B, ULBP-1) or ligands related to the death receptor signaling (Fas, DR-5) (308–310).

Immunomodulatory drugs (IMiDs), such as thalidomide, lenalidomide, and pomalidomide, significantly improved therapy outcome in the past two decades and represent indispensable agents that are used to treat MM (311). These agents exhibit pleiotropic anti-MM potential, including anti-angiogenic, anti-inflammatory, immunomodulatory, and anti-proliferative effects (312). In theory, earlier it was proposed that IMiDs could enhance impaired function of immune cells. As a matter of fact, studies confirmed that increased numbers of NK cells can be detected in patients receiving thalidomide therapy, and the positive effect of IMiDs on costimulation of T cells, NK cell proliferation, and their cytotoxic abilities was confirmed as well. Upregulation of IL-2 signaling, along with upregulation of PVR and MIC-A ligands, was discovered to participate in the IMiD-mediated stimulation of NK cells (312–314). Nonetheless, no positive effect of lenalidomide on NK cell activation, degranulation or secretion of IFN-γ or MIP1-β was observed in the study that was monitoring NK cell activity and functionality in 10 MM patients treated with lenalidomide-containing regimen and then maintained with lenalidomide. Progressive post-maintenance NK cell lineage normalization was observed, albeit this was possibly caused by the chemotherapy discontinuation (315). On the other hand, a positive effect of pomalidomide on innate lymphoid cells (ILCs), which are recently discussed lymphoid cells with antitumor potential, was described. Results indicate that pomalidomide leads to enhancement of ILC function through the stimulation of IFN-γ production as well as downregulation of Ikzf1 and Ikzf3, which are transcription factors essential for MM cell proliferation. Similar degradation by ubiquitination of Ikzf1 and Ikzf3 was confirmed by lenalidomide (316, 317).

DISCUSSION

Defects of NK cell cytokine production, chemotaxis, maturation, effector molecule expression, and related target recognition and killing are described in the context of MM BM or TME in general, although clearly there are large gaps in current knowledge. Unfortunately, some data are even contradictory probably due to the complexity and heterogeneity of the malignant niche and occurring interactions. An overall disruption of NK cell function was correlated with MM and cancer progression; thus, potentiating and restoring the proper NK cell abilities, maturation, and BM localization, as well as normalizing the BM niche, are for sure among the goals for future improvement of patients' survival and quality of life.

Data covering interactions between NK cells and individual cellular or non-cellular components of the MM niche, which would describe particular mechanisms of NK cell functional impairment, are extensively incomplete. Furthermore, additional information about disrupted NK cell chemokine signaling during MM progression are needed. Data describing the distribution of NK progenitors in the malignant marrow in general are missing completely. Similarly, there is only limited information about mechanisms behind the NK cell maturation distortion during disease progression in the BM. Restoring the generation of fully functional NK cells with normal chemotactic abilities may be critical for the future improvement of therapeutic options. Furthermore, data related to NK cell immune monitoring and expression profiles of surface effector molecules in the EMD are missing. These would provide critical information about the functional capacities of these cells, as well as about levels of potential targets.

Altogether, NK cells and their surface effector molecules represent a tempting therapeutic target in MM and other malignancies, although recent data suggest that combination with conventional protocols is needed in the present. Thus, further research that would uncover all the possible interactions between these receptors, their cognate ligands, as well as interfering factors and cells in the malignant niche is necessary. Moreover, all the data highlight the necessity of further research in the field of IMiDs as well as novel mAbs and proteasome inhibitors used for the treatment of MM. Their mechanisms of action or impact on NK cells or ILCs is still not fully understood. Promising results were published, but unfortunately, most of the available data were generated by *in vitro* or *in vivo* assays, and studies involving MM patients are scarce.

AUTHOR CONTRIBUTIONS

All authors made a substantial contribution to the manuscript preparation. OV prepared graphical figures, and conceived and

wrote the review. JB, BM, TJ, and RH commented and edited first versions of the manuscript and participated on the final version. All authors contributed to the article and approved the submitted version.

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REVIEW
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Burgeoning Exploration of the Role of Natural Killer Cells in Anti-PD-1/PD-L1 Therapy

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Antibodies targeting programmed death receptor-1 (PD-1)/programmed death ligand-1 (PD-L1) have been considered breakthrough therapies for a variety of solid and hematological malignancies. Although cytotoxic T cells play an important antitumor role during checkpoint blockade, they still show a potential killing effect on tumor types showing loss of/low major histocompatibility complex (MHC) expression and/or low neoantigen load; this knowledge has shifted the focus of researchers toward mechanisms of action other than T cell-driven immune responses. Evidence suggests that the blockade of the PD-1/PD-L1 axis may also improve natural killer (NK)-cell function and activity through direct or indirect mechanisms, which enhances antitumor cytotoxic effects; although important, this topic has been neglected in previous studies. Recently, some studies have reported evidence of PD-1 and PD-L1 expression in human NK cells, performed exploration of the intrinsic mechanism by which PD-1/PD-L1 blockade enhances NK-cell responses, and made some progress. This article summarizes the recent advances regarding the expression of PD-1 and PD-L1 molecules on the surface of NK cells as well as the interaction between anti-PD-1/PD-L1 drugs and NK cells and associated molecular mechanisms in the tumor microenvironment.

Keywords: tumor, immune checkpoint inhibitor, natural killer cell, programmed death receptor-1, programmed death-ligand 1

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1 INTRODUCTION

Antibodies targeting programmed death receptor-1 (PD-1) and programmed death ligand 1 (PD-L1) have been approved for the treatment of a variety of solid and hematologic malignancies; patients with various malignancies and even those with a very advanced disease showed durable responses to this treatment (1–3). However, only 10–20% of patients with different tumor types respond well to PD-1/PD-L1 blocking therapy (3, 4). In addition, treatment with anti-PD-1/PD-L1 monoclonal antibodies (mAbs) can lead to unexplained clinical responses of tumors with no or low expression of major histocompatibility complex (MHC) and/or PD-L1 (2, 4, 5). Therefore, a better understanding of the mechanisms of action of anti-PD-L1 (or anti-PD-1) mAb therapy and the impact of this therapy on each component of the tumor immune microenvironment will help improve the precision of cancer immunotherapeutics in the future. Most believe that antibodies targeting PD-1 and PD-L1 are largely only beneficial for eliciting T cell-driven responses, but accumulating evidence suggests that blocking the PD-1/PD-L1 axis may also improve

natural killer (NK)-cell function and activity and enhance antitumor cytotoxicity through direct/indirect but crucial mechanisms. For example, Hodgkin lymphoma cells with defective MHC class I expression responds well to anti-PD-1 mAb therapy, which suggests the presence of immune responses that are independent of cytotoxic $CD8^+$T cells, inhibited by PD-1, and rescued by anti-PD-1 therapy (5–7). NK cells show MHC-independent antitumor cytotoxicity, which allows them to exhibit killing effects on many tumor types with absent or low MHC expression and/or low neoantigen burden. Recently, some studies have reported evidence of PD-1 and PD-L1 expression in human NK cells (8, 9). *In vivo* mechanistic studies on whether and how PD-1 or PD-L1 plays a role in NK-cell response to tumors and whether and how PD-1/PD-L1 blockade mobilizes NK cell response remain scarce; however, with the development of multi-omics and high-throughput sequencing technologies, the understanding of this field has gradually deepened and progressed. Based on this knowledge, in this article, we comprehensively review the expression of PD-1 and PD-L1 molecules on the surface of NK cells and discuss the interactions between anti-PD-1/PD-L1 drugs and NK cells in the tumor microenvironment (TME) as well as the associated molecular mechanisms.

2 NK CELL FUNCTION AND PHENOTYPE

NK cells are mainly involved in killing microbes and malignantly transformed allogeneic and autologous cells without prior sensitization and exhibit non-MHC-restricted antitumor cytotoxicity (10). Activated NK cells exert a strong cytotoxic effect by inducing the secretion of cytotoxic mediators and the production of inflammatory cytokines and chemokines through integration of adhesion molecules and receptor signaling activation (11). According to the CD56 density on the cell surface, NK cells can be divided into $CD56^{dim}$ (~90%) and $CD56^{dim}$ (~10%) cells (12). $CD56^{bright}$ mainly performs immunoregulatory function by secreting cytokines, while $CD56^{dim}$ mainly performs cytolytic function by secreting granzyme B and perforin, which enhance the expression of immunoglobulin-like receptors and Fcγ receptor III (FcγRIII)/CD16 (13, 14). Induction and regulation of NK cell function is mediated by a range of activating or inhibitory surface receptors. In humans, the main activating receptors involved in target-cell killing include natural cytotoxic receptors (NCRs) (including NKp46, NKp30, and NKp44) and NKG2D (15). FcγRIII is also an activating receptor that is mainly expressed by $CD56^{dim}$NK cells and is essential for antibody-dependent cell-mediated cytotoxicity (ADCC) against IgG-coated target cells (16). Conversely, MHC class I antigen-specific inhibitory receptors on the surface of NK cells, namely, killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LIRs), and natural killer group 2 A (NKG2A), tightly regulate the cytotoxicity mediated by these cells and the production of lymphokines, by recognizing self MHC-I antigens (17, 18). In addition, several non-MHC-specific inhibitory NK receptors have been identified, including classical cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, and T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (TIGIT), CD96, lymphocyte-activation gene 3 (LAG-3), and T cell immunoglobulin-3 (TIM-3) (19). Sheffer M et al. (20) systematically defined the molecular signature of human tumor cells that determines their sensitivity to human allogeneic NK cells and found that the transcriptional signature of NK cell-sensitive tumor cells correlates with immune checkpoint inhibitor (ICI) resistance in clinical samples. The study has also applied genome-scale CRISPR-based gene editing screens in several solid tumor cell lines to functionally interrogate which genes in tumor cells regulate responses to NK cells. Tumor cells escape immune responses by regulating the expression of inhibitory receptors on NK cells, and in this context, PD-1/PD-L1 axis has been studied extensively; understanding the expression of NK cell surface receptors and their role in the functional activity of NK cells is essential for the development of effective immunotherapies.

3 PD-1/PD-L1 ON NK CELLS: EXPRESSION, REGULATION, AND EFFECTS ON CELL FUNCTION

3.1 PD-1 on NK Cells: Expression, Regulation, and Effect on Cell Function

PD-1 is a member of the immunoglobulin superfamily. It can be expressed on various immune cells, including T ($CD4^+$ and $CD8^+$) cells, B cells, bone marrow cells, NK cells, and other innate lymphocytes (ILCs) (21–23). When bound to ligands (PD-L1 and PD-L2) that may be expressed on tumor cells, PD-1 may play a role in impairing antitumor effects and facilitating tumor immune escape (21–23). In recent years, researchers have shown interest in targeting PD-1 to increase NK activity. However, PD-1 expression on NK cells is diverse and difficult to clarify. High expression of PD-1 on NK cells can be detected in the peripheral blood of approximately one-quarter of healthy individuals (22). PD-1, which is usually not expressed on $CD56^{bright}$ NK cells, is confined to fully mature NK cells of $NKG2A^-KIR^+CD57^+CD56^{dim}$ phenotype (24). In the TMEs of various cancers, such as ovarian cancer (ascites), Kaposi's sarcoma (peripheral blood), renal cell carcinoma, and multiple myeloma, the proportion of PD-1^+ NK cells and the expression of PD-1 on NK cells increase (25–28). PD-1 expression on peripheral and tumor infiltrating NK cells from patients with digestive cancers was increased (29). In addition, chronic infections such as those caused by human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human cytomegalovirus (HCMV) have also been shown to enhance PD-1 expression on NK cells (30, 31).

PD-1, an important checkpoint of NK activation, is more abundantly expressed in activated NK cells than in inactivated NK cells (9). Functional and phenotypic assays showed that PD-1^+ NK cells had the highest functional activity when stimulated and that most of these cells expressed activation markers (CD69

and Sca-1) (32). A higher frequency of circulating PD-1$^+$ NK cells (mean > 9%) was associated with a better overall survival (OS) in patients with head and neck cancers (HNCs) (33), which indicates that PD-1$^+$ NK cells are not necessarily inactive in PD-L1$^+$ tumors but may only be inhibited from killing tumor cells. The interaction of PD-1 with its ligands provides a negative signal for the activation of NK cells, which causes the NK cells to express a dysfunctional, exhausted phenotype (26) and an inhibited antitumor ability (29). Several murine tumor models have shown that PD-1$^+$ NK cells present at the site of PD-L1$^+$ tumors exhibit an exhaustive phenotype and that PD-1/PD-L1 interaction strongly inhibits NK cell-mediated antitumor immunity (32). Overall, PD-1$^+$ NK cells exhibit enhanced apoptotic sensitivity, reduced cytolytic activity, impaired cytotoxic and cytokine production efficiencies, and reduced proliferative capacity in PD-L1$^+$ tumors (24, 29). In addition, immune cells in TME can also affect NK cells by expressing PD-L1. For example, tumor-associated neutrophils (TANs) can impair the cytotoxicity and infiltration capacity of NK cells, and downregulation of CCR1 leads to diminished infiltration capacity of NK cells and reduced responsiveness of the NK-activating receptors NKp46 and NKG2D (34); CD56^{dim}PD-1$^+$ NK cells expressed in patients with Hodgkin lymphoma are efficiently inhibited by PD-L1-expressing myeloid cells (35).

The molecular mechanisms regulating PD-1 expression in human NK cells have not yet been elucidated. Signaling by cells and/or soluble factors in the TME may play a major role, and cytokines may mediate crosstalk between different immune checkpoints. For example, it has been demonstrated that tumor-derived interleukin (IL)-18 increases the immunosuppressive CD56^{dim}CD16$^{dim/-}$ NK cell fraction in patients with triple-negative breast cancer (TNBC) and induces the expression of PD-1 in these cells (36). The G-CSF/STAT3 pathway and IL-18 are responsible for upregulating PD-L1 expression on TANs and NK cells, respectively; transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ) impair NK cell cytotoxicity by upregulating the expressions of PD-L1 and PD-1 on tumor cells and NK cells, respectively (37). In addition, chemotherapeutic agents can upregulate PD-1 expression on NK cells and PD-L1 expression on tumor cells through nuclear factor kappa B (NF-κB) (38). In future studies, it is important to identify the mechanisms that lead to the expression of PD-1 on NK cells in the TME and their importance in NK cell-based immunotherapy.

3.2 PD-L1 on NK Cells: Expression, Regulation, and Effect on Cell Function

IFN-γ secreted by activated T cells can stimulate the upregulation of PD-L1 expression on the surface of tumor cells and transmit inhibitory signals to T cells after PD-L1–PD-1 binding, which results in T cell dysfunction and tumor immune escape (39). PD-L1 is reported to be expressed on tumor cells as well as immune cells within the TME, including antigen presenting cells (APCs) (mainly macrophages and dendritic cells [DCs]), activated/depleted T and B lymphocytes, regulatory T cells (Tregs), and NK cells (8, 40). According to a comprehensive review by Sun et al. (41), the regulation of PD-L1 expression and function occurs at different levels. Several inflammatory mediators, including TNF-α, IFN-γ, IL-10, IL-17, and C5a, are inducers of PD-L1 expression (42–44). The JAK/STAT, RAS/MAPK, and PTEN-PI3K/AKT pathways are involved in the control of PD-L1 gene expression through different downstream transcription factors, such as STAT1, STAT3, IRF1, IRF3, HIF-1α, MYC, JUN, BRD4, and NF-κB (45). Corresponding DNA-binding elements other than IRF3 have been described on the PD-L1 gene promoter (46–48). Other regulatory mechanisms include microRNA (e.g., miR-513, miR-34a, miR-200, and miR-570)-mediated post-transcriptional repression and the presence of soluble PD-L1 (sPD-L1) in the blood, which may compete with membrane-bound PD-L1 for binding to PD-1 to regulate cell surface PD-L1 expression (41, 49).

IFN-γ is one of the most studied inducers of PD-L1 expression in tumors, and NF-κB (a major transcription factor of inflammation and immunity) is involved in NK cell activation to regulate IFN-γ production (50). Researchers have proposed that NF-κB directly induces PD-L1 gene transcription by binding to the latter's promoter and that it post-transcriptionally regulates PD-L1 through an indirect pathway; thus, NF-κB may be a key positive regulator of PD-L1 expression in tumors (51). In addition, the PD-L1 promoter contains a hypoxia-inducible factor 1-alpha (HIF-1α) response element (52, 53), which drives the IKK-β gene transcription through a hypoxia response element present in the promoter while supporting NF-κB pathway activation by directly inducing p65 (54, 55); NF-κB can also induce HIF-1α transcription by directly binding to the HIF-1α promoter (56). Thus, both HIF-1α and NF-κB pathways can initiate and maintain PD-L1 expression and reinforce each other through positive feedback. Thus, the possibility that these signaling pathways can regulate PD-L1 expression in NK cells must be explored. A recent study confirmed that some myeloid leukemia cell lines and acute myeloid leukemia (AML) blasts from patients can induce PD-L1 signaling in NK cells through the PI3K/AKT/NF-κB pathway (8). Notably, the expression levels of two activating antigens, CD69 and CD25, were significantly higher in PD-L1$^+$ NK cells than in PD-L1$^-$ NK cells, which indicates that PD-L1$^+$ NK cells show an increase in antitumor cytotoxic effector function and IFN-γ-mediated CD69 expression. The study revealed that the higher the sensitivity of the target cells to NK cell cytotoxicity (due to its negative correlation with MHC-I molecule expression), the greater the directness of the cell-cell contact between NK cells and target cells, the higher the expression of PD-L1, and the stronger the activation of CD69 NK cells (8). Therefore, PD-L1 expression on NK cells may serve as an *in vivo* biomarker for sensitivity to NK cell lysis in patients with tumors. Nonetheless, the expression and function of PD-L1 on NK cells and the involvement of PD-L1$^+$ NK cells in anti-PD-L1 mAb therapy has not been comprehensively explored. An improvement in the understanding of PD-L1 expression on NK cells and signaling regulatory mechanisms is essential to understand tumor and immune cell biological characteristics and to develop NK cell-based antitumor immunotherapy.

4 EFFECTS OF ANTI-PD-1/PD-L1 ANTIBODIES ON NK CELL FUNCTION AND CORRESPONDING REGULATORY MECHANISMS

Existing literature has shown the potential benefits of anti-PD-1/PD-L1 therapy in rescuing and/or improving NK cell function and improving antitumor immune responses (28, 29, 33, 57). Researchers have found that disrupting the PD-1/PD-L1 interaction can enhance the killing effect of NK cells on tumor cells of mice with several cancers, and in some models, PD-1/PD-L1 blockers are completely ineffective when mouse NK cells are depleted (32). In humanized (Hu) NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice xenografted with dedifferentiated liposarcoma (DDLPS), abundance of hCD8$^+$ T subsets, such as hCD8$^+$ IFN-γ$^+$, hCD8$^+$PD-1$^+$, and hCD8$^+$Ki-67$^+$ cells, and hNK subsets, such as hCD56$^+$IFN-γ$^+$, hCD56$^+$PD-1$^+$, and hCD56$^+$Ki-67$^+$ cells, is functionally associated with anti-PD-1 effects (58). In addition, a significant increase in the number of activated hCD56$^+$NKp46$^+$NKG2D$^+$ NK cells was also detected, which indicates that NK cells play a pivotal role in the antitumor effect of anti-PD-1 therapy (58).

PD-1/PD-L1 blocking may rescue multiple aspects of NK cell-mediated antitumor immune activity (**Figure 1**). Firstly, most studies have shown that PD-1 expression on the surface of NK cells is upregulated in the TME, and this expression plays a negative immunoregulatory role after binding to the corresponding ligands and is associated with poor tumor prognosis (29, 32, 33). PD-1/PD-L1 blockade can activate NK cells by preventing inhibitory signals between PD-1$^+$ NK cells and PD-L1$^+$ target cells. Secondly, the activity of NK cells is determined by a series of activation and inhibition signals, and PD-1 blockade *via* antibodies activates some positive regulatory signaling pathways or prevents other intracellular inhibitory signals (8); furthermore, the unique ADCC effect of NK cells may be activated and enhanced by some PD-1/PD-L1 blockers, and this effect triggers strong antitumor activity (59). In addition, anti-PD-1/PD-L1 therapy may indirectly affect NK cell function through other immune cells in the TME (60, 61). NK cells may, in turn, enhance tumor immune responsiveness to PD-1 antibodies by affecting other immune factors in the TME. These mechanisms are reviewed in detail below.

4.1 Direct Interaction of Anti-PD-1/PD-L1 Antibodies With NK Cells

4.1.1 Anti-PD-1/PD-L1 Antibodies Block PD-1/PD-L1 Inhibitory Signaling in NK Cells

As discussed above, the binding of PD-L1 expressed by tumor cell surface to PD-1 expressed by activated NK cells potentially inhibits NK cell-dependent immune surveillance and mediates antitumor immune responses. However, studies on the mechanism by which PD-1 inhibits NK cell response against tumors and the possibility of inducing NK cell responses *via* PD-1/PD-L1 blockade are still scarce. In one study, CD16- or ILCs-activated NK cells showed upregulated expression of PD-1, as well as CD69, CD107a, IFN-γ, and granzyme B; however, the expression of these molecules was downregulated and that of CD16 surface density was significantly downregulated after the binding of PD-1 to PD-L1 (33). PD-1 blockade increased NK cell activation and cytotoxicity, but only in patients with HNCs showing high PD-L1 expression (33). Another study used anti-PD-1/PD-L1 antibodies to block the interaction between PD-1and PD-L1 and found that this strategy could help reverse PD-1 inhibition and the dysfunctional state of PD-1$^+$ NK cells. This blockade leads to a significant increase in NK cell cytotoxicity and cytokine production, inhibition of tumor growth *in vivo*, and

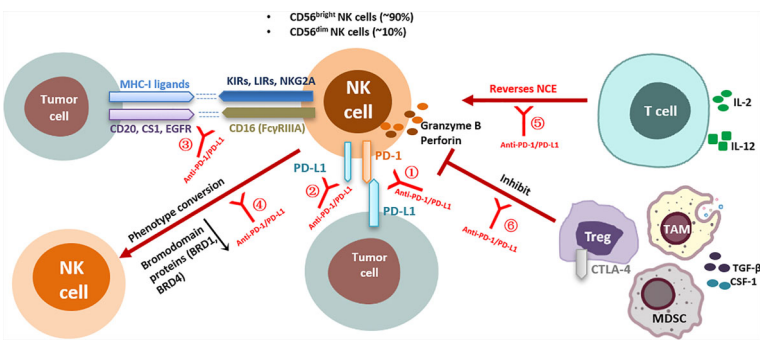

FIGURE 1 | Effects of anti-PD-1/PD-L1 antibodies on NK cell function and corresponding regulatory mechanisms. Direct interaction of anti-PD-1/PD-L1 antibodies with NK cells: ①Anti-PD-1/PD-L1 antibodies block PD-1/PD-L1 inhibitory signaling in NK cells; ②Anti-PD-1/PD-L1 antibodies enhance PD-L1$^+$ NK-cell antitumor activity; ③Anti-PD-1/PD-L1 antibodies enhance the ADCC effect of NK cells; ④Anti-PD-1/PD-L1 bispecific antibodies induce phenotypic transformation of NK cells; Indirect interaction of anti-PD-1/PD-L1 antibodies with NK cells: ⑤Anti-PD-1/PD-L1 antibodies indirectly reverse NK cell exhaustion by affecting CD8$^+$ T cell activity; ⑥Anti-PD-1/PD-L1 antibodies relieve the inhibition of NK cell function by affecting Tregs. KIRs, killer cell immunoglobulin-like receptors; LIRs, leukocyte immunoglobulin-like receptors; NKG2A, natural killer group 2 A; CTLA-4, cytotoxicT-lymphocyte-associated protein 4; IL, interleukin; NCE, NK cell exhaustion; PD-1, programmed death receptor-1; PD-L1, programmed death-ligand 1; MHC, major histocompatibility complex; TGF-β, transformation growth factor-β; IFN-γ, interferon-γ; Tregs, regulatory T cells; CSF-1, colony-stimulating factor 1; EGFR, epidermal growth factor receptor; NK cell, natural killer cell; TAMs, tumor-associated macrophages; MDSCs, myeloid-derived suppressor cells.

obvious improvement in NK cell-based antitumor response (32). Similarly, a previous study found that blocking PD-1/PD-L1 signaling on the surface of NK cells significantly enhanced IFN-γ production, CD107a expression, and cell degranulation, and inhibited NK cell apoptosis *in vitro*. More importantly, PD-1 blocking antibody was found to significantly inhibit the growth of xenograft tumors, and NK depletion completely abolished this inhibition of tumor growth (29). However, inhibition of tumor growth could be completely abolished by NK depletion. Furthermore, PD-1 may exert its inhibitory effect on NK cells by interfering with AKT activation, and PD-1/PD-L1 blockade activates NK cells by enhancing the PI3K/AKT signaling pathway in them (29). In addition, anti-PD-1/PD-L1 antibodies can enhance the secretion of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by inhibiting PD-1 in NK cells, effectively improving the antitumor activity of these cells (62). IFN-β has been shown to activate NK cells and induce cytotoxicity against tumor cells by upregulating NK cell-surface membrane-bound and soluble TRAIL expression, which leads to subsequent activation of the TRAIL receptor signaling pathway and apoptosis in nasopharyngeal carcinoma (NPC) cells (62). Subsequently, blocking the PD-1/PD-L1 checkpoint in the presence of IFN-β further increased the killing activity of NK cells against NPC cells, and this suggests that blocking PD-1 in activated NK cells may increase the secretion of soluble TRAIL and contribute to the killing of TRAIL NPC cells (62). This study revealed a new mechanism by which IFN-β and anti-PD-1 antibodies enhance the antitumor effect of NK cells and provide ideas for the therapeutic strategy of the combination of IFN-β and anti-PD-1.

Recently, researchers have found that repetitive irradiation can increase PD-L1 levels in non-small cell lung carcinoma (NSCLC) cells while reducing NKG2D ligand levels, which may reduce the sensitivity of lung tumor cells to the cytotoxic effect of NK cells and the possibility that tumor cells escape immune responses (63). Mechanistic studies revealed that IL-6-MEK/ERK signaling contributes most significantly to the upregulation of PD-L1 or downregulation of NKG2D ligand in radioresistant cells (63), while in another similar study, IL-6-JAK/STAT3 signaling was shown to contribute significantly to this process (64). Subsequently, researchers examined PD-1 levels in NK cells and found that PD-1 expression could not be detected in primary NK cells, but this expression increased when NK cells were exposed to tumor cells; in other words, there was a PD-L1/PD-1 interaction between tumor cells and NK cells, which inhibited the activity of NK cells (63). When neutralizing antibodies against PD-L1 were added to radioresistant cell/NK cell co-cultures, this resistance was reduced and the susceptibility of tumor cells to NK cell cytotoxicity increased, presumably because PD-L1/PD-1 interaction was blocked (63). In addition, the added PD-L1 antibodies effectively reversed NK cell activity by releasing PD-1 from the PD-L1–PD-1 complex; these antibodies also effectively reversed the expression of NKG2D ligand on tumor cells, which further enhanced the killing ability of NK cells against tumor cells (63). In summary, the PD-1/PD-L1 axis is an inhibitory/regulatory signal for the interaction between tumor cells and NK cells, and blocking of PD-1/PD-L1 interaction may be an effective antitumor immunotherapeutic strategy that is based on the reversal of NK cell dysfunction.

4.1.2 Anti-PD-1/PD-L1 Antibodies Enhance PD-L1$^+$ NK-Cell Antitumor Activity

Anti-PD-1/L1 mAbs may act on NK cells through other non-PD-1 dependent pathways in addition to the PD-1/PD-L1 pathway. Upon encountering and being activated by NK-susceptible tumor cells, NK cells not only secrete cytokines and cytolytic granules, but also upregulate the expression of PD-L1 on their own surface through the PI3K/AKT/NF-κB pathway (8). This population of PD-L1$^+$ NK cells is essential for the antitumor activity of PD-L1 mAbs. Upon application of atezolizumab, an mAb medication against PD-L1, PD-L1$^+$ NK cells express significantly increased levels of granzyme B, IFN-γ, and CD107a, which results in a significant increase in antitumor activity and ultimately a significant decrease in tumor burden in mice (8). Furthermore, it was confirmed that anti-PD-L1 mAbs directly activate PD-L1$^+$ NK cells through the p38/NF-κB pathway in PD-L1$^-$ tumors (8). Upregulation of PD-L1 by NK cells, as well as the direct effect of atezolizumab on NK cells, leads to NF-κB activation, which creates a positive feedback loop in the presence of excessive immunotherapeutic agents to consistently induce PD-L1 expression and further activate NK cells (8). In the loop, the binding of anti-PD-L1 mAbs to PD-L1 upregulates PD-L1 expression on the surface of NK cells, thus, increasing the number of binding sites for the drug; this, in turn, leads to sustained activation of p38, which further transmits strong activation signals to NK cells to maintain their cytotoxic and cytokine secretion characteristics (8). The study suggested that anti-PD-L1 mAb therapy has a unique therapeutic effect against PD-L1-negative tumors (8). Based on the fact that PD-L1$^+$ NK cells act through a PD-1-independent pathway, the discovery of new antitumor mechanisms provides insights into the activation of NK cells and a potential explanation for the response to anti-PD-L1 mAb therapy in some patients who lack PD-L1 expression.

4.1.3 Anti-PD-1/PD-L1 Antibodies Enhance the ADCC Effect of NK Cells

Most PD-1/PD-L1 inhibitors (including nivolumab, pembrolizumab, etc.) have human IgG4 which has low fragment crystallizable (Fc) effector activity. These would be expected to have low ADCC. Atezolizumab is aglycosylated and would be expected to have no ADCC. These all prevent potent ADCC against non-tumor cells expressing PD-L1. However, avelumab, a fully human IgG1 anti-PD-L1 mAb containing a wild-type Fc that induces ADCC (65), has shown toxicity and efficacy similar to those of Fc-modified anti-PD-1/PD-L1 mAbs in several phase I and II clinical trials (66). FcγRIIIA expressed on NK cells can activate NK cell-induced cytotoxicity by recognizing the Fc portion of tumor-bound antibodies and releasing cytotoxic factors and cytokines that recruit and activate other immune cells with specific antitumor

activity in the presence of tumor antigen-targeting antibodies (16). Investigators have demonstrated that avelumab triggers and enhances NK cell-mediated ADCC against TNBC cells expressing a certain level of PD-L1, which results in a significant increase in tumor cell lysis. Park et al. (59) also demonstrated that when NK cells were co-cultured with wild-type Fc anti-PD-L1 mAbs, which can induce ADCC effects on NK cells, the cytotoxicity against PD-L1-positive tumor cell lines was significantly enhanced. Therefore, some anti-PD-L1 mAbs may act as both tumor antigen-targeting antibodies and anti-PD-L1 inhibitors, which leads to the activation of NK cell and $CD8^+$ T cell and improvement in therapeutic efficacy.

The magnitude of the ADCC effect of NK cells evoked by anti-PD-L1 mAbs may be modulated by other factors. First, polymorphisms in FcγRIIIA may affect NK cell-mediated interindividual variability in the ADCC effect (67). Although this association was identified in studies involving different tumor antigen-targeting mAbs of the IgG1 isotype (rituximab, trastuzumab, and cetuximab), it may not be generalizable to different settings (68). Preliminary *in vitro* results showed that avelumab-mediated ADCC was more effective in patients with NK cells expressing the high-affinity CD16 valine (V) allele than in those with NK cells expressing the low-affinity phenylalanine (F) allele and F/F genotype (65); however, this difference in effectiveness needs to be verified in clinical studies. Second, the release of some cytokines, such as IL-2 and IL-15, as well as subsequently stimulated IFN-γ, can stimulate the enhancement of avelumab-triggered cytokine production and degranulation in NK cells while increasing lytic activity against tumor cells (67). In addition, in a previous study, investigators enhanced the ADCC of avelumab against many types of cancer cells through epigenetic priming of NK cells and tumors (69). This evidence suggests that therapeutic strategies that block PD-1/PD-L1 while inducing the ADCC action of NK cells may enhance existing immunotherapeutic efficacy. Considering that the magnitude of NK cell-based ADCC effects induced by anti-PD-L1 mAbs can be regulated by some factors such as immunomodulators (IL-15 or IL-2), combining drugs targeting these factors (67) may improve the effectiveness of immunotherapeutic strategy.

4.1.4 Anti-PD-1/PD-L1 Bispecific Antibodies Induce Phenotypic Transformation of NK Cells

Bromodomain proteins, such as BRD1 and BRD4, play a role in the development of immune and hematological cells and in the regulation of tumor inflammation (70–72). In a previous study on high-grade serous ovarian cancer (HGSC), BRD1 expression was found to be low in tumor cells and high in immune cells, which is associated with significant downregulation of T cell- and NK cell-surface activity markers (GZMA, GZMB, IFNG, and NKG7) and upregulation of the naïve T cell marker TCF7 (73). This study performed immune function and single-cell RNA-seq transcriptional profiling of novel HGSC organoid/immune cell co-cultures treated with unique bispecific anti-PD-1/PD-L1 antibodies versus monospecific anti-PD-1 or anti-PD-L1 antibodies (control) (73). It revealed that bispecific antibodies uniquely induced a transition from inert to more active and cytotoxic NK cell phenotypes and a transition from naïve to more active and cytotoxic progenitor-exhausted phenotypes of $CD8^+$ T cells after treatment. It was further found that these superior cell state changes were driven in part by the downregulation of BRD1 expression induced by bispecific antibodies in immune cells (73). The inhibitory effect of the small molecule inhibitor, BAY-299, on BRD1 partially leads to increased NK cell maturation, activation, and tumor cell killing *via* alteration of the chromatin pathway of key immune transcription factors (e.g., GATA3, TBX21, and TBXT), induces similar state transitions in immune cells *in vitro* and *in vivo* and demonstrates the *in vivo* efficacy of bispecific antibodies (73). Therefore, changes in the activity and cytotoxic status of NK cells and T cells may be the key to driving the induction of effective antitumor immune responses using bispecific antibodies, by partially eliminating some TME-driven dysfunction through epigenetic changes (BRD1 downregulation or inhibition).

4.2 Indirect Interaction of Anti-PD-1/PD-L1 Antibodies With NK Cells

4.2.1 Anti-PD-1/PD-L1 Antibodies Indirectly Reverse NK Cell Exhaustion by Affecting T Cell Activity

Regulative effects of NK and $CD8^+$ T cells on each other have been reported in many infection models (74, 75) and antigen-independent IL-2 models (76), and PD-1/PD-L1 inhibitors may indirectly alter NK cell function by affecting $CD8^+$ T cell activity. NK cell exhaustion (NCE) has been identified as a self-regulatory mechanism responsible for inducing dysfunctional phenotypes to prevent exacerbated immune responses under conditions of chronic stimulation (77), and it is a crucial mechanism involved in tumor or viral evasion of immune responses. IL-2 is a pleiotropic cytokine that activates T cells, NK cells, and dendritic cells. IL-2 binds to IL-2 receptors (IL-2Rs), including CD32 (IL-2Rγc), CD122 (IL-2Rβ), and CD25 (IL-2Rα). IL-2Rs showed different affinities for cytokines, with CD25 (IL-2Rα) having the highest IL-2 affinity. CD25 is constitutively expressed on Tregs; therefore, IL-2 treatment can lead to the expansion of these cells, thus mediating NK and $CD8^+$ T cell suppression through multiple mechanisms (76, 78, 79). A recent study evaluated the role of the PD-1/PD-L1 pathway in NK cell activation, function, and depletion in a mouse model of IL-2-dependent depletion (80) and found that anti-PD-1 therapy provides an activating advantage to $CD8^+$ T cells in the competition for IL-2 by promoting $CD8^+$ T cell expansion, activation, and functional phenotype; consequently, the quantity of stimulatory cytokines available to NK cells becomes limited, which results in a delayed NCE, improved NK cell activation, higher proliferative capacity, and enhanced granzyme B production. This evidence suggests that the phenotypic and functional benefits observed after the chronic stimulation of NK cells by anti-PD-1 therapy are indirectly mediated by the effect of anti-PD-1 antibodies on $CD8^+$ T cells, rather than by the direct effect of anti-PD-1 drugs on NK cells; this can be demonstrated by the reversal of the benefit of anti-PD-1 therapy on NK cells by the depletion of $CD8^+$ T cells.

Disruption of the balance between NK and $CD8^+$ T cells affects the homeostatic response to each other's stimuli, and this resource competition (i.e., IL-2) delays the onset of NCE. Thus, there is a delicate balance between $CD8^+$ T cells, Tregs, and NK cells that is partly regulated by their ability to respond to cytokines. Achieving a balance between these immune cells may be important for achieving long-term efficacy of immunotherapy. Although dual regulation between $CD8^+$ T cells and NK cells is caused by competition for space and resources, direct or indirect lysis, and functional inhibition, these two populations can also work together to mount a stronger response. Another study found that dual blockade of PD-1 and IL-10 enhanced cytokine secretion, NK degranulation, and killing target cell function of NK cells by restoring HIV-specific $CD4^+$ T cell function, thus establishing a previously unappreciated relationship between $CD4^+$ T cell injury and NK cell depletion in HIV infection (81). This important evidence also fully suggests that PD-1 blockade may enhance immunotherapy by improving collaboration between $CD4^+$ T cells and NK cells.

4.2.2 Anti-PD-1/PD-L1 Antibodies Relieve the Inhibition of NK Cell Function by Affecting Tregs

Immunosuppressive cells such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and Tregs in the TME inhibit NK cell proliferation, infiltration, and activation by secreting immunosuppressive cytokines (TGF-β and IL-10) or by interfering with NK cell receptor expression and activation; thus, these cells facilitate tumor immune escape and promote progression and metastasis (82, 83). It can, therefore, be hypothesized that PD-1/PD-L1 blockade prevents the induction of immunosuppression and improves NK cell efficacy to increase the survival of a tumor-bearing animal. A previous study showed that anti-PD-L1 therapy has no direct effect on the cytotoxicity or cytokine secretion of PD-1-negative PM21 particle-expanded NK cells in response to $PD\text{-}L1^+$ targets *in vitro*; however, secretion of a large quantity of IFN-γ by NK cells significantly improved antitumor efficacy, and long-lasting retention of their cytotoxic phenotype by NK cells can be observed *in vivo* (84). Thus, the investigators continued to explore the specific mechanism by which anti-PD-L1 therapy enhances the antitumor efficacy of NK cells. They found that PM21-NK cells are highly cytotoxic to tumor cells and secrete IFN-γ upon stimulation, which leads to the induction of PD-L1 expression in tumors and consequently to the induction and *in situ* proliferation of Tregs in the TME (84). Subsequently, blockade of PD-L1 mitigated the induction of Treg expansion and associated immunosuppressive responses, which in turn improved the NK cell phenotype and cytotoxic activity, as well as survival in treated animals (84). The number of $CD57^+$ NK cells has increased, a population with high cytotoxic capacity and responsiveness *via* CD16 binding, and the presence of these cells is correlated with better outcomes in patients with squamous cell lung cancer in other reports (85, 86). The expansion of Tregs may occur in the TME with PD-L1 expression, and Tregs have been reported to suppress the survival and cytotoxic function of NK cells through several mechanisms, including TGF-β surface presentation (87–89). Thus, anti-PD-L1 therapy may improve NK cell antitumor efficacy by reducing *in situ* Treg generation and preventing Treg induction.

5 NK CELLS ENHANCE ANTI-PD-1/PD-L1 ANTIBODY EFFICACY BY AFFECTING OTHER IMMUNE CELLS IN THE TME

NK cells and other immune cells in the TME mutually regulate each other; for example, $PD\text{-}L1^+$ liver-resident NK (LrNK) cells have an inhibitory interaction with $PD\text{-}1^+$ T cells (90). NK cells can indirectly affect the antitumor immune function by acting on these immune cells, thereby enhancing the response of tumors to anti-PD-1/PD-L1 antibodies. The large number of beneficial effects of tumor-infiltrating NK cells can be mediated by modulating the TME and promoting T cell recruitment and activation. An earlier study that used mixed lymphocyte cultures showed that NK cells are required for the differentiation and activation of $CD8^+$ T cells with cytotoxic functions (91). Another study that used *in vitro* co-culture and *in vivo* xenograft adoptive transfer experiments demonstrated that high-quality NK cells (iNK) derived from induced pluripotent stem cells (iPSCs) can recruit and activate T cells, allow them to respond to PD-1 blockade, and enhance inflammatory cytokine production and tumor elimination (92). NK cells in tumor-infiltrating and draining lymph nodes were reported to show upregulation of the inhibitory molecule PD-L1, which transmits an inhibitory signal by interacting with PD-1 expressed on DCs to limit the activation of these cells; this in turn leads to a decrease in the priming ability and memory response of tumor-specific $CD8^+$ T cells (61). When blocking the interaction between NK cells and DCs, a significantly higher frequency of $CD8^+$ T cells, level of IFN-γ production, and capacity for cytotoxicity were observed (61). In this model, tumor cells induced the modulation of DC activation *via* $PD\text{-}L1^{hi}$NK cells, which reduced the priming capacity of $CD8^+$ T cells. Conversely, PD-1/PD-L1 inhibitors may reverse the inhibitory effect of PD-1-PD-L1 interaction on $CD8^+$ T cells by disrupting the direct interaction pathway between NK cells and DC cells; this presumably activates NK cell activity and needs to be explored further in future studies. In addition to interacting with DCs through the PD-1/PD-L1 axis, a study has shown in human melanoma that NK cells stably form conjugates with stimulatory dendritic cells (SDCs) in mouse TME and positively regulate the abundance of SDCs in tumors by producing FLT3LG, the cDC1 formative cytokine. SDCs are important in stimulating cytotoxic T cells and driving anticancer immune responses (60). Although anti-PD-1 immunotherapy for cancer primarily targets T cells, NK cell frequencies correlate with protective SDCs in human cancers, with patient responsiveness to anti-PD-1 immunotherapy, and with prolonged overall survival (60).

In summary, blocking PD-1/PD-L1 may activate the systemic immune response by reversing the inhibitory effect of

NK cells on other immune cells or activating the antitumor capability of other immune cells by NK cells, which is an effective antitumor immunotherapy strategy. Therefore, a combination therapy based on NK cells and anti-PD-1/PD-L1 drugs can be established.

6 COMBINATION THERAPY OF NK CELLS AND IMMUNE CHECKPOINT INHIBITOR

Considering NK cell-mediated cytotoxicity does not require MHC class I and the unique effects of anti-PD-1/PD-L1 mAb therapy on NK cell function described above, adoptive transfer of autologous or allogeneic NK cells together with anti-PD-1/PD-L1 antibodies may potentially enhance the outcomes of patients receiving cancer immunotherapy. A previous study explored the effects of a combination of NK cells with PD-L1 blockers, regardless of PD-1 expression on NK cells or the initial PD-L1 status of the tumor cells (84). The OS of animals treated with a combination of anti-PD-L1 antibodies and PM21-NK cells was twice as higher than that of those treated with anti-PD-L1 antibodies alone (48 days vs. 24 days, $P = 0.0001$) (84). The first clinical study on the combination of anti-PD-1 antibodies (pembrolizumab) and allogeneic NK cells in patients with advanced NSCLC in China was recently published (93), and it reported the doubling of the number of NK cells, significant increases in the levels of cytokines such as IL-2, TNF-β, and IFN-γ, and significant decreases in the levels of multiple tumor markers such as circulating tumor cells (CTCs) after treatment in the combination therapy group. Patients in the combined therapy group had a significantly better overall response rate (36.5% vs. 18.5%) and a significantly better survival outcome (OS: 15.5 months vs. 13.3 months; PFS: 6.5 months vs. 4.3 months; all $P < 0.05$) than did those in the anti-PD-1 antibody alone group; moreover, the benefits were more significant in the combination therapy group [tumor proportion score [TPS] ≥ 50%] than in the latter group. In addition, patients who received multiple courses of NK cell infusion showed a better OS than those who received a single course of NK cell infusion (18.5 months vs. 13.5 months) (93). The treatment was well tolerated throughout the treatment. All adverse events were below grade 4, with grade 2 events comprising the majority of events. All symptoms were relieved after symptomatic treatment. Therefore, a combination therapy of anti-PD-L1 antibodies and NK cells can significantly enhance the antitumor effect, improve the survival benefit, and serve as new treatment regimen for previously treated patients with advanced PD-L1$^+$ NSCLC. In addition, the combination of anti-PD-1/PD-L1 antibody therapy and drugs that enhance the antitumor effects of NK cells through other mechanisms, including a combination of anti-NKG2A mAb (monalizumab) (94), KIR blockade (95), cytokine therapy (96), and therapies targeting other checkpoint receptors such as CTLA-4, LAG-3, CD96, and TIGIT, may have a synergistic effect (97–99); therefore, this strategy may be beneficial for improving the efficacy of immunotherapy and overcoming drug resistance.

7 SUMMARY AND PROSPECTS

The use of anti-PD-1/PD-L1 antibodies could be a promising option for the successful treatment of malignancies as they help overcome T-cell depletion by blocking the PD-1/PD-L1 signaling pathway in the TME. However, most patients and tumor types have shown low/no response to these therapies, or some patients with predicted no or low response to treatment have shown significant benefit; therefore, in-depth exploration of the mechanism of action and efficacy of ICIs is necessary to effectively screen for suitable patients to expand the benefits to them. Accumulating evidence suggests that immune cells other than T cells, such as NK cells, are also involved and play an important role in the PD-1/PD-L1 blocking process; these non-T cells have become an effective complement to T cell immune responses because of their unique advantages, especially against MHC-deficient tumors that show low antigenicity or are resistant to T cell recognition and cytolysis. At present, findings of studies on the expression of PD-1 and PD-L1 on NK cells and their functions are not completely consistent, and further evidence derived from human NK cell-based research is necessary. According to our review, PD-1/PD-L1 antibodies can rescue NK cell from multiple aspects of dysfunction caused by TME, revitalize the cytotoxic activity of these cells against tumors, and further initiate and enhance T cell-mediated adaptive antitumor immunity; NK cells can also indirectly enhance the efficacy of PD-1/PD-L1 blockade by affecting other immune cells in TME. On the basis of these findings, multiple combination strategies, such as the use of a combination of PD-1/PD-L1 inhibitors with drugs that promote NK cell infiltration, persistence, and activation in tumors (e.g.,cytokines, stimulator of IFN gene [STING] agonists) and resistance to inhibitory TMEs (e.g., anti-TGF-β mAbs), are under investigation in basis research studies or clinical trials; these strategies aim to further increase the antitumor activity of NK cells and improve the tumor response to immunotherapy. At present, our understanding of the role of NK cells during PD-1/PD-L1 blockade is still insufficient and needs to be further explored and validated using basic mechanistic studies. A comprehensive and in-depth understanding of the response mechanism of PD-1/PD-L1-based immunotherapy and the interaction mechanism between each immune cell in TME with another or with tumors can lay a foundation for optimizing the efficacy of existing treatments, developing new immunotherapies based on NK cells, and developing combination therapy strategies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Targeting Stress Sensor Kinases in Hepatocellular Carcinoma-Infiltrating Human NK Cells as a Novel Immunotherapeutic Strategy for Liver Cancer

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Natural killer (NK) cells may become functionally exhausted entering hepatocellular carcinoma (HCC), and this has been associated with tumor progression and poor clinical outcome. Hypoxia, low nutrients, immunosuppressive cells, and soluble mediators characterize the intratumor microenvironment responsible for the metabolic deregulation of infiltrating immune cells such as NK cells. HCC-infiltrating NK cells from patients undergoing liver resection for HCC were sorted, and genome-wide transcriptome profiling was performed. We have identified a marked general upregulation of gene expression profile along with metabolic impairment of glycolysis, OXPHOS, and autophagy as well as functional defects of NK cells. Targeting p38 kinase, a stress-responsive mitogen-activated protein kinase, we could positively modify the metabolic profile of NK cells with functional restoration in terms of TNF-α production and cytotoxicity. We found a metabolic and functional derangement of HCC-infiltrating NK cells that is part of the immune defects associated with tumor progression and recurrence. NK cell exhaustion due to the hostile tumor microenvironment may be restored with p38 inhibitors with a selective mechanism that is specific for tumor-infiltrating—not affecting liver-infiltrating—NK cells. These results may represent the basis for the development of a new immunotherapeutic strategy to integrate and improve the available treatments for HCC.

Keywords: immunometabolism, NK-cell, immunotherapy, hepatocellular carcinoma, oncoimmunology

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent type of primary liver cancer with high incidence and mortality rate (1, 2). The very early and early stages of HCC can be cured by surgery and/or loco-regional therapies. However, in the advanced stage, only a limited choice of effective strategies exists, and tumor recurrence can be very aggressive (1). Similarly to other cancers, immunotherapy will become a central strategy also for HCC (3). Despite the fact that immunotherapy has become the first-line treatment for several cancers in recent years (4, 5), the first immunotherapy for HCC, based on anti-PD-L1 in association with anti-VEGF, has been registered only last year after the failure of trials based on a single anti-PD-1 treatment (6).

Natural killer (NK) cells account for 25–50% of lymphocyte populations infiltrating the liver (7). Intrahepatic NK cells play a central role in innate immune response against liver pathogens and tumors (8–10). The results in lung cancer suggest that NK cells entering the tumor microenvironment display markers of activation and cytotoxicity but become rapidly exhausted after target recognition (10). Even though available data support the role of NK cells in the control of HCC progression, there is evidence of the rapid functional exhaustion of NK cells in the HCC microenvironment which is associated with tumor progression and poor clinical outcome (11–16). This suggests that NK cell-based immunotherapies may enhance the NK cell effector function. Among immunotherapeutic approaches, checkpoint blockade and adaptive transfer of cytokine-activated killer cells have demonstrated some clinical efficacy (17, 18). However, the limited knowledge of the mechanisms of NK cell exhaustion has not allowed the use of NK cell immunotherapy in HCC.

NK cell activation is regulated by the balance between inhibitory and activating signals, which determines whether an NK cell will be able to kill a target (19). Moreover, NK function is strongly influenced by the surrounding cytokine environment and by the interaction with other cells, such as regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages (20). NK cell activation is characterized by a high metabolic activity and upregulation of OXPHOS and glycolysis, with enhanced transport of nutrients, in particular glucose transporter, to support antitumor functions (21, 22).

A tumor is a hostile microenvironment for immune cells due to hypoxia, nutrient deprivation, and release of immunosuppressive cytokines such as TGF-β (23). The low oxygen concentrations induce the tumor cells to switch from aerobic metabolism to anaerobic glycolysis, which exploits most of the available glucose (24).

