IMMUNE REGULATION OF METABOLIC HOMEOSTASIS

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IMMUNE REGULATION OF METABOLIC HOMEOSTASIS

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EDITORIAL
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Editorial: Immune Regulation of Metabolic Homeostasis

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Editorial on the Research Topic

Immune Regulation of Metabolic Homeostasis

The worldwide prevalence of obesity, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes is reaching epidemic proportions, not only in Western societies but also in developing countries (1, 2). Among various pathophysiological underlying mechanisms, the obesity-associated chronic low-grade inflammation, also called meta-inflammation or metaflammation, contributes to the development of insulin resistance and dysregulated glucose/lipid metabolism, ultimately leading to type 2 diabetes, non-alcoholic steatohepatitis (NASH), and associated cardiovascular diseases such as atherosclerosis (3–5). During the last decade, landmark studies in the emerging field of immunometabolism have highlighted the central role played by the immune system in the regulation of metabolic homeostasis in both rodent and humans. Indeed, a growing repertoire of innate and adaptive immune cells have been reported to populate metabolic organs, including adipose tissue, liver, pancreas, skeletal muscle, intestine and some brain areas, and to contribute to tissue-specific maintenance of insulin sensitivity and/or biological functions through complex but yet incompletely understood crosstalk with metabolic cells. Furthermore, the tissue microenvironment (nutrients, metabolites, oxygen,…) also play an important role in the regulation of intrinsic metabolism and functions of resident and/or recruited immune cell subsets within metabolic organs (6, 7). This finely-tuned and dynamic homeostatic system is altered in the context of obesity and metabolic disorders, bringing up the idea that modulation of immune environment in metabolic organs could constitute an attractive new therapeutic approach for alleviating metaflammation and cardiometabolic diseases (8, 9).

The present Research Topic provides a collection of high-quality manuscripts focused on different aspects of the immune regulation of metabolic homeostasis, notably in the context of obesity and type 2 diabetes. This issue comprises twelve manuscripts, including 6 original research articles and 6 reviews, that could be divided in 3 interrelated parts.

The first section is mainly dealing with the role of tissue-resident immune cells, especially from the myeloid lineage, in the pathophysiological regulation of nutrient metabolism and insulin sensitivity in various metabolic organs. As the first one of a series of state-of-the-art reviews, Khan et al. summarize the current knowledge on the changes in immune cell composition in visceral adipose tissue during obesity and aging, and discuss the respective contribution of innate and adaptive immune cell types to tissue dysfunction. In the light of the recent development of single cell technologies that have revealed heterogenous immune cell populations within metabolic organs

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(10–15), Remmerie et al. provide an overview of the distinct macrophage subsets identified in both adipose tissue and liver, and their putative functions during the development of obesity and fatty liver disease. In an extensive review, Orliaguet et al. describe the main underlying molecular mechanisms by which tissue-resident macrophages can be reprogrammed by obesogenic micro-environment in pancreatic islets, adipose tissue and the liver, and how changes in their polarization state can affect organ functions and systemic insulin sensitivity. The role of micro-environment in shaping myeloid cell functions is expanded to dendritic cells by Brombacher and Everts who dissect how changes in local nutrients or oxygen tension could modulate intrinsic metabolic pathways and affect cell differentiation and immunogenicity in pathological conditions like cancer and type 2 diabetes. Focusing on the interaction between adipocytes and invariant natural killer T (iNKT) cells within adipose tissue, van Eijkeren et al. highlight the putative role played by local adipocyte-derived lipids in shaping tissue-resident iNKT cell functions. In an original study, Surendar et al. investigate the role of adipose tissue and liver macrophages in $CD8^+$ T cell functions during weight loss in diet-induced obese mice. Finally, in the last article of this section, Koc et al. report some differences in the transcriptional metabolic signatures of $CD14^+$ and $CD14^-$ peripheral blood mononuclear cells isolated from control subjects and age/weight-matched first-degree relatives of patients with type 2 diabetes.

In a second part, three original manuscripts describe various interventional approaches leading to improvements of metaflammation, insulin sensitivity and/or glucose homeostasis in mouse models of obesity and type 2 diabetes. Parasitic helminths are known to be master regulators of host immune responses through secretion of a portfolio of unique immunomodulatory molecules (16, 17). Interestingly, Khudhair et al. show that gastrointestinal helminth infection with the nematode *Nippostrongylus brasiliensis* induces a potent type 2 immune response in metabolic organs, and reduces body weight, systemic inflammation and hyperglycemia in high-fat diet-fed mice, a beneficial effect associated with changes in gut microbiota composition and increased fecal short chain fatty acid levels. In the second study, Romero-Zerbo et al. report the beneficial effects of the atypical cannabinoid Abn-CBD, a synthetic cannabidiol derivative with immunomodulatory properties, on both systemic and tissue-specific inflammatory parameters in diet-induced obese mice. Finally, Permyakova et al. describe how AN1284, a novel indoline derivative with antioxidant and anti-inflammatory activities, improves whole-body insulin sensitivity, reduces hepatic and renal inflammation and preserves kidney functions in BSK-*db*/*db* mice, a genetic model of diabetic obesity.

Finally, the last section of this Research Topic comprises two review articles focused on bone homeostasis. Benova and Tencerova summarize the current knowledge on the impact of obesity and lifestyle interventions on bone marrow microenvironment and subsequent remodelling of both hematopoietic and mesenchymal stem cells. In a mini-review, Cooney et al. discuss the role played by gut microbiota in the regulation of bone mass, notably through modulation of osteoclastogenesis by local immune cells, and how probiotics may influence this gut-bone axis.

Overall, the studies presented in this Research Topic highlight the central role played by the immune system in the (dys) regulation of many aspects of metabolic homeostasis, at both organ and systemic levels, especially during the development of obesity, type 2 diabetes and associated inflammatory-driven comorbidities. The expanding field of immunometabolism is expected to i) contribute to a better understanding of the role and functions of a growing numbers of tissue-specific innate and adaptive immune cell subsets, and ii) pave the way to the development of new immunomodulatory therapeutic strategies for the treatment of cardiometabolic diseases.

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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REVIEW
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Mechanisms of Macrophage Polarization in Insulin Signaling and Sensitivity

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Type-2 diabetes (T2D) is a disease of two etiologies: metabolic and inflammatory. At the cross-section of these etiologies lays the phenomenon of metabolic inflammation. Whilst metabolic inflammation is characterized as systemic, a common starting point is the tissue-resident macrophage, who's successful physiological or aberrant pathological adaptation to its microenvironment determines disease course and severity. This review will highlight the key mechanisms in macrophage polarization, inflammatory and non-inflammatory signaling that dictates the development and progression of insulin resistance and T2D. We first describe the known homeostatic functions of tissue macrophages in insulin secreting and major insulin sensitive tissues. Importantly we highlight the known mechanisms of aberrant macrophage activation in these tissues and the ways in which this leads to impairment of insulin sensitivity/secretion and the development of T2D. We next describe the cellular mechanisms that are known to dictate macrophage polarization. We review recent progress in macrophage bio-energetics, an emerging field of research that places cellular metabolism at the center of immune-effector function. Importantly, following the advent of the metabolically-activated macrophage, we cover the known transcriptional and epigenetic factors that canonically and non-canonically dictate macrophage differentiation and inflammatory polarization. In closing perspectives, we discuss emerging research themes and highlight novel non-inflammatory or non-immune roles that tissue macrophages have in maintaining microenvironmental and systemic homeostasis.

Keywords: macrophage, inflammation, type-2 diabetes, adipose tissue, liver, pancreas, immunometabolism

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INTRODUCTION: INFLAMMATION IN INSULIN SECRETION, SENSITIVITY AND RESISTANCE

Type-2 diabetes (T2D) is a disease with dual etiologies, inflammatory, and metabolic. Over the past 20 years, inflammation has gained increasing recognition for the important role it plays in increasing risk of insulin resistance and can be seen as an aetiological starting point for metabolic decline. Several studies have attempted to define the kinetics between inflammation and insulin resistance, where some report local insulin resistance preceding inflammation (1) and others reporting inflammation prior to insulin resistance (2). However, blunting inflammatory responses has consistently been reported as metabolically protective, mitigating the development of insulin resistance and T2D. Thus, inflammation is seen decisive factor in losing tolerance to metabolic

dysregulation. Insulin resistance in the liver, adipose tissue and skeletal muscle is initially met with a burst of activity from the pancreas that maintains normal levels of glycaemia (the pre-diabetic stage) (3–5). When this stage is prolonged and insulin production can no longer meet demands, frank T2D develops and predisposes individuals to a variety of complications and comorbidities (**Figure 1**). These complications and comorbidities are broadly hepatic and cardiovascular in nature and are directly related to increasing inflammation, hyperglycaemia, and dyslipidemia. The following review addresses the various mechanisms and roles of inflammation in the development of T2D with a particular focus on the liver, adipose tissue and the pancreas.

Inflammation and Metabolic Health

The first evidence linking inflammation to metabolic health dates back to 1993 when Gokhan Hotamisligil and Bruce Spiegelman discovered the increasing expression of pro-inflammatory cytokine tumor necrosis factor (TNF)-α in adipose tissue (AT) of rodent models of obesity (6). Neutralizing TNF-α in obese rats led to a significant increase in glucose uptake in response to insulin. Their study showed that blocking a single cytokine can restore insulin sensitivity. A decade later, macrophages were identified as the main source of TNF-α and other pro-inflammatory molecules (IL-6 and iNOS) in obesity (7). Moreover, macrophages drastically accumulate in adipose tissue during obesity and at the onset of insulin resistance. These early studies brought-to-light the contribution of inflammation to metabolic decline associated with insulin resistance and T2D.

Since these findings, the immune system has gained considerable attention as a major regulator of metabolic homeostasis. Innate immune cells, namely macrophages, reside in all the metabolic tissues that coordinate glycemic homeostasis, namely AT, liver, and pancreas. Tissue-resident innate immune cells form a *bona fide* tissue-specific immune niche, with each niche having its particularities to cope with microenvironmental cues. Macrophages are by far the most studied and proportionally numerous innate immune cell type [25% of AT innate immune cells (8), 20–35% of the non-parenchymal hepatic cells in the liver (9), up to 90% of immune cells in pancreatic islets (10)].

Macrophage Polarization: Regulation of Acute and Chronic Inflammation

Macrophages were firstly identified by Ellie Metchnikoff as phagocytic cells. They form part of the myeloid lineage and are capable of rapidly mounting non-specific responses to a wide range of pathogens. Phagocytosis is a cellular process associated with innate immune responses to pathogens, is critical in the clearance of cellular debris, tissue repair, and maintaining tissue homeostasis throughout the organism. Tissue-resident macrophages develop from progenitors in the yolk sac, fetal liver, and from circulating monocytes that originate in bone marrow (11). Under physiological conditions, tissue-resident macrophages play a key role in the maintenance of the integrity and homeostasis of their respective tissues.

Macrophages quickly respond to environmental cues and consequently adapt their function, they sense changes in their microenvironment through cell surface receptor engagement. The main receptors relaying environmental signals are toll-like receptors (TLRs), which form part of the larger family of pattern recognition receptors (PRRs). Ligation of TLRs/PRRs by damage- or pathogen-associated molecular patterns (DAMP/PAMPs) present in the microenvironment activates transcriptional programs in macrophages to mount an adapted functional phenotype (12). Whilst these transcriptional mechanisms have been well-described (and are addressed in this review), macrophages also extensively adapt their cellular metabolism to meet the bioenergetic needs and optimize effector function (13). The latter has gained much attention in recent research.

A dichotomy is currently used to describe macrophage polarization states: M1 as pro-inflammatory or classically activated vs. M2 as anti-inflammatory or alternatively activated (**Figure 2**). The nomenclature of these subsets derives from

Abbreviations: 2-DG, 2-deoxyglucose; ABCA/G1, ATP-binding cassette transporter; ACLY, ATP citrate lyase; AMPK, AMP-activated protein kinase; AP-1, Activator protein 1; APOA1, Apolipoprotein A1; ARG1, Arginase 1; AT, Adipose tissue; ATM, Adipose tissue macrophage; ATP, Adenosine triphosphate; CARKL, carbohydrate kinase like (sedoheptulose kinase); CCL2, Chemokine (C-C motif) ligand 2/monocyte chemoattractant protein 1; CCR2, C-C chemokine receptor 2; CD, Cluster of differentiation; Clec4F, C-type lectin domain family 4 member F; CLS, Crown-like structure; COX2, Cyclooxygenase 2; DAMP, Damage-associated molecular pattern; ETC, Electron transfer chain; EZH2, Enhancer of zeste homolog 2; FA, Fatty acid; FAS, Fatty acid synthase; FATP, Fatty acid transporter protein; FOXO, Forkhead box protein O; GC, Glucocorticoids; GLUT1, Glucose transporter 1; GPS2, G Protein Pathway Suppressor 2; GR, Glucocorticoid receptor; GRIP1, Glutamate receptor interacting protein 1; HDAC, Histone deacetylase; HFD, High-fat diet; HIF1, Hypoxia inducible factor 1; HK, Hexokinase; HRE, Hypoxia response element; HSC, Hepatic stellate cell; IFN, Interferon; IGFBP-7, Insulin-like growth factor binding protein 7; IKK, IκB Kinase; IL, Interleukin; iNOS, inducible nitrous oxide synthase; IRAK, Interleukin-1 receptor associated kinase; IRF, Interferon regulatory factor; IRS, Insulin receptor substrate; JAK, Janus Kinase; JNK, c-Jun N-terminal kinase; KC, Kupffer cell; KDM6B, Lysine demethylase 6B; KLF4, Kruppel-like factor 4; LPL, Lipoprotein lipase; LPS, Lipopolysaccharides; LXR, Liver-X-receptor; Ly6c/g, Lymphocyte antigen 6 c/g; MAPK, Mitogen activated protein kinase; miRNA/miR, Micro RNA; Mme, metabolically activated macrophage; MSR, Macrophage scavenger receptor; MyD88, Myeloid differentiation primary response 88; NADH, Nicotinamide adenine dinucleotide + hydrogen; NADPH, Nicotinamide adenine dinucleotide phosphate; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; NCoR, Nuclear receptor corepressor; NFκB, Nuclear factor-kB; NLRP3, NACHT, LRR and PYD domains-containing protein 3; NO, Nitrous oxide; NR, Nuclear receptor; OPN, Osteopontin; OXPHOS, Oxidative phosphorylation; PAMP, Pathogen associated molecular pattern; PD1, programmed cell death protein 1; PDGF-R, Platelet-derived growth factor receptor; PDH, Pyruvate dehydrogenase; PFK/uPFK, Phosphofructokinase/ubiquitous Phosphofructokinase; PGC-1a, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PKM2, Pyruvate kinase isozyme M2; PPAR, Peroxisome proliferator-activated receptor; PPP, Pentose phosphate pathway; PRR, Pattern recognition receptor; ROS, Reactive oxygen species; SDH, Succinate dehydrogenase; SLC1A5, Solute carrier family 1 member 5; SMRT, Silencing mediator for retinoid or thyroid-hormone receptor; SOCS, Suppressor of cytokine signaling; SREBP, Sterol regulatory element binding protein; STAT, Signal transducer and activator of transcription; T2D, Type-2 diabetes; TCA, Tricarboxylic acid; TGF, Transforming growth factor; Th, T-helper; TIRAP, Till-interleukin 1 receptor domain containing adaptor protein; TLR, Toll-like receptor; TNF, Tumor necrosis factor; TRAM, TRIF-related adaptor molecule; Treg, Regulatory T-cell; TREM2, Triggering receptor expressed on myeloid cells 2; TRIF, Toll-interleukin receptor adaptor inducing interferon-B; UDP-GlcNac, Uridine diphosphate N-acetylglucosamine; VASP, Vasodilator-stimulated phosphoprotein; VLDL, Very-low density lipoproteins.

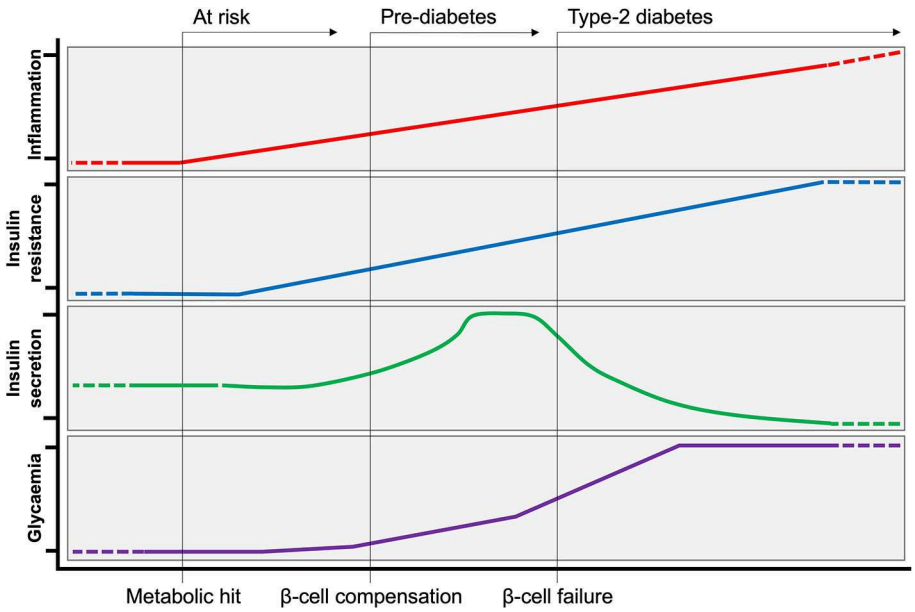

FIGURE 1 | Evolution of Type-2 Diabetes. Following a metabolic hit, inflammation is at the initial steps of developing type-2 diabetes (T2D). Peripheral insulin resistance develops in tandem with increasing inflammation. Insulin resistance is initially met by a compensatory response from the pancreas, producing more insulin to maintain normoglycemia (pre-diabetes). Over time, insulin producing β-cells can no longer cope with increased demand, and insulin production ceases (β-cell failure). At this stage of persistent hyperglycaemia T2D is established.

the type-1 or type-2 immune responses canonically associated with signaling molecules released upon polarization. Macrophage signaling also polarizes the adaptive immune compartment to maintain a chronic T helper (Th)1/17 or Th2 response. The M1 polarization state is associated with a type-1 response (Th1/17) and the production of pro-inflammatory mediators associated with bacterial or viral responses. M1 macrophages have strong microbicidal and antigen presenting capacities. They produce powerful pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and reactive oxygen species (ROS). M2 macrophages elicit type-2 signaling, typically in response to extracellular pathogens (helminths, parasites), producing anti-inflammatory mediators such as IL-10 and TGF-β. M2 polarization is also considered a pro-resolution response, associated with later stages of resolving inflammation. The adaptive immune system appropriately undergoes Th2 polarization producing regulatory and remodeling cytokines such as IL-4, IL-5, and IL-13. Accordingly, the immunoregulatory response has been attributed to the specialized regulatory T-cells (T_{Reg}) subpopulation. The pro-resolution response can manifest as scarring or tissue remodeling, which when aberrant causes tissue fibrosis, type-2 effector molecules also exacerbate allergic responses (14). Whilst the discrete M1 and M2 classification remains in use today, underlying this dichotomy exists a continuum of diverse responses and intermediate macrophage phenotypes. Novel functional classifications represent polarized macrophages along a sliding scale between M1 and M2 depending on chemokine/cytokine secretion, transcription factor engagement and more recently on the cellular metabolic phenotype (15). The rise of single-cell sequencing and of mass cytometry (CyTOF) are coming a long way to deciphering the functional diversity and plasticity of macrophages (16).

TISSUE MACROPHAGES IN METABOLIC PHYSIOLOGY AND PHYSIOPATHOLOGY

Efficient communication between insulin secreting and insulin target tissues (the pancreas, adipose tissue, the liver, and skeletal muscle) maintains metabolic homeostasis in response to physiological challenges that transiently vary glycaemia or lipaemia, such as feeding or fasting (3–5). Insulin resistance represents a partial breakdown in communication between these tissues, where insulin target tissues become resistant to insulin signaling, despite initial compensation by the pancreas. T2D represents a stage of complete to near-complete breakdown of communication where production of insulin no longer meets the body's requirement to regulate glycaemia. Each of these tissues has its specialized niche of macrophages with important physiological functions maintaining tissue integrity, more importantly the tissue macrophage population undergoes adaptation at each stage of developing T2D (3–5, 17, 18). The tissue macrophage responses have been shown to be extremely powerful mediators of insulin signaling, sensitivity, and resistance (**Figure 3**).

Pancreatic Islets Macrophages

Pancreatic islets, distributed within the exocrine pancreas, are micro-organs essential for systemic glucose homeostasis. β cells form the majority of the islet and respond to glucose, within

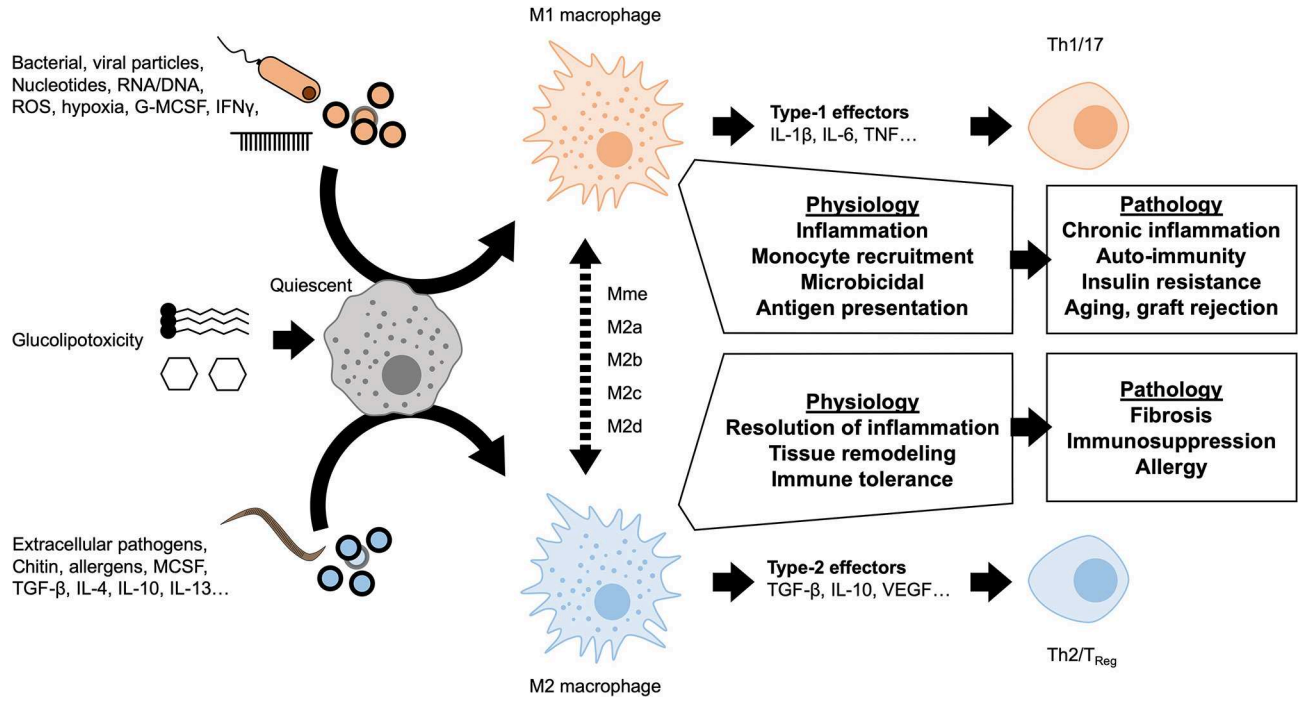

FIGURE 2 | Macrophage polarization and chronic inflammation. A variety of stimuli are known to induce either M1-like pro-inflammatory or M2-like anti-inflammatory polarization. These polarization states represent the extremes of a spectrum of activation profiles, dependent on nature of the stimulus and microenvironmental factors. Self-resolving inflammation is transient and considered a necessary physiological response in maintaining homeostasis and host-defense. If polarization is persistent, downstream signaling from macrophages also leads to lymphocyte polarization. Sustained dysregulated macrophage activation and lymphocytic polarization are important parts of a number of pathologies.

seconds, by secreting the appropriate amount of insulin required for optimal energy supply to insulin-sensitive tissues. Innate immune cells also form part of the pancreatic islet. Under steady-state, macrophages are the major innate immune cell in both mice and humans (10, 19–21). Over 20 years after their discovery, islet macrophage phenotype remains unclear. Unlike ATMs and liver macrophages, islet macrophages do not adhere to the M2 vs. M1 polarization paradigm associated with metabolic protection and dysfunction, respectively. Indeed, M1 markers (CD11c, MHC-II) are constitutively expressed by macrophages in healthy islets, they also highly express IL-1β, TNF-α, and the pro-inflammatory transcription factor interferon regulatory factor (IRF)-5 (10, 19, 22). Moreover, they do not express M2 markers (CD206), in contrast to stromal macrophages of the exocrine pancreas (19).

The role of macrophages in islet homeostasis has only begun to draw attention. *In situ* islet imaging revealed that macrophages are in close contact with both β cells and vasculature, in mice (23). Islet macrophages monitor β cell insulin secretion in response to glucose by detecting endogenous ATP that is co-released with insulin (24). In turn, macrophages may also directly provoke or enhance insulin secretion through production of factors such as retinoic acid (10). Interestingly, relative to any other tissue, β cells have the highest expression of the signaling IL-1 receptor 1 (IL-1R1), strongly indicating a physiological role for IL-1β in β cell function (25, 26). It is well-established that acute, but not chronic, exposure to IL-1β stimulates insulin secretion in mice and humans (27, 28). Underlying mechanisms remain unclear, but may involve an increase in insulin granule docking at the plasma membrane allowing enhanced exocytosis (27). Two studies confirm this hypothesis with transgenic murine models. β cell-targeted deletion of IL-1R1 impairs peripheral glucose tolerance *via* reduced glucose-stimulated insulin secretion (29). Other studies report that feeding induces a physiological rise in circulating IL-1β, potentiating postprandial insulin secretion (30). IL-1β secretion was attributed to peritoneal macrophages responding to glucose metabolism and bacterial products, released IL-1β in-turn acts on β cells (30). It has not been ruled out that islet-resident macrophages may also produce IL-1β post-prandially, indeed these macrophages may be the main source of IL-1β in the islet microenvironment. Taken together, these previous reports show that physiological IL-1β levels play a critical role in amplifying insulin secretion.

During obesity, increased production of insulin is required to maintain normal blood glucose levels. As a result, the number of β cells and islet size increase, mainly by local proliferation of pre-existing β cells (**Figure 3**). Therein, macrophages slowly accumulate and may play an important role in β cell adaptation to early weight gain and the development of insulin resistance. In that context, islet macrophages may license β cell mass expansion and the required angiogenesis during the first weeks of high fat diet and in early islet adaption of young Db/Db mice. Indeed, macrophage-depleted mice showed lower β cell replication rate,

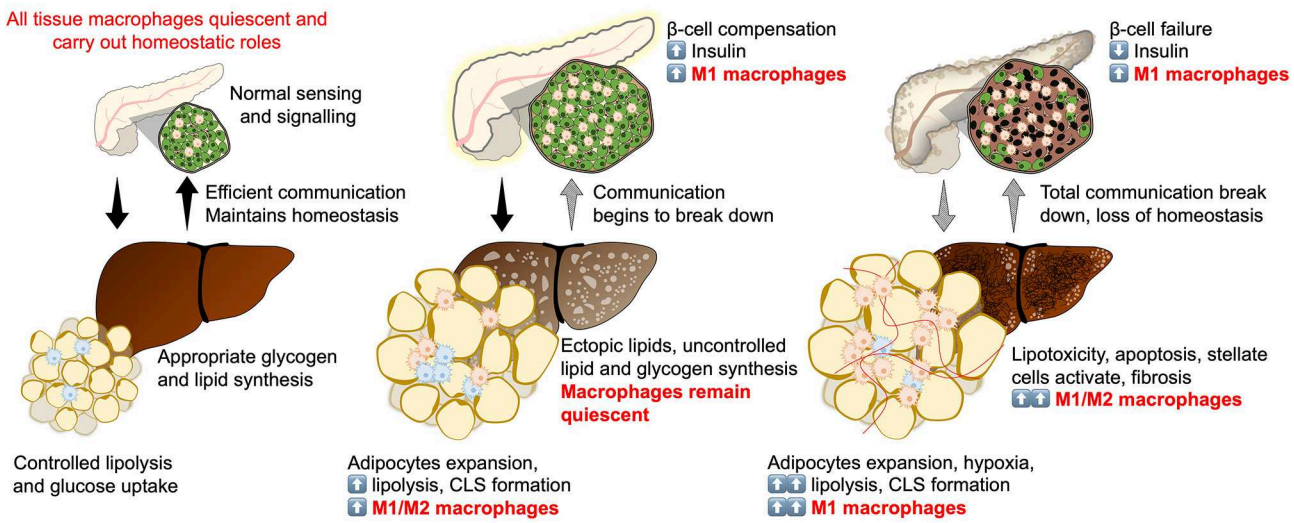

FIGURE 3 | Break down of insulin secretion and sensitivity in type-2 diabetes. At the physiological state glycaemic homeostasis is maintained by efficient communication between the insulin secreting organ, the pancreas, and insulin target organs (adipose tissue and liver). All tissues are populated by their respective tissue macrophages that participate in maintaining tissue homeostasis and physiological function. Insulin resistance is a breakdown of communication at insulin target tissues. At the onset of insulin resistance, macrophages accumulate in adipose tissue and pancreatic islets. Crown-like structures develop in adipose tissue with heterogeneously polarized macrophages and a decrease in adipocyte insulin sensitivity. Pancreatic islet size increases with increased β-cell number and increased macrophages. Increase in β-cell number allows compensatory insulin release to overcome insulin resistance. Increasing insulin resistance and systemic inflammation result in β-cell failure when insulin secretion no longer compensates for resistance and persistent hyperglycaemia develops. At the stage of type-2 diabetes, a complete breakdown in inter-organ communication occurs, insulin secretion drops and inflammatory macrophages permeate adipose tissue and the liver. Chronic inflammation, hyperglycaemia and dyslipidemia lead to the development of non-alcoholic steatohepatitis.

decreased insulin secretion and impaired glucose tolerance compared to controls (31). The promotion of β cell proliferation by islet macrophages could be mediated by the platelet-derived growth factor receptor (PDGF-R) signaling pathway (32).

When obesity becomes chronic, insulin secretion eventually no longer compensates for increased insulin demands, resulting in hyperglycemia and T2D. This β cell failure is associated with local islet inflammation and production of inflammatory effectors (IL-1β, TNF-α, CCL-2) (20, 26, 32–37). This phenomenon is associated with increased macrophages in the islet in diet-induced or genetically obese rodents and in patients with T2D (20, 31–33, 35, 37). Two distinct subsets of macrophages have been identified in the islet: resident macrophages and pro-inflammatory macrophages. Islet-resident macrophages ($CD11b^{+}Ly6C^{-}$ or $F4/80^{high}CD11c^{low}$) predominate at steady-state and pro-inflammatory macrophages ($CD11b^{+}Ly6C^{+}$ or $F4/80^{low}CD11c^{high}$) accumulate during the course of obesity (32, 33). While $CD11b^{+}Ly6C^{+}$ macrophages are recruited from monocytes, $F4/80^{low}CD11c^{high}$ macrophages proliferate *in situ*. In this context, chlodronate liposome macrophage depletion rescues glucose-induced insulin secretion in models of genetic obesity and in palmitate-infused mice (33). Interestingly, despite increasing islet macrophage number, diet-induced obesity does not markedly alter macrophage phenotype (19, 32). Another source of inflammatory factors that may participate in islet inflammation are endocrine cells themselves, including β cells. Indeed, RNA sequencing of islet cells from T2D patients revealed an inflammatory signature associated with β cell dysfunction relative to islet cells from healthy controls, this result was attributed not only to immune cells but also to endocrine cells fuelling local inflammation (38, 39). These results, somewhat contradictory, suggest that islet macrophages are not solely responsible for islet inflammation in obesity. More studies are required to fully define their phenotypes and to investigate the roles that other innate immune cells may play, such as innate lymphoid cells (ILC) and their potential role in regulating insulin secretion and β cell mass expansion (10).

Adipose Tissue Macrophages in Metabolic Homeostasis

AT is one of the first responders to alterations in energy balance. Physiologically AT regulates long term energy stores, appetite (through endocrine signaling) and body temperature (by providing insulation or even increasing thermogenesis in the case of brown adipose tissue). Adipose tissue macrophages (ATMs) generally present an M2 profile at steady state under physiological circumstances. They are characterized by expression of the mannose receptor CD206, CD301 alongside pan-macrophage markers such as F4/80 (in mice) CD14 (in humans) CD68 and CD11b. ATM homeostatic signaling includes expression of arginase 1 (ARG1), IL-10, and other type-2 effectors as well as catecholamines. The transcription factor peroxisome proliferator-activated receptor (PPAR)-γ is highly expressed in these cells and controls ATM oxidative metabolism and

capacity to cope with a lipid-rich environment. In this niche, ATMs interact with other immune cells and provide signals for activation or repression of B and T cells, neutrophils, natural killer cells, and ILCs (40).

ATMs maintain tissue homeostasis by removing dying adipocytes and debris from dead cells; this efferocytotic process maintains an anti-inflammatory environment. Indeed, murine adipose tissue presenting an excessive rate of dying adipocytes due to targeted activation of caspase 8 are characterized by an increased number of alternatively activated anti-inflammatory macrophages (M2, $CD206^+$), surrounding dead and dying adipocytes (41). This grouping of cells surrounding adipocytes in a ring-like structure are named crown-like structures (CLS). CLS are only occasionally found in lean AT. Under physiologic variation, AT homeostasis is challenged daily with periods of feeding, thus expansion and storage of lipids, or mobilization of stored-lipids during fasting or cold exposure. ATMs have enhanced lipid buffering capacities and this enables capturing lipids released from the dead adipocytes, also during physiological process such as weight loss, fasting-induced lipolysis (42), or thermogenesis (43). Interestingly, in obesity macrophage-mediated capture of excess lipids regulates systemic glucose tolerance. Lipids are stored within the macrophages and released into circulation in a controlled manner (44).

ATM lipid-buffering processes limit ectopic lipid storage, pro-inflammatory accumulation of lipids and systemic lipotoxicity/dyslipidemia. A program of lysosomal activity is activated in M2 ATMs to cope with environmental lipid overload. Interestingly, inhibition of lysosome biogenesis and consequently lipid accumulation and catabolism in ATMs decreases adipocyte lipolysis (45). More recently, novel pathways of lipid release independent of canonical lipolysis, have been described. Adipocytes release exosome-sized lipid-filled vesicles to be taken-up and stored by ATMs (46). The capture of lipids is facilitated by ATM expression of fatty acid transporter (CD36) and the lipid scavenger receptor MSR1 (45).

Much of the knowledge with regards to macrophage interactions with environmental lipids and their mechanisms of activation has come from the fields of atherosclerosis and the study of foam cells. Indeed, early studies carried out by Nagy et al. (47) brought to light the importance of such receptors as CD36, allowing macrophages to internalize oxidized lipids, which in turn act as nuclear receptor ligands (PPARγ in this case). The mechanisms described by Nagy et al. were amongst the earliest to elucidate links between metabolic stress, transcriptional regulation, and macrophage phenotypic plasticity.

The role of ATMs in thermogenesis is an emerging topic and pathways leading to the activating of ATMs are still under investigation (40). A novel population of macrophages involved in adipose tissue thermogenesis has been identified: sympathetic neuron associated macrophages (SAM) (48). These cells are morphologically different from ATMs and are located at fibers of the sympathetic nervous system in AT. Unlike ATMs, SAMs have the molecular machinery to uptake and catabolize norepinephrine which blunts catecholamine-induced lipolysis.

ATMs have also been associated with iron homeostasis, where intracellular iron is a source of free radicals and a cofactor for a number of proteins. Twenty-five percent of macrophages from lean adipose tissue are considered as ferromagnetic, i.e., iron-loaded and this proportion decreases with obesity (49). ATM iron recycling contributes to AT homeostasis, where an up-regulation of iron-related genes occurs during adipogenesis and an excess of iron contributes to adipocyte insulin resistance (50, 51).

ATMs play a more direct role in adipogenesis where alternatively activated macrophages form a niche for the development of adipocytes and in the vascularization of adipose tissue (52, 53). The accumulation of M2 ATMs in the CLS surrounding dead adipocytes leads to the recruitment of pre-adipocytes in response osteopontin (OPN). However, a recent study demonstrated that M2-like ATMs inhibit the proliferation of adipocyte progenitors through TGF-β signaling. A hallmark study by Buorlier et al. (53) characterized subcutaneous ATMs as being predominantly $CD206^+$, and to be the major source of matrix degrading enzymes, making them an essential part of tissue remodeling. In this same study, secreted factors from ATMs were found to promote angiogenesis and inhibit adipogenesis in stromal-vascular fraction progenitor cells (53, 54). Controlling angiogenesis is a key factor in the maintenance of tissue homeostasis as it limits the formation of hypoxic areas and insures appropriate irrigation supplying nutrients and oxygen to the microenvironment. In the light of the above work, the physiological phenotype of ATMs can be largely seen as protective and may, in the early stages of caloric excess, act to coordinate adipose tissue adaptation (53).

Finally, alternatively activated ATMs are characterized by their production of IL-10, an anti-inflammatory cytokine known for its important role as a modulator of insulin sensitivity (55). Indeed, acute IL-10 treatment improves global insulin sensitivity *in vivo* (56) and its expression is positively correlated with insulin sensitivity in humans (57). Surprisingly, the hematopoietic deletion of IL-10 does not promote obesity nor insulin resistance, suggesting that other factors and pathways are involved in the maintenance of AT metabolic health (58). Furthermore, ATMs can release exosomes containing miRNA, such as miR-155, that regulate insulin sensitivity. Such ATM-derived exosomes from lean mice improve glucose intolerance and insulin sensitivity when delivered to obese mice (59).

Adipose Tissue Macrophages and Metabolic Inflammation

Obesity is a complex pathology and a factor in the etiology of insulin resistance and T2D. The fundamental cause of obesity is chronic imbalance between energy expenditure and food intake leading to low-grade inflammation. Chronic low-grade inflammation is what is generally referred to when discussing metabolic inflammation, the starting point of which is the adipose tissue macrophage. An accumulation of inflammatory ATMs occurs in obesity and plays a key role in the pathogenesis of obesity-induced insulin resistance (**Figure 3**) (6, 7). Inflammatory ATMs correspond to the M1 subtype and are identified as $F4/80^+CD11b^+$ cells, also positive for CD11c and overexpressing IL-6, TNF-α, iNOS and the C-C chemokine receptor 2 (CCR2).

ATM accumulation in obesity occurs first due to *in situ* proliferation at CLS, and then by recruitment of circulating monocytes that differentiate into inflammatory macrophages (60). The first proliferative phase is driven by IL-4 signaling through Signal Transducer and Activator of Transcription (STAT)-6. Infiltrating macrophages increase upon CCL2 signaling to monocytes, several studies have demonstrated the importance of the CCR2/CCL2 axis in the recruitment of circulating monocytes (61). In addition, migratory capacity of macrophages is affected by obesity. Indeed, netrin-1, a laminin-related molecule known for its chemo-attractant/-repulsive properties, is induced by palmitate. It inhibits ATM migration to lymph nodes and consequently promotes ATM accumulation *in situ* (62).

The lipid-buffering capacity of ATMs is beneficial in early dysmetabolism and enhances a lysosomal program associated with M2 polarization (45), the abundance of lipids within ATMs impacts their polarization toward an M1 phenotype (63). Single-cell transcriptomic approaches confirm the heterogeneity of the ATMs, identifying three different macrophage populations in obese AT. Resident macrophages ($F4/80^{Lo}$) expressing CD206 are maintained in obese AT, whereas Ly6c expression characterizes the newly recruited macrophages (also $F4/80^{Hi}$). The pro-inflammatory subset of lipid-laden macrophages in CLS is characterized by the expression of CD9 (64). More recently, Jaitin and colleagues confirmed the phenotype and presence of $CD9^{+}$ lipid-laden macrophages at CLS. They report that $CD9^{+}$ cells counteract inflammation and adipocyte hypertrophy via the lipid receptor TREM2 (8). Proteomics analyses also identified specific ATM markers induced by stimuli reproducing the adipose tissue microenvironment with palmitate, insulin, and high levels of glucose (65). Such activation of ATMs gives rise to the metabolically activated macrophage (MMe), which is functionally and phenotypically distinct from classically activated M1 macrophages.

The importance of the pro-inflammatory capacity of the newly-recruited ATMs in the etiology of obesity is well established. Activated macrophages surround dead adipocytes and fuse to form multinucleate giant cells (66), an hallmark of chronic inflammation that correlates to insulin resistance (67). In 2008, Patsouris and colleagues demonstrated that the ablation of $CD11c^{+}$ cells during obesity restored insulin sensitivity by decreasing inflammatory markers (68). Interferon regulatory factor IRF5 is a pro-inflammatory transcription factor, commonly restricted to $CD11c^{+}$ cells, driving macrophage polarization toward an M1 phenotype (69), and is notably induced in ATMs in diet-induced obesity (70, 71).

Liver Macrophages in Metabolic Homeostasis

Liver resident macrophages, also called Kupffer cells (KCs), represent up to 80–90% of the whole body macrophage population and are characterized by the expression of canonical macrophage markers (F4/80, CD14, CD68, CD11b) as well as the C-type Lectin (Clec)-4F (5). Clec4f is the marker of *bona fide* KCs that are functionally distinct, specialized and self-renewing tissue-resident macrophages (72). KCs belong to the reticuloendothelial system of the liver, they are located close to blood vessels in lumen of hepatic sinusoids, they regulate hepatocyte proliferation and apoptosis upon injury and at steady-state they clear blood of aged erythrocytes and recycle iron by degrading hemoglobin (73). Their location is adapted to their function of clearance of the portal blood flow from pathogens, micro-organisms and cellular debris (74). KCs select and eliminate debris from blood through scavenger receptors and canonical PRRs expressed on the cell surface. Importantly, KCs impose immune tolerance in the liver, an organ constantly exposed to antigens and bacterial endotoxins from the intestine and portal blood. KCs maintain an anti-inflammatory environment by several mechanisms, secretion of IL-10, low expression of MHC-II and high expression of PDL-1, limiting antigen-presentation capacity and a powerfully inhibiting T-cells, respectively (75). Interestingly, even upon IFN-γ priming, KCs promote differentiation of T_{Regs}, a specialized immunoregulatory subset of T-cells that maintains immune tolerance (75, 76). At steady-state, KCs have limited interactions with distant non-immune cell types, because they are not typically motile cells. When microenvironmental communication is required, KCs secrete cytokines or signal to circulating monocytes to differentiate *in situ* (73).

Liver Macrophages in Metabolic Inflammation

Systemic extension of inflammation from AT is associated an increase of pro-inflammatory mediators in circulation and an increase in adiposity. Insulin resistance, persistent glucolipotoxicity, and systemic inflammation coincide in ectopic fat deposition, a major site of which is the liver. In obesity and T2D, the liver undergoes a spectrum of changes that range from benign steatosis to fibrosis and cirrhosis (77). This range of pathologies is known as non-alcoholic fatty liver disease (NAFLD), where lipotoxicity, inflammation and fibrogenesis characterize the more advanced stages of non-alcoholic steatohepatitis (NASH). Liver macrophages, KCs, are key actors in the progression of NASH, due to their pro-apoptotic and pro-inflammatory responses to lipotoxic hepatocytes and their capacity to activate matrix producing hepatic stellate cells (HSCs).

Ectopic fat deposition triggers activation of immune cells and an inflammatory environment which favors insulin resistance. Surprisingly, unbiased transcriptomic analysis revealed no differences in terms of expression of genes associated with a pro-inflammatory signature, between liver macrophages from lean and obese patients (similar data were obtained from mice fed an HFD for 9 weeks). Metabolic impairments are not associated with a pro-inflammatory activation of liver macrophages (78). However, the transcriptomic inflammatory signature is indeed variant between the stages of benign steatosis and NASH. At the transition between steatosis and NASH, liver macrophages target lipotoxic hepatocytes inducing their apoptosis and signal to HSCs to induce their activation (77). Chronic insults on the liver will result in fibrosis as an exuberant

scarring response to dead or dying hepatocytes, sustained fibrogenesis will in-turn affect liver function (77). Interestingly at the NASH stage, the liver macrophage pool is extremely heterogenous, with M1-like macrophages inducing hepatocyte apoptosis and M2-like macrophages promoting HSC activation and fibrogenesis (77, 79).

The pro-inflammatory transcription factor IRF5 has been shown to play a critical role in liver macrophages, mediating the transition between benign steatosis and NASH. Blunting IRF5 expression results in hepatoprotection through early upregulation of anti-apoptotic and immunoregulatory signaling, increasing T_{Reg} differentiation and IL-10 secretion upon hepatocellular stress (79). Recent research is delving into potential non-inflammatory or non-immune signaling efferent from KCs, notably effector molecules such as insulin-like growth factor-binding protein (IGFBP)-7, regulates insulin sensitivity in the context of obesity (80).

INITIATING AND SUSTAINING MACROPHAGE POLARIZATION IN T2D

Defining the extracellular metabolic and molecular signals associated with macrophage polarization in metabolic inflammation and insulin resistance is an area of active research. Candidate "metabolic" immunogens include lipids, hypoxia, cell death, and stress (42, 66, 81).

Ninety percent of ATMs are surrounding dead adipocytes in fat depots of genetically obese mice (82) suggesting that dead adipocytes are sources of DAMPs that lead to CLS formation and/or the accumulation of ATMs. Obese AT is also characterized by hypoxic areas and the expression of hypoxia-related genes, including HIF-1α. This transcription factor also promotes the pro-inflammatory capacities of ATMs in the context of obesity (83). Furthermore, lipolysis products and more generally lipids whose circulating levels are elevated in obesity, are extremely attractive candidates for the induction of an inflammatory response in ATMs. TLR-4 has been shown to be activated by nutritional fatty acids in macrophages, inducing pro-inflammatory signaling pathways (84). Macrophages can be activated by triglyceride-rich lipids, such as palmitate or very-low density lipoproteins (VLDL) which upregulate intracellular levels of ceramides and potentiate the pro-inflammatory response (85). Activation of the NLRP3-inflammasome by these mechanisms induces caspase-1-mediated cleavage of pro-IL-1β and pro-IL-18 into their active forms. Interestingly, saturated fatty acids such as palmitate have been shown to activate the NLRP3-inflammasome through an AMPK-autophagy-mitochondrial ROS signaling axis, leading to secretion of IL-1β and IL-18 (86). Importantly, IL-1β secretion *per se* is associated with insulin resistance. Indeed, IL-1β prevents insulin signaling through TNF-α-dependent and independent mechanisms (87). Once established, this pro-inflammatory environment favors the production of pro-inflammatory cytokines recruiting monocytes and other immune cells that sustain low-grade chronic inflammation.

Pro-inflammatory cytokines are key actors of the disruption of insulin signaling leading to insulin resistance (88). They act through paracrine mechanisms on insulin sensitive cells such as adipocytes. Physiologically, upon insulin binding to its receptor, the phosphorylation of tyrosine residues of insulin receptor substrate (IRS)-1 activates intracellular signaling pathways mediating insulin action (89). In the context of metabolic inflammation, JNK-1 and IKK are capable of interfering with insulin signaling by phosphorylating inhibitory serine/threonine residues of IRS-1. Insulin signaling is therefore disrupted (90). Similar pathways involving JNK-1 and IKK can be activated through the binding of fatty acids to TLRs. Moreover, IL-1β, which also signals through IKKβ and NFκB, favors insulin resistance by repressing IRS-1 expression at both transcriptional and post-transcriptional levels (91). Interestingly, IL-6 signaling inhibits insulin sensitivity through distinct mechanisms involving the JAK-STAT pathway that controls the transcription of its own suppressor, known as suppressors of cytokine signaling (SOCS), notably SOCS3. High levels of circulating IL6 induce increased expression of SOCS3 which physically interacts with tyrosine phosphorylated residues, and consequently inhibits IRS-1 binding to the insulin receptor (92).