HCC is a highly hypoxic tumor showing a particularly low median oxygen level (23, 25). The dramatic increase in glucose metabolism in tumor cells leads to decreased glucose availability and a dramatic increase in lactate level, which accumulates in the local microenvironment and induces the metabolic deregulation of infiltrating immune cells such as NK cells (22, 23).

Hypoxia and high intracellular lactate induce the dysfunction of hepatic cytotoxic lymphocytes, downregulating activating receptors and cytotoxic molecules or inducing apoptosis through reactive oxygen species (ROS) accumulation and mitochondrial damage, particularly in $CD56^{BRIGHT}$ NK cell subpopulation (21, 26, 27). In addition, TGF-β contributes to corroborate the glycolytic impairment. It has been shown, in lung cancer, that the aberrant expression of FBP1, an enzyme involved in gluconeogenesis, due to TGF-β and glucose depletion can inhibit NK cell glycolysis, thus determining ROS accumulation (28). Moreover, the combined effect of TGF-β and hypoxia can determine the excess of mitochondria fragmentation *via* fission, followed by decreased OXPHOS and an increase of ROS levels (28–30). In metastatic breast cancer patients, TGF-β blockade rescued NK cells from impaired glycolysis and mitochondrial respiration (31, 32). In the mouse model of murine cytomegalovirus infection, NK cells accumulate dysfunctional mitochondria and activate mitophagy as a rescue mechanism, thus facilitating memory formation (33). The role of TGF-β in shaping the NK cell metabolic rearrangement was also shown in the peripheral blood of patients with HCC, where TGF-β-specific targeting could partially restore the NK cell dysfunctions (34).

The opportunity of restoring NK cell function by targeting NK cell immunometabolism has become an active area of research in oncoimmunology (28, 35, 36). In this complex scenario, we may speculate that drugs modulating immune metabolism could provide new therapeutic strategies by enhancing the NK cell response against HCC.

To better define and validate the key dysregulated pathways associated with NK cell exhaustion in HCC, we applied genome-wide transcriptome profiling of infiltrating NK cells and targeted rescue strategies with functional and metabolic validation. Here we show that HCC-infiltrating NK cells are marked by a predominantly upregulated gene expression profile translating into the metabolic and functional impairment of glycolysis, OXPHOS, and autophagy. Targeting dysfunctional signaling *in vitro* can efficiently improve the functions of HCC-infiltrating NK cells and may thus represent a novel strategy to be implemented in HCC treatment.

MATERIALS AND METHODS

Patients and Biological Samples

The enrolled patients were featured by a class A liver cirrhosis (Child–Pugh) and an early stage of HCC (Barcelona Clinic Liver Cancer Stage A) diagnosed through ultrasonography/computed tomography or MRI in specific cases. All the patients were HBV- and HIV-negative. Additional information on the study population are shown in **Supplementary Table S1**.

Tumor and non-tumorous specimens were obtained from 11 HCC patients infected with hepatitis C virus, 6 patients with alcohol-associated HCC, one NASH and HCC patient, and control samples from the liver resection of 7 patients with colorectal metastasis. Functional and metabolic analyses of tissue-infiltrating NK cells were conducted on 12 matched HCC–non-tumorous samples and controls. Eight paired HCC– non-tumorous samples were used to evaluate the effect of *in vitro* treatment on cell metabolism.

The study underwent local ethical committee approval [Comitato Etico Indipendente Area Vasta Emilia Nord (AVEN) of the AOU of Parma, Parma, Italy]. Signed informed consent for each patient was obtained for them to take part in the study.

Gene Expression Profiling

Thawed liver- and tumor-infiltrating lymphocytes from the 11 HCC and HCV patients and from the 6 liver-resected control patients were stained with anti-CD3-PeCy7 (Biolegend, San Diego, CA, USA), anti-CD16 FITC (BD Bioscience, Franklin Lakes, NJ, USA), anti-CD56 PECF594 (BD), and then 7-amino-actinomycin D [7AAD PerCPCy5.5 vitality dye, for discriminating NK cells in normal liver-infiltrating NK cells (NLINK), tumor-infiltrating NK cells (TINK), and NK cells infiltrating hepatic tissue around HCC (LINK)]. NK cell sorting was performed with FACSAria III Cell Sorter (BD) by selecting CD3-negative, CD56-positive, CD16-positive, or CD16-negative subpopulations.

RNA was extracted from the isolated NK cells with the RNase Free DNase I Kit (Norgen Biotek), and the RNA concentration was measured using a Nanodrop spectrophotometer, in accordance with the manufacturer's instructions. The RNA integrity was detected with a Bioanalyzer2100 system (Agilent Technologies). Transplex Whole Transcriptome Amplification (WTA2) and GenElute PCR Clean-Up kits (Sigma) were employed to amplify and then purify the total RNA, respectively, following the manufacturer's protocol. cDNA labeling was performed by SureTag DNA Labeling kit (Agilent Technologies) and then hybridized to 60-bp oligonucleotide whole human genome arrays (Human GE 8x60K v2 SurePrint G3, Agilent Technologies), following the manufacturer's instruction. The microarrays were scanned with an Agilent dual-laser DNA scanner. The Agilent Feature Extraction software, v.7.5, was employed with default settings to achieve normalized expression values from raw data. The expression data are available at the National Center for Biotechnology Information Gene Expression Omnibus: GSE183349.

Microarray Statistical Analysis

GeneSpring software package GX 13.1 (Agilent Technologies) was employed for quality control, data normalization (75th percentile procedure), and preliminary microarray data analysis. The probe signals in at least four replicates for each condition were kept for subsequent analyses. Benjamini–Hochberg-corrected ANOVA for multiple testing [false discovery rate (FDR), ≤0.05] was employed to track differentially expressed genes (DEGs) by comparing LINK-, TINK-, and NLINK-derived samples. Student–Newman–Keuls *post-hoc* test was used to determine the DEGs in the three patient groups.

Median baseline transformation was applied before determining the unsupervised hierarchical clustering of the samples with Ward's linkage and Euclidean distance. In order to reduce data dimensionality, principal component analysis (PCA) was used with orthogonal transformation to cluster sample populations.

Gene Set Enrichment Analysis (GSEA) was employed on the detected probes in order to pinpoint significant pathways enriched in downregulated and upregulated probes and to analyze the gene profiles with other studies. Molecular Signature Database C2, canonical pathways version 6, was employed for statistical analysis with the permutation type ("gene set" for fewer than seven replicates); the default settings for all other options were kept. Significantly enriched gene sets were obtained with a FDR lower than 0.25, which was determined using 1,000 permutations of gene set and Signal2Noise as metric.

Assessment of Infiltrating NK Cell Metabolic Features

For all flow cytometry analysis, after the exclusion of cell aggregates, gating was performed on lymphocytes, and dead cells were subsequently excluded (7-AAD or live/dead as appropriate); CD3, CD56, and CD16 were used to select NK cells that were identified as CD3− CD56+.

Mitochondrial membrane potential was detected on infiltrating NK cells through the JC-1 probe to measure the mitochondrial membrane potential (ThermoFisher). Anti-CD3 APCCy7 and anti-CD56 APCR700 were used to stain LINK, TINK, and NLINK, and then the cells were treated for 10 min at RT with JC-1 (2.5 μg/ml) before the FACS analysis. Finally, viability probe 7-AAD was used for cell staining, and FACS Canto II was used to acquire samples. Mitochondrial depolarization was measured in NK cells by quantifying the percentage of FL1high/FL2low cells (JC-1 staining) detected in the different samples.

The uptake of fluorescent glucose was assessed in order to define the glycolysis capacity. After washing with 1× phosphate-buffered saline (PBS) to withdraw endogenous glucose, the infiltrating lymphocytes were marked for 30 min at 37°C with the analog 2-NBDG {2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose, ThermoFisher} at 40 μM in RPMI without glucose and added with 10% dialyzed fetal bovine serum, and then staining was caried out with anti-CD3, anti-CD56, and 7-AAD probes. The frequency and median fluorescence intensity (MFI) of 2-NBDG NK-positive cells were measured.

The autophagy potential was analyzed through the Cyto-ID Autophagy kit (Enzo Life Sciences, NY, USA) that detects autophagic vesicles and reveals autophagic flux in chloroquine diphosphate treatment to inhibit lysosomes and live cells (overnight at 30 μM), through a probe, which accumulate within the autophagic vacuole.

Changes in Cyto-ID expression were measured with or without chloroquine-mediated blocking of the autophagosome turnover, which hampers the fusion of autophagosome–lysosome vesicles, thus preventing their degradation.

On the following day, the NK cells were washed and resuspended in 1× PBS in order to eliminate any trace of phenol red. The Cyto-ID Autophagy kit was employed after anti-CD3 and anti-CD56 staining, according to the manufacturer's instructions, for the final acquisition on a FACS Canto II flow cytometer. Data were expressed as Cyto-ID MFI in NK cells from different groups.

Phosphorylation Status of p38 Protein in Infiltrating NK Cells

After thawing, the liver- and tumor-infiltrating NK cells (7 NLINK, 12 LINK, and 12 TINK) were stained with surface antibodies (anti-CD3-Alexa Fluor700 and anti-CD56-PerCp). Then, for anti-phosphorylated-p38 assay, Fixation/Permeabilization Solution Kit (Cytofix/Cytoperm BD) for intracellular staining was employed, in accordance with the manufacturer's protocol and the antibody specific for the phosphorylated form of the human p38 mitogen-activated protein kinase (MAPK; pT180/pY182)PE-Cy7 (BD). Samples were acquired with a FACSCANTO II BD and analyzed with DIVA (BD) and Flowjo software. Data were expressed as phosphorylated-p38 MFI in NK cells from different groups.

Functional Analysis of Infiltrating NK Cells

IFNγ and TNFα cytokine protein expression in NK cells was assessed after 4 h of phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml) stimulation. After 1 h, Brefeldin A (BFA, 10 μg/ml) was supplemented. Anti-CD3 PE (BD), anti-CD56 PECF594 (BD), and anti-CD16 FITC (BD) were used to stain the cells before fixing with medium A reagent and then permeabilization with medium B reagent (Nordic Mubio), according to manufacturer's instructions. Cytokine measurements were assessed by intracellular cytokine staining (ICS) with monoclonal antibodies specific for IFNγ (PerCp-Cy5.5, Biolegend) and TNFα (APC, Biolegend) and detected by FACS Canto II. In order to evaluate the activity of cytotoxic NK cells , CD107a degranulation marker was employed after PMA and ionomycin stimuli. Following the stimulus, the NK cells were incubated for 4 h with the antibody specific for CD107a (PE-Cy7 -BD) and the Golgi inhibitor, Brefeldin A (10 μg/ml). The results were displayed as the difference between cytokine- and CD107a-positive NK cells with or without stimulus. In order to study the adhesion ability of infiltrating NK cells toward K562 target cells, K562 cells were marked with a green fluorescent probe (CFSE, carboxyfluoroscein succinimidyl ester) and NK cells with antibodies specific for CD3 (APC-Cy7) and CD56 (APC-R700). To block CFSE staining, two volumes of cold fetal bovine serum were added, followed by washing thrice with Hanks' balanced salt solution. The NK cells have been joined to target cells in a ratio of 1:5, respectively, and then they have been mixed by gentle vortexing and centrifuged for 3 min at 4°C (at 300 rpm). The cells were incubated at different timepoints (0, 15, and 30 min) at 37° C, fixed with 1 ml of Fixation Permeabilization Concentrate and Diluent (eBioscience), and then measured on FACS Canto II (BD). The NK cells which adhered to the K562 cells were analyzed, and the results were shown as mean fluorescence intensity related to the NK lymphocytes gated on target cells.

Functional Restoration Assays

IFN-γ and TNF-α release from NK cells was assessed after IL-12 and IL-18 (5 ng/ml) overnight (O/N) stimuli in the presence or absence of specific p38 inhibitors. After 1 h of the stimulus, 10 μg/ml of BFA was supplemented for a further 3 h of culture. Anti-CD3 PE (BD), anti-CD56 PECF594 (BD), and anti-CD16 FITC (BD) were employed to stain the NK cells before fixing them with the medium A reagent and permeabilization with medium B reagent (Nordic Mubio) according to the manufacturer's instructions. Cytokine production measurements were assessed by ICS after staining with monoclonal antibodies for IFNγ (PerCp-Cy5.5, Biolegend) and TNFα (APC, Biolegend) and subsequently run on FACS cytometry. The cytotoxic activity of NK cells was monitored through CD107a degranulation staining. Specifically, IL-12 and IL-18 were employed to stimulate the inhibitor-treated NK cells overnight (as described above). The cells together with target cells (K562) were then incubated with BFA and CD107 antibody (PECy7, BD) for the last 4 h.

The results are displayed as a ratio (fold change) of cytokine-positive NK cells detectable in inhibitor-treated *vs* . untreated cells. As control, the percentage of dead cells was evaluated in each inhibitor-treated sample by FACS in order to assess the cell toxicity.

Metabolic Restoration Assays

Mitochondrial membrane potential (JC-1), glucose uptake, and autophagy potential by NK cells from LINK and TINK samples were evaluated, as described above, after O/N IL-12 + IL-18 (5 ng/ml) stimulation with or without the specific p38 inhibitors.

Data were presented as the ratio between the cells in the fluorescent channel FL1high and FL2low (JC-1 staining) and MFI (2-NBDG or Cyto-ID staining) detected in inhibitor-treated *vs* . untreated cells (fold change). As control, the percentage of dead cells was evaluated in each inhibitor-treated sample by FACS in order to assess cell toxicity.

The compounds tested were the SB203580 p38 inhibitor, used at 0.05–1 μM (Sellekchem), and doramapimod (BIRB-796), used at a concentration of 0.05–1 μM (Selleckem).

All assays were assessed on 8-color flow cytometer (FACS Canto II, BD), and the results were analyzed with FACS Diva and Flowjo software (BD). The frequency of $CD56^{DIM}$ and $CD56^{BRIGHT}$ NK cells was determined on the CD3-negative CD56-positive cells by evaluating the fluorescence intensity level of CD56 marker, and each parameter expression (carried out as percentage and MFI) was measured on total CD56 positive, $CD56^{DIM}$, or $CD56^{BRIGHT}$ NK cells .

Statistical Analysis

Statistics was assessed with GraphPad Prism v.7 software. Analyses of variance (*F* test) and of normality (Kolmogorov–Smirnov test) were initially evaluated. Subsequently, Mann–Whitney *U*-test, paired *t*-test, Wilcoxon matched-pairs test, and Pearson's correlation were used. Moreover, inhibitory drug experiments and fold change upon inhibitory treatments were statistically calculated by Wilcoxon signed rank tests (as compared to a theoretical median of 1).

All statistics were two-tailed, and significance was observed as $p < 0.05$.

RESULTS

Gene Expression Profiling of Tumor-Infiltrating NK Cells

NK cells ($CD3^-$, $CD56^+$) were purified by flow cytometric cell sorting from tumor and liver tissue samples of patients with HCC arising in HCV infection (LINK and TINK, $n = 11$) and

from liver control tissue samples (NLINK, $n = 7$). The analysis of variance on data of the detected probes (19,716 genes) identified 108 DEGs in NK cells from HCCs and controls. The hierarchical clustering of DEGs is depicted in **Figure 1A**. PCA showed a partial segregation among groups of patients and controls (**Figure 1B**).

We then focused on the comparison of NK cells infiltrating the HCC and the liver from the same patients and interrogated the Molecular Signatures database (http://www.broadinstitute.org/gsea). GSEA was conducted to understand the NK cell expression modifications in the tumor microenvironment. Gene sets related to mitochondrial functions and ROS detoxification system, glycolytic activity, ubiquitin-proteasome-mediated degradation system, and pathways related to cell cycle and DNA damage and repair (included the stress sensor p38-related pathway) appeared to be enriched and upregulated in TINK compared to LINK, as shown in **Figure 1C**.

Genes encoding the electron transport chain components and many subunits of complex I (NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome c reductase), IV (cytochrome c oxidase), and V (ATP synthase) were upregulated in HCC-infiltrating NK cells . Among the genes upregulated in tumor-infiltrating NK cells were genes related to glucose metabolism, particularly the aldehyde dehydrogenase family, aldolase complex, and phosphoglycerate kinase 1 (**Figure 1D**). Another group of transcripts upregulated in TINK encodes intracellular signaling and cell cycle control genes, such as MAPK14 and MAPK11 (subunits of p38 protein complex), TP53, p73, and p21 (**Figure 1D**).

Moreover, genes encoding the key components of the immune ubiquitin-proteasome pathway, including the 19S and 11S regulatory particles, the 20S proteolytic core, and the 26S proteasome subunits, resulted as upregulated in TINK. The detailed lists of genes may be found in **Supplementary Table S2**.

GSEA was also applied for the comparison between gene expression profiles of TINK and NLINK, leading to the identification of enriched upregulated genes related to the similar pathways described above in the previous comparison (**Supplementary Table S3**), confirming that tumor-infiltrating NK cells showed a misregulation in transcriptome profiles. The NK cells from tumor and liver tissue samples were analyzed by distinguishing the total NK cells from the $CD56^{DIM}$ and $CD56^{BRIGHT}$ subsets. A generalized upregulation thus appears to be the predominant feature of tumor-infiltrating NK cells .

Impaired Mitochondrial and Glucose Metabolism of Tumor-Infiltrating NK Cells

Because of similar data reported for tumor microenvironment (32), we hypothesized that HCC-infiltrating NK cells are in a condition of nutrient deprivation, which is required to meet the increased energy and biosynthetic demands for NK cell expansion and anti-tumor functions (37, 38). To evaluate the possible intra-tumor dysregulation of NK cell metabolism, we first focused on mitochondrion and glucose metabolism.

Mitochondrial membrane depolarization and glucose import were measured. As shown in **Figure 2A**, tumor-infiltrating NK cells displayed a defective mitochondrial functionality, indicated by increased depolarization of the mitochondria compared to the non-tumorous counterpart and the controls. On the left side is the frequency of JC-1-positive cells, while on the right side is the median fluorescence intensity, showing that tumor-infiltrating NK cells exhibited a higher content of depolarized mitochondria in all NK cell subsets (TOTAL, DIM, and BRIGHT).

Glucose uptake capacity, described by the 2-NBDG analysis, was reduced in TINK compared to NLINK and LINK cells, both in terms of the percentage of metabolically active NK cells and in terms of fluorescence intensity, confirming the existence of metabolic impairment at different levels (**Figure 2B**). $CD56^{BRIGHT}$ TINK showed the worst performance in glucose uptake capacity.

Stress Sensor p38 Kinase in Tumor-Infiltrating NK Cells

Then, we tried to clarify the mechanisms responsible for glycolytic impairment and to understand the upregulation of OXPHOS gene that was functionally ineffective. To this end, we performed a leading edge on GSEA data sets in order to extract a candidate list of regulatory genes. Notably, the majority of GSEA misregulated pathways highlighted an upregulation of MAPK11 and MAPK14 (α and β subunits of p38 complex).

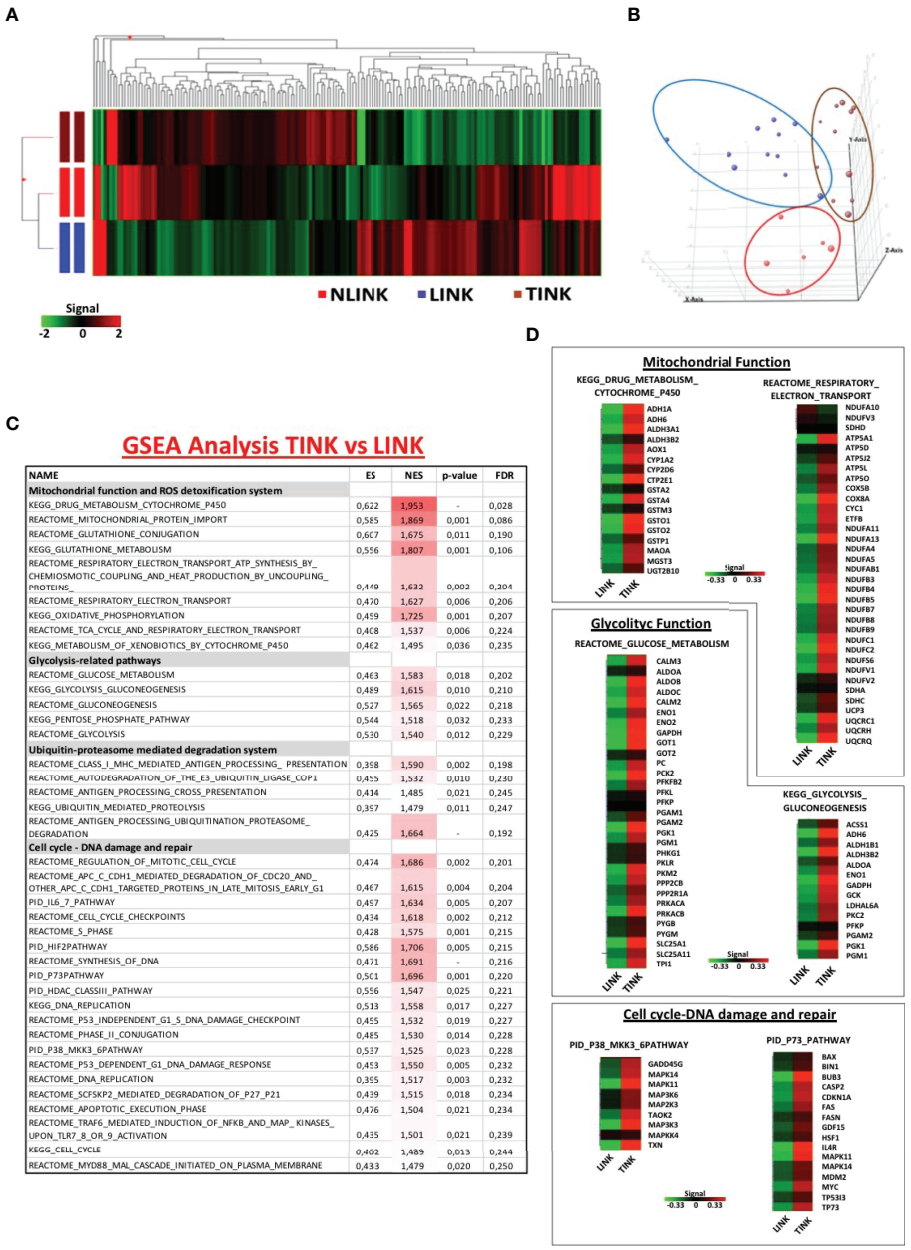
Indeed p38 protein is a stress sensor kinase from MAPK family that can be activated by nutrient deprivation in the microenvironment. Based on this observation, we analyzed the phosphorylation status of p38-MAPK protein in the three study groups. A higher level of phosphorylated p38, observed as median fluorescence intensity, was detected in total NK cells derived from tumor samples and was particularly enhanced in the $CD56^{BRIGHT}$ subset (**Figure 3A**). This observation confirms the activation of this specific pathway as indicated by the transcriptomic analysis.

A dimension reduction by t-distributed stochastic neighbor embedding (tSNE) on flow cytometry data of phosphorylated p38 MAPK (pT180/pY182) from the LINK and TINK groups was performed to represent the relative intensity of NK-cell p38 expression. Despite the fact that the distribution of events was almost similar in the total, $CD56^{BRIGHT}$, and $CD56^{DIM}$ subsets in the two groups (**Figure 3B**), the color shades representing the protein level of p38 marker showed a higher expression in $CD56^{BRIGHT}$ cells from the TINK samples (red indicating upregulation and blue indicating downregulation, **Figure 3C**).

Autophagy Deregulation in Tumor-Infiltrating NK Cells

In order to better understand the metabolic assessment of HCC-infiltrating NK cells , the autophagy potential was analyzed.

The results of the transcriptomic analysis and validation suggested an engulfment of ROS clearance and accumulation of damaged mitochondria (**Figures 1C, D**, **2A**). In addition, p38 has been implicated in autophagy repression in different cell types, including T cells, during starvation conditions such as those occurring in the tumor microenvironment (39–41).



NAME	ES	NES	p-value	FDR
Mitochondrial function and ROS detoxification system				
KEGG_DRUG_METABOLISM_CYTOCHROME_P450	0,622	1,953	-	0,028
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	0,585	1,869	0,001	0,086
REACTOME_GLUTATHIONE_CONJUGATION	0,667	1,675	0,011	0,190
KEGG_GLUTATHIONE_METABOLISM	0,556	1,807	0,001	0,106
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_ CHEMIOSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_ PROTEINS_	0,449	1,632	0,002	0,204
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	0,470	1,627	0,006	0,206
KEGG_OXIDATIVE_PHOSPHORYLATION	0,459	1,725	0,001	0,207
REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT	0,468	1,537	0,006	0,224
KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450	0,462	1,495	0,036	0,235
Glycolysis-related pathways				
REACTOME_GLUCOSE_METABOLISM	0,463	1,583	0,018	0,202
KEGG_GLYCOLYSIS_GLUCONEOGENESIS	0,489	1,615	0,010	0,210
REACTOME_GLUCONEOGENESIS	0,527	1,565	0,022	0,218
KEGG_PENTOSE_PHOSPHATE_PATHWAY	0,544	1,518	0,032	0,233
REACTOME_GLYCOLYSIS	0,530	1,540	0,012	0,229
Ubiquitin-proteasome mediated degradation system				
REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_ PRESENTATION	0,358	1,590	0,002	0,198
REACTOME_AUTODEGRADATION_OF_THE_E3_UBIQUITIN_LIGASE_COP1	0,425	1,532	0,010	0,230
REACTOME_ANTIGEN_PROCESSING_CROSS_PRESENTATION	0,414	1,485	0,021	0,245
KEGG_UBIQUITIN_MEDIATED_PROTEOLYSIS	0,357	1,479	0,011	0,247
REACTOME_ANTIGEN_PROCESSING_UBIQUITINATION_PROTEASOME_ DEGRADATION	0,425	1,664	-	0,192
Cell cycle - DNA damage and repair				
REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE	0,474	1,686	0,002	0,201
REACTOME_APC_C_CDH1_MEDIATED_DEGRADATION_OF_CDC20_AND_ OTHER_APC_C_CDH1_TARGETED_PROTEINS_IN_LATE_MITOSIS_EARLY_G1	0,467	1,615	0,004	0,204
PID_IL6_7_PATHWAY	0,457	1,634	0,005	0,207
REACTOME_CELL_CYCLE_CHECKPOINTS	0,434	1,618	0,002	0,212
REACTOME_S_PHASE	0,428	1,575	0,001	0,215
PID_HIF2PATHWAY	0,586	1,706	0,005	0,215
REACTOME_SYNTHESIS_OF_DNA	0,471	1,691	-	0,216
PID_P73PATHWAY	0,561	1,696	0,001	0,220
PID_HDAC_CLASSIII_PATHWAY	0,556	1,547	0,025	0,221
KEGG_DNA_REPLICATION	0,513	1,558	0,017	0,227
REACTOME_P53_INDEPENDENT_G1_S_DNA_DAMAGE_CHECKPOINT	0,455	1,532	0,019	0,227
REACTOME_PHASE_II_CONJUGATION	0,485	1,530	0,014	0,228
PID_P38_MKK3_6PATHWAY	0,537	1,525	0,023	0,228
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE	0,453	1,550	0,005	0,232
REACTOME_DNA_REPLICATION	0,395	1,517	0,003	0,232
REACTOME_SCFSKP2_MEDIATED_DEGRADATION_OF_P27_P21	0,439	1,515	0,018	0,234
REACTOME_APOPTOTIC_EXECUTION_PHASE	0,476	1,504	0,021	0,234
REACTOME_TRAF6_MEDIATED_INDUCTION_OF_NFKB_AND_MAP_ KINASES_ UPON_TLR7_8_OR_9_ACTIVATION	0,435	1,501	0,021	0,239
KEGG_CELL_CYCLE	0,402	1,489	0,013	0,244
REACTOME_MYD88_MAL_CASCADE_INITIATED_ON_PLASMA_MEMBRANE	0,433	1,479	0,020	0,250

FIGURE 1 | Gene expression pattern of infiltrating NK cells. **(A)** Hierarchical clustering representation of the 181 genes identified as differentially expressed in tumor-infiltrating NK cells (TINK, $n = 11$), surrounding liver-infiltrating NK cells (LINK, $n = 11$), and normal liver-infiltrating NK cells (NLINK, $n = 7$) by ANOVA with Benjamini–Hochberg correction ($p \leq 0.05$). Data were median-normalized before clustering; upregulated and downregulated genes are shown in red and green, respectively. **(B)** Principal component analysis of ANOVA-filtered data. **(C)** Enriched gene sets in TINK and LINK identified by Gene Set Enrichment Analysis (GSEA; MSigDB, C2 canonical pathways). NES, normalized enrichment score; FDR, false discovery rate. **(D)** Heat map of differentially expressed genes derived from GSEA in TINK and LINK related to mitochondrial and glycolytic function, cell cycle, and DNA damage/DNA repair. The upregulated genes are presented in red and the downregulated genes in green.

Similarly, we studied the lysosome degradation pathway by evaluating Cyto-ID cationic incorporation into autophagic vesicles (pre-autophagosomes, autophagosomes, and autophagolysosomes) with or without chloroquine treatment.

As presented in **Figure 4A**, TINK displayed a significantly lower basal Cyto-ID expression in all NK cell subsets compared to LINK and a limited one to $CD56^{BRIGHT}$ compared to NLINK. After treatment with chloroquine, the

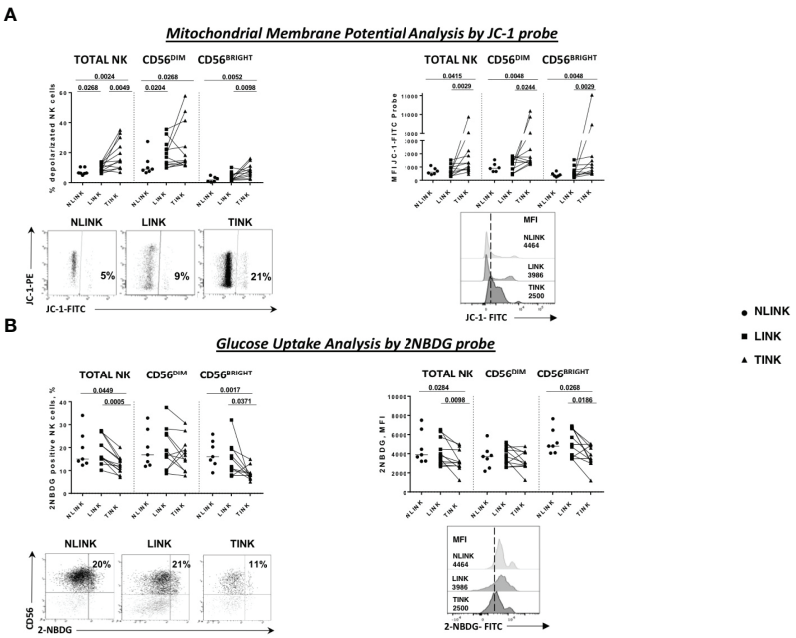


FIGURE 2 | Metabolism assessment of infiltrating NK cells. **(A)** Mitochondrial membrane potential measured by the potentiometric probe JC-1. After anti-CD3 and anti-CD56 staining, JC-1 was added to NK cells from the three compartments: NLINK (n = 7), LINK (n= 12), and TINK (n = 12). Afterwards, the cells were stained with 7-AAD for viability and then analyzed on a flow cytometer. Depolarized NK cells (upper left panel) were quantified by the percentage of FL1high/FL2low cells (JC-1 staining) detected in the different samples. Median fluorescent intensity (MFI) of JC-1 in the FITC channel were analyzed in all study groups in the NK cells subsets (TOTAL, $CD56^{DIM}$, and $CD56^{BRIGHT}$). In the lower panels, representative examples of the two analyses. **(B)** Glucose uptake assay was performed on LINK (n = 12), NLINK (n = 7), and TINK (n = 12). The cells were stained with the glucose analog 2-NBDG. The frequency of 2-NBDG-positive NK cells was evaluated (upper left panel) as well as the MFI of the probe (upper right panel). Representative dot plots and histograms show the 2-NBDG uptake in NK cells. Statistical analysis was performed by Wilcoxon matched pairs test (LINK *vs*. TINK) and Mann Whitney test (NLINK *vs*. TINK and NLINK *vs*. LINK). Horizontal lines represent median values.

reduced accumulation of autophagic vesicles was even more evident in HCC-infiltrating NK cells compared to NLINK and LINK (**Figure 4C**).

Cyto-ID expression was negatively correlated to p38 levels in TINK samples, suggesting a strong relationship between p38 and autophagy repression in TINK (**Figures 4B, D**).

Functional Impairment of Infiltrating NK Cells

In order to define the functional capacity of tumor-infiltrating NK cells, cytokine production and degranulation (CD107a expression) activity were assessed. IFN-γ production was not significantly impaired in TINK compared to LINK and NLINK (**Figure 5A**).

Conversely, TNF-α (**Figure 5B**) and CD107a (**Figure 5C**) expression was significantly downregulated in TINK compared to liver-infiltrating NK cells (LINK and NLINK) (**Figure 5D**), particularly in the $CD56^{BRIGHT}$ subset.

Dysfunctional NK adhesion to target K562 cells was detected in TINK, also suggesting an impairment in immunological synapse establishment by intratumor NK cells compared to NLINK and LINK (**Figure 5E**).

We then investigated the possible relationship between cytokine production/degranulation and metabolic functions (**Supplementary Figure S1**). IFN-γ, TNF-α, and CD107a expression did not correlate to the frequency of NK cells with depolarized mitochondria and autophagy function (**Supplementary Figure S1**, plots on the left) in LINK, whereas glucose uptake turned out to be positively correlated with IFN-γ production (**Supplementary Figure S1B**).

By contrast, the metabolic status of intratumor NK cells showed a strong association with cytokine production and degranulation capacity. In particular, the frequency of NK cells with depolarized mitochondria exhibited a negative correlation with respect to IFN-γ, TNF-α, and CD107a expression (**Supplementary Figure S1D**) suggesting that the mitochondrial functionality in TINK impacts on cytokine production and cytotoxic function. Glucose consumption and autophagy potential were also strictly connected to the immune functions of HCC-infiltrating NK cells (**Supplementary Figures S1E, F**).

Functional and Metabolic Restoration of Tumor-Infiltrating NK Cells by Targeting p38 Protein

To further analyze the relationship between p38 activation and TINK dysfunction, we assessed the effect on NK metabolic profile and anti-tumoral functions of two different chemical inhibitors targeting p38 (**Figures 6A–D**).

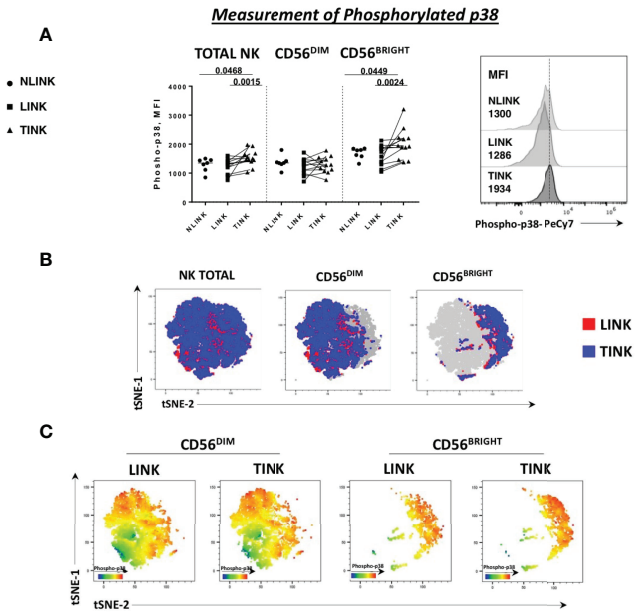


FIGURE 3 | Phosphorylation status of p38 protein in NK cells infiltrating hepatocellular carcinoma (TINK), HCC-surrounding liver (LINK), and normal liver tissue (NLINK). **(A)** On the left, median fluorescence intensity (MFI) of phospho-p38 protein in total, CD56^{DIM}, and CD56^{BRIGHT} NK-cell subpopulations from TINK (n = 12), LINK (n = 12), and NLINK (n = 7). Right panel: detail of phospho-p38 MFI values of total NK cells from different study groups. Statistical analysis was performed by Wilcoxon matched pairs test (LINK *vs*. TINK) and Mann-Whitney test (NLINK *vs*. TINK and NLINK *vs*. LINK). **(B)** To show the segregation of NK subsets, we generated a two-dimensional map of NK cells from paired TINK and LINK from all the experimental samples. tSNE was applied to flow cytometry data (single-cell expression values). **(C)** tSNE colored by the expression intensity of phospho-p38 protein in TINK and LINK samples. Red indicates upregulation, while blue indicates downregulation. Different color shades represent intermediate levels.

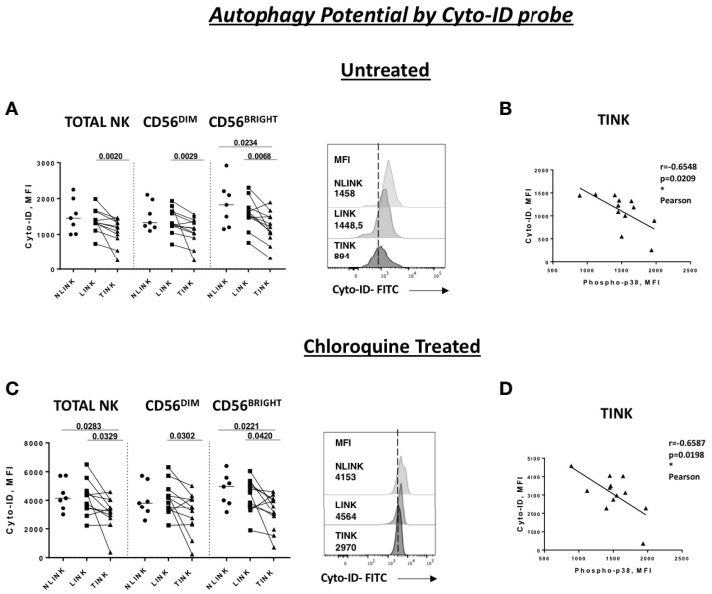


FIGURE 4 | Autophagy capacity in tumor-infiltrating NK cells (TINK), non-tumorous liver-infiltrating NK cells (LINK), and normal liver-infiltrating NK cells (NLINK). Cyto-ID median fluorescence intensity (MFI) values in TINK (n = 12), LINK (n = 12), and NLINK (n = 7) from untreated **(A)** and chloroquine-treated **(C)** samples (total, CD56^{DIM}, and CD56^{BRIGHT} NK cells). Examples are shown on the right of each panel. Statistical analysis was performed by Wilcoxon matched pairs test (LINK *vs* TINK) and Mann–Whitney test (NLINK *vs* TINK and NLINK *vs* LINK). Horizontal lines represent median values. Correlation between the Cyto-ID MFI values of untreated **(B)** and chloroquine-treated **(D)** and phospho-p38 protein in total NK cells from TINK samples. Statistics by Pearson correlation. *p < 0.05

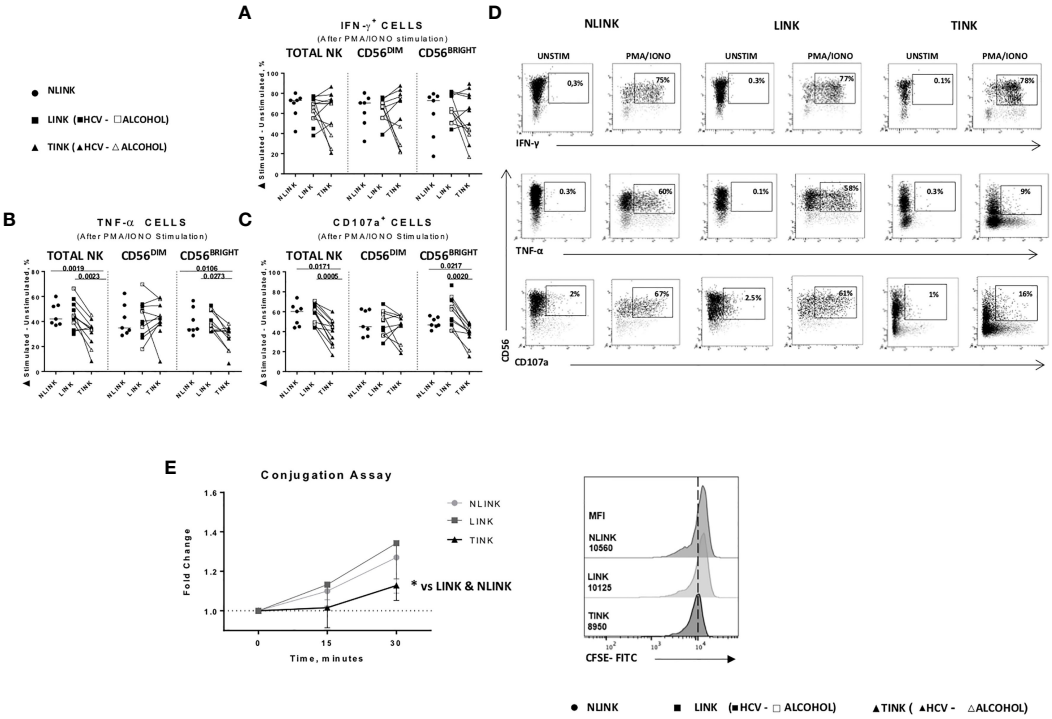


FIGURE 5 | Functional analysis of NLINK, LINK, and TINK. **(A, B)** IFN-γ and TNF-α production in infiltrating NK cells was evaluated with or without phorbol 12-myristate 13-acetate and ionomycin incubation for 4(h) **(C)** The cytotoxic potential of NK cells was measured by CD107a degranulation assay. **(D)** Representative dot plots showing IFN-γ, TNF-α, and CD107a expression in tumor- and liver-infiltrating NK cells from NLINK, LINK, and TINK upon PMA/ionomycin stimulation. **(A–C)** Data are presented as the delta between the frequency of cytokine$^+$ or CD107a$^+$ NK cells in unstimulated and stimulated samples. Horizontal lines indicate median values. **(E)** NK cell adhesion to target cells (K562) was evaluated by staining NK cells and K562 with two distinct fluorescent dyes (anti-CD3 and anti-CD56 and 0.5 μM CFSE, respectively). The effector-to-target ratio was 5:1. Cells were incubated at 37°C for 0, 15, and 30 min, followed by fixation. Median fluorescence intensity (MFI) ratio was measured at different time points (left panel). Cell conjugation is presented as MFI values of CD56$^+$ cells in CFSE-FITC target cells. Comparison of TINK, NLINK, and LINK at 30 min (right panel). **(A–E)** NLINK (n = 7), LINK (n = 12), and TINK (n = 12) *p < 0.05.

p38 blockade strongly increased the TNF-α (**Figure 6C**) and CD107a (**Figure 6D**) expression in TINK in all the NK subsets, whereas the effect on IFN-γ production was limited (**Figure 6A**). LINK and NLINK function did not benefit from p38 blockade, demonstrating the specificity of p38 inhibition in the tumor context. Representative dot plots showing CD107a, IFN-γ, and TNF-α expression in NK cells from NLINK, LINK, and TINK in untreated *vs*. p38 inhibitor-treated samples are shown in **Supplementary Figure S2**.

Interestingly, although IFN-y production was less affected than the other functions, TINK samples with impaired function were more affected by p38 inhibition (**Figure 6B**). Moreover, treatment with p38 inhibitors enhanced the adhesion to target cells by TINK compared to LINK, but the effect did not reach statistical significance (**Figure 6E**). Finally, p38 blockade strongly reduced the NK cells with depolarized mitochondria in the tumor, in particular in the CD56^{BRIGHT} subset, while p38 inhibition did not show any effect in modulating the mitochondrial membrane potential in the liver counterpart (**Figure 7A**). In addition, the NK autophagy potential was significantly enhanced by p38 inhibition, especially in the chloroquine-treated tumor-infiltrating NK cells (**Figure 7B**) in all NK cell subsets. Differently though, glucose import was not affected by treatment both in liver- and tumor-infiltrating NK cells (**Figure 7C**).

DISCUSSION

The understanding of the molecular and cellular characteristics of NK cell responses in patients with HCC has greatly improved in recent years (7, 11, 12). However, our knowledge of the mechanisms responsible for NK cell dysfunction in tumors and especially in HCC remains largely incomplete. Here we performed transcriptome, metabolic, and functional analyses targeting the identified dysregulated pathways of tumor-infiltrating NK cells . We found a predominantly upregulated gene expression profile in intra-tumoral NK cells compared to the liver counterpart. However, this extensive upregulation, involving mitochondrial components, glucose metabolism, and DNA damage stress responses, was not associated with an improved antitumor function but rather with a profound impairment at both functional and metabolic levels. This discrepant transcriptional and functional metabolic output was

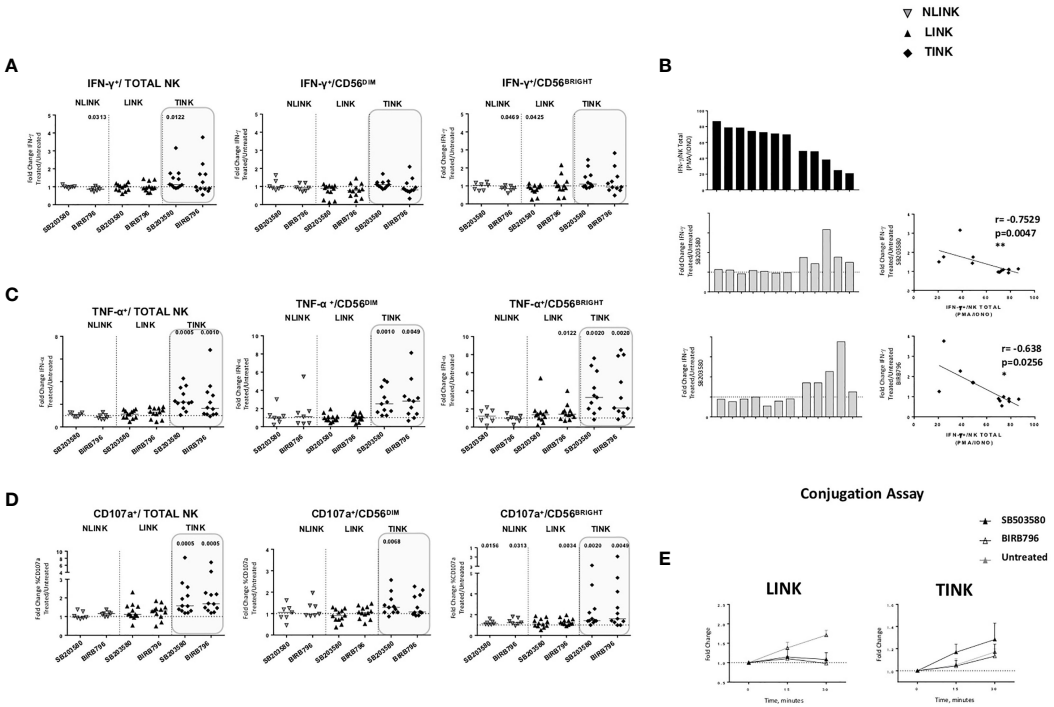


FIGURE 6 | Restoration assays of hepatocellular carcinoma-infiltrating NK cells. IL-12 + IL-18 O/n stimulation of NK cell stimulation with or without specific p38 inhibitors was followed by flow cytometry determination of IFN-γ **(A)**, TNF-α **(B)** and CD107a **(C)** production in study groups and in all NK cell subsets (total, $CD56^{DIM}$, and $CD56^{BRIGHT}$). Data are presented as the ratio between the frequency of cytokine and CD107a-positive NK cells tested in the presence of inhibitor or untreated cultures (fold change). Horizontal lines represent median values. **(D)** Bar graph showing IFN-γ production upon PMA/ionomycin stimulation from single TINK samples (upper panel). On the middle and lower panels, corresponding fold change values in the two p38 inhibitor-treated TINK samples. Right panels: correlation between IFN-γ production and response to p38 blockade. **(E)** Conjugation assay performed on LINK and TINK samples with or without p38 inhibition. **(A–C)** Statistical analysis by Wilcoxon signed-rank tests.**(D)** Statistics by Pearson's correlation. **(A–E)** LINK (n= 11) and TINK (n= 11). *p < 0.05, **p < 0.01.

especially clear for OXPHOS impairment, with an altered mitochondrial membrane potential, and for glucose metabolism, associated with a decreased glucose uptake capacity. OXPHOS was upregulated at the transcriptional level but functionally depressed, and glucose use and glycolytic functions were similarly upregulated at the mRNA level but translated into an energetic repression in HCC-infiltrating NK cells .

A similar discrepancy between metabolic, functional, and gene expressions that could reflect a failed compensatory attempt has been described in exhausted CD8+ T cells (42). In particular, defective OXPHOS and glycolytic activity have recently been described in several different models (33, 43), including early-exhausted CD8+ T cells reported in HCV infection (44).

Even if we do not have clear direct evidence, we can imagine that, in tumors as well as in chronic infections, NK cells may present an exhausted condition similar to exhausted T cells, with poor effector characteristics (45), and a progressive loss of function correlating to disease progression.

Even if NK cell metabolism studies are in an early phase, T and NK cells have common metabolic pathways for several functions from activation to regulatory function (46).

Indeed both cell types become metabolically quiescent in mature status, using mainly fatty acid β-oxidation and oxidative phosphorylation when a change in energetic needs is required (46). Although metabolic needs may be different upon activation by activating ligands, a common feature seems to be the use of glucose for enhanced glycolysis and OXPHOS. This metabolic shift is dictated by an increase of energy demands within a short period of time for ATP and protein synthesis in order to accomplish their cytotoxic functions (46).

Our GSEA results support the mechanism showing an upregulation of ROS detoxification system-related pathways. In line with this view, our data show an increase of depolarized mitochondria in HCC-infiltrating NK cells and a decrease in potential glucose utilization, especially in the $CD56^{BRIGHT}$ subpopulation, translated into an inefficient cytotoxic activity, as supported by the downregulation of TNF-α and CD107a expressions and by a poor ability to establish an immunological synapse with target cells (44).

While the production of inhibitory cytokines in the tumor microenvironment induces a profound immune impairment, immune cells react by activating the stress sensor-related pathways. Specifically, cellular stress induced by starvation leads to MAPK/p38 cascade activation. p38 subunits (such as

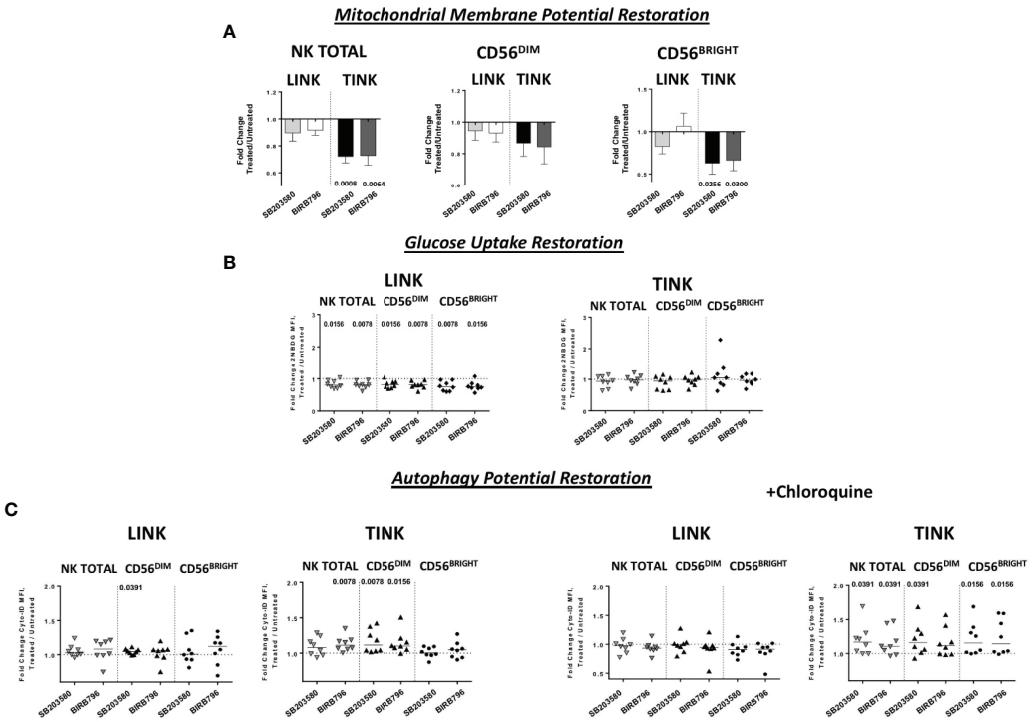


FIGURE 7 | Metabolic restoration of HCC (n = 8) and non-tumorous liver-infiltrating NK cells (n = 8). IL-12 + IL-18 overnight stimulation with or without specific p38 inhibitors was followed by flow cytometry determination of JC-1 **(A)** 2-NBDG **(B)** and Cyto-ID (with and without chloroquine) **(C)**. Data are presented as the ratio between the metabolic values in inhibitor-treated *vs*. untreated NK cells from liver and tumor counterparts (fold change). Horizontal lines represent median values. Statistics by Wilcoxon signed-rank tests.

p38α and MAPK14), after activation and phosphorylation, control a plethora of downstream functions. In particular, MAPK14 is responsible of the quick change from glycolysis to the pentose phosphate pathway (PPP) through modulation of the proteasome degradation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase 3. This leads to reduced autophagy and an increase in resistance to nutrient starvation (47). The results of NK-cell expression profiling support this view, suggesting the activation of the PPP pathway with an upregulation of PFK genes.

Under nutrient stress, there is activation of the catabolic process of autophagy with turnover of macromolecules and organelles (48). Since autophagy is considered a mechanism of cell survival, its deregulation does not lead to apoptotic cell death. It is known that a robust autophagic capacity appears in immature NK cells in order to lead to NK cell development and education (33, 49). During development, autophagy protects NK cells by removing damaged mitochondria that reduce the ROS levels (33).

It has already been described for CD8+ T cells that, under low nutrient conditions and high proliferative rate, p38 is phosphorylated *via* the AMP-activated protein kinase (AMPK) and the scaffold protein β-activated TAB1 (50). This leads to a metabolic reprogramming with glycolysis and autophagy reduction and with consequent blocking of mitophagy and accumulation of damaged and dysfunctional mitochondria. The final effect of p38 activity augmentation is metabolic engulfment and proliferation arrest (40, 51), which are associated to a senescent-like behavior with a negative effect on telomerase activity (40, 50). Similarly, in a previous study, we found that intra-tumoral NK cells show a profound reduction of intracellular transducers, including the proximal CD3 zeta chain (34, 52), and our current analysis also confirms a deregulation in the autophagy process related to p38 activation in tumor-infiltrating NK cells.

However, long-lasting activation of the p38α pathway can lead to impaired cell proliferation and cell death (53), which might explain the lower level of mature and cytotoxic intratumor NK cells (52). These profound metabolic derangements impair the capacity of NK cells to exert efficient anti-tumor functions (54) and to generate immunological memory, together with a lower basal level of autophagy, which are essential in NK cell development (33, 49). Targeting p38 may open to new strategies of immunotherapeutic intervention.

Interestingly, the $CD56^{BRIGHT}$ subset showed a higher level of p38 phosphorylation and functional impairment. This subset expresses liver residency markers such as CXCR6 and CD49a (52) at a higher degree, and it is easy to speculate that the NK cells in HCC have been recruited from the liver or from the periphery, and in a hypoxic tumor environment, they upregulate

CD56 (55). While the entire population of TINK is in a stress condition due to hypoxia and starvation, the $CD56^{BRIGHT}$ subset has been more profoundly impaired.

Despite the lack of direct supporting evidence in NK cells , a direct relationship between p38 upregulation and metabolic and functional impairment in HCC-infiltrating NK cells is likely, as already described for CD8+ T cells (40, 50).

For this reason, the pharmacological inhibition of p38 protein subunits could be a therapeutic option in patients with early HCC. Our data indicate that p38 modulation is highly specific for tumor-infiltrating NK cells, thus not affecting the functions of NK cells infiltrating liver tissue. In tumor NK cells, we observed that p38 blockade induced a strong restoration of TNF-α and CD107a production accompanied by increased IFN-γ expression in samples with a reduced production of this cytokine. These results suggest that NK cells might be exhaustion-oriented at different levels and that selective targeting of p38 might allow specific functional recovery. In addition, metabolic functions such as OXPHOS and autophagy were significantly improved by p38 inhibition, specifically in HCC-infiltrating NK cells, further indicating a positive impact of p38 blockade over NK cell fitness.

The results of this study are not sufficient to indicate a specific biochemical metabolic cascade of dysregulation of HCC-infiltrating NK cells and to define how OXPHOS derangement and impaired autophagy are linked to mitochondrial dysfunction and p38 activation. Given the functional recovery obtained with selected specific p38 inhibitors, the knowledge generated in this study altogether has relevant potential for novel and more effective strategies of NK cell reconstitution, thus able to improve immunotherapy for HCC patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/genbank/, GSE183349.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Indipendente Area Vasta Emilia Nord, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AZ: study concept and design, analysis, and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and statistical analysis. VB: acquisition of data, analysis and interpretation of data, and statistical analysis. AO: acquisition of data and technical or material support. EB: acquisition of data and technical or material support. CB: acquisition of data and critical revision of the manuscript for important intellectual content. PF: acquisition of data and critical revision of the manuscript for important intellectual content. IM: acquisition of data and technical or material support. CT: acquisition of data and technical or material support. RDV: acquisition of data, technical or material support, and critical revision of the manuscript for important intellectual content. CF: drafting of the manuscript and critical revision of the manuscript for important intellectual content. EC: study concept and design, drafting of the manuscript, and critical revision of the manuscript for important intellectual content. GM: study concept and design, drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained funding, and study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.875072/full#supplementary-material

Supplementary Table 1 | Demographic and clinical characteristics of study population.

Supplementary Table 2 | Detailed lists of misregulated genes derived from Gene Set Enrichment Analysis.

Supplementary Table 3 | Gene Set Enrichment analysis (GSEA). GSEA identified the list of enriched gene sets in TINK and LINK by MSigDB and C2 canonical pathways set. NES, normalized enrichment score; FDR, false discovery rate.

Supplementary Figure 1 | Correlation between TINK and LINK metabolism and functions. **(A)** Correlation between the frequency of depolarized mitochondria in LINK (n = 12) total NK cells and corresponding IFN-γ, TNF-α, and CD107a production.**(B)** Correlation between glucose uptake, described by 2-NBDG median fluorescence intensity, in LINK and corresponding IFN-γ, TNF-α, and CD107a production. **(C)** Correlation between the frequency of CD107a+, IFN-γ, and TNF-α and the Cyto-ID MFI values in LINK samples. **(D–F)**. Correlation between the percentage of CD107a+, IFN-γ, and TNF-α with mitochondrial function **(D)** glucose uptake **(E)** and autophagy potential in TINK (n= 12) **(F)**. Statistics by Pearson's rank-order correlation.

Supplementary Figure 2 | Flow cytometry analysis of NK cell functional and metabolic restoration. **(A)** Representative dot plots showing IFN-γ, TNF-α, and CD107a expression in NK cells from NLINK, LINK, and TINK in untreated *vs* . p38 inhibitor-treated samples. **(B)** Representative dot plots showing the percentage of depolarized mitochondria in tumor- and liver-infiltrating NK cells in untreated *vs*. p38 inhibitor-treated samples. **(C)** Histograms representing glucose uptake modulation in treated *vs*. untreated samples in LINK and TINK samples. **(D)** Representative histograms showing Cyto-ID staining in NK cells from LINK and TINK in untreated *vs*. p38 inhibitor-treated samples.

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Identification and Validation of a Novel Signature Based on NK Cell Marker Genes to Predict Prognosis and Immunotherapy Response in Lung Adenocarcinoma by Integrated Analysis of Single-Cell and Bulk RNA-Sequencing

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Natural killer (NK) cells, the effectors of the innate immune system, have a remarkable influence on cancer prognosis and immunotherapy. In this study, a total of 1,816 samples from nine independent cohorts in public datasets were enrolled. We first conducted a comprehensive analysis of single-cell RNA-sequencing data of lung adenocarcinoma (LUAD) from the Gene Expression Omnibus (GEO) database and determined 189 NK cell marker genes. Subsequently, we developed a seven-gene prognostic signature based on NK cell marker genes in the TCGA LUAD cohort, which stratified patients into high-risk and low-risk groups. The predictive power of the signature was well verified in different clinical subgroups and GEO cohorts. With a multivariate analysis, the signature was identified as an independent prognostic factor. Low-risk patients had higher immune cell infiltration states, especially $CD8^+$ T cells and follicular helper T cells. There existed a negative association between inflammatory activities and risk score, and the richness and diversity of the T-cell receptor (TCR) repertoire was higher in the low-risk groups. Importantly, analysis of an independent immunotherapy cohort (IMvigor210) revealed that low-risk patients had better immunotherapy responses and prognosis than high-risk patients. Collectively, our study developed a novel signature based on NK cell marker genes, which had a potent capability to predict the prognosis and immunotherapy response of LUAD patients.

Keywords: single-cell RNA-sequencing, NK cell marker genes, prognostic signature, immunotherapy, lung adenocarcinoma

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INTRODUCTION

Lung cancer is the global leading cause of cancer-related mortality (1), among which lung adenocarcinoma (LUAD) represents the main histological subtype, comprising nearly 50% of all lung cancers (2–4). Despite the significant advances in therapeutic strategies for LUAD, the 5-year overall survival (OS) of LUAD remains below 20% (5). Recently, the clinical application of immunotherapies targeting immune checkpoints has dramatically improved clinical benefits and shifted the treatment paradigm of LUAD (6, 7). Several biomarkers are now widely used in clinical practice to predict immunotherapy response, including PD-L1 expression and tumor mutation burden (TMB) (8). However, these biomarkers could not fully reflect the heterogeneous tumor microenvironment (TME) and clinical benefits from immunotherapy is still limited to a portion of LUAD patients (9). As a result, it is imperative to develop prediction models and identify new biomarkers to predict prognosis and therapeutic effect.

Tumor cells are surrounded by the TME, which is quite complex and comprises different immune cells, stromal cells, extracellular matrix molecules, and various cytokines (10, 11). Emerging evidence has demonstrated that the components of the TME are recognized to play vital roles in tumor initiation and progression. Furthermore, abnormal changes in TME not only impact the prognosis of patients but could also be used as a biomarker for immunotherapy (12). In the context of anti-tumor immunity, the focus is mainly on the adaptive T-cell response, while the role of innate immune cells has not yet received enough attention. Natural killer (NK) cells, a subtype of innate immune cells, can rapidly recognize and kill tumor cells (13). The efficient activity of NK cells depends entirely on a balance of inhibitory and activating receptors that can interact with ligands on target cells (14). NK cells can participate in anti-tumor immunity in the early presence of tumors by directly killing tumor cells and promoting adaptive T-cell immunological responses (15), thereby limiting tumor cell aggressiveness (16). NK and T cells work together to control cancer progression, indicating the importance of NK cells in shaping anti-tumor immunity, which has also been demonstrated by several previous studies. Reduced NK cell activity in the peripheral blood increases the risk of malignancy (17). Additionally, the higher abundance of tumor-infiltrating NK cells was significantly linked with better prognosis in different types of tumors (18–21). Given the roles of NK cells in immunity, previous studies have investigated the molecular characteristics of NK cells in infectious diseases and cancers (22–27), whereas a comprehensive molecular analysis of NK cells in LUAD is relatively poorly known.

The development of single-cell RNA-sequencing (scRNA-seq) technology and associated methods for data analysis has provided an unprecedented opportunity to unravel the molecular characteristics of diverse immune cell populations in the TME (28). Previous studies have reported that exploring gene expression signatures based on molecular characteristics of immune cells derived from scRNA-seq data might be a potent method to predict the prognosis and immunotherapy response of cancer patients (29, 30). In this study, we first performed a comprehensive analysis of scRNA-seq of LUAD to dissect the molecular characteristics of tumor-infiltrating NK cells and identify the marker genes of NK cells. Next, a NK cell marker gene signature (NKCMGS) was constructed for prognosis prediction of LUAD through bulk RNA-seq analysis. Furthermore, the predictive power of the NKCMGS was validated in six independent cohorts from the Gene Expression Omnibus (GEO) database, and the relationship between the NKCMGS and immunotherapy response in LUAD was investigated.

MATERIALS AND METHODS

Data Collection

Totally, 1,816 samples were enrolled in this study, namely, 11 LUAD samples with scRNA-seq data, 500 LUAD samples from the TCGA, 1,007 LUAD samples from six independent GEO cohorts (https://www.ncbi.nlm.nih.gov/geo/), and 298 samples treated with immunotherapy from the IMvigor210 cohort. Single-cell RNA-sequencing data from 11 primary LUAD samples of GSE131907 were obtained from the GEO database, and were used to determine the NK cell marker genes of LUAD. The Cancer Genome Atlas (TCGA) bulk tumor transcriptomic data (FPKM normalized) and clinical information of 500 patients with LUAD were downloaded from the UCSC Xena (https://xenabrowser.net/) for identifying survival-related genes and constructing prognostic signatures. Six independent microarray datasets, namely, GSE30219 (n = 83), GSE3141 fimmu.2022.850745(n = 58), GSE50081 (n = 127), GSE26939 (n = 115), GSE72094 (n = 398), and GSE31210 (n = 226), were also obtained from the GEO database for external validation. In this study, the TCGA RNA-sequencing data of were converted into transcripts per kilobase million (TPM) values, which are more comparable between TCGA samples and microarrays (31). Transcriptomic and matched clinical data of patients who received anti-PD-L1 treatment from the IMvigor210 cohort were collected from http://research-pub.gene.com/IMvigor210CoreBiologies to explore the value of NKCMGS in speculating on the immunotherapy response (32). The study used publicly available datasets with preexisting ethics approval from original studies.

Identification of NK Cell Marker Genes by scRNA-seq Analysis

We conducted an analysis of scRNA-seq data by R packages, including "Seurat" and "SingleR" (33). To retain high-quality scRNA-seq data, three filtering measures were applied to the raw matrix for each cell: only genes that were expressed in at least 5 single cells were included, cells that expressed less than 100 genes were eliminated, and cells with more than 5% of mitochondrial genes were also removed. We first used the "Seurat" R package to normalize scRNA-seq data by the "NormalizeData" function, setting the normalization method as "LogNormalize." Normalized scRNA-seq data were then transformed into a Seurat object, and the top 1,500 highly variable genes were identified using the "FindVariableFeatures" function. After that, we applied the "RunPCA" function of the "Seurat" R package to perform the principal component analysis (PCA) to

reduce the dimension of the scRNA-seq data based on the top 1,500 genes. We used JackStraw analysis to identify significant PCs, and we selected the first 15 PCs for cell clustering analysis according to the proportion of variance explained. The "FindNeighbors" and "FindClusters" functions in the "Seurat" package were used for cell clustering analysis. The k-nearest neighbor graph was constructed based on Euclidean distance in PCA using the "FindNeighbors" function to determine the closest neighbors of each cell. Then, t-distributed stochastic neighbor embedding (t-SNE) was performed using the "RunTSNE" function. Cell clustering was demonstrated using t-SNE-1 and t-SNE-2. The "FindAllMarkers" function in the "Seurat" package was used to calculate the differentially expressed genes (DEGs) of each cluster using Wilcoxon–Mann–Whitney tests. To identify the marker genes for each cluster, the cutoff threshold values, adjusted p-value <0.01 and | log2 (fold change)| >1 were used. For cluster annotation, we performed a reference-based annotation using reference data from the Human Primary Cell Atlas (34).

Construction and Validation of Prognostic Signature Based on NK Cell Marker Genes

A univariate Cox regression analysis was performed to evaluate the prognostic value of NK cell marker genes for OS in TCGA LUAD patients, and genes with p <0.01 were identified as prognostic genes. Next, to minimize overfitting, prognostic genes were assessed by least absolute shrinkage and selection operator (LASSO) Cox proportional hazards regression using the "glmnet" package. LASSO is a popular method for regression with high-dimensional predictors and is broadly applied to the Cox proportional hazard regression model for survival analysis (35). By using the function "cv.glmnet", 10-fold cross-validation was conducted to select the best model. The tuning parameter λ was chosen by 1 − SE (standard error). We got a list of genes with non-zero beta coefficients. Finally, based on the genes generated by LASSO Cox regression analysis, we used a stepwise multivariate Cox regression analysis to identify the prognostic values of specific gene signatures. The risk model was constructed by a linear combination of the mRNA expression of the genes and the relevant risk coefficient. Based on the median cut-off value, the patients were classified into the low-risk or high-risk groups. To validate the prognostic power of the NKCMGS, the area under the curve (AUC) was calculated using the "survivalROC" package (36). The Kaplan–Meier method was employed for survival analysis, and the log-rank test was used to determine the statistical significance of the differences using the R package "survminer" (37). The predictive ability of the signature was validated using survival analysis and AUC in 6 independent GEO datasets.

Pathway and Function Enrichment Analysis

We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis by using the R package "clusterProfiler" (38). GO analysis was performed using the enrichGO function of the R package "clusterProfiler" and GO annotations were based on genome-wide annotation packages (org.Hs.eg.db) released by the Bioconductor project (39). KEGG analysis was performed using the enrichKEGG function of the R package "clusterProfiler" and "clusterProfiler" queries the latest online KEGG database through a web API to obtain the pathway data and perform functional analysis. A p-value of < 0.05 was considered significant enrichment.

Immune Cell Infiltration Analysis and Gene Sets Variation Analysis (GSVA)

The CIBERSORT algorithm, a useful method for obtaining infiltrating characteristics of 22 immune cell types with gene expression profiles (40), was applied to dissect the proportion of immune cell infiltration in high-risk and low-risk groups. A seven-metagene (HCK, IgG, Interferon, LCK, MHC-I, MHC-II, and STAT1) has been extensively used to assess the inflammatory activity in TME (41). Therefore, we conducted GSVA analysis to investigate the associations between the NKCMGS and metagenes of inflammatory activities by using the "GSVA" package (42). Heatmap plots were generated using the "ComplexHeatmap" R package from Bioconductor (43).

Estimation of Stromal and Immune Scores

The ESTIMATE algorithm was employed to assess levels of stromal and immune cell infiltration using expression profiles by the "estimate" R package (44). Stromal score, immune score, ESTIMATE score, and tumor purity score were calculated using the RNA-sequencing data of the TCGA LUAD cohort and a Wilcoxon t-test was performed to compare these scores between different risk groups.

Immunotherapy Response Prediction

We first applied PD-L1 expression, tumor mutation burden (TMB), and TCR repertoire to predict the response to immune checkpoint blocking therapy. The PD-L1 mRNA expression of LUAD patients was collected from RNA-sequencing data of the TCGA LUAD cohort. Gene mutation data of LUAD patients were downloaded from the TCGA database and TMB was calculated using "maftools" package (45). TMB was determined as the number of somatic indels and base substitutions per million bases in the coding region of the genome detected, and was calculated as previously described (46). The richness and Shannon diversity indexes were used to characterize the diversity of the TCR repertoire. The richness measures the number of unique TCRs in the sample, while the Shannon diversity index reflects the relative abundance of the different TCRs. The richness values and Shannon diversity index valves of TCR in the TCGA LUAD patients were obtained from the Pan-Cancer Atlas study (47). Additionally, 298 urothelial carcinoma patients with both transcriptomic data and treatment response to immunotherapy from the IMvigor210 cohort were used for speculating the immunotherapy response of the signature.

Statistical Analysis

Categorized variables between different risk groups were compared by the Wilcoxon t-test. Univariate and multivariate Cox regression analyses were used to investigate the prognostic

value of the NKCMGS and different clinicopathological characteristics. P <0.05 was set as a significant threshold. Benjamini–Hochberg was implemented to adjust the P-value for multiple testing using the R function "p.adjust". For data analysis and generation of figures, R software version 4.1.0 (http://www.R-project.org) was used.

RESULTS

Identification of NK Cell Marker Gene Expression Profiles

Based on scRNA-seq data of GSE131907, we obtained gene expression profiles of 45,149 cells from 11 primary LUAD samples for further analysis (**Figure 1A**). We conducted PCA using the top 1,500 variable genes to reduce the dimensionality, and 17 cell clusters were then identified (**Figure 1B**). Subsequently, the cell identity of each cluster was annotated using a reference dataset from the Human Primary Cell Atlas, and cells in cluster 7 were defined as NK cells (**Figure 1C**). This cluster was also found to have distinct gene expression profiles, with 189 genes differentially expressed between the 17 clusters (**Figure 1D**), which were identified as LUAD-related NK cell marker genes (**Supplementary Table 2**). The functional enrichment, including GO and KEGG analysis, showed that the NK cell marker genes were mostly related to immune features, such as positive regulation of leukocyte activation, MHC protein complex, antigen binding, and hematopoietic cell lineage (**Supplementary Figure 1**).

Establishment of the Seven-Gene Prognostic Signature Based on NK Cell Marker Genes

To construct a prognostic signature based on the 189 NK cell marker genes, we first used the TCGA LUAD cohort as the training set to perform a univariate Cox regression analysis, and 25 NK cell marker genes were significantly related to OS (**Supplementary Table 3**). Next, LASSO Cox regression analysis with one standard error (SE) and 10-fold cross-validation was conducted on the 25 NK cell marker genes, and 16 genes were screened out (HPGDS, CTSG, SLC18A2, GCSAML, ADRB2, ACTG1, ACOT7, CLIC1,

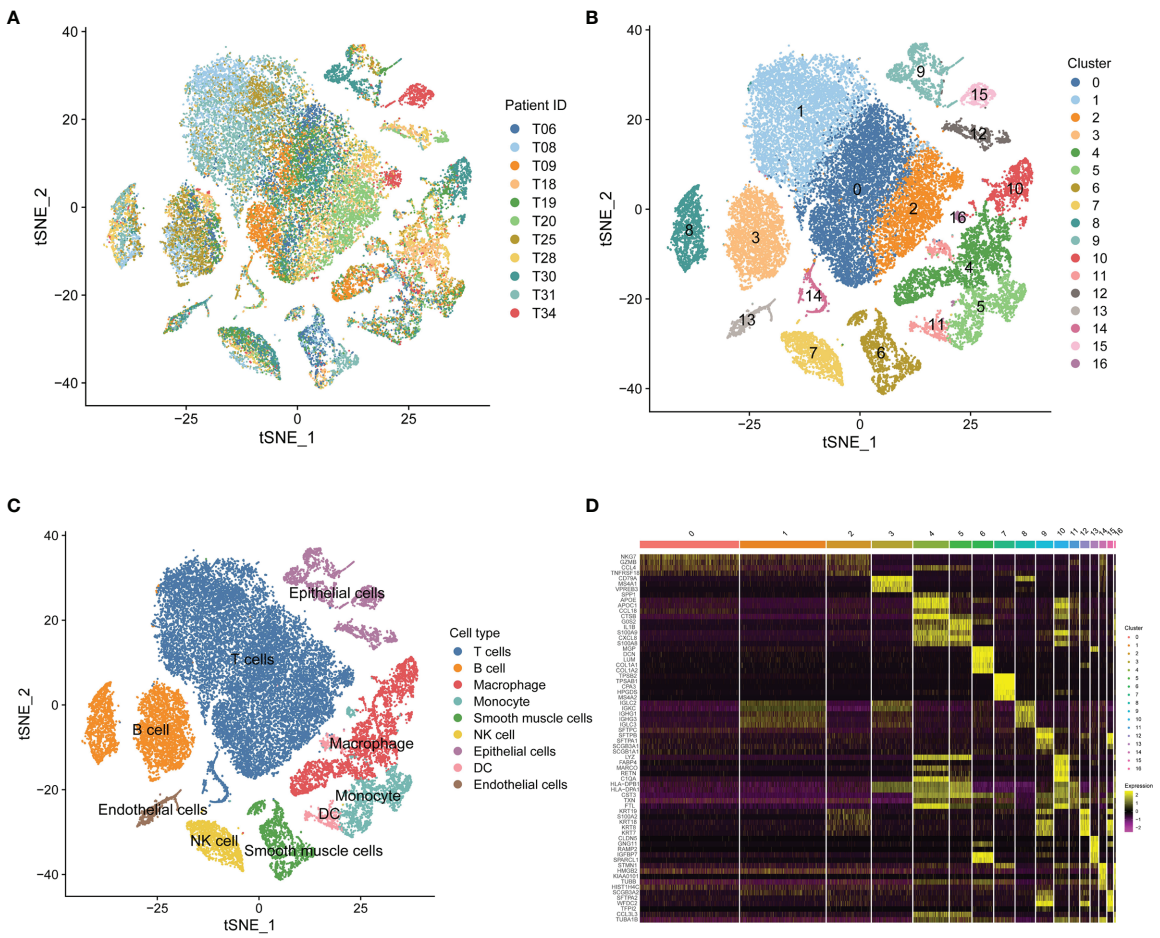

FIGURE 1 | Single-cell RNA-sequencing analysis identifies NK cell marker genes. **(A)** t-SNE plot of 45,149 cells from 11 primary LUAD samples. **(B)** t-SNE plot colored by various cell clusters. **(C)** The cell types identified by marker genes. **(D)** Heatmap showing the top 5 marker genes in each cell cluster.

SELENOK, PEBP1, BEX4, BIRC3, DDIT4, TRBC1, ACAP1, and S100A10) for further analysis (**Supplementary Figures 2A, B**). Finally, we used stepwise multivariate Cox regression analysis to optimize the prognostic signature to only include the 7 most predictive genes: Risk score = (−0.614 × GCSAML expression) + (1.893 × ACTG1 expression) + (1.022 × ACOT7 expression) + (−1.715 × SELENOK expression) + (−1.840 × PEBP1 expression) + (1.077 × BIRC3 expression) + (−1.180 × ACAP1 expression) (**Supplementary Figure 2C**). The relative expression of the 7 marker genes in various clusters is shown in **Supplementary Figure 3**, which indicates the specificity of the expression of the signature genes. The median risk score was 0.956 by ranking the risk score from high to low, which was used to classify patients into low-risk (n = 250) and high-risk (n = 250) groups. **Figure 2A** exhibited the distribution of risk scores and survival status, which suggested more deaths in the high-risk group. **Figure 2B** shows the expression details of the 7 NK cell marker genes.

Kaplan–Meier analysis demonstrated that patients with high-risk scores had significantly inferior OS than patients with low-risk scores (**Figure 2C**). To assess the predictive accuracy of the risk model, time-dependent area under the ROC curves for OS was calculated, and the 1-, 3-, and 5-year AUC values were 0.710, 0.725, and 0.730, respectively (**Figure 2D**). The performance of the model was then evaluated using a ten-fold cross-validation procedure, and the 1-, 3-, and 5-year mean AUC values were 0.669, 0.674, and 0.652, respectively.

Validation of the NKCMGS in Different Clinical Subgroups

The predictive value of NKCMG was first assessed in TCGA LUAD patients with different genders, ages, smoking histories, and tumor stages. The results revealed that high-risk score was significantly correlated with an inferior prognosis in the male ($P = 1.5 \times 10^{-4}$, **Supplementary Figure 4A**), female ($P = 3.563 \times 10^{-5}$, **Supplementary Figure 4B**), young ($P = 7.8 \times 10^{-4}$, **Supplementary Figure 4C**) or old ($P = 1.306 \times 10^{-6}$, **Supplementary Figure 4D**), non-smoker ($P = 0.016$, **Supplementary Figure 4E**), smoker ($P = 4.465 \times 10^{-6}$, **Supplementary Figure 4F**), early stage ($P = 1.709 \times 10^{-5}$, **Supplementary Figure 4G**) or advanced stage ($P = 0.0094$, **Supplementary Figure 4H**) LUAD patients. Next, we further evaluated the predictive performance of NKCMGS in the TCGA LUAD patients stratified by different molecular characteristics, including EGFR, KRAS, and TP53 mutations. Similarly, we observed that the NKCMGS showed robust predictive power in the EGFR wild-type (WT) ($P = 9.923 \times 10^{-7}$,

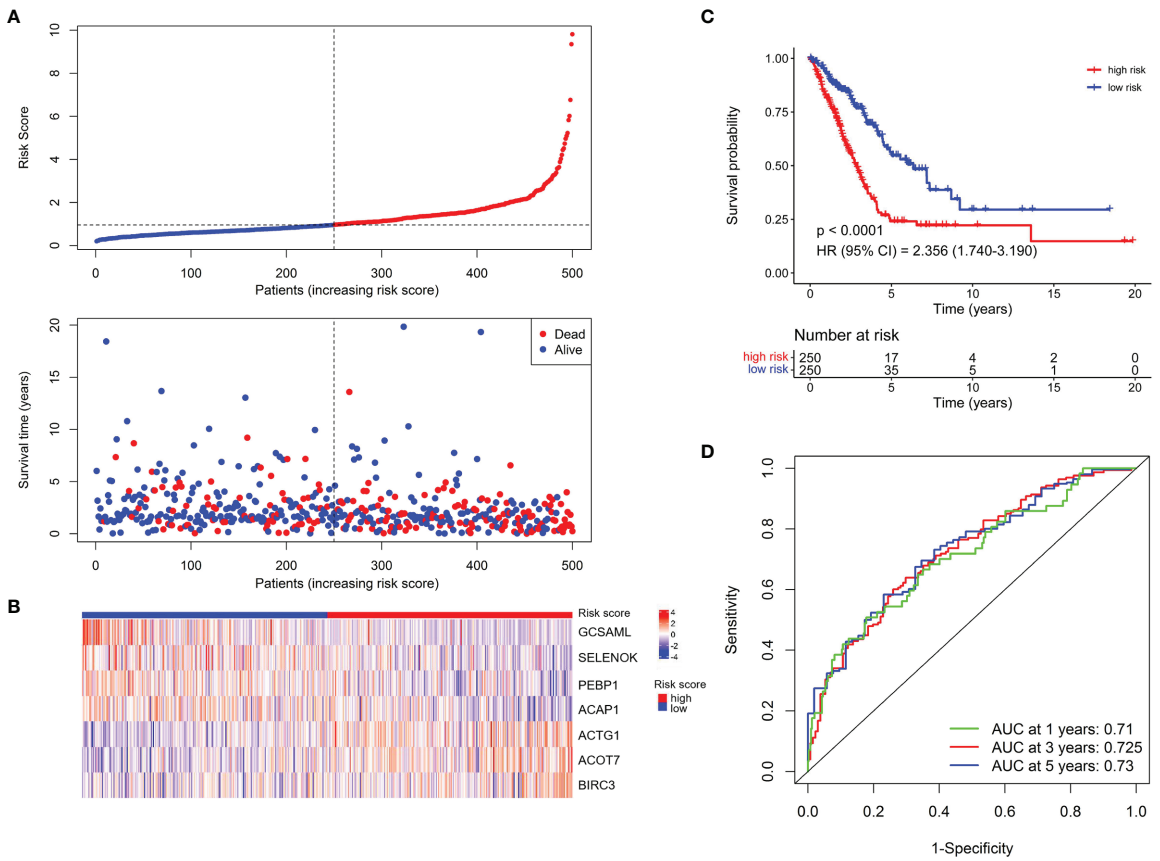

FIGURE 2 | Establishment of the NKCMGS in the TCGA LUAD cohort. **(A)** The distribution of risk score and survival status. **(B)** Heatmap showed the expression characteristics of the identified 7 NK cell marker genes. **(C)** Kaplan–Meier curves of survival analysis compared the overall survival of LUAD patients between high-risk and low-risk groups. **(D)** ROC curves of the NKCMGS for predicting the risk of death at 1, 3, and 5 years.

Supplementary Figure 5A), EGFR mutation (MUT) (P = 0.032, **Supplementary Figure 5B**), KRAS WT ($P = 1.23 \times 10^{-6}$, **Supplementary Figure 5C**), KRAS MUT ($P = 0.012$, **Supplementary Figure 5D**), TP53 WT ($P = 6.9 \times 10^{-4}$, **Supplementary Figure 5E**) or TP53 MUT ($P = 2.377 \times 10^{-5}$, **Supplementary Figure 5F**) subgroup.

External Validation of the Robustness of the NKCMGS in Six Independent Cohorts

To validate the robustness of the NKCMGS, we included 6 independent GEO cohorts in this study, and the clinical features of these 6 GEO cohorts are shown in **Table 1**. We used the same formula to calculate the risk score of each patient in 6 GEO cohorts. Patients were sorted into the high-risk and low-risk groups in each cohort by the median risk score. Kaplan–Meier analysis demonstrated that the high-risk group had inferior prognosis than the low-risk group in all 6 GEO cohorts, namely, GSE30219 (**Figure 3A**, HR: 3.557, 95% CI: 1.856–6.818, $P = 1.32 \times 10^{-4}$), GSE3141 (**Figure 3B**, HR: 3.064, 95% CI: 1.457–6.443, P = 0.002), GSE50081 (**Figure 3C**, HR: 1.932, 95% CI: 1.099–3.394, P = 0.02), GSE26939 (**Figure 3D**, HR: 2.312, 95% CI: 1.390–3.846, $P = 9.3 \times 10^{-4}$), GSE72094 (**Figure 3E**, HR: 2.038, 95% CI: 1.387–2.995, $P = 2.1 \times 10^{-4}$), and GSE31210 (**Figure 3F**, HR: 1.555, 95% CI: 0.789–3.063, P = 0.2). The ROC curves of the risk score in the 6 validation cohorts also showed good performance (**Supplementary Figure 6**). Additionally, a prognostic meta-analysis was performed by R package "meta" using the random-effects model to evaluate the integrated predictive value of NKCMGS in these 6 cohorts (48). The results of the meta-analysis indicated that NKCMGS was a significant prognostic indicator for patients with LUAD (HR: 2.227, 95% CI: 1.782–2.784, $P = 1.96 \times 10^{-12}$) (**Figure 3G**).

Independent Prognostic Role of the NKCMGS for Patients With LUAD

To further investigate whether the risk score can independently affect the prognosis of LUAD, we conducted univariate and multivariate Cox regression analysis using clinical features, molecular factors, and the risk score in the TCGA LUAD patients. As expected, the results of multivariate Cox regression analysis proved that the risk score was an independent prognostic factor (HR: 1.889, 95% CI: 1.373–2.599, $P = 9.37 \times 10^{-5}$) (**Table 2**). Meanwhile, we performed a LASSO Cox regression analysis with the risk score and all these clinical features to select the most predictive variables. The results demonstrated that the risk score and tumor stage were the best predictive factors for the prognosis.

Functional Enrichment Analysis of the NKCMGS Related Genes

To elucidate the potential mechanism of the excellent predictive capability of NKCMGS, we further investigated biological pathways related to NKCMGS. Firstly, the correlation analysis was performed using the TCGA LUAD dataset to identify the genes that were closely correlated with the risk score (Pearson $|R| > 0.4$, $P < 0.05$). As shown in **Supplementary Figure 7A**, 100 positively correlated genes and 24 negatively correlated genes were filtered out (**Supplementary Table 4**). Subsequently, we performed GO and KEGG enrichment analyses using the "ClusterProfiler" package for these selected genes. GO analysis revealed that these genes were mainly implicated in the biological processes of mitotic division, namely, chromosome segregation, mitotic nuclear division, and the G2/M transition of the mitotic cell cycle (**Supplementary Figure 7B**). KEGG analysis also verified that these genes were closely involved in the cell cycle pathway (**Supplementary Figure 7C**).

The NKCMGS Was Associated With the Immune Cell Infiltration of the TME

As NK cells play a vital role in anti-tumor immunity, we explored the relationship of the NKCMGS with immune cell infiltration in LUAD patients. By using the ESTIMATE algorithm, we found that high-risk patients had lower immune score, stromal score,

TABLE 1 | Clinical characteristics of lung adenocarcinoma from multiple cohorts.

Variables	TCGA N = 500	GSE30219 N = 83	GSE3141 N = 58	GSE50081 N = 127	GSE26939 N = 115	GSE72094 N = 398	GSE31210 N = 226
Age (year)							
Median	66	60	–	70	65	70	61
Range	33–88	44–84	–	40–86	41–90	64–77	30–76
Gender							
Male	230	65	–	65	53	176	105
Female	270	18	–	62	62	222	121
Smoking							
Yes	415	–	–	92	100	300	111
No	71	–	–	23	12	31	115
NA	14	–	–	12	3	67	0
TNM stage							
I and II	387	83	–	127	71	321	226
III and IV	105	0	–	0	16	72	0
NA	8	0	–	0	28	5	0
OS Status							
Alive	318	40	26	76	49	285	191
Death	182	43	32	51	66	113	35

NA, not available; OS, overall survival.

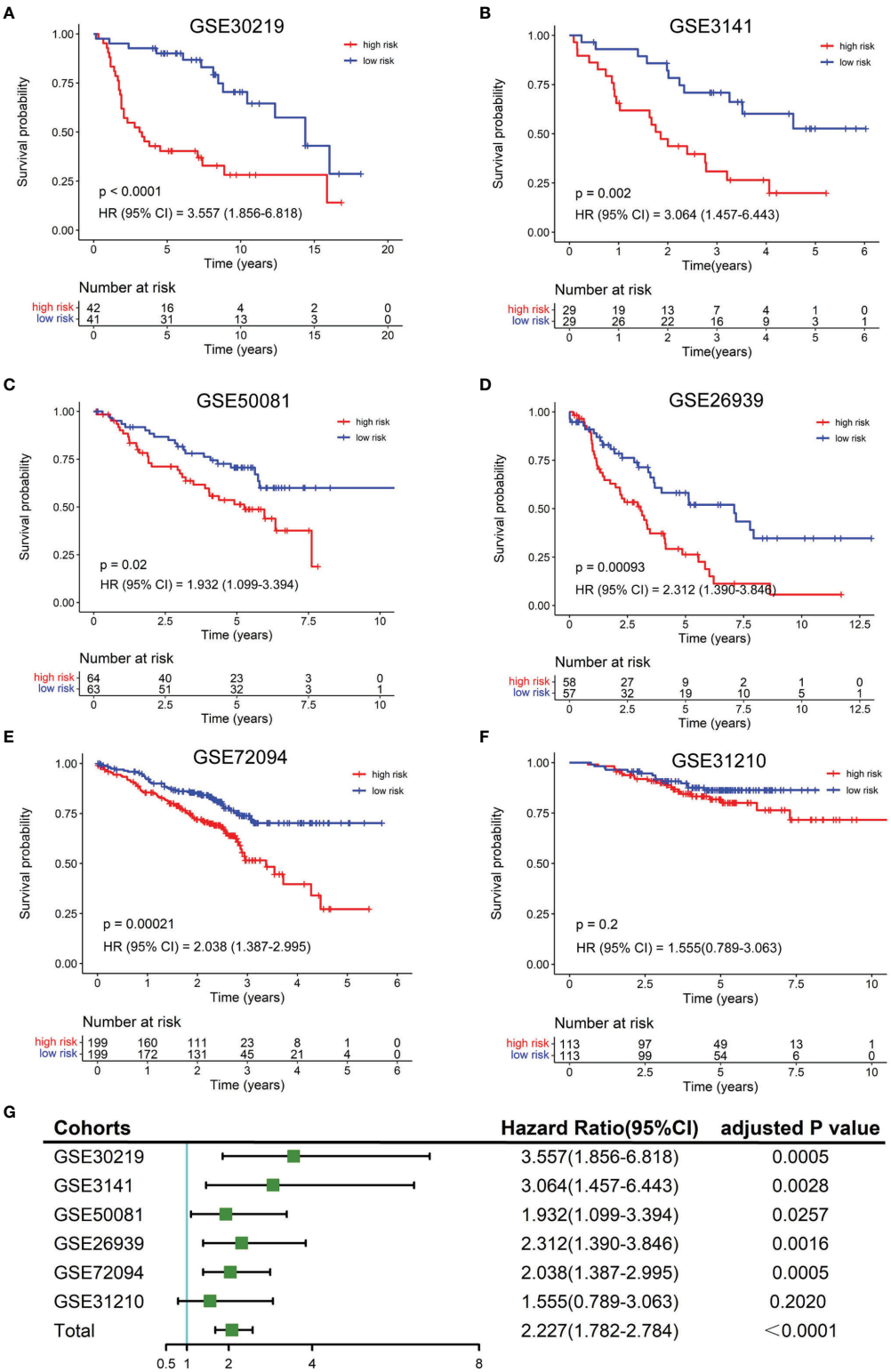

FIGURE 3 | Validation of the NKCMGS in six independent GEO cohorts. **(A)** GSE31210 (n = 226). **(B)** GSE30219 (n = 83). **(C)** GSE37745 (n = 106). **(D)** GSE50081 (n = 127). **(E)** GSE26939 (n = 115). **(F)** GSE42127 (n = 133). **(G)** A meta-analysis of six GEO cohorts and the P-value was adjusted for 7 hypothesis tests.

TABLE 2 | Univariable and multivariable Cox regression analysis of the NK cell marker genes signature in TCGA LUAD cohort.

Characteristics	Univariable analysis			Multivariable analysis		
	HR	95% CI	*P*-Value	HR	95% CI	*P*-Value
Age						
≤60	1.0 (ref)					
>60	1.217	0.906–1.635	0.192			
Gender						
Female	1.0 (ref)					
Male	1.049	0.784–1.405	0.747			
Smoking history						
No	1.0 (ref)					
Yes	0.881	0.583–1.330	0.546			
T stage						
T1 + T2	1.0 (ref)			1.0 (ref)		
T3 + T4	2.298	1.568–3.366	<0.001	1.646	1.077–2.515	0.021
Lymphatic metastasis						
No	1.0 (ref)			1.0 (ref)		
Yes	2.579	1.918–3.469	<0.001	1.903	1.326–2.733	<0.001
TNM stage						
I + II	1.0 (ref)			1.0 (ref)		
III + V	2.584	1.893–3.527	<0.001	1.337	0.885–2.022	0.168
EGFR mutation						
No	1.0 (ref)					
Yes	1.332	0.872–2.035	0.185			
KRAS mutation						
No	1.0 (ref)					
Yes	1.068	0.764–1.494	0.698			
TP53 mutation						
No	1.0 (ref)			1.0 (ref)		
Yes	1.413	1.054–1.893	0.021	1.152	0.851–1.561	0.359
Risk score						
Low	1.0 (ref)			1.0 (ref)		
High	2.356	1.740–3.190	<0.001	1.889	1.373–2.599	<0.001

HR, hazard ratio; CI, confidence interval; ref, reference category.

ESTIMATE score, and higher tumor purity than low-risk patients (**Figures 4A–D**), which suggested that the risk score was negatively correlated with the level of immune cell infiltration. Subsequently, we applied the CIBERSORT algorithm to estimate the infiltration level of different types of immune cells in the TME. The CIBERSORT analysis revealed that high-risk patients had a higher fraction of resting NK cells, M0 macrophages, M2 macrophages, activated dendritic cells, and activated mast cells, but had a lower fraction of plasma cells, $CD8^+$ T cells, follicular helper T cells, regulatory T cells, resting dendritic cells, and resting mast cells (**Figure 4E**). In **Figure 4F**, the fractions of different immune cells between high- and low-risk groups are shown. We further conducted a correlation analysis between the risk score and immune cell infiltration, which showed that the risk score was positively related to macrophages and neutrophils but was negatively related to T, B, and mast cells (**Supplementary Figure 8**).

Inflammatory and Immune Profiles of the NKCMGS

To figure out the relationship between NKCMGS and inflammatory activities, we explored the associations between the NKCMGS and 7 clusters of metagenes (HCK, LCK, IgG, Interferon, MHC-I, MHC-II, and STAT1), representing various inflammatory and immune activities as previously reported (41). **Supplementary Figure 9A** shows the expression details of these metagenes in the TCGA LUAD dataset. Next, we used Gene Sets Variation Analysis (GSVA) to calculate the expression of 7 gene clusters and the correlation between the NKCMGS and each cluster of metagenes is shown in **Supplementary Figure 9B**. The results showed that the risk score was negatively correlated with HCK, IgG, LCK, MHC-I, and MHC-II.

The NKCMGS Could Predict Immunotherapy Benefits in LUAD Patients

Given the important roles in anti-tumor immunity of NK cells and the promising treatment efficacy of NK cell-based immunotherapy, we explored whether the NKCMGS could predict responses of LUAD patients to immune checkpoint inhibitors. First, we analyzed the relationship between the NKCMGS and widely recognized immunotherapy biomarkers (PD-L1 expression and TMB) in the TCGA LUAD cohort. The results indicated that PD-L1 expression exhibited no significant difference between the low-risk and high-risk patients, but low-risk patients harbored a significantly lower TMB than high-risk patients (**Figures 5A, B**). In previous studies, the T-cell receptor (TCR) is in charge of the recognition of antigens presented by the MHC, and the repertoire analysis of TCR has been demonstrated as a useful biomarker for stratification and monitoring of patients on immunotherapy (49–51). Subsequently, we

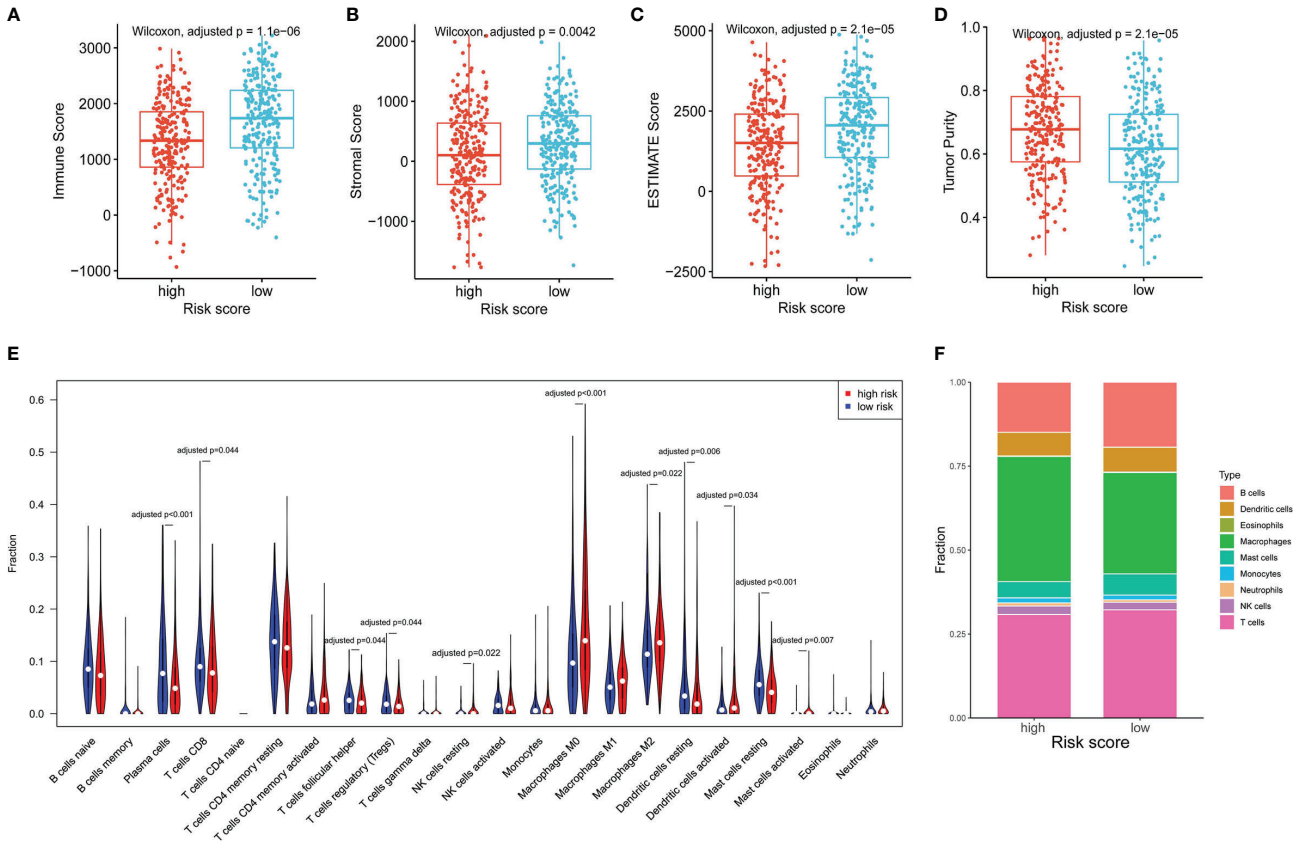

FIGURE 4 | The association between the NKCMGS and the immune cell infiltration in the TME. Differences among immune score **(A)**, stromal score **(B)**, ESTIMATE score **(C)**, and tumor purity **(D)** between high-risk and low-risk groups. The P-value was adjusted for 4 hypothesis tests. **(E)** The comparison of immune cells infiltration level of 22 immune cell types between high-risk and low-risk groups. **(F)** The fractions of different immune cells between high- and low-risk groups. The P-*value was adjusted for 22 hypothesis tests.

analyzed the TCR repertoire and found that the TCR richness and diversity of low-risk patients was significantly higher than that of high-risk LUAD patients (**Figures 5C, D**). Finally, to further explore the value of NKCMGS in predicting the immunotherapy response, 298 patients from the IMvigor210 cohort who received anti-PD-L1 treatment were enrolled in this study for analysis. Kaplan–Meier analysis showed an inferior survival rate for high-risk patients after immunotherapy (**Figure 5E**). Lower risk scores were associated with an objective response to anti-PD-L1 treatment (Wilcoxon test, P = 0.01; **Figure 5F**). The objective response rate of anti-PD-L1 treatment was significantly elevated in the low-risk group (two-sided chi-square test, P = 0.005; **Figure 5G**). The ROC curves showed that the combination of TMB, PD-L1, and risk score models could predict anti-PD-L1 response with 76.1% accuracy, which was superior to that of TMB (AUC = 0.728), PD-L1 (AUC = 0.569), or risk score (AUC = 0.603) alone (**Figure 5H**). Collectively, these findings indicate that patients with a low-risk score are more likely to benefit from immunotherapy, and the NKCMGS may be a useful biomarker to identify LUAD patients who may benefit from immunotherapy.