METABOLIC MECHANISMS OF MACROPHAGE POLARIZATION

As with any other cell, macrophages have their own metabolic requirements and depend on the same well-characterized bioenergetic pathways as non-immune cells; these pathways are broadly classified into glycolytic or mitochondrial (**Figure 4**). In addition to pro-inflammatory signaling and transcriptional control, cellular metabolism is gaining recognition for the key role it plays in macrophage terminal differentiation. Mobilizing metabolic pathways does not solely produce energy but also dictates the magnitude of macrophage effector function (13). Early studies in immunometabolism characterized fundamental mechanisms fuelling macrophage function in model systems with canonical activators. Such foundation studies allowed clear association of bioenergetic profiles to polarization states. Current research is expanding on these paradigms through investigating bioenergetic profiles and metabolic adaptation of tissue-specific macrophage niches under physiological and pathological conditions and in response to diverse stimuli. Interestingly, the metabolic classification of macrophages was one of the first to be made, with the initial observation that M2 macrophages are able to metabolize arginine (93).

Metabolic Adaptation of Pro-inflammatory Macrophages

The enhanced glycolytic activity of the pro-inflammatory macrophages was observed decades ago (94) but the mechanisms underlying this process and its physiological significance were only recently described. It is a hallmark metabolic response in the polarization of macrophages toward an M1 phenotype (**Figure 4**). Glycolysis corresponds to the metabolic pathway responsible for the conversion of glucose into pyruvate, through 10 sequential enzyme-catalyzed reactions. This pathway gives rise to the production of ATP and NADH.

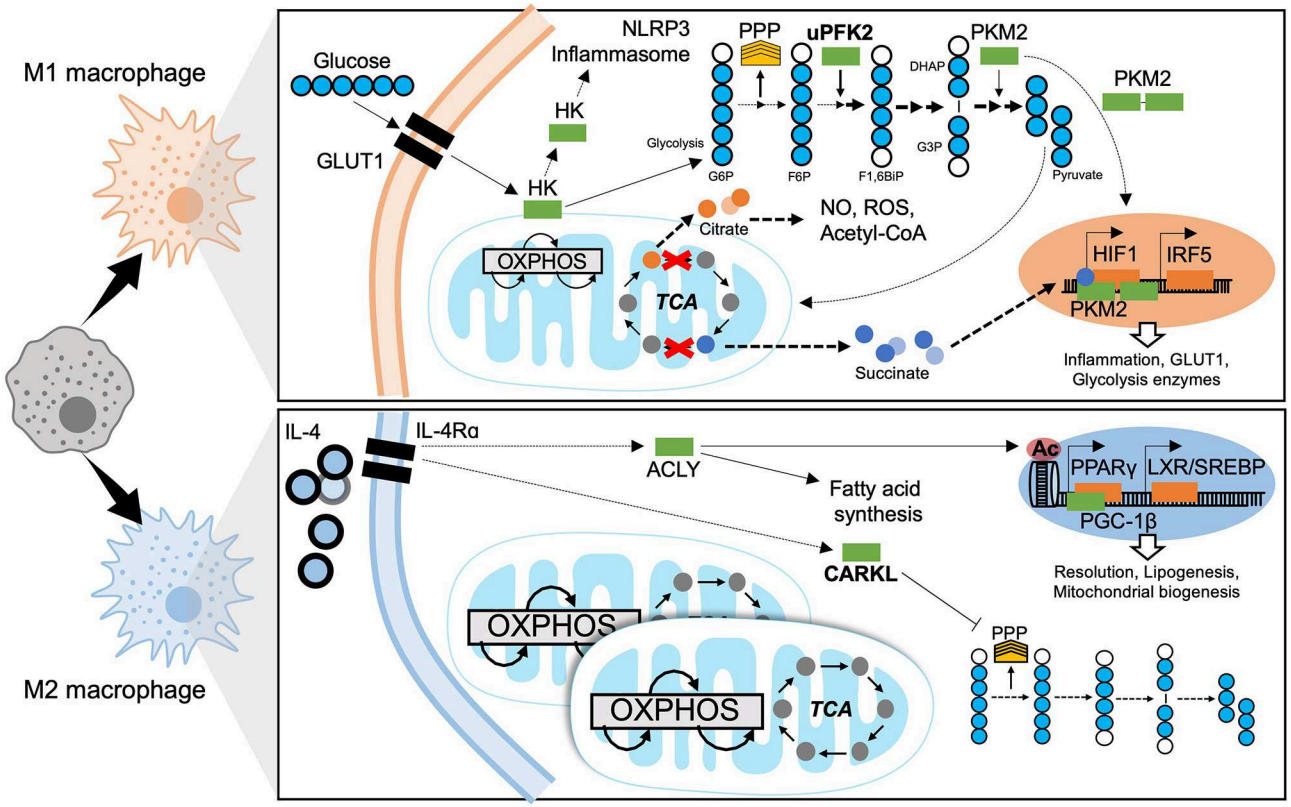

FIGURE 4 | Metabolic mechanisms of macrophage polarization. M1 macrophages are characterized by predominantly glycolytic metabolism. Glycolysis consists of breaking down a 6-carbon glucose molecule (where each carbon is depicted as a blue circle, white when phosphorylated) into 3-carbon sugars then into pyruvate, ATP, NADH, and H^+. The transcriptional programme that supports glycolysis is mediated by HIF1 and at least in part by IRF5. A Glucose substrate is provided by increased expression of the glucose transporter GLUT1. Meanwhile several glycolytic enzymes undertake non-canonical roles to support M1 effector functions. The mitochondrial tricarboxylic acid (TCA) cycle is disrupted, leading to accumulation of citrate and succinate which also enhance M1 effector function. The M2 macrophage has a fully intact TCA cycle, enhanced OXPHOS and increased mitochondrial biogenesis. ATP citrate lyase (ACLY) is activated downstream of IL4 signaling and enhances M2 effector functions through epigenetic mechanisms and producing substrates for lipogenesis. The sedoheptulose kinase (CARKL) represses the pentose phosphate pathway (PPP). Transcriptional programmes for M2 macrophage metabolism are mediated by PPARγ and LXR. GLUT1, Glucose transporter-1; HK, Hexokinase; NLRP3, NACHT, LRR, and PYD domains-containing protein; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle; PPP, pentose phosphate pathway; uPFK2, ubiquitous phosphofructokinase2; PKM2, pyruvate kinase isozyme 2; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BiP, Fructose-1,6-biphosphate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; NO, nitrous oxide; ROS, reactive oxygen species; CoA, Coenzyme A; HIF1, hypoxia-inducible factor 1; IRF5, interferon regulatory factor 5; IL-4, interleukin 4; IL-4Rα, IL-4 receptor alpha; ACLY, ATP-citrate lyase; CARKL, carbohydrate kinase like/sedoheptulose kinase; Ac, acetylation mark; PPARγ, peroxisome proliferator-activated receptor gamma; LXR, liver X receptor; SREBP, sterol regulatory element binding protein; PGC-1β, PPARγ coactivator 1-beta.

Glycolytic metabolism facilitates pro-inflammatory differentiation to enable efficient bacterial killing (95) and the secretion of pro-inflammatory mediators. Experimental inhibition of glycolysis with 2-deoxy-glucose (2-DG) limits the pro-inflammatory macrophage response to LPS (96). The rapid induction of glycolysis is enhanced by the upregulation of glucose transporter (GLUT)-1 expression (97). The switch toward glycolytic metabolism is dependent on the transcription factor HIF-1α (98). Its stabilization in hypoxic conditions promotes anaerobic metabolism and enhanced transcription of genes encoding glycolytic enzymes, such as pyruvate dehydrogenase kinase (PDK) and hexokinase (HK) which catalyse glucose phosphorylation. By HIF-1α-independent mechanisms, the ubiquitous isoform of phosphofructokinase-2 (uPFK2) is induced in M1 macrophages. Whilst uPFK2 is a more active isoform of PFK2, its induction enhances glycolytic flux and favors the formation of fructose-2,6P_2 which allosterically activates PFK1, the enzymes catalyzing commitment to glycolysis (99). As well as HIF-1α-dependent mechanisms, studies of IRF5 risk-variants report that gain-of-function single nucleotide polymorphisms of IRF5 (associated with auto-immune disease) increase glycolysis and inflammatory signaling, basally and in response to LPS (100).

Some glycolytic enzymes have non-canonical roles in macrophages. Notably, pyruvate kinase isoenzyme 2 (PKM2), induced by LPS (101), can be found as a dimer. This dimer can translocate to nuclei and act as a coactivator for HIF-1 (**Figure 4**). Consequently, PKM2 participates in a positive feedback loop with the up-regulation of pro-inflammatory and glycolytic genes in response to HIF-1 activation (102). Moreover, HK1 can be

inhibited by bacterial products and then dissociate from the mitochondria, which activates the NLRP3 inflammasome and the downstream production of pro-inflammatory cytokines (103). Mechanisms of resolution of glycolytic programming have not yet been brought to light; however, a recent study by Ip et al. demonstrates that IL-10 signaling exerts its anti-inflammatory effects by inhibiting the translocation of GLUT1 to the membrane (104). As well as being a substrate for glycolysis, glucose also fuels the pentose phosphate pathway (PPP), required for the synthesis of nucleotides and NADPH destined for ROS production by NADPH oxidase. The PPP is also induced upon LPS stimulation and M1 polarization (105).

The Krebs/tricarboxylic-acid (TCA) cycle is a mitochondrial metabolic pathway enabling ATP production and provision of substrates for the electron transport chain (ETC) that supports oxidative phosphorylation (OXPHOS) (**Figure 4**). In the context of pro-inflammatory macrophages, the TCA cycle is disrupted at two key steps: (i) accumulation of citrate due to a decrease in isocitrate lyase expression and (ii) the accumulation of succinate. Mitochondrial efflux of citrate is enhanced in M1 macrophages. Citrate accumulation has functional relevance to inflammatory polarization, being required for the production of ROS, NO, and prostaglandins (106). Citrate also acts a substrate for transformation into acetyl-CoA, feeding fatty acid synthesis through the ATP-citrate lyase (ACLY) (107). Interestingly, inhibiting fatty acid synthesis by silencing fatty acid synthase (FAS) in myeloid cells, has been shown protective in diet-induced insulin resistance, hindering ATM recruitment and chronic inflammation in mice. This underlies the importance of lipid metabolism in the polarization and function of macrophages, and notably synthesis and composition of the plasma membrane (108). Finally, the accumulation of citrate leads to a decrease in the levels of cis-aconitate which is the precursor of itaconate, a well-described anti-inflammatory intermediate. Itaconate exerts its anti-inflammatory effects by inhibiting succinate dehydrogenase (SDH), ROS production, and the release of pro-inflammatory cytokines.

An itaconate negative feedback loop has been described in the context of LPS and IFNγ stimulation, where itaconate shuts down the inflammatory response (109, 110). On the other side, the accumulation of succinate favors SDH activity and production of mitochondrial ROS (111). Succinate can trigger the expression of IL-1β through stabilizing HIF-1α (112). Consequently, pro-inflammatory macrophages are characterized by an increase of glycolytic activity and decreased OXPHOS. Interestingly, acute LPS treatment induces a burst of oxidative metabolism in macrophages which increases the pool of available of acetyl-CoA. This process supports histone acetylation and the downstream transcription of pro-inflammatory genes (113). The shutdown of oxidative metabolism, a hallmark of M1 macrophages, occurs following longer LPS treatments.

Finally, amino acids, the immunometabolism of which is relatively less known, can also be metabolized and influence macrophage polarization. For example, glutamine catabolism feeds the TCA cycle by giving rise to α-ketoglutarate, which acts as a co-factor for histone modifying enzymes implicated in macrophage differentiation (114). Arginine is also metabolized into L-citrulline simultaneously to the production of NO by iNOS, favoring the killing of bacteria.

Metabolic Adaptation of Anti-inflammatory Macrophages

Mitochondrial respiration dominates the M2 polarized state. M2 macrophages are characterized by an intact, fully functional TCA cycle and enhanced OXPHOS (**Figure 4**). Fatty acid oxidation (FAO) and mitochondrial biogenesis are increased in a PPAR-γ-coactivator-1β (PGC-1β)-dependent manner (115). With FAO being the main source of substrates, glycolysis-fuelled OXPHOS is not required to maintain the M2 phenotype (116).

The molecular mechanisms linking the metabolic adaptations of M2 macrophages to their functions in tissue homeostasis remain largely unexplored. Interestingly, IL-4 is known to activate ACLY enhancing substrate formation for histone acetylation. This epigenetic modification enables the transcription of specific M2-genes (117). Other proposed mechanisms implicate the carbohydrate kinase-like protein (CARKL), a sedoheptulose kinase that regulates PPP. CARKL is down-regulated in response to LPS and highly expressed upon IL-4 stimulation (118). CARKL activity inhibits the PPP in the M2 state (**Figure 4**).

Glutamine metabolism also plays an important role in M2 polarization. The expression of Slc1a5, a glutamine transporter, is increased upon IL-4 stimulation (119). Glutamine catabolism, in addition to glucose metabolism, leads to the formation of UDP-GlcNAc that supports N-glycosylation, a process required for the expression of several M2 markers (120).

Lipid synthesis, mediated by LXR, is central to M2 effector function and resolution of inflammation (121). Upon pro-inflammatory activation, LXR-dependent lipogenesis is inhibited. LXR being a the pro-lipogenic nuclear receptor and transcription factor later engages the master regulator of lipogenesis, SREBP1 to mediate the production of anti-inflammatory lipids (i.e., eicosanoids, resolvins) (122).

Deciphering Metabolic Adaptations of Tissue Resident Macrophages and Insulin Resistance

The above fundamental findings in macrophage bioenergetics were largely established using *ex vivo* modeling systems (such as murine bone marrow- or human monocyte-derived macrophages) and in response to known polarizing agents. Whilst these mechanisms apply to a large proportion of macrophages, typically infiltrating macrophages, responses to complex metabolic stimuli and the heterogeneity of tissue resident macrophages remains to be addressed. Tissue-resident macrophages face nutrient competition, normoxic and hypoxic areas and interactions with other cells. They respond to complex stimuli rather than unique stimuli. The bioenergetic adaptations of tissue-resident macrophages in obesity and insulin resistance remain to be thoroughly elucidated.

Interestingly, ATMs in obesity have a unique hypermetabolic profile with both increased glycolysis and OXPHOS compared to lean ATMs, whilst maintaining a pro-inflammatory phenotype

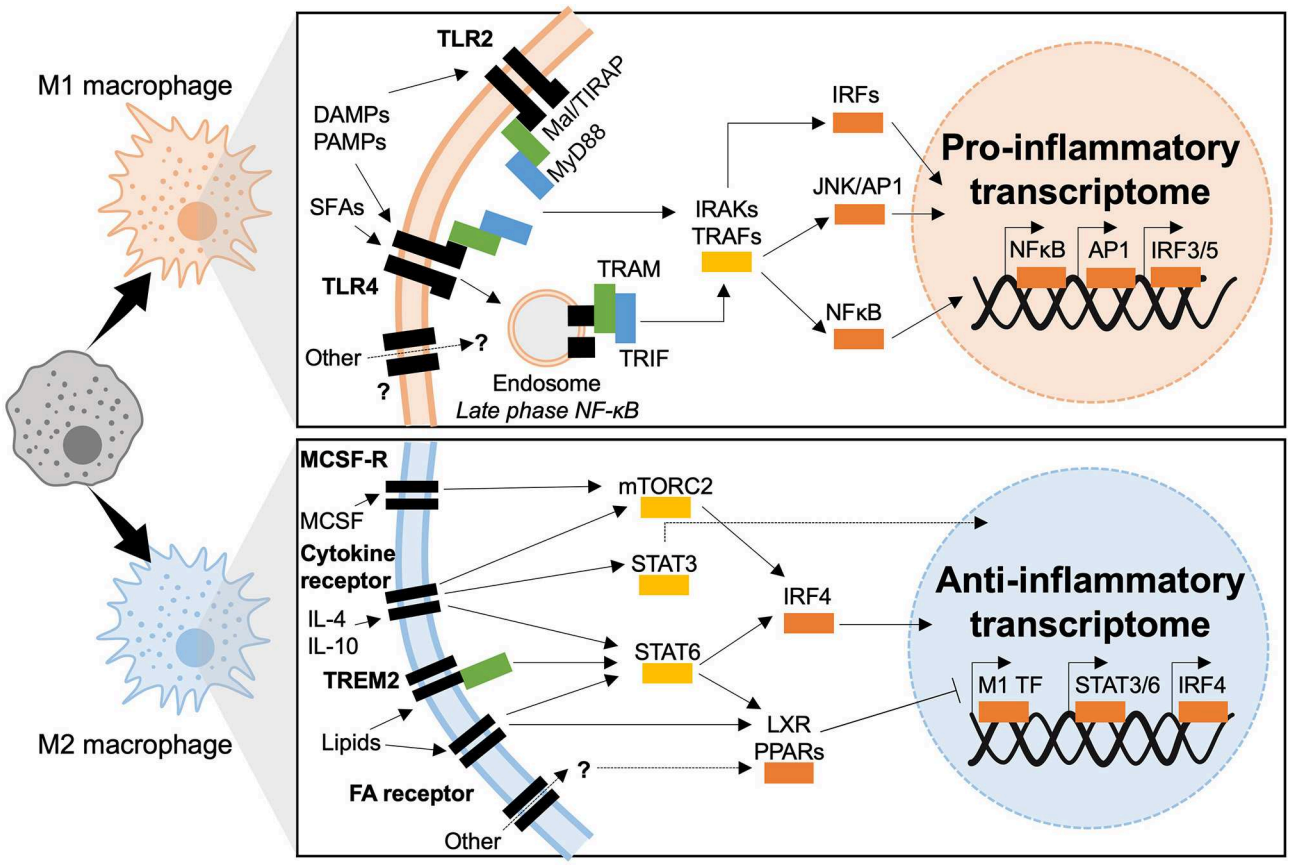

FIGURE 5 | Transcriptional mechanisms of macrophage polarization in T2D. M1 macrophages polarize in response to TLR2 or TLR4 ligation, downstream signaling is dependent of two adaptor complexes: Mal/TIRAP–MyD88 (TLR2 and TLR4) and TRAM–TRIF (TLR4). IRAK and TRAF signaling then dictate which transcriptional programmes are engaged, IRF3 IRF5, AP1, and NFκB have been shown to induce inflammatory polarization in T2D. M2 macrophages polarize downstream of cytokine and fatty acid receptor stimulation (also possible through other molecules such as TREM2). Intermediate signaling is largely through STATs and nuclear receptors (LXR and PPAR). STATs act as transcription factors or activate IRF4, whereas nuclear receptors act through transrepression of M1 transcription factors (M1 TF).

(123). More precisely, the pro-inflammatory capacity of the obese ATMs is mediated by glycolysis independently of HIF-1α (123). This bioenergetic profile is also distinct from peritoneal macrophages, despite the shared systemic glucolipotoxicity brought on by obesity. These observations underlie the specificity of metabolically activated macrophages and ATMs.

Hypoxic areas develop in AT upon inappropriate expansion in obesity and insulin resistance. Hypoxia and inadequate angiogenesis are attractive mechanisms leading to macrophage metabolic activation and their inflammatory polarization. Alternatively, the abundance of free fatty acids or lipolysis products in adipose tissue makes for a nutrient-/substrate-rich microenvironment. The effect of such lipid loading on macrophage metabolism and polarization remains to be investigated under iso- or hyper-caloric conditions. For example, the effect of obesity on macrophage glutamine metabolism remains to be investigated. Glutaminolysis is decreased in the AT of obese patients compared to lean subjects and glutamine levels in serum are decreased in patients with obesity or diabetes, suggesting an influential role for glutamine metabolism in ATM polarization (124).

TRANSCRIPTIONAL CONTROL OF MACROPHAGE POLARIZATION

Transcriptional control of macrophage polarization is well-characterized downstream of TLR ligation. Hallmark studies identified major TLR ligands as well as the key transcription factors that mediate inflammatory responses. Many of these pathways have been investigated in metabolic disease and are key mediators of macrophage activation in obesity, insulin resistance and T2D (**Figure 5**).

TLR-Dependent Inflammation in T2D

TLRs are highly-conserved transmembrane receptors expressed in and on macrophages. Their conservation is attributed to the evolutionary requirement to recognize structurally conserved

molecules and pathogens (125). Each TLR, from TLR1 to TLR13 recognizes specific ligands ranging from LPS, to nucleic acids, viral particles and chitin. Alongside their canonical roles in host-defense, several TLRs are implicated in metabolic inflammation and insulin resistance (126, 127). In this light, TLRs recognize not only infectious pathogens (through PAMPs) but also metabolic stressors or DAMPs associated with sterile inflammation and glucolipotoxicity.

The main TLRs implicated in diabetogenesis are TLR2 and TLR4. Engaging these two TLRs gives rise to chronic inflammation and insulin resistance through direct interference with insulin signaling (127–129). In macrophages TLRs 2 and 4 share common adaptor proteins, the myeloid differentiation primary response (MyD88) protein and Mal/TIRAP, that recruit IRAK kinases upon TLR engagement and dimerization. IRAK 1, 2, and 4 downstream signaling activates NFκB and Activator Protein (AP)-1. TLR4 also activates other downstream signaling. It is the only TLR that forms complexes with all adaptor proteins, Mal/TIRAP and MyD88, to initiate the early-phase NFκB response, the complex is then endocytosed and endosomal TLRs associate with TRAM and TRIF adaptors. Canonically, TRAM and TRIF set in motion the type-1 interferon response, transcriptionally mediated by Interferon Regulatory Factors (IRFs), AP-1 and late-phase NFκB activation. Both early and late phase action is required to sustain production of inflammatory cytokines (127–129). Co-ordinated action of TLRs, adaptor proteins and kinases result in the sustained activation of three major transcriptional programmes, headed by IRFs, AP-1, NFκB, and JAK-STAT.

Interferon Regulatory Factors

Initially characterized for their binding to virus-inducible enhancer elements on interferon coding regions, interferon regulatory factors (IRFs) are renowned for their control over innate immunity and type-1 interferon signaling. Also forming part of JAK-STAT signaling, IRFs respond to a number of DAMPs and PAMPs, mediate sterile inflammation (metabolic and auto-immune) and are also active in non-immune cells (e.g., adipocytes) (130, 131).

IRF family members are 300–500 amino acids long, share a conserved N-terminal DNA binding domain allowing binding to interferon sensitive regulatory elements. The C-terminal IRF association domain is variable and allows dimerization between the different IRFs (132). IRFs 1–5 and IRF9 control macrophage differentiation and polarization in response to PRR ligands, IRFs 3, 4, and 5 have been reported to play a role in metabolic inflammation (131).

IRF5 is responsible for M1 macrophage polarization, it is implicated in sterile inflammation and auto-immunity, namely rheumatoid arthritis where risk-variants contributing to the over-expression of IRF5 have been reported (131). In T2D, IRF5 contributes to macrophage activation and metabolic decline in both adipose tissue and in the liver.

In ATMs, IRF5 is highly expressed by $CD11c^{+}$ macrophages at CLS. Both CLS formation and IRF5 expression are strongly associated with AT inflammation, maladaptive adipocyte expansion and both local and systemic insulin resistance (70). Upon diet-induced obesity, mice with a myeloid-deficiency of IRF5 remain insulin sensitive despite increased adiposity. Visceral white adipose tissue in IRF5-deficiency is characterized by adaptive remodeling mediated by a *de facto* type-2 immune response, limiting adipocyte expansion and preventing loss of sensitivity to insulin's anti-lipolytic effect (70). Dysregulated expression of IRF5 is also causal in the progression to NASH. Throughout NAFLD, IRF5 mediates pro-apoptotic and inflammatory signaling from liver macrophages toward lipotoxic hepatocytes. Sustained inflammatory signaling and hepatocyte apoptosis result in scarring fibrogenesis in the liver (79).

IRF5 is the active transcription factor canonically downstream of TLR4. Interestingly, although the phenotypes of TLR4-deficiency and IRF5-deficiency are near identical under diet-induced obesity, the TLR4-IRF5 axis remains to be experimentally confirmed in the pathogenesis of T2D and its complications (133). Similarly downstream of TLR4, IRF3 promotes AT inflammation upon diet-induced obesity and inhibits adipose tissue browning. IRF3-deficient mice retain insulin sensitivity upon high-fat feeding and enhance AT browning (134).

In opposition to IRF3 and IRF5, IRF4 promotes macrophage M2 polarization and the resolution of inflammation (135). The metabolic phenotype observed in IRF4-deficient mice on HFD is accordingly aggravated (136). Myeloid-deficiency of IRF4 results in increased insulin resistance and adipose tissue inflammation when compared to IRF4-competent mice (136). Interestingly, IRF4 is nutritionally regulated by insulin signaling and by canonical transcription factors involved in metabolic signaling (e.g., FOXO1). Additionally, IRF4 regulates lipid handling in adipocytes, promoting lipolysis by facilitating lipase expression (130).

Activator Protein 1

AP-1 is a complex formed of the proto-oncogenes c-Jun and c-Fos that are essential for DNA binding. AP-1 activation responds to cytokine signaling and growth factors; it controls apoptosis, cell growth, and macrophage terminal differentiation to an M1-like phenotype (137).

AP-1 activity is dictated by post-translational modifications, notably translocation and/or dimerization of its subunits, by signaling from c-Jun N-terminal (JNK) and mitogen-activated protein kinases (MAPK). AP-1 activity is also regulated by the composition of its DNA binding dimer (Jun/Jun, Jun/Fos, bZIP) and through binding partners (138). AP-1 is canonically activated in response to PRR ligation, cytokine signaling and growth factors. In the case of metabolic inflammation AP-1 is responsive to saturated fatty acids (SFAs), namely palmitate (128). Macrophages exposed to palmitate release pro-inflammatory mediators in an AP-1 dependent manner (128).

AP-1 activity is also responsive in response to hormone signaling, where leptin increases binding of nuclear proteins to the AP-1 consensus sequence of the lipoprotein lipase (LPL) gene promoter. This activity increases macrophages expression of LPL, giving mechanistic insight into the

role of AP-1 in foam cell formation, atherogenesis and T2D (139).

The upstream kinases that activate AP-1 subunits have been extensively investigated in metabolic disease, namely JNKs. Mice-deficient for JNK1 and/or JNK2 remain metabolically healthy upon diet-induced obesity, mice gain less weight, are protected from insulin resistance and inflammation (140, 141). Interestingly, myeloid-specific deficiency of JNK, results in non-inflammatory obesity and a decrease in serum fatty acids. Studies indicate that myeloid-AP-1 is a key mediator of adipose tissue lipolysis upon diet-induced obesity (142).

Nuclear Factor-κB

NFκB is a transcription factor that promotes M1 polarization, it responds to a variety stress signals including: cytokines, redox stress, oxidized lipids, bacterial, or viral antigens (143–146). Dysregulated NFκB signaling occurs in a number of inflammatory conditions including T2D. NFκB is highly expressed in ATMs upon their M1/MMe differentiation and throughout the onset of insulin resistance. Furthermore, cytokines released by M1/MMe macrophages form an amplifying loop that recruits and polarizes other leukocytes at the site of inflammation.

Mice with a myeloid-deficiency of *Inhibitor of NFκB Kinase* (IKK-β), NFκB's canonical activator protein, display a diminished inflammatory response in diet-induced obesity and maintain systemic insulin sensitivity (147). Interestingly, hepatic deficiency of IKK-β only retains insulin sensitivity in the liver (not in muscle nor AT), indicating that the myeloid-derived IKK-β/NFκB is the main regulator of systemic metabolic homeostasis (147).

Signal Transducers and Activators of Transcription

A family of 7 transcription factors that regulate interferon signaling, Signal Transducers and Activators of Transcription (STATs), have well-established roles in apoptosis, proliferation, and differentiation of innate immune cells. Of note, STAT activity is particularly important in maintaining immune tolerance. STATs are activated downstream of cytokine, chemokine, and growth factor signaling. STAT dimerization and nuclear translocation is dependent on phosphorylation mediated Janus Kinase (JAK), together forming the JAK-STAT pathway.

STATs 1 and 5 promote M1-like signaling whereas STATs 3 and 6 promote M2-like signaling in macrophages (148–152). Interestingly the more recently described Mme phenotype is polarized independently of STAT1 activity (153).

STAT1 in macrophages is activated in response to high glucose and exerts pro-inflammatory signaling through epigenetic mechanisms. Of note, glucose-responsiveness of STAT1 has been reported in *in vitro* and *ex vivo* modeling, with little-to-no evidence being reported *in vivo* or from human studies of obesity, insulin resistance, and T2D (154, 155). To date no evidence links STAT5 activation *per se* to diabetic pathogenesis despite its known roles in inflammatory polarization.

STAT3 is strongly linked to the development of T2D and its complications, mainly with anti-inflammatory, metabolically protective properties. For example, STAT3 is a downstream target of the first-line T2D treatment, metformin. Metformin inhibits the differentiation of monocytes to macrophages and decreases their infiltration into atherosclerotic plaques through AMPK-mediated inhibition of STAT3 (156). Similarly, in insulin resistance and diet-induced obesity, protective effects of ABCA1/APOA1 activity are STAT3-dependent, as is the anti-inflammatory adipose tissue phenotype of mice with a myeloid-deficiency of JAK2 (157, 158).

STAT6, on its own, or in concert with the vasodilator-stimulated phosphoprotein (VASP) has immunoregulatory properties in the context of metabolic inflammation. The VASP-STAT axis has been described in mice with a myeloid-specific deficiency of VASP, mice were prone to hepatic inflammation and insulin resistance in a STAT6-dependent manner (159). Whereas, STAT6 deficiency predisposes mice to diet-induced obesity, oxidative stress, and adipose tissue inflammation (160).

Peroxisome Proliferator-Activated Receptors (PPARs)

PPARα, γ, and δ/β, are expressed at different levels in different tissues and vary across developmental stages. Highest expression levels are in the liver, skeletal, and cardiac muscle and in the spleen. PPARs are implicated in cellular metabolism, differentiation, development and more recently emerged as key regulators of inflammation.

In M1 macrophages PPAR-α inhibits the expression of pro-inflammatory mediators by negative regulation of AP-1 and NFκB. Several studies report the beneficial effects of PPAR-α activation in T2D and its complications. PPAR-α agonists have been applied in T2D patients and are beneficial in atherosclerosis, through inhibiting foam cell formation and inflammatory signaling. Beneficial effects are mediated by interfering with c-Fos and c-Jun interactions and by limiting lipid accumulation through repressing Fatty Acid Transport Protein (FATP)-1 (161–163).

PPAR-β/δ also acts on macrophage metabolism, regulating lipid efflux, fatty acid catabolism and beta-oxidation. PPAR-β/δ in macrophage regulates whole body energy dissipation and systemic responses to cholesterol; PPAR-β/δ activation occurs in response to dyslipidemia (164–166). In the pathogenesis of T2D, PPAR-β/δ plays a protective role controlling macrophage infiltration in adipose tissue and liver and promoting immune tolerance (M2 polarization) in ATMs acting downstream of STAT6 (167). Mice with a myeloid-deficiency of PPAR-β/δ display an aggravated metabolic phenotype upon diet-induced obesity.

PPAR-γ plays an important role in adipose physiology, adipocyte differentiation and maturation. Of the two known isoforms, PPAR-γ1 is expressed in macrophages and adipocytes whilst PPAR-γ2 is restricted to adipocytes (168). PPAR-γ1 enhances monocyte differentiation into M2 macrophages and is an inhibitor of inflammatory polarization, repressing MMP9,

IL-6, TNF-α, and IL-1β expression (161, 169, 170). In *in vitro* and *ex vivo* modeling, PPAR-γ inhibits M1 signaling associated with LPS+IFNγ stimulation, including iNOS, COX-2, and IL-12 (171–173). Importantly, macrophage PPAR-γ is also a downstream target of internalized lipids, and mediates expression scavenger receptors required for foam cell formation (47). Accordingly, PPAR-γ-deficient mice display impaired M2 maturation and develop exacerbated insulin-resistance and metabolic inflammation in diet-induced obesity (174, 175). Enhancing PPAR-γ activity with thiazolidinediones (TZDs) improves the metabolic phenotype in diet-induced obesity (176). Interestingly, reports of PPAR-γ overexpression demonstrate that mature adipocyte PPAR-γ is in-fact the main insulin-sensitizing component (overexpression phenotype is comparable to TZD treatment) (177). Little-to-no beneficial effects are observed upon diet-induced obesity when PPAR-γ is overexpressed in macrophage (177). Such over-/under-expression studies reveal divergent functions of PPAR-γ. Further mechanistic work is needed to precisely characterize the roles and regulation of this nuclear receptor and its different isoforms in different cell types and microenvironments.

With regards to mechanism of action, multiple mechanisms have been proposed, with the main one being *transrepression,* whereby PPAR-γ binds to active pro-inflammatory transcription factors and represses their function. Repressive mechanisms through interactions with nuclear receptor corepressor (NCoR) complexes have also been proposed (178).

Liver X Receptors

Liver X receptors (LXRs) exist in 2 isoforms, LXRα and LXRβ, both of which are lipid-activated and regulate macrophage inflammatory responses. To regulate transcription LXRs heterodimerise with Retinoid X Receptor (RXR) and bind to LXR response elements on the genome (179). LXRs, play important roles in T2D and in cardiovascular disease, promoting anti-inflammatory polarization and regulating macrophage lipid content.

LXR activation by oxysterol species and synthetic compounds allows cholesterol efflux from macrophages through the lipid transporters ACBCA1 and ACG1 (180). LXRs also directly repress transcription of pro-inflammatory genes and enhance transcription of anti-inflammatory genes in response to polarizing stimuli (181). Mechanistically, LXRs exert their effects by transrepression once they are sumoylated. This modification prevents LPS-dependent exchange of corepressors, thus maintaining LXR-mediated repression of inflammatory transcription factor activity (182). Several reports show the protective roles that LXRs have in metabolic inflammation and insulin resistance. Namely, LXR agonists act as insulin sensitizers and regulators of glycaemia through repressing hepatic gluconeogenesis (183–185).

Hypoxia Inducible Factor 1

Hypoxia inducible factor (HIF)-1 is a transcription factor with two subunits, α and β. HIF-1α is stabilized and its expression is increased in response to hypoxia, whereas HIF-1β is constitutively expressed and stabilized independently of oxygen levels (186). Under hypoxic conditions, HIF-1α translocates to the nucleus and dimerises with HIF-1β allowing binding to hypoxia response elements (HREs) on the genome and regulation of target gene expression (187). Under oxygen-poor conditions, HIF1 activation mediates a shift toward anaerobic respiration in cells where bioenergetic requirements are supported by glucose metabolism (188).

Myeloid-specific overexpression of HIF-1α leads to increased M1 polarization, inflammation and glycolysis in macrophages. Conversely, myeloid-specific deletion of HIF-1α impairs macrophage glycolysis and inflammatory polarization. In murine models of obesity, mechanisms of M1 polarization in adipose tissue macrophages are only partly dependent on HIF1 activation. Myeloid-specific deletion of HIF-1α results in decreased inflammatory signaling, decreased CLS formation and an ameliorated metabolic phenotype upon diet-induced obesity (189, 190).

EPIGENETIC CONTROL OF MACROPHAGE POLARIZATION

Epigenetic mechanisms control chromatin structure and conformation, factors that dictate the accessibility of genetic loci to transcription factors. Epigenetic remodeling, through transcriptional coregulators and epigenetic modifying enzymes (such as histone deacetylases or HDACs), regulates transcription factor activity. Understanding underlying epigenomic regulatory mechanisms can help develop new therapies, for example, by blocking an unwanted pathway or reprogramming macrophages to a more beneficial phenotype.

Rapid induction of an inflammatory transcriptional profile is a hallmark of macrophage activation required for an effective immune response. Under steady state, coregulator complexes bind to genomic regions of a broad repertoire of inflammatory genes to maintain macrophages in a quiescent state, this mechanism avoids deregulated inflammatory gene induction.

Coregulators function by first recognizing transcription factor activity and they then modulate this activity by establishing interactions with transcriptional machinery and chromatin (191, 192). Coregulators can be categorized as either coactivators or corepressors. Coactivators recognize and promote active transcription; corepressors however recognize and repress inactive transcription. However, this categorization of coregulator activity does not truly reflect the physiological or physiopathological situation, since coregulator activating or repressive function is highly context dependent. Coregulators establish cell type-dependent and ligand-dependent epigenomes by forming large multiprotein complexes that "write," "erase," or "read" reversible chromatin modifications associated with transcriptional activity (**Figure 6**). Although underlying mechanisms remain to be elucidated, convincing evidence places altered function or expression of coregulators at the center of dysregulated transcription inherent to disease-specific epigenomes.

Two such corepressors are the Nuclear Receptor Corepressor (NCoR1) and silencing mediator of retinoic acid and thyroid

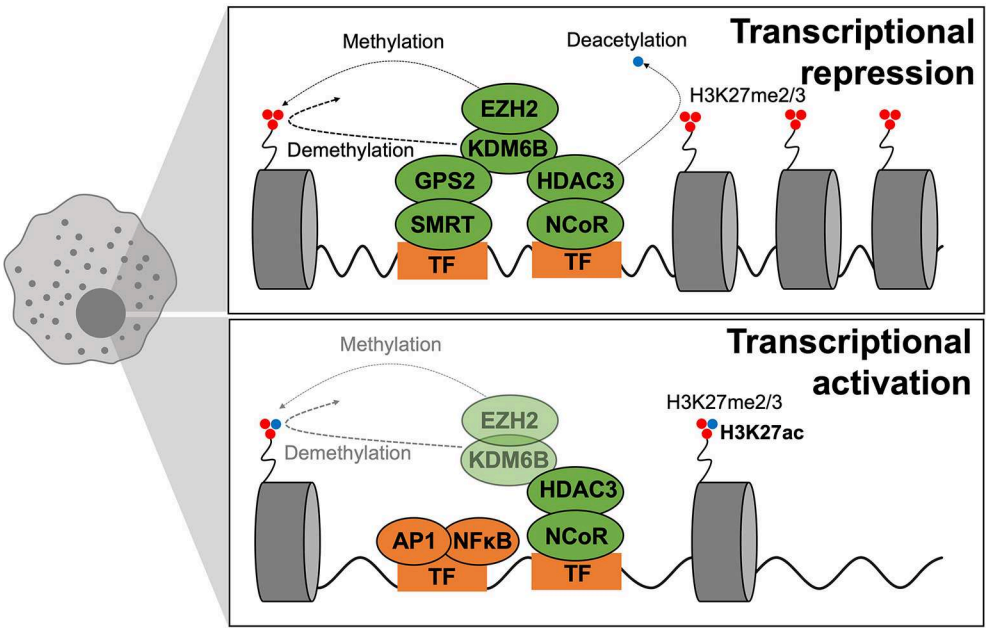

FIGURE 6 | Epigenetics of macrophage polarization in T2D. Epigenetic mechanisms that modulate transcription act on chromatin remodeling and altering DNA accessibility to transcriptional machinery. Modifications are in dynamic exchange where methylation and acetylation status of H3K27 dictate repression or activation of transcription, respectively. When chromatin is closed, methylation is dynamically altered by EZH2 (adding methyl groups) and KDMB6 (removing methyl groups), and the active transcription mark (H3K27ac) is removed through HDAC3 activity. GPS2–SMRT also participate in gene repression. During active transcription, the active transcription mark (H3K27ac) is maintained whilst EZH2–KDM6B play smaller roles. GPS2–SMRT are not present to exert repressive effects.

hormone receptor (SMRT or NCoR2) that interact with inflammatory transcription factors such as AP-1 and NFκB and in-turn bind to specific *genomic* regions to regulate transcription (192, 193). The classical view was that upon TLR4 stimulation, the NCoR complex is released from promoter/enhancer regions of inflammatory genes to promote, or de-repress, their transcription (194). However, in many cases this distinction does not truly reflect the *in vivo* situation, with context-specific microenvironmental cues dictating coregulator properties. In the context of macrophage polarization and T2D, the specific deletion of NCoR in macrophages caused the transcriptional activation of LXR, leading to the induction of lipogenic genes, which in-turn causes local anti-inflammatory effects by repressing NFκB (185). NCoR exerts pro-inflammatory actions in macrophages. Similarly, to NCoR, it was surprising that macrophages from HDAC3-deficient phenotypes were anti-inflammatory in two independent studies (195, 196). These findings are not consistent with earliest studies showing that HDAC3 and NCoR were shown to assemble a repressive complex *via* interaction with the NFκB subunit p50, necessary for the TLR tolerance phenomena where sustained TLR4 activation represses inflammatory gene expression (192). More mechanistic insights are required to better understand the specific action of NCoR1 and HDAC3.

In contrast, anti-inflammatory functions have been attributed to the SMRT/GPS2 (G-protein pathway suppressor-2) subunit/complex. In our recent study, we demonstrated that macrophage specific knockout of the GPS2 subunit exacerbates metabolic inflammation, aggravating glucose homeostasis under metabolic stress (197). The phenotype is associated with genomic features of the GPS2-repressive pathway, involving direct repression of the c-Jun subunit of AP-1. Considering all the recent studies, 2 sub-complexes may have different functions: GPS2/SMRT may have anti-inflammatory actions whilst NCoR/HDAC3 may act as pro-inflammatory machinery. This could explain the contradictory phenotypes of the respective KO models, despite both being initially classified as corepressor complexes.

Subcomplex specificities would allow controlling transcription of distinct gene clusters in response to a variety of signals and likely result from differential interactions with TFs, coregulators, and chromatin components (e.g., histones). This is exemplified by GPS2 actions in others cell types. In fact, the anti-inflammatory action of GPS2 is conserved in adipocytes by repressing CCL2 and IL6 (198, 199). While the main action of GPS2 in hepatocytes is to repress the metabolic nuclear receptor PPAR-α action (200). Hepatocyte-specific KO of GPS2 is then protective upon inflammatory stimulus while adipocyte- or macrophage-specific GPS2 deficiency is deleterious for whole body glucose homeostasis and exhibits exacerbated inflammation. These opposite functions are also observed in humans (197, 200, 201). These correlations are of importance because they point at the possibility that inappropriate GPS2 function could be linked to macrophage pathways that drive adipose tissue dysfunction and insulin resistance.

Other coregulators, such as Glutamate receptor-interacting protein (GRIP)-1, regulate macrophage programmed responses to IL-4 by acting as a coactivator for Kruppel-like factor (KLF)-4, a known driver of tissue-resident macrophage differentiation (202). Obese mice with conditional macrophage-specific deletion of GRIP1 develop inflammation and substantial macrophage infiltration in metabolic tissues, fatty livers, hyperglycemia and insulin resistance; recapitulating metabolic disease through GRIP-1's glutamate receptor-independent actions. Thus, coregulators such as GPS2, GRIP-1, NCoRs, and HDAC3 are critical regulators of macrophage reprogramming in metabolic disease. Their co-ordinated actions engage transcriptional mechanisms that coordinate the balance between macrophage polarization states and subpopulations to maintain metabolic homeostasis.

Epigenetic remodeling of specific histones is also a mark of macrophage activation states. Macrophage activation can be regulated by trimethylation of lysine residue 27 on histone 3 (a modification annotated as H3K27me3) via the action of lysine-specific demethylase 6B (KDM6B; also known as JMJD3). The histone mark H3K27me3 is represses transcription and is deposited by histone-lysine *N*-methyltransferase (EZH)-2, a subunit of the Polycomb Repressive Complex 2. Whereas, removal of this histone mark is mediated by the H3K27me3 demethylases KDM6A and KDM6B. Zhang et al. reported the critical role of the EZH2 histone methyltransferase modification in altering macrophage phenotype (203). EZH2 controls H3K27me3 deposition on the promoter of SOCS3, that encodes a cytokine signaling repressor. Accordingly, mice with a myeloid-specific deficiency of EZH2 exhibit attenuated macrophage activation and reduced inflammation under models of autoimmune disease. These findings make EZH2 an attractive target for other inflammatory diseases such as T2D.

The role of KDM6B in macrophage polarization is unclear. Pioneer studies have proposed that KDM6B is not necessary for the polarization of the pro-inflammatory macrophage phenotype in mice but is required for a proper anti-inflammatory response via the removal of H3K27me3 from the IRF4 promoter (204, 205). The absence of KDM6B completely blocks the induction of M2 macrophages in mice challenged with helminths or chitin, indicating that the role of KDM6B must be greater in M2 than in M1 macrophages (206). In contrast, Pro-inflammatory TLR4 gene activation was decreased in KDM6B-deficient macrophages. In line with these results, targeting KDM6B H3K27me3 demethylases with small-molecule inhibitors impairs inflammatory responses in human primary macrophages and could thus be of high pharmacological interest for the treatment of inflammatory diseases including T2D (207, 208). Interestingly, KDM6B also modulates expression of chemokines dependent of GM-CSF stimulation, which normally acts via STAT5-mediated and IRF5-mediated induction of a pro-inflammatory phenotype. Epigenetic signatures differ in disease states of chronic inflammation, such as T2D. KDM6B is one of the few epigenetic modifiers that could be directly involved in altering the epigenetic signature of macrophages. Gallagher et al. were the first to report on the role of KDM6B in controlling macrophage expression of IL-12 in a diabetic context (208). Proof-of-principle of these findings was achieved in a recent study where macrophages treated with a selective KDM6B inhibitor showed altered expression of pro-inflammatory cytokines (209). What remains to be determined are the degrees of contribution of nutrient overconsumption and obesity, insulin resistance, or hyperglycaemia to observed changes in histone methylation. A recent study proposes that altered DNA methylation is predominantly a consequence of adiposity, rather than a cause (210).

CONCLUSIONS: TOWARD FUNCTIONAL CLASSIFICATION, BIOENERGETICS AND NON-IMMUNE SIGNALING

Important advances have been made in the past decades characterizing the role of tissue macrophages in the development of insulin resistance. Indeed, macrophages are now seen as central actors in maintaining tissue and organism homeostasis in response to daily challenges of transient over- and under-nutrition; from inflammatory signaling necessary for insulin secretion, to the housekeeping roles they play in buffering AT lipolysis and their non-inflammatory signaling in NAFLD.

To date studies have largely focused on deciphering the molecular mechanisms that control macrophage responses to dysmetabolism, with a relatively restrictive categorization into M1-like vs. M2-like macrophages. Recent technological advances of single cell sequencing have allowed a much more in-depth characterization of tissue macrophage subsets that do not neatly adhere to the previously proposed dichotomies. Indeed in other fields of study, namely immune cell ontogeny, single cell sequencing has led to a thorough functional reclassification of innate immune subtypes (16). Such studies have particular value in characterizing macrophages in tissue niches that have been overlooked until recently, like pancreatic islet macrophages or sympathetic nervous system associated macrophages.

Such a shifting paradigm in macrophage functional classification can also be extended to their metabolic characterization, their bioenergetic requirements and adaptations to the specific challenges of insulin resistance. Numerous studies in infection and immunity have largely embraced bioenergetic adaptation as *bona fide* immune cell activation. Tissue macrophage bioenergetics remains to be elucidated, at the developmental stage, at steady state and at the onset of insulin resistance. Macrophage metabolism represents an attractive therapeutic target that will modulate inflammation without drastically altering effector functions by turning the immune response "on" or "off."

Following recent discoveries of non-immune and non-inflammatory signaling from macrophages, the scientific community has gained insight into non-canonical roles of the innate immune system. Further investigation into such homeostatic non-inflammatory signaling must be carried out in macrophages as well as related innate immune cells, such as dendritic cells, NK cells, and ILCs. As innate immunity, in all its diversity, is known to maintain homeostasis without necessarily engaging inflammation, steady state characterization,

and responses to physiological variation must be mapped to gain more basic insight into the deregulation of innate immune effector function that leads to metabolic pathology.

Despite consistently strong associations and mechanistic links between inflammation and insulin resistance there have been relatively few successful translational advances. Current anti-diabetic treatments aim to normalize glycaemia through various mechanisms and have been shown to also buffer systemic inflammation (e.g., TZDs, DPP-4 inhibitors, GLP-1 RAs). Such positive effects attribute improvement in the inflammatory profile to improved metabolic responses (211). Considering the overwhelming evidence that macrophage polarization is central to T2D pathology seemingly few clinical trials target inflammation in T2D.

To date anti-inflammatory strategies in clinical trials have targeted cytokines with neutralizing antibodies (e.g., anti-TNF, anti-IL1) or have applied agents with uncharacterised mechanisms (e.g., chloroquine, diacerein). Studies on these drugs have been promising, improving insulin sensitivity, secretion, or fasting blood glucose (212–214). The main obstacles to their routine application are the lack of long-term studies to evaluate efficacy and safety. Other hurdles to the translatability of anti-inflammatory approaches is the fact that inflammation in T2D is multifactorial, and the disease itself predisposes patients to a slew of complex complications and comorbidities (in which case the rise of precision medicine aims to identify mechanisms of response or those at-risk). Technical barriers also affect translational potential, for example clinical trials evaluate inflammation based on relatively non-specific circulating markers, such as CRP, which at best reflect systemic inflammation. Whereas, in preclinical studies scientists tend to evaluate tissue-specific inflammation, the extrapolation of which to human studies represents a substantial technical hurdle. Specific drug delivery to macrophages also represents a technical challenge and bypassing the cell-specificity leaves the door open to unexpected or unwanted side-effects. In light of the above work, promising approaches are slowly but surely increasing the translational potential of targeting inflammation in metabolic disease, for example the repurposing of well-tolerated drugs from other pathologies or fields, as was the case with anti-malarial chloroquine and hydroxychloroquine, and diacerein used to treat arthritis. In basic research, increasing attention is being placed earlier in disease course, where mechanisms that may delay or negate the natural course of T2D are being described and will soon provide bases for novel therapeutic targets. The development of small-molecule inhibitors or anti-sense oligonucleotides are increasingly attractive when targeting epigenetic or transcriptional pathways and are proving of increasing value to the clinical research community. Similarly, the search for metabolic immunogens or characterization of circulating immune cell populations will allow the development of predictive biomarkers of susceptibility to disease or risk-proxies of disease progression once insulin resistance has been established.

AUTHOR CONTRIBUTIONS

LO, ED, KD, NV, and FA wrote the review.

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The Atypical Cannabinoid Abn-CBD Reduces Inflammation and Protects Liver, Pancreas, and Adipose Tissue in a Mouse Model of Prediabetes and Non-alcoholic Fatty Liver Disease

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Background and Aims: The synthetic atypical cannabinoid Abn-CBD, a cannabidiol (CBD) derivative, has been recently shown to modulate the immune system in different organs, but its impact in obesity-related meta-inflammation remains unstudied. We investigated the effects of Abn-CBD on metabolic and inflammatory parameters utilizing a diet-induced obese (DIO) mouse model of prediabetes and non-alcoholic fatty liver disease (NAFLD).

Materials and Methods: Ten-week-old C57Bl/6J mice were fed a high-fat diet for 15 weeks, following a 2-week treatment of daily intraperitoneal injections with Abn-CBD or vehicle. At week 15 mice were obese, prediabetic and developed NAFLD. Body weight and glucose homeostasis were monitored. Mice were euthanized and blood, liver, adipose tissue and pancreas were collected and processed for metabolic and inflammatory analysis.

Results: Body weight and triglycerides profiles in blood and liver were comparable between vehicle- and Abn-CBD-treated DIO mice. However, treatment with Abn-CBD reduced hyperinsulinemia and markers of systemic low-grade inflammation in plasma and fat, also promoting white adipose tissue browning. Pancreatic islets from Abn-CBD-treated mice showed lower apoptosis, inflammation and oxidative stress than vehicle-treated DIO mice, and beta cell proliferation was induced. Furthermore, Abn-CBD lowered hepatic fibrosis, inflammation and macrophage infiltration in the liver when compared to vehicle-treated DIO mice. Importantly, the balance between hepatocyte proliferation and apoptosis was improved in Abn-CBD-treated compared to vehicle-treated DIO mice.

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Conclusions: These results suggest that Abn-CBD exerts beneficial immunomodulatory actions in the liver, pancreas and adipose tissue of DIO prediabetic mice with NAFLD, thus protecting tissues. Therefore, Abn-CBD and related compounds could represent novel pharmacological strategies for managing obesity-related metabolic disorders.

Keywords: cannabinoids, inflammation, prediabetes, NAFLD, obesity, liver, islets of Langerhans, adipose tissue

INTRODUCTION

Western diet and sedentary lifestyle increase prevalence of obesity worldwide. Obesity is a risk factor for developing diabetes and cardiovascular and liver diseases among others, reducing disease-free years as obesity becomes more severe (1). Obesity-related disorders arise progressively; prediabetes and non-alcoholic fatty liver disease (NAFLD) are early stages of pancreatic and liver damage. Although these pathologies can be diagnosed, there are no treatments at the level of the underlying molecular mechanisms. Current biomedical research focuses on the study of the early stages of obesity-related diseases for the generation of treatments aimed at stopping its progression and complications.

It is becoming evident that dysfunction of the molecular integration of the immune and metabolic systems underlies metabolic diseases such as type 2 diabetes (2). In fact, low-grade chronic inflammation (meta-inflammation) that occurs in obesity is considered an important factor in many disorders related to obesity, including type 2 diabetes (3). Several studies have detected changes in inflammatory cytokines in people with prediabetes (4–6). The adipose tissue has been determined to be the source of a plethora of inflammatory signals that, once in circulation, induce activation of lymphocytes. Indeed, accumulation of triglycerides in non-adipose tissues triggers inflammation, macrophages infiltration and apoptosis. Sustained high blood glucose level is associated with an increase in oxidative stress and intracellular inflammation that ultimately leads to loss of beta cell mass (7, 8). In the liver, accumulation of triglycerides leads to NAFLD that eventually ends in liver failure (9).

Cannabinoids are known to modulate the metabolism of lipids and glucose as well as inflammatory processes (10). In fact rimonabant, a cannabinoid type 1 receptor (CB1R) antagonist, has been in the market for the treatment of complicated obesity, although central side effects finally led to its withdrawal (11). Since then, the development of a second-generation family of cannabinoid-based drugs without side effects for treating metabolic diseases has been the focus of intense research. Atypical cannabinoids are ligands that do not target the canonical cannabinoid receptors CB1R and CB2R. The prototype of this kind of molecules is the phytocannabinoid cannabidiol (CBD) that lacks psychoactive effects and is one of the main components of *Cannabis sativa* plant. Most synthetic atypical cannabinoids derive ultimately from CBD and include abnormal CBD (Abn-CBD), O-1602, O-1918, and O-1821 (12). CBD and some synthetic atypical cannabinoids have been reported to display anti-inflammatory and anti-oxidant properties, including potential anti-diabetic actions (13–16). Unfortunately, clinical trials with CBD have failed to demonstrate improvements in glycemic and lipid parameters in patients with type 2 diabetes (17). However, effects of synthetic atypical cannabinoids on obesity-related inflammation and early stages of related diseases remain largely unexplored.

Abn-CBD results from the transposition of the phenolic hydroxyl group and the pentyl side chain of CBD (18). *In vitro* findings point to Abn-CBD displaying modulatory actions on neutrophils in inflammatory conditions such as experimental colitis and atherogenesis (19, 20). Indeed, Abn-CBD was found to improve glucose tolerance in streptozotocin-induced diabetic mice (15). In agreement with these findings we recently reported that Abn-CBD decreases cytokine-induced apoptosis in mouse and human isolated islets while promoting beta cell proliferation (21). However, the impact of Abn-CBD on obesity-related meta-inflammation and its relationship with prediabetes and NAFLD remains unstudied.

Here we aimed to investigate the metabolic effects and the anti-inflammatory properties of Abn-CBD in the liver and pancreas of a mouse model of diet-induced prediabetes and NAFLD.

MATERIALS AND METHODS

Study Design and Generation of Diet-Induced Prediabetic and NAFLD Mice

The European Union recommendations (2010/63/EU) on animal experimentation were followed. All animal experimentations were approved by the Ethic Committee of the University of Malaga (authorization no. 2012–0061A), and followed the 3R's principle. Ten-week-old C57BL/6J male mice were purchased from Charles River (France) and were acclimatized to the animal facility for one week with food and water available *ad libitum* and lights on between 8:00 and 20:00 h. Mice were then fed a 10% fat diet (control) or a 45% fat diet (45% of Kcal from lard, saturated fats, HFD) for 15 weeks (n = 10 and 30 mice, respectively). Body weight was monitored twice a week and glucose and insulin tolerance assessed by intraperitoneal glucose tolerance (GTT) and insulin tolerance test (ITT), respectively. Briefly, GTT was performed after overnight fasting by injecting 2 g/kg D(+)glucose (Sigma-Aldrich, St. Louis, MO). Blood glucose was monitored from the tail vein at baseline and 15, 30, 60, and 90 min using a glucose meter (Accu-check, Roche Diagnostic). For the ITT, mice were fasted for 6 hours and then injected with 0.5

U/kg of insulin (Humulin, Lilly, France). Blood samples were collected from the tail vein as above and blood glucose was measured at the same time points with the glucometer. Glucose and insulin area under the curve (AUC) was calculated from their corresponding graphs using IMAGEJ software (National Institutes of Health, Bethesda, MA, USA). Once HFD-fed mice showed greater body weight than control mice, glucose intolerance and insulin resistance, animals were randomized to vehicle or treatments and 10 mice were treated with the synthetic cannabinoid Abn-CBD. The herein study with Abn-CBD represents a subset of a larger study that also included the cannabinoid ligand LH-21. The pre-diabetic phenotype of these mice (HFD-vehicle vs. SD-vehicle) has been previously described (22). A detailed scheme of Abn-CBD study design is depicted in **Figure 1A**.

Subchronic Treatment With Abn-CBD and Monitoring of Body Weight and Food Intake

HFD mice were treated daily for the last 2 weeks of the experimental design with 0.05 mg/kg of Abn-CBD (Cayman Chemical, Ann Harbor, MI) or vehicle. Dosage was selected based on previous literature (15, 23). The stock solution of Abn-CBD was dissolved in ethanol, aliquoted, and stored at −20°C until use. Every day the dose to inject was freshly prepared by diluting an aliquot in saline-Tween80 (1% final ethanol content, 5% Tween80) and then intraperitoneally administered. Body weight and food intake were monitored daily, and the amount of food ingested was converted to kilocalories. Food efficiency was calculated as body weight variation (in grams) over caloric intake (in Kcals). Five days before euthanasia all groups of mice were also daily injected with 75 mg/kg of 5-bromo-2′-deoxyuridine (BrdU) dissolved in saline (Sigma-Aldrich). At the end of the treatment period, mice were euthanized by cervical dislocation and tissues collected for further histological and biochemical analysis. Samples from adipose tissue, pancreas and liver were fixed in 4% PFA by overnight immersion and then paraffin-embedded for histochemistry and immunohistochemistry. In parallel, plasma, adipose, pancreatic and hepatic samples were also snap-frozen and stored at −80°C for biochemistry analysis. The number of animals used for each experiment is detailed in figure legends.

Adipokines and Cytokines Determination

Plasma was obtained by centrifuging (2,000 × g, 4°C, for 10 min) whole blood in EDTA-coated tubes. Plasma levels of insulin and several inflammatory cytokines (IFN-γ, IL-5, IL-6, CXCL1, IL-10) were measured using the Meso Scale Discovery Multi array (Meso Scale Diagnostics, Rockville, MA, USA) in an MSD instrument (SECTOR S 600) equipped with multi-array electrochemiluminescence detection technology (Meso Scale Diagnostics). Plasma levels of leptin (BioVendor, Czech Republic) and HMW Adiponectin (Shibayagi Co., Japan) were measured by ELISA assay in accordance with manufacturer's instructions.

Biochemical Analysis in Plasma Samples

The plasma lipid profile (Triglycerides, total cholesterol, HDL-c, LDL-c, NEFA, and glycerol), blood glucose level and the liver marker alanine transaminase (ALT) were determined by routine laboratory methods using a Cobas Mira autoanalyzer (Roche Diagnostic System, Basel, Switzerland) and reagents from Spinreact (Spinreact S.A.U., Girona, Spain) and Biosystems (Biosystems S.A., Barcelona, Spain). Plasma insulin levels were measured using a commercial ELISA assay according to manufacturer's instructions (Mercodia, Uppsala, Sweden).

Lipid Peroxidation Measurement

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS). Malondialdehyde (MDA), a natural bi-product of lipid peroxidation reacts with thiobarbituric acid (TBA) to generate a MDA-TBA adduct that can be easily quantified at 532 nm in a spectrophotometer. Tissue samples were homogenized with ice-cold Tris-HCl buffer (150 mM KCl, 50 mM Tris, pH 7.4) supplemented with butylated hydroxytoluene to avoid artificial peroxidation during the test. The supernatant was incubated with MDA to obtain the TBARS. The absorbance was measured (VERSAmax, Molecular Devices LLC, San Jose, CA, USA) and interpolated in a standard curve using malondialdehyde-bisdiethyl-acetal (MDA, Sigma-Aldrich). The final values were expressed as nanomoles of TBARS per milligram of tissue.

Glucose-Stimulated Insulin Secretion (GSIS)

Islets of Langerhans were isolated from two mice in each group by using the collagenase digestion method, as previously described (24). Islets were then cultured for 20–24 h in RPMI-1640 medium supplemented with 11 mM glucose (Invitrogen, CA, USA), 2 mM glutamine, 200 IU/ml penicillin, 200 μg/ml streptomycin and 8% fetal bovine serum stripped with charcoal-dextran (Invitrogen). For GSIS experiments islets were pre-incubated at 37 °C for 2 h in Krebs-bicarbonate buffer solution containing 14 mM NaCl, 0.45 mM KCl, 0.25 mM $CaCl_2$, 0.1 mM $MgCl_2$, 2 mM HEPES and 3 mM glucose, and equilibrated with 95% O_2: 5% CO_2 at pH 7.4. Size-matched islets, five in each well from a 24-well plate, were seeded in 0.5 mL fresh buffer containing 3 mM glucose or 11 mM glucose. Then islets were incubated for 1 h at 37°C, 5% CO_2. After incubation, 1% bovine albumin was added to each well, and the plate was cooled at 4°C for 15 min to stop insulin secretion. Media were then collected and stored at −20°C until insulin measurement by ELISA (Mercodia, Uppsala, Sweden), according to the manufacturer's instructions.

Islet Morphometric Analysis

Number of islets and islet area were assessed by morphometric analysis in pancreatic sections. Paraffin-embedded pancreases from each mouse were cut at four different levels and stained with haematoxylin and eosin. Low-magnification photomicrographs were taken in an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan) and analyzed by the ImageJ software.

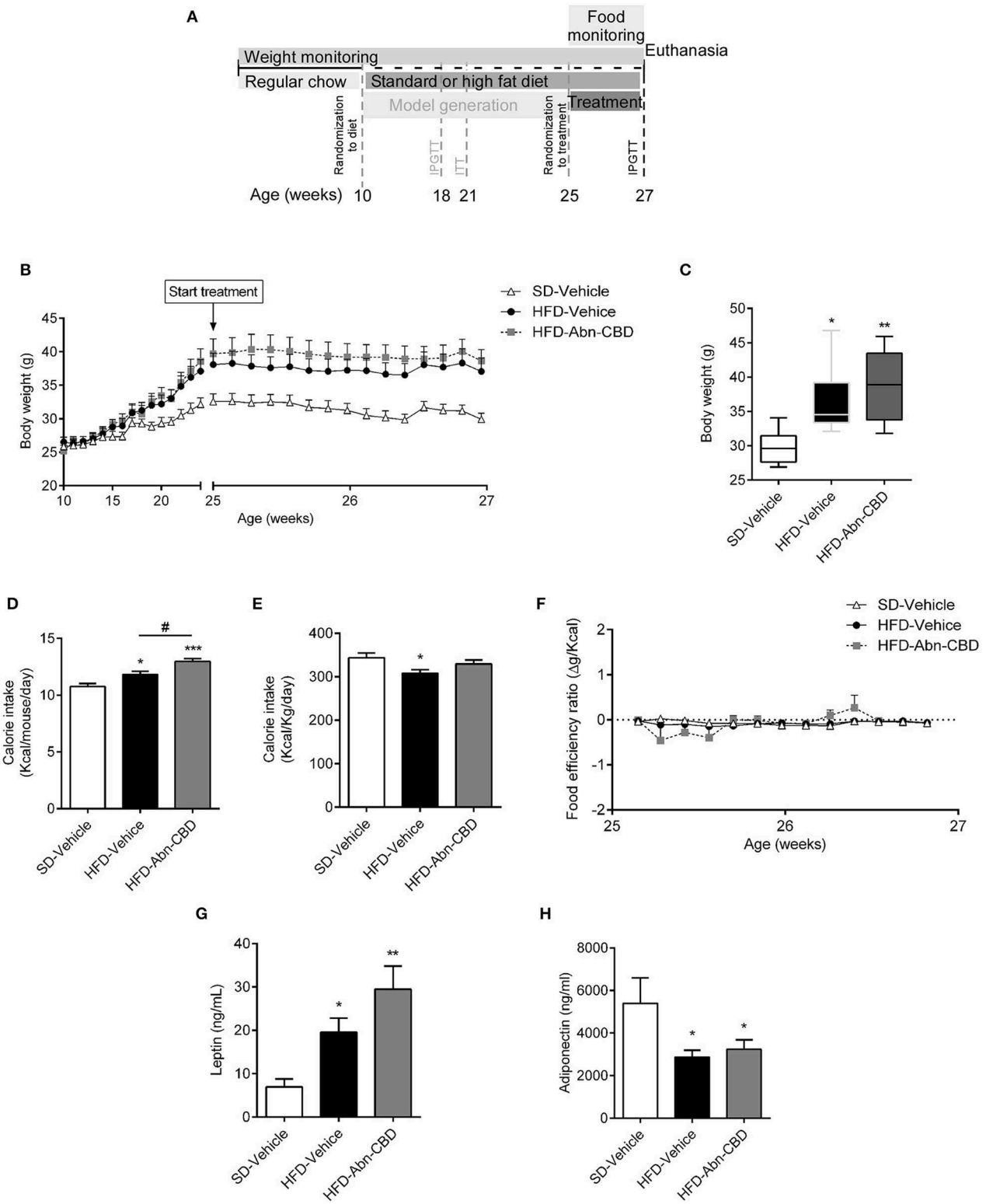

FIGURE 1 | Effect of Abn-CBD on body weight and food intake. Mice fed a standard diet (SD) or high fat diet (HFD) for 15 weeks were randomized to vehicle or Abn-CBD for further 2 weeks **(A)**. Body weight was measured weekly during the first 15 weeks and daily during treatment **(B)**. **(C)** Body weight after the 2-week treatment with vehicle or Abn-CBD. **(D)** Average of daily calorie intake per mouse and **(E)** per Kg of weight during the 2-week treatment and **(F)** food efficiency. Leptin **(G)** and adiponectin **(H)** plasma levels at the end of the study. Data show mean ± S.E.M except **(C)** that shows median ± min to max. $n = 6$ SD-Vehicle, $n = 7$ HFD-Vehicle and $n = 8$ HFD-Abn-CBD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ compared to SD-Vehicle; $^{\#}p \leq 0.05$ compared to HFD-Vehicle.

Liver Glycogen Measurement

Determination of hepatic glycogen was performed according to previous reports with some modifications (25, 26). Briefly, the liver samples (300–500 mg) were transferred to test tubes containing 30% KOH (w/v) and boiled for 1 h until complete homogenization. Na_2SO_4 was then added, and the glycogen was precipitated with ethanol. The samples were centrifuged at 800 g for 10 min, the supernatants were discarded, and the glycogen was dissolved in hot distilled water. Ethanol was added and the pellets obtained after a second centrifugation were dissolved in distilled water in a final volume of 25 ml. Glycogen content was measured by treating a fixed volume of sample with phenol reagent and H_2SO_4. Absorbance was then read at 490 nm with a spectrophotometer (VERSAmax, Molecular Devices LLC).

RNA Isolation and Real Time PCR

Tissues (100 mg of adipose tissue) were dissected and mRNA isolated using Trizol Reagent (Sigma-Aldrich) and RNeasy Mini Kit (Qiagen) following manufacturer's instructions. Retrotranscription was performed using SuperScript IV RT (Thermo Fisher Scientific Inc., Waltman, MA, USA) and mRNA expression were analyzed in an Applied Biosystems® 7500 fast using Fast Advanced Master Mix (all from Thermo Fisher Scientific) and appropriate FAM-labeled Taqman primers and probes for *Cxcl1*, *Il10,* and *Ucp1*. VIC-labeled primers and probe were used for housekeeping genes.

Oil Red O Staining of Liver Ectopic Lipid Deposition

Frozen liver samples were sliced in a cryostat, attached to microscope slides, and air-dried at room temperature for 30 min. Liver sections were then stained in fresh Oil red O for 10 min, rinsed in distilled water and immediately counterstained with haematoxylin for 1 min. Photomicrographs were taken on an Olympus BX41 microscope and the Oil red O staining intensity was quantified by using Image J software.

Masson's Trichrome Staining of Liver Fibrosis

Liver fibrosis was evaluated at the histological level by staining collagen fibers with Masson's trichrome staining. For that purpose, paraffin-embedded sections were hydrated and stained by using the Masson's trichrome kit (Casa Álvarez Material Científico S.A., Madrid, Spain) according to manufacturer's instructions.

Immunohistochemistry and Immunofluorescence Staining

Paraffin-embedded tissue sections (3 μm) were dewaxed, hydrated, and treated with antigen unmasking solution, citric acid based (Vector Laboratories Inc., Burlingame, CA, USA) for 20 min in a steamer and then 20 min to cool down. Sections were washed thrice with phosphate buffered saline (PBS). Endogenous peroxidase was quenched with 2% H_2O_2 in PBS for 30 min with agitation and endogenous biotin, biotin receptors, and avidin binding sites were blocked by avidin/biotin blocking kit according to manufacturer's instructions (Vector Laboratories Inc.). Immunohistochemistry was performed by incubating overnight at 4°C with primary antibody 1/100 (anti-insulin, Sigma-Aldrich; anti-F4/80, Abcam, Paris, France; anti-pNFKB, Abcam), rinsed thrice with PBS, followed by HRP polymer-conjugated Goat anti-Rat/Mouse polyclonal antibody (1 h) and finally rinsed thrice again and developed with diaminobenzidine substrate. Slides were rinsed in tap water, lightly counterstained with Mayer's haematoxylin, rinsed in ammonium chloride dehydrated and mounted with DPX medium (Shandon, Pittsburgh, Pennsylvania, USA). Specific primary antibodies were substituted with PBS or non-immune isotype-matched sera as the negative control.

For immunofluorescence primary antibody (anti-insulin 1/100 overnight, Santa Cruz Biotechnology Inc., Dallas TX, USA; anti-5-bromo-2′-deoxyuridine 1/100 overnight, Sigma-Aldrich; anti-αSMA, 1/100 overnight, Santa Cruz Biotechnology) incubation was followed by anti-rabbit IgG-AlexaFluor488 and/or anti-mouse IgG-AlexaFluor568 (1/1000; Thermo Fisher Scientific), for 1 h at room temperature. Slides were coverslipped and protected from photobleaching by Fluoroshield Mounting Media (Sigma-Aldrich). Photomicrographs were taken on an Olympus BX41 and further processed by Image J software to quantify signal intensity.

Apoptosis Assessment

Apoptosis was evaluated by the TUNEL technique using the *in situ* apoptosis detection kit (Roche) according to manufacturer's instructions. Images were analyzed using ImageJ software. Number of TUNEL-positive cells were normalized to islet area.

Data Analysis

Data are expressed as mean ± standard error of the mean (S.E.M.) for data fitting a normal distribution and median ± min to max for non-normal distributions. The statistical significance of differences in mean or median values was assessed by Student *t*-test or analysis of variance (ANOVA) followed by Tukey's multiple comparison test for normal distributions and by Mann-Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparison test for non-normal distributions. All analyses were performed with GraphPad Prism 6.07 or 7.04 (GraphPad Software, San Diego, CA, USA). A $p < 0.05$ was considered significant.

RESULTS

ABN-CBD Does Not Affect Body Weight

Ten-week-old male C57Bl/6J mice were fed a high fat diet (HFD) or standard diet (SD) for 15 weeks. Mice fed HFD weighed significantly more than SD-fed mice (37.9 ± 0.9 vs. 32.6 ± 1.2 g respectively; **Figure 1B**). Diet induced obese (DIO) mice were glucose intolerant and insulin resistant but they did not show impaired fasting glucose [103 ± 7 vs. 108 ± 4 mg/dl, SD- and HFD-mice, respectively (22)]. DIO mice were then randomized to daily intraperitoneal injections of vehicle (HFD-vehicle mice) or 0.05 mg/kg of Abn-CBD (HFD-Abn-CBD mice) for 2 weeks (**Figure 1A**). Dosage for

TABLE 1 | Systemic lipid markers.

Systemic lipid markers	SD	HFD-vehicle		HFD-Abn-CBD	
Triglycerides (mg/dl)	104.6 ± 7.9	103.4 ± 9.5	n.s.	112.4 ± 9.96	n.s.
Total cholesterol (mg/dl)	136.4 ± 10.7	174.8 ± 12.6*	*	170.3 ± 13.8	*
HDL-c (mg/dl)	42.4 ± 3.3	64.0 ± 4.1	**	65.1 ± 4.6	**
LDL-c (mg/dl)	14.7 ± 1.4	16.8 ± 2.82	n.s.	16.8 ± 2.5	n.s.
NEFA (mM)	2.1 ± 0.3	3.5 ± 0.1	*	3.6 ± 0.1	*
Glycerol (mg/l)	5.7 ± 2.9	10.0 ± 4.4	n.s.	12.9 ± 2.8	n.s.

*$p \leq 0.05$ and **$p \leq 0.01$ compared to SD-vehicle. n.s., non-significant.*

Abn-CBD was selected based on an ipGTT on lean mice (**Supplementary Figure 1**) and previous literature (15). After 2 weeks of treatment, body weights of HFD-vehicle and HFD-Abn-CBD mice were comparable (36.5 ± 0.8 and 38.9 ± 1.7 g, respectively), while SD-fed mice weighed 29.8 ± 1.8 g (**Figures 1B,C**). Both HFD-Abn-CBD and HFD-vehicle mice remained glucose intolerant (**Supplementary Figure 2**). Calorie intake per mouse was significantly higher in the HFD-Abn-CBD than in the HFD-vehicle group (**Figure 1D**), although calorie per gram (**Figure 1E**) and food efficiency was comparable in both groups (**Figure 1F**). In obesity, adiposity positively correlates with leptin plasma levels. HFD-vehicle mice had a 2.8-fold increase in plasma leptin levels compared to lean mice (**Figure 1G**). In agreement with their increased body weight and food consumption (**Figures 1C,D**), HFD-Abn-CBD mice had greater leptin levels (4.2-fold higher compared to lean mice) although differences found between HFD-vehicle and -Abn-CBD mice did not reach statistical significance ($p = 0.07$) (**Figure 1G**). Adiponectin, a hormone secreted from the adipose tissue to regulate glucose homeostasis, is known to be reduced in obesity (27). Plasma adiponectin levels were significantly lower in HFD-fed mice compared to SD-fed mice, independently of treatment (**Figure 1H**).

Abn-CBD Does Not Alter the Plasma Lipid Profile

As DIO is associated with elevated triglycerides, free-fatty acids and cholesterol in circulation, we therefore analyzed the plasma lipid profile of SD-fed, HFD-vehicle, and HFD-Abn-CBD mice. Although plasma triglyceride levels in HFD-fed mice were similar to those found in SD-fed mice, total cholesterol was 1.3-fold higher in HFD-fed compared to SD-fed mice (**Table 1**). High density lipoprotein (HDL) and non-esterified fatty acid (NEFA) content in plasma were also significantly higher in HFD-fed mice than in plasma from SD-fed mice (1.5- and 1.6-fold, respectively; **Table 1**). There were no significant differences in low density lipoprotein (LDL) levels in plasma, neither of glycerol levels between HFD-fed and SD-fed mice (**Table 1**). The lipid profile found in plasma from HFD-Abn-CBD mice was comparable to the one found in HFD-vehicle mice, including high cholesterol, elevated HDL and NEFA (1.25-, 1.5- and 1.6-fold higher, respectively, than in SD-fed mice; **Table 1**).

ABN-CBD Ameliorates Hyperinsulinemia, Protects Beta Cells From DIO-Induced Apoptosis, Decreases Inflammation, and Stimulates Beta Cell Proliferation

After 2 weeks of treatment with vehicle or Abn-CBD, HFD-vehicle mice were hyperinsulinemic compared to SD-fed mice (**Figure 2A**) while blood glucose levels were comparable (**Figure 2B**). These data suggest that mice remained insulin resistant as before starting the treatment but had not yet developed diabetes. Remarkably, mice treated with Abn-CBD had a similar insulinemia than SD-vehicle (**Figure 2A**) while maintaining blood glucose at the same level as SD- and HFD-vehicle mice (**Figure 2B**). We analyzed the pancreatic islets of Langerhans both at the morphometric and functional level (**Figures 2C–F**). No changes in number of islets were detected among groups (**Figure 2E**). However, mice fed a HFD showed a strong tendency toward larger islets than SD-fed mice ($p = 0.06$) while this hypertrophy was absent in HFD-Abn-CBD islets (**Figure 2D**). Treatment with Abn-CBD had no effect on total insulin secretory capacity, as shown by a static *in vitro* glucose-stimulated insulin secretion assay (**Figure 2F**). In DIO, loss of beta cell mass is associated with increased inflammation and oxidative stress in beta cells that leads to beta cell death. Accordingly, we found increased intra-islet apoptosis (**Figure 3A**), phosphorylation of p65 (p-NFκB) (**Figure 3B**) and a slight non-significant increase in pancreatic TBARS (**Figure 3D**) in HFD-vehicle when compared to SD-Vehicle. Importantly, Abn-CBD significantly reduced pancreatic content of TBARS (**Figure 3D**), intra-islet pNFκB staining (**Figure 3B**) and macrophage infiltration (**Figure 3C**), greatly lessening apoptosis (**Figure 3A**). Moreover, Abn-CBD induced beta cell proliferation, as measured by BrdU positive staining of beta cells, compared to SD-fed mice (**Figure 3E**). Thus, Abn-CBD protected beta cell mass without altering beta cell function.

Abn-CBD Reduces DIO-Induced Meta-Inflammation

Low-grade chronic inflammation is associated with DIO-related complications. Accordingly, plasma of HFD-vehicle mice showed significantly higher levels of interleukin 6 (IL-6; 3.2-fold increase; **Figure 4A**) and CXCL-1 (2.4-fold increase; **Figure 4B**) compared to SD-fed mice, while interleukin 5 (IL-5) levels trended to be 1.6-fold higher (**Figure 4C**). Of importance, treatment with Abn-CBD restored the levels of IL-6, CXCL-1, and IL-5 to those found in SD-fed mice (**Figures 4A–C**). HFD-fed mice did not show any change in either interferon gamma (IFNγ; **Figure 4D**) nor interleukin 10 (IL-10; **Figure 4E**) compared to SD-fed mice, independently of the treatment.

Since obesity is strongly associated to inflammation at white adipose tissue (WAT) (28), we analyzed the inflammation occurring in visceral (VAT) and subcutaneous adipose tissue (SAT). HFD-fed mice showed an expected increase in adipocyte size compared to SD-fed mice, independently of treatment (**Figure 5A**). We found an increase in the presence of crown-like structures in HFD-vehicle WAT upon staining with F4/80, which was significantly decreased in WAT from Abn-CBD-treated mice

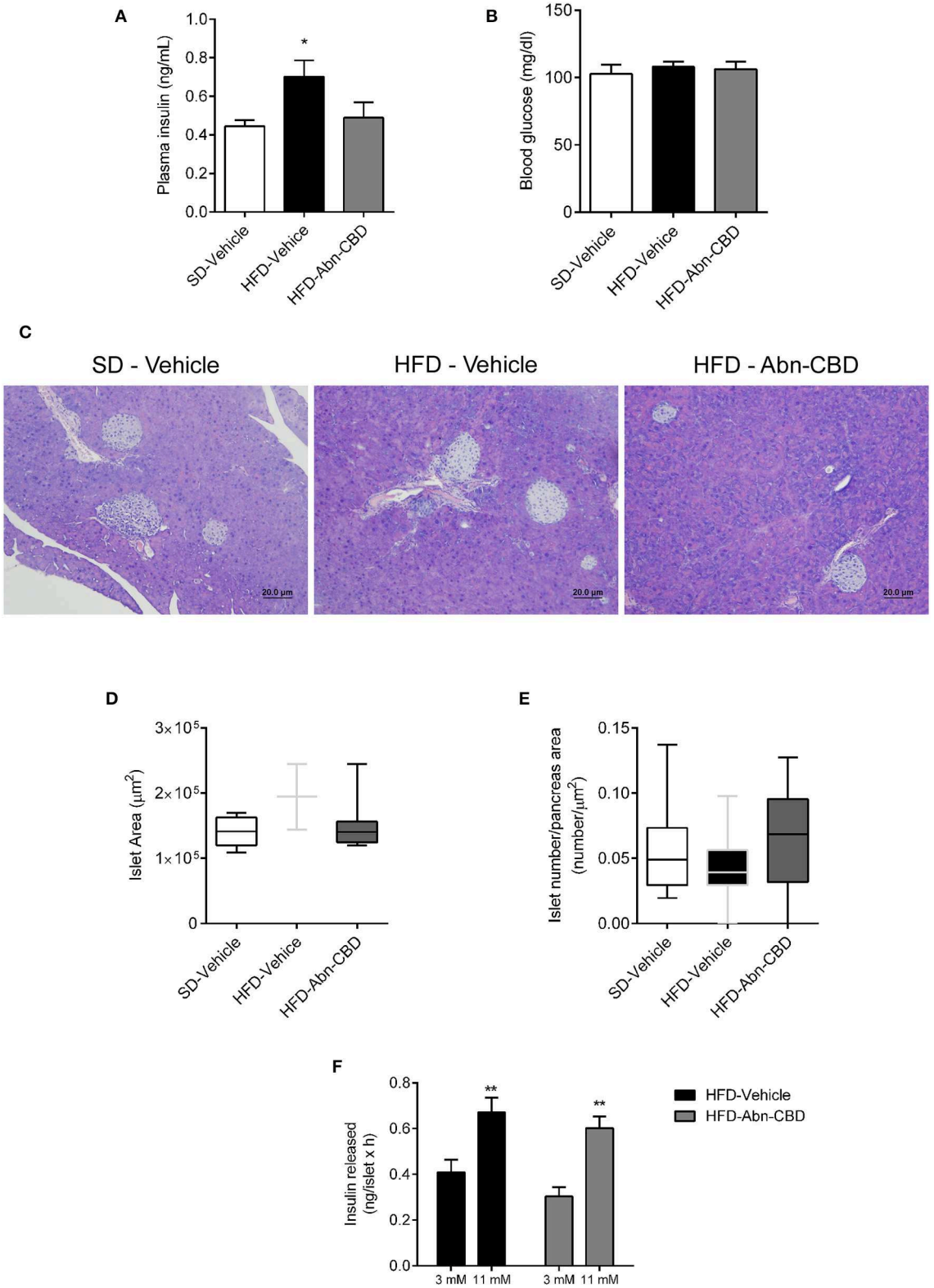

FIGURE 2 | Effect of Abn-CBD on glucose homeostasis, islet morphology and functionality. **(A)** Fasted (overnight) plasma insulin and **(B)** blood glucose levels at the end of the study. **(C)** Representative photomicrographs of islets. **(D,E)** Morphometric analysis of islets. Data show mean ± S.E.M. except **(D,E)** that show median ± min to max. $n = 6$ SD-Vehicle, $n = 7$ HFD-Vehicle and $n = 8$ HFD-Abn-CBD except **(D)**, $n = 51$–66 islets from 4 mice each group, **(E)** $n = 52$–81 islets from 4 mice each group. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ compared to SD-Vehicle. **(F)** Static glucose-stimulated insulin secretion in HFD-Vehicle and HFD-Abn-CBD isolated islets. $N = 11$–12 wells each condition, islets from 2 mice each group.

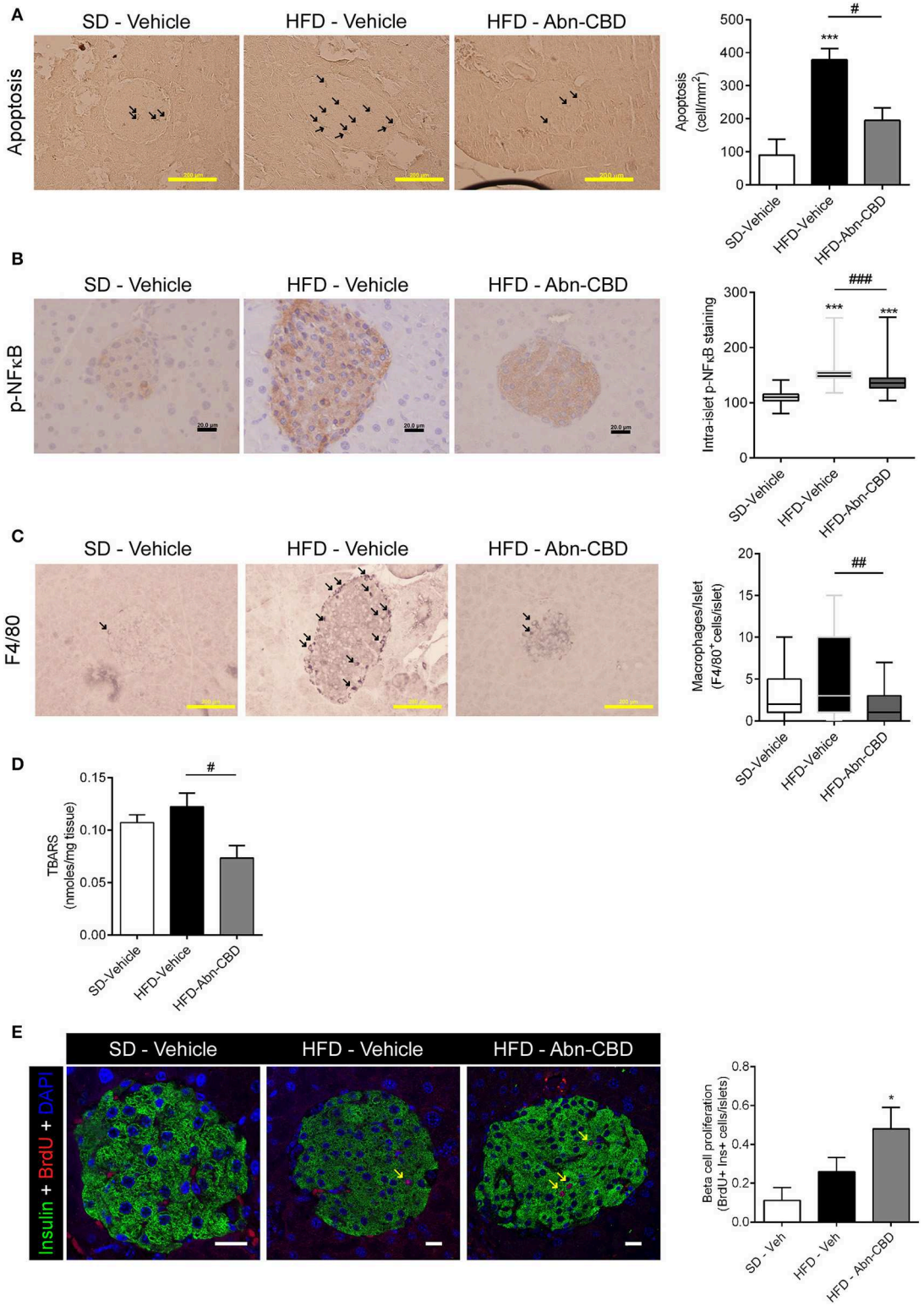

FIGURE 3 | Effect of Abn-CBD on pancreatic beta-cell viability and intra-islet inflammation. Representative photomicrographs and quantification of islets immunostained for apoptosis **(A)** and p-NFκB **(B)**; arrows indicate apoptotic cells; p-NFκB immunostaining was counterstained with haematoxylin. **(C)** F4/80 staining; arrows indicate macrophages. **(D)** Quantification of lipid peroxidation by TBARS production. **(E)** Representative photomicrographs of double insulin (green) and BrdU (red) immunostaining in islets and quantification of BrdU+/Insulin+ cells; arrows indicate proliferative beta cells; scale bar is 20 μm. $n = 6$ SD-Vehicle, $n = 7$ HFD-Vehicle and $n = 8$ HFD-Abn-CBD. $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$ compared to SD-Vehicle; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, and $^{\#\#\#}p \leq 0.001$ compared to HFD-Vehicle.

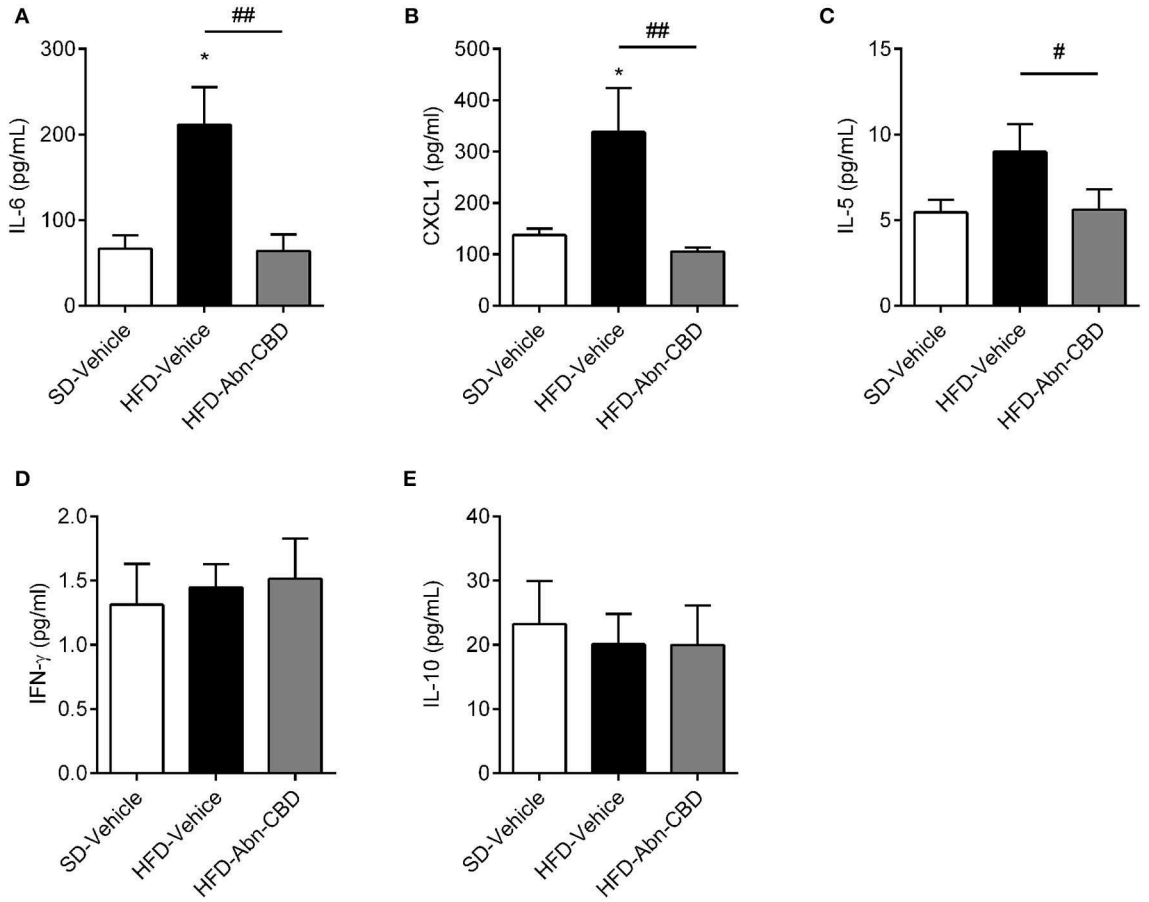

FIGURE 4 | Effect of Abn-CBD on circulating inflammatory cytokines. Plasma levels of IL-6 **(A)**, CXCL1 **(B)**, IL-5 **(C)**, IFN-γ **(D)**, and IL-10 **(E)** after the 2-week treatment of mice. $n = 6$ SD-Vehicle, $n = 7$ HFD-Vehicle and $n = 8$ HFD-Abn-CBD. $^{*}p \leq 0.05$, compared to SD-Vehicle; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ compared to HFD-Vehicle.

(**Figure 5B**). Analysis of cytokine expression showed a significant reduction of *Il10* expression in VAT (**Figure 5C**), and a significant increase in the expression of *Cxcl1* in both VAT ($p = 0.07$) and SAT from HFD-vehicle mice (**Figures 5D,E**, respectively). Interestingly, treatment with Abn-CBD prevented the alterations observed in cytokine expression in VAT (**Figures 5C,D**) and SAT (**Figure 5E**), showing that Abn-CBD protects WAT from diet-induced inflammation. We also wanted to explore whether Abn-CBD could promote a shift toward browning in WAT. For this purpose we analyzed *Ucp1* expression in BAT, VAT, and SAT. While no significant differences were found in brown adipose tissue among groups (**Figure 5F**), HFD-vehicle mice showed a significant decrease in *Ucp1* expression in VAT and SAT that was counteracted by Abn-CBD (**Figures 5G,H**).

Abn-CBD Does Not Protect Against NAFLD

In the liver the accumulation of triglycerides leads to NAFLD that eventually leads to liver failure (9). Herein, DIO mice accumulated triglycerides in liver independently of treatment, displaying 2-fold increase in lipid content compared to lean mice, as shown by oil red-O staining (**Figure 6A**). Furthermore, liver from HFD-vehicle mice exhibited fibrosis, as determined by a significant increase in collagen content compared to SD-fed mice (**Figure 6B**) as well as early makers of liver fibrosis, such as α-SMA (**Figure 6C**). Interestingly, the liver from HFD-Abn-CBD mice had significantly lower collagen than HFD-vehicle mice (**Figure 6B**) and reduced staining of α-SMA (**Figure 6C**). Moreover, liver glycogen content was comparable in both HFD- and SD-fed vehicle-treated mice (**Figure 6D**). Although not reaching statistical significance ($p = 0.07$), HFD-Abn-CBD mice displayed a 2.2-fold higher content of hepatic glycogen than HFD-vehicle mice (**Figure 6D**). In order to assess liver damage, we measured plasma levels of alanine aminotransferase (ALT). Levels of ALT in DIO mice were comparable to those found in lean mice, independently of treatment (**Figure 6E**).