DISCUSSION

With the rapid development of scRNA-seq technologies, researchers are increasingly exploring molecular characteristics of tumor-infiltrating immune cells in the TME. However, most current studies have focused on adaptive immune cells, and the roles of innate immune cells have not yet received enough attention, which may markedly affect the prognosis and treatment response, especially with immunotherapy. The abundance of tumor-infiltrating NK cells is tightly associated with the prognosis of patients with various solid tumors (18–21). Recently, Cursons et al. developed a transcriptional signature based on NK cell marker genes to evaluate nature killer (NK) cell infiltration in the TME, and an increased NK score significantly stratified patients with superior prognosis in metastatic cutaneous melanoma (21). Inspired by this research, we attempted to explore the NK cell marker genes of LUAD through scRNA-seq analysis in our study. A novel prognostic prediction signature based on NK cell marker genes (NKCMGS) was developed for LUAD patients in the TCGA database and well verified in 6 independent cohorts from the GEO dataset.

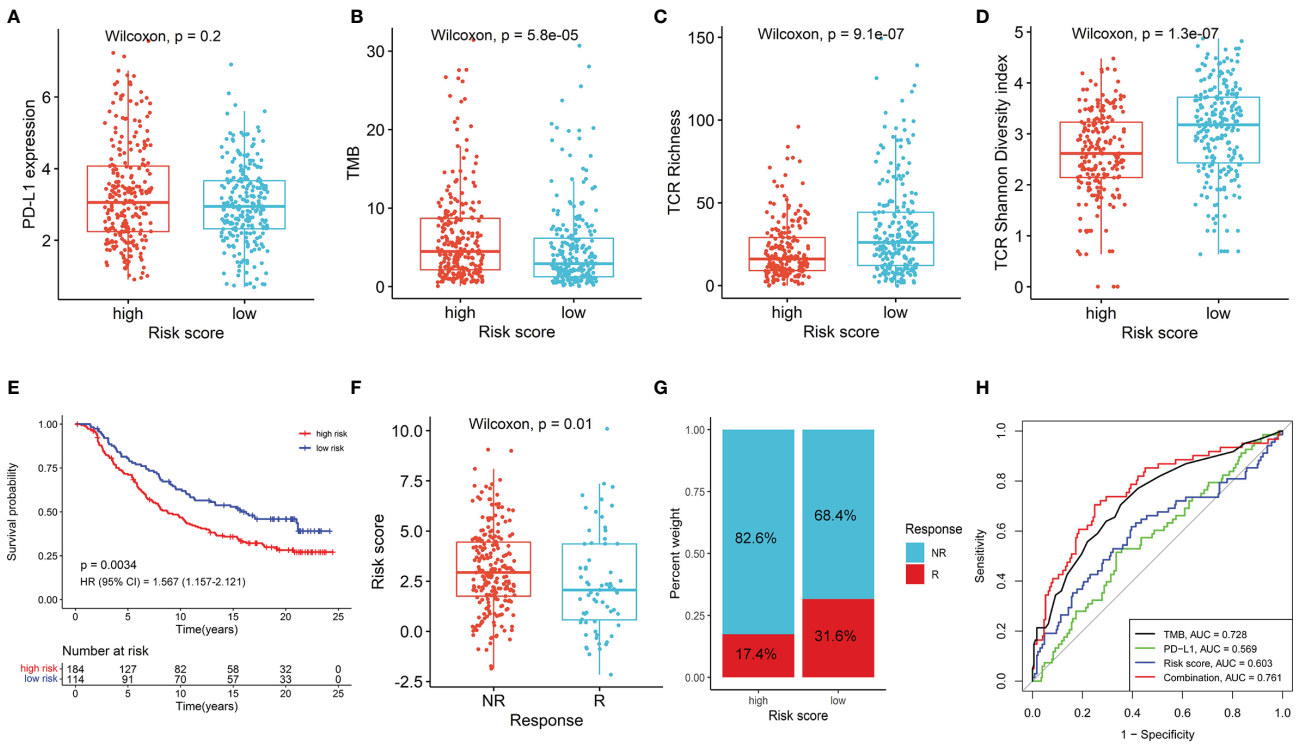

FIGURE 5 | The role of NKCMGS in predicting immunotherapeutic benefit. The comparison of PD-L1 expression **(A)**, tumor mutation burden (TMB) **(B)**, TCR richness **(C)**, TCR diversity **(D)** between high-risk and the low-risk patients. **(E)** Kaplan–Meier curves for patients with high-risk and low-risk scores in the IMvigor210 cohort. **(F)** The comparison of risk scores in groups with different anti-PD-L1 treatment response status in the IMvigor210 cohort. NR represents progressive disease (PD)/stable disease (SD); R represents complete response (CR)/partial response (PR). **(G)** Treatment response rates of anti-PD-L1 immunotherapy in high- and low-risk groups in the IMvigor210 cohort (P = 0.005). NR represents SD/PD; R represents CR/PR. **(H)** ROC curves evaluating the predictive accuracy of the TMB, PD-L1, risk score, and combination of TMB, PD-L1, and risk score in the IMvigor210 cohort.

Low-risk scores of NKCMGS were closely correlated with abundant infiltration of immune cells and a high level of TCR richness and diversity. Furthermore, we discovered that the immunotherapy response rate of patients with low-risk scores was dramatically higher than that of patients with high-risk scores, indicating that immune checkpoint blockade therapy is more appropriate for low-risk patients.

In this study, NKCMGS was composed of 7 NK cell marker genes (GCSAML, ACTG1, ACOT7, SELENOK, PEBP1, BIRC3, and ACAP1), most of which are correlated with the prognosis of LUAD patients or the activity of NK cells. ACTG1 encodes γ-actin, which is a component of the cytoskeleton. Increased expression of ACTG1 was linked to the enhanced ability of cell migration in lung adenocarcinoma (52) and upregulated expression of ACTG1 was also markedly associated with poor prognosis in patients with lung cancer (53). As one of the acyl-CoA metabolic enzymes, ACOT7 was implicated in the progression of LUAD by regulating the cell cycle through the p53/p21 signaling pathway (54), which is consistent with our findings that the signature was closely related to the cell cycle pathway. ACOT7 expression levels were found to be high in LUAD and were linked to impaired prognosis (54). SELENOK encodes a membrane selenoprotein (SelK), which is expressed abundantly in NK cells and is involved in regulating the function of NK cells (55, 56). SELENOK may modulate cell proliferation and migration through regulating Ca^{+} flux (56), and the expression of SELENOK was also associated with a poor prognosis in LUAD (57). Moreover, PEBP1, also known as Raf kinase inhibitory protein (RKIP), was downregulated in LUAD tissues compared with normal adjacent tissues (58). Low PEBP1 expression led to reduced survival in LUAD, and *in vitro* experiments demonstrated that upregulation of PEBP1 expression can suppress the proliferation and invasion of LUAD cells, which indicates that PEBP1 may act as a tumor suppressor gene (58). BIRC3 is a hallmark of tumor-infiltrating NK cells, and upregulation of BIRC3 can inhibit NK cell activity (59). Besides, BIRC3 expression was dramatically higher in LUAD tissues, and higher BIRC3 expression was correlated with a poorer prognosis (60). These reports indicated that genes identified in the NKCMGS might provide potential targets for experimental design in the laboratory to illuminate molecular mechanisms in LUAD.

In this study, the NKCMGS prognostic signature proved to be a powerful predictive tool for the prognosis of patients in both training and validation cohorts. The excellent predictive ability of the NKCMGS inspired us to investigate the potential

underlying mechanism. We first performed GO and KEGG analyses to explore the enriched pathways for genes closely related to the NKCMGS and found that these correlated genes were mostly enriched in the biological processes of cell division and cell cycle pathway. Hence, the inferior prognosis of patients with high-risk scores may be partly attributed to the abnormal regulation of the cell cycle, which is intimately linked to tumor proliferation and progression (61). Besides, tumor-infiltrating immune cells in the TME play a vital role in tumor development and significantly affect prognosis (62). We then compared the abundance of immune cell infiltration between high-risk and low-risk groups by ESTIMATE and CIBERSORT algorithms. The results revealed that high-risk tumors had a lower infiltration level of immune cells, especially T and B cells, which suggested that tumors with a high-risk score were characterized as so-called "cold tumors" with decreased anti-tumor activity (63). The low level of immune cell infiltration can promote tumor cell escape from immune surveillance and facilitate tumor progression, which may partly account for the significantly decreased survival of high-risk LUAD patients.

Furthermore, the NKCMGS was evaluated in relation to immune and inflammatory activities by analyzing immune-related metagenes, and the risk score was found to correlate negatively with HCK, IgG, LCK, MHC-I, and MHC-II clusters. HCK is pivotal in innate immunity by regulating the phagocytosis of neutrophils and macrophages (64). LCK, a Src-related protein linked to CD8 and CD4 molecules, is required for the maturation and stimulation of T cells (65). MHC-I and MHC-II are closely associated with the function of antigen-presentation and tumor cells can escape T-cell killing by losing the expression of MHC-I and MHC-II (66). Therefore, high-risk patients showed an immunosuppressive microenvironment, which may be partly responsible for the significantly inferior prognosis. Collectively, according to all the findings above, we inferred that the potential mechanism of the powerful predictive ability of the NKCMGS may lie in the dysregulation of the cell cycle and immunosuppressive microenvironment.

The discrepancy in immune cell infiltration and inflammatory activities between different risk groups prompted us to explore the value of the NKCMGS in predicting immunotherapy response. We first analyzed the association between the NKCMGS and the well-recognized biomarkers, including PD-L1 expression TMB. The results revealed that PD-L1 expression showed no significant difference between high-risk and low-risk patients, but low-risk patients harbored a significantly lower TMB, which indicated the low immunogenicity of low-risk tumors. TCR is a unique molecule on the T-cell surface that recognizes antigens presented by MHC. Several studies have recently used high-throughput TCR sequencing to analyze the characteristics of T-cell repertoires in patients with diverse cancer types, demonstrating that TCR repertoires could act as a potent tool to predict immunotherapy response (49–51). We evaluated the richness and diversity of the TCR repertoire and discovered that low-risk patients were associated with a higher level of TCR richness and diversity, which reflected higher functionality of T cells in recognizing antigens and killing tumor cells in low-risk LUAD patients. The success of immune checkpoint blockade therapy was associated with many factors, namely, the immunogenicity of the tumor, the abundance and functionality of tumor-infiltrating T cells, and the expression of immune checkpoints. In this study, although low-risk tumors had lower immunogenicity, the abundance and functionality of tumor-infiltrating T cells in low-risk groups was dramatically elevated compared with that in high-risk groups. Therefore, an immunotherapy cohort was needed to verify the predictive value of the NKCMGS. By using an immunotherapy cohort (Imvigor210), we explored the ability of NKCMGS to predict immunotherapeutic efficacy and observed that low-risk patients were more sensitive to anti-PD-L1 therapy response, which demonstrated that the impact of the abundance and functionality of tumor-infiltrating T cells on immunotherapy response is more important than tumor immunogenicity. Taken together, low-risk patients were more likely to benefit from immunotherapy. With further validation, NKCMGS might act as a reliable biomarker for predicting immunotherapy response.

Despite the promising findings obtained, this study has several limitations. First, the expression and prognostic role of the genes in NKCMGS at protein-level warrant further investigation. Second, the candidate genes involved in our study were restricted to the NK cell marker genes, and the tumor immune microenvironment is highly spatially heterogeneous. Hence, the prognosis-predicting ability of the signature was limited. Lastly, all the mechanistic analysis in our study was descriptive. Future research must explore the underlying mechanism between the expression of genes in NKCMGS and the prognosis of LUAD.

In conclusion, a seven-gene signature based on NK cell marker genes was identified and validated to have powerful performance to predict prognosis and immunotherapy response in LUAD patients. It might serve as a prognostic biomarker for clinical decision-making regarding individualized prediction and facilitate the selection of appropriate patients who can benefit from immunotherapy.

DATA AVAILABILITY STATEMENT

The results shown here are in whole based on data generated by the TCGA Research Network: https://www.cancer.gov/tcga, GEO database: https://www.ncbi.nlm.nih.gov/geo/ under the accession numbers GSE131907, GSE30219, GSE3141, GSE50081, GSE26939, GSE72094, GSE31210, and IMvigor210 cohort: http://research-pub.gene.com/IMvigor210CoreBiologies.

AUTHOR CONTRIBUTIONS

SG and JH supervised the project and designed this study. WL organized the public data and prepared all the figures and tables. LG conducted the data analysis. PS drafted the manuscript. JY revised the manuscript. All authors listed have made a

substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.850745/full#supplementary-material

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REVIEW
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Neoantigens and NK Cells: "Trick or Treat" the Cancers?

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Immunotherapy has become an important treatment strategy for cancer patients nowadays. Targeting cancer neoantigens presented by major histocompatibility complex (MHC) molecules, which emerge as a result of non-synonymous somatic mutations with high immunogenicity, is one of the most promising cancer immunotherapy strategies. Currently, several therapeutic options based on the personalized or shared neoantigens have been developed, including neoantigen vaccine and adoptive T-cell therapy, both of which are now being tested in clinical trials for various malignancies. The goal of this review is to outline the use of neoantigens as cancer therapy targets, with an emphasis on neoantigen identification, clinical usage of personalized neoantigen-based cancer therapy agents, and the development of off-the-shelf products based on shared neoantigens. In addition, we introduce and discuss the potential impact of the neoantigen–MHC complex on natural killer (NK) cell antitumor function, which could be a novel way to boost immune response-induced cytotoxicity against malignancies.

Keywords: neoantigen, T cell, NK cell, cancer vaccine, adoptive T cell therapy

INTRODUCTION

According to immune surveillance theory, the immune system's job is to keep the bodily environment stable and free of malignancies by detecting and destroying "non-self" tumor cells (1, 2). These tumor cells, on the other hand, try to evade immune surveillance in several ways, such as immunologic sculpting during tumor formation (1, 3, 4). The purpose of cancer immunotherapy is to increase the activity of the patient's immune system to fight against cancer cells by natural mechanisms (5, 6). Checkpoint inhibitors, adoptive T cells, cancer vaccines, and antibody-based therapies are among the most clinically investigated immunotherapies thus far. T cells and natural

killer (NK) cells are two of the most essential effector cells for recognizing and destroying tumor cells. The positive signals provided by precise identification of tumor antigens and the negative signals presented by immunological checkpoints can both be used to determine tumor-specific T-cell activation (7). Similarly, the activation of NK cells also relies on the integration of activating and inhibiting signals (8). As a result, tampering with the balance by blocking negative signals and boosting positive signals for T cells and NK cells may be advantageous to patients with cancer. To date, there have been many reports on therapies that block T- and NK-cell inhibitory receptors such as the checkpoint molecules CTLA-4 or PD-1/PD-L1. However, several pieces of evidence show that these strategies only bring benefits to a limited number of tumor-bearing patients, and the majority of patients still experience disease progression (9–12). As a result, therapies that can safely and effectively enhance the function of tumor-specific cytotoxic lymphocytes are required. Cancer neoantigens, which emerge as a result of non-synonymous somatic mutations in tumor cells and can be displayed by the major histocompatibility complex (MHC) molecules on the cell surface, may serve as a primary target for tumor-specific immune cells (13, 14). Indeed, neoantigen-based cancer vaccines and neoantigen-specific adoptive T-cell treatment alone or combined with immune checkpoint blockade (ICB) have had some progress (15–17). In this review, we primarily discuss the possibility of using neoantigens as cancer therapy targets by triggering the antitumor function of T cells, mainly focusing on neoantigen identification, clinical usage of personalized neoantigen-based cancer therapy agents, and the development of off-the-shelf products based on shared neoantigens. In addition, we introduce and discuss the potential impact of the neoantigen–MHC complex on the antitumor function of NK cells, which could be a novel way to boost immune response-induced cytotoxicity against malignancies.

CANCER NEOANTIGENS AND THEIR ROLE IN CANCER IMMUNOTHERAPY

Cancer neoantigens are non-autologous antigens arising from non-synonymous somatic mutations occurring in tumor cells and that have the potential to be recognized in the context of MHC by T cells (15). These mutations, mainly containing single-nucleotide variants, splice variants, mutational frameshifts, and gene fusions, can generate aberrant proteins as malignancies grow (18–21). Varying people and cancer types have different types and numbers of mutations, and more neoantigens are expected to be formed in tumors having more mutations, whereas fewer tend to be generated in tumors having fewer mutations. The ability of T cells to identify mutant peptides in human tumors has been demonstrated for more than 20 years (22, 23). While CD4+ T cells recognize neoepitopes shown by MHC II molecules, CD8+ T cells identify neoantigens in the context of MHC I molecules expressed by tumor cells, which triggers T-cell cytotoxicity and tumor cell killing (24). Evidence suggests that the frequency of neoepitope-specific CD8+ T cells in tumor-infiltrating lymphocytes (TILs), as well as the presentation of neoantigens by MHC I molecules and the load of neoantigens on the surface of tumor cells, has a positive relationship with prognosis in patients with solid tumors (20, 25, 26). Thus, approaches of boosting T-cell responses specifically against neoantigens could be greatly beneficial to cancer patients in terms of clinical outcomes.

As for taking advantage of neoantigens in cancer immunotherapy, two main strategies have been employed in clinical trials (**Figure 1**). The first is T-cell adoptive treatment, which involves isolating immune cells from a patient's tumor tissue and then injecting them back into the patient following *ex vivo* modification and amplification, primarily neoantigen-specific T cells (27). Another option is to design and produce a tailored vaccine against tumor cells that targets neoantigens to expand preexisting T-cell responses or induce new antitumor T-cell clones (28, 29). Some common mutations, such as TP53 and RAS family mutations, have been found in patients with the same or different types of malignancies (30–32). The possibility of off-the-shelf products based on neoantigens from these common mutations is being investigated, such as shared neoantigen-based vaccines and bispecific diabodies (**Figure 1**) (33, 34).

IDENTIFICATION OF IMMUNOGENIC NEOANTIGENS

The development of next-generation sequencing (NGS) combined with *in silico* analysis in recent years has revolutionized the quick detection of neoantigens in cancer patients. To discover immunogenic neoantigens, the current framework often includes the following steps (**Figure 1**): obtaining patient tumor specimens and finding somatic mutations by NGS, predicting and selecting the possible neoantigens that can be presented by an MHC molecule of a given patient through *in silico* analysis or/and protein mass spectrometry (MS) analysis, and lastly testing the immunogenicity of the candidate neoantigens.

After NGS has revealed the mutations in clinical tumor specimens, computational techniques can be used to identify possible neoantigens. Whole-exome sequencing (WES) data of the cancerous and non-cancerous DNA can be used to map tumor-specific genetic abnormalities, and data from the RNA sequence can be compared with WES data to evaluate whether the mutant genes are expressed in tumors (35). To predict the binding affinity of the HLA (human leukocyte antigen, the MHC molecules in human) alleles with processed mutant peptides from cancer patients, several computational techniques have been developed, the majority of which rely on machine learning algorithms trained by extensive experimental datasets of HLA-binding peptides (21). NetMHCpan and NetMHCIIpan are two of the most often used algorithms for predicting peptide binding with HLA I and HLA II, respectively (36, 37). Meanwhile, MS analysis has been employed to scan the peptide repertoire shown on MHC I and II directly (38). *In silico* neoantigen

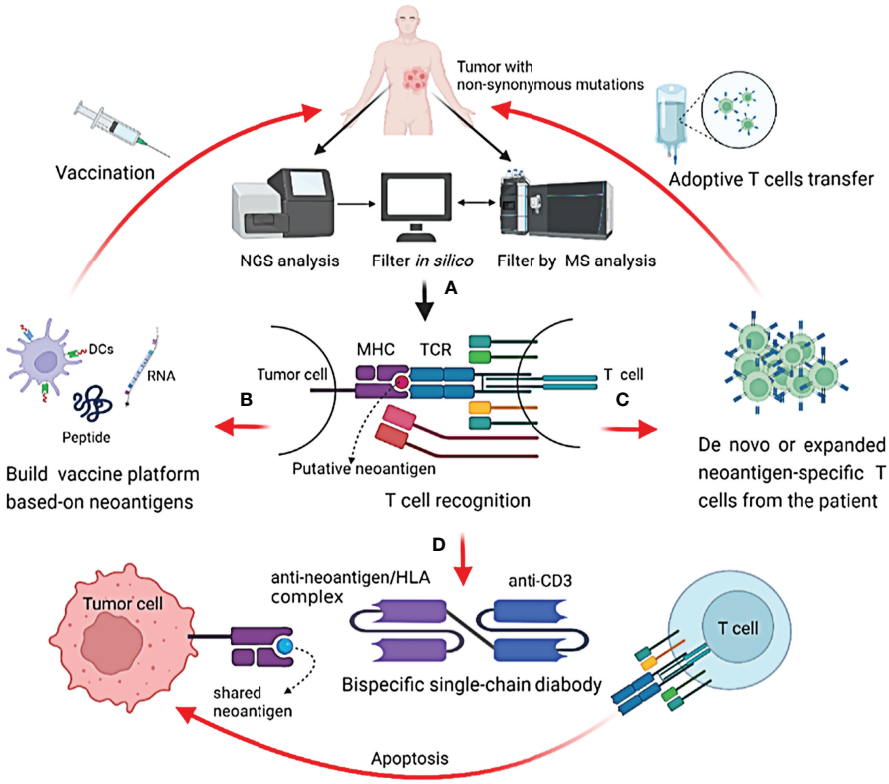

FIGURE 1 | Identification of neoantigens and strategies to target them in cancer patients. **(A)** Identify potential neoantigens; **(B)** neoantigen-based cancer vaccine therapy; **(C)** neoantigen-specific T cell adoptive transfer; **(D)** mechanism of action of shared neoantigen-based bispecific diabody.

predictions can be complemented with MS analysis of MHC-displayed peptide repertoire to limit possible neoantigens and increase prediction efficiency. Finally, the immunogenicity of predicted neoantigens should be determined experimentally, by testing their ability to induce T-cell activation, as not every mutant peptide displayed by MHC molecules can induce an immune response (39). The potential neoantigens can be employed in the development of immunotherapies. So far, several neoantigens have been discovered in preclinical and clinical investigations, some of which are derived from shared mutations in a range of malignancies (30, 31, 40–47) (**Table 1**).

Therapeutic Use of Personalized Neoantigens in Cancer Immunotherapy

Neoantigens are largely patient-specific due to each patient's unique mutation repertoire. Autologous adoptive T-cell therapy

TABLE 1 | Potential shared neoantigens from common mutations in solid tumors.

Gene	AA mutation	Neoantigens	HLA restriction	Reference
TP53	p. R175H	HMTEVVR**H**C	HLA-A*02:01	(30, 44, 46)
	p. R248W	SSCMGGMN**W**R	HLA-A*68:01	(31)
	p. Y220C	VVP**C**EPPEV	HLA-A*02:01	
KRAS	p. G12D	VVVGA**D**GVGK	HLA-A*11:01	(41)
	p. G12V	VVVGA**V**GVGK	HLA-A*11:01	
	p. G12D	GA**D**GVGKSA(L)	HLA-C*08:02	(43)
	p. Q61L	ILDTAG**L**EEY	HLA-A*01:01	(45)
	p. G12V	VVVGA**V**GVGK	HLA-A*03:01	
EGFR	p. L858R	KITDFG**R**AK	HLA-A*11:01	(47)
	p. T790M	LTSTVQLI**M**	HLA-C*15:02	
PIK3CA	p. H1048R	EALEYFMKQMNDA**R**HGGWTTKMDWIFH	HLA-DRB1*04:05	(42)
IDH1	p. R132H	GWVKPIIIG**H**HAYGDQYRAT	HLA-DRB1*01:01	(40)

and cancer vaccines based on individualized neoantigens are the two most common cancer immunotherapy methods studied in clinical trials and have indicated clinical advantages for solid tumors.

Adoptive T-Cell Therapy

Although tumor cells can be recognized and killed by TILs, adoptive transfer of T cells specific for certain neoantigens expressed by tumor cells may be more advantageous than an infusion of randomly isolated TILs after *ex vivo* amplification. The process of adoptive T-cell therapy (ACT) includes identifying neoantigens, expanding antitumor TILs *via* neoantigens, identifying or modifying neoantigen-reactive T cells, and lastly infusing the T cells back into the patient. Infusion of autologous neoantigen-reactive T cells in patients with a variety of solid tumors resulted in long-term regressions (48–54).

A phase II trial showed that 40 days after infusion of *ex vivo* amplified TILs that contained specific CD8+ T cells targeting neoepitope derived from the KRAS mutation G12D, a patient with metastatic colorectal cancer encountered regression of all the metastatic lung lesions, suggesting an important role of neoepitope-reactive CD8+ T cells in cancer therapy (51). Further research shows that TIL adoptive treatment is linked to an increase in neoepitope-specific CD8+ T cells (55). On the other hand, TIL-based ACT responders retained a subset of stem-like neoantigen-specific CD8+ T cells that show self-renewal and superior growth capacity *in vitro* and *in vivo*, highlighting the relevance of T-cell phenotypes in ACT response (56). In another case, after treating a widely metastatic cholangiocarcinoma patient with TILs containing Th1 cells specifically targeting neoantigens derived from the mutated erbb2 interacting protein (ERBB2IP), an obvious tumor size reduction was observed. Moreover, the size reduction of lesions in the lung and liver was shown again after retreatment with a pure population of neoepitope-reactive Th1 cells, suggesting the potential role of neoantigen-reactive CD4+ T cells in cancer treatment (50). Meanwhile, two HPV+ metastatic cervical carcinoma patients achieved total tumor regression that is ongoing for 44 months after adoptive transfer of TILs including neoantigen-targeted T cells and a relatively lower proportion of HPV-targeted T cells. This offered a new paradigm for immunotherapy of virally associated cancers: targeting neoantigens (52). Another clinical trial in a metastatic breast cancer patient found that adoptive transfer of TILs reactive against neoepitopes derived from four proteins combined with interleukin-2 and ICB resulted in complete durable regression for over 22 months (53), implying clinical benefits of combining ACT with other immunomodulators such as the checkpoint inhibitor. At the same, after adoptive transfer of enriched neoantigen-specific TILs combined with anti-PD-1 antibody pembrolizumab in a phase II pilot trial, three of the six patients with metastatic breast cancer showed objective tumor regression, including one complete response that was ongoing for more than 5.5 years (54).

In addition to *ex vivo* expanded antitumor T cells, ACT with modified T-cell receptor (TCR) or chimeric antigen receptor (CAR) has been demonstrated effective for cancer patients (57–59). One of the challenges for engineered T-cell therapies is to target tumor-specific antigens without destroying normal tissues (60, 61). Therefore, neoantigens may be a good target for engineered T-cell therapies. Recently, the molecular signatures of neoantigen-reactive antitumor T cells have been identified by single-cell RNA sequencing and TCR sequencing in a variety of solid tumors, and both neoepitope-targeted CD8+ and CD4+ T cells harbor distinct transcriptomic signatures compared with bystander T cells (62–66). This may provide new opportunities for cancer treatment by harnessing reprogrammed autologous T cells with enriched neoepitope specificities. In a recent report, a patient with progressive metastatic pancreatic cancer was treated with a single infusion of autologous T cells that had been genetically engineered to clonally express two allogeneic HLA-C*08:02-restricted TCRs targeting mutant KRAS G12D expressed by the tumors. This patient had regression of visceral metastases, and the response was ongoing at 6 months (67).

In general, using specific T cells to target neoantigens found only in tumor cells is a promising way to stimulate the activity of a patient's immune system against cancer cells while minimizing the risk of toxicity, and many clinical trials are currently underway to investigate ACT as a monotherapy or in combination with checkpoint inhibitors (68).

Personalized Cancer Vaccines

Cancer vaccines utilizing tumor antigens have long been thought to be promising strategies for cancer treatment. Unlike traditional cancer therapeutic vaccines, which mainly focus on tumor-associated antigens (TAAs) that are abnormally expressed in tumor cells but can also be detected in normal tissues, neoantigen-based cancer vaccines have the capacity to amplify endogenous repertoire of T-cell responses specifically targeting neoantigen-expressed tumor cells, potentially reducing the risk of vaccine-induced tolerance or autoimmune responses (69). CD8+ T cells can be primed by antigen-presenting cells (APCs) expressing the neoantigen-MHC I complex after immunization, enhancing their cytotoxicity against neoantigen-expressing cancer cells. Dendritic cells (DCs, which are professional APCs), peptides, DNA, and RNA are the most common vaccination platforms. It is conceivable that immune responses induced by neoantigen-based vaccines could offer immunological memory and establish long-term protection against cancer recurrence. The viability and safety of tailored neoantigen vaccines have been demonstrated in human patients with solid tumors in clinical trials (70–76).

Three patients with stage III melanoma accepted an autologous DC vaccine including seven projected customized neoantigens as well as peptides from TAA-gp100 in a phase I trial (70). The vaccine can produce *de novo* T-cell responses while also increasing the response of preexisting neoantigen-reactive CD8+ T cells, indicating a broadening and diversification of T-cell responses (70). Similarly, 12 patients with advanced lung cancer received a tailored neoantigen-pulsed DC vaccine in another clinical trial. The disease control rate was

75%, and the median progression-free survival was 5.5 months. All treatment-related adverse events were grades 1–2, and there were no dose delays due to toxic effects (74). In another phase I trial, patients with high-risk melanoma were vaccinated with a lengthy peptide spanning 20 mutations each following the first curative-intent surgery. Following vaccination, neoantigen-reactive CD4+ and CD8+ T cells that had previously been undetectable were activated, with CD4+ T cells accounting for a larger frequency of the response. While four patients did not show disease recurrence for a median of 25 months after vaccination, two of the six patients who experienced recurrence a few months after vaccination were then treated with the anti-PD-1 antibody. Both of the patients showed complete clinical responses, highlighting the potential of combining ICBs and neoantigen therapeutic vaccines (69). Further research revealed that these patients developed memory T-cell responses with cytolytic capabilities *in vivo* that persist in the peripheral blood for years (75). In a phase Ib glioblastoma trial using a similar method with multi-epitope customized neoantigen peptide immunization, patients generated circulating neoantigen-specific T-cell responses, implying that neoantigen-targeting vaccines may have benefits in glioblastoma patients, which normally have a low mutation load (73). Meanwhile, the safety and function of neoantigen-based mRNA vaccines were tested in a study of patients with gastrointestinal cancer, although no clinical responses were observed in three of four individuals (77), while in another case, T-cell responses were elicited when patients with stage III/IV melanoma were given RNA vaccines that encoded neoantigens derived from specific mutations (10 per patient). Among the 13 patients, two had objective clinical responses, and one showed a complete response after combined with PD-1 blocking therapy (72).

According to these findings, tailored neoantigen vaccinations can trigger specific T-cell responses, and neoantigen-based vaccines in combination with ICBs could produce improved clinical results. Thus, personalized neoantigen-based vaccinations are being investigated in several clinical trials as monotherapy or in combination with checkpoint inhibitors (78).

Therapeutic Strategies Based on Shared Neoantigens

Although personalized neoantigen discovery leads to attractive personalized therapeutics, high prices and time delays limit their use, and the challenges of predicting and identifying optimal neoantigens for each patient still remain. Despite each patient's unique neoantigen repertoire, some neoepitopes can be found in various patients with the same or even in distinct forms of malignancy. Thus, ongoing research is being done to evaluate off-the-shelf anticancer therapeutics based on shared neoantigens, which could benefit multiple patients.

The mutation in isocitrate dehydrogenase 1 (IDH1) is common in diffuse glioma, and the most prevalent IDH1 mutation (R132H) generates a neoepitope presented by MHC class II molecules (40). In a phase I trial, 32 patients with IDH1 (R132H)+ astrocytomas were given an IDH1(R132H)-specific peptide vaccination and the vaccine-induced immune responses were observed in 93.3% of individuals, with vaccine-related adverse effects limited to grade 1. The progression-free rate after 2 years was 0.82 for patients who had immune responses (33). Similarly, immunogenic frameshift peptide (FSP) neoantigens resulting from mutations in coding microsatellites are shared by the majority of mismatch repair (MMR)-deficient malignancies, indicating that these FSP neoantigens may be utilized as targets to induce or amplify antitumor T-cell responses (79). Patients were subcutaneously vaccinated with shared FSP neoantigens (derived from mutant AIM2, HT001, and TAF1B genes) combined with montanide ISA-51 VG in a clinical phase I/IIa trial. All immunized patients showed immune responses to the vaccine. Three patients suffered grade 2 local injection site responses, but no vaccine-related serious adverse events happened (80). More preclinical and clinical investigations are being conducted to determine the effectiveness of shared neoantigen-based vaccines (**Table 2**).

TABLE 2 | List of clinical trials employing off-the-shelf neoantigen vaccines.

Strategy	Tumor type	Drugs	Phase	ClinicalTrials.*gov identifier*
Vaccine	Melanoma	A vaccine made of 6MHP and NeoAg-mBRAF (a peptide BRAF585-614-V600E)	Phase I/II	NCT04364230
Vaccine	KRAS-mutated pancreatic ductal adenocarcinoma and other solid tumors	ELI-002 2P (Amph-modified KRAS peptides, Amph-G12D and Amph-G12R admixed with admixed Amph-CpG-7909)	Phase I	NCT04853017
Vaccine with checkpoint inhibitor	Intrinsic pontine glioma, intrinsic midline glioma (H3 K27M-mutant)	rHSC-DIPGVax vaccine Balstilimab Zalifrelimab	Phase I	NCT04943848
Vaccine with checkpoint inhibitor	Lung cancer, colorectal cancer, pancreatic cancer, other shared neoantigen-positive solid tumors	GRT-C903/GRT-R904 (shared neo antigen- based vaccine) Nivolumab Ipilimumab	Phase I/II	NCT03953235
Vaccine with checkpoint inhibitor	Malignant glioma	IDH1R132H peptide vaccine Avelumab	Phase I	NCT03893903
Vaccine with checkpoint inhibitor	Unresectable or metastatic deficient mismatch repair (dMMR) or MSI-H colorectal cancer, gastric or gastro-esophageal junction tumors	GAd-209-FSP MVA-209-FSP Pembrolizumab	Phase I/II	NCT04041310

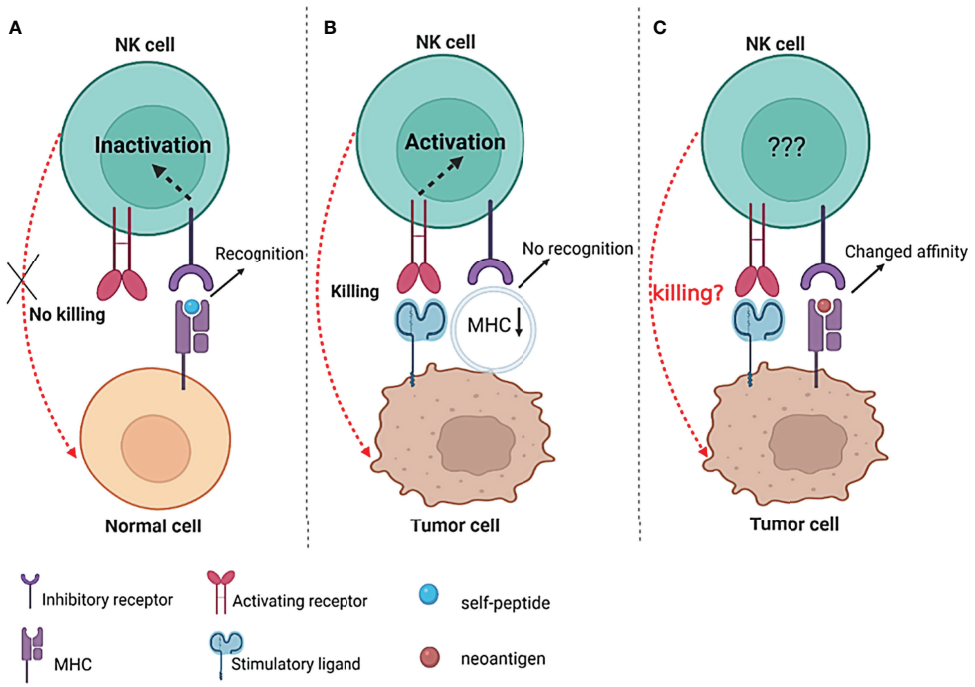

FIGURE 2 | The possible influence of neoantigens on NK-cell function. **(A)** The interactions between inhibitory receptors and their specific MHC/self-peptide ligands inactivate NK cells, thus preventing cytolytic activity against healthy cells; **(B)** decrease or even lose the expression of MHC I on the surface of tumors, resulting in the "missing-self "recognition of NK cells to kill tumor cells; **(C)** the neoantigen presented by MHC I molecules on the surface of tumor cells may change the interaction affinity of MHC I and inhibitory receptors (such as KIRs) and finally influence the activity of NK cells.

In addition to neoantigen-based off-the-shelf vaccines, other strategies for the treatment of patients with common mutations are being tested, such as TCR-mimic antibodies, a type of agents that target the peptide–HLA complex in tumor cells (81, 82).

RAS oncogene mutations are found in different types of malignancies, and neoepitopes from RAS mutations are shared by several patients. Single-chain variable fragments specific for KRAS G12V mutation and NRAS Q61L mutation-generated peptide–HLA complexes were identified *via* phage display and transformed to bispecific single-chain diabodies (scDbs). *In vitro*, the scDbs were able to activate T cells to kill target cancer cells that expressed low endogenous amounts of the KRAS G12V or NRAS Q61L neoepitope–HLA complexes. In the mouse model, the scDbs employed *in vivo* similarly demonstrated a capacity to decrease the growth of tumors with KRAS G12V or NRAS Q61L mutation (45). Similarly, a bispecific scDb targeting the neoantigen produced from the R175H mutation of TP53, one of the most commonly mutated cancer driver genes, was developed and demonstrated an excellent affinity for the R175H mutant-derived neoepitope–HLA complex. Despite the low expression of TP53 R175H-derived neoantigen–HLA complex on the cancer cell surface, T cells were successfully activated to kill the cancer cells by the scDb *in vitro* as well as in mouse models (46). Recently, a TCR-mimic monoclonal antibody that could target a range of phospho-neoantigens displayed by HLA-A*02:01 in various tumor cells has been generated and has shown the capacity to induce T cells to kill tumor cells. The phospho-peptides derived from dysregulated protein phosphorylation in different types of tumor cells may serve as shared tumor-specific neoantigens (83). Based on these findings, a TCR-mimic antibody that can selectively target shared neoantigens while boosting T-cell function may theoretically be utilized to target malignancies with common mutations and could work as an off-the-shelf agent in cancer immunotherapy, although more research is required to testify this idea.

The Possible Transformation of NK Cells' Antitumor Function Based on Neoantigens

Human NK cells are the first line of antitumor lymphocytes, and lower NK-cell cytolytic activity has been linked to a higher tumor incidence (84–86). NK cells are cytotoxic toward tumor cells without prior activation and can regulate various immune responses by secreting immune-regulatory cytokines as well as chemokines (87–89). The combination of activating and inhibiting signals modulates the antitumor action of NK cells. Killer cell immunoglobulin-like receptors (KIRs) and natural killer group 2 A (NKG2A) are the two primary inhibitory receptors, both of which recognize HLA molecules (90). KIRs are transmembrane receptors of type I that majorly detect classical HLA I, which include HLA-A, HLA-B, and HLA-C (91, 92). KIR genes have several alleles, and variability within each gene allows the complex KIR repertoire to recognize changes in HLA I expression, which is itself highly

polymorphic (93). KIRs with long intra-plasmatic tails and immunoreceptor tyrosine-based inhibitory motifs (ITIMs), such as KIR2DL1, KIR2DL2, and KIR2DL3, can interact with HLA-C, while others, including KID3DL1 and KIR3DL2, engage with HLA-A and -B (94). NKG2A, which forms a heterodimer with CD94, is a type II transmembrane receptor that can interact with a non-classic HLA molecule HLA-E (95).

Unlike T cells that detect the peptide in an MHC-restricted manner, NK-cell receptors that can recognize MHC molecules tend to bind to MHC itself and may be less specific for the provided peptide. However, the peptide could modify the interaction affinity of MHC with NK receptors (96). KIR3DL2 can interact with HLA-A3 and HLA-A11, and the interaction affinity appears to be highly dependent on the peptide displayed by HLA, with residue 8 playing a key role in recognition (97). Similarly, the interaction of KIR2DL2/3 with HLA-C was peptide selective. The bound peptide, particularly residues 7 and 8, can increase or abrogate the binding specificity of KIR2DL2/3 to HLA-C (98). Furthermore, peptides displayed by HLA I can operate as changed peptide ligands and effectively diminish KIR-mediated inhibition, indicating that alterations in the peptide presented by HLA I can influence NK-cell function (99). A peptide deriving from the core protein of hepatitis C virus (HCV) presented on HLA-C*03:04 modulates the function of NK cells by engaging the inhibitory receptor KIR2DL3 in a sequence-dependent manner, further implying that the binding of KIRs with HLA molecules and the function of NK cells can be influenced by the peptide (100). This specific peptide-dependent binding of KIR with HLA provides a potential mechanism for pathogens and self-peptides to influence NK-cell activation by varying inhibitory levels. The creation of wholly unique regions of amino acid sequence that can bind to MHC molecules should be conceivable as a result of the DNA changes accumulated in tumor cells. Following the malignant transformation of cells, the repertoire of peptides displayed by MHC molecules changes (13). Based on the fact that HLA–KIR interaction affinity is peptide-dependent and can influence the effector function of NK cells (97, 99–101), the altered peptide repertoire displayed by MHC molecules in cancer cells may be considered to influence the function of NK cells. As a result, it would be interesting to see if the neoantigens displayed by MHC molecules on tumor cells, especially for those that do not induce the downregulation of MHC I, may change the binding affinity of KIR-MHC and finally modify NK-cell activation (**Figure 2**).

The synergistic function of many NK-cell surface receptors determines the status of NK cells. Tumor cells can be rendered "invisible" to NK cells by upregulating inhibitory signals or/and downregulating activating signals on NK cells. The function of NK cells may even be modified by alteration in the affinity of the KIR–MHC interaction through the diverse peptide repertoire given by MHC molecules. Interfering with the activating and inhibitory signals has been utilized in several therapeutic techniques to boost NK-cell function (102–104). However, the possibly changed interaction affinity of the KIR-peptide/MHC complex resulting from the varied repertoire of a peptide given by MHC molecules in tumor cells may require more research.

Perspectives

So far, strategies to boost the antitumor function of T and NK cells have yielded encouraging results. As knowledge of the neoantigens presented by MHC molecules expands, the research and clinical implementation of neoantigen-based therapeutic methods, including adoptive T-cell therapy and cancer vaccines, are full of potential in the clinical applications. However, there are still many questions required to be answered. For example, how may neoantigens should be identified and therapeutic strategies developed for cancers with modest mutation loads? Even though the discovery of neoantigen has the potential to lead to such appealing tailored treatments, high prices and time constraints limit their use. Thus, how can neoantigen-based therapeutics be developed faster and at a lower cost? Meanwhile, while neoantigen-targeted therapy for cancer patients is based on each patient's unique neoantigen repertoire, the obstacles to predicting and selecting the best neoantigens for each patient remain. Because of that, can we better investigate some off-the-shelf cancer therapies based on common neoantigens and HLA allotypes? Furthermore, because neoantigen-based vaccines and adoptive T-cell treatment may be limited in their ability to overcome immune suppression caused by regulatory cells or tumor-derived factors as a monotherapy, they might be combined with other therapies to fully utilize their potential. Many studies indicated that the immune response can be boosted by conventional radiotherapies, chemotherapies, and targeted therapies (105–107). It remains to be determined, however, how and when combination therapy should be applied. Finally, now that intriguing customized treatment options based on neoantigen-reactive T cells have been brought, may new therapeutic approaches also be developed in NK cells against malignancies with a distinct peptide–MHC complex repertoire? All in all, a better understanding of the mechanisms underlying the activation of immune cells against cancer cells using neoantigens will undoubtedly aid the development of effective new mono- or combination cancer therapeutic strategies.

AUTHOR CONTRIBUTIONS

DL collected the data, drew the figures, and wrote the manuscript. HS proposed the idea and modified, supervised, and approved the final version of the manuscript. MK helped to edit the manuscript. ZL and YG provided professional pieces of advice in the minor revision previously. All authors contributed to the article and approved the submitted version.

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Natural killer cells: the next wave in cancer immunotherapy

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Immunotherapies focusing on rejuvenating T cell activities, like PD-1/PD-L1 and CTLA-4 blockade, have unprecedentedly revolutionized the landscape of cancer treatment. Yet a previously underexplored component of the immune system - natural killer (NK) cell, is coming to the forefront of immunotherapeutic attempts. In this review, we discuss the contributions of NK cells in the success of current immunotherapies, provide an overview of the current preclinical and clinical strategies at harnessing NK cells for cancer treatment, and highlight that NK cell-mediated therapies emerge as a major target in the next wave of cancer immunotherapy.

KEYWORDS

natural killer (NK) cells, immune checkpoint, cancer, immunotherapy, NK cell therapy, iPSC-NK

Introduction

NK cells, a subset of lymphocytes that are principally innate immune cells, arise from common lymphoid progenitors and constitute the third lymphoid lineage in addition to T-cell and B-cell lineages (1). NK cells were initially discovered and named based on their ability to kill cancer cells *in vitro* (2). They express a broad repertoire of activating and inhibitory receptors, the "net weight" of which controls the final outputs. The biology of NK cells has been extensively reviewed elsewhere (3, 4). In this review, we mainly focus on the therapeutic potential of NK cells as the next wave in cancer immunity. We will discuss the prognostic roles of NK cells in cancers, summarize the contributions of NK cells in the success of immune checkpoint blockade (ICB) therapies and approaches including cell therapies to harness NK cells in the cancer treatment.

NK cells in cancers

The immune surveillance role of NK cells in human cancers was first implicated in 1980s by reports revealing higher incidence of cancers in patients with NK cell defects (5, 6) and low NK cell activities in cancer patients or their families (7–11). Subsequently, a landmark 11-year following-up study reported a positive correlation between impaired

NK cell functions and higher risk to develop numerous types of cancers (12). Meanwhile, the critical role of NK cells in control of tumor growth and metastasis was demonstrated in mice models in early studies (13, 14). However, due to the paucity of NK cells usually overserved in primary tumors in clinic, questions have been raised – as to whether NK cells play an important role in tumor control and prognosis, and whether NK cells contribute to therapies such as targeted antibody therapies, despite the role of NK cells in immune surveillance.

Subsequent to early findings, accumulating evidence have reported impaired functions of NK cells in chronic myelogenous leukemia (CML) (15) and acute myeloid leukemia (AML) (16, 17). Intriguingly, NK cells in AML patients have been reported to significantly down-regulate activating receptor NKp46 and up-regulate inhibitory receptor NKG2A compared to those in healthy age-matched controls (17). Furthermore, lower NKp46 expression on NK cells (18), phenotypic and functional defects of NK cells (17) or defective NK cell maturation (19) have been reported to be associated with adverse clinical outcomes in AML patients treated with allogeneic stem cell transplantation (allo-SCT) (18) or chemotherapy (17, 19).

Furthermore, the prognostic role of NK cells has not only been observed in chemotherapy-based studies in hematopoietic cancers, but also observed in targeted antibody therapy-based studies, in both liquid and solid tumors (Table 1). In diffuse large B-cell lymphoma (DLBCL) patients treated with Rituximab-CHOP (20), breast cancer patients treated with anti-HER2 monoclonal antibody (mAb) and chemotherapy (22), and in colorectal cancer patients treated with anti-EGFR mAb and chemotherapy (24), the tumor-infiltration of NK cells have been reported to positively correlate with clinical responses. Moreover, high baseline of antibody-dependent cellular cytotoxicity (ADCC) has been reported to correlate with a complete response (CR) and a long overall survival (OS) in head and neck cancer patients treated with anti-EGFR mAb and radiotherapy (23). Those evidence suggested a role of NK cells in targeted antibody therapy, probably mediated by ADCC, and support the development of tools harnessing ADCC activities of NK cells for enhanced anti-tumor efficacy. We will expand the discussion in later sessions of the review.

Another intriguing observation related to the prognostic and predictive role of NK cells comes from the studies on immune checkpoint blockades (ICBs) therapies. Higher NK cell infiltration has been found in responders to anti-PD-1 treatment compared to non-responders from independent studies (25, 26), and thus raise the question whether NK cells contribute to the success of ICBs.

NK cells contribute to the success of ICBs

Many inhibitory receptors including PD-1, LAG3, TIM3, TIGIT, NKG2A etc. are expressed and mediate inhibition on both NK cells and T cells (27) (Table 2). To date, anti-PD-1/PD-L1 therapies have achieved remarkable efficacy in a wide spectrum of cancers (28). Moreover, ICBs targeting LAG3 (29) and TIGIT (30) are displaying great potentials to further improve clinical outcomes in combination with anti-PD-1 therapy. Basically, the efficacy has been attributed to

TABLE 1 Clinical correlations of NK cells with patient outcomes.