Abn-CBD Protects Hepatocytes From DIO-Induced Immune Cell Infiltration

As HFD-Abn-CBD mice displayed reduced liver fibrosis concomitant with lower levels of several circulating pro-inflammatory cytokines compared to HFD-vehicle mice, we further assessed the degree of liver inflammation. Liver from HFD-vehicle mice had increased p-NFκB compared to liver

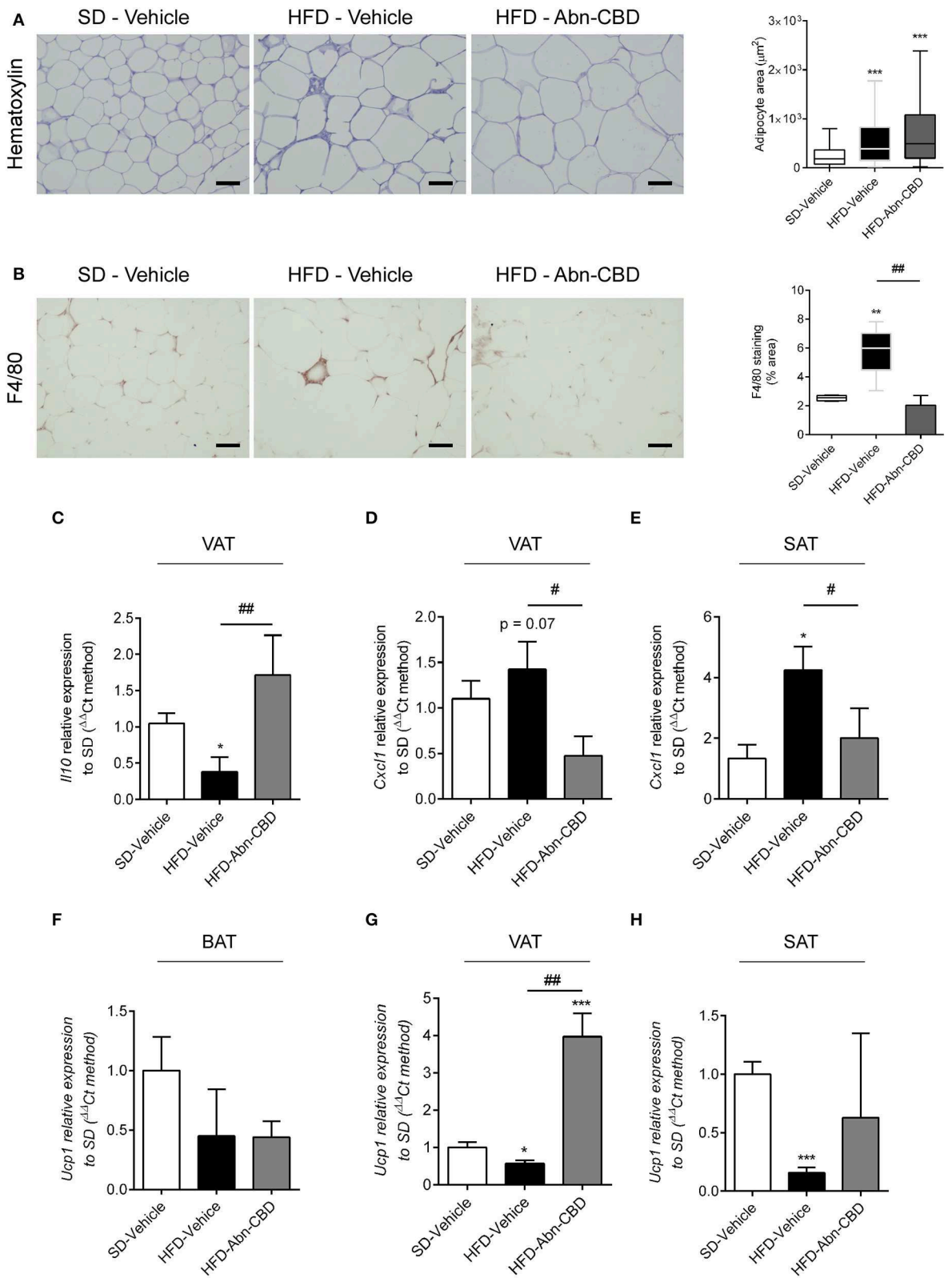

FIGURE 5 | Effect of Abn-CBD on inflammation in white adipose tissue. Representative photomicrographs and quantification of **(A)** adipocyte size and **(B)** crown-like structures in white adipose tissue. Relative expression of **(C)** *Il10* and **(D)** *Cxcl1* in visceral adipose tissue and of **(E)** *Cxcl1* in subcutaneous adipose tissue. Relative expression of *Ucp-1* in **(F)** brown adipose tissue, **(G)** visceral adipose tissue, and **(H)** subcutaneous adipose tissue. β-actin was used as reference gene. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ compared to SD-Vehicle; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ compared to HFD-Vehicle.

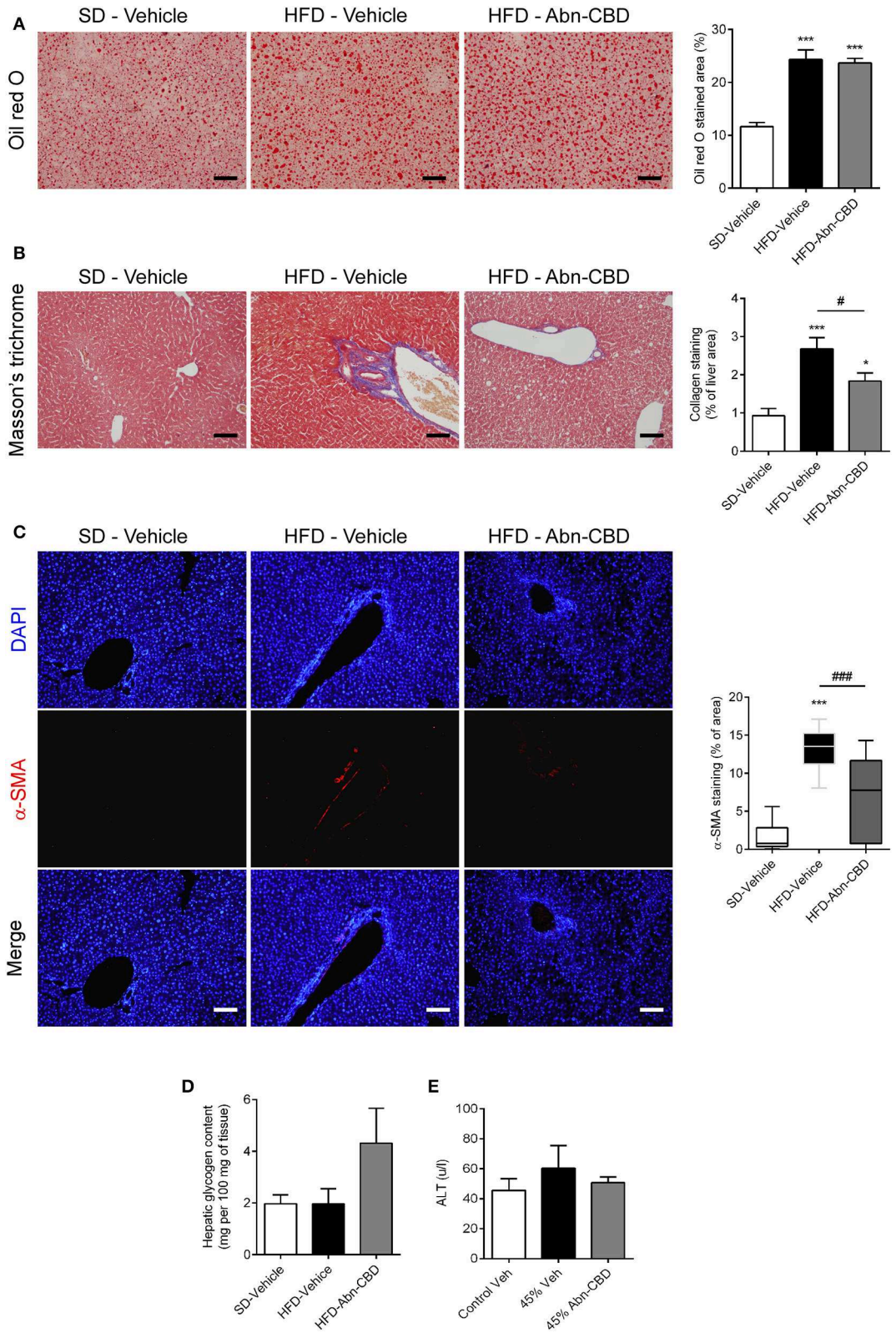

FIGURE 6 | Effect of Abn-CBD on liver structure and function. Representative photomicrographs and quantification of livers stained for lipid droplets **(A)** and collagen fibers **(B)**, scale bar is 100 μm. Representative photomicrographs of immunofluorescence in liver for α-SMA (red), counterstained with DAPI (in blue) **(C)**. Biochemical determination of hepatic glycogen content **(D)** and circulating levels of alanine aminotransferase **(E)**. $n = 7–8$ SD-Vehicle, $n = 5–8$ HFD-Vehicle and $n = 6–8$ HFD-Abn-CBD. $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$ compared to SD-Vehicle; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$ compared to HFD-Vehicle.

from SD-fed mice, while liver from HFD-Abn-CBD mice had significantly reduced p-NFκB levels comparable to those in liver from SD-fed mice (**Figure 7A**). Additionally, liver from HFD-vehicle mice showed a significant 3.3-fold increase in the number of macrophages (F4/80$^+$ cells) compared to SD-fed mice (**Figure 7B**). Treatment with Abn-CBD greatly reduced the number of macrophages to the levels found in SD-fed mice (**Figure 7B**). We then determined hepatocytes viability measured as apoptosis and proliferation. Hepatocytes from HFD-vehicle mice were undergoing significantly more apoptosis than those from SD-fed mice, which was reverted upon Abn-CBD treatment (**Figure 7C**). In addition, Abn-CBD increased hepatocytes proliferation when compared to HFD-vehicle, but not over those levels found in hepatocytes from SD-fed mice (**Figure 7D**).

DISCUSSION

CBD and Abn-CBD lack psychoactive effects, and therefore bestow them as attractive candidates in medicinal cannabis research. Here, we investigated the *in vivo* actions of Abn-CBD in the metabolic and inflammatory dysfunctions that arise in the context of obesity, prediabetes, and NAFLD. At the biochemical and cellular level, C57Bl6/J mice fed a 45% HFD (saturated fats from lard) for 15 weeks displayed, dyslipidaemia, hyperleptinemia, hypoadiponectinemia, hyperinsulinemia, and increased islet cell apoptosis. In addition, mice developed fatty liver with macrophage infiltration, increased hepatocyte apoptosis and decreased hepatocyte proliferation. However, we did not detect impaired liver function, underlining the validity of this mouse model to assess new therapies tackling early stages of obesity comorbidities.

Overall, subchronic treatment with Abn-CBD in this mouse model improved low-grade inflammation, reverted hyperinsulinemia and decreased liver fibrosis without altering body weight or ectopic accumulation of fat in the liver.

Although Abn-CBD did not impact either body weight or the lipid profile, it increased leptin levels. CBD has been described to decrease food intake in acute tests (29, 30) but its impact on body weight is poorly investigated and controversial (31, 32). Previous data using a streptozotocin (STZ)-induced diabetic mouse model showed that Abn-CBD lowered food intake (15), which was associated with restored plasma insulin levels. The discrepancies of our results with these findings may lay on the obvious differences between STZ-model and DIO-model. Indeed STZ-model does not induce obesity, insulin resistance and hyperinsulinemia, suggesting that underlying mechanisms for Abn-CBD-driven changes in food intake are different in insulin resistant model and in the insulin production deficient model.

Inflammation and insulin resistance are intimately related processes, and currently there are evidence suggesting that inflammation is driven by insulin resistance (33). Unfortunately, we could not measure insulin tolerance or insulin sensitivity after Abn-CBD treatment, but the absence of hyperinsulinemia without increase in fasting glucose levels indirectly suggest that Abn-CBD might improve insulin sensitivity in our mouse model. However, gold-standard techniques such as euglycemic hyperinsulinemic clamp would be necessary in order to unequivocally establish the impact of Abn-CBD in insulin tolerance/sensitivity. Given that direct anti-inflammatory properties have been ascribed to Abn-CBD and other GPR55 agonists (19, 34, 35), decreased inflammation in our model could be related to improved insulin sensitivity, direct actions on receptors such as GPR18 and GPR55, or both. Further studies in this regard would be required to decipher the molecular mechanism that underlies the role of Abn-CBD in insulin sensitivity toward inflammatory processes.

We and others have previously demonstrated that Abn-CBD can directly stimulate insulin secretion *in vitro* (21, 23) as well as *in vivo* in a mouse model of mild type 1 diabetes (15). However, to the best of our knowledge it has not been assayed in a mouse model of obesity and prediabetes. Herein, Abn-CBD did not seem to interfere with islet function as it did not change islet sensitivity to glucose as assessed by GSIS *ex vivo* experiments. Although subchronic Abn-CBD treatment in prediabetes does not enhance insulin secretion, we report here that hyperinsulinemia as well as the trend to increase islet area in HFD-vehicle mice were reverted, suggesting that subchronic Abn-CBD treatment in prediabetes reduces the inflammatory state that leads to beta cell death.

Notwithstanding, immunohistological analysis of pancreas showed that treatment with Abn-CBD greatly lowered islet cell apoptosis while induced beta cell proliferation, thus promoting preservation of beta cell mass. These findings are in agreement with our previous work showing a proliferative and protective role of Abn-CBD against cytokine-induced apoptosis on isolated islets from lean mice and human (21). Similar observations were also obtained in STZ-induced diabetic mice by another group (15). Importantly, cannabinoids and cannabinoid receptors have been previously shown to modulate islet viability in DIO mice (36, 37). Herein, we also found a significant reduction in oxidative stress in pancreas isolated from HFD-Abn-CBD mice compared to HFD-vehicle mice. Moreover, Abn-CBD significantly lowered levels of phosphorylated (i.e., active) NFκB corroborating with lower cell apoptosis in islets from HFD-Abn-CBD treated mice. Thus, Abn-CBD preserves islet viability and beta cell mass *in vivo* by reducing islet cell apoptosis and enhancing beta cell proliferation.

Importantly, the pro-adiposity effect found with Abn-CBD treatment was not only devoid of an extra pro-inflammatory component but rather accompanied by decreases in plasma levels of cytokines involved in systemic low-grade inflammation such as IL-6, IL-5, and CXCL1 levels. In fact, IL-6 is one of the primary mediators of low-grade inflammation in obesity (3) and both CXCL-1 as well as IL-5 have been found to be altered in prediabetes (6, 38). There are some evidence pointing at Abn-CBD as a modulator of the inflammatory response (19) and the two main G-protein coupled receptors (GPCRs) that so far have been related to Abn-CBD effects, i.e., GPR18 and GPR55, have been widely involved in inflammation and related processes (39, 40). Comparable to our data, the GPR55 agonist O-1602 significantly reduced the levels of IL-6 both in plasma and pancreas tissue in mice with cerulein-induced pancreatitis (34).

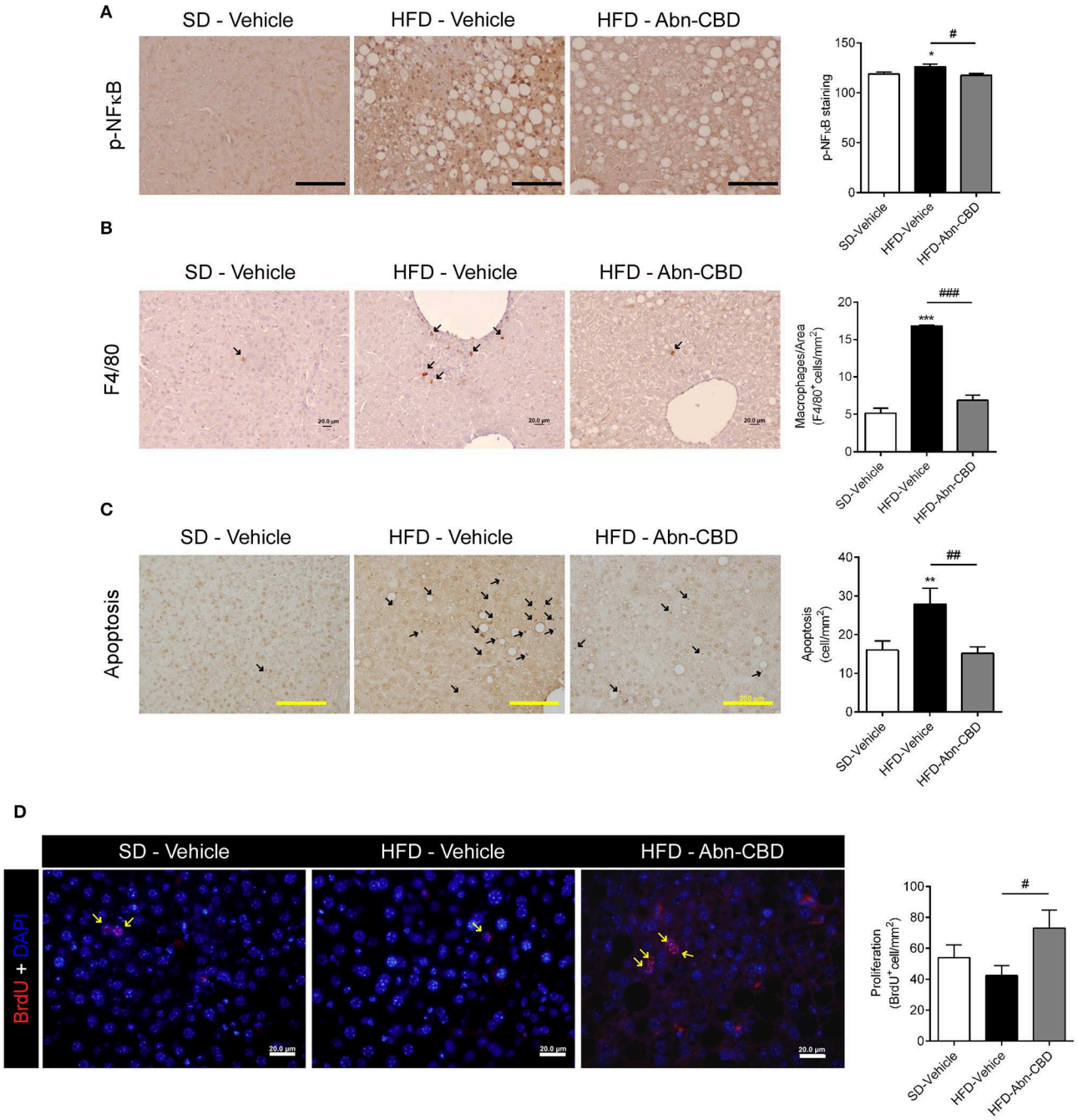

FIGURE 7 | Effect of Abn-CBD on hepatocyte viability and liver inflammation. **(A)** Representative photomicrographs and quantification of livers stained for the inflammatory marker p-NFκB **(A)**, the macrophage marker F4/80 **(B)** and apoptotic cells **(C)**; arrows indicate stained cells; scale bar is 100 µm **(A)**, 20 µm **(B)**, and 200 µm **(C)**. **(D)** Representative photomicrographs and quantification of BrdU immunofluorescence (in red) in the liver, counterstained with DAPI (in blue). Arrows indicate proliferating cells. $n = 7$–8 SD-Vehicle, $n = 5$–8 HFD-Vehicle and $n = 6$–8 HFD-Abn-CBD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ compared to SD-Vehicle; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, and $^{\#\#\#}p \leq 0.001$ compared to HFD-Vehicle.

Therefore, our results support an anti-inflammatory effect of Abn-CBD in prediabetes and NAFLD. The putative involvement of Abn-CBD in browning processes has not been explored so far. Our results show that Abn-CBD potently increases *Ucp1* expression in VAT, also counteracting HFD-induced decrease in SAT. This is suggestive of Abn-CBD promoting browning in WAT, what potentially could be contributing to decreased meta-inflammation. This first evidence warrants further investigation on the role that Abn-CBD may have on energy expenditure. NAFLD is an increasing concern in obesity-related comorbidities

as it worsens metabolic syndrome, development of insulin resistance and cardiovascular disease (41). As expected, HFD induced a prominent increase of ectopic fat in the liver of our mouse model. Although Abn-CBD treatment did not resorb fat depot, it decreased collagen staining, suggesting a reduce fibrosis, most probably by reducing the pro-inflammatory environment. Importantly, Abn-CBD treatment did not affect liver function since both its capacity to store glycogen and the marker of liver function ALT were not compromised. Previously, CBD has been found to protect the liver from both alcohol-induced and non-alcohol-induced steatosis by mechanisms including inhibition of oxidative stress, increase in autophagy and decrease of lipid accumulation (42, 43). In contrast, a recent report found a deleterious effect of chronic administration of the atypical cannabinoids O-1918 and O-1602 in the liver of DIO Sprague-Dawley rats (44). However, administered doses were 100-fold higher than those used in our study, which could account for increased off-target effects and chemical toxicity. We also found that Abn-CBD decreased apoptosis and preserved proliferation in liver cells. An enhanced hepatocyte proliferation could carry the risk of developing hepatocellular carcinoma, which is related to the onset of NAFLD (45). In fact, high levels of IL-6 suppress hepatocyte proliferation in obesity (46), maybe as a protective mechanism. However, Abn-CBD increased hepatocyte proliferation only when compared to HFD-vehicle but proliferative levels were not above those found in healthy mice (SD-fed mice). This agrees with Abn-CBD preserving hepatocytes proliferation without inducing tumorogenesis. Interestingly, Abn-CBD has been reported to have anti-tumoral activity rather than promoting uncontrolled proliferation (47, 48). Given that Abn-CBD also decreased IL-6 levels in our mice, the beneficial effects of Abn-CBD on preserving hepatocytes proliferation might be mediated through reductions in IL-6 levels. The number of macrophages infiltrating the liver and activation of p-NFκB pathway was also diminished, suggesting a reduced intra-liver inflammation. Interestingly, IL-6, for which DIO-mediated increases were reverted by Abn-CBD in our study, has been found to play an important role in obesity-induced liver inflammation (49, 50). CBD was also shown to protect the liver by modulating inflammation, oxidative stress and cell death (51). Of note, CBD acts as a functional antagonist of CB1R (52), whose blockade was reported to revert liver steatosis in DIO mice (53). Moreover, O-1918, considered an antagonist of the Abn-CBD receptor, increased the levels of circulating pro-inflammatory cytokines in DIO rats (44). Taken together, these results point to Abn-CBD promoting a healthier cellular environment also in the liver.

In summary, we herein provide evidence that the atypical cannabinoid Abn-CBD is able to induce beneficial metabolic and anti-inflammatory actions at both systemic and tissue level in a mouse model of diet-induced prediabetes and NAFLD. Considering the sex limitation of our study -performed in males only-, further studies to confirm these effects in females are warranted. These results warrant further investigation on the potential this compound and/or related molecules may have to treat early stages of obesity-induced metabolic diseases.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethic Committee of the University of Malaga (authorization no. 2012–0061A).

AUTHOR CONTRIBUTIONS

SR-Z and FB-S conceived, designed, and supervised the study. SR-Z, IG-M, MG-F, VE-J, MP-M, AE-S, LS-S, EL, NC-V, and AR performed the experiments. SR-Z, GR-M, GO, BG, IG-M, and FB-S analyzed the results. FB-S, SR-Z, and IG-M wrote the manuscript. FB-S was the guarantor of this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00103/full#supplementary-material

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A Novel Indoline Derivative Ameliorates Diabesity-Induced Chronic Kidney Disease by Reducing Metabolic Abnormalities

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Both diabetes and obesity (diabesity) contribute significantly to the development of chronic kidney disease (CKD). In search of new remedies to reverse or arrest the progression of CKD, we examined the therapeutic potential of a novel compound, AN1284, in a mouse model of CKD induced by type 2 diabetes with obesity. Six-week-old BKS Cg-Dock 7^{m}+/+ $Lepr^{db}$/J mice with type 2 diabetes and obesity were treated with AN1284 (2.5 or 5 mg kg^{-1} per day) via micro-osmotic pumps implanted subcutaneously for 3 months. Measures included renal, pancreatic, and liver assessment as well as energy utilization. AN1284 improved kidney function in BSK-*db/db* animals by reducing albumin and creatinine and preventing renal inflammation and morphological changes. The treatment was associated with weight loss, decreased body fat mass, increased utilization of body fat toward energy, preservation of insulin sensitivity and pancreatic β cell mass, and reduction of dyslipidemia, hepatic steatosis, and liver injury. This indoline derivative protected the kidney from the deleterious effects of hyperglycemia by ameliorating the metabolic abnormalities of diabetes. It could have therapeutic potential for preventing CKD in human subjects with diabesity.

Keywords: obesity, diabetes, chronic kidney disease, indoline derivative, insulin resistance, fatty liver

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INTRODUCTION

Over the past decades, diabetes and obesity have evolved into global epidemics, affecting social groups and ages in both developed and developing countries. Diabesity, a new term used to describe the co-existence of these two conditions (1), results in the death of over a million people worldwide each year, and its global prevalence exceeds 347 million. It is also the principal cause of cardiovascular disease, stroke, dementia, cancer, non-alcoholic fatty liver disease (NAFLD), and chronic kidney disease (CKD) (2). The latter remains the leading cause of end-stage renal disease (1) and is responsible for the rise in mortality among diabetic patients (3, 4). CKD is characterized by a decrease in glomerular filtration rate, glomerular and tubular hypertrophy, albuminuria, renal inflammation, and tubulo-interstitial fibrosis (5). While current treatments have been shown to delay the progression of the disease and temporarily improve the patient's quality of life, CKD eventually results in dependence on dialysis or kidney transplantation (6). Therefore, there is a critical need to develop more effective, long-lasting treatments for CKD.

Recently, we described a novel series of indoline derivatives with potent antioxidant and anti-inflammatory activities (7). These compounds act by inhibiting p38 MAPK, reducing the degradation of IκBα and the nuclear translocation of NF-κB and AP-1 (8, 9) and have been shown to prevent cellular damage in a mouse model of acute liver disease (10). The aim of the current study was to examine the effect of AN1284 in ameliorating the development of CKD in a murine model of diabesity and its potential to mitigate metabolic abnormalities. Our data show that the compound was able to preserve kidney morphology, ameliorate the development of inflammation and fibrosis, and decrease proteinuria. In addition, AN1284 improved the metabolic profile of the mice, resulting in weight loss via increased fat oxidation, decreased hepatic steatosis, and enhanced insulin sensitivity.

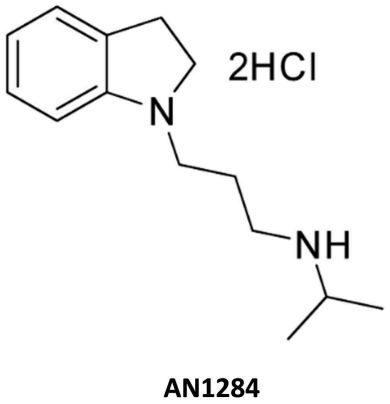

FIGURE 1 | The chemical structure of AN1284.

MATERIALS AND METHODS

Animals

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem (AAALAC accreditation #1285; Ethic approval number MD-17-15302-3) and are in compliance with the ARRIVE guidelines (11). All the animals used in this study were housed under specific pathogen-free conditions, up to five per cage, in standard plastic cages with natural soft sawdust as bedding. The animals were maintained under a controlled temperature of 22–24°C, humidity at 55 ± 5%, and alternating 12-h light/dark cycles and provided with food and water *ad libitum*.

Induction of Type 2 Diabetes

Male 6-week-old BKS Cg-Dock7^{m}+/+Lepr^{db}/J mice (BKS *db*; Jackson Laboratory, Cat# 00642) were randomly divided into the experimental groups, 8–10 mice per group, and treated chronically (3 months) with AN1284 [2.5 or 5 mg kg^{-1} per day; **Figure 1**; prepared as described by Zeeli et al. (7)], by a subcutaneously implanted micro-osmotic pump (Model 1004; alzet®). A new pump was implanted each month. One group of obese BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J mice and non-obese controls underwent sham surgery in an identical manner to the treatment groups but without pump insertion. Body weight and glucose levels were monitored weekly. Twenty-four-hour urine output and water consumption were measured using the CCS2000 Chiller System (Hatteras Instruments). Body composition was determined by EchoMRI-100H™ (Echo Medical Systems). Mice were euthanized by cervical dislocation under anesthesia; trunk blood, kidneys, pancreas, liver, muscle, and fat pads were collected, and samples were either snap-frozen or fixed in buffered 4% formalin for further analyses.

Multi-Parameter Metabolic Assessment

Mice with free access to food and water were housed under a standard 12-h light/12-h dark cycle, which consisted of a 48-h acclimation period followed by 24 h of sampling. Measures of mouse activity and respirometry were made with the Promethion High-Definition Behavioral Phenotyping System (Sable Instruments, Inc., Las Vegas, NV, USA) as described previously (12). Respiratory gases were measured with a GA-3 gas analyzer using a pull-mode, negative-pressure system. Airflow was measured and controlled by FR-8, with a set flow rate of 2,000 ml/min. Water vapor was continuously measured, and its dilution effect on O_2 and CO_2 was mathematically compensated. Total energy expenditure (TEE) was calculated as $VO_2 \times (3.815 + 1.232 \times RQ)$, normalized to effective body mass, and expressed as kcal/h/kg$^{0.75}$. Fat oxidation (FO) was calculated as $FO = 1.69 \times VO_2 - 1.69 \times VCO_2$ and expressed as g/d/kg$^{0.75}$. Ambulatory activity and position were monitored simultaneously with the collection of the calorimetry data using XYZ beam arrays with a beam spacing of 0.25 cm. Meal size and total food intake during a period of 24 h were measured automatically by the system.

Blood, Kidney, and Urine Biochemistry

Serum insulin and urine albumin were measured by ELISA (Millipore and Bethyl Laboratories, respectively). Serum urea, glucose, alanine transaminase (ALT), alkaline phosphatase (ALP), urine glucose and creatinine, total triglycerides, cholesterol, and high- and low-density lipoprotein (HDL and LDL, respectively) were determined using the Cobas C-111 bio-analyzer (Roche, Switzerland). Blood urea nitrogen (BUN) was calculated by serum urea levels (BUN mg/dl = Urea mM × 2.801). Blood glucose was measured with the Elite glucometer (Bayer, Pittsburgh, PA). Glucose and insulin levels were used to calculate the homeostasis model assessment-insulin resistance index (HOMA-IR) as glucose (mg/dl) × insulin (U/L) 22.5. Whole kidney proteins were extracted and the levels of the following markers (MCP1, TNFα, TGFβ, and IL18) were measured using ELISA kits (Abcam; Cat# ab100721, ab46105, ab119557, and ab216165, respectively). Liver proteins were extracted and the levels of TNFα were measured using the same ELISA kit used for the kidney.

Hepatic Triglyceride and Cholesterol Contents

The liver was extracted as described previously (13), and its triglyceride and cholesterol contents were determined using the

Cobas C-111 bio-analyzer (Roche, Switzerland) and normalized to wet tissue weight.

Histopathology

Paraffin-embedded kidney sections (2 μm) from each mouse were stained with Periodic Acid–Schiff (PAS), followed by hematoxylin. Paraffin-embedded liver sections (5 μm) from each mouse were stained with H&E. Kidney and liver images were taken from 10 random 40× and 20× fields with an AxioCam ICc5 color camera mounted on an Axio Scope.A1 light microscope (Zeiss, Germany). The mesangial expansion, glomerular, and Bowman's space cross-sectional areas were quantified in a blinded manner using ZEN BLUE software (Zeiss, Germany). The hepatic fat area was quantified in the same manner, using Adobe Photoshop CS3 software.

Immunohistochemistry

Kidney and liver tissues were fixed in buffered 4% formalin for 48 h and then embedded in paraffin. Sections were deparaffinized and hydrated. Heat-mediated antigen was retrieved with 10 mM citrate buffer pH 6.0 (Thermo Scientific, IL, USA). Endogenous peroxide was inhibited by incubating with a freshly prepared 3% H_2O_2 solution in MeOH. Unspecific antigens were blocked by incubating sections for 1 h with 2.5% horse serum (VE-S-2000, Vector Laboratories). For assessment of inflammation and fibrosis, the sections were stained with rabbit anti-mouse MCP-1 (Abcam; Cat# ab25124, 1:5,000), IL-18 (Abcam; Cat# ab71495, 1:2,000), TNFα (Abcam; Cat# ab6671, 1:800), TGFβ (Abcam, Cat# ab66043, 1:2,000), Collagen-1 (Abcam; Cat# ab34710, 1:1000), and α-SMA (Abcam; Cat# ab5694, 1:5000) antibodies, followed by incubation with horse anti-rabbit HRP conjugate (MP-7401, ImmPRESS™, Vector laboratories). Color was developed after an incubation with 3,3'-diaminobenzidine (DAB) substrate [ImmPACT DAB Peroxidase (HRP) Substrate, SK-4105, Vector Laboratories], followed by hematoxylin counterstaining and mounting (Vecmount H-5000, Vector laboratories). Stained sections were photographed as mentioned above. Positive areas for each marker were calculated using color thresholding and measuring area fractions by ImageJ software, with a minimum of 10 random kidney and liver sections per mouse. Images are presented in the figures showing the animal with the median value for each group.

For assessing the number of Langerhans β-islets and their composition, pancreatic sections were stained with guinea pig anti-insulin (Dako; Cat# A0564, 1:1,600) antibody, followed by biotinylated secondary antibody and VECTASTAIN ABC reagent (VECTASTAIN ABC-Peroxidase Kit, Vector laboratories). Color was developed as described in the preceding section followed by hematoxylin counterstaining and mounting (Vecmount H-5000, Vector Laboratories). Stained sections were photographed as described above. Panoramic images were taken for the entire section using ZEN BLUE software (Zeiss, Germany) and the islets-to-pancreas ratio and the β-cell-to-islet ratio were calculated.

Real-Time PCR

Total mRNA from liver and muscle was extracted with Bio-Tri RNA lysis buffer (Bio-Lab, Israel) followed by DNase I treatment (Thermo Scientific, IL, USA) and reverse transcribed using the Qscript cDNA Synthesis Kit (Quanta Biosciences, MA). Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, CA) and the CFX connect ST system (Bio-Rad, CA), and gene expression quantification was done using the 2∧(-ΔΔCT) method. The primers were as follows: *Col1α1* (forward-5′-TTCTCCTGGCAAAGACGGACTCAA-3′, reverse-5′-GGAAGCTGAAGTCATAACCGCCA-3′), *Tgfβ1* (forward-5′-GCGGACTACTATGCTAAAGAGG-3′, reverse-5′-GTAGAGTTCCACATGTTGCTCC-3′), *Glut4* (forward-5′-ATTTGGGGCCCTAGGTTGTT-3′, reverse-5′-ATACAGCAGCCCTTGGGTTT-3′), *Cxcl10* (forward-5′-GGATGGCTGTCCTAGCTCTG-3′, reverse-5′-TGAGCTAGGGAGGACAAGGA-3′), *Stat6* (forward-5′-AGCCCAGAAACAAAGCCTCTT-3′, reverse-5′-TTCGAGCATTAACACCCCACT-3′), *Klf4* (forward-5′-AGAAGTGTGACAGGGCCTTTT-3′, reverse-5′-TCGTGGGAAGACAGTGTGAAA-3′), *Acc* (forward-5′-GGACCACTGCATGGAATGTTAA-3′, reverse-5′-TGAGTGACTGCCGAAACATCTC-3′). QuantiTect Primer (Qiagen) was used to evaluate the expression of *Scd1* (QT00291151), and *Cd36* (QT0105825). Normalization was performed against *Ubc* (forward-5′-GCCCAGTGTTACCACCAAGA-3′, reverse-5′-CCCATCACACCCAAGAACA-3′) and *Gapdh* (forward-5′-AGGTCGGTGTGAACGGATTTG-3′, reverse-5′-TGTAGACCATGTAGTTGAGGTCA-3′).

Extraction of AN1284 From Liver and Kidney

Liver and kidney samples from *db/db* mice treated for 3 months with AN1284 were homogenized (100 mg ml^{-1}, Polytron, Kinematiea GmbH, Germany) in 1× PBS. To 180 μl of tissue homogenate was added 20 μl of rivastigmine (750 ng ml^{-1}) as an internal standard. Proteins were precipitated with 300 μl of ice-cold HPLC-grade MeOH; the mixture was vortexed for 5 min and then allowed to stand for 5 min. This procedure was repeated three times. The samples were centrifuged at 20,800 × *g* at 10°C for 15 min. The supernatant (200 μl) was filtered through 0.25 μM GHP membranes and stored at −80°C until analyzed. Calibration curves for the compounds in liver and kidney homogenates were prepared from untreated mice.

Quantification of AN1284 by Mass Spectroscopy

Tandem liquid chromatography–mass spectrometry (LC-MS/MS) enables the analysis of complex mixtures and therefore can be applied in pharmacology research. Herein, we analyzed and quantized indoline along with his oxidative major indole metabolite, revealed in the spectrum of the organisms' extractions. A similar indoline-to-indole metabolic conversion has been reported earlier (14). To verify the identity of this metabolite, the compound was prepared as shown in Zeeli et al. (7) (compound 19a).

The targeted molecules were quantified by extracted-ion chromatogram (EIC) of exhibited characteristic parent ions MH^+ 219.185 and 217.169 m/z, respectively, sharing identical product ion of 132.080.

Analytical system: UHPLC system: Agilent 1260 Technologies consisted of a quaternary pump (G4204A), autosampler (G4226A), column heater (G1316C). System control and data analysis were made by Mass Hunter software. Mass Spectrometer was an Agilent 6545 QTOF LC-MS with "AJS ESI" ion source in ESI + ve mode. Quantitation was performed by monitoring the product ions of the parent molecular ions for the different analytes. LC conditions: column, Poroshell EC-C18, 4.6 × 50 mm, 2.7-micron LC column (Agilent); column temperature, ambient; flow rate, 0.5 ml/min; injection volume, 20 μl; mobile phase, A: water with 0.1% formic acid, B: acetonitrile with 0.1% formic acid.

Statistics

Values are expressed as the mean ± SEM. Unpaired two-tailed Student's *t* test was used to determine differences between saline and drug-treated groups. Results in multiple groups and time-dependent variables were compared by ANOVA, followed by Tukey's *post hoc* analysis (GraphPad Prism v6 for Windows). Significance was set at $P < 0.05$.

RESULTS

AN1284 Preserves Kidney Morphology and Function in Type 2 Diabetic Mice

At the beginning of the experiment, the *db/db* mice exhibited higher body weight and blood glucose levels than did their healthy controls (**Figures 2A,B**), indicating an early onset of obesity and type 2 diabetes, which further progressed during the experiment. At the end of 3 months, the diabetic mice developed complete CKD, manifested by renal dysfunction and morphology. AN1284 treatment lessened the renal damage, as seen by a reduction in albuminuria and albumin-to-creatinine (ACR) ratio (**Figures 2C,D**). AN1284 treatment prevented the severe glomerular enlargement and that of Bowman's capsule in the diabetic mice (**Figures 2G–I**), but not the rise in urine glucose levels (**Figure 2E**) or the polyuria (**Figure 2F**). We showed that type 2 diabetes in this model is associated with renal inflammation, as indicated by the increased protein expression of inflammatory markers MCP1, TNFα, TGFβ, and IL-18. This up-regulation was significantly reduced by AN1284 (**Figures 3A–H**).

AN1284 Mitigates the Increased Body Weight via Increasing Fat Oxidation

Although AN1284-treated mice were significantly heavier than the normal controls, they did not reach the same level of obesity as the untreated BSK *db* mice (**Figure 4A**) and their weight no longer increased after 8 weeks of treatment (**Figure 4B**). As a result, analysis of body composition confirmed that the arrested weight gain is due to a lower body fat accumulation with no change in lean body mass (**Figures 4C,D**). Full metabolic assessment revealed that AN1284 increased TEE due to increased fat oxidation during both the light and dark periods without affecting the animals' activity (**Figures 4E–G**). Interestingly, meal size was significantly reduced in *db/db* mice treated with the lower dose of AN1284 (**Figure 4H**); however, it did not significantly affect total daily food intake measured while the animals were housed in the metabolic chambers for 24 h (**Figure 4I**).

AN1284 Increases Insulin Sensitivity

All the *db/db* mice displayed extreme hyperglycemia (**Figure 5A**), but a reduction in hyperinsulinemia and HOMA-IR was found in the AN1284-treated groups (**Figures 5B,C**). Histological analysis of the pancreas revealed an increase in the area of the Langerhans islet in the untreated BSK *db* mice. This was completely normalized by treatment with the higher dose of AN1284 (**Figure 5D**). A diabetic pancreas also undergoes massive β cell degeneration (15). AN1284-treated mice exhibited a higher percentage of functional β cells and the ability to secrete insulin than the untreated animals (**Figures 5E,F**). One reason for the development of a loss of insulin sensitivity is the downregulation of expression of the GLUT4 transporter, as shown in *db/db* mice (16). This was demonstrated in the skeletal muscle of the untreated diabetic mice, and GLUT4 mRNA and protein expression levels were improved by AN1284 treatment (**Figures 5G,H**). Taken together, these findings suggest that AN1284 has the ability to increase insulin sensitivity.

AN1284 Ameliorates Hepatic Steatosis, Liver Injury, and Fibrosis

AN1284 was able to decrease hepatocellular damage and hepatic steatosis, as manifested by a lower liver weight (**Figure 6A**), serum levels of ALT and ALP (**Figures 6B,C**), as well as hepatic triglyceride and cholesterol contents (**Figures 6D,E**). AN1284 reduced serum triglyceride levels and increased HDL/LDL ratio, but did not affect total serum cholesterol (**Figures 6F–H**). AN1284-treated *db/db* mice showed a reduced accumulation of fat in hepatocytes (**Figures 6I,J**). The latter was most likely mediated through the ability of AN1284 to downregulate the hepatic expression of several key genes involved in *de novo* lipogenesis (*Acc* and *Scd1*; **Figures 6K,L**, respectively) and fatty acids transport (*Cd36*; **Figure 6M**). Although only a trend toward reduction in hepatic TNFα levels was noted in *db/db* mice treated with AN1284 (**Figure 6N**), the levels of protein expression of IL-18 and MCP1 were significantly decreased (**Figures 6O,P**). This effect is most likely mediated by the ability of AN1284 to induce a shift in macrophages toward a predominantly M2 phenotype (**Figures 6Q,R**). Moreover, AN1284 also reduced the elevated mRNA and protein expression of the hepatic fibrogenic markers, α-SMA, and Collagen 1 (**Figures 7A–D**), suggesting that AN1284 has anti-fibrogenic activity in the liver.

DISCUSSION

Obesity commonly precedes type 2 diabetes and results in the metabolic syndrome and hypertension, which increases pressure on the kidney. Following weight gain, hyper-filtration develops to meet the increased metabolic demand (17). About half of the patients with type 2 diabetes and one third of those with

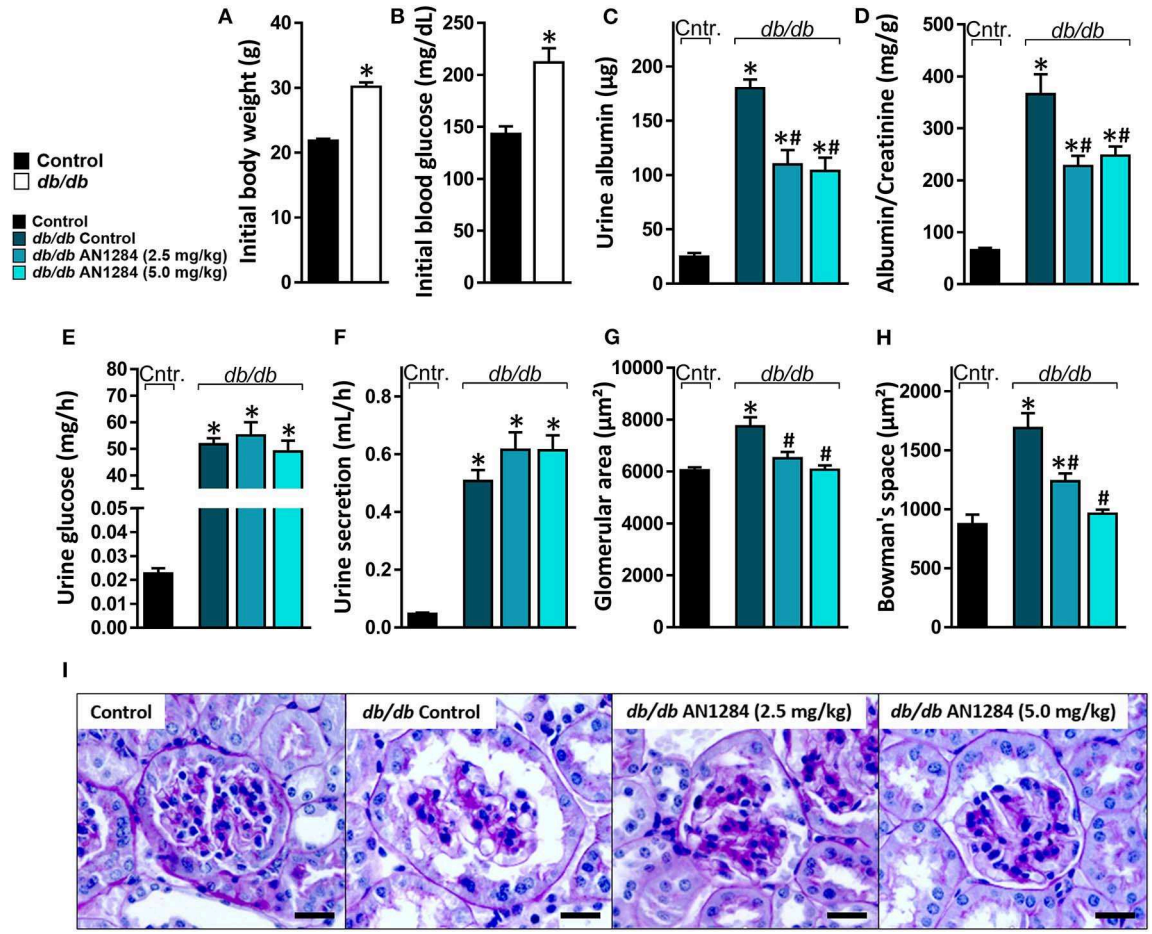

FIGURE 2 | AN1284 preserves kidney morphology and function in type 2 diabetic mice. Compared to the healthy controls, *db/db* mice exhibited increased body weight **(A)** and hyperglycemia **(B)** at the beginning of the experiment. AN1284 significantly reduced the elevated levels of urine albumin **(C)** and the albumin-to-creatinine ratio **(D)**, but not the urine glucose levels **(E)** and the urine secretion volume **(F)**. AN1284-treated *db/db* animals exhibited a reduction in the glomerular area **(G,I)**, and in Bowman's space area **(H,I)**. Scale bar, 20 µm. Data represent the mean ± SEM from 8 to 10 mice per group. $^{*}P < 0.05$ relative to control non-diabetic mice, $^{\#}P < 0.05$ relative to *db/db*-Veh-treated control mice.

type 1 diabetes develop nephropathy (18, 19). Although the molecular mechanisms contributing to the development of CKD in diabetes and obesity are quite different, it is accompanied in each condition by glomerular hypertrophy and transient hyper-filtration, glomerular basement membrane thickening, and mesangial matrix expansion, resulting in albuminuria (20, 21). Together with renal inflammation (22) and oxidative stress (23), these changes may lead to renal fibrosis, and ultimately to a progressive decline in glomerular filtration rate (5). Normalization of blood pressure and glucose levels can protect the kidneys and reduce the likelihood of developing CKD. However, a significant number of patients with diabetes may still progress to end-stage renal disease (24), stressing the importance of developing novel drugs to treat CKD. Here, we show that the indoline derivative, AN1284, has a therapeutic potential to ameliorate CKD in type 2 diabetic conditions in mice. The *db/db* mouse model of type 2 diabetes was chosen for this study rather than a high-fat diet-induced model of metabolic disorder because we were primarily interested in trying to prevent the development of CKD, which is greater in *db/db* mice due to hyperglycemia and hyperlipidemia.