Cancer type	Treatments	Correlation of NK phenotypes with clinical outcomes	References
AML	Chemotherapy	Phenotypic and functional defects of NK cells associate with poor response.	(17)
	Conventional chemotherapy with or without the addition of anti-CD33 mAb	Patients with hypomaturation profile had reduced OS and progression-free survival (PFS) rates.	(19)
	Allo-SCT	$NKp46^{high}$ phenotype at diagnosis is associated with better PFS and OS.	(18)
DLBCL	Rituximab-CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone)	Lack of NK cell infiltration associate with poor survival.	(20)
CML	Imatinib	NK cell counts are associated with molecular relapse-free survival after imatinib discontinuation.	(21)
Breast cancer	All patients received a neoadjuvant combination treatment of standard chemotherapy and anti-HER2 mAbs	Tumor-infiltrating NK cells associate with pathological CR and disease-free survival.	(22)
Head and neck cancer	anti-EGFR and radiotherapy	High baseline of ADCC correlates with a CR and a long OS.	(23)
Colorectal cancer	A first-line anti-EGFR based chemotherapy	Tumor infiltrating $CD56^{+}$ cells are correlated with PFS and response.	(24)
Melanoma	Anti-PD-1 mAbs	Higher NK cell infiltration in responding *vs* non-responding patients.	(25)
	Anti-PD-1 mAbs	Up-regulated NK signatures and higher NK cells infiltration in tumors in responding *vs* non-responding patients.	(26)

TABLE 2 Selected shared immune checkpoint receptors between NK cells and T cells.

Receptor	Cell distribution	Drugs approved or in advanced clinical trials	Phase
PD-1	NK cells, T cells, B cells, myeloid cells	Pembrolizumab	FDA Approved
		Nivolumab	FDA Approved
		Cemiplimab	FDA Approved
		Dostarlimab	FDA Approved
		Tislelizumab	Phase III in US; approved in China
TIGIT	NK cells, T cells	Tiragolumab	Phase III
		Vibostolimab	Phase III
		Ociperlimab	Phase III
TIM3	NK cells, T cells, DCs, monocytes, macrophages, mast cells	MBG453	Phase II
		BGB-A425	Phase I/II
		TSR-022	Phase II
NKG2A	NK cells and T cells	Monalizumab	Phase III

unleashing T cell responses, leaving the contributions of NK cells yet to be fully explored. Recently, growing evidence is suggesting a prognostic role of NK cell activation status and tumor infiltration in the success of ICB (25, 26, 31), thus raising considerable interests to fill the conceptual gap with respect to whether and how NK cells play a role in the ICB practice.

First, NK cells may contribute to the ICB success by restraining the emergence of cancer cell clones that have escaped T cell attack through inactivation of antigen presentation. There is growing evidence that loss of genes associated with antigen presentation serves as an important mechanism of acquired resistance to ICB (32). In pre-clinical models, Nicolai et al. and Das et al. showed that NK cells mediate the rejection of $CD8^+$ T cell resistant $B2m^{-/-}$ tumors (33, 34). It is in line with the longstanding observations that NK cells express inhibitory receptors binding to MHC-I, thereby maintaining "self-tolerance" to normal cells. When cancers down-regulate MHC-I on their surface to escape T cell attack, the "missing-self recognition" by NK cells is triggered, thus initiating NK cell mediated cytotoxicity against the "escapers" (35).

Second, the ICB may confer a direct modulation on NK cell activity. One study reported that PD-1 is upregulated on circulating and intra-tumoral NK cells in patients of Hodgkin lymphoma. $PD\text{-}L1^+$ myeloid cells efficiently suppress the function of $PD\text{-}1^+$ NK cells *in vitro*, while anti-PD-1 treatment can effectively reverse the suppression (36). Further evidence for the PD-1/PD-L1 signaling in NK cells comes from studies describing PD-1 upregulation in NK cells in non-small cell lung cancer (NSCLC) and head and neck cancer (HNC) patients. PD-L1 beads or $PD\text{-}L1^+$ target cells impaired $PD\text{-}1^+$ NK cell function, while anti-PD1 or anti-PD-L1 treatment significantly activated $PD\text{-}1^+$ NK cells *in vitro* (37, 38). Moreover, there is evidence in *in vivo* mouse models that PD-1 is up-regulated on most activated tumor-infiltrating NK cells, and NK cells mediate full therapeutic efficacy of PD-1/PD-L1 blockade (39). Nevertheless, to what extent the anti-PD-1/PD-L1 therapies could directly activate NK cells in patients and thereby contribute to the efficacy remains an open question that needs to be further explored. Another shared checkpoint between T cells and NK cells, TIGIT, is constitutively expressed on PBMC-derived NK cells as well as *in vitro* activated human NK cells (40–43). In a recent publication, we have demonstrated the direct activation of NK cells by the therapeutic TIGIT blocking antibody ociperlimab (BGB-A1217) in an *in vitro* NK-cancer cell co-culture assay (44). Remarkably, the full Fc effector function of ociperlimab further elevated NK cell function in addition to checkpoint blockade (44), probably through the synergy between FcγRIIIa (CD16a) signaling and release of TIGIT mediated suppression on NK cells (45). Another immune checkpoint, TIM3, has been found to be up-regulated on NK cells from patients with melanoma (46), gastric cancer (47) and lung adenocarcinoma (48), and blockade of TIM3 has been reported to release the exhaustion of NK cells from advanced melanoma patients *in vitro* (46).

Recently, the NKG2A/CD94 blockade seems to carve a new path in the adoption of ICB in the cancer treatment *via* unleashing both T cells and NK cells. Pre-clinical data suggest a dual role of NKG2A blockade on NK cells and T cells (49–51). In clinic, monalizumab (49), a humanized IgG4 ICB targeting the NKG2A/CD94 receptor, blocking its interaction with HLA-E, is being investigated in the treatment of solid tumors. Encouraging results from a large, randomized Phase II trial showed monalizumab in combination with durvalumab, a PD-L1 blockade antibody, improved PFS and objective response rate (ORR) compared to durvalumab alone in patients with unresectable, stage III NSCLC. The 12-month PFS rate was 72.7% for durvalumab plus monalizumab, versus 33.9% with durvalumab alone (52).

From another perspective, it is noteworthy that the immune checkpoint blockade antibodies can activate NK cells through

the Fc effector functions, as we reviewed previously (53). Direct evidence for this hypothesis comes from the Fc-competent TIGIT antibody Ociperlimab. TIGIT expression is highly expressed on Treg cells, relative to effector T cells, and is further elevated on Tregs in tumor microenvironment (44, 54). Our data have shown that the ligation of TIGIT on Tregs and Fcγ receptors on NK cells by Ociperlimab directly promoted NK cell activation and induced ADCC against cancer patient PBMC derived Tregs *in vitro*. In the CT26 mouse model, we also observed the decrease of intratumor Treg numbers (44). It is of great interest to further explore the potential mechanisms in clinical settings. Another T cell checkpoint, CTLA-4, is also expressed on cancer cells such as melanoma, leading to potential NK cell mediated ADCC against CTLA-4$^+$ cancers induced by anti-CTLA-4 treatment (55). Nevertheless, CTLA-4 is also expressed on CTLs, thus rendering the overall mechanisms complicated.

Third, emerging evidence have suggested an essential role of NK cells in checkpoint therapy response through an NK-dendritic cell (DC) axis (25, 56). Conventional type 1 dendritic cells (cDC1) are a subtype of DC that stimulate robust T cell response to cancer. They adept at taking up dead cells and cross-present tumor antigen to CD8$^+$ T cells (57, 58), attract T cells into tumor (59), and elicit tumor-specific T cell responses (60). Intriguingly, work from Bottcher et al. revealed a strong correlation between cDC1 signatures and NK cell signatures in cancers including skin cutaneous melanoma (SKCM), breast invasive carcinoma (BRCA), head and neck squamous cell carcinoma (HNSC) and lung adenocarcinoma (LUAD) (56). Moreover, NK cell signatures were found positively associated with patient survivals in all those cancers. Furthermore, they discovered in pre-clinical models that intratumor NK cells recruit cDC1 into tumors to promote tumor control (56). Similarly, Barry et al. observed that NK cell signatures positively correlate with stimulatory dendritic cells (SDC; intratumor cDC1) in melanoma, patient response to anti-PD-1 therapy and overall survival. In line with the data from Bottcher et al., they also uncovered a role of NK cells in the control of CD103$^+$ SDC in a mouse tumor model (25).

Taken together, NK cells may contribute to ICBs success through multiple aspects. However, one caveat is that one should be cautious to interpret the data from pre-clinical models and translate from laboratory to clinic. Gaps exist between mouse models and human cancers, e.g., FcγRIII on mouse NK cells is actually not the homologue of FcγRIII on human NK cells. Human FcγRIIIa is functionally similar with a unique mouse FcγR – FcγRIV (61). Nonetheless mouse FcγRIV is not expressed on mouse NK cells, but abundantly on macrophages (53). In addition, syngeneic or xenograft tumor models may not truly mimic the NK cell infiltration status in human tumors, thereby suggesting translational gaps between pre-clinical tumor models and cancer patients.

Approaches to harness NK cells

NK cells express a broad range of activating and inhibitory receptors. Whether NK cells attack a target cell depends on the net equilibrium of the activating and inhibitory signals. Here we focus on emerging novel modalities for NK cell targeting, e.g., ADCC enhanced antibodies, bi- or tri-specifics, and iPSC-derived NK cells (iPSC-NK) therapies.

ADCC-enhanced antibodies

In humans, FcγRIIIa is the major type of FcγRs expressed on NK cells (62). Binding of Fc portion of human IgG to FcγRIIIa can trigger NK cell ADCC against mAb-opsonized target cells, as has been firmly established. Two alleles encode different FcγRIIIa variants that differ at the position 158, with either a valine (V) or phenylalanine (F). Between the two isoforms, FcγRIIIa-V_{158} exhibits higher affinity to IgG1, and mediated more efficient ADCC (63). In clinic, the FcγRIIIa dimorphism was strongly associated with the outcome of patients treated with anti-EGFR or anti-CD20 antibodies (64–69). Although it remains controversial about the relative contributions of different immune cells or effectors in the therapeutic efficacy of tumor-targeting mAbs (70–73), multiple studies have suggested a positive correlation of NK cell infiltration and activity with the response to tumor-targeting mAb treatment (22–24), and again, caution is warranted on the interpretation of mechanistic studies in mice given the discrepancies of FcγRs expression profiles between human and mice. Therefore, several strategies have been employed to develop ADCC-enhanced mAbs for harnessing NK cell functions.

Removal of core fucose from N-glycans attached to human IgG1 significantly enhances the binding affinity of IgG1 to FcγRIIIa and ADCC (74, 75), and has been the most widely adopted approach to harness the mAb mediated ADCC response in clinical practice (76). As of today, three afucosylated mAbs have been marketed for the treatment of human cancers: Obinutuzumab, a CD20-directed afucosylated antibody approved for the treatment of chronic lymphocytic leukemia (CLL); Poteligeo (mogamulizumab), a CCR4-targeting afucosylated mAb, approved for the treatment of Mycosis Fungoides (MF) and Sézary Syndrome (SS); and Fasenra (benralizumab), an afucosylated IL-5Rα targeting mAb for the treatment of patients with severe eosinophilic asthma. In addition, Rybrevant (amivantamab), an anti-EGFR and anti-cMet bispecific low fucose antibody with enhanced Fc function, have been approved for the treatment of NSCLC. Blenrep (belantamab mafodotin-blmf), consisting of an afucosylated humanized anti-BCMA IgG1 mAb conjugated to the tubulin inhibitor, monomethyl auristatin F (MMAF), for the treatment of adult patients with relapsed or refractory multiple myeloma, is

the only FDA approved ADC with an afucosylated antibody (77). Nowadays, numerous afucosylated mAbs targeting a diverge range of receptors are actively in clinical development, with the outcomes yet to be revealed (78).

Fc engineering represents another approach to enhance ADCC (62, 79). Several Fc-enhanced mAbs through the genetic engineering approach are being investigated in clinical trials, with only one approved by FDA till now, Margenza (margetuximab), for the treatment of metastatic HER2-positive breast cancer. It is noteworthy that exploratory PFS analysis by FcγRIIIa genotype suggested that presence of a FcγRIIIa-F_{158} allele may predict margetuximab benefit over trastuzumab. Margetuximab provided no clinical benefit in FcγRIIIa-V_{158} homozygotes compared with trastuzumab (80). Since the Fc engineering of margetuximab-cmkb increases affinity for both FcγRIIIa allotypes, and FcγRIIIa-V_{158} per se has higher affinity to IgG1, the none-benefit in FcγRIIIa-V_{158} homozygotes might be attributed to the rapid cleavage and downregulation of FcγRIIIa due to stronger binding of the antibodies to FcγRIIIa-V_{158}. From another perspective, strong binding to FcγRIIIa may induce enhanced antibody internalization by FcγRIIIa expressing cells, thus promoting the anti-drug antibody (ADA) production, to compromise the efficacy. The exact underlying mechanisms are yet to be elucidated.

NK cell engagers

There are a broad range of activating and inhibitory receptors on NK cells. The integration of signals for activation and inhibition determines the final outputs of NK cells. The loss of inhibitory signaling, like downregulation of MHC-I expression on tumor cells, renders tumor cells susceptible to NK cell cytotoxicity. Alternatively, NK cells can attack cancer cells that retain full expression of MHC-I if activating receptors on NK cells are engaged.

Recently, bi-specific or tri-specific antibodies targeting NK cell activating receptors are emerging as novel approaches to harness NK activity. Preclinical results provide the rationale for developing multi-specific NK cell engagers through ligation of tumor antigens and activating NK receptors. Examples include those targeting NKp46 (81), NKp30 (82), NKG2D (83, 84), and FcγRIIIa (CD16a) (85, 86). Encouraging data comes from a Phase I clinical study in which an anti-CD16/anti-CD30 bispecific NK-cell engager combined with pembrolizumab has shown an ORR of 83% and a CR rate of 46% in patients with relapsed or refractory Hodgkin Lymphoma (HL) (87). However, it should be noted that most of the activating receptors are not exclusively expressed on NK cells, instead are often shared with T cells or myeloid compartments. Targeting the activating receptors on NK cells may synergically augment both NK cells and other immune effectors.

NK cell-checkpoint blockades

A wide range of immune checkpoints are expressed on NK cells. As with the activating receptors, NK checkpoints are usually shared with other immune components (27). As discussed in the earlier part of the review, some blockades that can target checkpoints on both T cell and NK cells have obtained remarkable success or promising preliminary clinical responses (Table 2), albeit the contributions of NK cells therein are yet to be fully understood. In addition, blockers that target inhibitory killer Ig-like receptors (KIRs) have been investigated in clinical settings. KIRs are a group of receptors on NK cells that bind to HLA molecules to mediate inhibitory or activating signaling (88). Clinical evidence have suggested that adoptive NK cell transfer has the potential to improve outcomes of KIR ligand-mismatched recipients even further (89–91). Lirilumab, a humanized IgG4 mAb, binds to KIR2DL-1, KIR2DL-2 and KIR2DL-3 and thereby blocks their inhibitory signaling mediated by both HLA-C C1 and HLA-C C2 subtype molecules (92). It has shown good safety profiles in phase I trials, however, the phase II trial in patients with smoldering multiple myeloma failed to demonstrate clinical efficacy (93). The minimal efficacy may result from lack of the KIR matched HLA types from patients, and existing of other dominant inhibition signals (94). However, this does not rule out the possibility that inhibitory KIR blockers could synergistically work together with other ICBs or NK cell therapies to induce a combination efficacy.

NK cell-based cell therapies

Adoptive cell therapies, basically chimeric antigen receptor T (CAR-T) cell therapies, have exhibited remarkable clinical responses in treating hematologic malignancies, and thus spawned an explosion in the CAR-T field. As of today, six CAR-T cell therapies have been approved by FDA, wherein four targeting CD19, and two targeting BCMA. Although the efficacies have been notable (95–101), limitations are still obvious. First, as a highly personalized therapy, autologous CAR-T cells have to be individually prepared for each patient in a time- and material- consuming process that carries the risk for failure and demanding logistics. Patients who have already received multiple rounds of chemotherapy may not be able to mobilize enough T cells for the CAR-T cell preparation. Additionally, during the time waiting for CAR-T cells manufacturing, patients may experience disease progression. As such, the therapy results in low scalability and remains unacceptable and unaffordable to most of patients. Second, severe toxicity associated with CAR-T cells hampers the broad applicability of the treatment. In several patients, CAR-T cell treatments have been associated with substantial toxicity

including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), thus decreasing the feasibility due to demanding toxicity management, and inconvenient administration (102).

In contrast, NK cell therapies harbor high potential to overcome those hurdles. Firstly, the activating machineries of NK cells differ from the TCR system of T cells. NK cells do not require HLA matching to exert cytotoxicity against tumor cells. This allows for using NK cells in an allogeneic way. On the other hand, allogeneic NK cells do not result in graft versus host disease (GvHD), even in the setting of substantial HLA disparity between adoptive CAR-NK cells and the recipients (103), and thus can be provided as complete "off-the-shelf" products, significantly lowering the cost of manufacturing and logistics. Secondly, both autologous and allogeneic NK cells have exhibited excellent safety profile, without severe toxicity such as CRS or ICANS (103, 104). Compared to their T cell counterparts, NK cells present a safer cytokine profile, and differ in the crosstalk with myeloid cells (4). This property confers the feasibility of the NK cell therapy when specialized care units are unavailable. Thirdly, from the efficacy perspective, NK or CAR-NK cell therapies have shown inspiring clinical outcomes in early phase clinical trials when used alone (103) or in combination with other therapies (104), encouraging more endeavors in the field. Last but not least, repeated doses can be administrated given the short lifetime of NK cells, and NK cells from different donors can be sequentially dosed to circumvent rejection of donor NK cells by recipient memory T cells recognizing allo-antigens on the same donors.

NK cells for cell therapy can be generated from different sources and by a variety of methods (Table 3). Peripheral blood derived, and ex vivo expanded autologous NK cells have been well tolerated in clinical trials, whereas efficacy has been limited (105, 106). The low efficacy may be attributed to the suppression of autologous NK cells by self-HLA molecules. As such, allogeneic NK cells serve as a promising alternative approach to overcome the resistance. In a seminal study by Miller et al., a complete remission induced by haploidentical allogeneic NK-cell infusions in 5 of 19 poor-prognosis AML patients was observed (90). In a subsequent study in pediatric AML, all patients who received adoptive haploidentical NK cells remained in remission with a median follow-up time of 964 days (91). Later on, 53% complete remission was observed in AML patients treated with haploidentical NK cells combined with an IL-2 diphtheria toxin fusion protein, which was used to deplete host regulatory T cells (107); 32% complete remission was observed in AML patients treated with haploidentical NK cells combined with IL-15 (108), and 44% complete remission was observed in AML patients treated with allogeneic cytokine-induced memory-like NK cells, in separate studies (109).

Besides peripheral blood-derived NK cells (PB-NK), an alternative approach to generate functional NK cells is to obtain NK cells from umbilical cord blood and expand *ex vivo* (110, 111). A recent clinical study by Liu et al. has presented inspiring results to show the remarkable efficacy and excellent safety profile of engineered umbilical cord blood derived NK cells (UCB-NK) in the treatment of CD19 positive relapsed or refractory lymphoid tumors. The HLA-mismatched UCB-NK cells were transduced with a retroviral vector encoding anti-CD19 CAR, IL-15, and inducible caspase 9 as a safety switch. Of the 11 patients who were treated, 8 (73%) had a response and 7 (64%) had a complete remission. Notably, no severe toxicity including CRS, neurotoxicity or GvHD were observed (103). Another method to derive NK cells from umbilical cord blood is to differentiate them from $CD34^+$ hematopoietic progenitor cells (HPC) (112). In the first-in-human study, $CD34^+$ HPC derived NK cells (HPC-NK) were administrated to 10 older AML patients after lymphodepleting chemotherapy without cytokine boosting. Preliminary data showed that HPC-NK cells were well tolerated, with neither GvHD nor toxicity observed. Notably, 2 of 4 patients with minimal residual disease (MRD) in bone marrow before HPC-NK cells infusion became MRD negative, which lasted for 6 months (113).

Albeit the encouraging efficacy achieved by those clinical trials using PB-NK or UCB-NK, limitations exist due to the requirement for collection from a donor by apheresis or from umbilical cord blood, the variability of NK cell yield influenced by donor variability, and the challenge in generic manipulation

TABLE 3 Comparison of clinical-scale NK cells generated from distinct sources.

Attributes	NK-92	PB-NK	UCB-NK	HPC-NK	iPSC-NK
Source	NK-92 cell line	Peripheral blood of donors	Cryopreserved umbilical cord blood (UCB) in UCB unit	$CD34^+$ hematopoietic progenitor cells from UCB	iPSC
Tumorigenicity	High (need to be irradiated before infusion)	Low	Low	Low	Low
Accessibility	Easy	Easy	Less easy	Less easy	Easy
Homogeneity	High	Low	Low	Low	High
Genetic engineering	Easy	Less easy	Less easy	Less easy	Easy
Cell number sufficiency of a uniform cell population for repeated doses	High	Low	Low	Low	High

on differentiated cells with low proliferation capacity. To overcome those limitations, a number of studies used NK-92, a NK cell line originally established from a patient with non-Hodgkin's lymphoma (114–116). There are several advantages of NK-92 cell line as a source for NK cell therapy – it provides a homogeneous master cell bank, can be expanded indefinitely and served as an uniform "off-the-shelf" product, is more amenable to genetic modification and allows sufficient cells for cell therapy (117). However, on the other hand, albeit NK-92 lack expression of most known KIRs and exhibit broad cytotoxicity against numerous cancers, it loses expression of typical activating receptors including NKp44, NKp46 and notably, FcγRIIIa, which mediated ADCC (117). Additionally, as a lymphoma cell line, NK-92 holds inherent draw backs such as potential tumorigenicity and latent infection by Epstein-Barr Virus (EBV). Thus, for safety considerations, NK-92 must be irradiated before administration to patients. The irradiation limits the proliferation and persistence of NK-92 *in vivo*, and eventually may impede the long-term anti-tumor efficacy. And this may account for the observed limited efficacy of NK-92 cells in clinic (118, 119).

In recent years, iPSC-NK technology emerges as a breakthrough innovation in the NK cell therapy field, offering the potential to overcome challenges often seen with other source-derived NK cells. Serial seminal studies from Kaufman et al. have significantly optimized the protocols to derive NK cells to a clinical-scale from embryonic stem cells (hESCs) or iPSC (120), and demonstrated for the first time that CAR-NK cells can be derived from iPSCs expressing CAR (121). Since pluripotent stem cells have the potential to grow indefinitely in an undifferenced state (122, 123), the iPSC can serve as a stable cell bank for uniform NK cell generation and allows for sufficient cell numbers for cell therapy. As such, the iPSC-NK can serve as a standardized "off-the-shelf" product. In addition, iPSC is amenable to genetic engineering. Once the genetically modified clones are selected, it can be expanded for a production of a uniform pool of iPSC-NK cells. Multiple genetical modifications, such as ectopic expression of IL-15/IL-15R fusion protein (124, 125), CAR (121), high-affinity non-cleavable variant of CD16a (125, 126), deletion of CISH (127) or CD38 (125) have been successfully introduced on iPSC-NK to achieve enhanced expansion, better *in vivo* persistence or greater cytotoxicity. The difference between iPSC-NK, PB-NK and UCB-NK are yet to be fully understood, yet some pre-clinical evidence have suggested that iPSC-NK may have comparable or superior activities relative to PB-NK or UCB-NK (128–130). To date, iPSC-NK cell therapies have entered phase I clinical trials, used alone or in combination with therapeutic monoclonal antibodies (mAbs) for the treatment of hematopoietic lymphomas or solid tumors (131–136). Remarkably, the first in human results are encouraging (137, 138). In a phase I trial, FT516, an iPSC-NK cell therapy using iPSC-NK cells engineered with a high-affinity, non-cleavable CD16a (hnCD16) that enables tumor targeting and enhanced ADCC in combination with a therapeutic mAb, was combined with rituximab to treat patients with relapsed or refractory B-cell lymphoma (BCL) (132). Eight of the 11 pts (73%) treated with ≥90 million FT516 cells achieved an objective response. Seven (64%) patients achieved CR, including 2 patients with prior CD19 CAR T-cell therapy (139). FT596 (140), is an iPSC-derived CAR-NK cell therapy armed with three modalities: a CD19-targeting CAR, a hnCD16, and IL15/IL-15 receptor fusion which promotes NK cell persistence by the autonomous cytokine. In a phase I trial, FT596 was administrated as monotherapy or in combination with rituximab or obinutuzumab for the treatment of relapsed or refractory BCLs and CLL (141). At single-dose levels of ≥90 million cells, 8 of 11 (73%) efficacy-evaluable patients achieved ORR, including 7 (64%) CR. Of 4 patients with prior CAR T-cell therapy treated at ≥90 million cells, 2 achieved CR (142). Notably, no dose-limiting toxicities, CRS, ICANS, or GvHD of any grade were observed for FT516 or FT596, and repeated doses were allowed (139, 142).

NK cell-based combination strategies

Nowadays, NK cell-based combination strategies have been investigated in the cancer immunotherapy and represent an important direction in the future.

NK cell therapies in combination with ICBs

As we have discussed in the previous sessions, activated or intra-tumor NK cells up-regulate checkpoint molecules (e.g., PD-1, TIGIT, TIM3, NKG2A) and blocked of those molecules unleash NK cell activity. In a pre-clinical model, iPSC-NK cells in combination with T cells and an anti-PD-1 antibody have been reported to eliminate tumors in a xenograft ovarian cancer mouse model (143). Combination of ICBs and adoptive NK cell therapy would be a promising approach to achieve optimized NK functions, and in concert with T cells.

NK cell therapies in combination with tumor targeting mAbs or NK cell engagers

Adoptive NK cell therapy in combination with tumor-targeting mAbs or other NK cell engagers represent an approach to fully augment the tumor-specific NK cell cytotoxicity. Impressive results have been obtained from a phase I clinical study that combine CAR-iPSC-NK cell therapy with anti-CD20 (139). Moreover, the combination of CB-NK and a bispecific CD30/CD16 antibody is being actively investigated in a phase I/II study (144, 145). As disclosed by Affimed on the 2022 AACR meeting, as of the cut-off date, the study had enrolled 22 patients with relapsed or refractory $CD30^+$ Hodgkin and non-Hodgkin lymphoma having received a median of seven prior lines of therapy. Out of the 13 patients

treated at the recommended phase 2 dose (RP2D), 13 patients (100%) achieved objective response, and 8 (62%) patients achieved CR after two cycles of treatment (146).

CAR-NK and CAR-T cell combinations

Sequential infusions of CAR-NK cells and CAR-T cells would be a good strategy to achieve better efficacy and safety. CAR-NK cells should rapidly decrease the tumor burden, particularly for patients with high tumor load. This may decrease the CRS and neurotoxicity risk imposed by CAR-T cells. Then subsequent CAR T cell infusion may eliminate residual tumor cells and provide a lasting anti-tumor effect through memory T cells that survive and persistence.

Other attempts to target NK cells

In addition to the approaches discussed above, strategies targeting cytokines, such as IL-12, IL-15 and IL18 (109, 124, 147), targeting intracellular checkpoints, such as CISH (127, 148), Cbl-b (149) GSK3 (150) and CDK8 (151, 152), or targeting tumor cells which can indirectly trigger NK cell surveillance through non–cell autonomous mechanisms (153) may also effectively augment NK cell functions and eventually result in novel therapeutic candidates.

Conclusions and prospects

Taken together, NK cell-based therapies have attracted intense interest and shown great potential in the treatment of cancers, emerging as the next wave in cancer immunotherapy. Multiple approaches, including checkpoint blockades, ADCC enhanced antibodies, agonist antibodies and multi-specific NK cell engagers, and adoptive NK cell therapies (particularly engineered iPSC NK cell therapies) have significantly widened the pool of potential clinical options. However, challenges exist with the opportunities. As a heterogenous population, NK cells are still not fully understood. It is crucial to continue to delineate the NK cell biology and characterize the differences of NK cells derived from distinct sources and methods. In addition, although NK cell-based therapies have demonstrated great potentials in the treatment of hematopoietic cancers, the advances in solid tumors remain limited. It is important to further understand NK homing capacities and the reasons underlying their poor infiltrations in solid tumors, which may eventually lead to the development of novel approaches to overcome the barriers. Furthermore, the questions about the persistence of NK cells, and the durability of the response, and the affordable cost for patients need to be considered. Along with the advancing of new technologies and methods, NK cell-based therapies will continue to evolve, and get closer to benefit patients with otherwise no treatment options. In summary, NK cell-mediated therapies have emerged as the next wave in cancer immunotherapy.

Author contributions

XC and LJ drafted the manuscript. XL reviewed the manuscript, and all authors were involved in revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

XC is a Senior Principal Investigator at the company BeiGene (Beijing) Co., Ltd. LJ is a Senior Scientist at the company BeiGene (Beijing) Co., Ltd. XL is the Vice President, Head of Biology at the company BeiGene (Beijing) Co., Ltd.

Publisher's note

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B7-H3-targeting Fc-optimized antibody for induction of NK cell reactivity against sarcoma

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Natural killer (NK) cells largely contribute to antibody-dependent cellular cytotoxicity (ADCC), a central factor for success of monoclonal antibodies (mAbs) treatment of cancer. The B7 family member B7-H3 (CD276) recently receives intense interest as a novel promising target antigen for immunotherapy. B7-H3 is highly expressed in many tumor entities, whereas expression on healthy tissues is rather limited. We here studied expression of B7-H3 in sarcoma, and found substantial levels to be expressed in various bone and soft-tissue sarcoma subtypes. To date, only few immunotherapeutic options for treatment of sarcomas that are limited to a minority of patients are available. We here used a B7-H3 mAb to generate chimeric mAbs containing either a wildtype Fc-part (8H8_WT) or a variant Fc part with amino-acid substitutions (S239D/I332E) to increase affinity for CD16 expressing NK cells (8H8_SDIE). In comparative studies we found that 8H8_SDIE triggers profound NK cell functions such as activation, degranulation, secretion of IFNγ and release of NK effector molecules, resulting in potent lysis of different sarcoma cells and primary sarcoma cells derived from patients. Our findings emphasize the potential of 8H8_SDIE as novel compound for treatment of sarcomas, particularly since B7-H3 is expressed in bone and soft-tissue sarcoma independent of their subtype.

KEYWORDS

sarcoma, B7-H3, mAb, Fc-optimized, immunotherapy, NK cells

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Introduction

Sarcomas are malignancies of mesenchymal origin with relatively rare occurrence that are classified depending on the tissue origin (1, 2). They comprise more than 100 distinct subtypes with different biological behavior which eventually results in differing responses to treatment (3, 4). Five-year survival rates of 60–80% have been reported for patients undergoing surgical resection with subsequent chemotherapy (5). Outcome for patients with metastatic disease at time of diagnosis or for patients with recurrence of disease is considerably worse (6, 7). Even though therapeutic options have significantly increased over the last years, there is an urgent need for new treatment approaches that are desperately needed to improve patient outcomes (8, 9).

Treatment outcome in various types of cancer has been significantly improved by introduction of immunotherapy with monoclonal antibodies (mAbs). Prominent examples are Herceptin and Rituximab, which are now established standard treatment options for patients with Her2 expressing breast cancer and B-cell non-Hodgkins lymphoma, respectively (10, 11). Nevertheless, there is still plenty of room for improvement regarding the efficacy of so far available tumor-targeting mAbs, and in many disease entities including sarcoma no immunotherapeutic mAbs are yet available. Antitumor mAbs elicit therapeutic efficacy to a substantial part by induction of antibody-dependent cellular cytotoxicity (ADCC). The major effector cell population which in humans mediates this fundamental mAb function are natural killer (NK) cells (12, 13). To reinforce therapeutic efficacy of tumor-targeting mAbs, modification of the antibody Fc part is one possible approach. Affinity to the Fc receptor CD16 on NK cells can be increased by genetically engineering the glycosylation motifs or the amino-acid sequence of the Fc part. This Fc optimization potentiates the capability of mAbs to engage Fc receptor expressing immune effector cells like NK cells (14, 15). To engraft mAb treatment for additional disease entities, identification of suitable target antigens that are widely expressed on tumor cells while ideally being not expressed on healthy cells is inevitable. In sarcomas, this is particularly challenging due to lack of well-established target antigens, among others because of differences between the many subtypes (8).

B7-H3 (CD276) belongs to the B7 protein family and is classified as an integral transmembrane protein (16–18). So far, the immunoregulatory role of B7-H3 is still under discussion, alike its role in cancer pathogenesis (19–21). Expression of B7-H3 has been reported for a multitude of human cancers which include glioma, acute myeloid leukemia (AML), lung adenocarcinoma, ovarian cancer, neuroblastoma, pancreatic cancer, and also certain sarcomas, whereas it is largely absent in healthy tissues (22–27). In addition, B7-H3 is expressed on the tumor vasculature in many cancers (28–30). Overexpression of B7-H3 is linked to unfavorable disease course and poor prognosis for patients (25, 31, 32) and has been suggested to impair antitumor reactivity of T cells and NK cells (19, 29, 33). Based on these findings, B7-H3 appears to be an attractive target antigen for immunotherapy, as (i) B7-H3 expression is almost exclusively limited to tumor tissue, (ii) expression on tumor vasculature allows for additional targeting of both cancer cells and vasculature, allowing for dual mode of anticancer action and (iii) blocking of B7-H3 might reduce its immunosuppressive properties. Accordingly, several B7-H3-directed therapeutics are presently under investigation.

In the present study, we report on the overexpression of B7-H3 in various sarcoma subtypes and the development of an Fc-optimized B7-H3 mAb, which was characterized with regard to its potential to induce ADCC of NK cells against sarcoma cells.

Material and methods

Cells

Isolation of healthy donor peripheral blood mononuclear cells (PBMC) was carried out by density gradient centrifugation (Biochrom, Berlin, Germany). PBMC were collected from healthy donors of mixed age and gender and randomly selected, for each experiment. Cryopreserved cells were cultured at 37°C overnight in media prior the use in functional experiments. In all cases, written informed consent, in accordance with the Helsinki protocol, was given. The study was conducted according to the guidelines of the local ethics committee.

The sarcoma cell lines RD-ES (rhabdomyosarcoma), SaOs (osteosarcoma), SW1353 (chondrosarcoma), SW872 (liposarcoma) and SW982 (synovial sarcoma) were purchased from ATCC (American Type Culture Collection) and cultured as previously described (34). Cell lines used in experiments were cultivated for a maximum of two months. To validate cell authenticity, the respective immunophenotype provided by the supplier was examined. To exclude contamination of cultured cells with mycoplasma, cells were tested regularly every three months. Patient-derived sarcoma cells from patients diagnosed with liposarcoma, chondrosarcoma, rhabdomyosarcoma, osteosarcoma or synovial sarcoma were obtained from outgrowth cultures of resected primary tumors as previously described (34).

Production of antibodies

Generation of 8H8_SDIE and 8H8_WT as well as corresponding controls was carried out by chimerization (human immunoglobulin G1/κ constant region) of the anti-B7-H3 mAb 8H8 and control mAb MOPC21, respectively and Fc-optimization (S239D/I332E modification) for mAbs as described previously (35). Briefly, respective light chain (LC) and heavy chain (HC) plasmids were received using the EndoFree Plasmid Maxi kit from Qiagen (Hilden, Germany)

as described in the manufacturer's instructions. For antibody production, the ExpiCHO cell system (Gibco, Carlsbad, CA) was used according to the manufacturer's recommendations. mAbs were purified from media by protein A affinity chromatography (GE Healthcare, Chicago, IL) followed by preparative size exclusion chromatography (HiLoad 16/60 Superdex 200; GE Healthcare). To ensure quality and purity of produced antibodies, analytical size exclusion chromatography (Superdex 200 Increase 10/300 GL; GE Healthcare) and 4–12% gradient SDS-PAGE gels (Invitrogen; Carlsbad, CA) was performed using the gel filtration and Precision Plus standard from Bio-Rad (Hercules, CA), respectively.

Expression of B7-H3 mRNA based on TCGA database analysis

Data on relative expression of B7-H3 mRNA for tumor tissue and normal tissue samples was obtained from the Cancer Genome Atlas (TCGA) database and the GTEx project utilizing the Gene Expression Profiling Interactive Analysis (GEPIA) web server as described previously (26). Data sets for 5 different tumor subtypes (275 tumor/349 normal tissue) colon adenocarcinoma, (286/60) kidney renal papillary cell carcinoma, (179/171) pancreatic adenocarcinoma, (486/338) lung squamous cell carcinoma, and (262/2) sarcoma samples were downloaded from TCGA (http://www.oncolnc.org) and analyzed employing the online web server GEPIA (http://gepia.cancer-pku.cn).

PCR

B7-H3 primers were QuantiTect Primer Assay Hs_CD276_1_SG (Qiagen), GAPDH primers were 5′-AGCCACATCGCTCAGACAC-3′ and 5′-GCCCAATACG ACCAAATCC-3′. 1-2 x 10^6 cells were used for total RNA isolation utilizing the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) followed by cDNA synthesis using FastGeneScriptase II (NIPPON Genetics Europe, Düren, Germany) as described in the manufacturer's instructions, respectively. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously (36, 37). Quantitative PCR (qPCR) was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences Beverly, MA) with a LightCycler 480 (Roche) instrument.

Flow cytometry

For analysis of B7-H3 surface expression, fluorescence-conjugates of B7-H3 mAb or isotype control (Biolegend, San Diego, CA) were used. For dose titration and binding experiments, cells were incubated with 8H8_WT, 8H8_SDIE, Iso_WT and Iso_SDIE followed by anti–human PE conjugate (Jackson ImmunoResearch West Grove, PA).

To stain CD16 positive NK cells, fluorescence-conjugates CD3-APC/Fire, CD14-BV785, CD16-APC, CD19-FITC and CD56-PECy7 (all from Biolegend) were used.

Quantitative analysis of immunofluorescence to determine the number of B7-H3 molecules on the cell surface was performed using a murine B7-H3 Hybridoma-derived antibody and the QIFIKIT (Dako, Hamburg, Germany) as described previously (35).

For staining of intracellular IFNγ, cells were stained with CD3-FITC and CD56-PECy (both from BioLegend) followed by fixation and permeabilization and staining with mouse anti-human IFNγ-BV421 (clone b27) in 1:25 dilution using the Fixation/Permeabilization Solution Kit with BD GolgiPlug from BD Biosciences according to manufacturer's instructions.,

Flow cytometry-based determination of target cell lysis was conducted as previously described (34). In brief, sarcoma cells were loaded with 2.5 µM CellTrace™ Violet cell proliferation dye (Thermo Fisher Scientific, Waltham, MA) prior to seeding in cocultures with PBMC of healthy donors in the presence or absence of the antibodies (1 µg/mL each). Measurement of equal assay volumes was allowed by using beads (Sigma). The percentage of living target cells was calculated as follows: 7-AAD$^-$ cells upon treatment/7-AAD$^-$ cells in control × 100.

7-AAD (BioLegend) staining (1:200) was used to exclude dead cells from flow cytometric analysis or LIVE/DEAD™ Fixable Aqua (Thermo Fisher Scientific). All samples were analyzed using the BD FACS Canto II or BD FACSCalibur (BD Biosciences). Data analysis was performed using FlowJo software (FlowJo LCC, Ashland, OR).

Analysis of NK cell activation and degranulation

To determine activation and degranulation of NK cells within healthy donor PBMC, 20,000 sarcoma cells or 5,000 patient-derived sarcoma cells were cocultered with PBMC (E:T ratio 2.5:1) in the presence or absence (untreated) of treatment (1 µg/mL). For analysis of degranulation, Brefeldin A (GolgiPlug, BD Biosciences) was added into the coculture. Cells were harvested after 4 h and stained for CD107a expression followed by flow cytometric analysis. After 24 h, cells were harvested and stained for CD69 expression followed by flow cytometric analysis. Analysis of CD25 expression was performed after 72 h by FACS analysis. NK cells were selected as CD3$^-$ CD56$^+$ cells within PBMC.

Analysis of cytokine expression and secretion

For analysis of cytokine secretion, healthy donor PBMC were cultured with 20,000 sarcoma cells or 5,000 patient-derived

sarcoma cells (E:T ratio 2.5:1) with or without (untreated) the indicated mAbs (1 µg/mL each). After 24 h, coculture supernatants were analyzed for secretion of Granzyme A, Granzyme B, Perforin, Granulysin, sFasL, TNF, IL-2, IFNγ, IL-4 and IL-10 by Legendplex assays (BioLegend) according to the manufacturers protocol. For analysis of intracellular cytokine expression, PBMC were handled as described above and cultured for 4 h in the presence of Brefeldin A (GolgiPlug, BD Biosciences) and Monensin (GolgiStop, BD Biosciences). After incubation, cells were stained for flow cytometry-based analysis. NK cells were selected as $CD3^-$ $CD56^+$ cells within PBMC.

Analysis of target cell lysis

Cytotoxicity of PBMC against sarcoma cells was analyzed by BATDA Europium assays after 2 hours as described previously (38). Percentage of specific lysis was calculated as follows: 100 × [(experimental release) – (spontaneous release)]/[(maximum release) – (spontaneous release)].

To perform long-term cytotoxicity analyses, the IncuCyte® S3 Live-Cell Analysis System (Essenbioscience, Sartorius, Göttingen) was used. Sarcoma cells were seeded in 96-well plates and cocultured with PBMC of healthy donors (E:T ratio 5:1) with or without the indicated mAbs (1 µg/mL each). To determine the confluence of sarcoma cells, images were taken with 10x magnification every 4 h. To quantify living cells, confluences were normalized to the respective measurement at T=0 h. Cell confluence at T=0 h was set to 100%.

Statistical analysis

Data are represented as mean ± standard deviation of replicates or individual values. Statistical analyses were performed utilizing the GraphPad Prism software (version 9). Significant differences were calculated using the Student's t tests, one-way ANOVA, nonparametric Mann–Whitney test, or log-rank test. P values are represented as: $*p < 0.05$.

Results

B7-H3 is expressed in bone and soft-tissue sarcoma independent of subtype

So far, B7-H3 expression has been reported for many solid tumors (39), but little is known regarding its expression in the multiple subtypes of bone and soft-tissue sarcoma. As a first step, B7-H3 mRNA expression level data sets derived from TCGA of tumor and corresponding normal tissues were analyzed for relative B7-H3 expression. Analysis included data sets for 275/349 (tumor/normal tissue) colon adenocarcinoma, 286/60 kidney renal papillary cell carcinoma, 179/171 pancreatic adenocarcinoma, 486/338 lung squamous cell carcinoma, and 262/2 sarcoma. Compared with RNA expression in normal tissues, the expression of B7-H3 was profoundly increased in all analyzed data sets (Figure 1A). Based on these findings, we analyzed expression of B7-H3 mRNA in rhabdomyosarcoma (RD-ES), osteosarcoma (SaOs), liposarcoma (SW872), synovial sarcoma (SW982) and chondrosarcoma (SW1353) cells. Ubiquitous expression of B7-H3 with varying expression intensity in all tested soft-tissue and bone sarcoma cell lines was observed (Figures 1B, C). As a next step, we studied surface expression of B7-H3 on sarcoma cells. FACS based analysis of different sarcoma cell lines and patient-derived sarcoma cells derived from multiple subtypes revealed a varying extent of B7-H3 surface expression (Figures 1D, E). B7-H3 molecule counts were found to range between 2,960 (SaOs) and 23,797 (SW872) (Figure 1F).

Generation and characterization of Fc wildtype and Fc-optimized B7-H3 mAbs

From a panel of mAbs directed to B7-H3 (described in patent application EP3822288A1), a humanized mAb with suitable binding characteristics (clone 8H8) was selected for generation of our B7-H3-targeting mAbs with either a human IgG1 wildtype Fc part (8H8_WT) or a human IgG1 part, which contains the amino-acid substitutions S239D/I332E (8H8_SDIE) described to increase the affinity to the Fc receptor CD16, which mediates ADCC (Figure 2A). As controls served wildtype and Fc optimized mAbs with non-relevant target specificity termed Iso_WT and Iso_SDIE (40). The mAbs were then produced as described in the material and methods section. To biochemically characterize the produced mAbs, SDS-PAGE and gel filtration was conducted and revealed the expected molecular weights for LC, HC, and full mAb, for both 8H8_WT and 8H8_SDIE, and confirmed the lack of aggregates (Figure 2B). Next, dose titration experiments (range 3-30,000 ng/ml) with the five different sarcoma cell lines were performed using flow cytometry. We observed saturated binding of both 8H8_WT and 8H8_SDIE at concentrations of about 1000 ng/ml. Specificity and affinity of the B7-H3 mAbs was not affected by the Fc optimization (Figure 2C), and the concentration of 1000 ng/ml was used for further analyses. Binding of 8H8_WT and 8H8_SDIE to sarcoma cells was confirmed with samples derived from patients with sarcomas of various subtypes (Figure 2D). Next, we analyzed the binding of both mAbs to CD16 on NK cells within resting PBMC. 8H8_WT bound to NK cells, while 8H8_SDIE displayed substantially more pronounced binding (Figure 2E; Supplementary Figures 1, 2).

Binding of an antibody to its target molecule often results in a dose-dependent modulation of target antigen expression. This in turn impairs therapeutic efficacy (41). Therefore, we analyzed the antigen shift induced by our 8H8_SDIE on sarcoma cells.

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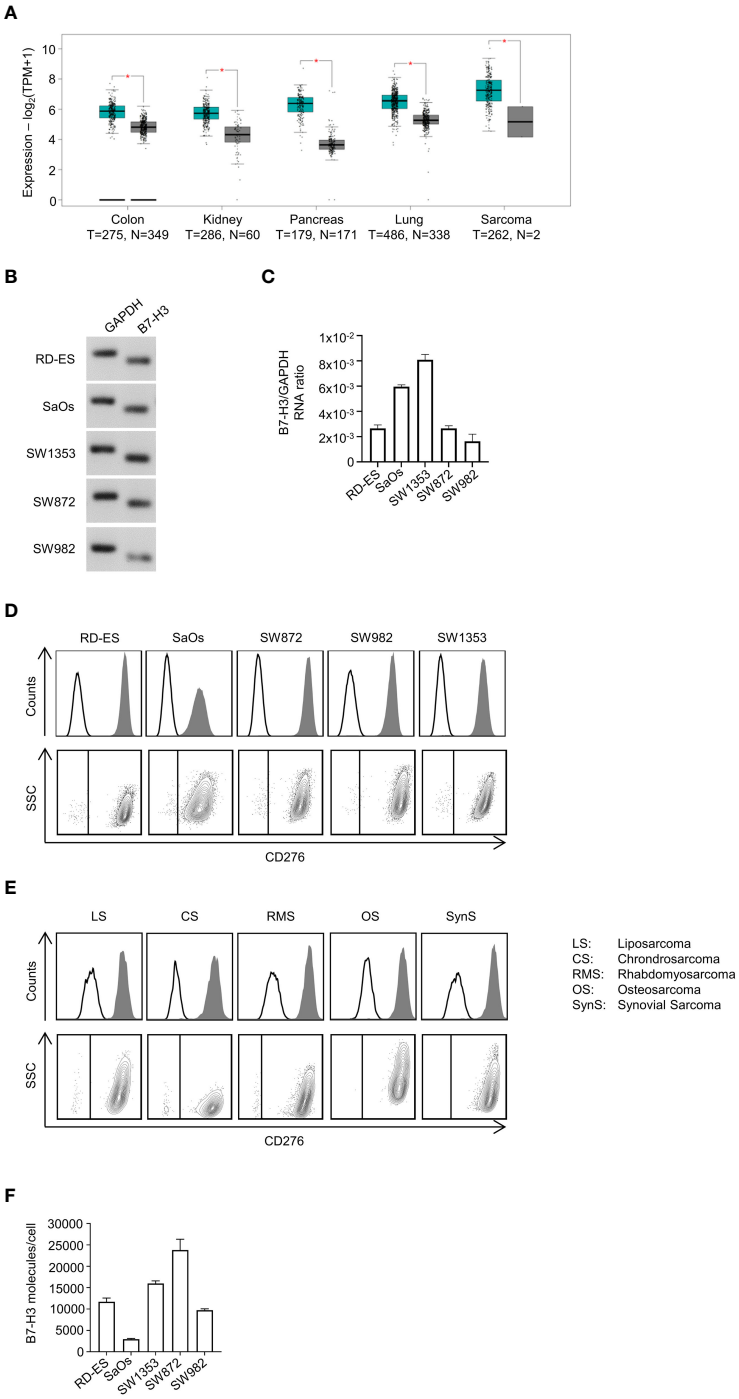

FIGURE 1
Characterization of B7-H3 expression in sarcoma cell lines and patient-derived sarcoma cells. **(A)** Relative mRNA expression of B7-H3 in indicated tumor and corresponding normal tissues was analyzed using the online web server GEPIA. T, tumor tissue; N, normal tissue **(B)** B7-H3 mRNA expression of sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 was determined *via* RT-PCR with GAPDH serving as control. PCR products were visualized by agarose gel electrophoresis. **(C)** mRNA expression analysis of B7-H3 mRNA relative to GAPDH mRNA in five different sarcoma cell lines. Results for n=3 experiments are shown. **(D, E)** Surface B7-H3 expression on the indicated sarcoma cells was analyzed by flow cytometry using mAb against B7-H3 (shaded peaks) and corresponding isotype control (open peaks). Exemplary histograms (upper panels) and dot plots (lower panels) from one representative experiment of a total of three with similar results are shown. B7-H3 expression of **(D)** sarcoma cell lines and **(E)** patient-derived sarcoma cells of different subtypes dissociated from primary tumor samples are shown, respectively. **(F)** B7-H3 molecule counts on sarcoma cell lines were determined by FACS. Results for two independent experiments are shown.