We have previously reported that a series of novel indoline derivatives possess antioxidant and anti-inflammatory properties (7, 8). The therapeutic potential of these novel molecules (with or without a carbamate moiety) was tested in murine models of acute lung injury (9), ulcerative colitis (25), and acute liver injury (10). The current study demonstrates that one of them, AN1284, is also effective in mitigating the development of diabetes-induced CKD, which is a chronic condition. Although we do not know the identity of specific targets activated by AN1284, we previously reported that AN1284 reduced the phosphorylation of p38 MAP kinase and the nuclear translocation of activator protein 1 in LPS-activated macrophages and in the liver of mice with acute liver injury (10). We are currently performing RNA seq analysis and high-throughput bioassay screening including *in silico* and data-mining methodologies to try to

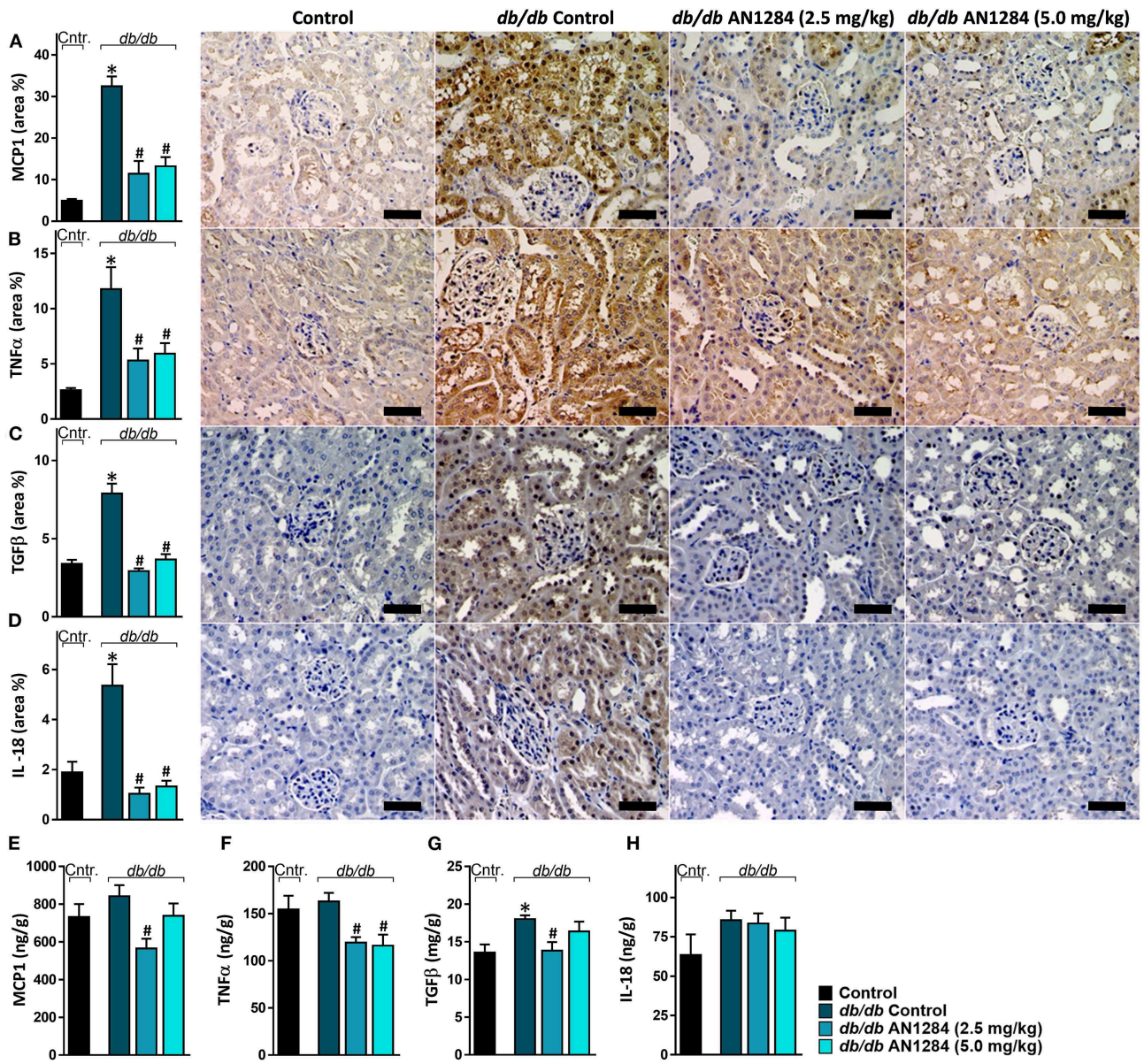

FIGURE 3 | AN1284 prevents the renal inflammatory response in type 2 diabetic mice. The elevated renal protein expression levels of the inflammatory markers MCP-1 **(A,E)**, TNFα **(B,F)**, TGFβ **(C,G)**, and IL-18 **(D,H)** in *db/db* mice were normalized by AN1284 treatment. Scale bar, 50 μm. Data represent the mean ± SEM from 8 to 10 mice per group. $^{*}P < 0.05$ relative to control non-diabetic mice, $^{\#}P < 0.05$ relative to *db/db*-Veh-treated control mice.

decipher specific targets for AN1284. Here, we administered AN1284 by subcutaneously implanting mini-pumps for 12 weeks, and the treatment was started after confirming the presence of hyperglycemia. Although the drug was unable to reduce blood glucose levels, it improved renal function and its histopathological appearance and reduced renal inflammatory and fibrogenic markers. The concentrations of the drug found in the kidney and liver (**Table 1**) were sufficient to cause an anti-inflammatory and antioxidant effect (7). AN1284 was very effective in reducing renal inflammation in the kidney at both doses tested here. The higher dose of AN1284 caused significantly greater polyuria than that seen in the untreated mice. It is possible that the added diuretic effect is mediated by another mechanism, such as inhibition of sodium reabsorption in the renal tubule cells, which is also seen in human subjects given Indapamide (26) that also contains an indoline group. However, AN1284 lacks the thiazide group of Indapamide and of other potent diuretics, making this less likely.

Other current therapies for attenuating the progression of diabesity-induced CKD focus on ameliorating risk factors, such as visceral obesity, insulin resistance, and NAFLD. Evidence shows that lifestyle modifications resulting in weight

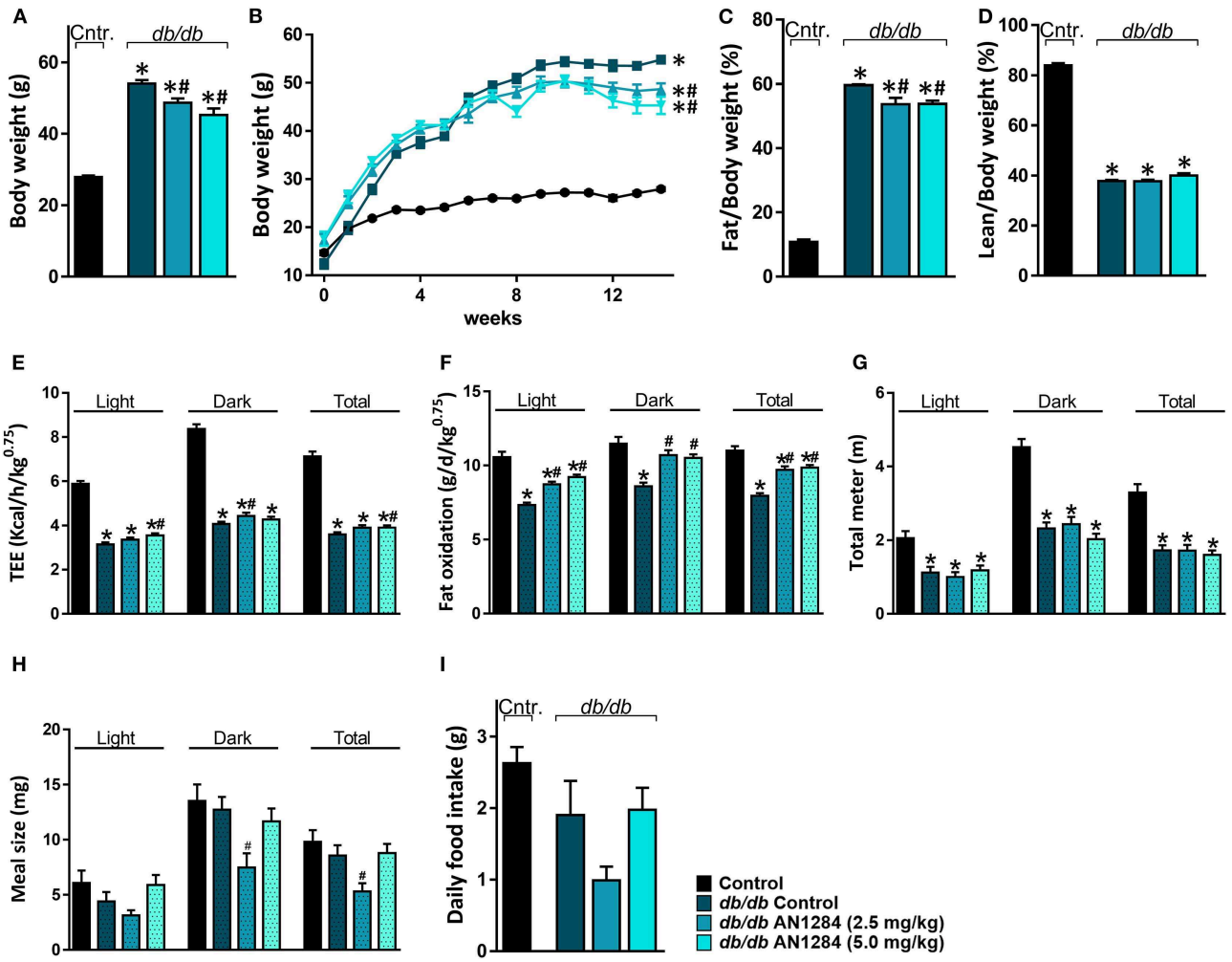

FIGURE 4 | AN1284 mitigates increased body weight in *db/db* mice via increasing fat oxidation. Chronic treatment with AN1284 significantly attenuated the increased body weight **(A,B)** and fat mass **(C)** without affecting lean mass **(D)** in *db/db* mice. Significant increases in total energy expenditure (TEE; **E**) and fat oxidation **(F)** were measured in *db/db* mice chronically treated with AN1284. No effect was found on mouse activity as measured by the total distance the mice moved in their cages **(G)**. *db/db* mice treated with the lower dose of AN1284 exhibited decreased meal size **(H)**, without a significant effect on daily food intake **(I)**. Data represent the mean ± SEM from 8 to 10 mice per group. $^{*}P < 0.05$ relative to control non-diabetic mice, $^{\#}P < 0.05$ relative to *db/db*-Veh-treated control mice.

loss, bariatric surgery, and pharmacotherapy may arrest renal dysfunction in patients with type 2 diabetes [reviewed in Docherty et al. (27)]. Moreover, a modest weight loss has been reported to decrease proteinuria in such patients (28). We also observed a significant body weight loss, together with up to 11% fat mass loss in the AN1284-treated mice. These effects can be explained by the increased TEE and fat oxidation, and not by drug-induced changes in the activity of the animals.

Insulin resistance, assessed by HOMA-IR, is present in patients with mild to severe CKD (29). In a few cross-sectional studies in humans, insulin resistance has been linked to the risk of developing diabetes-induced CKD (30, 31). On a molecular basis, both the direct and indirect effects of hyperinsulinemia play a key role in kidney function. Under type 2 diabetic conditions, pancreatic islets respond not only by increasing insulin secretion but also by expanding β cell mass (32). The latter was completely normalized by treatment with the AN1284 (5 mg kg^{-1} per day). Hyperinsulinemia *per se* may induce glomerular hyperfiltration, endothelial dysfunction, and increased vascular permeability, which together may lead to albuminuria (33). In addition, insulin resistance is associated with altered renal cellular metabolism, mesangial expansion, renal inflammation, and lipid deposition in the renal proximal tubule cells, all established contributors to nephropathy (34). Together with the critical role of insulin in secreting different adipokines and chemokines, which are known to modulate kidney function, increased insulin sensitivity may attenuate the development and progression of CKD. Tejada and colleagues have shown that podocytes isolated from *db/db* mice are unable to respond to insulin (35). Tiwari et al. reported downregulation of the renal insulin receptor in several type 2 diabetes models (36), and that its complete deletion in the proximal tubule cells results in albuminuria (37). AN1284 may

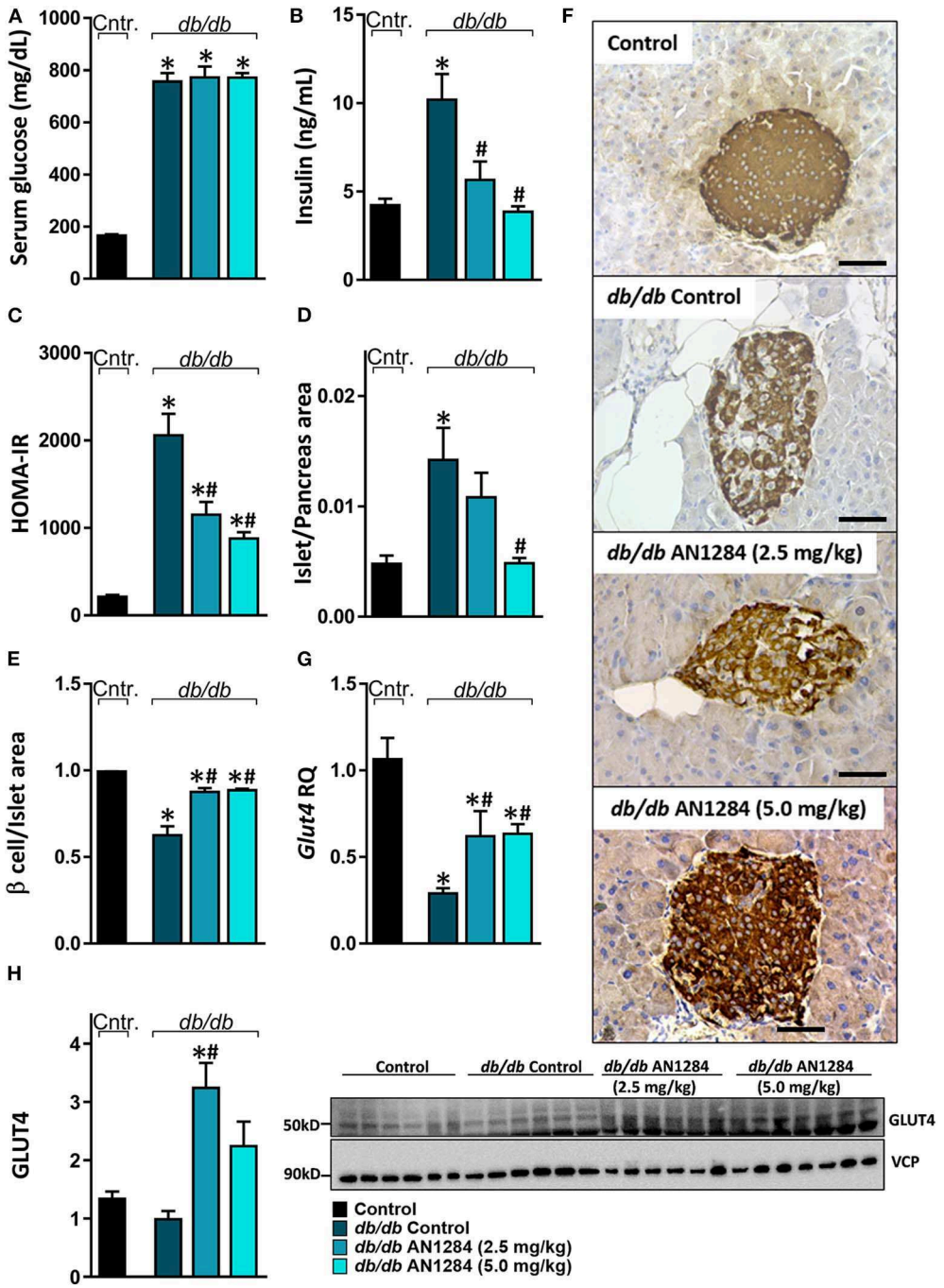

FIGURE 5 | AN1284 increases insulin sensitivity in *db/db* mice. The *db/db* mice exhibit hyperglycemia and hyperinsulinemia (**A,B**, respectively), compared with healthy controls. AN1284 treatment significantly reduced serum insulin levels without changing those of serum glucose. A reduced HOMA-IR **(C)** and Langerhans islet-to-pancreas tissue ratio **(D)**, and an increased β cell-to-Langerhans islet area **(E)** and *Glut4* mRNA **(G)** as well as protein expression **(H)** were found in *db/db* mice treated with AN1284. **(F)** Representative insulin staining of the pancreas from each treatment group. Original magnification, ×40. Scale bar, 50 mm. Data represent the mean ± SEM from 8 to 10 mice per group. $^{*}P < 0.05$ relative to control non-diabetic mice, $^{\#}P < 0.05$ relative to *db/db*-Veh-treated control mice.

improve renal function by reducing insulin levels and the increase in pancreatic β-cell mass in *db/db* mice. Thus, AN1284 reversed renal insulin resistance by decreasing the direct effect of insulin on podocytes or tubular cells, which express all elements of the insulin-signaling cascade (38). Nevertheless, as the ability of AN1284 to reverse insulin sensitivity directly was not fully characterized here (by assessing whole-body glucose utilization in live animals), we cannot conclude whether AN1284 suppresses

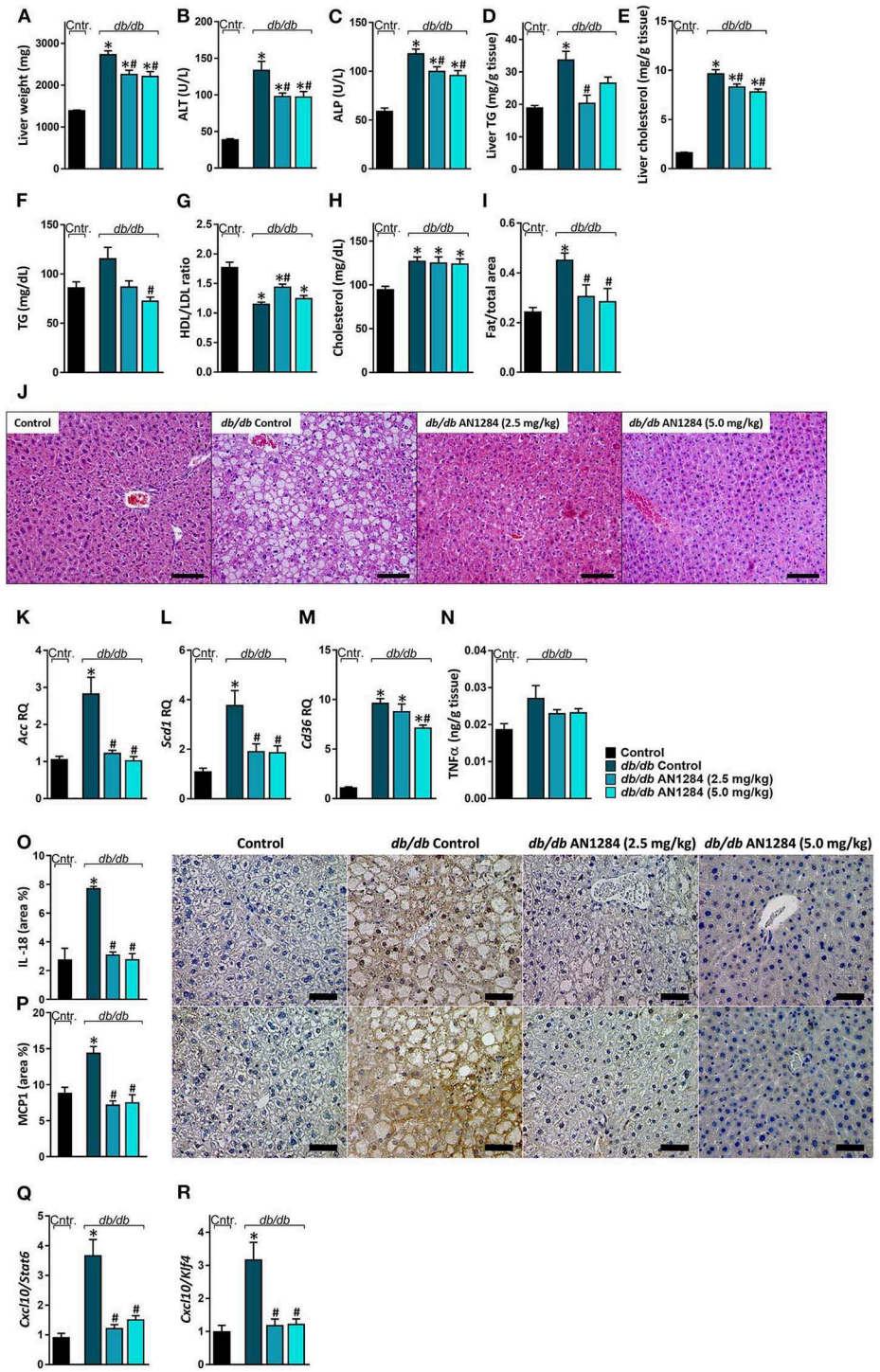

FIGURE 6 | AN1284 ameliorates hepatic steatosis and inflammation in *db/db* mice. The *db/db* mice exhibit increased liver weight **(A)**, serum ALT **(B)**, and ALP **(C)** levels as well as hepatic triglyceride and cholesterol contents **(D,E)**. These parameters were prevented or ameliorated by AN1284 treatment. AN1284-treated mice also displayed decreased serum triglyceride levels **(F)**, increased HDL/LDL cholesterol ratio **(G)**, without an effect on total cholesterol levels **(H)**. Quantification of hepatic fat content revealed an increase in *db/db* animals, which was normalized by AN1284 treatment **(I)**. Representative liver images demonstrating macrovesicular steatosis in H&E-stained sections from *db/db* mice. Scale bar, 100 μm **(J)**. The elevated hepatic mRNA expression levels of *Acc* **(K)**, *Scd1* **(L)**, and *Cd36* **(M)** in *db/db* mice were normalized by AN1284 treatment. Insignificant changes in hepatic TNFα protein levels were noted in non-treated and treated mice **(N)**. Reduced protein expression of IL-18 **(O)**, and MCP1 **(P)** in AN1284-treated *db/db* mice was documented. Scale bar, 50 μm. M1-to-M2 macrophage marker ratios displayed high content of M1 macrophages in *db/db* animals, which was diminished by AN1284 treatment **(Q,R)**. Data represent the mean ± SEM from 8 to 10 mice per group. $^{*}P < 0.05$ relative to control non-diabetic mice, $^{\#}P < 0.05$ relative to *db/db*-Veh-treated control mice.

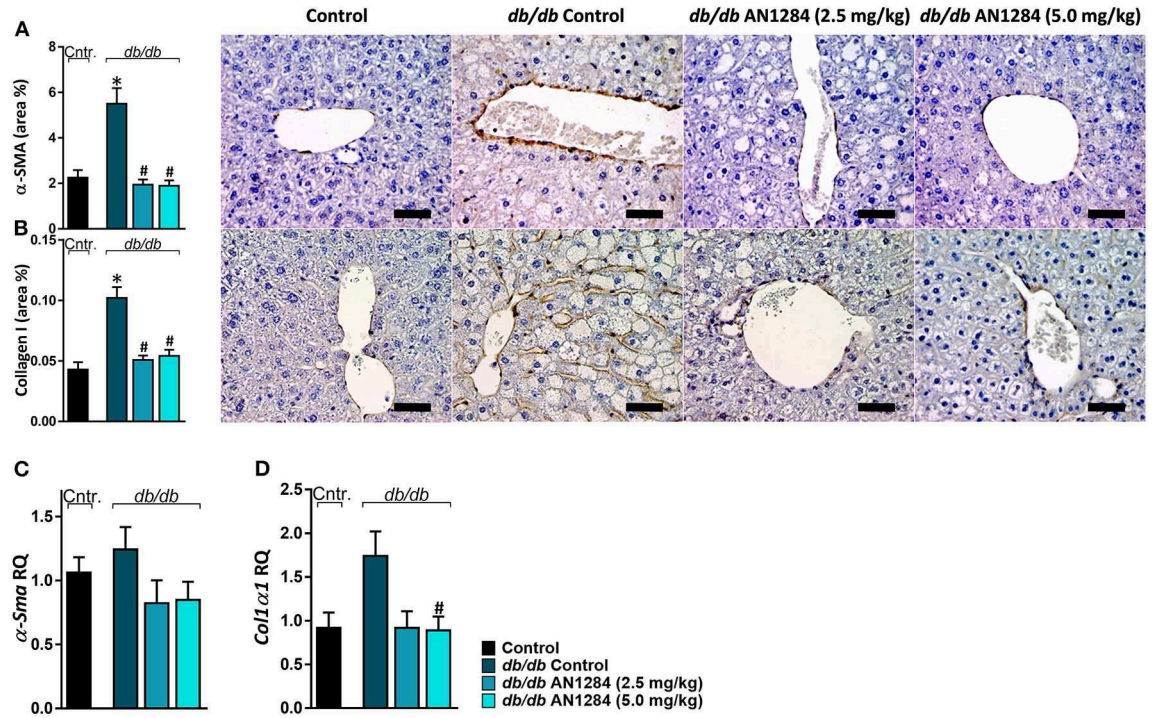

FIGURE 7 | AN1284 mitigates hepatic fibrosis in *db/db* mice. Untreated *db/db* animals exhibited increased protein and mRNA expression levels of the fibrotic markers α-SMA **(A,C)** and Collagen I **(B,D)**, which were normalized in AN1284-treated mice. Scale bar, 50 μm. Data represent the mean ± SEM from 8 to 10 mice per group. $^{*}P < 0.05$ relative to control non-diabetic mice, $^{\#}P < 0.05$ relative to *db/db*-Veh-treated control mice.

TABLE 1 | Concentration of AN1284 and its metabolite in liver and kidney.

Dose (mg kg^{-1} per day)	Concentration ± STD (ng/g)			
	Kidney		Liver	
	AN1284	Metabolite	AN1284	Metabolite
2.5	140 ± 69	116 ± 30	36.2 ± 7.3	13.7 ± 2.7
5.0	298 ± 52	181 ± 17	40.5 ± 3.7	17.0 ± 5.6

hepatic glucose production or improves skeletal or white adipose tissue insulin sensitivity to improve HOMA-IR reported here. These issues as well as the underlying molecular mechanisms by which AN1284 ameliorates insulin tolerance are important questions that will need to be addressed in the near future.

Several lines of evidence suggest that NAFLD promotes type 2 diabetes. While NAFLD is present in 20–30% of the general population (39), it reaches a prevalence of 50–75% of patients affected by diabesity (40). Conversely, insulin resistance, which occurs in 66–83% of patients with NAFLD, increases the flux of free fatty acids from adipocytes to the liver and promotes hepatic lipid accumulation and liver injury. Once diabesity is fully developed, it further contributes to the development of steatosis and also to hepatic inflammation and fibrosis (41). To date, there is evidence linking NAFLD, as a causative player, to the development and progression of CKD (42). Furthermore, several large cross-sectional studies of patients with NAFLD show a prevalence of CKD between 4 and 40% and a positive correlation between the severity of NALFD and CKD [reviewed in (43)]. Whereas, the complex "crosstalk" among adipose tissue, the liver, and kidneys makes it difficult to decipher the specific pathway underlying NAFLD as a cause of CKD, it is not surprising that these diseases may be linked, and therefore the amelioration of NAFLD may also contribute to the reversal of CKD. Importantly, hepatic steatosis, liver injury, dyslipidemia, inflammation, and fibrosis present in *db/db* mice were significantly ameliorated by chronic treatment with AN1284. With its ability to mitigate acute liver injury via reduced inflammation (10), our current data strongly support the idea that an anti-inflammatory agent like AN1284 could provide a therapeutic intervention for the treatment of NAFLD and may also assist in the prevention of diabesity-induced CKD. At present, we cannot differentiate between direct or indirect effects of AN1284 since no specific target(s) have been identified. AN1284 positively affects kidney, liver, muscle, and pancreatic functions. Based on the very marginal effect that AN1284 had on body weight (the animals still remained obese), we can only postulate that the improvements in multiple processes may be mediated via a general anti-inflammatory effect and specific actions of AN1284 on various renal cells (podocytes, tubular,

and mesangial cells), hepatocytes, myocytes, and pancreatic beta cells. Further work is needed to be done to understand the molecular mechanism(s) by which AN1284 improves diabesity-induced CKD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the https://patents.google.com/patent/WO2017125932A1/en.

ETHICS STATEMENT

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem (AAALAC accreditation #1285; Ethic approval number MD-17-15302-3).

AUTHOR CONTRIBUTIONS

AP conducted the experiments and analyzed the data. AG and LH assisted in experiments. MWeit conducted the LC-MS/MS analysis. MWein and JT designed and supervised the experiments, and analyzed the data. AP, MWein, and JT wrote the manuscript.

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frontiers in Endocrinology

ORIGINAL RESEARCH
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Macrophages Mediate Increased CD8 T Cell Inflammation During Weight Loss in Formerly Obese Mice

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Even after successful weight reduction, obese adults tend to quickly regain the lost weight. This raises the question of whether weight loss improves the underlying chronic adipose tissue inflammation characteristic of obesity. In order to improve our understanding of the mechanisms that reshape metabolic organs during weight loss, we investigated the macrophage and T cell function of the liver and adipose tissue on reversing high fat diet (HFD) mice to normal control diet (NCD). Obese mice that were switched to NCD showed an improvement in their metabolic profile that included enhanced glucose and insulin tolerance, decreased cholesterol, triglyceride, serum glutamic-oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) levels that were comparable to NCD controls. However, despite weight loss, increased frequencies, but not total numbers, of IL-17+ and IL-22+ CD4+ T cells, IFN-γ+ and TNF+ CD8+ T cells and IL-17+ and IL-22+ CD8+ T cells were observed in the adipose tissue of mice switched from HFD to NCD compared to NCD and even HFD fed mice. Further, in the liver, IFN-γ+ and TNF+ CD8+ T cell, IL-17+ and IL-22+ CD8+ T cell, macrophage frequencies and their expression of antigen presenting molecules were increased. To determine if macrophages are the major determinants of the sustained inflammation observed during weight loss, we depleted macrophages, which significantly reduced IFN-γ+, TNF+, IL-17+, and IL-22+ CD8+ T cell frequencies in the liver and the adipose tissue. In conclusion, we show that although weight loss improves the metabolic profile, there is an active and ongoing CD8+ T cell inflammation in liver and adipose tissue mediated by macrophages.

Keywords: obesity, inflammation, T cell, macrophages, weight loss, adipose, liver, glucose

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INTRODUCTION

Obesity is a major public health problem, which causes the death of at least 2.8 million people every year worldwide. It is causally linked to type 2 diabetes, cardiovascular diseases and cancers (1). In order to achieve weight reduction, various intervention strategies like anti-obesity drugs, bariatric surgery, and lifestyle interventions exist (2). Among these, decreasing caloric intake and physical exercise are by far the most widely adopted methods of choice to promote weight reduction. However, despite successful weight reduction, there is often a quick regain of weight and persistent inflammation in adipose tissue (3, 4). It is therefore important to delineate the underlying mechanisms that contribute to disease risk despite successful weight reduction.

Although it is well-known that obesity is a state of chronic adipose tissue inflammation, little is known about the modulation of this inflammatory state during weight loss. Particularly, the question of how weight loss modulates the specific aspects of obesity-associated inflammation like T cell inflammation is not addressed in detail. A few earlier studies have shown that weight loss did not improve the inflammatory markers' gene expression within the adipose tissue (5). Schmitz et al. (4) have shown that despite improving glucose tolerance, weight loss could not successfully ameliorate adipose tissue inflammation and improve insulin sensitivity. Some reports have also demonstrated an accentuated inflammatory profile after weight loss (6). However, these results are controversial, as other studies have shown that weight loss improves the inflammatory profile of obese subjects (7) and attenuates inflammation in skeletal muscle and liver, but not in adipose tissue (8). Effects may be proportional to the degree of weight loss, as weight loss of 11–16%, but not 5%, resulted in a decrease in adipose tissue inflammation. Furthermore, the increase and persistence of liver and adipose tissue inflammation was also shown to be gender-dependent, with male mice showing a persistent inflammation (9). Of note, weight loss resulted in dynamic effects on macrophages in adipose tissue, with an initial increase followed by a decrease in macrophage numbers (10). Taking into consideration the crucial role of T lymphocytes in obesity related inflammation and the existing literature stating an incomplete resolution of inflammation after weight loss, we investigated the effects of weight loss on type-1 (IFN-γ and TNF) and type-17 (IL-17 and IL-22) cytokines from T cell subsets in the liver and adipose tissue. We report a potent increase in frequencies of cytokine producing CD8 T cells during weight loss from the liver and adipose tissue, which was in part mediated by macrophages.

MATERIALS AND METHODS

Mice

All mice were maintained in ventilated cages with a 12-h day/night cycle, food and water *ad libitum*. The experiments were carried out on male C57BL/6J mice purchased from Janvier Labs (Le Genest-St.-Isle, France). Mice were maintained at the animal facilities of the University Hospital Bonn. Starting at 6 weeks of age, mice received either a normal control diet (NCD, 15% fat), a high fat diet (HFD, 60% kilocalories from fat; Research Diets, Inc., Brogaarden, Denmark), or a HFD which was switched after 16 weeks to NCD for an additional 4 weeks (**Figure 1A**). All mice in the comparative studies were age matched within individual experiments. Animal housing conditions and the procedures were conducted according to European Union animal welfare guidelines. Study protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany (84-02.04.2016.A331).

In a separate experiment, after switching to NCD, macrophages were depleted by intravenous (i.v.) injection of 150 µl of clodronate liposomes (Clodronate Liposomes Foundation; Netherlands; http://clodronate.liposomes.com) and the control mice received equal volumes of PBS liposomes.

Glucose Tolerance and Insulin Tolerance Test, Lipid Profile and Liver Enzymes

Glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were carried out as described elsewhere (11). In brief, 6 h after fasting, mice were intraperitoneally (i.p.) injected with 1 g/kg body weight of glucose solution. At 0, 30, 60, and 120 min blood glucose levels were measured by a glucometer (AccuCheck Advantage; Roche Diagnostics GmbH, Mannheim, Germany).

Four hours after fasting, ITT was performed. Briefly, human insulin (Sanofi-Aventis, Frankfurt, Germany) 1 U of insulin/kg body weight was i.p. injected and at 0, 30, 60, and 120 min blood glucose levels were measured. The area under the curve (AUC) was derived by calculating the area between the x-axis and a given curve using GraphPad Prism software (version 8.3; GraphPad Software, San Diego, Calif., USA).

Lipid profiles and liver enzymes—serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)—were measured using Reflotron (Roche Diagnostics GmbH) according to the manufacturer's protocol.

Isolation of Stromal Vascular Fraction From Adipose Tissue and Leucocytes From Liver

Mice were deeply anesthetized by i.p. injection of 10 mg/kg xylazin (Rompun® Bayer, Germany) + 100 mg/ml ketamine (Ratiopharm GmbH Germany). Mice were intracardially perfused with 1x PBS for 5 min to remove circulating and non-adhered blood leukocytes from the organs (12).

After perfusion, adipose tissue stromal vascular fraction (SVF) and liver lymphocytes were isolated. In brief, the excised epididymal adipose tissue from the mice was digested with 0.2 mg/ml of collagenase (Sigma-Aldrich; Taufkirchen, Germany) in DMEM medium at 37°C for 40 min. After the digestion, the adipocytes were removed and SVF pellet was filtered by passing through a 40 µm filter after red blood cell lysis (Invitrogen, Thermo Fisher Scientific; Carlsbad, CA, USA).

To isolate cells from the liver, the liver was minced into small pieces followed by digestion with 0.5 mg/ml collagenase A (Roche, Basel, Switzerland) at 37°C for 30 min. After the digestion single cell suspension was generated by passing the digested tissue through a 70 µm filter. Lymphocytes were enriched from the homogenate using a percoll gradient.

Cell Culture

After cell enumeration from SVF and liver single cell suspension, isolated cells were cultured in 12-well tissue culture at concentrations of 1×10^6 cells/ml in the presence of phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (1 µg/ml) for 6 h in RPMI-1640 medium (Gibco, Thermo Fischer scientific) at 37°C. After 2 h, Golgi Stop/Golgi Plug (BD Biosciences, Heidelberg, Germany) was added and cells were harvested 4 h later.

Flow Cytometry

After *in vitro* stimulation, cells were harvested and incubated in fixation/permeabilization buffer overnight (eBiosciences; Darmstadt, Germany). Next, cells were blocked with PBS/1% BSA including 0.1% rat IgG for 30 min (Sigma-Aldrich). After

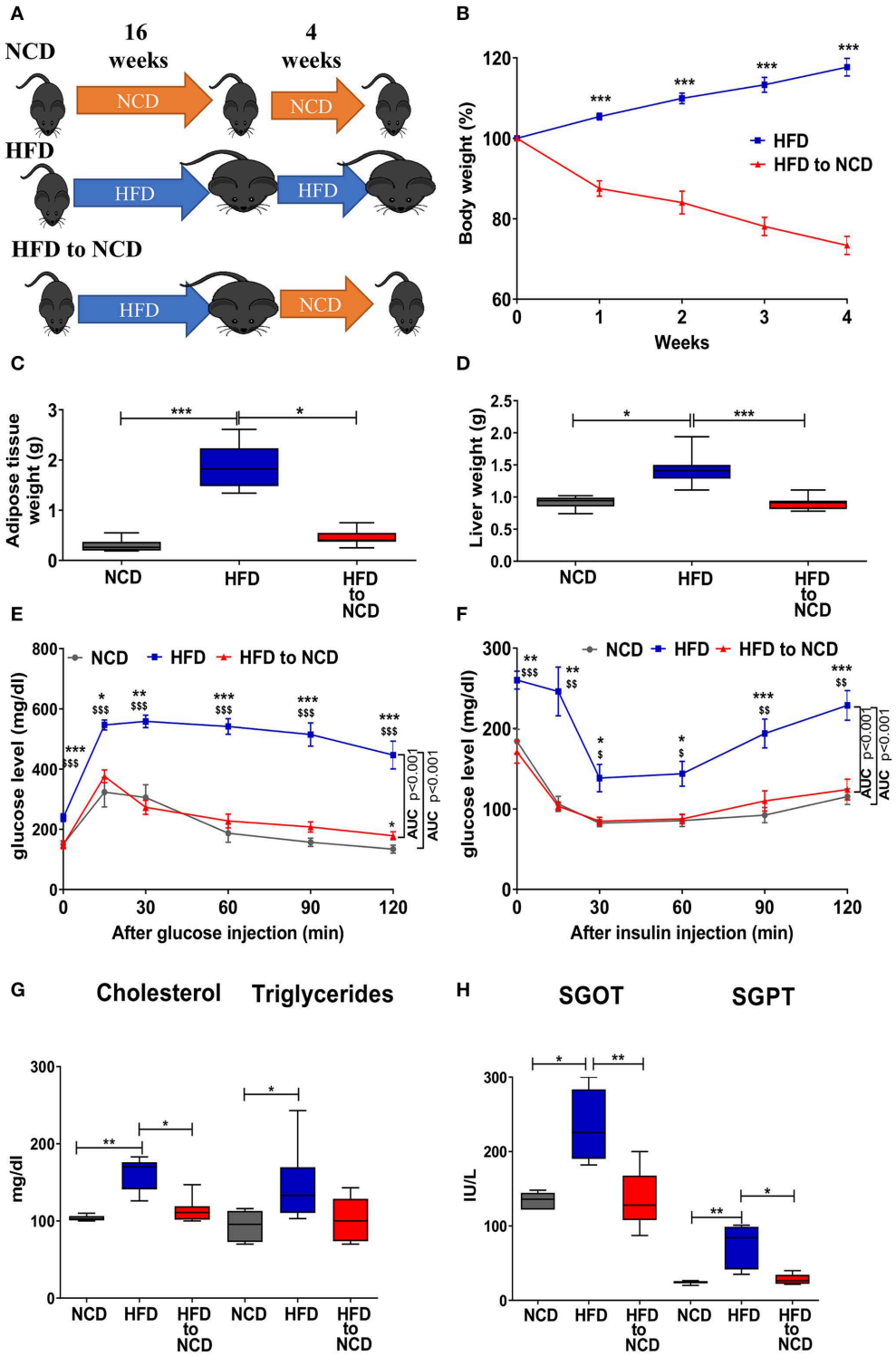

FIGURE 1 | Weight loss in formerly obese mice improves the metabolic profile. **(A)** Schematic experimental design. **(B)** Change in body weight, **(C)** adipose tissue, and **(D)** liver weight 4 weeks after switching from a high fat diet (HFD) to a normal control diet (NCD). **(E,F)** Blood glucose levels over time after i.p. glucose (GTT, **E**) or insulin (ITT, **F**) challenge. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to NCD. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared to HFD to NCD. **(G)** Serum lipid profile and **(H)** and liver function enzymes between NCD, HFD and HFD to NCD groups. Pooled data from $n = 2$–3 experiments, 3–5 mice each. Statistical significance was tested by Kruskal-Wallis followed by Dunn's test **(C,D,G,H)** or 2-Way ANOVA followed by Tukey's multiple comparisons test **(B,E,F)**.

a washing step, the cells were incubated with permeabilization buffer (eBioscience) for an additional 20 min. After washing, cells were stained for flow cytometry with antibodies against CD4-PE/Cy7, CD8-PerCP/Cy5.5 and intracellular cytokines IFN-γ-PE, TNF-FITC or IL-17A-PE, IL-22-APC. For quantification of regulatory T cells, cells were permeabilized and stained with CD4-PE/Cy7 and FoxP3-FITC for 30 min. For identification of macrophages, the whole cell population was selected and doublet cells were excluded by FSC-W and SSC-A characteristics followed by gating of F4/80-PerCP/Cy5.5+ and CD11b-APC/Cy7+ cells as macrophages. Macrophages were shown as percentage of total SVF cells. All antibodies were purchased from eBioscience (Darmstadt, Germany) or Biolegend (Fell, Germany). Data were acquired with a BD FACS Canto System (BD Biosciences) and analyzed using FlowJo 10 (Flowjo LLC; Ashland, Oregon) software. During analysis, gates were set using the FMO (fluorescence minus one) approach.

Real Time PCR

From adipose tissue RNA was extracted using the RNeasy mini kit (Qiagen). RNA was reverse transcribed with the Omniscript RT Kit (Qiagen) according to the manufacturer's instructions with oligo-d(T) primers (Roche; Penzberg, Germany). Real-time PCR was performed with the Thermo Fisher QuantStudio 5 using the TaqMan universal PCR master mix (Thermo Fisher Scientific). TaqMan probes for arginase-1 (*Arg-1*), resistin-like molecule alpha (*RELM-α*) and *Tnf* were analyzed and hypoxanthine-guanine phosphoribosyltransferase (*hprt*) was used as an endogenous control (Thermo Fisher Scientific). The relative CT (threshold cycle at the exponential phase of amplification) method was used to calculate the qPCR results. Delta CT was calculated as CT (gene of interest)—CT (*hprt*). The fold change was calculated as $2^{-\Delta CT}$.

Statistics

GraphPad Prism software version 8.3 was used for statistical analyses (GraphPad Software, San Diego, CA, USA). Data were analyzed for statistical significance by Kruskal Wallis followed by Dunn's test for multiple comparisons and Mann Whitney *U*-test for comparison of two groups. Changes in GTT and ITT as well as body weight over time were analyzed by 2-Way ANOVA followed by Tukey's multiple comparisons test. $P < 0.05$ were considered statistically significant.

RESULTS

Weight Loss Improves Insulin Sensitivity, Lipid Profile and Liver Enzymes

Mice were fed a high fat diet (HFD) for a period of 16 weeks to induce weight gain and were then maintained on HFD or switched to normal control diet (NCD) for 4 weeks to induce body weight reduction (**Figure 1A**). HFD to NCD switching (formerly obese mice) resulted in a significant reduction in body weight (**Figure 1B**), adipose tissue weight (**Figure 1C**), and liver weight (**Figure 1D**).

After 20 weeks of HFD, impaired glucose and insulin tolerance were observed, whereas formerly obese mice that were switched to a NCD for the last 4 weeks had a glucose and insulin tolerance that was comparable to NCD controls (**Figures 1E,F**). Further, circulating levels of cholesterol (**Figure 1G**), triglycerides (**Figure 1G**) and the liver enzymes SGOT (**Figure 1H**) and SGPT (**Figure 1H**) were significantly increased in HFD fed mice compared to NCD control mice and formerly obese mice.

Increased Relative Numbers of Pro-inflammatory Cytokine-Producing T Cells in Adipose Tissue and Liver Are Sustained Despite Weight Loss

We next investigated the effect of weight loss on frequencies of type-1 and type-17 cytokine positive CD4+ and CD8+ T cells in the liver and adipose tissue. The gating strategy for T cells is shown in **Supplementary Figure 1**. In the adipose tissue, switching from HFD to NCD resulted in decreased frequencies of IFN-γ+ ($p > 0.05$) and TNF+ CD4+ T cells ($p<0.05$) compared to HFD controls (**Figure 2A**). On the contrary, frequencies of IL-17+ and IL-22+ CD4+ T cells (**Figure 2B**), IFN-γ+ and TNF+ CD8+ T cells (**Figure 2C**) and IL-17+ and IL-22+ CD8+ T cells (**Figure 2D**) were highest in the HFD to NCD group. In the liver, frequencies of TNF+ CD4+ and IL-17+ CD4+ T cells were found to be decreased (**Figures 2E,F**), whereas the percentages of IFN-γ+ and TNF+ CD8+ T cells (**Figure 2G**) and IL-17+ and IL-22+ CD8+ cells (**Figure 2H**) were highest in the formerly obese group. With regard to total numbers, HFD significantly increased the number of CD4+ type-17 (IL-17 and IL-22) (**Supplementary Figure 2B**), CD8+ type-1 (IFN-γ+ and TNF) (**Supplementary Figure 2C**), CD8+ type-17 (**Supplementary Figure 2D**) and by trend CD4+ type-1 (**Supplementary Figure 2A**) cytokine positive cells compared to the NCD group in adipose tissue. Reversal of the diet from HFD to NCD significantly reduced IFN-γ+ CD8+ T cell (**Supplementary Figure 2C**) and IL-17+ CD8+ cell numbers (**Supplementary Figure 2D**) in adipose tissue. Total numbers of liver CD4+ type-1 and type-17 cytokine positive T cells increased in the HFD group compared to the NCD group and switching diet from HFD to NCD significantly reduced CD4+ type-1 and type-17 cytokine positive T cells compared to the HFD group (**Supplementary Figures 2E,F**). HFD further increased liver CD8+ type-1 and type-17 cytokine positive T cells compared to the NCD group (**Supplementary Figures 2G,H**). Frequencies of FoxP3+ regulatory T cells were significantly reduced after HFD in both adipose tissue and liver and remained low after the reversal of the diet (**Supplementary Figures 3A,B**).

Macrophage Depletion Attenuates CD8+ T Cell Cytokine Production in Adipose Tissue and Liver

Intriguingly, the frequencies of CD11b+ adipose tissue macrophages (**Figure 3A**) and the expression of MHCI and MHCII on adipose tissue macrophages were highest in HFD animals that were switched to a NCD (**Figure 3B**).