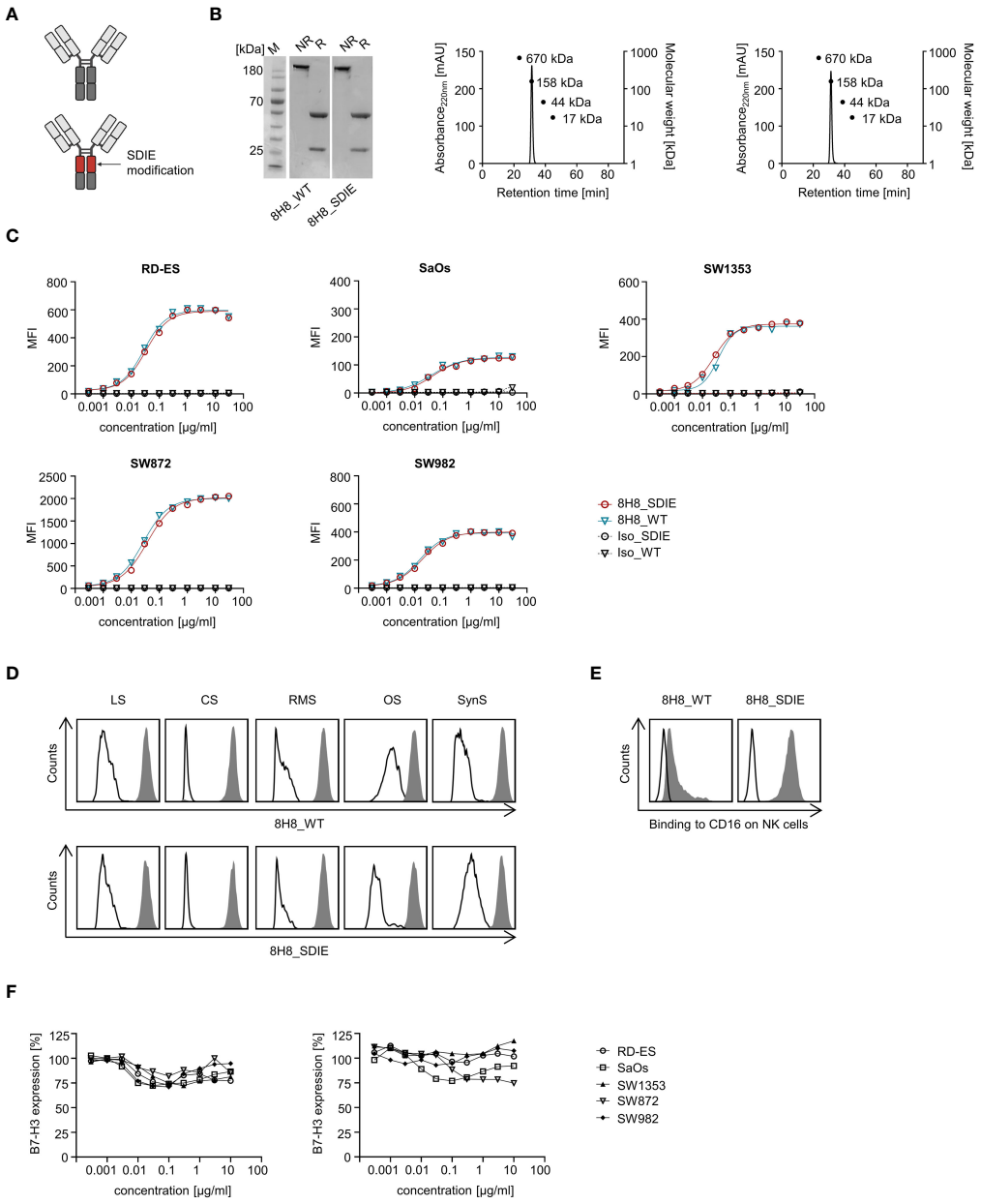

FIGURE 2
Generation and binding characteristics of B7-H3 specific antibodies. **(A)** Schematic illustration of the generated B7-H3 specific antibodies, either with wildtype Fc part (8H8_WT) (top) or Fc optimized part to enhance affinity to CD16 (8H8_SDIE) (bottom). Created with BioRender.com. **(B)** Exemplary results of an SDS PAGE (left panel) for both B7-H3 antibodies and of size exclusion chromatography for 8H8_WT (middle panel) and 8H8_SDIE (right panel) **(C)** Sarcoma cell lines were incubated with the indicated concentrations of 8H8_WT, 8H8_SDIE or the corresponding isotype controls followed by an anti-human PE conjugate and analyzed by flow cytometry. Exemplary data for mean fluorescence intensity (MFI) levels from one representative experiment of a total of three with similar results are shown. **(D)** Binding of 8H8_WT and 8H8_SDIE or the corresponding controls (1 µg/ml) to the surface of patient-derived sarcoma cells was analyzed by flow cytometry using the 8H8 antibodies (shaded peaks) and the corresponding isotype controls (open peaks), respectively. **(E)** Specific binding of 8H8 mAbs to CD16 on NK cells was analyzed by flow cytometry using NK cells ($CD3^-$ $CD56^+$) within healthy donor PBMC incubated without (open peaks) or with the 8H8 mAbs (shaded peaks) followed by an anti-human PE conjugate. **(F)** Sarcoma cells RD-ES, SaOs, SW1353, SW872 and SW982 were incubated with indicated concentrations of 8H8_WT, 8H8_SDIE or the corresponding controls for 24 h (left panel) or 72 h (right panel), respectively. Then, cells were washed and reincubated with 1 µg/ml of 8H8_SDIE, followed by an anti-human PE conjugate (1:100) and then analyzed by flow cytometry. Relative surface expression of B7-H3 was calculated by defining the mean fluorescence intensity of cells preincubated without antibody as 100%. Exemplary data from one representative experiment of a total of three are shown.

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Upon exposure to different concentrations of 8H8_SDIE (range 3–10 000 ng/ml) for 24 h or 72 h, only marginal reduction of B7-H3 on the cell surface was observed (Figure 2F).

B7-H3-targeting mAbs induce NK cell reactivity against sarcoma cells

Next, we determined whether and how our B7-H3-targeting mAbs induce NK cell anti-sarcoma reactivity. For these studies, healthy donor PBMC bearing NK cells as immune effector cells were cultivated with sarcoma cell lines with or without 8H8_WT, 8H8_SDIE or the corresponding isotype controls. Analysis of NK cells within PBMC for CD69 expression after 24 h by flow cytometry revealed that 8H8_WT already enhanced NK cell activation, and NK cell activation was further significantly enhanced upon treatment with 8H8_SDIE for all sarcoma cell lines. The control mAbs with nonrelevant target specificity had no significant effect (Figure 3A). Since $CD25^+$ NK cells exhibit a higher proliferative activity (42), we analyzed induction of the activation marker CD25 on NK cells. CD25 expression was significantly increased upon incubation of PBMC with all sarcoma cell lines for 72 h with 8H8_SDIE, but not with 8H8_WT for cocultures with all cell lines or controls (Figure 3B). CD107a serves as surrogate marker for degranulation of NK cells. Flow cytometry analysis of CD107a expression revealed that presence of 8H8_WT in cocultures with sarcoma cells RD-ES, SaOs and SW1353 already significantly enhanced CD107a expression, and a significantly more pronounced effect was observed for all cell lines upon treatment with 8H8_SDIE, whereas control mAbs had no relevant effect (Figure 3C). Analysis of IFNγ secretion into culture supernatants of PBMC and sarcoma cell lines by Legendplex assays showed an increase in cytokine release after treatment with 8H8_WT with a significantly higher effect upon incubation with 8H8_SDIE (Figure 4A). IFNγ is a cytokine that mediates various immunomodulatory effects including direct anti-tumor effects, but also enables NK cells to shape subsequent adaptive immune responses (43). Analysis by intracellular flow cytometry confirmed that induction of IFNγ was significantly increased in NK cells upon treatment with both 8H8_WT and 8H8_SDIE, with again superior effects of 8H8_SDIE (Figure 4B). Finally, we analyzed the release of effector and immunomodulatory molecules mediating NK cell effector functions in supernatants after coculturing PBMC and sarcoma cells using Legendplex assays. The presence of 8H8_WT already enhanced levels of analyzed molecules, whereas a clear tendency for enhanced levels of Granzyme B, Perforin, Granulysin, sFasL and IFNγ was observed for 8H8_SDIE, whereas levels of Granzyme A, TNF, IL-4 and IL-10 were significantly increased (Figure 4C).

Induction of target cell lysis by B7-H3-targeting mAbs 8H8_SDIE and 8H8_WT

Next, we investigated whether the enhanced NK cell activity was mirrored in analyses of cytotoxicity. To this end, we determined the capacity of 8H8_SDIE and 8H8_WT to induce target cell lysis by NK cells. Cocultures of bone and soft tissue sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 with healthy donor PBMC revealed that 8H8_WT enhanced target cell lysis in short-term cytotoxicity assays. Sarcoma cell lysis induced by 8H8_SDIE was clearly superior to 8H8_WT for all different sarcoma cells lines, whereas presence of control mAbs had no effect on target cell lysis (Figure 5A). In line, analysis in long-term FACS based lysis assays over 72 h showed pronounced efficacy of 8H8_SDIE against sarcoma cells (Figure 5B) compared to 8H8_WT. Despite the heterogenous morphology and growth rates of the different sarcoma cell lines, the superior capacity of 8H8_SDIE to induce target cell lysis compared to 8H8_WT was additionally confirmed in extended analyses of sarcoma cell lysis observed for 120 h by live cell imaging (Figure 5C, Supplementary Figure 3). Of note, 8H8_WT also showed a clear tendency to induce NK cell reactivity against sarcoma targets, but upon treatment with 8H8_SDIE, profound sarcoma cell lysis was observed with all five cell lines displaying varying levels of B7-H3 on the cell surface.

B7-H3-targeting mAbs induce NK reactivity against patient-derived sarcoma cells

Finally, we investigated the efficacy of our B7-H3-targeting mAbs in comparative analyses of 8H8_SDIE versus 8H8_WT and respective controls to induce NK reactivity against sarcoma cells derived from patients diagnosed with liposarcoma, chondrosarcoma, rhabdomyosarcoma, osteosarcoma or synovial sarcoma. Cocultures of healthy donor PBMC containing NK cells as effector cells with B7-H3 expressing patient-derived sarcoma cells revealed, alike our flow cytometric analyses with sarcoma cell lines, that treatment with 8H8_WT already enhanced NK cell activation and degranulation (Figures 6A, B). Treatment with 8H8_SDIE caused a pronounced and significant increase in activation and degranulation as compared to 8H8_WT, whereas presence of control antibodies Iso_WT and Iso_SDIE had no relevant effect. In line, the findings for 8H8_SDIE on NK cell activation and degranulation resulted in potent induction of ADCC and ultimately tumor cell lysis. Short-term cytotoxicity assays confirmed that treatment with 8H8_WT and 8H8_SDIE induced a clearly target-antigen restricted lysis, whereas 8H8_SDIE induced superior killing as observed with all tested

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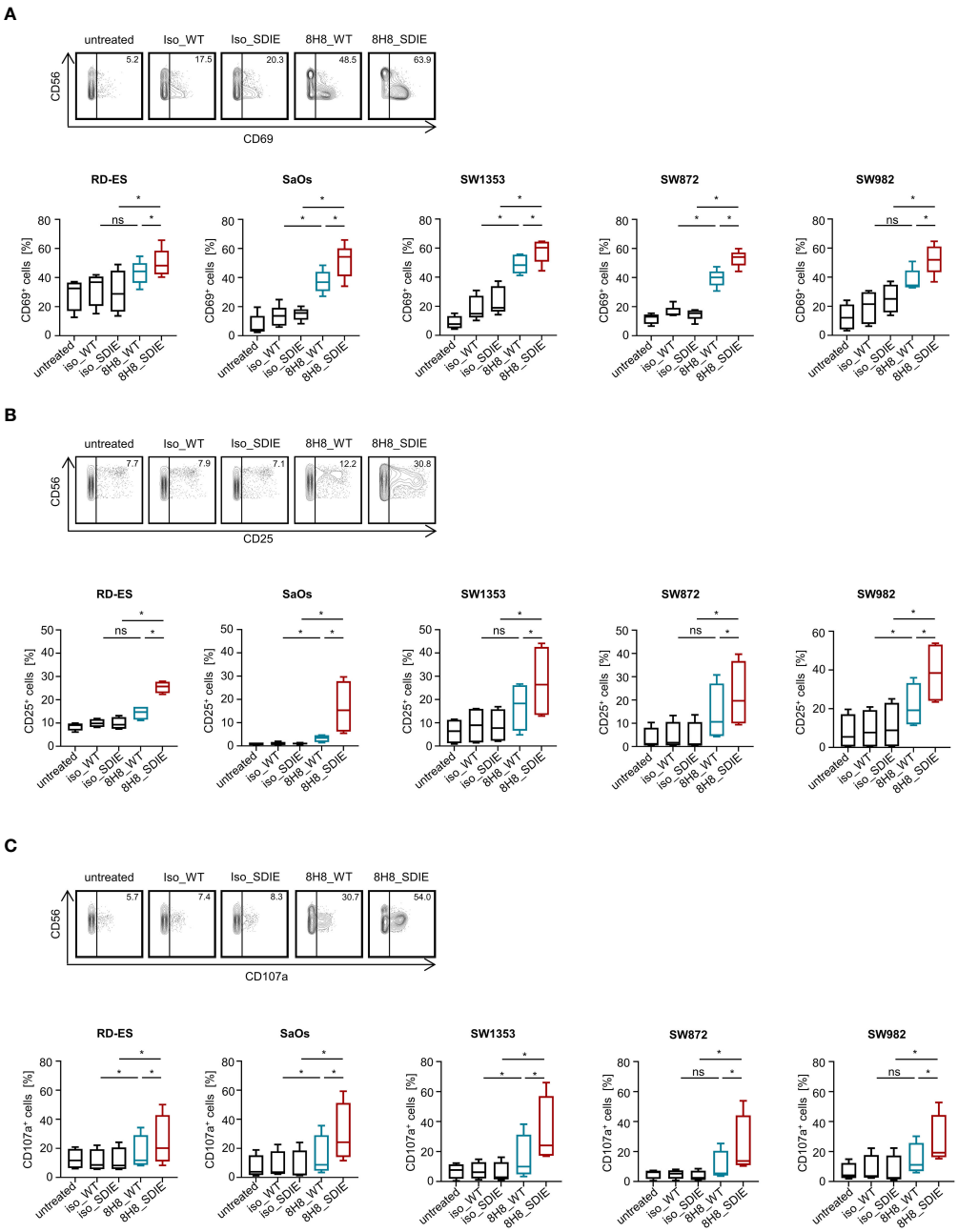

FIGURE 3
Induction of NK cell reactivity by B7-H3 antibodies against sarcoma cells. PBMC of healthy donors were cultured with or without sarcoma cells at an E:T ratio of 2.5:1 in the presence or absence of B7-H3 antibodies or the corresponding isotype controls (1 µg/mL). **(A)** Activation of NK cells was determined by expression of CD69 after 24h. In the upper panels, exemplary flow cytometry results obtained with SaOs and in the lower panels data for sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 (n=5) with PBMC of 4 different donors are shown. **(B)** Activation of NK cells was determined by expression of CD25 after 72h. In the upper panels exemplary flow cytometry results obtained with RD-ES and in the lower panels data with sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 with PBMC of 4 different donors are shown. **(C)** Degranulation of NK cells was determined by expression of CD107a after 4h. In the upper panels exemplary flow cytometry results obtained with SW872 and in the lower panels data with sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 with PBMC of four independent donors are shown. ns, not significant; *statistically significant differences (p-value < 0.05).

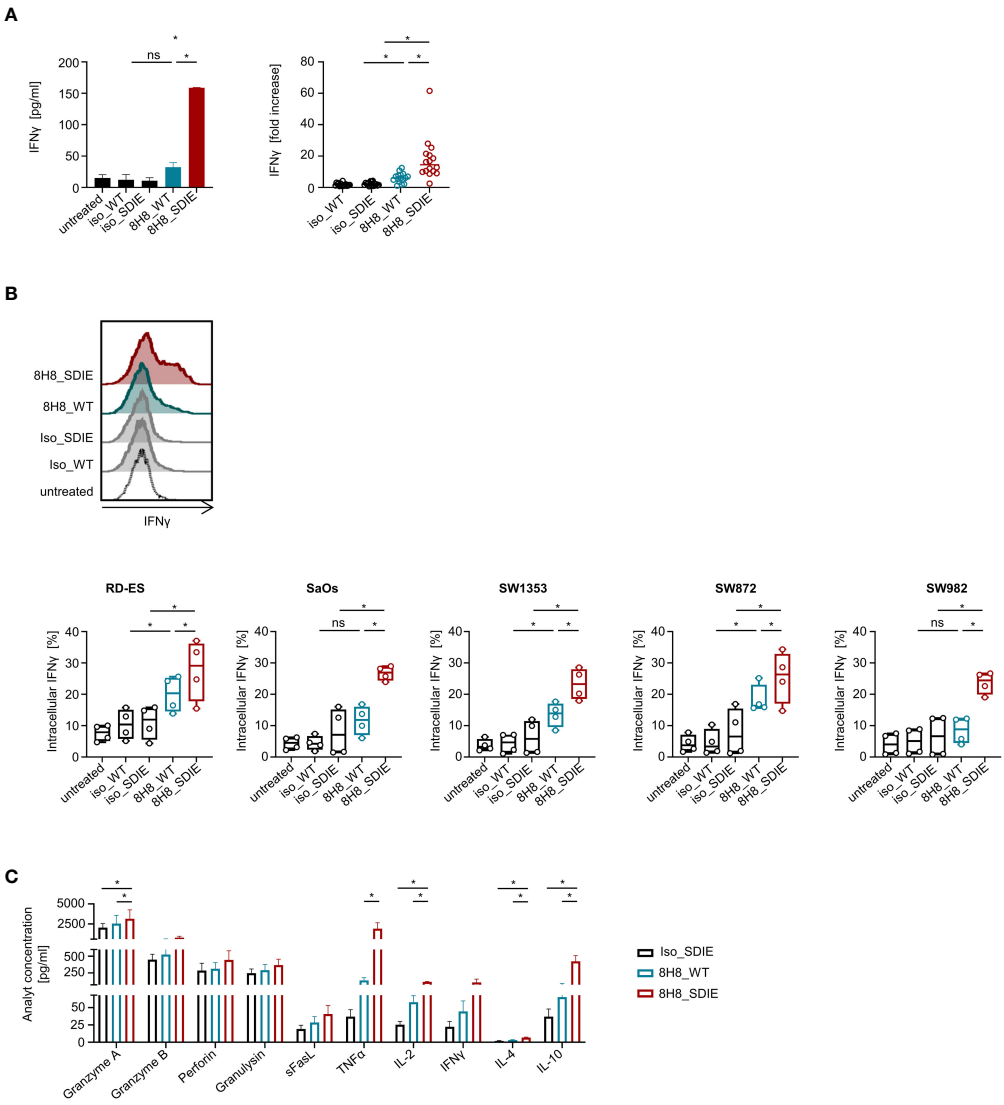

FIGURE 4
Induction of immunoregulatory molecules by B7-H3 antibodies against sarcoma cells. Healthy donor PBMC were cultured with sarcoma cells at an E:T ratio of 2.5:1 in the presence or absence of 8H8_WT, 8H8_SDIE or the corresponding isotype controls (1 µg/mL) **(A,B)** Supernatants and NK cells within PBMC were analyzed for IFNγ. **(A)** Release of IFNγ after 24 h was analyzed in supernatants of cocultures by Legendplex assays. In the left panel, exemplary results with SW1353 are shown, in the right panel results obtained with sarcoma cell lines RD-ES, SaOs, SW1353 and SW872 and with PBMC of four independent donors are shown. **(B)** Intracellular expression of IFNγ in NK cells within PBMC identified by counterstaining with $CD3^-$ $CD56^+$ was analyzed after 4 h by flow cytometry. In the upper panel, exemplary results obtained with SaOs and in the lower panel data obtained with sarcoma cells (RD-ES, SaOs, SW1353, SW872 and SW982) and with PBMC of four independent donors are shown. **(C)** Supernatants were analyzed for effector molecules Granzyme A, Granzyme B, Perforin, Granulysin and sFasL and for release of immunoregulatory molecules TNFα, IL-2, IFNγ, IL-4, IL-10 after 4 h by Legendplex assays. Shown are pooled results with sarcoma cell lines SW1353 and SW872 and with PBMC of two independent donors. ns, not significant; *statistically significant differences (p-value < 0.05).

patient-derived sarcoma cells of different origin (Figure 6C). Likewise, B7-H3 mAB induced profoundly higher and long-lasting lysis of sarcoma cells. Hence, 8H8_SDIE is able to potently elicit NK cell immunity against primary sarcoma cells regardless of their subtype.

Discussion

Therapeutic modalities for sarcoma patients have improved in recent years, yet treatment and especially cure of sarcomas remains a challenge. So far, FDA-approved antibody-based approaches in

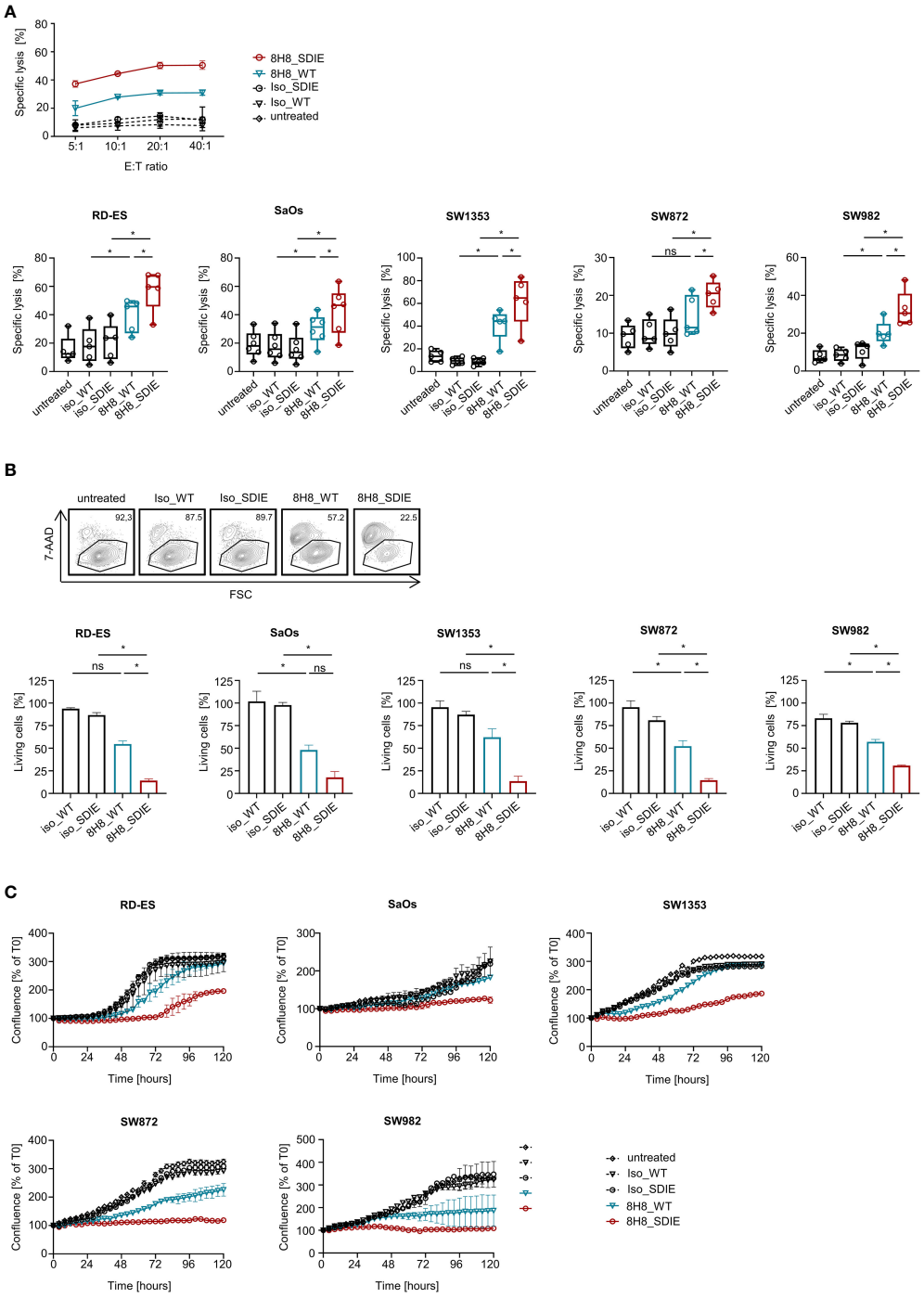

FIGURE 5
Induction of target cell lysis by Fc optimized B7-H3 antibody. PBMC of healthy donors were incubated with different sarcoma cell lines (n = 5) and treated without or with indicated B7-H3 antibodies or corresponding isotype controls (1 µg/mL). **(A)** Lysis of sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 (n=5) was analyzed by 2 h Europium cytotoxicity assays. In the top panel, exemplary data obtained with SW982 and one PBMC donor with different E:T ratios and in the bottom panel, pooled data for each cell line obtained with PBMC of healthy donors (n=5) at an E:T ratio of 20:1 are shown. **(B)** Lysis of sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 (n=5) was determined after 72 h by flow cytometry-based lysis assays at an E:T ratio of 10:1. In the top panel exemplary dot plots with SW872 and one PBMC donor are shown, the bottom panel depicts combined results for each cell line with PBMC (n=5) of healthy donors. **(C)** Cell death of sarcoma cells was determined using a live cell imaging system. Sarcoma cell lines RD-ES, SaOs, SW1353, SW872 and SW982 were incubated with PBMC of two healthy donors at an E:T ratio of 10:1 for 120h. T=0 h corresponds to a confluence of 100%. Results are shown as mean ± SD. ns, not significant; *statistically significant differences (p-value < 0.05).

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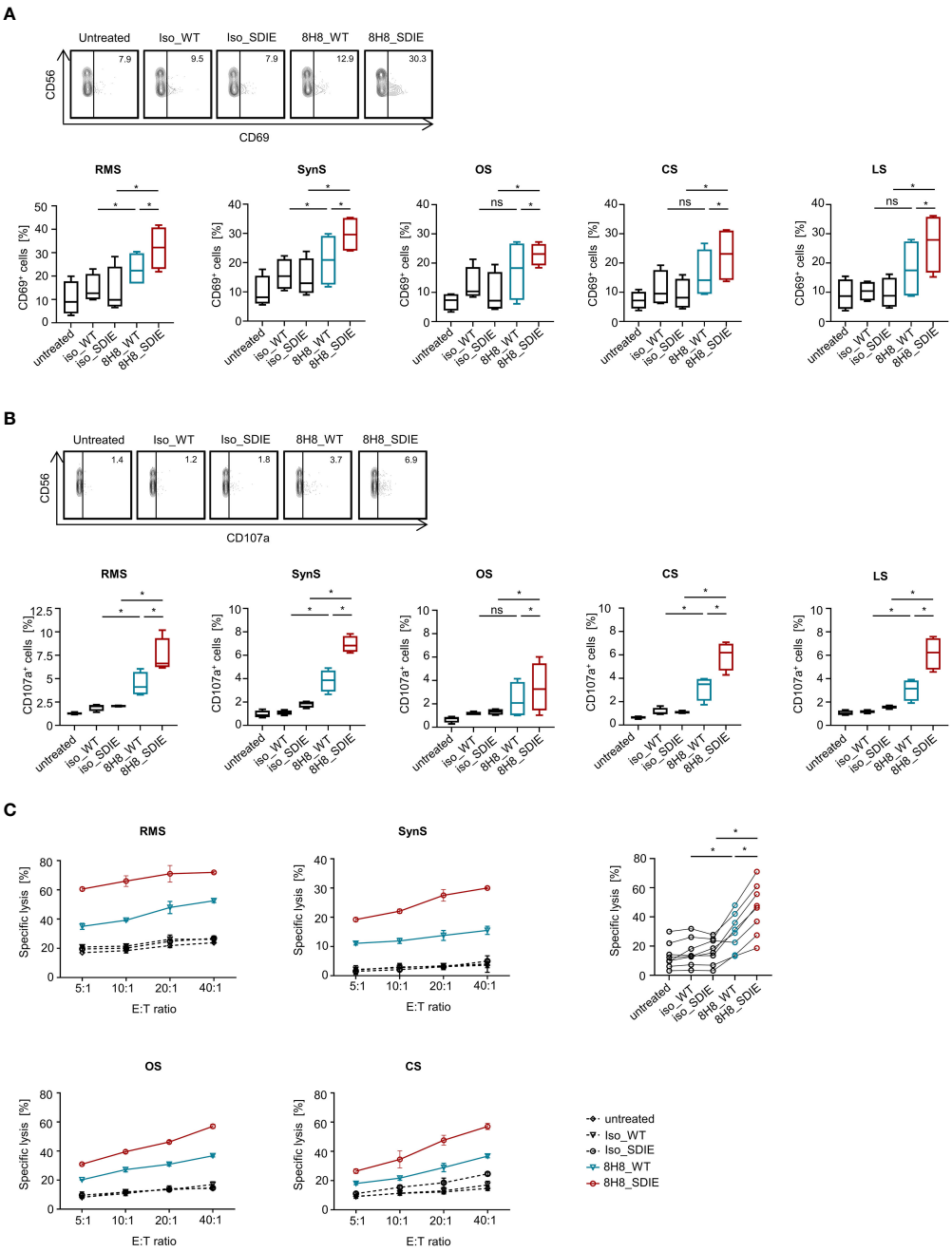

FIGURE 6
8H8_SDIE induces NK cell reactivity and cytotoxicity against patient-derived sarcoma cells. PBMC of healthy donors were incubated with patient-derived sarcoma cells of different subtypes and treated with the indicated B7-H3 antibodies or the corresponding isotype controls (1 µg/mL). **(A)** Activation of NK cells was determined by expression of CD69 after 24h. In the top panels, exemplary flow cytometry results obtained with patient-derived synovialsarcoma cells and one PBMC donor and in the bottom panel, combined data with patient-derived sarcoma cells (n=5) and with PBMC of four different donors (n=4) are shown. **(B)** Degranulation of NK cells was determined by expression of CD107a after 4h. In the upper panels, exemplary flow cytometry results obtained with patient-derived rhabdomyosarcoma cells and one PBMC donor and in the lower panel, data with patient-derived sarcoma cells (n=5) and with PBMC of four independent donors (n=4) are shown. **(C)** Killing of patient-derived sarcoma cells (n=4) was analyzed by 2 h Europium cytotoxicity assays. In the left panels, exemplary data obtained with PBMC of one healthy donor and patient-derived rhabdomyosarcoma, osteosarcoma, synovialsarcoma and chondrosarcoma cells as indicated with different E:T ratios and on the right, pooled data obtained with PBMC of healthy donors (n=2) and patient-derived sarcoma cells (n=4) at an E:T ratio of 20:1 are shown. ns, not significant; *statistically significant differences (p-value < 0.05).

sarcoma like PD-1/PDL-1 checkpoint inhibition (Dostarlimab and Pembrolizumab) or Denosumab for treatment of a subset of bone sarcomas are restricted to a minority of patients (8). Accordingly, new therapeutic concepts are urgently needed. Interestingly, sarcomas were already treated with Coley's toxins about 100 years ago, the latter in retrospective being a precursor of modern immunotherapy (44).

In the presented study, we report the preclinical characterization of an Fc-optimized B7-H3-targeting mAb termed 8H8_SDIE for treatment of sarcoma. Sarcoma cell lines of different origin as well as primary sarcoma cells from patients diagnosed with soft-tissue and bone sarcomas were identified to express substantial quantities of B7-H3. Our optimized mAb 8H8_SDIE showed optimal binding characteristics with all sarcoma cells and NK effector cells. No relevant downregulation of B7-H3 expression (antigen shift) that can impair efficacy of mAb treatment (41) was observed upon incubation with 8H8 mAbs. The anti-tumor activity induced by 8H8_SDIE against sarcomas was superior to its counterpart with a wildtype Fc part, and this was confirmed in multiple experimental settings using sarcoma cell lines as well as patient-derived sarcoma cells.

An important subset of cytotoxic lymphocytes are NK cells, which largely contribute to cancer immune surveillance. Their efficacy is based on their ability not only to mediate direct cytotoxicity, but also to influence subsequent immune responses of the adaptive immune system. Accordingly, numerous attempts are currently aiming at using NK cells for treatment of cancer (45, 46). Application of antitumor antibodies which induce ADCC represents a promising therapeutic approach for many cancers, as demonstrated for example by the clinical success of rituximab. The effect of the latter mAb is mainly based on induction of ADCC. Meanwhile, rituximab is well established for treatment of various B cell malignancies (47). To further enhance ADCC induced by therapeutically utilized antibodies and thus to increase efficacy, several strategies currently aim at generation of improved antitumor mAbs using the approach of Fc-optimization to increase affinity for CD16. Besides modifying glycosylation motifs (15), increased affinity to CD16 can also be attained by changing the amino acid sequence in the CH2 domain of the Fc part for example by the S239D/I332E substitutions (SDIE modification) (14) that is also contained in our B7-H3-targeting mAb 8H8_SDIE. The Fc optimization resulted in a significant increase in NK-mediated ADCC against sarcoma cells as compared to 8H8_WT that contains a wildtype Fc part. At present, many Fc-optimized mAbs that comprise the SDIE modification successfully undergo clinical evaluation, for example FLYSYN (anti-FLT3; NCT02789254), margetuximab (anti-HER2; NCT01828021), BI 836858 (anti-CD33; NCT02240706, NCT03013998), and MEN1112 (anti-CD157; NCT02353143) or FDA approved tafasitamab (anti-CD19).

In previous studies, we evaluated various mAbs and fusion proteins containing the SDIE modification for improved induction of ADCC, e.g. in leukemia, colorectal cancer, breast cancer as well as sarcoma, some of them until the stage of clinical application (34, 35, 38, 40, 48–51). Here we set out to develop an B7-H3 directed Fc-optimized mAb for treatment of sarcomas based on the reasoning that B7-H3 is reportedly overexpressed in many solid tumors (23, 25), whereas expression in healthy tissues is limited (39, 52), and that sarcomas are NK cell-sensitive cancer types (45). Our efforts were further prompted by our observation that B7-H3 is overexpressed in bone and soft tissue sarcomas independently of subtype, as tumor associated antigens with homogenous overexpression are a prerequisite for success of immunotherapeutic treatment. Of note, in some recent reports the B7-H3 protein has been characterized as a checkpoint molecule that exerts immunosuppressive and tumor promoting activity (53, 54). There is also first evidence that the B7-H3 positive cell fraction within cancer cells potentially represents cancer stem cells (55–57). Based on these findings, it is not surprising that multiple immunotherapeutic approaches directed against B7-H3 are currently under clinical investigation. This includes, but is not limited to strategies like antibody-drug conjugates (MGC018: NCT03729596; DS7300a: NCT04145622), Fc-optimized mAbs (MGA271, enoblituzumab: NCT02923180, NCT02475213, NCT04634825; DS-5573a: NCT02192567, clinical trial terminated), radiolabeled mAbs (^{131}I-8H9: NCT03275402, NCT04022213; ^{177}Lu-DPTA omburtamab: NCT04315246, NCT04167618), and bispecific antibodies (MGD009: NCT026285351, clinical trial terminated) (58). Our work expands this armamentarium to Fc-optimized mAbs for potent induction of NK cell ADCC.

Regarding toxicity/side effects expected by targeting B7-H3, it must be considered that B7-H3 is not only (inducible) expressed on antigen-presenting cells (16), but basal expression is also reported for endothelial cells, resting fibroblasts, amniotic fluid stem cells and osteoblasts (52, 59). However, in preclinical studies which used B7-H3 as therapeutic target including B7-H3-targeting chimeric antigen receptor (CAR) T cells, significant anti-tumor effects in preclinical models (23, 26, 27, 60, 61), but no toxicity was observed, likely due to profoundly lower B7-H3 antigen levels in healthy tissue (27). In line, the first evaluations of B7-H3-targeting immunotherapeutics in clinical studies like anti-B7-H3 antibodies and B7-H3 CAR-T cells did not reveal any unbearable toxicity and off-tumor effects against healthy B7-H3 expressing cells (58, 62). Nevertheless, this issue requires further elucidation.

In conclusion, 8H8_SDIE showed powerful anti-sarcoma effects in a preclinical setting. Of note, our treatment approach was not restricted to a distinct sarcoma entity, as different sarcoma cells lines as well as patient-derived sarcoma cells including osteosarcoma, rhabdomyosarcoma, synovial sarcoma liposarcoma and chondrosarcoma were sensitive to treatment with our B7-H3-targeting mAbs. 8H8_SDIE could thus constitute an immunotherapeutic option for sarcoma patients. Although future studies including *in vivo* experiments are

certainly warranted to fully characterize 8H8_SDIE, the data presented in this study underscore the potential of our B7-H3-targeting Fc-optimized mAb for sarcoma treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by IRB (ethics committee of the Faculty of Medicine of the Eberhard Karls Universitaet Tuebingen and of the University Hospital Tuebingen) and was conducted in accordance with the Declaration of Helsinki; reference number 13/2007V and 612/2010BO2. The patients/participants provided their written informed consent to participate in this study.

Author contributions

IH designed and performed the experiments, analyzed and interpreted data, and wrote the manuscript. ME designed and performed experiments. TM provided the B7-H3 mAb. MM contributed to the study design and contributed to writing of the manuscript. CH provided patient samples and contributed to writing of the manuscript, GJ contributed to study design and writing of the manuscript. HS contributed to the study design, critically revised the manuscript, and co-supervised the study. LZ designed and supervised the study and contributed to writing of the manuscript. All authors approved the submitted version of the manuscript.

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Conflict of interest

GJ, HS, LZ, and TM are listed as inventors on the patent application "Antibodies targeting, and other modulators of, the CD276 antigen, and uses thereof," EP3822288A1, applicant German Cancer Research Center, Heidelberg, Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1002898/full#supplementary-material

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New insights into iNKT cells and their roles in liver diseases

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Natural killer T cells (NKTs) are an important part of the immune system. Since their discovery in the 1990s, researchers have gained deeper insights into the physiology and functions of these cells in many liver diseases. NKT cells are divided into two subsets, type I and type II. Type I NKT cells are also named iNKT cells as they express a semi-invariant T cell-receptor (TCR) α chain. As part of the innate immune system, hepatic iNKT cells interact with hepatocytes, macrophages (Kupffer cells), T cells, and dendritic cells through direct cell-to-cell contact and cytokine secretion, bridging the innate and adaptive immune systems. A better understanding of hepatic iNKT cells is necessary for finding new methods of treating liver disease including autoimmune liver diseases, alcoholic liver diseases (ALDs), non-alcoholic fatty liver diseases (NAFLDs), and liver tumors. Here we summarize how iNKT cells are activated, how they interact with other cells, and how they function in the presence of liver disease.

KEYWORDS

NKT cells, cytokine, chemokine, liver diseases, immune

Introduction

Natural killer T (NKT) cells are a group of innate immune cells first recognized in the 1990s (1). These cells feature surface receptors of both T cells and NK cells (e.g., NK1.1 in mice or CD161+/CD56+ in humans). The activation and deactivation of NKT cells are closely tied to our immune activities, such as pathogen elimination, tumor surveillance, and autoimmune responses (2–4). NKT cells can be divided into two subtypes, namely type I and type II. Type I NKT cells, usually referred to as invariant NKT (iNKT) cells, express a semi-invariant mouse Vα14-Jα18/Vβ8 or human Vα24-Jα18/Vβ11 T cell-receptor (TCR) α chain, hence the name. Type I NKT cells are able to recognize lipid antigens (such asglycosphingolipids, glycerophospholipids, lysophospholipids, and cholesterol ester) in the context of CD1d, a non-polymorphic MHC class I-like

molecule (5–8). Researchers have found that the injection of α-galactosylceramide(α-GalCer) activates type I NKT cells (9). Type II NKT cells, in contrast, express a relatively diverse range of TCR receptors, and are reactive to a self-glycolipid sulfatide (10). Studies preliminarily suggest contradictory functions for the two types of NKT cells: type I NKT cells are likely pro-inflammatory, while type II are anti-inflammatory (6, 11). Note that type II NKT cells have not been broadly studied due to a lack of distinctive surface characteristics. In this review, we mainly focus on iNKT cells, with also a few contents talking about type II NKT cells, and "NKT cells" will stand for iNKT cells unless otherwise stated.

The liver is a vital part of the human digestive system, and functions as the center of metabolism and detoxification. Though not seen as a primary immune organ, the liver is not to be neglected when we talk about immune reactions. In addition to parenchymal cells (i.e., hepatocytes), the liver also hosts non-parenchymal cells, such as liver sinusoidal endothelial cells, Kupffer cells (macrophages), lymphocytes, and stellate cells. Interestingly, the liver has the highest NKT cell/conventional T cell ratio in the body (12), suggesting that this organ might play an important role in NKT immunology. In this review, we mainly focus on two points: 1) how NKT cells are activated, and 2) how NKT cells interact with other cells (e.g. Kupffer cells, T cells, hepatocytes) in the presence of liver disease.

NKT-cell activators

Briefly, it is known that NKT cells can be activated by lipid antigens (especially α-GalCer) (13–15) cytokines (such as IL-2 (interleukin-2) and IL-18) (16–18) chemokines (including CXCR6) (19), and other substances, but the type and common characteristics of these NKT stimulators remain poorly elucidated. Here we summarize three types of molecules that lead to NKT-cell activation (Figure 1), and briefly discuss their roles in the process of liver diseases.

α-GalCer and analogues

Previous studies have shown that lipid antigens are presented to NKT cells through CD1d located on the surface of dendritic cells. Among all the lipid antigens, α-GalCer (also known as KRN7000), a synthetic component, is the first to be experimentally confirmed to potentiate NKT cells, both *in vitro* and *in vivo* (5, 13, 14).This glycolipid was discovered in an extract of the marine sponge Agelasmauritianus, and its effect on NKT in both *in vitro* and *in vivo* activation is widely reported. After α-GalCer administration, there are detectable increases in the number of NKT cells (15)and NKT-derived cytokines (14) (TNF (tumor necrosis factor), IFN-γ (interferon-γ), IL-12, etc.)

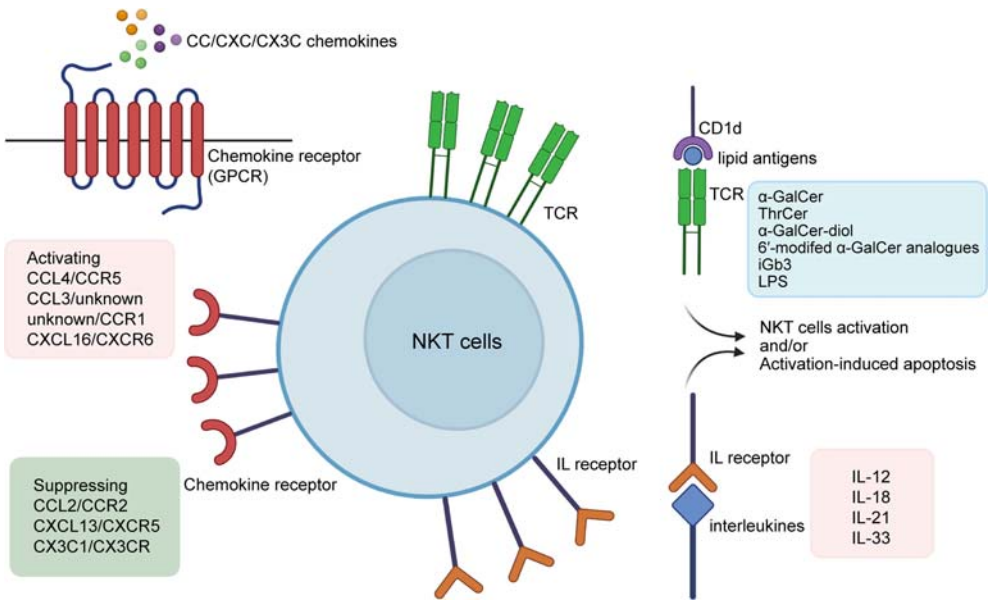

FIGURE 1
Activators of NKT cells. α-Galcer and some of its analogues (e.g.ThrCer, α-GalCer-diol, 6'-modifed α-GalCer analogues), iGb3, and LPS are among lipid antigens proven to activate NKT cells *via* the CD1d-dependent pathway; interleukins such as IL-2, IL-12, IL-15, IL-18, IL-21, and IL-33 promote NKT cells by binding directly to their interleukin receptors; CC/CXC/CX3C chemokines are associated with the recruitment and proliferation of NKT cells, with non-exclusive matches with their receptors that come in the form of GPCRs. Also, α-Galcer and some interleukins may also lead to activation-induced apoptosis of NKT cells. α-GalCer, α-galactosylceramide; ThrCer, threitolceramide; iGb3, isoglobotrihexosylceramide; LPS, lipopolysaccharide; IL, interleukin.

as well as a degranulation marker (CD107a) (20), and symptoms of experimental animals improve or worsen accordingly (21, 22). Some researchers have also proposed an α-GalCer-based therapy for infection and autoimmune diseases (23, 24), but this proposal has been met with the opposing argument that administration of α-GalCer is also likely to induce hepatocyte damage (15)and NKT-cell anergy (25).

In many types of liver diseases, administration of exogenous α-GalCer changes their pathophysiological process. For example, α-GalCer-induced NKT activation is responsible for exacerbation of ALDs (26). The anti-tumor activity of α-GalCer in the liver is also demonstrated in mice experiments (27). Interestingly, in the mouse model of CCl_4-induced acute liver injury, natural activation of NKT cells ameliorates liver damage and inflammation, possibly by suppressing HSC (hepatic stellate cell) activation, while α-GalCer-induced NKT activation accelerated acute liver injury, inflammation and fibrosis (28). Considering its dual effects on liver diseases and hepatic toxicity, further trial of α-GalCer is needed before clinical use.

Furthermore, analogues of α-GalCer are studied for their potential to activate NKT cells. In 2008, Jonathan D. Silk et al. (29)reported that threitolceramide- (ThrCer-) induced activation of NKT cells. In addition, researchers have managed to createα-GalCer analogues artificially, including α-GalCer-diol (with added hydroxyl groups in the acyl chain compared to α-GalCer, Juan Ma et al., 2020) (30)and6′-modified α-GalCer analogues (Matthias Trappeniers et al., 2008) (31). Hopefully, with careful design, these analogues will be applicable as preventative and therapeutic vaccine adjuvants (32, 33).

However, it is worth noting that not allα-GalCer analogues have the potential to activate NKT cells. For instance, in 2005, Jochen Mattner et al. (8)found that injection of α-glucuronosylceramide (PBS 30) or galacturonosylceramide (PBS 59) in mice led to the proliferation of NKT cells, but β-glucuronosylceramide (PBS 50) did not.

Other lipid antigens that activate NKT cells include glycosphingolipid isoglobotrihexosylceramide (iGb3), an endogenous antigen synthesized in the endoplasmic reticulum (ER) and Golgicomplex (34, 35), and bacteria-derived lipopolysaccharide (LPS), a ligand for Toll-like receptor 4 (TLR4) expressed on NKT cells, which corresponds with the roles that NKT cells play during exogenous infection (8, 36, 37).

Interleukins

Interleukins are a group of cytokines that are partially secreted by NKT cells, and some of them have biological effects on NKT cells. In brief, interleukins can activate NKT cells include IL-2, 12, 15, 18, 21, 27, and 33 (Table 1).

Interleukins activate NKT cells by binding to receptors on the cell surface, and many of the working mechanisms of functioning interleukins remain elusive. Activated NKT cells secrete large amounts of Th1/Th2 cytokines, which could be modulated by administration of the interleukins mentioned above, indicating that these interleukins have a profound impact on NKT-cell activation.

IL-2 is found effective for stimulating NKT cells both *in vitro* and *in vivo* in many studies focused on mice. Co-administration of IL-2, 12 and 18 results in a stronger ability of NKT cells to secrete IFN-γ (38). Small amounts of IL-2 cDNA (complementary DNA) increases the number of NKT cells *in vivo* (39), and potentiates the effect of α-GalCer (50). Also, studies found that IL-2 enhances the effect of NKT activation by α-GalCer, but administration of IL-12 alone is not enough to potentiate NKT cells (40). In addition, exogenous IL-2 and/or IL-15 partially overcome the hyporeponsiveness of iNKT cells in chronic HBV patients (51).

Murine models showed that IL-15, partly from Kupffer cells, facilitates the proliferation and maintain the homeostasis of NKT cells (42, 43). IL-15 can potentiate the α-GalCer-stimulated NKT expansion (40). Some researchers have found that IL-15-related NKT activation is associated with the NF-κB signaling pathway, but the exact mechanism remains controversial. Vallabhapurapu S et al. claimed that IL-15-related NKT activation is dependent on the NF-κB signaling pathway, because they found that RelA, a member of Rel/NF-κB family, controls IL-15 signaling by regulating IL-15Rα chain and γC chain, and deficiency of RelA blocks NKT activation by administration of IL-15 (44). However, Locatelli I et al. believed NF-κB deficiency might stimulate NKT recruitment by promoting IL-15 activity (45).Mice experiments showed that the IL-18/IL-18R

TABLE 1 Reported interleukin-induced NKT cells activation.

Interleukins	Mechanism	Effects	References
IL-2	No data found	Increased number of NKT cells and enhanced secretion of IFN-γ	(38–40)
IL-12	No data found	Enhanced Th1 responses	(41)
IL-15	NF-κB signaling	Enhanced proliferation and homeostasis	(40, 42–45)
IL-18	NF-κB signaling	Enhanced Th1 and Th2 responses	(16–18)
IL-21	Autocrine	Enhanced Th2 responses	(46, 47)
IL-27	Modulate IL-12 secretion of DCs	Enhanced maintenance and recruitment of NKT cells	(48)
IL-33	IL-33/ST2L interaction	Enhanced secretion of IFN-γ and FasL expression	(49)

(IL-18 receptor) axis functions *via* a rapid NF-κB signaling pathway, directly enhancing IL-4 production by NKT cells. Unlike IL-12,which mainly promotes Th1 response (41), IL-18 stimulates Th1 and Th2 responses simultaneously (16–18); hence the two kinds of cytokines are sometimes co-administered for their combined activation effects (52). Also, we would like to point out that continuous stimulation of IL-18 may result in impaired long-term NKT activation, which is important during clinical practice.

Intriguingly, we noticed an autocrine phenomenon with regard to IL-21, namely that not only does IL-21 enhance the survival of NKT cells, it is also secreted by NKT cells after CD3 and CD28 administration. NKT cells activated by IL-21 exhibit higher granzyme and IL-4 expression (46). Some researchers also report witnessing less IFN-γ and TNF production by NKT cells, indicating that IL-21 leads to "Th1 to Th2" cytokine transformation (47), though this conclusion needs more support. Moreover, α-Galcerhas been found to coordinate with interleukins, including IL-18 and IL-21 (17, 46).

IL-27 and IL-33 also contribute to activation of NKT cells. IL-27 modulates IL-12 secretion of dendritic cells, thus indirectly enhancing maintenance and recruitment of NKT cells (48). IL-33 binds with ST2L (the suppressor of tumorgenicity 2 ligand, and also the receptor of IL-33) on NKT cells to promote IFN-γ secretion as well as FasL expression (49).