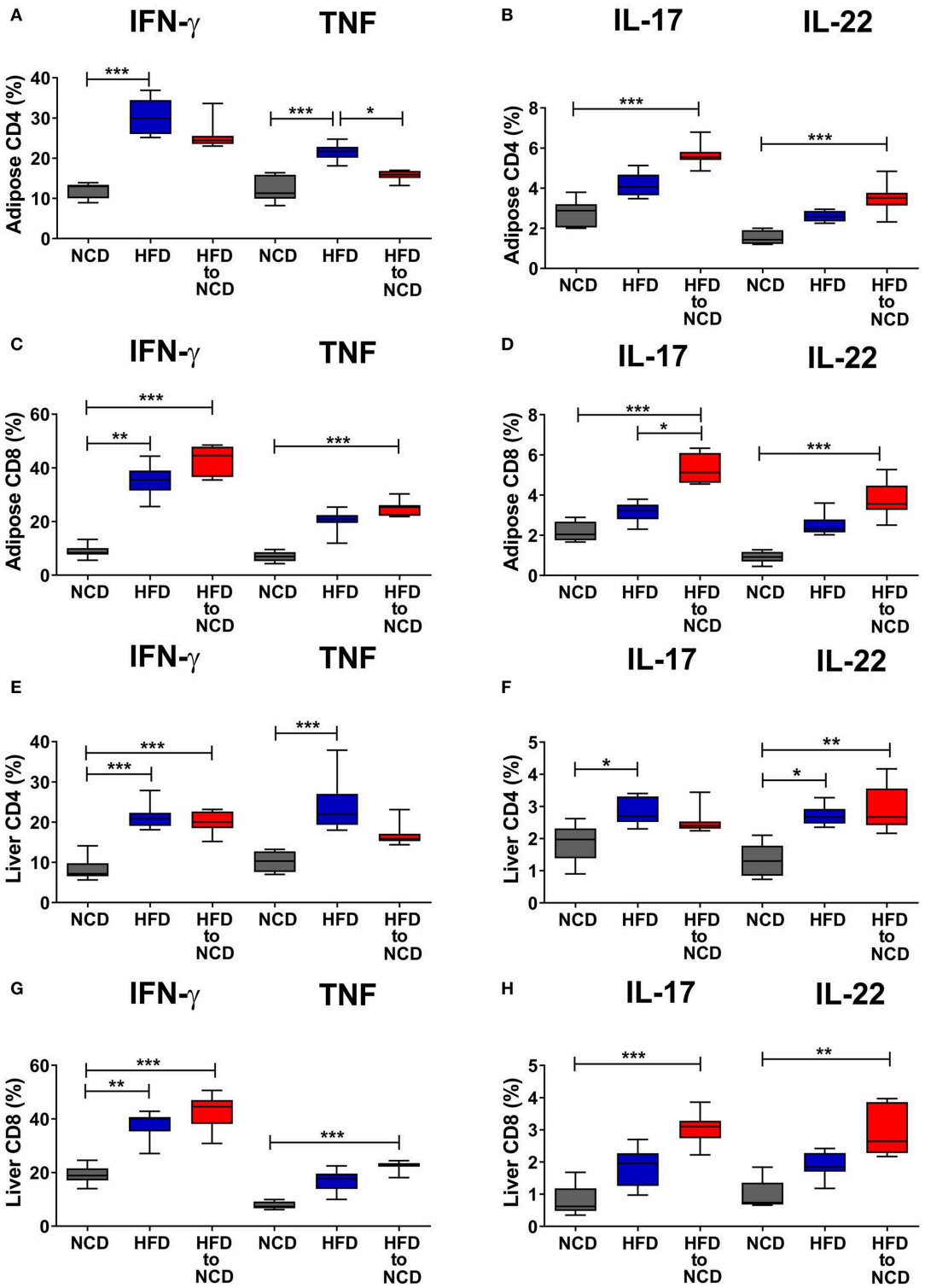

FIGURE 2 | Switching obese mice to a normal control diet increases frequencies of pro-inflammatory cytokine producing CD8+ T cells in adipose tissue and liver. Animals were fed for 20 weeks on a high fat diet (HFD), normal control diet (NCD) or were switched after 16 weeks of a HFD to a NCD for 4 weeks. Frequency of IFN-γ+ and TNF+ **(A,C)**, IL-17+ and IL-22+ **(B,D)** CD4+ T cells **(A,B)**, and CD8+ T cells **(C,D)** within the adipose tissue. Frequency of IFN-γ+ and TNF+ **(E,G)**, IL-17+ and IL-22+ **(F,H)** CD4+ T cells **(E,F)**, and CD8+ T cells **(G,H)** within the liver. Cytokine expression of T cells was determined following PMA/Ionomycin stimulation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Pooled data from $n = 2$–3 experiments with 3–5 mice each. Statistical significance was tested by Kruskal-Wallis followed by Dunn's test.

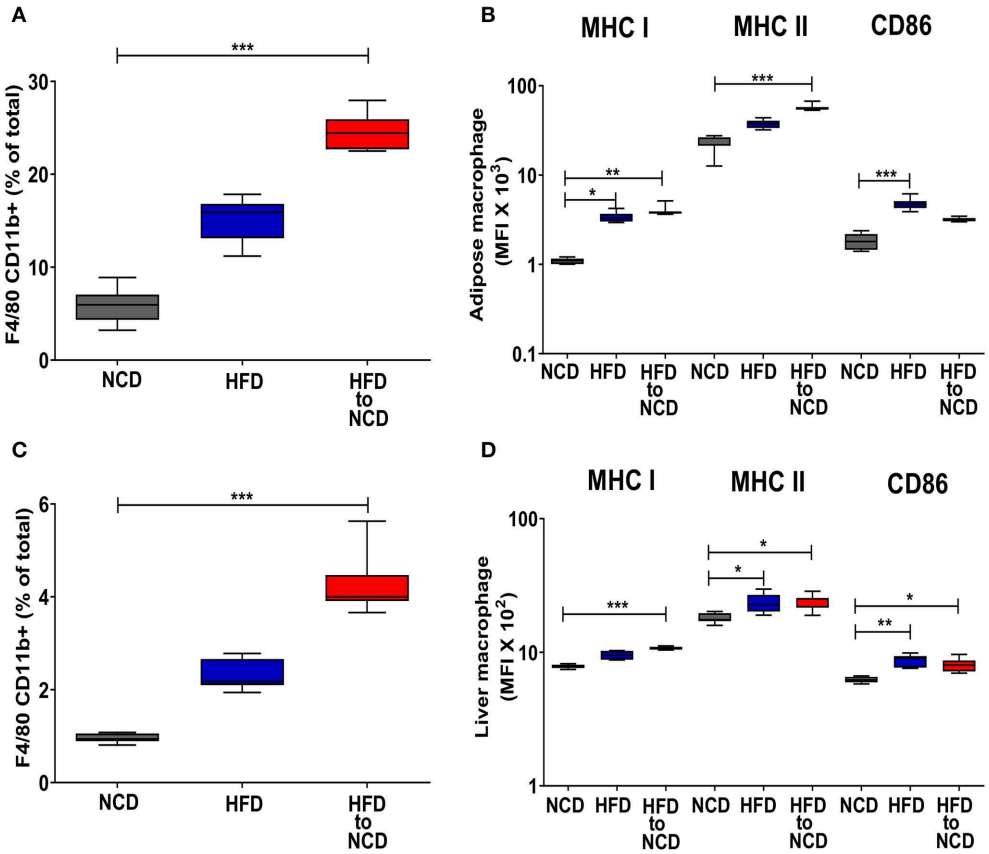

FIGURE 3 | Switching obese mice to a normal control diet leads to an increased frequency of adipose tissue and liver macrophages and increased expression of antigen presenting molecules. Animals were fed for 20 weeks on a high fat diet (HFD), normal control diet (NCD) or were switched after 16 weeks of a HFD to a NCD for 4 weeks. **(A)** Frequency of macrophages in the visceral adipose tissue and **(B)** mean fluorescence intensity (MFI) of MHCI, MHCII and CD86 on the adipose tissue macrophages. **(C)** Frequency of macrophages in liver and **(D)** MFI of MHCI, MHCII and CD86 on the liver macrophages. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Pooled data from $n = 2$–3 experiments with 3–5 mice each. Statistical significance was tested by Kruskal-Wallis followed by Dunn's test. NCD, normal control diet; HFD, high fat diet, HFD to NCD–HFD switched to NCD.

A similar pattern on macrophages was observed in the liver as shown by increased liver macrophage frequencies (**Figure 3C**) and the elevated expression of MHCI on liver macrophages (**Figure 3D**). The total number of macrophages in adipose tissue and liver were highest in the animals that were maintained on a HFD and remained significantly increased following reversal of the diet in comparison to the NCD group (**Supplementary Figures 4A,B**). The macrophage gating strategy is shown in **Supplementary Figure 5**. The expression of the alternatively activated macrophage markers Arginase-1 and RELM-α were reduced in the adipose tissue of the HFD group in comparison to the NCD group ($p > 0.05$) and tended to increase after the reversal of the diet ($p > 0.05$; **Supplementary Figures 6A,B**). HFD increased the TNF expression in adipose tissue compared to the NCD group and switching the diet reduced the TNF expression (**Supplementary Figure 6C**). Collectively, there was no clear increase of alternatively activated macrophage-associated gene expression in adipose tissue macrophages 4 weeks after the reversal of diet.

As we observed that frequencies of T cell cytokine positive cells are in parallel with macrophage accumulation and their expression of antigen presenting molecules in adipose tissue and liver, we set out to determine if macrophages are a major determinant of sustained inflammation observed during weight loss. Macrophage depletion was confirmed by flow cytometry (**Supplementary Figure 5**), but did not affect frequencies of CD4+ cytokine positive cells (**Figures 4A,B**), but significantly diminished the frequency of IFN-γ+, TNF+ (**Figure 4C**) as well as IL-17+ and IL-22+ CD8+ T cells (**Figure 4D**) in the adipose tissue. Similar to adipose tissue, depletion of macrophages had no effect on frequencies of IFN-γ+, TNF+ (**Figure 4E**) as well as IL-17+ and IL-22+ CD4+ T cells (**Figure 4F**) in the liver. However, frequencies of IFN-γ+, TNF+ (**Figure 4G**) and IL-17+ and IL-22+ CD8+ T cells (**Figure 4H**) in the liver were significantly decreased upon macrophage depletion compared to the control group. Macrophage depletion did not affect any of the metabolic parameters such as body weight (**Supplementary Figure 7A**), adipose (**Supplementary Figure 7B**), and liver weight

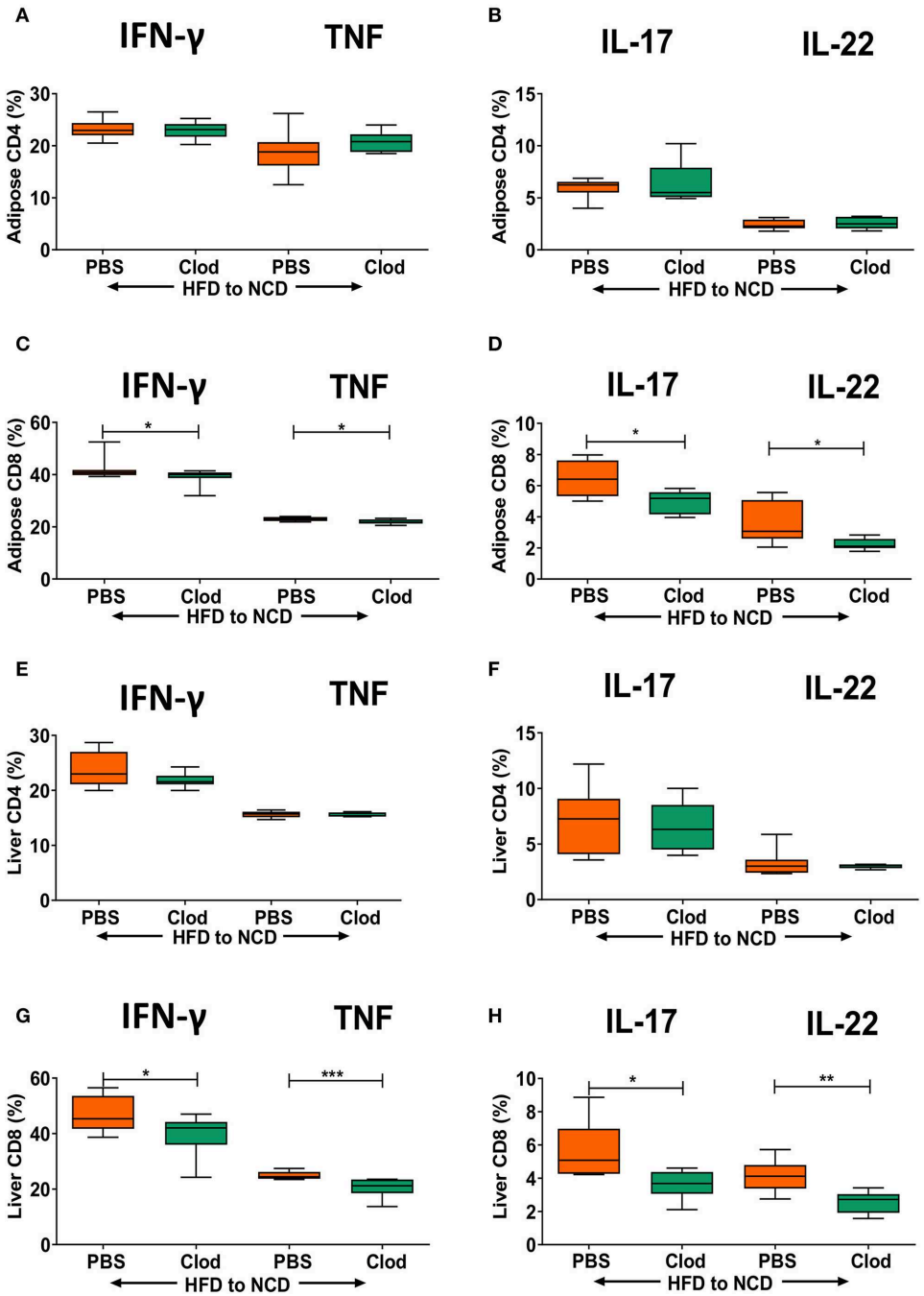

FIGURE 4 | Depletion of macrophages reduces frequencies of pro-inflammatory cytokine-producing CD8+ T cells in formerly obese mice. Animals were fed for 16 weeks on a HFD and switched to a NCD for 4 weeks, macrophages were depleted by clodronate liposomes (Clod) injection immediately after switching to the NCD diet. Controls receiving the same switch from a HFD to a NCD were treated with PBS liposomes (PBS). **(A–D)** Frequency of IFN-γ+ and TNF+ **(A,C)**, IL-17+ and IL-22+ **(B,D)** CD4+ T cells **(A,B)** and CD8+T cells **(C,D)** in adipose tissue. **(E–H)** Frequency of IFN-γ+ and TNF+ **(E,G)**, IL-17+ and IL-22+ **(F,H)** CD4+ T cells **(E,F)** and CD8+T cells **(G,H)** in liver. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Pooled data from $n = 2$ experiments, 3–5 mice each. Mann Whitney *U*-test was used to assess the statistical significant differences.

(**Supplementary Figure 7C**), GTT (**Supplementary Figures 7D,E**), and ITT (**Supplementary Figures 7F,G**), lipid profiles (**Supplementary Figures 7H,I**), and liver enzymes (**Supplementary Figures 7J,K**).

DISCUSSION

A combination of physical exercise and dietary changes is considered as the most effective non-invasive intervention

strategy for obesity. However, weight loss programs have been shown to bring about only an average weight reduction of 10% and despite the weight loss, the majority of patients quickly regain the lost weight (13). This leads to the question of whether weight loss improves the adverse metabolic and inflammatory profile underlying obese conditions. In order to better understand the biological mechanisms that reshape metabolic organs during weight loss, we investigated the macrophage and T cell function of the liver and adipose tissue on reversing HFD mice to NCD. Although an improvement in insulin sensitivity, lipid profile, and liver enzymes were evident after weight loss, the adipose tissue, and liver macrophage frequencies were increased and the expression of antigen presenting molecules on the macrophages were also increased. Total macrophage numbers increased in the metabolic organs during HFD and remained significantly increased following the reversal of the diet. Depletion of macrophages was sufficient to specifically limit the specific type 1 and type 17 CD8+ T cell frequencies, but not frequencies of type 1 and type 17 positive CD4+ T cells, from the adipose tissue and liver. Our findings are in agreement with the results of Zamarron et al. (5), who showed increased infiltration of macrophages in the adipose tissue up to 6 months after diet intervention. Further, Fischer et al. (14) have shown the presence of an inflammatory imprint in liver and perigonadal fat even after normalization of the metabolic parameters. Taken together these results argue in favor of liver and adipose tissue intrinsic memory of previous obesity. However, there are other reports that have shown that weight loss improves the inflammatory profile of obese subjects (7, 8).

When we probed into T cell alterations in our study, HFD increased type 1 cytokine positive CD4+ T cell frequencies in metabolic organs and switching HFD mice to NCD reduced them. In contrast to our findings, Zamarron et al. showed that HFD reduces frequencies of IFN-γ+ CD4+ T cells in adipose tissue and this phenotype was reversed after switching to a NCD (5). Regarding type-17 cells, reversing HFD to NCD increased the frequency of IL-17+ and IL-22+ CD4+ T cells in the adipose tissue and IL-22+ CD4+ T cells in the liver. Nonetheless, macrophage depletion did not rescue this effect of the reversed diet. Therefore, further studies are needed to identify the factors that regulate Th17 cells in the metabolic organs during weight loss. Intriguingly, the frequencies, but not total numbers, of type 1 and type 17 cytokine positive CD8+ T cells increased after weight loss. As increased macrophage accumulation was accompanied by increased frequencies of cytokine positive T cells in liver and adipose tissue, we depleted macrophages, which significantly reduced the frequencies of IFN-γ+, TNF+, IL-17+, and IL-22+ positive CD8+ T cells of the liver and the adipose tissue. It was previously shown that formerly obese mice regain body weight much faster than constantly lean counterparts (6). CD4+ T cells could be essentially involved as $H2A^{-/-}$ mice, which lack CD4+ T cells, did not develop an obesity memory and an accelerated weight regain occurred when CD4+ T cells of formerly obese mice were introduced to $Rag1^{-/-}$ mice (6). However, we did not see a significant impact of macrophages depletion on systemic parameters in obese mice. In contrast to our finding, Feng et al. showed that macrophage depletion modulates systemic metabolic parameters even in lean mice (15). We speculate that the reason for the lack of effect on metabolism in our study could be the shorter follow up period of 4 weeks after macrophage depletion and/or type of diet composition. There were slight but non-significant reductions in body weight, adipose tissue weight, and SGPT levels upon macrophage depletion. Thus, a longer follow up time might have resulted in a significant improvement of metabolic parameters.

One limitation of this study is that we have not validated our findings in humans and further studies are needed to investigate the impact of macrophages on adipose tissue inflammation and systemic metabolic parameters during weight loss in humans. It is not clear how and why weight loss leads to pro-inflammatory T cell responses. It is possible that the inflammation might have some beneficial effects as a low level of inflammation has been shown to be a pre-requisite for metabolic homeostasis (16) and the increased adipose tissue macrophages could help in resolving the excess deposition of extracellular matrix (17).

In conclusion, we show that although weight loss improves the metabolic profile, there is a robust increase in macrophage frequencies and antigen presentation accompanied by an active and ongoing/augmented CD8+ T cell inflammation both in liver and adipose tissue for at least 4 weeks after stopping a HFD. Depletion of macrophages reduced the expression of CD8+ T cell cytokines, suggesting that macrophages are major mediators of CD8+ T cell inflammation during weight loss. Therefore, it is possible that the sustained, increased CD8+ T cell inflammation in liver and adipose tissue could be the reason for the quick regaining of body weight.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany (84-02.04.2016.A331).

AUTHOR CONTRIBUTIONS

JS and MH: conceptualization and project administration. JS and SF: formal analysis. JS, IK, SF, and MK: methodology. AH and MH: resources and supervision. JS, IK, and MH: writing—original draft. JS, IK, SF, AH, and MH: writing—review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00257/full#supplementary-material

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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.

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REVIEW
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Macrophage Subsets in Obesity, Aligning the Liver and Adipose Tissue

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The increasing prevalence of obesity is accompanied by a rising incidence in metabolic syndrome and related pathologies such as non-alcoholic fatty liver disease. Macrophages are hypothesized to play central roles in these diseases, through their role as inflammatory mediators and as such are thought to be potential targets for future therapies. Recently, single cell technologies have revealed significant heterogeneity within the macrophage pool in both liver and adipose tissue in obesity. Thus current efforts are focused on dissecting this heterogeneity and understanding the distinct functions of the individual subsets. In this review, we discuss the current knowledge regarding macrophage heterogeneity, ontogeny and functions in the context of obese adipose tissue and fatty liver disease and attempt to align the distinct populations described to date.

Keywords: macrophages, adipose tissue, non-alcoholic fatty liver disease (NAFLD), single-cell, heterogeneity, liver, NASH (non-alcoholic steatohepatitis)

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INTRODUCTION

The transition to a more sedentary lifestyle coupled with a higher caloric intake has led to an enormous rise in incidences of obesity over the past decades. Simultaneously, this has led to a dramatic increase in the number of patients suffering from numerous obesity-linked metabolic disorders including insulin resistance and type 2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD). In fact, since 1975, obesity levels have almost tripled worldwide and according to the World Health Organization (WHO) this accounted for ∼8% of deaths in 2017 largely due to cardiovascular disease. Chronic low-grade inflammation has been suggested to be related to many of the co-morbidities of obesity including type 2 diabetes, NAFLD, steatohepatitis and cardiovascular disease [reviewed in (1)], leading to the concept of "metainflammation" (2). This is characterized by abnormal cytokine production, activation of inflammatory signaling pathways and increased acute phase reactants (3). Macrophages, as cells of the innate immune system, are thought to contribute significantly to metainflammation across obese tissues [reviewed in (4)]. Here, macrophages have been suggested to alter their phenotype toward a more pro-inflammatory profile and this has been proposed to be detrimental in the long-term, driving fibrosis and tissue damage (5, 6). More recently, the idea that these macrophages may be generated as (initially) a tissue protective mechanism, to deal with the increased lipid load has also been proposed (7). In recent years, it has become evident that there are multiple subsets of macrophages present in both obese adipose tissue and the fatty liver that may contribute differently to the pathogenesis of disease. What remains unclear is whether these represent an altered phenotype of tissue-resident macrophages (Res-macs) or newly recruited populations of macrophages. In the

latter case how these recruited macrophages relate to their tissue-resident counterparts also remains unclear. Answering these questions will be crucial to deciphering the different functions of the macrophages and understanding how best to target them therapeutically. While our understanding of macrophage heterogeneity, has been greatly improved through the use of single cell technologies including single cell RNA sequencing (scRNA-seq), there is often little consensus between studies with authors often using expression of different genes to identify subsets and giving the subsets identified distinct names. In this review, we will discuss what is currently known regarding the macrophage subsets present in the liver in non-alcoholic fatty liver disease and in obese adipose tissue, their origins and their specific functions. In addition, where possible, we attempt to align the different populations identified to date. For the most part the studies discussed here have been conducted in murine models unless stated otherwise (**Table S1**).

TISSUE-RESIDENT MACROPHAGES

Under homeostatic conditions, tissue-resident macrophages (Res-macs) represent the majority of macrophages in the body. These are generated alongside their tissue of residence, typically during embryogenesis and as such derive, at least initially, from embryonic progenitors including yolk-sac macrophages and fetal liver monocytes [reviewed extensively in (8–10)]. As the tissues grows in the 1st weeks of life, bone-marrow derived monocytes can also engraft in some tissues and generate Res-macs (8, 11). In most tissues, once organ growth has ceased, the Res-macs are maintained independently from any significant input from circulating progenitors, but rather through local proliferation of existing macrophages, although there are a few exceptions to this including the gut, dermis, heart and lung interstitial macrophages which are continuously replaced from the BM throughout life [reviewed in (8, 9, 12)]. Additionally, in old-age, BM monocytes may also start to contribute again to the Res-mac pools in some tissues, with a slight increase in BM-derived macrophages being observed in the spleen and peritoneal cavity between 36 and 46 weeks after tamoxifen labeling (13). Notably, in the lung and liver, the specific origin of the Res-macs, termed alveolar macs (Res-AMs) and Kupffer cells (Res-KCs) respectively, whether yolk-sac macrophages, fetal liver monocytes or bone marrow monocytes, does not appear to significantly affect the transcriptional profile of these macrophages or their ability to self-renew provided they are generated under homeostatic conditions (11, 14). This conditioning of progenitors enabling the development of Res-macs under homeostatic conditions, is proposed to result from the interactions between the progenitors/macrophages and the specific cells in their local environment or "niche" including stromal cells, endothelial cells and structural cells (8, 15) and indeed crosstalk between macrophages and fibroblasts has already been demonstrated *in vitro* under both healthy and fibrotic conditions (16, 17), while *in vivo*, many of the signals driving monocyte recruitment and differentiation into Kupffer cells following depletion have already been elucidated including Il1β, TNFα, DLL4, NOTCH, LXRα, and BMPs (18, 19).

Regarding their functions, Res-macs fulfill central roles in tissue homeostasis and immunity through their constant surveillance and their ability to clear foreign substances, dead cells and debris. This clearance is mediated through recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) present on their cell surface. In addition to physically clearing the tissue of pathogens, dead/dying cells and other debris, Res-macs can also express multiple cytokines which contribute to the activation of other innate immune cells and the adaptive immune system. Notably, through their unique transcriptomic profiles imprinted by signals from the local niche (8, 15), Res-macs are also proposed to play additional roles in maintaining tissue homeostasis, performing several "accessory" functions depending on the needs of their tissue of residence (20). These tissue-specific transcriptional profiles have been shown to be driven through expression of "master" transcription factors, which are imprinted in the macrophages by signals from their local niche (21, 22). For example, the transcriptional profiles of Res-KCs in the liver and Res-AMs in the alveolar space of the lung are both enriched for genes associated with a lipid metabolism function (21, 23), with the lipid metabolism profile of KCs being driven at least in part through expression of LXRα (23), which is imprinted on KCs by liver sinusoidal endothelial cells and hepatic stellate cells (18, 19). Similarly, Res-macs in the liver and spleen are enriched for genes necessary for iron processing, a function regulated by the master transcription factor SpiC (23–25).

With the identification of the substantial role played by the niche in regulating Res-mac gene expression and hence functionality, one of the key questions in the macrophage field today is how does the niche change in inflammation and how does this impact Res-mac survival and function? Notably, a population of microglia with a distinct transcriptional and functional profile have recently been described in aged mice (20 months). These microglia contain lipid droplets and were hence termed lipid droplet-containing aged microglia (LDAMs) (26). As there is limited input of HSCs to the microglia pool 46 weeks after tamoxifen administration (13) these LDAMs are suggested to be a population of Res-microglia, suggesting that Res-macs may indeed respond to changes in their local environment as occurs in aging or inflammation. However, as the origin of LDAMs was not formally tested in this study, it remains to be seen if these are indeed altered Res-macs or a newly recruited population (26). Nevertheless, understanding how the niche changes and how this may alter the macrophages is relevant question especially in the context of obesity and metabolic disease, where, for example, changes in hepatic stellate cells and myofibroblasts in the liver have been already been reported in NASH and fibrosis in both mouse and human samples (27, 28).

RECRUITED MACROPHAGES

Alongside Res-macs, when homeostasis is perturbed, for example due to inflammation and/or infection, bone marrow monocytes are recruited to the tissue, where they subsequently differentiate into macrophages. In some instances these cells represent a

short-lived transient population that are recruited during the inflammation/infection but are typically lost a few days after return to homeostasis (29–32), thus from herein these will be termed "temporary macrophages" (Temp-macs). However, bone marrow monocytes can also engraft and become long-lived resident macs under non-homeostatic conditions (32–36). When this happens in the brain due to genetic models or irradiation substantial differences are observed between the profiles of these recruited Res-macs compared with the embryonically-derived Res-microglia, including expression of the microglia-identity gene *Sall1* (33–35). Thus, we propose to call these Res-macs recruited under non-homeostatic conditions inflammatory Res-macs (infRes-macs). Whether these differences in brain microglia are due to timing and hence a potentially altered niche (for example adulthood vs. embryo) or due to intrinsic differences in the progenitors remains to be investigated. Notably, BM-derived KCs engrafting after genotoxic injury also display some (albeit minor) differences in their transcriptional programs compared with those engrafting under homeostatic conditions following DT mediated depletion (11, 37), suggesting the altered niche may be the most important factor at play. In the lung in the context of influenza infection, Res-AMs were found to be largely unchanged by the virus but reduced in numbers, which led to the development of BM-derived infRes-AMs during the infection with distinct functions, transcription and epigenetic profiles as compared with the Res-AMs (36). Notably, with time spent in the lung niche following recovery from infection, these differences were gradually lost and thus the infRes-AMs more closely resembled the Res-AMs with time (36). In the liver in a model of NASH, we also observed BM-monocytes differentiating into infRes-KCs that were maintained for at least 4 weeks after return to homeostasis (32). However, how similar these infRes-KCs were to the Res-KCs was not investigated (32) and remains an open question. Given the observation that infRes-macs can have significantly altered profiles and hence altered functionality compared with Res-macs, these macs should be discriminated from one another. This could be especially important in disease settings where one might aim to promote Res-macs over newly recruited infRes-macs or *vice versa* depending on their specific functions.

The recruitment of both Temp-macs and infRes-macs to supplement the original Res-macs in inflammation raises multiple questions. For example, why do we recruit macrophages in non-homeostatic conditions? Does this mean that Res-macs are not plastic enough to be able to deal with the insult? Do Res-macs alter their profiles as has been previously suggested or are changes within the total macrophage pool due solely to the recruitment of Temp-macs and infRes-macs? Indeed the influenza study would suggest the original Res-macs are not substantially altered by the virus but rather cannot maintain their numbers and hence require input from the BM (36). Is this true in obese tissues? Are infRes-macs required to maintain macrophage numbers as some Res-macs are lost and proliferation rates are not sufficient to replenish the pool? If so, how similar are these infRes-macs to their original Res-mac counterparts? Moreover, how distinct are infRes-macs and Temp-macs? Do these represent distinct populations of macrophages entering the tissue or can some Temp-macs also become infRes-macs? In the context of obesity, it will be important to address these questions to understand (i) which subsets are important in the pathology, (ii) which signals drive the recruitment and differentiation of the different subsets, and (iii) how we could target the required subsets therapeutically. Crucially, it will be important to understand which subsets are found across multiple labs, models, and indeed species to be able to understand which populations could be clinically relevant. With this in mind, below we will discuss the current knowledge regarding the distribution and origins of different macrophages subsets and their functions in obese tissues and attempt to align the subsets identified by different groups. However, as will become clear, this is not always straight-forward and as a result there are still many unanswered questions regarding these cells in obesity.

MACROPHAGE SUBSETS IN NAFLD AND NASH

NAFLD is the hepatic manifestation of metabolic syndrome. It currently represents the most common etiology of chronic liver inflammation in the western world. Due to the lack of treatment options available, it is predicted to be the primary reason for liver transplantation in the western world by 2030 (38–41). The current lack of therapies for patients with NAFLD stems from the fact that NAFLD is a complex disease, consisting of different stages ranging from simple steatosis (retention of fat in the liver) to non-alcoholic steatohepatitis (NASH), cirrhosis and even hepatocellular carcinoma (HCC). While patients with simple steatosis are largely asymptomatic, the progression to NASH, fibrosis and eventual cirrhosis leads to the need for liver transplantation. Notably, this progression from NAFLD to NASH does not occur in all patients and the mechanisms underlying this transition remain largely unknown. It has been proposed that multiple "hits" coming from the gut and adipose tissue may account for this progression (42), with one of the hits being the ensuing inflammation potentially driven by macrophages in response to the increased lipid load and hepatocellular death.

During steady state, the predominant macrophage population present in the liver is the resident Kupffer Cells (Res-KCs). They are one of the largest populations of Res-macs present in the body and reside with part of their body in the liver sinusoids where they are in close contact with liver sinusoidal endothelial cells (LSECs) and part in the liver parenchyma where they are in close contact with hepatic stellate cells (HSCs) and hepatocytes (19). In mice, KCs can be identified by their specific expression of the C-type lectin, CLEC4F (11). In addition, once resident in the liver, KCs also express TIM4 and thus the combination of CLEC4F and TIM4 is very useful to discriminate between Res-KCs ($CLEC4F^{+}TIM4^{+}$) and recently recruited KCs, on their way to becoming resident ($CLEC4F^{+}TIM4^{-}$) and Temp-macs ($CLEC4F^{-}TIM4^{-}$) that can either differentiate into KCs or be lost from the tissue upon return to tissue homeostasis (11, 32). In humans, the best markers of Res-KCs compared with the other mac populations present in the liver are still being elucidated,

although *CD163, TIMD4,* and *MARCO* expression have been suggested to identify Res-KCs (28, 43).

In the context of NAFLD, Res-KCs have been proposed to drive the progression from NAFLD to NASH through their role as inflammatory mediators [recently reviewed in (6)]. This hypothesis stems from studies suggesting the balance between M2- and M1-like hepatic macrophages is altered in NAFLD and that promoting more M2-like macrophages may be beneficial in NAFLD (44–48). In addition, studies depleting macrophages using models including LysM-driven genetic models and clodronate liposomes have further fueled the hypothesis that Res-KCs drive the development of hepatic steatosis, insulin resistance, liver damage and inflammation (49–53). However, two main findings are now altering this line of thinking. Firstly, it is now clear that the M1/M2 polarization states are not sufficient to describe the complex milieu of signals which imprint macrophage phenotypes and functions *in vivo* (54). Indeed, under homeostatic conditions, the transcriptional profile of Res-KCs and other tissue Res-macs do not resemble those of either M1 or M2 macrophages (**Figure 1**, **Table S2**). Moreover, it has become clear that Res-KCs are not the only population of macrophages in the fatty liver, with a number of Temp-macs being recruited from the bone marrow in a CCR2-dependent manner that are also thought to play a role in the progression to steatohepatitis and fibrosis (56–58). Thus, it is not yet clear if Temp-macs or Res-KCs are contributing to the phenotypes observed when total macrophages are manipulated using such non-KC-specific models as described above and hence the exact roles played by Res-KCs in NAFLD/NASH remain to be understood. In addition, in mice fed a methionine-choline deficient (MCD) diet to induce NASH, we demonstrated that CLEC4F$^-$ Temp-macs are recruited to the liver and that some of these give rise to CLEC4F$^+$TIM4$^-$ infRes-macs (32). Thus, potentially, there could be at least 3 distinct subsets of macs with different transcriptional profiles and functions in NAFLD/NASH. However, as mice fed the MCD diet develop NASH symptoms but do not gain weight (32), whether these infRes-Macs are also found in more biologically relevant models of NAFLD/NASH or indeed in human NAFLD/NASH remains to be seen as discussed below.

With these caveats of earlier studies in mind, what have we learnt in recent years regarding the subsets and roles of liver macrophages in NAFLD/NASH? Intriguingly and contrary to the widely accepted hypothesis, a recent study has found that neither obese human macrophages nor high-fat diet fed (12 weeks) murine liver macrophages (purified as adherent cells following tissue digestion, percoll gradient and plating) altered their expression of a standard panel of activation associated genes including *Tnfa, Il1b, Il6, Il10, Tgfb1, Ccl2, Ccl5, Itgax, Cd80, Socs3*, *Chil3, Arg1,* or *Egr2* (59). This therefore raises the question whether Res-KCs do respond to the fatty liver environment and mediate the inflammation as originally proposed or if perhaps they behave more like the Res-AMs in the lung during influenza infection (36). However, as here there was no discrimination between Res-KCs and other liver macs (59) it is difficult to draw any conclusions regarding the roles of these cells, perhaps Res-KCs are not activated or perhaps they are but their numbers are reduced in NAFLD leading to the majority of total macs being Temp-macs without an overtly activated profile. Thus, further work is required to answer these questions.

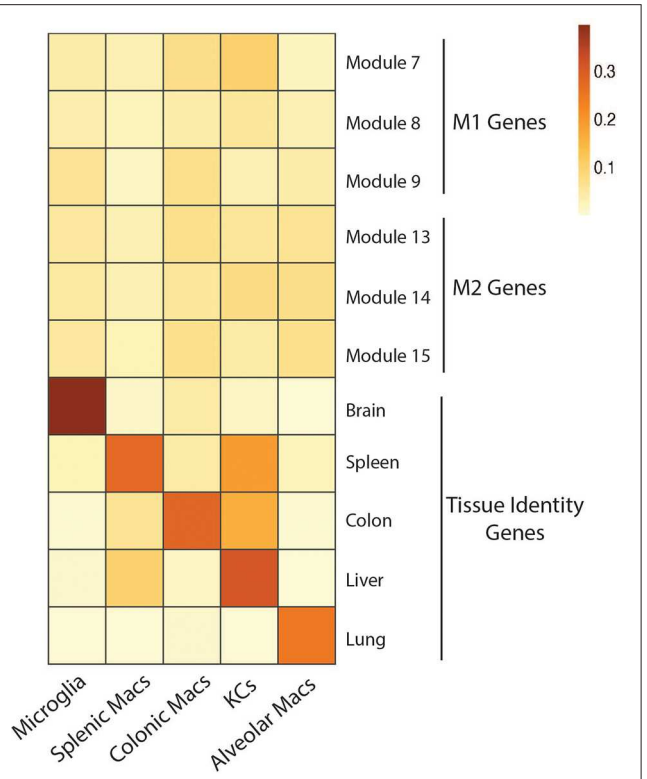

FIGURE 1 | No overt M1 or M2 activation profile in tissue resident macrophages in homeostatic conditions. Expression of gene modules associated with M1 (module 7, 8, 9) and M2 (module 13, 14, 15) human macrophages (55) by microglia, splenic macrophages, colonic macrophages, Kupffer cells and Alveolar macrophages isolated from mice under homeostatic conditions (23). Expression of core identity genes of each tissue resident macrophage population (23) were also visualized as a control (Genes associated with each module and population can be found in **Table S2**). To generate the heatmap, mouse gene symbols were used as input for the AUCell R package. In brief, AUCell allows you to identify cells with active gene sets (e.g., signatures, gene modules) in single cell RNA-seq data by first ranking the genes from highest to lowest value per cell. Next, area under the curve (AUC) is calculated to determine whether the gene set is enriched at the top of the gene-ranking for each cell. The output is a matrix with an AUC score for each gene set in each cell. For the heatmap we calculated the mean AUC of all cells.

The recent advances in scRNA-seq studies have started to shed light on the heterogeneity of macrophages present in the NAFLD/NASH liver. One recent study employing scRNA-seq of CD45$^+$Ly6G$^-$ myeloid cells isolated from mice fed a western diet supplemented with fat, sugar and cholesterol for 16 weeks identified 3 distinct clusters of recruited macrophages (termed Mo-MFs) and a cluster of likely Res-KCs (57). All of these macrophage populations, as well as cDC2s and bone marrow monocytes in these mice expressed lower levels of *S100a8* and *S100a9* encoding the inflammatory protein Calprotectin compared with the same populations in control mice fed a normal diet, suggesting they may have anti-inflammatory

properties (57). Fitting with this, challenge of these mice with an overdose of paracetamol as a model of acute liver injury resulted in attenuated disease compared with normal diet fed controls (57). While a similar response of recruited macrophages and Res-KCs as observed here (57), would be consistent with the relative unresponsiveness of total liver macrophages observed by the Aouadi lab (59), there are a few remaining questions surrounding this study. The presence of recruited macrophage populations in the control mice (albeit at lower frequencies than the NAFLD mice) is rather unexpected especially as some of these populations seem to be more abundant than the KCs (57). This raises the question if the control mice are completely healthy? If so, the question becomes what are these recruited macrophage populations in the control mice and where are they located? In addition, a key question is whether the distinct clusters of recruited macrophages identified truly represent distinct subsets of macrophages or rather one population existing along a gradient of activation and/or developmental stages? Regarding the relative paucity of Res-KCs, while the authors attribute this to digestion and cell isolation, it raises the question as to whether the Res-KCs isolated are representative of the total Res-KC pool in NAFLD/NASH? Moreover, as the origins of these cells were not studied it remains to be seen if these are truly Res-KCs or if any infRes-KCs may also be found in the NAFLD/NASH liver.

A second recent study also employing scRNA-seq but this time using mice fed an amylin (ALMN) diet for 20 weeks to induce NASH identified distinct clusters of macrophages in the NASH liver (27). Here one population of recruited macrophages alongside two KC subsets were identified. The two KC subsets were identified as KCs based on expression of *Cd5l* and the two subsets were distinguished from each other based on expression of *Trem2, Cd9,* and *Gpnmb* (27). The KC population expressing all of these genes was only present in the NASH condition and was therefore termed NASH-associated macrophages or NAMs (27). The expression profile of these NAMs was significantly different from healthy Res-KCs (27), but the healthy Res-KC profile was largely maintained in the non-NAM KCs in the ALMN diet (27), further supporting the idea that at least some of the Res-KCs may not be altered in NAFLD/NASH. As the relationship between the two KC subsets identified in the ALMN-fed mice was not studied (27), it is not clear if the NAMs represent a recruited population of infRes-KCs, while the non-NAM KCs represent Res-KCs that do not alter their phenotype in NASH or if the NAMs are a population of Res-KCs that have altered their transcriptome due to the NASH environment. This will of course be important to address as targeting such macrophages either to assess their functions or later on in the clinic would require different approaches in each case. Notably while further study is required to understand the nature of these macrophage subsets, macrophages with a phenotype similar to the NAMs have also recently been identified in fibrotic human livers (28) indicating the potential relevance of these macs in human disease although not necessarily restricted to NASH as the name NAMs would suggest. The macrophages identified in fibrotic human liver tissue were rather termed scar-associated macrophages (SAMs) and were specifically located around the fibrotic scars (28). Interestingly the SAMs were distinct from KCs further highlighting the need to investigate the designation of NAMs as KCs in the mouse (27).

Despite the advances in understanding macrophage heterogeneity, an unfortunate feature of these studies is that it is not always easy to link the subsets identified in the different studies. This is further complicated by each study giving a different name to the macrophage populations they identify. A crucial next step will therefore be to understand what subsets and profiles are shared across NAFLD/NASH models and between species and how best to identify them for downstream functional studies which are still lacking. To try to align these studies, we have downloaded the data from the two reports and recreated the tSNE plots (27, 57). While the tSNE plots are not exact replicates, the same clusters identified by the two groups could be identified in our recreations based upon the data provided and the gene lists published for each population (**Figure 2**). Expression of a set of markers proposed as Res-KC markers (*Clec4f, Timd4, Cd5l*), general macrophage markers (*Adgre1*, *Fcgr1*), monocyte markers (*Ccr2 and Ly6c2*) and the markers proposed by the studies (*S100a8*, *S100a9*, *Cd9*, *Trem2,* and *Gpnmb*) were assessed (**Figure 2**). Interestingly, the subsets identified in both studies as recruited macrophages expressed relatively low levels of the macrophage markers *Adgre1* (encoding F4/80) and *Fcgr1* (encoding CD64) while still expressing the monocyte genes *Ly6c2* (encoding Ly6C) and *Ccr2*, thus perhaps some of these populations may represent monocytes or monocytes transitioning to macrophages rather than fully differentiated macrophages (**Figure 2**). This would also explain their presence in the healthy controls in the study from the Tacke lab (57). In terms of expression of the KC-specific markers, it appears that some of the NAMs do not express these genes suggesting they may indeed represent Temp-macs (**Figure 2**). While further studies will be required to confirm this, this could indeed suggest that Res-KCs are not significantly altered in NAFLD/NASH but rather that recruited macs in NASH have a distinct phenotype from Res-KCs. Regarding the presence of infRes-macs, in both studies a population of KCs exist that do not express *Timd4* (encoding TIM4), a marker of long-term residence of KCs in the liver (**Figure 2**). This could suggest that like in the MCD-induced NASH model, infRes-KCs may be generated with an altered profile to Res-KCs. However, as coverage of *Timd4* expression is not always 100% even in homeostatic conditions (23) and we do not yet fully understand the signals driving *Timd4* expression in KCs, this will need to be confirmed with fate-mapping studies. While *S100a8* and *S100a9* expression was not seen in the study from Xiong and colleagues (**Figure 2**), what is notable is that the *Trem2*, *Cd9*, *Gpnmb* signature of NAMs (27), that is also found in macrophages from human fibrotic tissue (28) can also be found in some cells in one of the recruited macrophage clusters (mo-mf-II) in the study from Krenkel et al., notably, in the cells expressing lower levels of *Ly6c2* and higher levels of *Adgre1* and *Fcgr1* compared with the other populations designated as recruited macs (**Figure 2**), suggesting that this may be a truly conserved signature of Temp-macs in fibrosis. Thus, understanding the functions of these cells represents an important question for future studies.

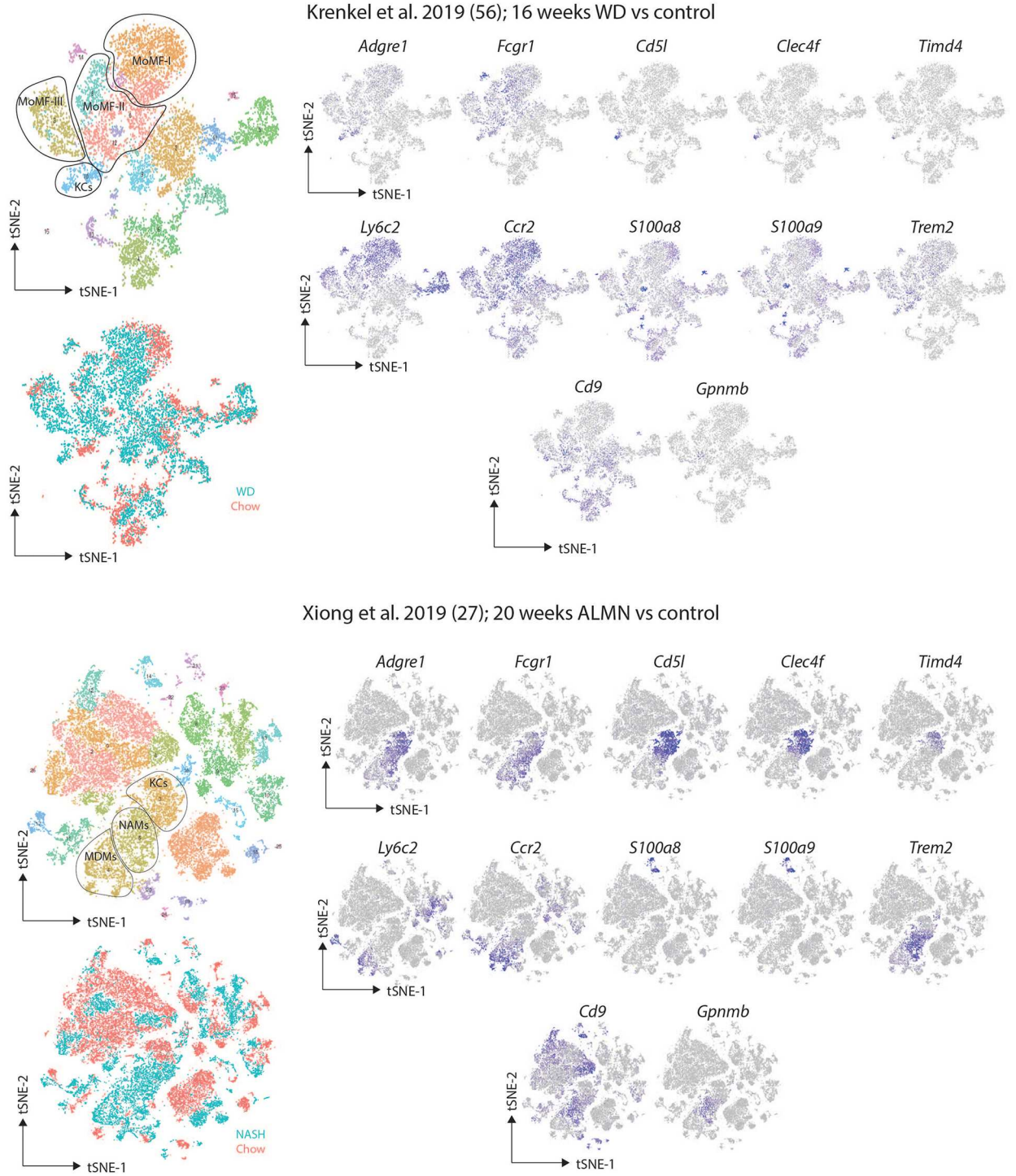

FIGURE 2 | Comparison of murine scRNA-seq datasets on macrophages in NAFLD/NASH. Publicly available scRNA-seq datasets from (27, 57) were downloaded and tSNE maps recreated as close to the original as possible with the information provided. The distinct clusters of macrophages and KCs as identified in the original studies (27, 57) were identified and expression of the indicated markers assessed.

Another key question is if Res-KCs are not functioning as inflammatory mediators in NAFLD as these recent studies may suggest, do they play any role? One possibility is that Res-KCs may function to metabolize the ectopic lipid in NAFLD, as under homeostatic conditions Res-KCs have been shown to express a module of genes associated with lipid/cholesterol

metabolism (11), although this remains to be tested directly. In line with other roles for Res-KCs, Morgantini et al. proposed that liver macrophages would function in NAFLD through their production of non-inflammatory factors including *Igfbp7* regulating liver metabolism (59). While an interesting concept that should be followed up, it will also be important to determine which of the liver macrophage subsets in NAFLD express *Igfbp7* (Res-KCs or recruited macs) and how specific this expression is across the liver. For example, do any of the niche cells of the liver such as HSCs and LSECs which express higher levels of *Igfbp7* than Res-KCs under homeostatic conditions (unpublished data) also express *Igfbp7* in NAFLD/NASH and what role does this play?