Some researchers have studied the functions of interleukins on liver NKT cells and examined them as possible treatment methods. For example, administration of IL-18 potentiates the cytotoxicity of hepatic NKT cells in a perforin-dependent way (53). Co-administration of IL-12 and IL-18 triggers higher IFN-γ release from NKT cells than either administered alone, which demonstrates a higher efficiency for killing liver tumors (54). However, extra work is urgently needed to investigate the effects of interleukins on hepatic NKT cells and their clinical values.

Chemokines

The chemokine superfamily was first discovered in the late 1980s to play a role in inflammation. The protein superfamily consists of four groups, namely XC, CC, CXC, and CX3C, a categorization based on the discrete location of cysteine residues on the initial sequence of the molecules. Chemokine receptors are defined as a group of seven transmembrane-spanning G-protein-coupled receptors (GPCRs), having no one-on-one match with their ligands (55–57). Chemokines are tightly associated with the maturation and localization of NKT cells. The effective ligands and receptors are summarized in Table 2.

Of the four chemokine subgroups listed in Table 2, XC and CX3C have seldom been studied since their discovery; thus, there are very few articles concerning their functions on NKT cells. XC, interestingly, has not been reported so far to potentiate NKT cells. In contrast, CX3C1/CX3CR1 is considered to take part in NKT-cell trafficking within the thymus, but this function may not be of vital importance as CX3CR1-deficient mice do not show NKT-cell developmental disability (67). Also, some researchers hold opposing views on the NKT-cell-activating function of CX3C1/CX3CR1 (68). However, CX3CR1 expression on the cell surface can be utilized to define NKT subtype present in the thymus and peripheral organs (67).

Unlike XC and CX3C, the functions of the other two subgroups are known in more details. In mice models, CCL2, also known as MCP-1, recruits NKT cells to peripheral organs such as the spleen (58) and exerts an anti-inflammatory effect by interacting with CCR2 to prevent IL-4 secretion of NKT cells, which demonstrates a hepatoprotective effect in the liver (59). CCL3 and CCL4, secreted by activated dendritic cells, also attract NKT cells. This effect is accompanied by CXCR3 ligands (CXCL9-11) which derive from the same dendritic cells (61). CCL4 has also been found to induce distinct chemotaxis in different NKT subgroups, attracting CCR5-expressing cells in particular (69). Lack of CCR5 in mice promotes fulminant liver failure because of exacerbated inflammatory responses related to a higher amount of IL-4 from NKT cells that fail to go through apoptosis after activation (62), suggesting a role for CCR5 in NKT-cell regulation. Another CC chemokine receptor on NKT cells surface is CCR1, which together with CCR5 recognizes ligands that come from activated macrophages and dendritic cells (60).

The last type, CXC, is the most comprehensively studied at present, especially CXCR6 and its ligand CXCL16. In short,

TABLE 2 Different chemokines on NKT cell activation/inactivation.

Chemokine subgroups	Ligands/Receptors	Functions	References
XC	No data found	No data found	/
CC	CCL2/CCR2	Suppress NKT cells (by recruiting NKT cells to spleen and preventing IL-4 secretion)	(58–60)
	CCL3/unknown	Recruit NKT cells	(61)
	CCL4/CCR5	Recruit NKT cells; activation-induced apoptosis	(61, 62)
	Unknown/CCR1	Recruit NKT cells	(60)
CXC	CXCL16/CXCR6	Recruit NKT cells; promote IFN-γ and IL-4 secretion of NKT cells	(63–65)
	CXCL13/CXCR5	Suppress NKT cells	(66)
CX3C	CX3C1/CX3CR1	Enhance NKT cells trafficking; define NKT sublineages	(67)

CXCR6/CXCL16 functions on the distribution rather than maturation of NKT cells. Animal research shows that CXCR6 expression of NKT cells is elevated upon NKT-cell activation, but is not indispensable for NKT-cell development within the thymus, as CXCR6-challenged mice do not show reduced numbers of thymic NKT cells. However, CXCR6 is closely related to localization of NKT cells because of its interaction with CXCL16, which resides on target organs such as the spleen and liver (70). As a result, CXCR6-deficient mice possess fewer NKT cells in their livers, making them more susceptible to infection (63, 70). The CXCR6/CXCL16 reaction boosts IFN-γ and IL-4 release from NKT cells, enhancing inflammatory response (64, 65). In particular, CXCR6/CXCL16 is involved in many liver diseases. For example, CXCR6/CXCL16 expressions greatly increase during liver inflammation (71). Hepatocytes produce CXCL16 in non-alcoholic fatty liver disease (NAFLDs), which ameliorates inflammation and fibrosis (72, 73). In contrast, CXCR5, along with its ligand CXCL13, is reported to reduce NKT-cell activation (66).

The relationship between NKT cells and other cells in liver disease

In the liver, NKT cells have close connections with other cells including hepatocytes (normal liver cells), dendritic cells, macrophages (Kupffer cells), T cells, and B cells, and are able to regulate their functions during innate and acquired immune reactions. This connection is achieved through either direct contact or secretion of cytokines. Evidence shows the significance of this connection because changes in how NKT cells interact with other cells can be found in liver disease and may lead to severe dysfunction of the organ. Here we summarize the ways in which NKT cells coordinate with other liver-resident cells.

NKT cells and hepatocytes

As mentioned above, NKT cells are activated by lipid antigens through CD1d molecules which, in the liver, are expressed on macrophages, dendritic cells, and hepatocytes. CD1d then presents the antigens to the TCR on NKT cells. A decrease in CD1d on hepatocytes results in dysfunction of NKT cells (74). Some studies based on HBV transgenic mice find that during liver diseases such as HBV infection, CD1d expression is elevated on injured hepatocytes, rather than macrophages (75). Meanwhile, hepatocyte-derived IL-7 is also important in the maintenance of NKT cells, which indicates that hepatocytes play a role in the development and maintenance of the immune system (76).

NKT cells attack hepatocytes by expressing FasL, perforin, and granzymes, but their main effects on hepatocytes are achieved by producing Th1 cytokines, especially TNF-α and IFN-γ. Upon activation, NKT cells start to release more TNF-α that directly interacts with TNF receptor 1 (TNFR1)expressed on hepatocytes, on which this molecule has a dual effect, either promoting hepatocyte death or regeneration indifferent contexts (77). Increased level of NKT-derived TNF-α is responsible for exacerbation of α-GalCer-induced liver damage (26). However, in mice that underwent partial hepatectomy, TNF-α promotes regeneration of hepatocytes (78). The interactions between NKT cells and hepatocytes are also tightly associated with a wide range of liver diseases. For instance, in autoimmune liver diseases, NKT cells release death signals to hepatocytes through FasL pathway, and secrete TNF-α, perforin and granzymes in synchronization, promoting the process of autoimmune liver diseases (79). In ALDs, NKT cells also play the role of killing hepatocytes (80). In 2014, Monika Julia Wolf et al. (81)found that TNFSF14 (TNF superfamily 14, also referred to as LIGHT) secreted by NKT cells is responsible for enhanced lipid uptake of hepatocytes as well as liver damage, causing an enhanced possibility of NAFLDs in mice (Figure 2A).

IFN-γ expression is also increased in activated NKT cells. During HCV infection, IFN-γ induces liver sinusoidal endothelial cells to produce CXCL9 and CXCL10 that bind to and recruit CXCR-positive T cells. As a result, more T cells start to locate in the infected liver and negatively affect hepatocytes (82). Moreover, IFN-γ stimulates hepatocytes to express a higher number of Fas, causing liver cell apoptosis after binding with FasL on NKT cells (Figure 2B) (83).

NKT cells and B cells

The interactions between NKT cells and B cells mainly lead to strengthened capacity of B cells. Animal research suggested that CD1d loaded with lipid antigens from B cells surface is a source for NKT-cell activation (84, 85). In return, NKT cells offer helper signals for B cells by expression of CD40L and CD28, which bind to CD40 and CD80/86, respectively, on B cells (86). Secretion of IL-21 (87) and IFN-γ (86) also play a role in B-cell activation. In addition, NKT cells can indirectly enhance B cells by communicating with dendritic and CD4+ T cells (84, 85, 88). Interestingly, stimulation of NKT cells also leads to recruitment of regulatory B cells to the liver that suppress inflammation (Figure 2C) (89). However, up to now, it remains poorly studied how the interactions between NKT and B cells contribute to the pathogenesis of hepatic diseases.

NKT cells and macrophages (Kupffer cells)

The interaction between NKT cells and macrophages is relatively complex as it involves multiple surface and secreted molecules. As one of the main APCs in the liver, macrophages

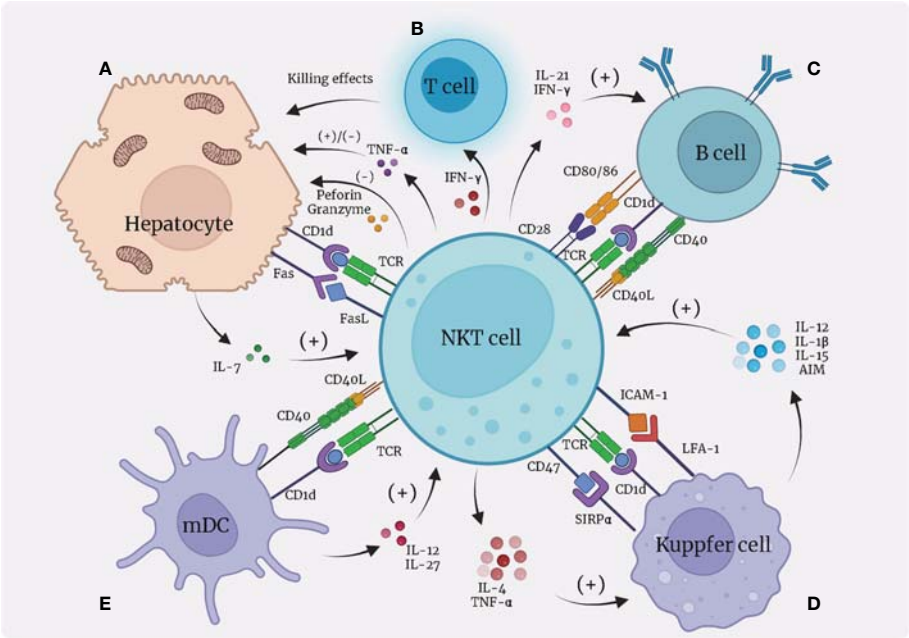

FIGURE 2
Interactions between NKT cells and hepatocytes, dendritic cells, Kupffer cells, and B cells. **(A)** Hepatocytes present lipid antigens to TCR on NKT cells *via* the CD1d-dependent pathway; hepatocytes release IL-7 to activate NKT cells; NKT cells secrete TNF-α that has dual functions on hepatocytes; NKT cells express FasL and induce Fas expression on hepatocytes, leading to apoptosis of hepatocytes. **(B)** NKT cells secrete IFN-γ to recruit T cells to kill hepatocytes. **(C)** B cells present lipid antigens to NKT cells through CD1d; NKT cells and B cells contact each other directly through CD40L/CD40 and CD28/CD80, 86; NKT cells secrete IL-21 and IFN-γ to promote B cells. **(D)** Kupffer cells present lipid antigens to TCR on NKT cells *via* the CD1d-dependent pathway; Kupffer cells express LFA-1 and SIRPα, which bind with ICAM-1 and CD47, respectively, on NKT cells to activate NKT cells; Kupffer cells secrete IL-12, IL-1β, IL-15, and AIM to recruit and promote NKT cells; NKT cells in return produce pro-inflammatory IL-4 and IFN-γ to function on Kupffer cells. Notably, over-stimulation of NKT cells by Kupffer cells leads to apoptosis of NKT cells. **(E)** Dendritic cells (especially myeloid dendritic cells, mDC) present lipid antigens *via* CD1d towards NKT cells to activate NKT cells; dendritic cells secrete IL-27 and IL-12 to activate NKT cells; NKT cells express CD40L to bind with CD40L and reciprocally benefit dendritic cells. mDC, myeloid dendritic cells; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; SIRP, signal regulatory protein; LFA, lymphocyte function-associated antigen; ICAM, intercellular adhesion molecule; AIM, apoptosis inhibitor expressed by macrophages.

(Kupffer cells) connect with NKT cells in a CD1d-restricted manner. The CD1d molecule located on the surface of Kupffer cells presents exogenous lipid antigens to TCR on NKT cells, leading to NKT-cell activation (90).

Another pivotal means of Kupffer/NKT interaction is through the LFA-1/ICAM-1 pathway. LFA-1 (lymphocyte function-associated antigen 1) and ICAM-1 (intercellular adhesion molecule 1) are resident on the surface of Kupffer cells and NKT cells, respectively, and bind to each other with high affinity. Aside from NKT-cell activation, Kupffer cells are also reported to show quicker iNOS (inducible nitric oxide synthase) and NO synthesis, indicating mutual activating functions between NKT cells and Kupffer cells (91). Notably, over-stimulation of NKT cells by Kupffer cells can result in activation-induced apoptosis and necrosis of NKT cells (90). Signal regulatory protein α (SIRPα) on Kupffer cells binding to CD47 on NKT cells also enhances the function of NKT cells (92).

Kupffer cells secrete many types of cytokines that have biological functions on NKT cells, mostly different interleukins. Kupffer-cell-derived IL-12, IL-1β, and IL-15 are thought to recruit NKT cells, promote NKT-cell activation, and participate in the maintenance of NKT cells (42, 93–95). AIM ("apoptosis inhibitor expressed by macrophages", also referred to as CD5L), a protein that is normally considered to inhibit apoptosis of CD4+CD8+ double-positive thymocytes, is secreted by Kupffer cells to protect NKT cells from apoptosis (96). On the other hand, NKT cells are capable of producing large quantities of pro-inflammatory IL-4 and IFN-γ, which are associated with granuloma formation around infected Kupffer cells (97).

In conclusion, we believe the relationship between NKT cells and Kupffer cells is reciprocal, enhancing both NKT and Kupffer cells (Figure 2D). In hepatic diseases like inflammation (93, 98), alcoholic liver injury (94) and infection (92), the interactions between Kupffer cells and NKT cells play an indispensable part.

NKT cells and dendritic cells

As a type of APC, dendritic cells participate in lipid antigen (such as α-Galcer) presentation *via* CD1d towards NKT cells, which leads to NKT-cell activation (99, 100), just like hepatic macrophages do. Dendritic cells also secrete cytokines including IL-27 (48) and IL-12 (101) that both have a positive impact on NKT cells. Notably, only myeloid dendritic cells (mDC), not all dendritic cells, mediate activation of NKT cells, whereas plasmacytoid dendritic cells (pDC) are likely to cause tolerance of NKT cells in a way concerning the activation of type II NKT cells (6). In addition, NKT cells also function as promoters of dendritic cells by expressing CD40L that binds with CD40 on the surface of dendritic cells, forming a reciprocal activating loop (5, 101). It has been observed that dendritic cells respond to TLR stimulation more actively in the presence of NKT cells (Figure 2E) (102, 103).

The positive influence of dendritic cells on NKT cells might provide an insight into treatment of liver diseases. In 2007, Tomohide Tatsumi et al. (104)demonstrated with mice models that α-GalCer-pulsed dendritic cells suppressed liver tumor by activating NK cells, and they proposed that NKT cells might also take a part. Hopefully, future research might provide a more explicit answer.

NKT cells and T cells

T cells consist of a wide range of different cell subgroups including CD8+ T cells, CD4+ T cells, and regulatory T cells (Tregs), each having distinct immune bioactivity.

The interaction between NKT cells and CD8+ T cells seems confusing as researchers have obtained contradictory experimental results. Some people believe NKT cells boost CD8+ T cells just like they do CD4+ T cells, *via* CD40/CD40L signaling and secretion of cytokines such as IL-4 and IL-13. Activated CD8+ T cells then secrete IFN-γ, a pro-inflammatory cytokine (105, 106). However, other researchers have discovered an inhibitory effect of NKT cells on CD8+ T cells in animal experiments. IFN-γ secreted by activated CD8+ T cells allows NKT cells to produce IL-4 and IL-13, which in turn inhibit CD8+ T cell activity by harassing their chemotaxis to CCL5. Also, NKT cells indirectly suppress CD8+ T cells by potentiating regulatory T cells (Tregs) (107).

The effects of NKT cells on CD4+ T cells are mainly positive, leading to enhanced IFN-γ secretion (105, 108). Normally, CD4+ T cells are the main producers of IL-10, a cytokine that has anti-inflammatory effects, but their IL-10 secretion is profoundly inhibited after activation from NKT cells (105). This indicates that activation of CD4+ T cells by NKT cells may be pro-inflammatory. Dendritic cells are also said to participate in NKT/T crosstalk, enhancing both CD4+ and CD8+ T cells (106). Moreover, NKT-cell-induced Treg activation plays an important role in the depletion of CD4+ T cells (109).

Regulatory T cells (Tregs), on the other hand, are a special subgroup of T cells that can suppress immune reactions. Some studies show a negative feedback relationship between NKT cells and Tregs. Activated NKT cells stimulate activation of Tregs by secreting higher amounts of cytokines such as IL-2, IL-10, and TGF-β. Tregs then suppress the proliferation and functions of NKT cells, reducing their cytotoxic activity. Interestingly, the inhibitory effects of Tregs on NKT cells are achieved in a CD1d-dependent manner that requires direct cell-to-cell contact (110, 111), rather than TGF-β and IL-10, as the suppression of NKT cells continues even after neutralization of the two cytokines (109, 111). This feedback mechanism is likely to prevent over-stimulation of NKT cells and the disastrous cascade immune reactions that could ensue (see Table 3 and Figure 3).

The interactions between NKT and T cells play a pivotal role in liver diseases. For example, during HBV infection in a transgenic mouse model, NKT cells promotes the proliferation of HBV-specific CD8+ T cells (112), and blockade of NKG2D expression prevents hepatitis induced by T cells (113). These results respond with the investigation which found that HCV-specific T cell response comes in accordance of NKG2D expression on NKT cells in healthcare workers who were exposed to small amounts of HCV but showed no obvious liver damage (114). In autoimmune liver disease and NAFLDs, NKT cells contribute to the recruitment of T cells, and potentiate their biological functions (79, 115, 116). As a result, NKT cells are considered as a factor for exacerbation of these two diseases. However, in autoimmune liver diseases, NKT cells promote the activation of Tregs, which ameliorates the killing effect of T cells (117).

TABLE 3 Interactions between NKT cells and different subgroups of T cells.

T cell subsets	Interaction with NKT cells	Results on T cells	Results on NKT cells	References
CD8+ T cells	CD40/CD40L, IL-4, IL-13	Enhanced IFN-γ secretion, inhibited chemotaxis to CCL5	Enhanced IL-4 and IL-13 secretion	(105–107)
CD4+ T cells	CD40/CD40L, IL-4, IL-13	Enhanced IFN-γ secretion, reduced IL-10 secretion	Decreased suppression of IL-10 on NKT cells	(105, 106, 108)
Regulatory T cells (Treg)	Cell-to-cell contact	Enhanced secretion of IL-2, IL-10 and TGF-β (which functions negatively on CD8+ and CD4+ T cells)	Impaired proliferation	(110, 111)

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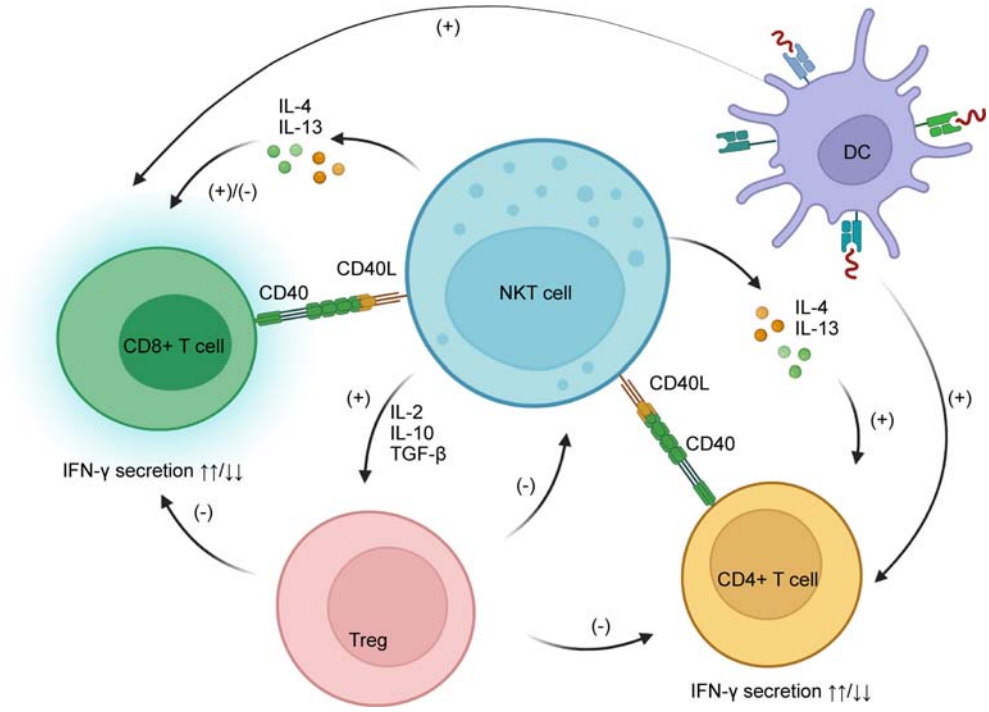

FIGURE 3
NKT cells interact with CD8+ and CD4+ T cells through CD40/CD40L and secretion of IL-4 and IL-13. The main functions of NKT cells on CD4+ T cells are stimulatory, while on CD8+ T cells they are both stimulatory and inhibitory, as NKT cells can harass their chemotaxis. NKT cells potentiate Tregs by secreting IL-2, IL-10, and TGF-β; Tregs have a negative impact on CD8+ and CD4+ T cells as well as NKT cells. IL, interleukin; TGF, tumor growth factor; Treg, regulatory T cells.

NKT cells and other cells

Besides hepatocytes, dendritic cells, macrophages (Kupffer cells), T cells, and B cells, hepatic NKT cells also have close relationship with neutrophils, hepatic stellate cells, and NK cells. These interactions have a significant influence in modulating inflammation as well as immune tolerance, and play a role in the processes of liver cirrhosis and cancerous proliferation. We outline these cell interactions as follows.

NKT cells secrete IL-4 to recruit and promote accumulation of neutrophils, thus enhancing hepatitis and liver fibrosis (115, 118). Additionally, NKT-cell-derived IFN-γ acts as a potent suppressor of neutrophils by inducing apoptosis (118). This may be to prevent over-activation of pro-inflammatory responses.

HSC activation is among the causes of liver fibrosis as it enhances synthesis and accumulation of collagen and extracellular matrix (119). NKT cells can either have a stimulatory or inhibitory effect on HSCs. Normally, NKT cells enhance the growth of HSCs *via* Hh (Hedgehog) signaling pathway and secreting OPN (osteopontin) (81, 120, 121), but under certain circumstances NKT cells induce HSCs apoptosis *via* FasL (122). (See 4.3"alcoholic liver diseases (ALDs)" and 4.4"non-alcoholic fatty liver diseases (NAFLDs)").

Generally, NKT cells are considered to enhance the activation of NK cells. Tomonori Iyoda et al. (123)found activated NKT cells induce NKG2D and DNAM-1 (also known as CD226) expression on NK cells that are necessary for the anti-tumor effects of NK cells. NKT-cell activation also leads to improved cytokine production (e.g IFN-γ) and killing activity of NK cells through the mTOR (mechanistic target of rapamycin) pathway, which brings about enhanced anti-pathogen capacity (124, 125). However, some researchers have also reported an inhibitory effect of NKT cells on IFN-γ secretion of NK cells after alcohol intake (126).

NKT cells in liver diseases

Given all the functions of NKT cells on other cells within the liver, it is easily deduced that NKT cells make an enormous contribution to the pathogenesis and progression of many kinds of liver disease ranging from autoimmune hepatitis to hepatoma. With more insights into how NKT cells work in these diseases, hopefully new methods to treat or cure liver diseases will be discovered. For example, tazarotene, a RAR-γ (retinoic acid receptor-γ) agonist that inhibits NKT-cell proliferation, as well

as cytokine release, is tested in mice for treatment of liver steatosis and fibrosis (127). Below, we discuss how NKT cells play their role in autoimmune liver diseases, alcoholic liver diseases (ALDs), non-alcoholic fatty liver diseases (NAFLDs), and liver tumors.

HBV and HCV infection

HBV and HCV infections are two important reasons for liver damage all across the world, affecting approximately 250 million (128) and 80 million (129) people respectively. Thus, research aimed at fighting against viral hepatitis has become a global task. In this chapter, we will review the roles that NKT cells play during the pathophysiological process of HBV and HCV infection.

During HBV infection, hepatocytes that are invaded by virus would present lipid antigens, namely lysophosphatidylethanolamine, to NKT cells *via* CD1d (130). This leads to NKT activation, causing an elevated amount of IFN-γ, which mediates anti-viral effects (131) but also results in liver damage (132) in mice models. IFN-γ inhibits the proliferation of hepatocytes by inducing apoptosis and negatively regulating cell cycle (75). In addition, NKT cells inhibit HBV by promoting the activation of cytotoxic T lymphocytes (CTL) (112). Blockade of NKG2D is found to ameliorate acute HBV hepatitis both *in vitro* and *in vivo* (113). In a retrospective investigation in 2009, India, researchers found that the amount of NKT cells is smaller in fulminant HBV liver failure than acute HBV patients, indicating the role that NKT cells play in limiting HBV infection (133). Notably, any factors that hinder the presentation of lipid antigens of hepatocytes to NKT cells, such as deficiency of NKT cells or CD1d or dysfunction of ER-associated lipid transfer, would result in a delayed anti-viral reaction (130).

However, data collected from clinical patients demonstrated that there is a decreased density (134) and down-regulated function (135) of NKT cells for chronic HBV infection compared to acute HBV infection. Both animal and human studies suggested that NKT cells are associated with over-activation of HSC and excessive healing during HBV infection, which increases the possibility of liver cirrhosis (136, 137). Moreover, the number of NKT cells is positively correlated with the quantity of HBV during chronic HBV infection, and a decrease in NKT number is witnessed after effective anti-viral treatment. This indicates NKT density as a potential marker for evaluating anti-HBV treatments (138).

To this day, plenty of novel NKT-related treating methods of HBV have been experimented on cells or clinical trials. For example, α-GalCer was found to inhibit HBV replication by directly activating NKT cells in mice (131), but clinical trials showed pessimistic results, as administration of α-GalCer alone even decreases NKT density and does not influence density of HBV DNA (139). Also, the function of α-GalCer on NKT activation decreases during chronic HBV infection, but this phenomenon is partly reversible after administration of exogenous IL-2 and/or IL-15 (51). Other ways to activate hepatic NKT cells and inhibit HBV replication include IL-18 (140), thymosin-α1 (141), β-glycosphingolipids (142), CD28/CD80 (143) activation and PD-1/PD-L1 blockade (143), but these methods have not received clinical confirmation yet.

NKT cells also participate in the process of HCV infection. Elevated expression of CD1d on infected biliary cells promotes the activation of NKT cells (144), leading to secretion of cytokines including IL-4 which recruits T cells and perforin and granzyme which mediate liver damage (145). Notably, some researchers claimed that the number of NKT cells in peripheral blood decreases in patients infected with HCV (146, 147), but more researchers did not find significant changes in number of NKT cells (148–150), although hepatic NKT cells showed enhanced activity (147), producing a higher amount of IL-13 that has a pro-Th2 effect (150). The functions of NKT cells during HCV are associated with macrophages and T cells. Macrophages (Kupffer cells) in the liver secrete significantly more IL-15 that boost NKT activation (95). In healthcare workers who were continuously exposed to small amounts of HCV but did not develop symptoms, NKT cells were found to be activated in a way related to specific T cells activation, indicating the protective effect of NKT cells against HCV is partly associated with T cells (114). In patients with chronic HCV, the sustained response to IFN plus ribavirin therapy is associated with elevated dynamism of NK and NKT cells, suggesting NKT cells play a vital role in anti-HCV reaction (151). In addition, in pregnant women infected by HCV, density of NKT cells increase in placenta tissues, which is thought to be responsible for preterm birth (152).

Preclinical studies proposed novel NKT-related anti-HCV therapy including the administration of IFN-α (153) and IL-2/OKT3 (a CD3-specific mAb) (154), which leads to NKT activation and up-regulated IFN-γ expression that inhibit virus replication in mice. Moreover, a clinical trial experimented oral administration of hepatocyte-extracted proteins and HBV or HCV proteins to figure out their anti-viral functions in chronic HBV or HCV patients. Results showed that all patients experienced increased number of NKT cells for at least 2-fold, and histological necro inflammatory score improved in 4/13 (30.7%) and 2/12 (17%) patients of HBV and HCV, respectively (155).

Autoimmune liver diseases

There are three main types of autoimmune liver disease, namely autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC), which are associated with destruction of hepatic parenchyma, small intrahepatic bile ducts, and large bile ducts, respectively. Liver NKT cells primarily reside in liver sinusoids. Numerous studies

have found that NKT cells are closely associated with all three types of autoimmune disease (79, 115, 117, 156).

Interestingly, liver NKT cells can either promote or combat autoimmune liver diseases depending on their down-stream target cells (Table 4). Besides, NKT cells promote the development of hepatocyte injury in three ways (1): NKT cells directly kill hepatocytes by expressing FasL and secrete TNF-α, perforin and granzyme B; (2) NKT-derived IFN-γ induces Th0-cell transduction into Th1 cells and CD8+ T-cell transduction into CTLs that bind to the MHC I molecule on the surface of hepatocytes; (3) NKT cells secrete IL-4 that turns Th0 cells into Th2 cells, enhancing B-cell-producing autoimmune antibodies (79). Furthermore, TNF-α and IFN-γ are involved in recruitment of functional T cells, and IL-4 probably promotes neutrophil infiltration within the liver (115). NKT cells are also potent activators of Tregs, which have a negative effect on immune response, thus mitigating autoimmune liver injury (160). Statistics show that simultaneous suppression of NKT cells and promotion of Tregs is helpful for mitigating autoimmune liver injuries in experimental animal models (161).

Apart from inflammation, NKT cells are likely to play a role in liver fibrosis resulting from autoimmune liver diseases. IL-4 and IL-13 from activated NKT cells promote liver fibrosis, suggesting a role for NKT cells in cirrhosis resulting from chronic autoimmune liver injury (77). However, this fibrinogenic effect requires further examination (115).

Given the roles that NKT cells play during autoimmune liver diseases, researchers have been experimenting on modulating NKT cells to find treatments for the disease. In recent years, several substances have been found effective in mice for alleviating concanavalin A (Con A)-induced autoimmune hepatitis partly by inhibiting NKT cells and related production of inflammatory cytokines, including mitochondrial-targeted ubiquinone(MitoQ) (162), diammonium glycyrrhizinate (161) and secoemestrin C (163). Gene modulation such as C6orf120 knockout (164) is also used as a therapeutic target. However, these experiments were only done on mice, and lack of clinical statistics limits extensive application.

Other studies also suggested the role that type II NKT cells plays in autoimmune liver diseases. The number of type II NKT cells was up-regulated in both peripheral blood and liver during autoimmune liver diseases (165). Increased CD1d expression on T cells during autoimmune liver diseases results in activation of type II NKT cells and favors Th1 cytokine production over Th2 within type II NKT cells (166). It is important to understand the physiology of type II NKT cells as they might influence iNKT cells. For example, Ramesh C. Halder et al. reported that activation of type II NKT cells and pDCs are associated with recruitment of anergic iNKT cells (167).

TABLE 4 Functions of NKT cells in autoimmune liver diseases, alcoholic liver diseases (ALDs) and non-alcoholic fatty liver diseases (NAFLDs).

Disease type	Role of NKT	Mechanisms	References
HBV	Inhibitors of HBV replication	Inhibit hepatocyte proliferation (by secreting IFN-γ that induces apoptosis and negatively modulates cell cycle)	(131, 132)
		Promote the activation of CTL	(112)
	Destructive factors	Cause liver damage	(132)
		Cause over-activation of HSCs and excessive healing, promoting cirrhosis	(136, 137)
HCV	Inhibitors of HCV replication	Death of infected liver cells (by perforin and granzyme)	(145)
		Recruit and activate T cells	(145)
Autoimmune liver diseases	Promoters of autoimmune liver diseases	Kill hepatocytes (via FasL, TNF-α, perforin, granzyme B)	(79)
		IFN-γ (induce Th0→Th1 and CD8+ T cells →CTL transformation, recruit T cells)	
		IL-4 (induce Th0→Th2 transformation that promotes B cells to produce antibodies, recruit neutrophils)	
		TNF-α (recruit T cells)	(115)
	Inhibitors of autoimmune liver diseases	Activate Tregs	(117)
Alcoholic liver diseases (ALDs)	Promoters of ALDs	NKT cells recruit neutrophils (via TNF-α, etc.)	(94, 157, 158)
		NKT cells mediate death of hepatocytes (via FasL)	(80)
		NKT cells inhibit IFN-γ secretion of NK cells	(126)
	Inhibitors of ALDs	NKT cells suppress HSCs (via FasL, IFN-γ) in early stage of ALDs	(122)
Non-alcoholic fatty liver diseases (NAFLDs)	Promoters of NAFLDs	NKT cells improve insulin resistance	(81)
	Inhibitors of NAFLDs	NKT cells enhance lipid intake of hepatocytes (via secretion of LIGHT)	(81, 120, 121)
		NKT cells activate HSCs (via OPN, Hh pathway)	(116)
		NKT cells recruit CD8+ T cells and macrophages	(120, 159)

Alcoholic liver diseases

It is widely known that excessive consumption of alcohol ranks high among the risk factors for liver pathogenesis. Alcohol-induced liver diseases include alcoholic hepatitis, steatosis, cirrhosis, and hepatocellular carcinoma (HCC).

ALDs are closely linked to enhanced immune activation (168). Activated inflammatory responses are noticed quickly after alcohol intake with a higher NKT-cell concentration within the liver as well as mesenteric lymph nodes (169–171). Conversely, the absolute number of NKT cells in the whole body is decreased (172), which may be the result of NKT-cell recruitment to the liver and vast consumption of these cells.

Generally, NKT cells are thought to contribute to the development of ALDs, in contrast to type II NKT cells that are considered to attenuate ALDs (157). Binge-feeding with ethanol results in accumulation and activation of NKT cells combined with a higher expression of inflammatory and fibrotic genes in wild-type mice compared to their NKT-deficient counterparts (158). Evidence shows that alcohol potentiates α-Galcer stimulation of NKT cells by facilitating CD1d loading (171). IL-1β from Kupffer cells is also required for hepatic NKT-cell accumulation during ALDs (94).

Activated NKT cells recruit neutrophils by secreting TNF-α and up-regulating expression of neutrophil-attracting MCP-1 (monocyte chemoattractant protein-1, also known as CCL2) (94, 157), ICAM-1 (intercellular adhesion molecule-1), E-selectin, MIP-1α (macrophage inflammatory protein-1α, also known as CCL3), MIP-2, and osteopontin (OPN) (158). Also, NKT cells mediate apoptosis of hepatocytes by expression of FasL (80). Some researchers consider NK cells to be protective against ALDs by secretion of IFN-γ, while NKT cells inhibit this process. However, a subgroup of IL-10-secreting NKT cells (thus called NKT10) facilitates the protective effect of NK cells in ALDs (126).

Controversially, in the early stages of ALDs, NKT cells are likely to play an anti-fibrotic role by suppressing HSCs. The negative influence of NKT cells on HSCs is achieved through direct killing *via* the Fas/FasL pathway and IFN-γ production (122). Taken together, we conclude that with a few exceptions, NKT cells are mainly promoters of ALDs (Table 4).

Up to now, we have not found any results of NKT-based clinical trial for ALD therapy, but there are experiments done on mice, showing several promising molecules with therapeutic potential, including retinoids and sulfatide (157). These two substances alleviate ALDs by inhibition of NKT cells. Additionally, researchers found that prednisolone, a drug widely used to antagonize inflammation, exacerbates ALDs by inhibiting phagocytosis mediated by macrophages and neutrophils and hepatic regeneration, which provide an insight into management of steroid therapy (173).

Non-alcoholic fatty liver diseases

As the name suggests, the most significant feature of non-alcoholic fatty liver diseases (NAFLDs) is abnormal lipid storage in the liver. NAFLDs include simple stenosis, non-alcoholic steatohepatitis (NASH), cirrhosis, and even liver cancer. In many studies that focus on NAFLDs, a high-fat feeding model is used. Statistics have demonstrated that a high-fat diet, especially one with high concentrations of saturated fatty acids and monounsaturated fatty acids rather than polyunsaturated fatty acids, is associated with liver inflammation, insulin resistance, and NAFLDs (64, 174, 175).

During NAFLDs, the number of NKT cells within the liver decreases. This is because: (1) endothelium stress leads to fewer CD1d's and impaired lipid presentation (74); (2) Kupffer cells mediate apoptosis and necrosis of over-activated NKT cells and secrete IL-12 to suppress NKT cells (64, 90); (3) normally Kupffer cells-derived IL-15 is stimulating for NKT cells, but in NAFLDs IL-15 secretion is down-regulated (45, 176) and (4) norepinephrine (NE) concentration decreases (176). However, some studies also report an increase in the number of NKT cells in the late stages of NAFLDs, probably because of enhanced activating functions of Kupffer cells *via* the CD1d-dependent pathway, which is inconsistent with many other study results (120).

Notably, the effects of NKT cells on the development of NAFLDs are rather controversial. While NKT cells ameliorate NAFLDs, probably by improving insulin resistance (120, 159), they are also likely to play a pro-inflammatory role in NAFLDs. Some studies show that NKT cells secrete LIGHT (TNFSF14), which significantly enhances lipid intake of hepatocytes (81). Also, NKT cells lead to activation of HSCs in two ways: (1) they facilitate OPN (osteopontin) secretion; and (2) they promote the Hedgehog (Hh) signaling pathway (note that NKT cells are both inducers and targets of the Hh signaling pathway). HSC activation is associated with exacerbation of liver fibrosis or cirrhosis (81, 120, 121). Activated NKT cells recruit CD8+ T cells and macrophages, too (116). Overall, NKT may have a protective effect in the early stages of NAFLDs but a destructive effect in later stages (Table 4).

In 2017, a published clinical trial said oral administration of β-glucosylceramide improved the hepatic fat content by 14% in NASH patients, which is associated with a decrease in CD4+ and NKT cells, suggesting NKT cells as a possible therapeutic target (177). Mice models also indicate that oral administration of liver-extrated proteins (178), immunoglobulin G-enhanced colostrums (179) and PRX-106 (180) (a recombinant anti-TNF-αfusion protein). The number of hepatic NKT cells was increased in all these mice models.

Liver tumors

Liver tumors are a global health problem that deprives millions of people of their lives. primary hepatic carcinoma (HCC) is the main type of liver tumor. Many studies have shown the tumor-suppressing effect of hepatic NKT cells. Understanding of the roles that NKT cells

play in the pathogenesis of both primary and metastatic liver cancer is helpful for finding effective ways of treatment.

NKT cells participate in anti-tumor immune responses mainly by producing IFN-γ. Mice studies found that not only can activated NKT cells secrete IFN-γ, they also stimulate IFN-γ production from NK cells (12, 181). IFN-γ then functions on hepatic T cells and Kupffer cells, enhancing the cytotoxicity and phagocytosis of T cells and Kupffer cells, respectively (182, 183). NKT cells also participate in chemotaxis of T cells as they secrete T cell-recruiting chemokines such as MIP-1α, MIP-1, and IL-8. IFN-γ up-regulates CXCR3 expression of T cells, potentiating T-cell recruitment. Moreover, NKT cells directly mediate the death of tumor cells through FasL, perforin, and granzyme (183). On the other hand, some researchers do not perceive NKT cells as necessary in anti-tumor immunity (184).

Activation of NKT cells in the background of hepatic cancer is closely associated with dendritic cells and Kupffer cells. Dendritic cells communicate with NKT cells in a CD40/CD40L-dependent way. Up-regulated expression of CD40L in NKT cells potentiates DC cells, leading to secretion of IL-12 that in turn activates NKT cells (101). Kupffer cells are also sources of IL-12 (182). Importantly, IL-12-induced NKT activation is linked to reduced primary hepatic tumor and less metastasis to the liver (185) As a result, IL-12-based therapy has been proposed and examined by many researchers (186, 187).

Interestingly, activation of NKT cells is also dependent on CXCR6/CXCL16 interaction, as deficiency of CXCR6 or neutralization of CXCL16 cause hepatic cancer to deteriorate. CXCR6 is expressed on the surface of liver sinusoid epithelium cells, while CXCL16 is a characteristic molecule of NKT cells. However, the deficiency of CXCR6 can be compensated for by systemic NKT-cell activation through other methods (181, 188).

Given the significant functions of NKT cells in development of liver tumors, many novel treatments of hepatic cancer based on NKT cells have been invented in recent years. Mice experiments showed that exogenous IL-12 and α-Galcer (186), direct transfer of ex vivo modulated NKT cells (189), tumor antigens (190, 191), and even antigens of some microorganisms (e.g. LPS from bacteria (182) and some recombinant oncolytic viruses (192)are used to potentiate the anti-tumor effect of NKT cells. Low protein diet (193) and blockade of PD-1/PD-L1 axis (194)are also found useful in suppressing hepatic tumors. Notably, clinical trials have confirmed the effectiveness of some NKT-related treatments. For example, in 2021, Tian-Tian Li et al. (195) reported that stereotactic body radiotherapy had positive effects on peripheral NKT cells in HCC patients, which is associated with a higher overall survival. These results indicate NKT cells as a very promising therapeutic target.

Perspectives and conclusion

In this review, we mainly discussed how NKT cells are activated and the functions of NKT cells during the pathogenesis and development of some liver diseases. It is widely acknowledged that the role NKT cells play in the immunity of the liver and even the whole body is indispensable, and treatment focused on modulating NKT-cell activity is becoming more and more promising. However, considering the dual functions NKT cells have in many liver diseases, the treatment tactics should be studied thoroughly.

In addition, since this review mainly focuses on the physiology of type I NKT cells (iNKT cells), more work needs to be done for a better understanding of type II NKT cells which are more abundant in the human liver than in mice (196). Type II NKT cells are normally considered to be anti-inflammatory, and regulate type I NKT cells (iNKT) and other immune cells and favor tumor growth (101, 197). However, some also report the role that Type II NKT cells play in promoting chronic inflammation (166). Further studies are needed to better demonstrate the functions of Type II NKT cells and how these cells interact with type I NKT cells as well as other participants in our immune system.

Recently, gene analysis has cast new light on NKT researches. Single-cell RNA sequencing indicates distinct populations of functional NKT cell subsets with differences on gene and epigenetic levels (198, 199). This offers a deeper understanding, and is likely to guide future studies within this field.

Author contributions

XG, QC and XM have equal contributions to this study. XG and HZ designed the whole study. JW and CC conducted the statistical analysis. XG, QC, and XM draft the manuscript. JG, YR, and SW made the relevant edits to the manuscript. XG, QC and XM revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Overcoming the challenges in translational development of natural killer cell therapeutics: An opinion paper

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Introduction

Cellular therapies have attracted huge research and clinical attention lately (1, 2). Natural killer cells (NKCs) are a class of innate immune lymphoid cells (ILP) mainly derived from bone marrow lymphoid stem cells (3). They are mainly distributed in peripheral blood (PB) and peripheral lymphoid tissues, accounting for about 10% of the total lymphocytes in PB (4–7). Presently, NK cell immunotherapeutics utilize cells derived from many sources including PB, umbilical cord blood (8), immortalized NK cell lines, and more recently, induced pluripotent stem cells (iPSC) (9–11). They are characterized by rapid response and non-specific cytotoxic effect without prior antigen sensitization, and are independent on antibodies or complements (12, 13). NKCs induce apoptosis of target cells by secreting perforin, granzyme, cytokines and chemokines (14, 15). NKCs also selectively attacks foreign and diseased cells through expression of killer-cell immunoglobulin-like receptors (KIRs) and Fc receptor (CD16) where the later mediates antibody-dependent cell-medicated cytotoxicity (ADCC) (16–18).

Human NKCs are defined by the $CD3^{-}CD56^{+}$ surface phenotype (19, 20). According to their surface expression of CD56, they are divided into two main subpopulations: CD56bright and CD56dim, which differ significantly in biological characteristics (20–22). Briefly, CD56bright NKCs are immature cells and the progenitor for effector cells with high expression of CD56 and low expression of CD16 and KIRs (21), and accounts for about 5- 10% of the total NKCs. Also, they are weakly immunoregulatory and rely mainly on the secretion of cytokines, growth factors and chemokines (22). On the contrary,

CD56dim subset accounts for about 90-95% of the circulating NKCs. They are characterized by low expression of CD56 and high expression of CD16, KIRs, FcγRIII and a variety of NK cell inhibitory receptors. They exhibit intrinsic cytotoxicity and ADCC but have weak cytokine secretion ability (23, 24).

Many studies have revealed the role of NKCs in pathologies like autoimmune diseases (25, 26), leukemia (25), pregnancy-related conditions (27), liver diseases (28–30), HIV (31), HPV (32), atherosclerosis (33) and in many age-related diseases. Aging is usually associated with increased susceptibility to infectious diseases and cancers due to immunosenescence which is particularly reflected in the biological changes in the population and subsets of NKCs throughout life (34). As the functioning of the immune system decreases with age, NKCs have difficulties in initiating adaptive immune responses and mobilizing effective immune molecules, leading to the occurrence of diseases related to aging, such as infections and tumors (35–38). The proportion of CD56dim NKCs in the elderly increased with age, while the CD56bright NKCs decreased significantly, suggesting that the increased CD56dimNK : CD56brightNK ratio is significantly age related (39, 40). Studies have also shown that the mortality risk in the elderly with low NK cell counts is three-fold higher than that in the elderly with high NK cell counts (41). In addition to the decreased CD56bright NKCs in older adults (42–44), Sagiv et al. demonstrated that the decline in perforin-mediated NK cytotoxicity is similarly age-related, and may hinder the ability of NKCs to clear senescent cells in the elderly (45). Similarly, NKCs from geriatric population have synonymous reduction in proliferative response to interleukin 2 (IL-2) and expression of the CD69 activation antigen (46–49). With immunosenescence being almost inevitable, many are willing to explore therapies to escape the negative consequences of aging especially tumor development which is increasingly prevalent. Therefore, undergoing NK cell therapy in early old age may help particularly in alleviating cancers.

Studies have shown that the therapeutic efficiency of NK cell therapeutics while encouraging in hematopoietic malignancies, is unsatisfactory in solid tumors as it is problematic for NKCs to infiltrate tumor sites (50, 51). The function, activation, and persistence of NKCs are significantly diminished by the tumor microenvironment (TME), leading to their dysfunction or exhaustion. In this paper, we wish to draw the attention of researchers to the fact that NK cellular products are highly promising in the fight against cancer and other age-complicated diseases and if they must be applied safely, more efforts should be directed towards addressing the bottlenecks. Broadly, NK cell infiltration, solid tumor targeting, *in vivo* persistence and resistance to TME must be improved, and reproducible and standardized protocols must be developed for the generation and expansion of NKCs. Here in, we highlight the strategies employed in tackling the challenges as this will serve as guide to the research trend and future directions considered in the development of clinical grade NK biotherapeutics.

Improving tumor targeting, microenvironment resistance and solid tumor infiltration

Clinical trials involving NKCs is generally not a new topic as they have been in progress for over two decades from where preliminary data regarding the safety and efficacy of NKCs have been obtained following their adoptive transfer to treat hematologic malignancies (25, 52). Nevertheless, some limitations have been encountered in the application of NK cell therapy to solid tumors largely because the TME harbors suppressive ligands, metabolites and cytokines which threatens the survival of NKCs (53). Additionally, the tumor itself possesses other defense mechanisms against attack by NKCs. Therefore, enormous research efforts are directed towards producing or modifying NKCs to be more resistant to attack from tumors and TME while harnessing their cytolytic effect after tumor penetration (54, 55). One of such approaches include blocking of inhibitory receptors with monoclonal antibodies (mAbs) like monalizumab (56).

Increasing the efficacy of tumor cell recognition is achievable *via* genetic modification (57) (58). For example, CAR-NKCs have enhanced cytolytic activity attributable to the synergistic effect of targeted specificity against tumor associated antigens and intracellular signaling of receptors (59, 60). CAR-NKCs can also be fashioned with receptors for a wide range of antigens with the CAR expression permitting carrier cells to recognize antigens on tumor-cell surfaces without major histocompatibility complex restriction (9, 58, 61). Also, unlike CAR-T cell therapies, CAR-NKCs possess reduced risk of cytokine release syndrome, neurological complications and better potential for allogeneic applications (62).

Transfection efficiency for primary NKCs is a key obstacle to the large-scale manufacture of genetically modified CAR-NKCs and different techniques like viral transduction and non-viral electroporation are underway to addressing this challenge (63, 64). Kumar et al. has recently led the production and evaluation of CRISPR-engineered NK-92 cell constitutively expressing Cas9 or dCas9 which have shown good prospects for further research and possible clinical application (65).

Pharmacokinetics and pharmacodynamics of natural killer cell therapeutics

NK and other cellular therapeutics are different from conventional (chemical) drugs therefore great disparity exists in their pharmacokinetics and pharmacodynamics properties. There is substantial evidence from clinical studies regarding the safety and efficacy of NK cell therapeutics. Nonetheless, more research is on

demand to explore the sensitivity a wider variety of tumors to NK cell therapy, determine the mechanism(s) of action (cytotoxic response) against different types of tumors and identify possible contraindications. Some of the identified issues are as thus:

1. Allogenic NKCs from PB are relatively safe and satisfactorily effective against tumors but are susceptible to rejection by the host (66, 67).
2. Although several studies show the relationship between high NKCs, their receptors/ligands levels and better overall survival in patients with hepatocellular carcinoma (HCC), the underlying mechanism of remains unclear (13).
3. In obese patients, significant numbers of NK and T cells are recruited to the visceral adipose tissue at the expense of successful tumor infiltration and eradication (68), thus posing a serious challenge for the application of NK cell therapy in certain comorbid situations.
4. Combination therapy with NK and T cells or other tumor therapy strategies need to be confirmed with large-scale clinical trials as the clinical outcome can vary between tumor types.