Taken together, this significant level of heterogeneity within the macrophage pool in NAFLD/NASH (**Figure 3**) highlights the need to understand the specific functions of the different macrophage populations identified. In addition, with the possibility that some monocytes infiltrating the liver in NAFLD/NASH may give rise to infRes-KCs (**Figure 3**), this brings into question the strategy to target all recruited cells in NAFLD through CCL2 inhibitors (60), as potentially infRes-KCs and Temp-macs may play distinct roles in disease pathology. Thus, further investigation into the biology of this heterogeneity is warranted, especially in terms of understanding the functional contribution of the conserved subset of putative Temp-macs expressing *Cd9*, *Trem2,* and *Gpnmb* found across multiple models and in human fibrotic tissue as well as the location of these subsets in the tissue and their interactions with the local niche. Of note, one study in *Trem2* KO mice fed a western diet found reduced pathology in the KOs compared with WT controls (61). While this was attributed to macrophages in the adipose tissue (61) (see below) it is also possible that this phenotype was due to the lack of *Trem2*-expressing macrophages in the fatty liver, further highlighting the need for functional studies with these populations.

MACROPHAGE SUBSETS IN OBESE ADIPOSE TISSUE

The adipose tissue consists mainly of adipocytes and functions as an energy store but also produces hormones, for example those regulating lipid metabolism and hunger satiety. Adipose tissue macrophages (ATMs) are spread throughout the adipose tissue with a significant proportion in lean mice being found associated with the vasculature and hence recently termed vasculature-associated macrophages or VAMs (62). VAMs are identified on the basis of their expression of CD206 and CD11b and can be subdivided into two subpopulations (VAM1 and VAM2) based on MHCII and TIM4 expression, with VAM2s expressing higher levels of TIM4 (62). In addition a population of $CD206^{int}CD11b^{+}CD64^{+}CD11c^{-}$ pre-VAMs and $CD206^{int}CD11b^{+}CD11c^{+}CD64^{+}$ double positive (DP) macrophages were also identified in this study although their localization was not investigated (62). Regarding ATM origins, although initially not included in macrophage fate mapping studies, recently, it was suggested that, under homeostatic conditions, Res-ATMs derive from yolk-sac progenitors (63). However, BM progenitors were also shown to engraft and give rise to Res-ATMs as ∼40% of ATMs were derived from the BM following congenic donor BM transplantation (63) moreover whether fetal liver monocytes can contribute to the Res-ATM pool remains to be investigated. This contribution of BM monocytes to the total homeostatic Res-ATM pool in lean mice has also recently been confirmed by fate mapping of ATMs using *Ms4a3*-tagging (61). However, how similar the BM derived Res-ATMs are compared with the embryonic-derived Res-ATMs remains to be investigated. Notably, upon discriminating between VAMs, pre-VAMs and DP adipose tissue macs, Silva et al. identified VAMs to be primarily embryonically derived with only minimal input from BM monocytes while pre-VAMs and DP macrophages were largely derived from the BM (62), suggesting that the turnover of the VAMs is relatively slow with between 1 and 10% of the VAMs being monocyte-derived 60 days after shielded irradiation and congenic donor BM transplant when donor BM input in blood monocytes is normalized to 100% (62). Given the relationship between TIM4 expression and residency time of Res-KCs in the liver (11), as VAM2s had lower levels of chimerism than VAM1s and also expressed TIM4 it is tempting to speculate that VAM2s may be the Res-ATMs that have been in the tissue the longest, while $TIM4^{lo}$ VAM1s may be younger recruits (62). Notably, Res-ATMs were also found to proliferate locally in lean mice (63). In addition to local proliferation, Res-ATM numbers are also maintained through the action of cytotoxic type 1 ILCs in adipose tissue which control ATM numbers (64).

In obese adipose tissue, adipocytes enlarge and subsequently die. In response, the number of ATMs is dramatically increased from ∼10% of all immune cells in lean mice to ∼50% (65, 66), of which, many are found localized with dead adipocytes forming crown-like structures (CLS) in both humans and mice, allowing the macrophages to surround and engulf the adipocytes (67–69). The increase in ATMs derives partially from the recruitment of new macs in a CCL2 dependent manner, and as in NAFLD, it was these recruited macs that were thought to drive local inflammation and insulin resistance (70–72). In addition, local proliferation of ATMs, specifically those located in the crown-like structures (CLS) has also been reported to contribute to the increase in ATM number in obesity (73–75). Until recently the exact nature of these ATMs in obese adipose tissue was unclear. It had been established that obesity led to a switch in macrophage phenotype from a more M2-like phenotype to a M1-like phenotype (76), with the caveats of using this nomenclature *in vivo* withstanding (**Figure 1**). However, it was unclear if this represented the recruitment of a distinct population of Temp-ATMs or infRes-ATMs with an M1-like phenotype in obesity or if this also represented plasticity of Res-ATMs. In addition, M1-like cells were typically associated with increased inflammation and a negative outcome however, it is also possible that the recruited ATMs in obese adipose tissue may be beneficial for the tissue, adopting a profile that enables the clearance of dying adipocytes (7). Recently, many groups have begun to address these questions using scRNA-seq and multi-parameter flow cytometry approaches (61, 62, 77–80). Hill and colleagues

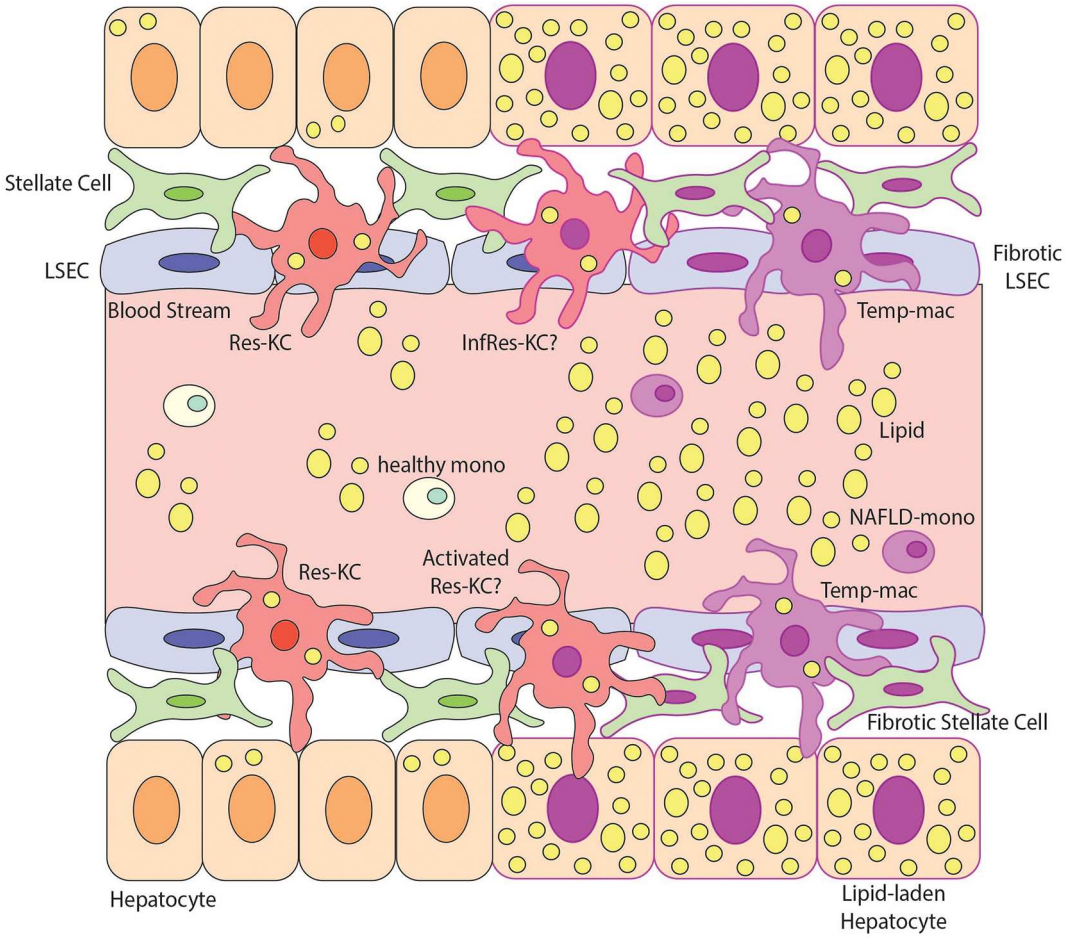

FIGURE 3 | Macrophage Heterogeneity in NAFLD and NASH. This figure demonstrates possible macrophage heterogeneity present during NAFLD/NASH. During steady state the main macrophage population present in the liver is resident Kupffer Cells (Res-KCs) which are in close contact with the liver endothelial cells (LSECs), stellate cells and hepatocytes. Healthy monocytes pass through the blood flow of the liver sinusoids daily and typically do not engraft unless the niche becomes available. During NAFLD there is an increase in lipids in the liver and subsequently hepatocytes become lipid laden. The lipemic environment also contributes to stellate cell and endothelial cell activation likely altering the macrophage niche. Multiple populations of macrophages have been identified in the NAFLD liver but the relationship between them remains unclear. For example, does the lipemic environment activate the Res-KCs (activated Res-KCs) or do monocytes (which have been shown to have an altered transcriptional profile) recruited to the liver in NAFLD engraft and give rise to infRes-KCs, with a similar profile to Res-KCs but showing signs of the altered environment. Additionally, in NAFLD, monocytes have been shown to engraft and generate Temp-macs, although how distinct these are from either Res-KCs, activated Res-KCs or InfRes-KCs remains to be studied. For example, an interesting population of macrophages expressing *Cd9, Trem2,* and *Gpnmb* in murine NASH and human fibrotic livers have been described (27, 28) but whether these represent activated Res-KCs, infRes-KCs or Temp-macs remains to be formally examined.

identified 2 subsets of F4/80$^+$CD64$^+$ ATMs distinguished on the basis of CD9 expression as well as a population of Ly6C$^+$CD11b$^+$ monocyte-like cells (78). While the monocyte-like cells were increased in frequency in obese compared with lean adipose tissue, CD9$^+$ ATMs were restricted to the adipose tissue of the mice fed a HFD and were also identified in obese patient adipose tissue (78). Notably, some of the Ly6C monocytes also expressed CD9 suggesting these could be the precursors for the CD9$^+$ ATMs (78). Both populations were shown to derive from the BM using a chimera model, however the origins of the CD9$^-$ ATMs were not assessed (78). The authors found the CD9$^+$ ATMs to be located in the CLSs, where they had an increased lipid content and a transcriptional profile enriched for proinflammatory and metabolic genes when compared with the Ly6C monocytes. However, how this compared with the CD9$^-$ ATMs was not investigated. Fitting with the more complex *in vivo* environment, the CD9$^+$ ATM transcriptional profile was clearly distinct from M1 or M2 profiles (78).

After identifying the VAM, pre-VAM, and DP mac populations in lean mice using flow cytometry, Silva and colleagues then put the mice on a HFD for 20 weeks. This led to an increase in all ATM populations identified with the largest increase observed in the DP macs (62). Interestingly, these DP macs were reported to correlate with the CD9$^+$ macs identified by Hill and colleagues (62), however, Silva and colleagues also reported increased CD9 expression in the VAMs upon HFD

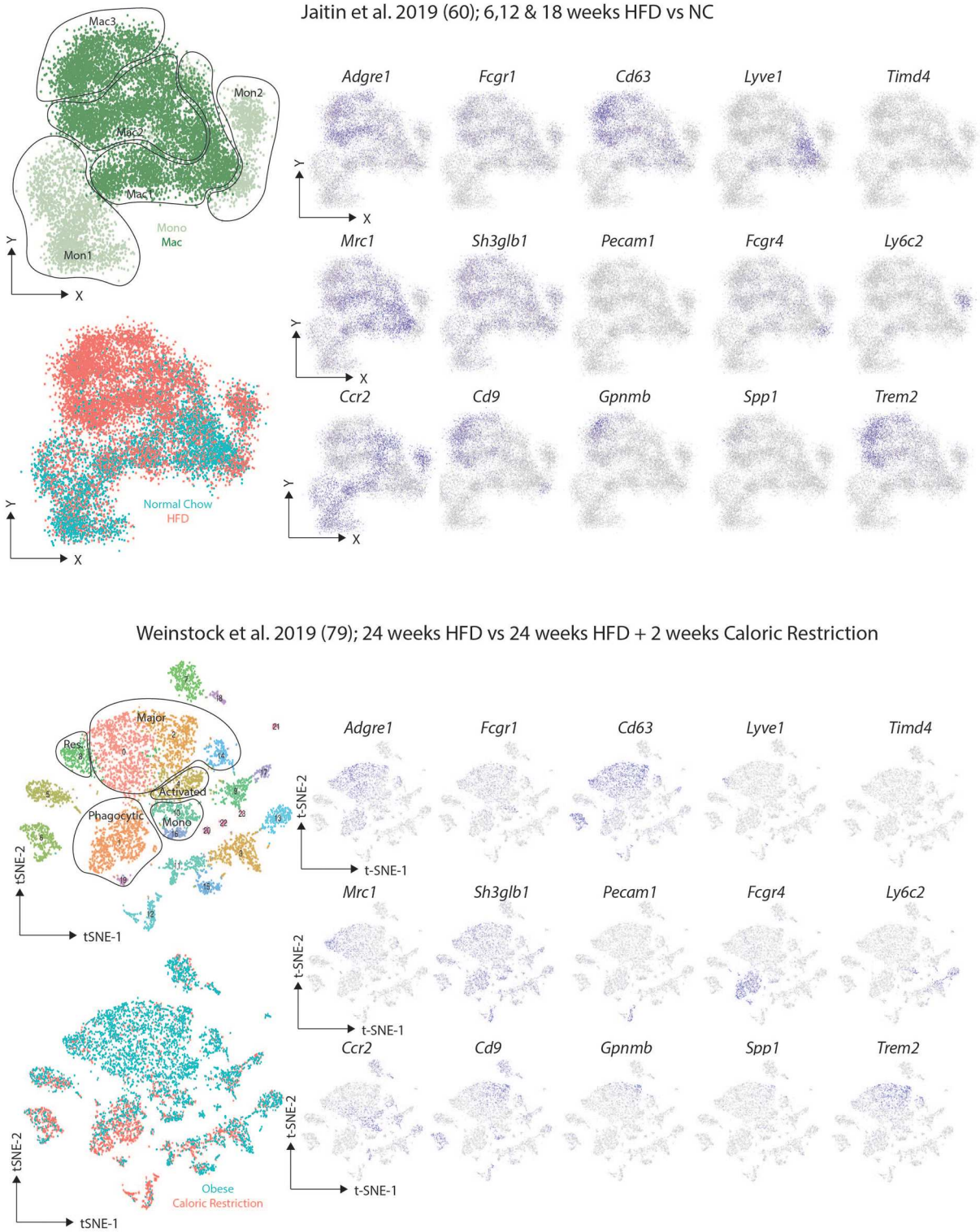

FIGURE 4 | Comparison of murine scRNAseq datasets on macrophages in obese adipose tissue. Publicly available scRNA-seq datasets from (61, 80) were downloaded and tSNE maps recreated as close to the original as possible with the information provided. The distinct clusters of macrophages and KCs as identified in the original studies (61, 80) were identified and expression of the indicated markers assessed.

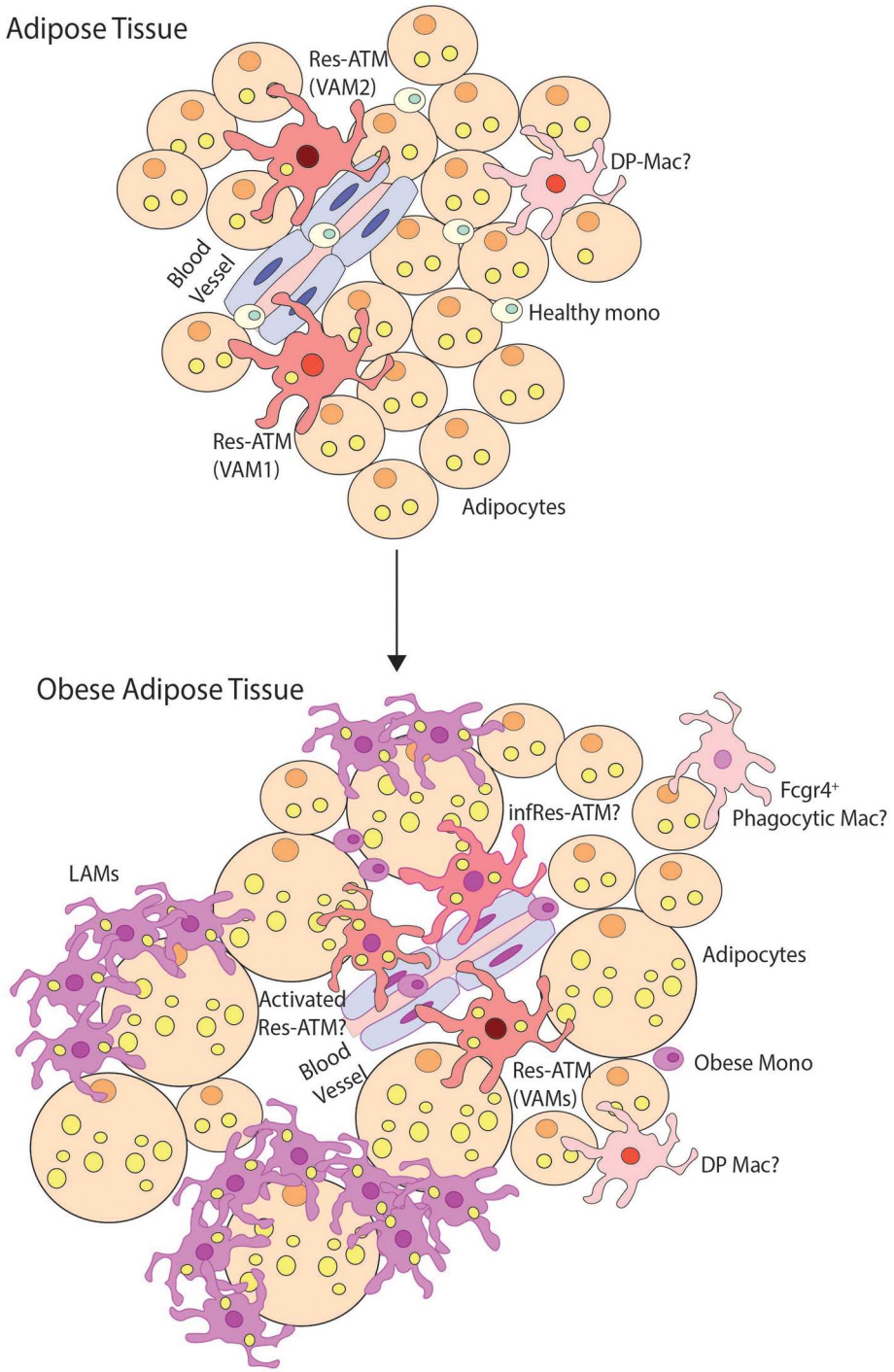

FIGURE 5 | Macrophage heterogeneity in obese adipose tissue. This figure demonstrates possible macrophage heterogeneity present in healthy and obese adipose tissue. In healthy tissue, resident macrophages (Res-ATMs) have been described which express genes suggesting they would be found close to vasculature (61, 62, 80). In one study these were hence termed vascular associated macrophages (VAMs) and could be identified in two subsets those expressing TIM4 and intermediate levels of MHCII (VAM2) and those with a $TIM4^{lo}MHCII^{hi}$ profile (VAM1) (62). In addition a population of macrophages deriving from monocytes and expressing CD11c and CD64 termed double-positive macrophages (DP-macs) were also described, however their precise nature remains to be seen as a distinct subset of such macrophages was not observed in healthy adipose tissue in scRNA-seq studies (61, 80). Monocytes are also found in healthy adipose tissue. In obese adipose tissue, the number of monocytes and macrophages dramatically increases. In terms of subsets, Res-ATMs remain, however, exactly how distinct these are from those in healthy tissue remains a question. Some changes in transcriptional profile were identified in VAM1s and VAM2s but whether these represent activated

(Continued)

FIGURE 5 | Res-ATMs or infRes-ATMs remains to be seen. Whether some Res-ATMs exist that do not react to the obese environment also remains a question but notably in both scRNA-seq studies, Res-ATMs were identified in the same cluster with those isolated from lean mice (61, 80) suggesting that at least a proportion of the population may not react extensively to the environment. In addition, a population of *Cd9, Trem2 Gpnmb, Spp1* expressing lipid-associated macrophages (LAMs) have also been identified forming crown-like structures in obese adipose tissue. These represent a unique population in the scRNA-seq studies found only in obese adipose tissue. In addition a population of phagocytic macrophages have also been described in obese adipose tissue and being expanded calorie restricted obese mice (80) that have a phenotype similar to DP-macs but also show some overlap with $CD9^+$ Temp-macs (non-LAMs) in obese adipose tissue. Thus, the exact nature of these cells also requires further analysis.

feeding suggesting that the $CD9^+$ population identified by Hill may include these two subsets (62). In another study, Jaitin et al. identified 3 $CD63^+$ ATM clusters using scRNA-seq (61). One cluster, termed Mac1, were the only ATM population present in lean mice and hence were identified as the Res-ATMs (61). This Mac1 cluster contained $Cd9^-$ ATMs that expressed genes previously associated with perivascular macrophages in the lung interstitium including *Cd163*, *Lyve1,* and *Retnla* (61, 81), and thus likely correspond to the steady state VAMs (62). Indeed upon downloading the data and regenerating the plots to compare the studies (**Figure 4**), expression of the proposed VAM markers *Mrc1* (encoding CD206), *Shglb3* and *Timd4* (62) was observed in this *Lyve1*-expressing Mac1 population, although *Shglb3* expression was not restricted to these cells (**Figure 4**). Notably no second cluster of DP macs was identified in lean conditions again questioning if these truly represent a distinct subset of macrophages or rather a distinct developmental stage/monocyte intermediate given that BM monocytes were found to engraft (albeit slowly) into the VAMs (61, 62). The other two clusters (Mac2 and Mac3) identified by Jaitin et al. expressed *Cd9* and were only found in obese mice, either due to feeding a HFD or genetic defects (db/db obese mice) (61). The Mac2 cluster also expressed the genes encoding surface markers proposed for VAMs (**Figure 4**) and thus could represent the $CD9^+$ VAMs reported to be present in obese adipose tissue (62). However, the Mac2 cluster lacked expression of the genes associated with being located in a vascular niche and rather $CD9^+$ macrophages (Mac2 and Mac3) were found to be located in the CLSs (61). If the Mac2 cluster do represent $CD9^+$ VAMs, they could be seen as either activated Res-ATMs or infRes-ATMs. As fate-mapping studies using the *Ms4a3*-CrexTdTomato reporter mice found an increased contribution of monocytes to the total $CD63^+$ ATM pool after 16 weeks of HFD feeding compared with mice fed normal chow for the same period (61), this could suggest that these would be infRes-ATMs, however, this needs to be tested. Interestingly, the Mac3 cluster, while expressing some genes encoding VAM markers, also expressed *Itgax* (encoding CD11c) and *Mrc1* expression (encoding CD206) was lower than in other Mac populations which could suggest that these are the DP macs identified by Silva and colleagues but then restricted to the obese adipose tissue in this study (61, 62). Overall, while there is potential to align these populations, it is clear that further analysis combining protein and mRNA expression at the single cell level will be necessary to completely align the subsets/clusters identified in these studies and to determine which represent infRes-Macs and Temp-macs in the obese tissue. Further analysis of the differences between the 3 ATM clusters in the study by Jaitin et al. identified the Mac3 cluster to be enriched for a number of genes associated with lipid metabolism and phagocytosis, including *Trem2*, *Gpnmb*, *Lipa*, *Lpl*, *Cd36,* and *Fabp4* and thus these cells were termed lipid-associated macrophages (LAMs) (61). Although some *Trem2* expression was also observed in the Mac2 population (61). Moreover, the LAMs also expressed the chemokine osteopontin (encoded by *Spp1*) and crucially were also found in human obese adipose tissue (61).

Simultaneously, two additional studies performed scRNAseq studies in obese adipose tissue. These studies identified multiple clusters of macrophages in obese mice including those they termed major macs, activated macs, resident macs and phagocytic macs (79, 80). Major macs were the subset that were most significantly expanded in obese conditions (79, 80) while phagocytic macs were only found in obese adipose tissue and were further expanded upon caloric restriction in obese mice (80). Intriguingly, a cluster within the major macs were enriched for expression of *Cd9, Trem2, Gpnmb,* and *Spp1* (80) (**Figure 4**), markers of the LAMs identified by Jaitin et al. (61). As lean controls from the same facility were not included in this study it will be interesting to examine if this cluster of major macs are indeed restricted to obese conditions. Similarly, it will be interesting to determine the longevity of this cluster and expression of these genes in more long-term caloric restriction studies as some macs with this LAM phenotype were also found in this study after 2 weeks of caloric restriction (**Figure 4**) (80).

The presence of phagocytic macs in obese and caloric restricted mice but not lean mice is also interesting (80). A comparison between these and the macrophage subsets identified by Silva and colleagues (62), found that these most closely resembled the DP macs (80), a population of cells difficult to accurately identify in the dataset from Jaitin et al. (61). The two markers proposed to identify the phagocytic macs based on mRNA expression but also at the protein level were *Pecam1* (encoding CD31) and *Fcgr4* (80). While *Pecam1* expression was low in both datasets (**Figure 4**), *Fcgr4* was indeed enriched in the phagocytic macs from the Weinstock study and was also expressed by the Mac2 cluster in the study from Jaitin et al. (**Figure 4**). As this cluster is only found in obese adipose tissue fitting with the description from Weinstock et al. perhaps these represent the phagocytic macrophages however, this Mac2 subset does not express *Itgax* (encoding CD11c) suggesting they are likely not the DP macs as described above (at least based on mRNA expression), and thus further comparison of these datasets is warranted. As the phagocytic macs are expanded in caloric restriction, it is also possible that this subset does not exist in the dataset from Jaitin et al., but then the suggestion that the phagocytic macs could be the DP macs should be further

examined (61, 62, 80). Notably, some monocytes in the study by Jaitin et al. also expressed *Fcgr4* further questioning if the DP macs in lean adipose tissue represent a distinct subset or rather a developmental intermediate (**Figure 4**).

While there is still much to learn regarding the macrophage clusters in obese adipose tissue, there is clearly heterogeneity within adipose tissue macs (**Figure 5**) and a consensus for identifying these populations has not yet been reached. Nevertheless, the LAMs represent a very interesting population. Firstly, they do not appear to be restricted to adipose tissue, as the LAM profile was found to be highly similar to disease-associated microglia (DAMs) found in the brain in Alzheimer's disease (82) suggesting this may be a general of phenotype of macrophages that have to process significant levels of ectopic lipid. Indeed the transcriptional profiles of NAMs (27) and SAMs (28) identified in the NASH or fibrotic liver also bear some resemblance to the LAMs, although exactly how similar these are remains to be studied. Moreover, a population of macrophages in CLSs with a similar transcriptional profile to LAMs including *Spp1*, *Fabp5, Lpl, Lipa,* and *Cd36* expression was identified during *de novo* lipogenesis induced by β3-adrenergic receptor activation using scRNAseq (77). *De novo* lipogenesis in WAT requires macrophages to clear dead/dying white adipocytes suggesting that this profile could be driven by uptake of adipocytes and dying cells and a need for lipid metabolism. Interestingly, this LAM profile is distinct from Res-KCs despite their expression of genes involved in lipid metabolism (21, 23) and LDAMs, which were identified on the basis of their lipid content and unique transcriptional profiles (26), thus perhaps the signature is governed by more than the presence of lipid. It will be intriguing to understand which other diseases induce such a profile in the recruited macs across tissues. In addition, functionally the LAMs appear to be an interesting population. Given their expression of *Trem2* within the adipose tissue, the role of *Trem2* in the LAMs was also investigated using *Trem2*-deficient mice (61). Notably, LAMs were not identified in the adipose tissue of *Trem2*-deficient mice fed the HFD, with ATMs retaining the signatures of the macs found in clusters 1 and 2, suggesting that TREM2 signaling is specifically required for the LAM profile (61). Notably, the $Trem2^{-/-}$ ATMs also had decreased lipid content and the formation of CLSs was abrogated in the absence of *Trem2*-expressing ATMs (61). This correlated with adipocyte hypertrophy and increased weight gain/body fat accumulation and increased signs of metabolic syndrome including glucose intolerance, elevated serum insulin levels and hypercholesterolemia (61). Conversely, deletion of Netrin-1 in macrophages (driven by LysM-Cre) resulted in a partial protection from diet-induced obesity and improved insulin sensitivity coupled with an increase in adipocyte beiging markers (79). This improved outcome correlated with alterations in the macrophage populations including an increase in the proportion of major macrophages compared with the other mac populations (although the number of macrophages in total was reduced compared with WT mice fed the HFD) (79). While an increase in genes associated with lipid handling in the macrophage pool and a decrease in pro-inflammatory lipid mediators were attributed to the improved outcome on the diet (79), as the major mac population appears to include the LAM population (80) and macs of this phenotype have been implicated in *de novo* lipogenesis (77), it is tempting to speculate that this population maybe also plays a role in the improved response to the HFD in the myeloid-specific Netrin-1-deficient mice (79). While this remains to be investigated, it is plausible that, contrary to the original idea that these recruited macs would be detrimental for disease, LAMs may be required to help restrict disease progression. Given that these results were obtained in either full body KOs or chimeras (*Trem2*) or in all myeloid cells (*Netrin-1*) and as macs with similar profiles have been identified in other tissues, it will now be important to examine the role of TREM2 and the expression and function of NETRIN-1 in the distinct macrophage populations in adipose tissue and liver to determine exactly which macrophage populations are important for these differences in disease pathology.

CONCLUDING REMARKS

Taken together, it is clear that there is significant heterogeneity within the macrophage pool in both obese adipose tissue and the fatty liver. This heterogeneity is made up of different subsets (Res-macs, infRes-macs and Temp-macs) but also likely include developmental intermediates and/or different activation states of the same cell subset. While single-cell technologies have helped us to understand this heterogeneity, questions regarding the specific functions of these different subsets and/or the precise definition of distinct subsets remain which will be important to understand in the coming years. Studies using full body KOs and chimeras are helping to shed light on the functions of the different populations, but further work is still required using cell-type specific KOs before we can truly assess the roles of one population over another so that we can better understand which populations we may need to target with new therapeutic approaches.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00259/full#supplementary-material

Table S1 | Summary of scRNA-seq studies.

Table S2 | Genes associated with M1/M2 modules and Res-macs.

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REVIEW
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Obesity-Induced Changes in Bone Marrow Homeostasis

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Obesity is characterized by low-grade inflammation, which is accompanied by increased accumulation of immune cells in peripheral tissues including adipose tissue (AT), skeletal muscle, liver and pancreas, thereby impairing their primary metabolic functions in the regulation of glucose homeostasis. Obesity has also shown to have a detrimental effect on bone homeostasis by altering bone marrow and hematopoietic stem cell differentiation and thus impairing bone integrity and immune cell properties. The origin of immune cells arises in the bone marrow, which has been shown to be affected with the obesogenic condition via increased cellularity and shifting differentiation and function of hematopoietic and bone marrow mesenchymal stem cells in favor of myeloid progenitors and increased bone marrow adiposity. These obesity-induced changes in the bone marrow microenvironment lead to dramatic bone marrow remodeling and compromising immune cell functions, which in turn affect systemic inflammatory conditions and regulation of whole-body metabolism. However, there is limited information on the inflammatory secretory factors creating the bone marrow microenvironment and how these factors changed during metabolic complications. This review summarizes recent findings on inflammatory and cellular changes in the bone marrow in relation to obesity and further discuss whether dietary intervention or physical activity may have beneficial effects on the bone marrow microenvironment and whole-body metabolism.

Keywords: bone marrow microenvironment, bone marrow mesenchymal stem cells, hematopoietic stem cells, immune responses, obesity, life-style interventions

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INTRODUCTION

Bone marrow (BM) is a soft tissue localized inside of the bones and represents ∼5% of total body mass in healthy individuals (1). BM is primary recognized as a hematopoietic organ supporting the production of new blood cells (2). However, it has also a mechanical and immune function as it comprises bone marrow mesenchymal stem cells (BMSCs), important building blocks for bone formation, and hematopoietic stem cells (HSCs) responsible for producing several types of immune cells crucial for immune responses (3, 4). While BMSCs promote bone tissue regeneration by osteoblast differentiation and neo-vascularization thereby supporting growth of a new tissue, HSCs are quiescent cells (5, 6). However, in response to external cues HSCs can mobilize to the site of inflammation, A majority of HSCs reside in BM and 0.01% of them can migrate into circulation (7). Circulating HSCs in peripheral blood are attracted by several biochemical factors and cytokines including SCF, CXCL12, or IL-8 (8, 9).

The initiation of hematopoiesis starts in the fetal liver, where HSCs proliferate and then migrate to BM. Later during adulthood HSCs continuously migrate from BM to peripheral blood, which maintains steady hematopoiesis (10). In the process of HSC migration from BM, stem cells leave proliferative niches and migrate to more oxygenated and vascularized regions in BM (11). In cases of stress, injury or pharmacological intervention, alterations in HSC niche formation and interaction with BMSCs lead to HSC mobilization and egress. These processes are affected by the metabolic status of an organism, which is altered by caloric restriction, obesity and type 2 diabetes (12). However, it is not well-documented how the composition of BM, interaction between HSCs and BMSCs, and the inflammatory status in this organ are affected in metabolic complications.

Thus, the purpose of this review is to give an overview of the latest literature on inflammatory changes in the BM microenvironment in relation to bone homeostasis. Also, we will discuss how BM composition and secretory function change in different metabolic states and whether dietary intervention or physical activity may have beneficial effects on the BM microenvironment and whole-body metabolism.

BONE MARROW AS AN IMMUNE AND ENDOCRINE ORGAN

BM is a heterogeneous immune organ, which consists of various cell types with different immune functions, including HSCs (myeloid and lymphoid precursors), which are important for immune cell production and BMSCs with immunosuppressive properties (3, 4, 13). It has been reported that 8–20% of BM mononuclear cells belong to lymphocyte lineage (T cells, B cells, Tregs) (14, 15) and approximately 1% represent plasma cells contributing to antibody production (16). Also located in the BM are natural killer T cells (NKT) (cca 0.4–4%) (17), dendritic cells (1–2%) (18), myeloid progenitor cells (giving rise to osteoclasts), megakaryocytes important for platelets (thrombocytes) production via thrombopoietin (1%) (19), neutrophils (8–15%), eosinophils (0.5–2%), and basophils (0.01–2%) (**Figure 1**). Importantly, BM represents a major reservoir of neutrophils and provides migration of these cells into circulation as a first host defense in response to infection and stress (20). Neutrophils are also cleared in BM; once they are senescent, they are phagocytosed by stromal BM macrophages (21). Thus, BM is a home of immune and progenitor cells, whose composition can be changed with age, metabolic status, or inflammatory condition.

Further, BM is well-vascularized with blood vessels and sinusoids, which create a barrier between BM and peripheral circulation (22, 23). This microvasculature allows a release of proliferating progenitor cells and secreted molecules from BM into blood stream in order to reach peripheral tissues depends on the stimulatory signals or physiological condition, which modulate a local microenvironment of the target tissue (24).

Early in life, many bones contain red BM with a high hematopoietic activity, which decrease and turn red BM into yellow "fatty" BM with aging (25). In adults, there are few bones with red BM (e.g., sternum, vertebrae, ribs, or pelvic bone) contributing to hematopoiesis (26). Thus, the bone homeostasis at different body sites is affected by BM composition of HSCs and BMSCs, which contribute to bone integrity, and mechanical and immune properties (4, 27). The crosstalk between these cells activate several processes, including proliferation, migration, and differentiation of stem cells, which are accompanied with production of various bioactive molecules creating the BM microenvironment (3, 4, 25). The maintenance of this microenvironment is important for healthy cell development, immune system function and metabolism.

INTRINSIC REGULATORS OF HEMATOPOIETIC STEM CELL AND BONE MARROW MESENCHYMAL STEM CELL DIFFERENTIATION

HSCs and BMSCs represent multipotent stem cells, which can differentiate into different cell types based on the regulation via intrinsic (e.g., transcription factors and cofactors, posttranscriptional and posttranslational modifications) and extrinsic factors (e.g., secretory molecules, BM microenvironment, metabolic cues) (28). HSC differentiation is coordinated by transcription factors such as c-Myc, PU.1/Spi-1, GATA1-3, TNFβ, EGR1, BMI1, Gfi1, FoxO3, and others (29). c-Myc, for example, regulates the balance between HSC

Abbreviations: AT, Adipose tissue; BATF, Basic leucine zipper transcription factor, ATF-like; BM, Bone marrow; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; BMP, Bone morphogenic protein; BMSC, Bone marrow mesenchymal stem cell; C/EBP, CAAT enhancer binding protein; CD25 (CD80, CD86, CD40, CD69), Cluster of differentiation 25, 80, 86, 40, 69; Cdc42, Cell division control protein 42 homolog; CTSK, Cathepsin K; CTHRC1, Collagen triple helix repeat containing 1; CXCL12, C-X-C motif chemokine 12; Del-1, Developmental endothelial locus 1; DNMT1, DNA methyltransferase 1; FO, Fish oil; GATA 1-3- GATA-binding protein 1-3; G-CSF, Granulocyte–colony stimulating factor; GFI1- Zinc-finger protein GFI1; H4K15ac, Acetylation on lysin 16 of histone 4; HGF, Hepatocyte growth factor; HSC, Hematopoietic stem cell; IDO, Indoleamine 2,3-dioxygenase; IFNα, Interferon alpha; IFNβ, Interferon beta; IFNγ, Interferon gama; IGF-1, Insulin-like growth factor 1; IL-1 (IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-15, IL-21), Interleukin 1-21; LCN2, Lipocalin 2; LepR, Leptin receptor; LFA-1, Lymphocyte function-associated antigen 1; LIF, Leukemia inhibitory factor; MCP1, Monocyte chemoattractant protein 1; MIF, Macrophage stimulating factor; M-CSF, Macrophage colony-stimulating factor; NKT, Natural killer cell; OPG, Osteoprotegerin; OPN, Osteopontin; PGE_2, Prostaglandin E_2; PPARγ, Peroxisome proliferator-activated receptor gama; PUFA– Poly unsaturated fatty acid; RANKL, Receptor activator of nuclear factor-κβ ligand; RUNX2, Runt-related transcriptional factor 2; S100A8/A9, Neutrophil-derived S100 calcium-binding proteins A8/A9; SCF, Stem cell factor; SDF-1, Stromal cell-derived factor 1; sFRP1, secreted Frizzled-related protein 1; TGF-β_1, Transforming growth factor beta 1; TLR4, Toll-like receptor 4; TN-C, Tenascine-C; TNFα, Tumor necrosis factor alpha; TNFβ, Tumor necrosis factor beta; TRAP, Tartrate-resistant acid phosphatase; Treg, Regulatory T cell; VLA-4 (VLA-5, VLA-7), Very late antigen 4, 5, 7; WBV, Whole body vibration; Zfp432, Zinc finger protein 432; Zfp521- Zinc finger protein 521.

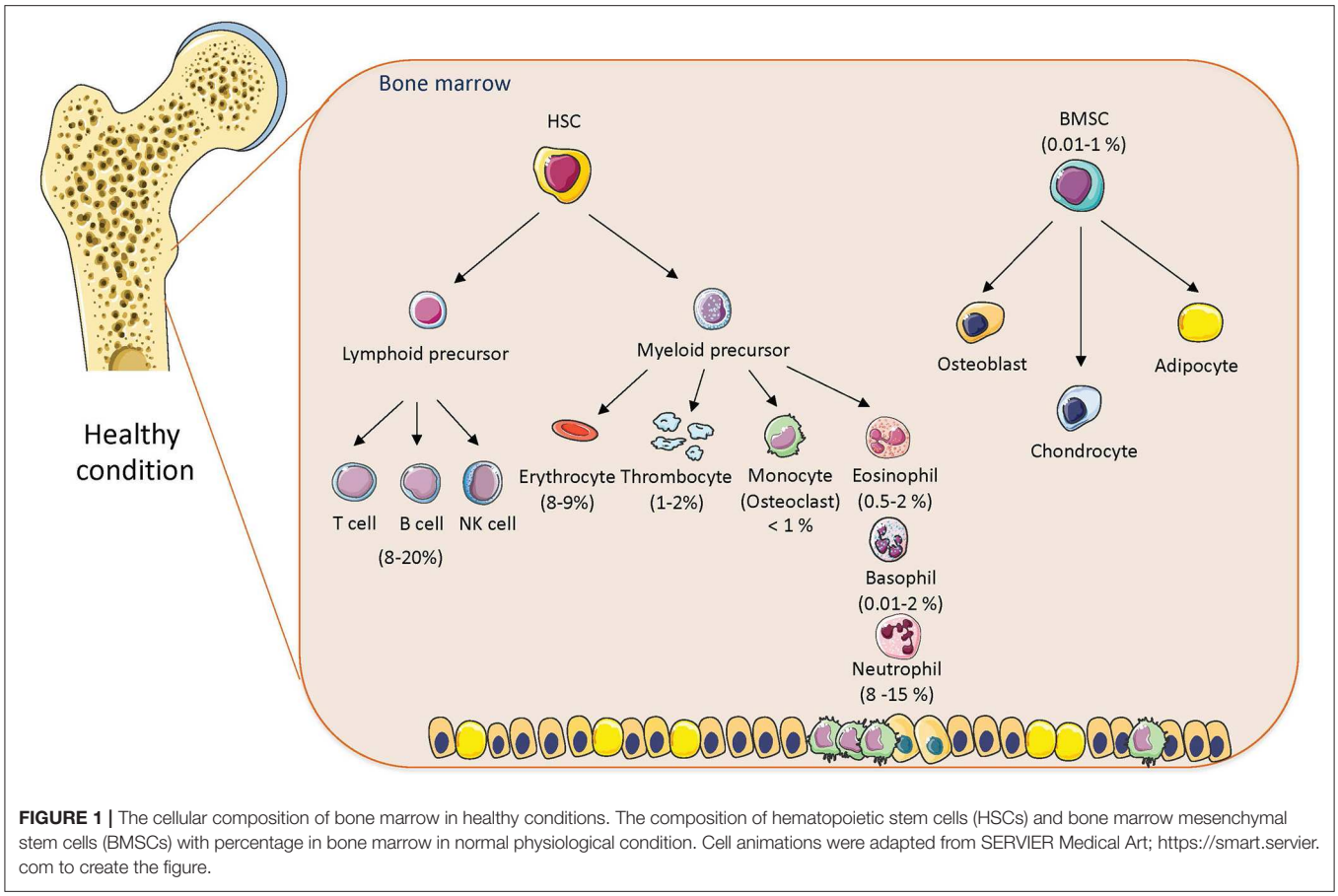

FIGURE 1 | The cellular composition of bone marrow in healthy conditions. The composition of hematopoietic stem cells (HSCs) and bone marrow mesenchymal stem cells (BMSCs) with percentage in bone marrow in normal physiological condition. Cell animations were adapted from SERVIER Medical Art; https://smart.servier.com to create the figure.

self-renewal and differentiation (30). PU.1/Spi-1 is involved in myeloid lineage determination via regulation of target genes, including granulocyte colony-stimulating factor receptor (31), granulocyte-macrophage colony-stimulating factor receptor (32) and macrophage colony-stimulating factor receptor (33). Some studies showed that PU.1/Spi-1 expression can direct stem cell differentiation to myeloid lineage if Notch signaling is reduced [reviewed in Rothenberg et al. (34)]. Another regulatory molecule of HSC differentiation is Ikaros, which displays a crucial function as a transcription activator promoting lymphocyte differentiation. Impairment of this protein leads to hypoplasia, absence of secondary lymphoid organs or absence of B- and T- cell precursors (35). Basic leucine zipper transcription factor, ATF-like (BATF) is an important factor promoting lymphoid lineage differentiation (36), while TNFβ serves as a negative regulator of HSC self-renewal (37). Further, the HSC cell fate determination is regulated by GATA1-3, zinc finger transcription factors, which coordinate development of diverse hematopoietic lineages (38), and B lymphoma Mo-MLV insertion region 1 homolog (BMI1), which is important for the multilineage potential of HSCs and their replating capacity (39, 40). Lee et al. recently identified a role of a transcriptional repressor, known as Gfi1 in the regulation of HSC quiescence and self-renewal, which is modulated by metabolic status (i.e., upregulated with obesity and decreased with weight loss) (41). Besides transcriptional regulation, HSC renewal and differentiation are under control of posttranslational modifications, including DNA methylation, acetylation, or ubiquitination, which can be modulated by aging or metabolic diseases (42). Recent findings documented that increases in H4K16Ac levels results in inhibition of Cdc42, which leads to restoration of the B cell lineage output in aged HSCs (43). Further, G9a/GLP methyltransferase is responsible for increased levels of H3K9me2 pattern associated with HSC lineage commitment. On the other hand, inhibition of G9a/GLP decrease differential potential of stem cells and improves HSC maintenance (44). Additionally, methylation by DNA methyltransferase 1 (DNMT1) permits efficient hematopoietic differentiation (45). All above-mentioned transcription factors and posttranslational modifications are only part of the HSC regulatory network, which shows together the complexity of stem cell differentiation process.

Differentiation of BMSCs toward osteoblasts and adipocytes is regulated by specific transcription factors: Runt-related transcription factor 2 (Runx2) (46), osterix (47), GATA2 (48, 49) (responsible for osteoblast lineage determination), and

TABLE 1 | Transcription factors and determinants of HSC and BMSC differentiation.

Intrinsic regulators	Cell type	Function	Obesity	Life-style interventions
BATF	HSC	Transcription factor regulating lymphoid differentiation (36)	–	–
BMI1	HSC	Transcription factor regulating multilineage potential of HSCs (39, 40)	–	–
TNFβ	HSC	Negative regulator of HSC self-renewal (37)	–	–
c-Myc	HSC	Transcription factor regulating balance between HSC self-renewal and differentiation (15545632)	–	–
Pu.1/Spi-1	HSC	Transcription factor regulating myeloid lineage differentiation (31)	↑ (54, 55)	–
Ikaros	HSC	Transcription activator of lymphoid differentiation (35)	–	–
Notch	HSC	Signaling molecule enhancing self-renewal and regenerative capacity of HSCs (56)	–	–
GATA1-3	HSC	Transcription factors regulating HSC lineage determination (38)	↑ GATA 3 (57)	–
Gfi1	HSC	Transcription factor regulating HSC quiescence and self-renewal (58)	↑ (59)	↓ Weight loss (59)
DNMT1	BMSC	DNA methyltransferase promoting HSC differentiation to myeloid lineage (45)	–	–
Runx2	BMSC	Transcription factor promoting osteoblast differentiation (46)	= (60)	↑ Vibration (61)
Osterix	BMSC	Transcription factor promoting osteoblast differentiation (47)	↓ (62)	↓ Low magnitude high frequency vibration (63)
PPARγ	BMSC	Transcription factor regulating adipogenesis (50)	↑ (60)	↑ Low magnitude height frequency vibration (64)
GATA2	BMSC	Transcription factor regulating adipogenesis and osteogenesis (48, 49)	–	–
C/EBP	BMSC	Transcription factor regulating adipogenesis (4)	↑ (65)	↑ Low magnitude height frequency vibration (64)
TGF-β_1	BMSC	Negative regulator of adipogenesis (4)	↓ (66)	–
BMP-2	BMSC	Positive regulator of osteoblast differentiation (51)	↓ (67)	= Calorie restriction (68)
Zfp521	BMSC	Regulator supporting osteoblast differentiation (53)	–	–

peroxisome proliferated-activated receptor gamma (PPARγ) (50), CAAT enhancer binding protein (C/EBP) family (4) (responsible for adipocyte lineage determination). The activation of these transcription factors can be controlled by Wnt signaling, transforming growth factor β_1 (TGF-β_1) and bone morphogenic proteins (BMPs) [reviewed in Tencerova and Kassem (4)]. The regulation of BMSC differentiation is also accompanied by epigenetic modifications. For example, histone deacetylation in genes involved in transcriptional regulation, cellular survival, growth and proliferation of BMSCs. Increased acetylation during osteoblast differentiation results in increased expression of Runx2, BMP-2, osterix and osteopontin (OPN), which are important for osteoblast maturation (51, 52). A recent study by Addison et al. identified Zfp521 as a key regulator of lineage specification in progenitor cells, regulating BMP-induced MSC differentiation coupled with histone modification at Zfp423 promoter (53).