Hence, several research efforts are geared towards unravelling possible NK mechanism of cytolysis like mitochondrial apoptosis (64) and release of perforin and granzymes (69); factors that may increase their cytotoxicity such as E26 transformation-specific transcription factor ELK3 expression by cancer cells (70); factors that attenuate cytolytic function like increased transforming growth factor-beta 1 (TGF-β1) (32), inhibition of O-GlcNAcylation (71), low surface expression and impaired function of transient receptor potential melastatin 3 (TRPM3) (32, 71–75); and addressing the complications accompanying rejection-prone cellular products (67, 76, 77).

Expansion and activation of natural killer cells

Another obstacle to the manufacture of clinical grade NK cell therapeutics is the large-scale expansion of NKCs without loss of their cytotoxic activity (78). The expansion of NKCs can be *ex vivo* or *in vivo* followed by isolation by CD3+ cell depletion and subsequent positive selection of CD56+ cells. Other strategies involve a single step depletion of CD3+ and CD19+ cells using magnetic beads (79), and differentiation of functional NKCs from enriched CD34+ progenitors present in cord blood and bone marrow (80, 81). While good manufacturing practice (GMP) guidelines have been established, inconsistencies exist between the cytotoxicity, expansion rate, receptor expression, cytokine secretion and phenotype based on their respective source and expansion method (80, 82–84) which may influence their therapeutic activity. iPSCs derived NKCs possess improved expansion rate, cytokine secretion and cytotoxic compared to those from PB. They can also be genetically functionalized to harness tumor targeting, cytolytic activity and persistence in the TME (85). They have been clinically tested for different diseases including graft versus host disease, Parkinson's disease and heart failure (86, 87). Synergistic activity of iPSCs-derived NKCs with other effector T-cells and successful *in vitro* tumor infiltration has been described in animal studies (86). Other studies in this regard are focused on developing efficient methods and designing biomaterials for activation, expansion and isolation of NKCs (85, 88–91). Gao et al. recently classified the biological and transcriptomic signatures of cord blood and placenta-derived NKCs revealing the cellular/molecular level similarities and differences existing between the NK cell types (92). More of such studies are needed as they serve as database for cell-based immunotherapy and will be beneficial to understanding and categorizing the mechanism of action of different NKCs.

Optimizing persistence and cytolytic activity of natural killer cells

There is a huge need of novel technologies to enhance the activity of NKCs and their interaction with tumors. Consequently, several methods have been proposed. For instance, concomitant use of NK cell adoptive transfer and other therapeutic methods, including T-cells, chemotherapeutic agents, cytokines and immunomodulatory drugs could fortify NKCs against the TME and be synergistic in tumor immunotherapy (93–95). The biological targets of these supplementary molecules like cytokines and drugs vary from those of NKCs providing synergy (96) but their safety must also be assured before clinical application. Biber et al. describes the design of a non-viral lipid nanoparticle-based delivery system that encapsulates small interfering RNAs which targets NKCs *in vivo*, silences inhibitory molecules, and activate NK cell anti-tumor activity (97). Park et al. reports that *Aurantii Fructus Immaturus*, a commonly used herb in traditional medicine enhances the anticancer efficacy of NK (98), Bispecific killer cells engagers (BiKEs) and trispecific killer cells engagers (TriKEs) improve *in vitro* secretion of cytokines and efficiently induce the cytotoxic effects of NKCs (99, 100). Moving forwards, optimizing and improving these formulations to avoid undesirable side effects are vital steps toward their clinical application.

Conclusion

The role of NKCs in neutralizing senescent, stressed and malignant cells has attracted enormous research attention aimed at producing clinical grade NKCs for adoptive cell immunotherapy. Currently, some clinical studies are designed to determine the safety and efficacy of ex vivo activated and expanded NKCs while others test the effect of administering the NKCs in combination with other immune molecules. Major advances, including the development of efficient *ex vivo* expansion systems, prolonged *in vivo* persistence and genetic manipulation strategies involving CARs are currently explored to facilitate clinically applicable NK cell therapeutics. However, additional research effort is needed to enhance tumor targeting, overcome immune suppression by inhibitory signals or cells and exhaustion in the TME, increase persistence in allogeneic settings, facilitate expansion in patients, sustain *in vivo* surveillance against tumor relapse, and increase the applicability of NK cell therapy to a wider range of life-threatening diseases especially those marked by depletion in NK cell function. Finally, iPSC-NKCs hold great prospects and further refinement of their differentiation protocol is necessary to match the phenotypic properties of PB NKCs.

Author contributions

Conceptualization, HQ, CY, FY, KT, CX, and ME. Writing—original draft preparation, HQ, ME, RZ, and ST. Writing—review and editing, FY, ME, KT, CX, and ST. Supervision and approval, HQ, CY, and ST. All authors contributed to the article and approved the submitted version.

Conflict of interest

Authors HQ, CY, FY, KT, CX were employed by company NanHua Bio-medicine CO., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Combined analysis of bulk and single-cell RNA sequencing reveals novel natural killer cell-related prognostic biomarkers for predicting immunotherapeutic response in hepatocellular carcinoma

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Introduction: Natural killer (NK) cells play an irreplaceable and important role as a subtype of innate immune cells in the contemporary setting of antitumor immunity.

Methods: We chose a total of 1,196 samples for this analysis from the public dataset's six separate cohorts. To identify 42 NK cell marker genes, we first carried out a thorough study of single-cell RNA sequencing data from the GSE149614 cohort of hepatocellular carcinoma (HCC).

Results: Using the NK cell marker genes in the TCGA cohort, we next created a seven-gene prognostic signature, separating the patients into two categories with distinct survival patterns. This signature's prognostic prediction ability was well verified across several validation cohorts. Patients with high scores had higher TIDE scores but lower immune cell infiltration percentages. Importantly, low-scoring patients had superior immunotherapy response and prognosis than high-scoring patients in an independent immunotherapy cohort (IMvigor210). Finally, we used CD56 and TUBA1B antibodies for immunohistochemical labeling of HCC tissue sections, and we discovered a lower number of CD56+ cells in the HCC tissue sections with high TUBA1B expression.

Discussion: In summary, our research created a unique prognostic profile based on NK cell marker genes that may accurately predict how well immunotherapy would work for HCC patients.

KEYWORDS

NK, biomarker, HCC, immunotherapy, TUBA1B

1 Introduction

It is generally recognized that a diverse tumor microenvironment (TME) surrounds tumor cells in hepatocellular carcinoma (HCC) (1). The TME, which has a very complicated makeup, is crucial to the development and growth of tumors. Additionally, the interaction between immune cells and tumor cells in the TME not only influences a patient's prognosis but may also alter a patient's response to immunotherapy (2). The importance of innate immune cells has not gotten enough attention in the contemporary setting of antitumor immunity, which has mostly focused on adaptive T-cell responses, like CD4+ CD25+ Foxp3 regulatory T cells (Tregs), cytotoxic T lymphocytes (CTLs) et all (3, 4). By specifically identifying and eliminating tumor cells and encouraging adaptive T-cell immunity responses, natural killer (NK) cells are a subtype of innate immune cells that can reduce the proliferative and invasive potential of tumor cells at an early tumor stage (5). The balance of inhibitory and activating receptors that can interact with ligands on target cells determines how well NK cell function. NK cells can collaborate with T cells to control the spread of cancer and play a crucial part in the development of antitumor immunity. Cancer risk is increased by decreased NK cell activity in peripheral blood (6). Additionally, more tumor-infiltrating NK cells are strongly linked to improved prognosis across a variety of tumor types (7). Given the function of NK cells in immunity, earlier research has focused on their molecular features in cancer and infectious disorders (8, 9), but little is known about their complete molecular analysis in HCC.

Unprecedented chances to unveil the molecular properties of various immune cell populations in TME have been made possible by the advent of single-cell RNA sequencing (scRNA-seq) technology and related data processing methodologies (10). The prognosis and immunotherapeutic response in cancer patients may be accurately predicted by examining gene expression patterns based on molecular characterization of immune cells acquired from scRNA-seq data, according to previous research (11, 12). In this work, we first carried out a thorough examination of scRNA-seq data in HCC to characterize the molecular properties of tumor-infiltrating NK cells and to identify NK cell flag genes. Then, using bulk RNA-seq analysis, NK cell marker gene-related signatures for predicting the prognosis of HCC were created. Additionally, the link between NKCMGS and HCC immunotherapy response was examined, and the predictive ability of NKCMGS was verified in three separate cohorts from the ICGC and the Gene Expression Omnibus (GEO) database. Multiple datasets from TCGA, GEO and ICGC cohort were analyzed in our study for constructed NK cell-related genetic signature. We obtained a more parsimonious gene signature over existing studies, which contains seven genes, and provided better prediction for immunotherapeutic effect and drug sensitivity.

Abbreviations: NK, Natural killer; HCC, hepatocellular carcinoma; TME, tumor microenvironment; scRNA-seq, single-cell RNA sequencing; GEO, Gene Expression Omnibus; TCGA, the Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; ICB, immune checkpoint blockade; IC50, half-maximal inhibitory doses; NMF, non-negative matrix decomposition; GSEA, Gene Set Enrichment Analysis; TMB, tumor mutational burden; TIDE, Tumor Immune Dysfunction and Exclusion; PD, progressing disease; SD, stable disease; CR, complete response; PR, partial response; HPA, Human Protein Atlas; ICIs, immune checkpoint inhibitors.

2 Methods

2.1 Data collection and pre-processing

A total of 1196 samples, 31396 cells, including 10 HCC samples with single-cell RNA-sequencing (scRNA-seq) data from the GSE149614 cohort, 342 HCC samples from the Cancer Genome Atlas (TCGA) cohort (https://xenabrowser.net/), 230 HCC samples from the International Cancer Genome Consortium (ICGC) cohort (https://dcc.icgc.org/), 221 HCC samples from the GSE14520 cohort, 95 HCC samples from the GSE76427 cohort, and 298 samples treated with immunotherapy from the IMvigor210 cohort (http://research-pub.Gene.com/imvigor210corebiologies/), were enrolled in this study. In the GSE149614 dataset, with each gene expressed in at least three cells and each cell expressing more than 250 genes, single cells were initially screened for scRNA-seq data. The percentage of mitochondria and rRNA was then calculated by the Seurat package (13). Further testing of the single cells involved caused each one to express at least 5000 genes with a UMI > 100. The mitochondrial content was no more than 30%. In the end, 31396 cells were still present for identifying the NK cell marker genes of HCC. To find survival-related genes and create prognostic signatures, the bulk transcriptome data (FPKM normalized) and clinical details of HCC patients in the TCGA were employed. For external validation, three separate datasets were acquired: ICGC, GSE14520, and GSE76427.

2.2 Identification of NK cells and their hub genes

The GSE149614 dataset contains scRNA-seq data from 10 HCC samples, which we again examined. Following log normalization of the expressed genes, uniform flow-form approximation and projection techniques were used to reduce nonlinear dimensionality. We used the FindNeighbors and FindClusters () algorithms to arrange individual cells into 17 separate subgroups at dim=50 and resolution=0.1. Three marker genes, including CD3D, CD3E, and NKG7, were identified in NK cells. Using the FindAllMarkers program with logFC=0.5, minpct=0.25, and adjusted p-values less than 0.05, marker genes were found for each NK subpopulation. A univariate Cox regression analysis with P less than 0.05 was then used to further identify the genes among these NK marker genes that are associated with prognosis. We used the LASO-Cox regression to compress the number of genes and created a risk profile based on the outcomes of the multivariate Cox model using the equation: Risk score =∑iCoefficient (Genei)*Expression (Genei). Depending on their risk assessments, patients were separated into high- and low-risk groups. The receiver operating characteristic curve (ROC) analysis

and Kaplan-Meier survival analysis were used to examine the risk profile's ability to predict survival outcomes. The validation cohort underwent a similar examination. GSEA was used to examine KEGG, GO, and HALLMARK elements that had drastically changed across the different categories.

2.3 Analysis of the immune landscape

Based on the gene expression patterns of HCC patients, the stromal and immune scores were computed using the ESTIMATE software (14). The abundance ratio of immune cells was evaluated using the CIBERSORT (15), MCPcounter (16), and TIMER (17) databases to learn more about the TME.

2.4 Response to immune checkpoint blockade (ICB) and analysis of the sensitivity to potential therapeutic drugs

The TIDE database (http://tide.dfci.harvard.edu/), which estimates how often immunotherapy for HCC patients will be effective, was first performed. We also retrieved the matched clinical and transcriptome data from the IMvigor210 cohort of patients who were receiving anti-PD-L1 medication. Additionally, we evaluated multiple immune checkpoint gene expression changes such as PD1, PD-L1, CTLA4, and PD-L2 in different subgroups. Finally, we searched the Cellminer database for delicate medications that successfully address this risk profile (18). If a drug's adjusted P-value was less than 0.001 and its Pearson correlation coefficient was larger than 0.3, it was categorized as tumor-sensitive. The discrepancies in the half-maximal inhibitory doses (IC50) of several classes of tumor-sensitive medications were then studied.

2.5 Evaluation of TUBA1B expression in clinical samples

Using 30 samples of HCC and related paracancerous tissues that had undergone standard pathological evaluation in our pathology department, a validation cohort was developed. The clinicopathological information for all patients were shown in Table 1. Following the tissue wax blocks' serial sectioning, sample sections were gathered and stored for subsequent use in a 4°C freezer. We next used TUBA1B (Abcam, ab108629) and CD56 (Abcam, ab75813) antibodies in IHC experiments on formalin-fixed de-paraffinized slices and captured pictures using microscopy as previously reported (19).

2.6 Statistical analysis

R software was used to conduct all statistical analyses (v4.1.2). Pearson correlation was used to calculate the correlation analysis. For comparisons between the two groups, the chi-square test and grouped t-test were used, respectively. Kaplan-Meier survival analysis and a Log-rank test were used to assess survival differences between groups. Using the RMS software, a nomogram was produced following the signature. Statistics were deemed significant when the P value was less than 0.05.

TABLE 1 Characteristics of patients and tumor samples studied (n=30).

Clinicopathological characteristic	
Age, median (range) Female	56.5 (38–74) years old12/30 (40%)
T stage of primary tumor	
T1	2/30 (6.67%)
T2	5/30 (16.67%)
T3	16/30 (53.33%)
T4	7/30 (23.33%)
N stage of primary tumor	
N0	6/30 (20%)
N1	15/30 (50%)
N2	9/30 (30%)
Lymphovascular invasion present in primary tumor	17/30 (56.67%)
Perineural invasion present in primary tumor	11/30 (36.67%)
Synchronous metastasis (unknown for n=2)	13/28 (46.43%)
Underlying liver disease etiology (unknown for n=6)	
HBV, HCV and hepatocirrhosis	14/24 (58.33%)
Fatty liver and diabetes mellitus	4/24 (16.67%)
Alcohol Hereditary liver cancer	5/24 (20.83%) 1/24 (4.17%)

3 Results

3.1 Identification of NK cells in the scRNA-seq samples

Figure S1 displays the full outcomes of data preparation. After log-normalization and dimensionality reduction, 17 clusters were found. The TSNE plots showing the distribution of the 17 clusters are displayed in Figure 1A. As shown in Figure 1B and S2A, based on the expression of three marker genes (CD3D, CD3E, NKG7, CXCR3 and IL2RB), two NK cell subsets were discovered (Figure 1C). The fact that neither of the two NK cell clusters expressed the epithelial cell-specific gene (PECAM1) proves that NKs were correctly identified (Figure 1D). Further analysis of CD19 and CD14 expression in 17 clusters was performed for ruled out the interference of other cell types (Figure S2B). The expression of the top 10 DEGs in the two clusters is shown in Figure 1E. The 2 NK cell clusters contained 42 DEGs (marker genes recognized as NK clusters). The percentage of the two clusters in each sample was

shown in Figure 1F. Furthermore, we computed the ssGSEA scores for the marker genes of each NK cluster (the top 10 DEGs of the NK clusters) in the TCGA cohort to examine the connection between NK clusters and prognosis. The samples in the high ssGSEA score group in the NK 0 cluster had a better prognosis than those in the low ssGSEA score group, while the opposite finding was seen in the NK 1 cluster (Figure 1G). Finally, to further analyze the function and mechanism of NKs marker genes in HCC, we performed molecular subtype identification analysis of the TCGA dataset by non-negative matrix decomposition (NMF) algorithm. The HCC samples were split into two subclasses based on the NKs marker genes after it was found that two clusters were the ideal number (Figures 2A, S3). Significant disparities in patient survival existed between the two subgroups (Figure 2B). Additionally, the two subgroups' TME features were contrasted. Figures 2C–E demonstrates that as compared to samples from cluster 2, samples from cluster 1 had higher immunological, stromal, and ESTIMATE scores. HCC patients in Cluster 1 had a larger percentage of immune cell infiltration in their TME, as shown by the results of the TIMER (Figure 2F), MCPcounter (Figure 2G), and CIBIS. ORT (Figure 2H).

3.2 Screening for NK-associated hub genes

Using univariate Cox regression analysis, the prognostic value of these DEGs was evaluated to create a signature, with 10 genes displaying prognostic values (Figure 3A). To reduce the number of genes, Lasso-Cox regression analysis was used (Figure 3B). Seven genes were left with a lambda value of 0.0139 (Figure 3C). After multivariate Cox regression analysis, we finally included these seven genes (CREM, PFN1, KLRB1, TUBA1B, APOC1, ACTG1, and HSPA1A) in the signature. Following is the final seven-gene signature formula: Score = (0.1574×CREM) + (0.4582×PFN1) - (0.3804×KLRB1)+(0.1094×TUBA1B)-(0.0821×APOC1) +(0.3267×ACTG1) + (0.1718×HSPA1A). After each sample's score was determined, the groups of high- and low-risk individuals were created (Figure 3D). The association between score and clinical characteristics was first evaluated and found that higher scores were associated with HBV infection, advanced TNM stage, later grade, later T stage, and recurrence (Figure S4). In both the TCGA, ICGC, and the GEO cohort, Kaplan-Meier survival analyses showed that high-risk patients had considerably worse survival outcomes than low-risk patients (Figures 3E, 4A). The

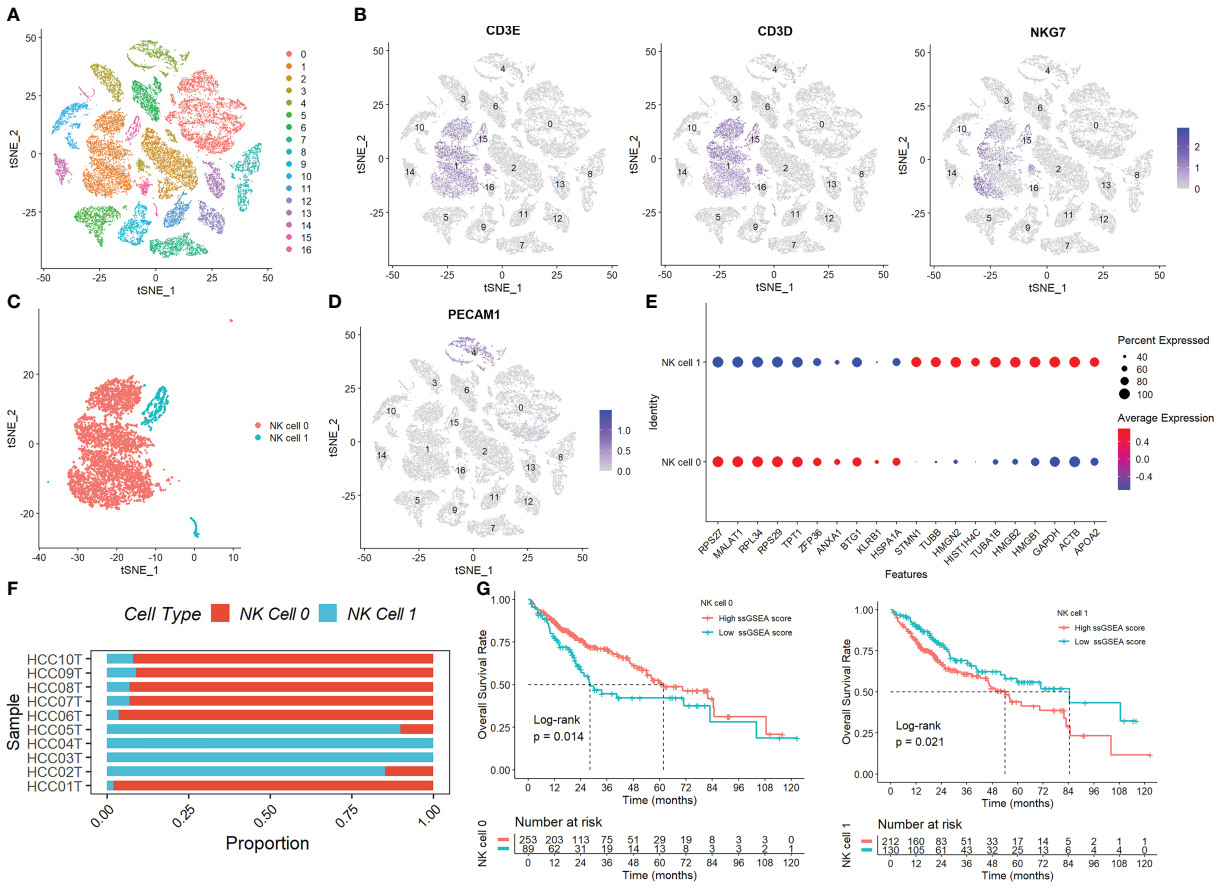

FIGURE 1
Identification of NK cells in the scRNA-seq samples. **(A)** The TSNE plots showed the distribution of the 17 clusters. **(B)** The expression of three marker genes (CD3D, CD3E, and NKG7) in the 17 clusters. **(C)** Two NK cell subsets were discovered. **(D)** The expression of PECAM1 gene. **(E)** The expression of the top 10 DEGs in the two clusters. **(F)** The percentage of the two clusters in each sample. **(G)** The samples in the high ssGSEA score group in the NK 0 cluster had a better prognosis than those in the low ssGSEA score group, while the opposite finding was seen in the NK 1 cluster.

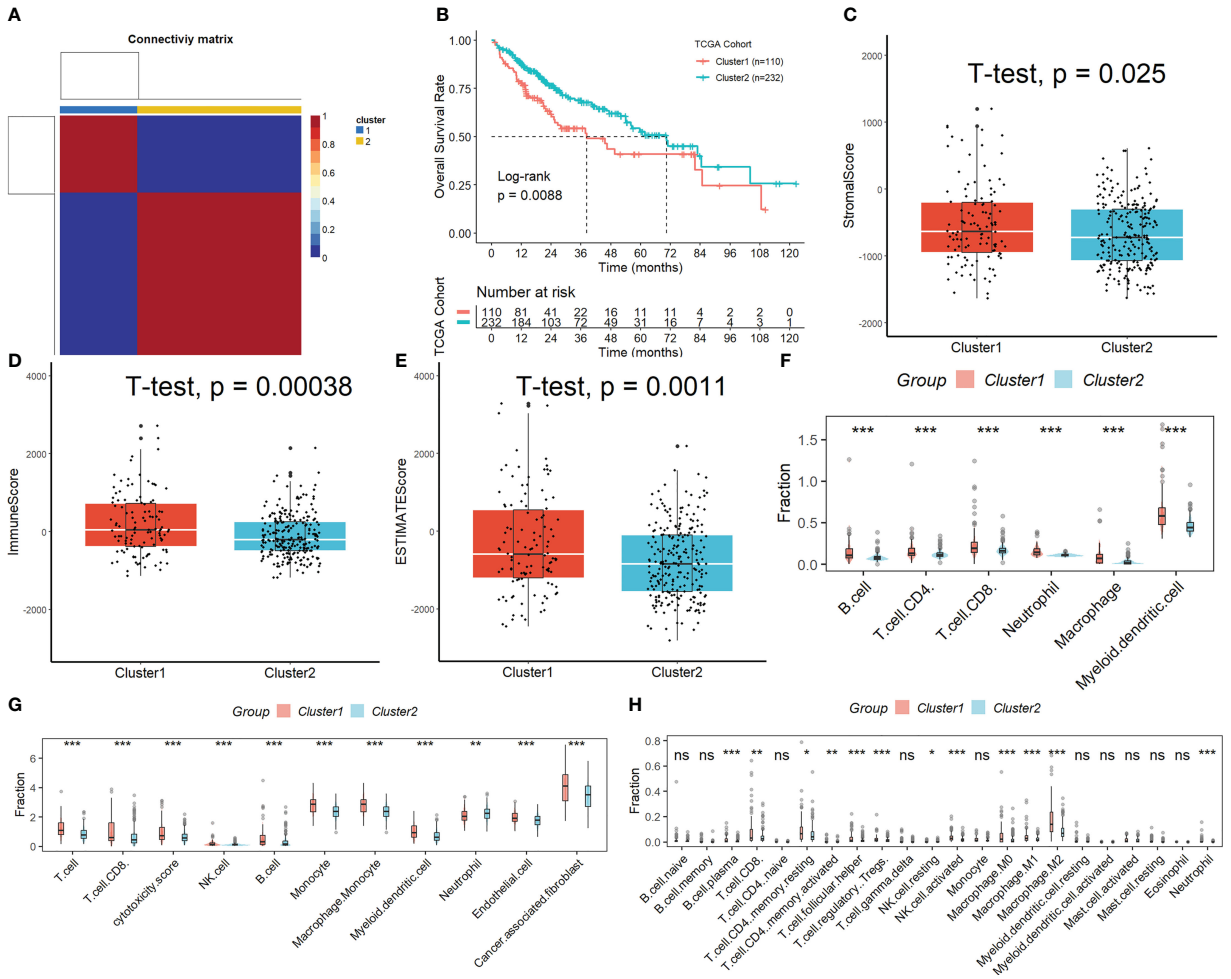

FIGURE 2
Subtypes identification by NMF algorithm based on NKs marker genes. **(A)** Samples were split into two subclasses. **(B)** Significant disparities in patient survival existed between the two subgroups. **(C-E)** Difference of immunological, stromal, and ESTIMATE scores in different molecular subtypes. The difference in the percentage of immune cell infiltration in different molecular subtypes was analyzed by the TIMER **(F)**, MCPcounter **(G)**, and CIBISORT **(H)**. ns, not significant; *p < 0.05; **p <0.01; ***p < 0.001.

TCGA cohort's AUC values for the model for 1- to 3-year survival range from 0.71 to 0.77 (Figures 3F), whereas those for the ICGC and GEO cohorts ranged from 0.57 to 0.72 (Figures 4B).

3.3 Mutation and functional enrichment analysis of the signature

The results of Gene Set Enrichment Analysis (GSEA) revealed that the majority of the impacted HALLMARK, GO, and KEGG components were engaged in DNA synthesis and replication, mitosis, chromosome segregation, and other biological processes related to the cell cycle (Figure S5). Studies on genetic modification that focused on significantly altered genes showed that the mutation rates in the two groups were very different from one another (Figure S6A). After tumor mutational burden (TMB) values for each HCC patient were analyzed, we found that patients in the high-score group with greater TMB values had the lowest overall survival rates, while the opposite results were found in patients in the low-score group with lower TMB values (Figure S6B).

3.4 Correlation analysis between the signature and immunity

Figure 5A showed that samples in the low-scoring group had higher immune, stromal, and ESTIMATE scores compared to samples in the high-scoring group. As shown in the results of TIMER (Figure 5B), MCPcounter (Figure 5C), and CIBISORT (Figure 5D), a greater percentage of immune cell infiltration was found in the TME of HCC patients in the low-scoring group.

3.5 The signature's response to PD-L1 blockade immunotherapy

The Tumor Immune Dysfunction and Exclusion (TIDE) analysis showed that, although the exclusion scores had the opposite impact, the TIDE and dysfunction scores were significantly greater in the group with higher risk scoring than in the group with lower risk scoring (Figure 6A). When the projected

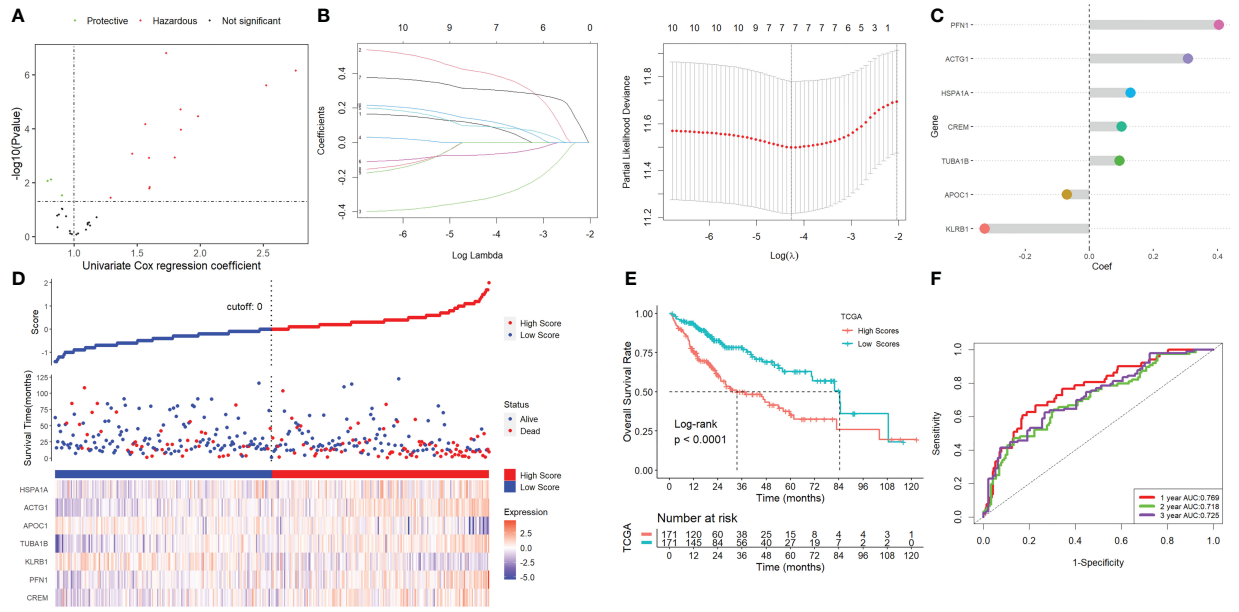

FIGURE 3
Screening for NK-associated hub genes. **(A)** Using univariate Cox regression analysis. **(B)** Lasso-Cox regression analysis. **(C)** Seven genes were left with a lambda value of 0.0139. **(D)** After each sample's score was determined, the groups of high- and low-risk individuals were created. **(E)** Kaplan-Meier survival analysis. **(F)** ROC analysis.

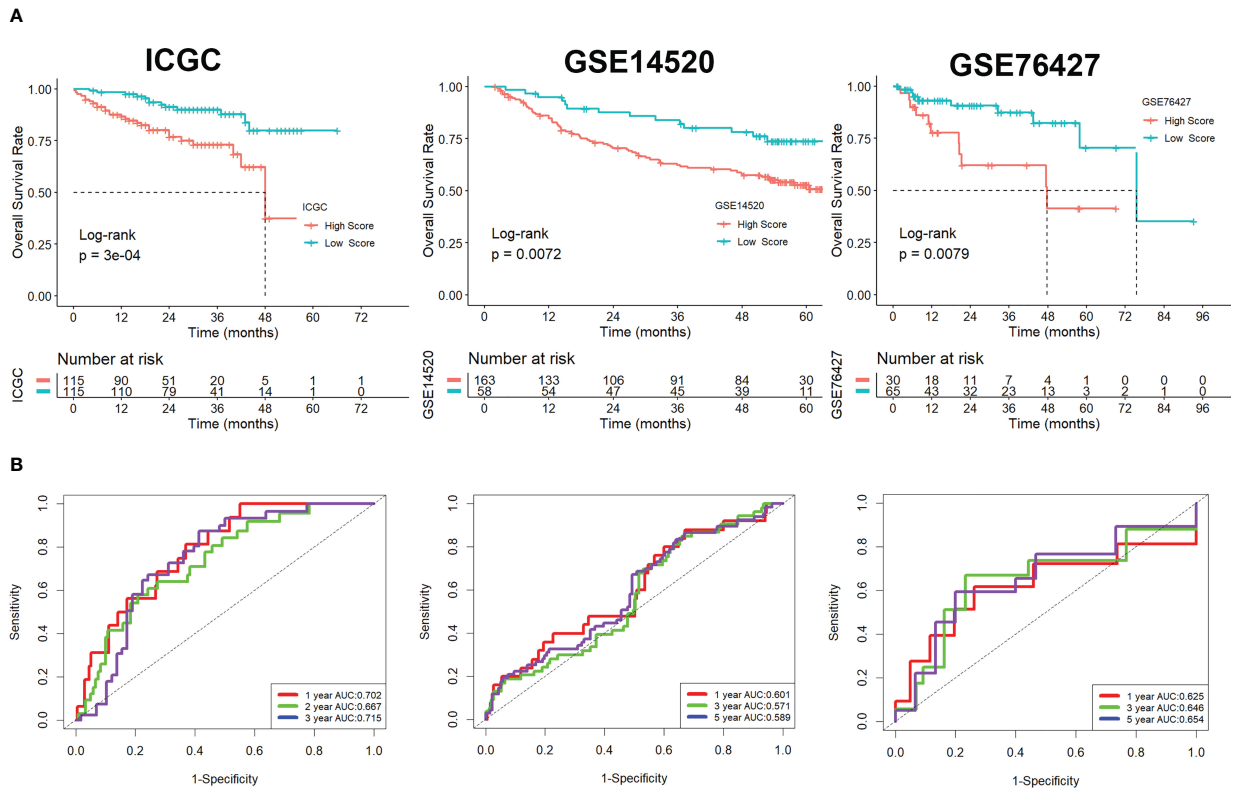

FIGURE 4
Validation of the signature in the ICGC and GEO cohorts. **(A)** Kaplan-Meier survival analysis. **(B)** ROC analysis.

immunotherapy response rate was included, the proportion of "respond" was lower in the high-risk group (Figure 6B). Then, using data from the IMvigor210 cohort, we assessed the predictive efficacy of immune checkpoint treatment risk factors. In comparison to the high-scoring group, patients in the low-scoring group saw notable clinical benefits and considerably longer overall life (Figure 6C). Figure 6D showed that patients with progressing disease (PD) or stable disease (SD) had greater risk ratings than those who had a complete response (CR)/partial response (PR). Finally, we discovered that the levels of the genes PD-1, PD-L1, PD-L2, CTLA4, CD4, CXCR4, LAG3, and LL6 were higher in patients with lower scores than in patients with higher scores (Figure 6E), suggesting that these ICIs may be more beneficial for patients with lower scores.

3.6 Construction of a nomogram model and exploration of potential drug sensitivity

As shown in Figure S7A, we created a nomogram incorporating clinical features and the signature to maximize the predictive performance of risk characteristics. The calibration plots demonstrated that the nomogram was capable of accurately forecasting the final survival rate (Figure S7B). In addition, we identified 7 drugs with tumor sensitivity (Figure S8A). As shown in Figure S8B, we also found that the IC50 for Cladribine, Fludarabine, and Clofarabine was lower in patients with higher scores (20–22).

3.7 TUBA1B expression in HCC

We first initially investigated the expression and prognostic value of these seven genes in HCC in the GEPIA database (23). We found that only TUBA1B showed differential expression in HCC and normal tissues (Figure S9A), although several genes including KLRB1, TUBA1B, APOC1, ACTG1, and HSPA1A had high prognostic values (Figure S9B). We then focused our main attention on TUBA1B. Using the Human Protein Atlas database (HPA) (24), we found significant variability in the protein expression of TUBA1B in normal and HCC tissues (Figure 7A). This phenomenon was confirmed in clinical HCC and normal tissue sections (Figures 7B, C). Last but not least, we found differential expression of CD56 in these HCC tissues (Figure 7D) and a lower number of CD56-positive cells in the HCC tissue sections with high TUBA1B expression (Figure 7E).

4 Discussion

Researchers are learning more about the variety and heterogeneity of TME as well as the molecular properties of tumor-infiltrating immune cells thanks to the quick development of scRNA-seq technology (25). However, the majority of recent research has concentrated on adaptive immune cells, and the function of innate immune cells has not received enough attention, which may have a significant impact on the prognosis

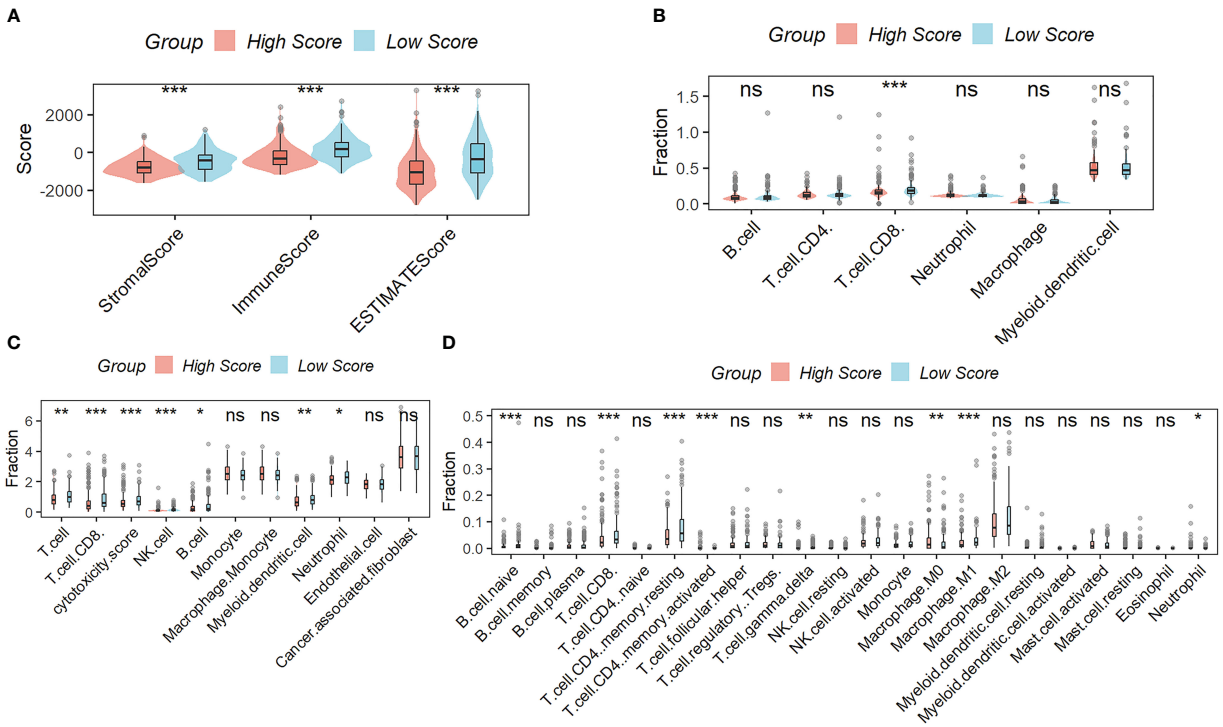

FIGURE 5
Correlation Analysis between the signature and immunity. **(A)** samples in the low-scoring group had higher immune, stromal, and ESTIMATE scores compared to samples in the high-scoring group. The difference in the percentage of immune cell infiltration in different molecular subtypes was analyzed by the TIMER **(B)**, MCPcounter **(C)**, and CIBISORT **(D)**. ns, not significant; *p < 0.05; **p <0.01; ***p < 0.001.

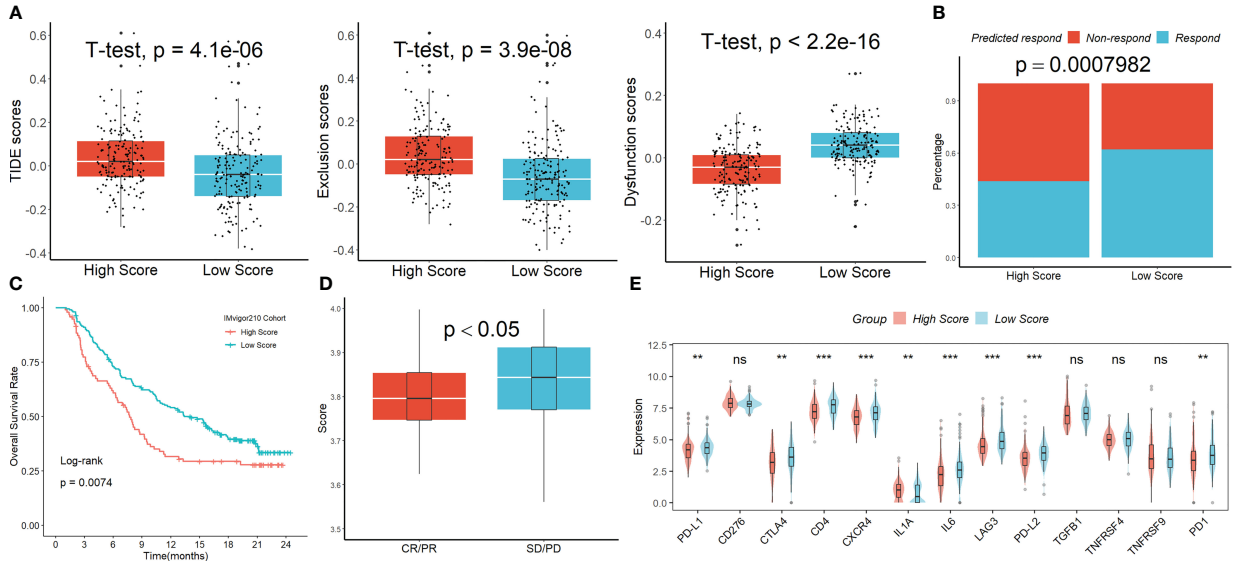

FIGURE 6
The signature's response to PD-L1 blockade immunotherapy. **(A)** The TIDE analysis. **(B)** When the projected immunotherapy response rate was included, the proportion of "respond" was lower in the high-risk group. **(C)** In comparison to the high-scoring group, patients in the low-scoring group saw notable clinical benefits and considerably longer overall life in the IMvigor210 cohort. **(D)** Patients with PD/SD had greater risk ratings than those who had a complete response CR/PR. **(E)** The expression levels of the ICIs genes in the two groups. ns, not significant; **p <0.01; ***p < 0.001.

and effectiveness of immunotherapy in patients with tumors (26). If neighboring cells exhibit surface markers linked to oncogenic transformation, NK cells can quickly destroy many of them by improving antibody and T-cell responses (27). The prognosis of patients with various tumors, including lung adenocarcinoma (28), gastric cancer (29, 30), liver cancer (31), melanoma (32), and colorectal cancer (33), is highly correlated with the number of tumor-infiltrating NK cells. The overall survival of HCC following hepatectomy is significantly impacted by the low frequency of NK cells relative to myeloid and other lymphocytes seen in HCC tumor tissue (34). The number of NK cells inside the TME, which has a favorable correlation with patient survival, also influences how well patients respond to sorafenib therapy (35). In the current work, we aimed to investigate NK cell marker genes in HCC by bulk and scRNA-seq analysis as well as to create a transcriptional signature based on NK cell marker genes to evaluate NK cell infiltration in TME. By boosting NK scores, we discovered a substantial classification of HCC patients' prognosis that was well verified across three separate cohort datasets. Additionally, we discovered that immunotherapy response rates were much greater for patients with low NK scores than for patients with high NK scores, indicating that immune checkpoint blockade treatment is better suitable for individuals with low NK scores.

Immune cells that invade tumors and contribute considerably to tumor growth might have a negative impact on a patient's prognosis if they have HCC (36). By using the TIMER, MCPcounter, and CIBERSORT algorithms to estimate and compare the abundance of immune cell infiltration between high and low NK score populations, we discovered higher levels of immune cell infiltration, particularly T and B cells, in low NK score tumors, indicating that low NK score tumors are referred to as "hot tumors" with increased antitumor activity (37). The greater survival rate of patients with low NK scores may be partially explained by the strong immune cell infiltration, which may promote tumor cell attenuation to avoid immune monitoring and impede tumor development.

Taking into account that variations in immune cell infiltration between different NK score subgroups affect the effectiveness of immunotherapy that patients receive, we first examined the clinical response to immunotherapy in HCC patients using the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm (38). We found significantly higher TIDE scores in the higher NK score group than in the lower NK score group, and a lower proportion of "respond" in the high NK score group. Subsequently, we validated the predictive power of our NK score with an immunotherapy cohort (Imvigor210). We found that patients with progressing disease (PD) or stable disease (SD) had greater risk ratings than those who had a complete response (CR)/partial response (PR). In light of the possibility that complex TME can cause HCC cells to develop resistance to immune checkpoint inhibitors (ICIs), which could affect the efficacy of immunotherapy, we also looked at the differences in the expression levels of various immune checkpoint genes between high- and low-NK scores subgroups. It has been demonstrated that patients with lower scores had larger prevalences of the genes PD-1, PD-L1, PD-L2, CTLA4, CD4, CXCR4, LAG3, and LL6 than people with higher scores. In conclusion, NK scores may be a valid biomarker for predicting response to immunotherapy, and patients with low NK scores are more likely to benefit from it.

Finally, utilizing the CellMiner database, we discovered seven medicines that are tumor sensitive. Since individuals with higher

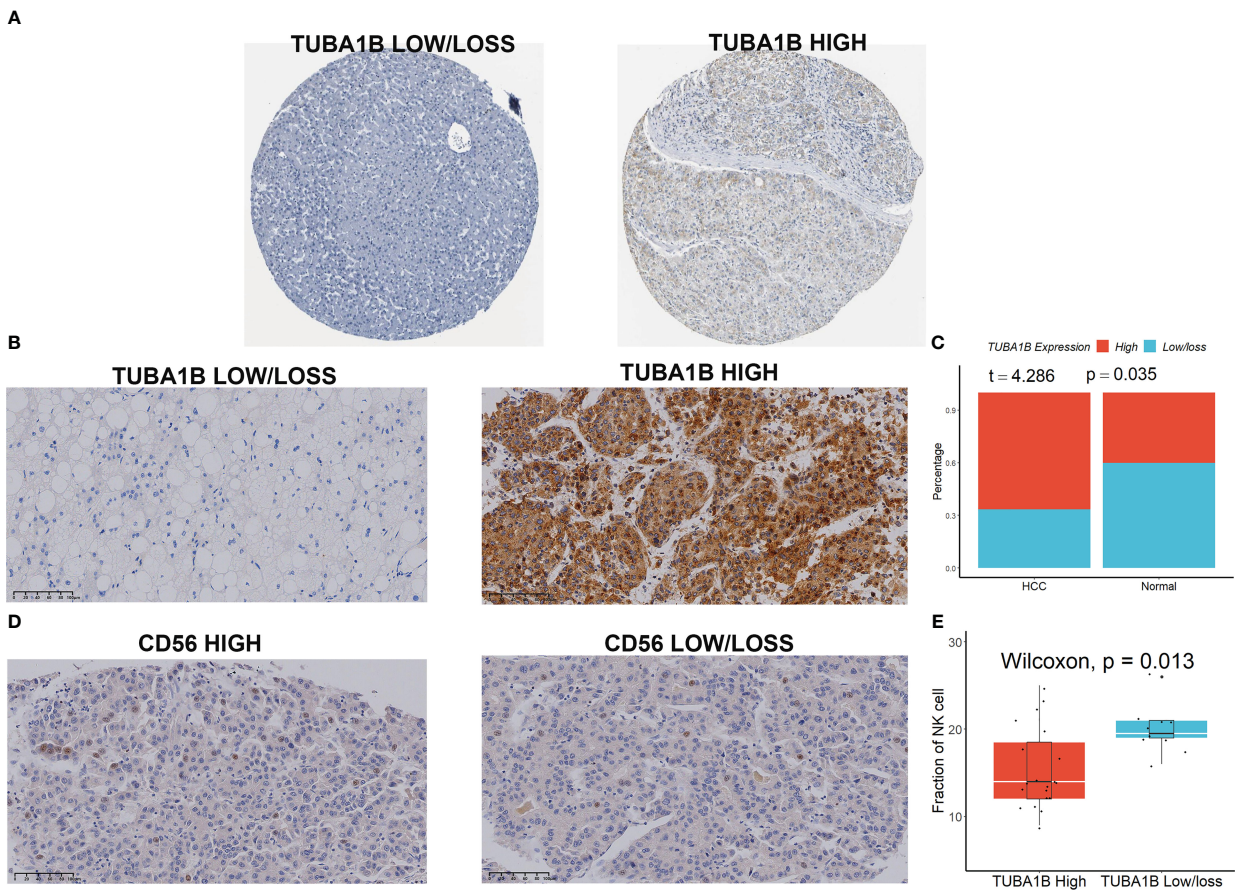

FIGURE 7
TUBA1B expression in HCC. **(A)** TUBA1B expression explored in HPA database. **(B-C)** TUBA1B expression explored by IHC in clinical samples. **(D)** CD56 expression explored by IHC in HCC samples. **(E)** A lower number of CD56-positive cells in the HCC tissue sections with high TUBA1B expression.

NK scores had lower IC50s for Cladribine, Fludarabine, and Clofarabine, it is obvious that these patients are more susceptible to these medications. Cladribine and Clofarabine are nucleoside analogs that are frequently used to treat hematologic cancers and target B and T cells (39, 40). Cladribine has been successfully utilized as a first-line therapy for hairy cell leukemia for some time now (41). Unfortunately, when used to treat multiple sclerosis, cladribine can lead to acute, specific liver harm in individuals (42). Additionally, clofarabine is employed as an anticancer therapy for several solid tumors, including bladder (43), stomach (44), and breast malignancies (45). Since fludarabine dramatically reduces the release of HBV progenitor DNA, it has been used to treat HBV infection and enhance the prognosis of HCC that is related to HBV (46). Combining fludarabine with fusion proteins comprising the poliovirus receptor (PVR) and the programmed death-1 (PD-1) extracellular structural domain improves long-term tumor-specific immunosurveillance and CD8+ T cell-mediated anticancer effects (47). However, additional research is required to confirm if these drugs can eventually increase tumor cell death by targeting NK cells.

Despite the encouraging findings, there are several limitations to this study. First, the candidate genes involved in our study were limited to NK cell marker genes, and the tumor immune microenvironment is highly spatially heterogeneous; second, a sizable multicenter randomized controlled trial will be needed in the future to evaluate this signature. Finally, all mechanistic analyses in our study were descriptive. Future studies must explore the potential mechanisms between NK marker gene expression and HCC prognosis.

5 Conclusion

In summary, a prognostic seven-gene signature built on NK cell marker genes was discovered and proven to have a strong performance in predicting immunotherapy response in HCC patients. It may be used as a prognostic biomarker to aid in the selection of suitable individuals who would benefit from immunotherapy and support therapeutic decision-making about customized prediction.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found within the article/Supplementary Materials.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committees of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

KZ designed this work and analyzed the data; EY performed experiments and analyses; KZ helped for providing tumor samples; KZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1142126/full#supplementary-material

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