These data demonstrate that HSC and BMSC differentiation are complex processes under the control of specific transcription factors, whose activity is further epigenetically modulated. These intrinsic factors contribute to the regulation of the BM homeostasis and are changed by obesity and dietary interventions. **Table 1** summarizes key factors and determinants regulating HSC and BMSC differentiation and associated signaling pathways.

SECRETORY FACTORS OF BONE MARROW MESENCHYMAL STEM CELLS AFFECTING BONE HOMEOSTASIS AND IMMUNE CELL PROPERTIES

BMSCs represent around 0.01–0.1% of total BM cells in adults and are capable of differentiating into different cell types such as osteoblasts (bone formation), adipocytes (adipose tissue formation) or chondrocytes (cartilage formation), all of which are important for maintaining of bone homeostasis (4, 13). BMSCs are also known for their immunosuppressive properties as they express human leucocyte antigen (HLA) class I and costimulatory molecules CD80, CD86, or CD40 important for regulation of T cell proliferation and activation (69, 70). Recent findings suggest that BMSCs mediate their immunoregulatory function via cell-cell interactions and secretion of soluble molecules (70, 71). The BMSC secretory profile of prostaglandin E_2 (PGE_2), hepatocyte growth factor (HGF), transforming growth factor (TGF)-β_1 and indoleamine 2,3-dioxygenase (IDO) show profound immunosuppressive properties inhibiting T cell activation and proliferation without affecting expression of early activation markers such as CD25 and CD69 (72). Indeed, BMSCs can modulate function of several immune cells without being recognized by immune cells. BMSCs inhibit proliferation and antibody production of B cells (73), differentiation of

TABLE 2 | Secretory factors of BMSCs contributing to BM microenvironment and bone homeostasis.

Secretory factors	Function	Obesity	Life-style interventions
PGE_2	Anti-inflammatory, inhibition of T cell proliferation (72)	↓ (59)	Omega 3 dietary intervention ↓ (82)
HGF	Anti-inflammatory, inhibition of T cell proliferation (72)	–	–
TGFβ	Anti-inflammatory, inhibition of T cell proliferation (72)	↓ (66)	–
IL-7	B cell development (83)	↓ (84)	–
IL-15	T cell homeostasis (85)	↓ (86)	–
IL-21	NKT cells maturation (87)	–	–
TNFα	HSC proliferation and activation (88)	↓ (60), ↑ (89)	–
CXCL12/SDF-1	Stem cell migration (90, 91)	↓ (92)	↑ Exercise, caloric restriction (68, 93)
Thrombopoietin	HSC quiescence (94, 95)	↑ (96)	↓ thrombopoiesis (97)
Angiopoietin	HSC quiescence (98)	↑ (99)	–
M-CSF	Myelopoiesis (100)	= (101)	–
G-CSF	Myelopoiesis (100)	↑-(101, 102)	↑ Sleeve gastrectomy (100)
RANKL	Osteoclast differentiation (103)	↑ (104)	= Exercise (105)
OPG	Osteoclast differentiation (103)	↓ (104)	= Exercise (105)
LCN2	HSC proliferation, inhibition of senescence (106)	↓ (60)	–

HSC progenitors into dendritic cells (74), and they promote anti-inflammatory cytokine production of myeloid cells while inhibiting the cytotoxic activity of NKT cells (75–77). Although there is limited information on the exact mechanism of BMSC immunoregulation in relation to immune cell interaction, they represent an important tool in stem cell therapy. BMSCs have been used in several clinical trials for tissue regeneration and healing (78–81).

The secretory profile of BMSCs may differ depend on developmental, immune or metabolic challenges they are exposed to. A brief overview of BMSC secretory factors is listed in **Table 2**, describing their functions and changes in metabolic complications and life-style interventions. BMSCs secrete IL-7, which is important for early B cell development (83, 84), IL-15 for T cell homeostasis (85) and IL-21 for maturation of NKT cells (87). Further, expression of CXCL12/SDF-1 mediates the interaction of BMSCs with BM endothelial cells in order to contribute to the maturation of megakaryocytes and thrombopoiesis. SDF-1 also initiates trans-endothelial migration of BMSCs in homing process via activation of integrins (LFA-1, VLA-4, and VLA-5) (90, 91). Moreover, BMSC secretory products, including leukemia inhibitory factor (LIF), macrophage stimulating factor (MIF), granulocyte-colony stimulating factor (G-CSF), OPN, IL-6, tumor necrosis alpha (TNFα) affect immune cell behavior (102, 107, 108). Costa et al. showed that osteoblast-derived lipocalin 2 (LCN2), with its anti-senescent function, regulates HSC progenitors and their proliferation capacity (106). Functional studies indicate that thrombopoietin and angiopoietin secreted by osteoblasts promote HSC quiescence (94, 98, 109), while CXCL12 regulates HSC migration in BM (110, 111). In addition, osteoblasts may regulate the activity of osteoclasts (derived from myeloid precursors in BM) in order to attract them to the site of resorption, thereby maintaining bone homeostasis (112). Osteoblasts produce receptor activator of nuclear factor-κβ ligand (RANKL) and osteoprotegerin (OPG), two critical factors in osteoclast differentiation and activation (103). Osteocytes (mature osteoblasts) modulate myelopoiesis via activation of Gsα-dependent signaling, which regulates secretion of G-CSF (100, 113). These data point out the importance of maintaining BM homeostasis, which is based on the molecular interactions among different cell types present in BM. And changes in local BM microenvironment induced by metabolic status of organism may shift this balance in favor of action of specific progenitors, which disrupt the priming of immune cell progenitors arising in BM in their function when they reach circulation.

SECRETORY FACTORS OF HEMATOPOIETIC STEM CELLS AFFECTING BONE HOMEOSTASIS AND IMMUNE CELL PROPERTIES

Multipotent HSCs represent another cellular component of BM, which are recognized as the ancestors of blood cells (114–116). Traditionally, HSCs differentiate into myeloid lineage (e.g., erythrocytes, granulocytes, macrophages, monocytes, and platelets) or lymphoid lineage [e.g., B lymphocytes, T lymphocytes, and natural killer (NK) cells] (117). While myeloid cells mature in the BM, human lymphoid cells must

TABLE 3 | Secretory factors of HSCs contributing to BM microenvironment and bone homeostasis.

Secretory factors	Function	Obesity	Life-style interventions
IL-1β	HSC activation (128)	↓ (60)	↓ Dietary restriction-reduced intake of amino acids (129)
MCP1	HSC activation (128)	↓ (60)	–
TNFα	HSC activation (128, 130)	= (60)	–
Wnt10b	Bone formation (131)	↓ (132)	–
CXCL16	Osteoblast migration (133)	–	–
TRAP	Osteoclast activation (134)	= (60) ↑(135)	↓ Caloric restriction (136)
LIF	Osteoblast migration (133)	–	–
CTSK	Collagen degradation (137)	–	–
CTHRC1	Bone formation (138)	–	–
Del-1	HSC proliferation and differentiation (139)	–	–
TN-C	Bone remodeling and bone renewal (140)	↑ (141)	–

migrate to other lymphoid organs (e.g., thymus) in order to complete their maturation. In most of the experimental models, multipotency of HSCs is coupled with self-renewal abilities (118). HSCs together with endothelial cells (119), $LepR^+$ stromal cells (120), megakaryocytes (121), sympathetic nerves, non-myelinating Schwan cells (122) and secreted bioactive molecules (123–126) create a dynamic BM microenvironment (127). **Table 3** summarizes HSC secretory factors contributing to BM homeostasis along with their functions and changes in metabolic complications and life-style interventions. The BM microenvironment mediates signals for HSCs to differentiate into particular cell type in response to infection or blood cell destruction (142, 143). Interactions between HSCs and BMSCs are tightly interlinked by secreted signals and regulatory factors affecting the quiescence, self-renewal or mobilization of stem cells. HSCs are capable of receiving and producing signals that directly dialogue with the immune system. A recent study by Mitroulis et al. identified developmental endothelial locus-1 (Del-1) glycoprotein secreted by several components of HSC niche such as endothelial cells, reticular cells as a regulator of long-term HSC proliferation and differentiation toward the myeloid lineage (139). Another protein expressed in extracellular matrix of BM is tenascine-C (TN-C), important for active bone remodeling and HSC renewal in the endosteal region in conditions of hematopoietic stress (140, 144, 145).

In the context of inflammation, HSCs are recognized as primary responders to infection, and the secretion of pro-inflammatory cytokines during infection is important for HSC regulation. This cascade of pro-inflammatory cytokines and signaling molecules includes IL-1, IL-2, IL-8, TLR4 (146), TNFα (147), IFNα, β, and γ (148) to activate T cells, NKT, and IL-4 and IL-6 to activate B cells [reviewed in King and Goodell (149)]. These cytokines are required for the maintenance of the appropriate proliferation and differentiation of HSCs in the steady-state and stress-induced condition.

Another cell type derived from the myeloid lineage are osteoclasts ("bone macrophages"), which are key players in process of bone resorption. Osteoclasts are specialized multinucleated cells derived from monocyte fusion containing from 2 to 12 nuclei per cell (150). The process of osteoclast differentiation is regulated via main activators of osteoclast formation, RANKL and M-CSF. In addition, RANKL promotes osteoclast resorption activity (151). In healthy conditions, osteoclasts play an important role in replacing of old or damaged bone matrix (bone resorption), which is followed by osteoblasts forming a new mineralized bone matrix (bone formation). This renewal process of bone matrix is also known as bone remodeling, which is energetically demanding (152). During this process, osteoclasts communicate with osteoblasts through cytokines such as TGF-β and IGF-1, which promote migration of BMSCs to newly resorbed tissue (153, 154). TGF-β can also induce expression of CXCL16, LIF, and Wnt10b by osteoclasts, which induce mineralization and recruitment of osteoclasts to osteoblasts (131, 133). Activated osteoclasts further produce secreted factors supporting their resorption activity, including cathepsin K (CTSK), sphingosine-1-phospate (137), tartrate-resistant acid phosphatase (TRAP) (134). CTSK is cysteine protease secreted by osteoclasts with an essential function in degradation of matrix collagen and activation of TRAP (155, 156). Mutation in CTSK leads to pycnodysostosis, rare autosomal recessive skeletal dysplasia, during which osteoclasts function is defected. Animal models with this deficiency showed reduced bone resorption, which together with normal or increased bone formation led to osteopetrotic phenotype (137). TRAP is a phosphatase expressed by osteoclasts and macrophages participating in skeletal development, collagen synthesis, and degradation or mineralization of bone matrix (134). Another molecule secreted by osteoclasts is collagen triple helix repeat containing 1 (CTHRC1), which serves as a positive regulator of osteoblastic bone formation (138, 157). These data provide further evidence that HSCs are capable of producing several inflammatory molecules, which contribute to creation of the BM microenvironment. Importantly, HSC differentiation in process of building active immune cells is under control of several bioactive molecules and signaling pathways, which need to be tightly regulated in response to metabolic or inflammatory stressors.

OBESITY-INDUCED CHANGES IN BONE MARROW

Obesity is characterized by low-grade inflammation, challenging the immune cell responses in peripheral tissues. Further, the obesogenic condition increases BM cellularity 20–30% (101), changes BM composition of HSC and BMSC subpopulations and affects their differentiation capacity and increases white and red blood cell counts (**Table 4**) (96, 158, 164, 165, 172). Conditions associated with metabolic dysregulations, including hyperglycemia and hypercholesterolemia, have been linked to hematopoietic disruption and particularly to myeloid skewing (84, 165, 183).

TABLE 4 | The changes in cellular composition of hematopoietic stem cells and bone marrow mesenchymal stem cells in bone marrow in obesity, exercise and dietary interventions.

Cell type	Obesity	Exercise	Dietary intervention
Erythrocytes	↑ (158)	↓ (159)	↑ (160) ↓ (161)
Lymphocytes	↓ (162)	↑ (162, 163)	↑ (160)
Monocytes (Osteoclasts)	↑ (164–166)	↓ (167)	↓ (167)
Eosinophils	↓ (168, 169)	–	↑ (169)
Basophils	↑ (170)	↑ (171)	–
Neutrophils	↑ (164, 165, 172)	↓ (173, 174)	↓ (174)
Thrombocytes	↑ (96)	–	↓ (97)
Chondrocytes	↓ (175, 176)	↑ (177)	↑ (178)
Osteoblasts	↓(60)	↑ (162, 163)	↑ (179, 180)
Bone marrow adipocytes	↑(60)	↓ (162, 163)	↓ (181, 182)

Hyperglycemia drives myelopoiesis and activation of neutrophils in the BM of obese mice (164, 165). Moreover, HFD-induced changes in bone architecture and immune cell homeostasis showed bone loss and a shift of HSC differentiation in myeloid over lymphoid progenitors (60, 162, 184). Further, morbid obesity elevated neutrophils in circulation and primed their immune function and metabolic activity, suggesting a higher inflammatory response in obesity-related diseases associated with impaired whole-body glucose metabolism (172). Another study by Kraakman et al. demonstrated that an obesogenic condition coupled with high glucose levels promotes increased thrombopoiesis via interaction of neutrophil-derived S100 calcium-binding proteins A8/A9 (S100A8/A9) and thrombopoietin in hepatocytes, which in turn leads to megakaryocyte activation and thrombocyte maturation in BM (96). Also, eosinophils with their anti-inflammatory activity have been shown to be affected by obesity, evidenced by decreased accumulation in AT and enhanced trafficking from BM to lung during allergic asthma (168, 185). Obesity-induced changes have been attributed also to basophils, which participate in lung inflammation and allergic reaction associated with metabolic complications (170).

It has been shown that differentiation capacity of BMSCs is changed by obesity in favor of increased adipocyte differentiation and impaired osteoblast and chondrocyte differentiation, which contributes to impairment of bone homeostasis and production of secretory factors affecting the function of neighboring cells in BM (60, 175, 176, 186). Liu et al. (54) recently reported an impairment of BMSC mobilization and selective migration of specific immune cells from BM into circulation in obesity. Further, Ferraro et al. showed a negative effect of diabetes on HSC mobilization capacity by changing the BM microenvironment (92). Not only proportion of immune cells in BM, but also secretion of inflammatory cytokines is modified by obesity (see some examples in **Table 2**). For instance IL-15 with its anti-obesity effect, TGF-β and IL-7 with their immunosuppressive properties are decreased with obesity in BM (66, 84, 86).

Previous studies in rodents under HFD condition have demonstrated increased pro-inflammatory BM microenvironment (e.g., TNFα, IL-6, and IL-1β) measured in BM or bone lysates (89, 104, 187). Our recent publications have reported that obesity does not induce increased inflammatory responses in BMSCs and HSCs of HFD mice or obese individuals compared to lean, which is accompanied with no change or decrease in osteoclast resorption activity (60, 188). This finding was also found in the study by Trotter et al., showing no changes in the mRNA levels of inflammatory markers in BM of HFD mice compared to lean (101). Further, obesity was identified as a negative factor of bone homeostasis in relation to osteoclast formation (104, 166, 189). Halade et al., using 12 months old female mice fed with 10% corn oil as a model of age-associated obesity, showed that increased adiposity enhances pro-inflammatory cytokine production (e.g., IL-1β, IL-6, and TNFα) and was associated with a higher differentiation of osteoclasts (104, 190). Another animal study using 5 weeks old male mice found higher rates of osteoclast precursors, as well as elevated osteoclast formation, bone resorption activity and increased expression of RANKL, TNFα, and TRAP (166). In addition, acute exposure to dietary fatty acids increased osteoclastogenic activity in circulating monocytes and increased secretion of cytokines (191). However, this study did not investigate the osteoclast in BM and their resorption activity. In our animal study using a HFD model (60% calories from fat) in 12 weeks old C57BL/6 male mice, we did not observe any significant changes in osteoclast activity or number (60). In clinical study (188) examining obese subjects, we found decreased bone resorption and bone formation activity, suggesting a slowing of bone turnover. The discrepancies between studies may be explained by using different animal models, length/composition of the diet, or different source of bone cells for measurement of inflammatory condition in BM.

In terms of HSC secreted molecules (e.g., CXCL16, CTSK, Del-1, LIF, or CTHRC1), which play an important role in bone homeostasis and metabolism (**Table 3**), there is very limited information about expression changes in the BM during obesity. Thus, these observations suggest that further studies are needed in order to investigate the inflammatory status of BM cells and their microenvironment in obesity in relation to bone and whole-body metabolism.

However, it raises further questions whether obesogenic condition activates immune cells in BM or immune cells need to migrate through the circulation into the target tissue, i.e., adipose tissue (AT), skeletal muscle, liver to activate their inflammatory status. This would suggest that BM is a primary site of immune cell production and plays an important role in immune cell mobilization into circulation, whereby these cells are directed to traffic into the sites of inflammation.

LIFE-STYLE INTERVENTIONS: DIETARY AND PHYSICAL ACTIVITY INTERVENTIONS IMPROVE OBESITY-INDUCED CHANGES IN BONE MARROW HOMEOSTASIS

In obesity and type 2 diabetes, several approaches have been applied to treat or prevent the detrimental effects of metabolic complications. These include physical activity, as well as dietary or pharmacological treatment. As a lot of investigations have been focused on the metabolic and inflammatory improvements in peripheral tissues, there is limited information on these parameters in relation to BM homeostasis (**Tables 1–4**).

Dietary supplementation with long-chain n-3 (ω-3) PUFAs, supplied as fish oil (FO), which is known for its anti-inflammatory effects, demonstrated to be beneficial for skeletal health, as evidenced by increased osteogenesis and decreased osteoclastogenesis (179, 180). A recent study by Cao et al. (192) reported that 6 months of a FO diet increased bone density and microstructure. However, they did not investigate bone adiposity or inflammatory responses in BM in these conditions.

Exercise also showed a positive effect on bone homeostasis. In rodents and humans, exercise has been shown to increase bone density, decrease bone adiposity, and improve chondrogenesis in HFD mice and humans (177, 193, 194). Further, increased physical activity has been shown to promote HSC proliferation and differentiation and modulate immune cell composition in circulation (159, 171, 173, 195–197) (**Table 3**). An additional effect of exercise on the bone is increased mechanical stress for skeletal system induced by whole body vibration (WBV), which has been shown to improve bone density by reducing bone marrow adiposity in mice and humans and restoring lymphopoiesis (increased number of B cells). WBV also showed an effect on immune cells in circulation and induced lower infiltration in AT (162, 163). A key strategy to prevent obesity and its complications, including bone health, is a combination of exercise and a well-balanced diet. A study by Garbiax et al. using 11 months old male rats showed that exercise along with a caloric restricted diet (low fat and low sucrose) decreased bone resorption and osteoclast number in the obese state (167). However, the inflammatory properties of immune cells in BM also have not been investigated following these interventions.

The effects of caloric restriction on bone health is still poorly understood. Generally, caloric restriction, accompanied with weight loss, has a positive effect on systemic glucose tolerance and inflammatory status of immune cells and their count (neutrophils) (169, 174). A recent study by Collins et al. (160) showed that dietary caloric restriction protects BM and optimizes immunological responses of immune cells by enhanced accumulation of memory T lymphocytes in BM, erythropoiesis and bone marrow adiposity. However, caloric restriction or starvation in growing mice leads to increased accumulation of bone marrow fat even though peripheral adipose tissue (AT) mass is decreased. Further, it causes decreased bone density and increased bone resorption (161, 198, 199). Caloric restriction had a similar effect in patients with anorexia nervosa (200). However, in the obese condition, caloric restriction may have a positive effect, as evidenced by reduced bone adiposity and improvement of bone density and chondrogenesis. Although no results on inflammatory components in BM have been measured in this setting (178, 181, 182).

In the context of inflammatory cytokine production, a recent publication showed that reduced intake of amino acids may inhibit secretion of pro-inflammatory mediator IL-1β mediated by myeloid precursors in BM (See also examples in **Table 3**) (129). However, for most of the above-mentioned inflammatory proteins (**Table 3**) (e.g., MCP1, TNFα, LIF, CTSK, CXCL16, CTHRC1, Del-1, or TN-C), there is a lack of information in the literature about the modulation of their secretory activity in dietary interventions in BM, which indicates that this area of research needs to be further investigated. Based on the recent publications in relation to life-style interventions and bone health, it suggests that further studies are needed to dissect the role of inflammatory components in BM homeostasis and how these may contribute to local BM and systemic metabolic regulation.

CONCLUSIONS AND PERSPECTIVES

BM is an important immune organ, whereby immune and progenitor cells with different functions interact with each other and affect local and systemic immune conditions in response to metabolic and inflammatory stressors, including obesity. Obesity leads to a pro-inflammatory state, which influences metabolic function in insulin-responsive tissues including bone and its immune compartment, BM. Further, the obesogenic condition induces BM hyperplasia defined by increased number of immune cells (monocytes, neutrophils, thrombocytes etc.) migrating into the circulation, which are usually primed in higher inflammatory responses to activate inflammation in peripheral tissues.

How can we define an inflammation in BM? Is it a process of bone resorption defined by activation and expansion of osteoclasts in BM or a process accompanied by increased secretion of inflammatory cytokines, which we know from definition of inflammation in peripheral tissues? And how is it affected in metabolic complications? Most studies have reported changes on the level of osteoclast resorption activity, but not much on secretory properties of immune cells in BM niche. Another aspect of inflammatory status in BM is the immunosuppressive properties of BMSCs, which also contribute to immune regulation in BM microenvironment through cell to cell interactions and secretory bioactive molecules to maintain BM homeostasis.

Undoubtedly, metabolic stressors such as obesity interrupt the existing balance among BMSC and HSC functions, which further affect systemic whole-body immune regulation in relation to metabolic status of organism. Many bone cell-secreted molecules have been found to play an important role in the regulation of AT development (e.g., RANKL, CTSK, and CTHRC1) (201–203) (**Figure 2**). Therefore, studying their function in relation to bone and fat metabolism is of interest. However, more studies are

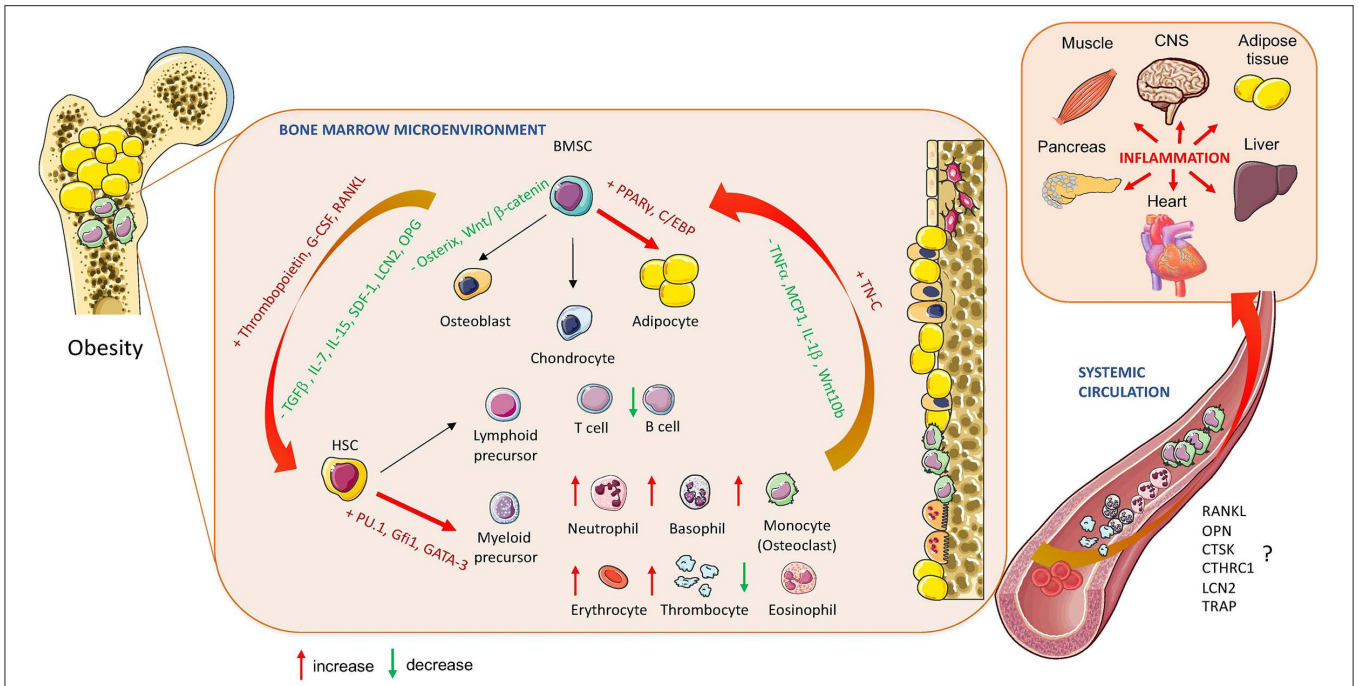

FIGURE 2 | Obesity-induced changes in bone marrow homeostasis. The effect of obesity on BM cellular composition and secretory profile of bioactive molecules produced by hematopoietic stem cells (HSCs) and bone marrow mesenchymal stem cells (BMSCs) in relation to systemic changes affecting whole-body metabolism and inflammation (RANKL, Receptor activator of nuclear factor-κβ ligand; OPN, Osteopontin; CTSK, Cathepsin K; CTHRC1, Collagen triple helix repeat containing 1; LCN2, lipocalin 2; TRAP, Tartrate-resistant acid phosphatase; OPG, Oteoprotegerin; TGF-ß, Transforming growth factor beta; IL-7, Interleukin 7; IL:15, Interleukin 15; IL1-ß, Interleukin 1 beta; SDF-1, Stromal cell derived factor; TNFα, Tumor necrosis factor alpha; MCP1, Monocyte chemoattractant protein 1; TN-C, Tenascine C; PPARγ, peroxisome proliferated-activated receptor gamma; C/EBP, CAAT enhancer binding protein; Gfi1, Zinc finger protein Gfi1; GATA3, GATA binding protein 2). Cell animations were adapted from SERVIER Medical Art; https://smart.servier.com to create the figure.

needed to understand the role of inflammatory changes and crosstalk between immune cells and BMSCs in BM in response to obesity and how these changes can be modulated with targeted therapies focused on treatment for bone and metabolic complications.

AUTHOR CONTRIBUTIONS

AB and MT researched data and wrote the manuscript. MT reviewed and edited the manuscript.

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REVIEW
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The Immune Landscape of Visceral Adipose Tissue During Obesity and Aging

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Obesity and aging represent major health burdens to the global adult population. Both conditions promote the development of associated metabolic diseases such as insulin resistance. The visceral adipose tissue (VAT) is a site that becomes dysfunctional during obesity and aging, and plays a significant role during their pathophysiology. The changes in obese and aging VAT are now recognized to be partly driven by a chronic local inflammatory state, characterized by immune cells that typically adopt an inflammatory phenotype during metabolic disease. Here, we summarize the current knowledge on the immune cell landscape of the VAT during lean, obese, and aged conditions, highlighting their similarities and differences. We also briefly discuss possible linked mechanisms that fuel obesity- and age-associated VAT dysfunction.

Keywords: metabolism, obesity, aging, diabetes, insulin resistance, immunology, immunometabolism, visceral adipose tissue

INTRODUCTION

Obesity and aging represent two of the largest global health issues of our time. Obesity currently affects over a third of the world's population. Alarmingly, 57.8% of the global adult population are estimated to be overweight or obese by 2030 (1–3). Obesity is part of a condition known as Metabolic Syndrome (MetS), which is defined as a cluster of metabolic risk factors such as abdominal obesity, dyslipidemia, hyperglycemia and insulin resistance (IR) (4). MetS is characterized by a state of low-grade inflammation that is implicated in the development of chronic diseases such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) (5–7).

The biological processes of aging and senescence are accelerated during MetS and the risk of developing chronic diseases increases with age (8). In the years to come, aging is expected to represent an enormous challenge for social and health systems. Without adjusting for the rise in longevity, the median age of the world's population is expected to increase from 26.6 to 37.3 years between 2000 and 2050 and to 45.6 years by 2100 (9). This increase in aging will be likely associated with an elevated risk of developing chronic diseases that will aggravate the functional abilities and quality of life of the elderly (10). The changes in the immune system associated with aging are characterized by an imbalance between inflammatory and anti-inflammatory pathways, leading to low-grade inflammation and a greater susceptibility to chronic disease (11).

Obesity and aging contribute to the development of chronic low-grade inflammation in multiple tissues. This low-grade inflammation is recognized as an important factor that promotes downstream consequences of obesity and aging, including the control of whole-body metabolism. During obesity and aging, cells of the innate and adaptive systems accumulate inside the VAT where they alter the local inflammatory environment and impact insulin sensitivity (12). Indeed, the transition from lean to obese or aged states is accompanied by distinct and associated immune cell-driven inflammatory processes. This review will focus on the immune changes that occur in adipose tissue and the means by which immune cells shape the progression of VAT-specific obesity-associated inflammation (OAI) and age-associated inflammation (AAI).

VAT PLASTICITY AND REMODELING

The adipose tissue is considered an endocrine organ that becomes remodeled during metabolic diseases and is involved in the progression of obesity- and aging-associated IR. There are two distinct types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT acts an energy store by accumulating free fatty acids whereas BAT has the capacity of undergoing thermogenesis to dissipate energy (13, 14). WAT can be further divided into two major depots: subcutaneous and visceral. Subcutaneous WAT forms a layer under the skin in the hypodermis while visceral WAT surrounds the inner organs in the abdominal cavity and mediastinum (14, 15). VAT is deposited in certain locations such as the mesenteric fat between the intestines, and the retroperitoneal fat surrounding the kidneys; each VAT store consists of adipocytes and the stromal vascular fraction (SVF) (14). The mesenteric fat pads in mice are the most analogous to human VAT but are not well-studied due to limitations in surgical access (16). Perigonadal fat pads in mice are the most accessible and are used in the majority of mouse VAT studies; however, humans do not have such identical fat depots as mice and as such, these differences should be considered when comparing humans and mice (16). While adipocytes are tightly packed unilocular cells that are supported by a dense network of capillaries, the SVF consists of extra-cellular matrix (ECM) that holds together various cells such as pre-adipocytes, stem cells, fibroblasts, vascular endothelial cells and immune cells (13, 17–19).

During obesity, adipose tissue expansion is characterized by adipocyte hyperplasia (increase in adipocyte numbers) and hypertrophy (increase in adipocyte size), which results in increased adipocyte hypoxia, dysregulation of fatty acid fluxes, increased chemokine secretion, adipocyte cell death, and the recruitment of pro-inflammatory cells (20–23). In turn, pro-inflammatory cytokine release mediated by immune cells induces serine phosphorylation of insulin receptor substrate-1 (IRS-1) leading to local and systemic insulin resistance, which is thought to contribute toward whole body glucose and fatty acid metabolic dysregulation (24, 25) Furthermore, the obese adipose tissue can exhibit a pro-fibrotic phenotype characterized by an increased expression of ECM proteins such as collagen, which can impact adiposity, glucose homeostasis, and susceptibility to metabolic disease (26–29). On the other hand, aging results in an increase in body fat percentage, expansion of the VAT due to a shift in lipid storage from the SAT to the VAT, increased accumulation of senescent cells, and an altered pre-adipocyte cell phenotype (30). During aging, the accumulation of senescent preadipocytes in the adipose tissue leads to increased production of pro-inflammatory cytokines in a process dependent on the JAK pathway (31). It has been hypothesized that an elevated presence of senescent cells and reprogrammed pre-adipocytes in the aged VAT results in the generation of chemokines, pro-inflammatory cytokines, and ECM modifiers which contribute to VAT inflammaging (30).

The innate and adaptive immune systems are now widely accepted as forces that respond to and participate in the remodeling processes taking place in the VAT during metabolic diseases. While inflammatory changes in fat are likely required for proper adipose tissue remodeling and expansion, it is the chronic nature of the inflammation which ultimately drives metabolic disease during obesity and aging (32). In this review, we will discuss the role of immune cells in these two conditions with a focus on their function in the VAT.

INNATE IMMUNE CELLS

Macrophages

Homeostasis

Macrophages are a fundamental component of the innate immune system, with the ability to phagocytose harmful pathogens and apoptotic/necrotic cells. Historically, macrophages have been broadly categorized into two categories: the pro-inflammatory "classically activated" M1-like and the anti-inflammatory "alternatively activated" M2-like macrophages. However, macrophages are now thought to exist on a spectrum of functionalities based on their resident and recruited status (33). Macrophages were the first immune cell to be characterized inside VAT during obesity and, until recently, have been the primary focus of most studies. Under homeostatic conditions, the VAT is home to a group of macrophages known as Adipose Tissue Macrophages (ATMs), which represent roughly 5–10% of the stromal vascular fraction, display an M2-like phenotype, depend on the expression of peroxisome proliferator activated receptor γ (PPARγ), and secrete anti-inflammatory IL-10 (34–36). Fate mapping techniques have revealed that not all tissue resident macrophages terminally differentiate from monocyte precursors or emerge from adult hematopoiesis; indeed, some ATMs may develop from bone marrow (BM)-independent progenitors in the embryonic yolk sac and possess the ability to self-renew (37, 38).

OAI

During obesity, the population of ATMs within the VAT increases up to 40–50% of the stromal vascular fraction, become metabolically activated, secrete pro-inflammatory cytokines, and engage in "inflammatory cross-talk" with other immune cells, notably CD4+ T cells (34, 35, 39–43). Obesity-associated ATMs accumulate primarily at crown like structures (CLS), characterized by ATMs surrounding dead

or dying adipocytes (44). This accumulation of ATMs is the result of increased infiltration due to chemo-attractive gradients and higher proliferation, both partly driven by the monocyte chemoattractant protein 1 (MCP1) (45–47). It is possible that these mechanisms of ATM accumulation are reflective of the presence of distinct self-renewing yolk sac-derived and BM monocyte-derived ATMs. Efforts are in progress to reconcile these observations. Recent work has implicated secreted molecules in skewing ATM population phenotypes. In addition to pro-inflammatory cytokines such as TNFα and IFNγ, the transmembrane activator and calcium modulator and cyclophiliin ligand interactor (TACI) has been implicated in the skewing of macrophages toward an M1-like phenotype. Indeed, ATMs from TACI$^{-/-}$ mice were biased toward an M2-like phenotype and their adoptive transfer into obese mice rescue their dysregulated metabolic parameters (48, 49). Hormones and/or growth factors, such as insulin growth factor 1 (IGF1), can also impact the balance between M1- and M2-like phenotypes in ATMs (50).

However, as mentioned earlier, segregating macrophages into M1-like and M2-like subsets is not reflective of macrophage heterogeneity and efforts are being made to avoid this categorization (51). The markers and phenotypes of VAT monocytes, macrophages and DCs in the context of obesity have been described (52, 53). Further, macrophage classification is moving toward a genomics approach to identification in order to delineate the many heterogeneous cell subsets (54). Conditions during obesity may produce a "metabolically-activated' phenotype in macrophages that is mechanistically distinct from classical activation, which may explain the complexity in macrophage phenotypes seen in mice and humans (55). In light of this consensus, it was recently reported that the obese VAT contains a heterogenous group of macrophages, with BM-derived CD9+ and Ly6C+ ATMs representing the predominant populations (56). CD9+ ATMs exhibit pro-inflammatory gene signatures, localize in the CLS, are lipid-laden, secrete pathogenic exosomes, and induce a pro-inflammatory response in lean adipose tissue upon adoptive transfer (56). In contrast, Ly6C+ ATMs reside outside the CLS, express factors that support vascular development and organization, contain less lipids, and activate gene programs typical of normal adipocyte physiology upon adoptive transfer into lean mice (56). An expansion of the monocyte-derived CD9+ ATM population was recently confirmed via unbiased single-cell RNA sequencing. This population also displays a transcriptional signature associated with lipid metabolism and phagocytosis, consequently being termed lipid-associated macrophages (LAMs) (54). LAMs express Trem2, depletion of which resulted in their loss, decreasing the formation of CLSs observed during diet-induced obesity (54). However, in the absence of Trem2, mice fed a HFD displayed worsened metabolic parameters, implicating a role of these CD9+ Trem2+ LAMs in "buffering" against CLS-associated lipid plaques (54). While an increase in CLS surrounding lipid metabolizing CD9+ ATMs was reported in both of these studies, there is a need to reach a consensus with regards to their inflammatory profiles, their role in VAT inflammation, and whether lipid metabolism can directly regulate the inflammatory output of LAMs. Recent reports also propose the accumulation of sympathetic neuron/nerve-associated macrophages (NAMs) during diet-induced obesity which mediate the clearance of norepinephrine, a catecholamine that has been implicated in lipolysis and fat mass reduction (57).

AAI

In aged mice, the proportion of M2-like ATMs reportedly decreases, M1-like macrophages remains unchanged, and CD11c CD206 double negative (DN) ATMs trended to increase, suggesting that aging also skews ATM phenotypes toward a pro-inflammatory phenotype (58). These changes contrast to obesity where a large increase in numbers of VAT macrophages are seen, though aged macrophages still show an overall inflammatory shift. Similar to obesity, aged ATMs display an elevated expression of the chemokine receptor CCR2, possessed enhance secretion of pro-inflammatory cytokines IL-6, MCP-1, and TNFα, and a decrease in expression of PPARγ, which mechanistically accounts for the loss in M2 ATMs. (58). Recent studies propose that although there is a decrease in the proportions of ATMs in aged mice, aged ATMs lack M1- or M2-like polarization and showed a diversity of activation states (59). Furthermore, NAMs are also detected within the aged VAT, which were activated in an NLRP3-dependent manner to regulate lipolysis and fatty acid release through altering catecholamine bioavailability, thereby affecting VAT lipolysis (59). NLRP3 activity was further linked to the production of cytokines IL-1β and IL-18, which contributes to B cell accumulation in the VAT, as discussed below (60). Notably, NLRP3-deficient mice had restored proportions of ATMs which was postulated to reflect an exhausted senescent-like profile driven by chronic activation of the NLRP3 inflammasome (59). It remains to be determined whether levels of CD9+ LAMs and Ly6C+ATMs are altered in the aged VAT; future research in aging should focus on the heterogeneity of the ATM population within the adipose tissue, similar to what has been done for studies in obesity.

Innate Lymphoid Cells

Homeostasis

Innate lymphoid cells (ILCs) play a crucial role in providing defense against a wide array of pathogens such as parasites, microbes, and viruses as well as demonstrating cytotoxic activity toward tumors (61). ILCs are widely categorized into three groups based on their functional differences: group 1 ILCs (ILC-1), group 2 ILCs (ILC-2), and group 3 ILCs (ILC-3). In mice, ILC2 and ILC3 are the most abundant ILC subsets, and often residing in WAT and the intestine, respectively. In humans, however, ILC1 and ILC3 subsets are the predominant ILC subsets which are found in higher frequencies in mucosal and lymphoid sites such as the colon and ileum (62).

ILC-1s are defined by their ability to produce the pro-inflammatory cytokine IFNγ and have been widely studied in natural killer (NK) cells and mixed ILC-1 cells, which develop from different progenitors and possess unique tissue distribution (63). Under homeostatic conditions, ILC-1s are present in lean VAT, where they are resident cells and rely less on infiltration from the periphery, unlike splenic ILC-1s that

constantly recirculate (64, 65). Interestingly, compared to their peripheral counterparts, murine NK1.1+ NKp46+ VAT ILC-1s express low levels of Ly49 and consist primarily of three major populations: immature NK (iNK cells), mature NK (mNK) cells, and mixed ILC-1s (64, 65). Parabiosis studies have revealed that while mNK cells recirculate, mixed ILC-1s and iNK cells are mostly resident within the VAT (64, 65). Under homeostatic conditions, VAT ILC-1s were observed to surround ATMs and regulate their numbers by killing both M1-like and M2-like ATMs (65).

ILC-2s are known for producing T helper 2 (Th2) cytokines such as IL-5 and IL-13, and a large body of work conducted on ILC-2s has focused on their role in promoting "beiging" within the adipose tissue (61). Beiging is a process whereby fat-accumulating white adipose tissue is converted into energy-dissipating brown-like adipose tissue (66, 67). Under homeostatic conditions, the presence of ILC-2s in the VAT is maintained by IL-33. ILC-2s have been implicated in promoting the beiging of adipocytes and the accumulation of eosinophils and M2-like macrophages in an IL-4/IL-13-dependent manner (68–70). Additionally, ILC-2s promote beiging of white adipose tissue through the production and release of methionine-enkephalin peptides that upregulate UCP1 expression in adipocytes (71).

ILC-3s contain various populations of RORγt-expressing ILCs that can produce T helper 17 (Th17) cytokines such as IL-17 and IL-22 (61). It remains to be determined whether ILC-3s are found within the adipose tissue, whether they are tissue resident or recruited from the periphery, and finally, what their role is in the context of diet-induced obesity, aging, and chronic inflammation in the VAT.

OAI

In mice, during the early stage of diet-induced obesity, there is a transient increase in VAT ILC-1s, although their proportion decreases within the VAT during chronic obesity (64, 65). ILC-1s from chronically obese VAT show a reduced ability to regulate ATMs which may contribute to their uncontrolled expansion (65). Additionally, IFNγ secretion by VAT ILC-1s is also elevated during short-term HFD feeding in an IL-12/STAT-4-dependent manner, which further contributes to the polarization of macrophages to an M1-like phenotype (65). Furthermore, VAT ILC-1s, and potentially their IFNγ, have been shown to promote adipose fibrogenesis by activating pro-inflammatory $CD11c^+$ macrophages and the TGF-β1 pathway in adipocytes. These changes may serve to impair adipose function and glycemic tolerance in both mice and humans (72).

The early stage expansion of ILC-1s is primarily a consequence of the recruitment of mNK cells from the periphery followed by a smaller contribution of local proliferation (64, 65). A pathogenic role for the accumulation of mNK cells is likely, as their depletion improved metabolic parameters and decreased macrophage infiltration in obese mice (73–75). During obesity, adipocytes increase their expression of NK cell activating receptor (NCR1) which may be responsible for the expansion of local VAT NK cells and their IFNγ production (74, 76, 77). While increased IFNγ secretion by NK cells drives metabolic dysfunction during early-stage obesity, TNFα secretion by NK cells may play a larger role in driving inflammation and metabolic dysfunction during chronic obesity (78). During chronic obesity, NK cells have been shown to have an impaired capacity to degranulate or produce pro-inflammatory cytokines, which is linked to their uptake of lipids and subsequent metabolic reprogramming (79).

Chronic obesity also promotes the expansion of a distinct IL-6 receptor (IL-6R)- and colony-stimulating factor 1 receptor (csf1r)-expressing myeloid-signature NK cell (myNK) subpopulation in the perigonadal adipose tissue (PGAT) and blood circulation (80). Specific depletion of myNK cells led to reduced inflammation, obesity, and systemic insulin resistance, which could also be recapitulated through abrogation of IL-6 and STAT-3 signaling that is required for the formation of these unique cells (80). Future research should focus on determining the factors responsible for the change in ILC-1 functionality in the transition between early and late chronic obesity. Identifying the factors involved in NK cell recruitment early in obesity is needed as inhibiting NK cell accumulation within the VAT may serve as a therapeutic avenue to prevent elevated IFNγ levels within the adipose tissue; as well, understanding mechanisms to spare NK cells from becoming metabolically dysfunctional due to the lipid-laden adipose environment during chronic obesity.

ILC-2s have been shown to decrease in frequency and numbers in the obese WAT of humans and mice, which may impair the thermogenic ability of fat (68). As such, ILC-2s, along with eosinophils, discussed below, represent potential targets that can be manipulated to promote beiging and thermogenesis of white adipose tissue.

AAI

The role of VAT ILCs in aging is largely unknown. As myNK cells are among the cell types that potently respond to IL-6, a cytokine that is elevated in the VAT and circulation during aging, it is possible that aging affects the number and function of these cells (81). However, a large study in humans recently cataloged the different ILC subsets in humans with obesity and age (62). While age showed only marginal mixed up and down correlations of ILC-1/2/3s in abdominal and mesenteric fat, there was a significant correlative decrease in the presence of intestinal ILC-3s with age, highlighting tissue specific changes of these cells during aging (62). More work is needed to tease out how aging impacts VAT ILC accumulation and their inflammatory potential in mice and humans.

Neutrophils

Homeostasis

Neutrophils are responsible for providing protection against pathogens through the release of secretory granules containing a diverse array of antimicrobial proteins and pro-inflammatory mediators (82). Neutrophils can release reactive oxygen species (ROS) and cytokines to kill extracellular bacteria and recruit additional leukocytes to the region of inflammation (82, 83). Neutrophils can also kill extracellular bacteria through the generation of a web of extracellular fibers known as neutrophil extracellular traps (NETs), which are composed of DNA, histones, and antimicrobial proteins (82–84). Much of the work

that has been conducted on VAT neutrophils has focused on their role in obesity. Their frequency inside VAT is very low (<1% of non-adipocyte cells) in the lean state and their role in homeostatic functions in fat is unclear (85).

OAI

Neutrophils have been shown to enter the adipose tissue during early high fat diet (HFD) feeding in mice. The frequency of VAT neutrophils increase from <1% of non-adipocyte cells in lean mice to ∼2% within 1 week after initiating HFD feeding (85). Their crosstalk with adipocytes is sustained through neutrophil CD11b and adipocyte ICAM-1 interactions (85, 86). VAT neutrophils show increased production of the serine protease, elastase, which promotes inflammation in a toll like receptor (TLR)-dependent manner, and deletion of elastase *in vivo* in mice results in improved metabolic parameters (85). It has also been shown that elastase can decrease the amounts of proteins involved in the insulin signaling pathway such as IRS-1 (87, 88). In humans, clinical evidence points toward increased numbers and activation of neutrophils in obese patients. Neutrophils from obese patients possessed enhanced chemotactic activity and produced elevated amounts of superoxide molecules (87, 89, 90).

HFD-fed mice showed increased release and decreased clearance of NETs and increased autoantibodies against nuclear antigens (86). The excess nucleic acids and related protein antigens worsened metabolic parameters through the activation of VAT macrophages and plasmacytoid dendritic cells in the liver through a TLR-dependent manner while treatment of HFD-fed mice with inhibitors against TLR7/9 or NET formation improved metabolic parameters (86). Future work should aim to understand mechanisms and subsequently design therapies that can be used to reduce the accumulation of these cells within the adipose tissue or inhibit their ability to secrete NETs or elastase during obesity and metabolic disease.

AAI

There are no data regarding the role for neutrophils in the adipose tissue during the aging process, though few studies have explored the effect of aging in neutrophils. Neutrophils show age-related impairments in phagocytosis, degranulation, ROS generation, migration, and neutrophil microbicidal activity, which can contribute to the poor resolution of infections in the elderly (91–97). Future research should aim to address what factors contribute to the dysregulation of neutrophils in aged individuals, and whether these changes manifest inside fat.

Dendritic Cells

Homeostasis

Dendritic cells (DCs) are considered the bridge between the innate and adaptive immune system due to their antigen presentation role to prime T cells (98). There are two main subsets of DCs that have been well-studied: antigen presenting classical or conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (98). pDCs are significantly less efficient at presenting antigen and stimulating T cells as compared to cDCs but can secrete copious amounts of type 1 interferon (IFN-1) when activated (98). Recently, it was suggested that pDCs emerge from lymphoid progenitors that are distinct from the myeloid lineage and hence share a different ontogeny from cDCs (99).

Two main populations of cDCs are found under homeostatic conditions in murine VAT, namely CD103+ cDC-1s and CD11b+ cDC-2s, both of which promote a tolerogenic, anti-inflammatory environment in the VAT (100). cDC-1s primarily activate the Wnt/β-catenin pathway whereas VAT cDC-2s upregulate the PPARγ pathway. Depletion of β-catenin and PPARγ in VAT cDCs stimulates a pro-inflammatory response in a mouse model of obesity, suggesting a role of these pathways in cDCs in delaying the onset of metabolic disease (100).

OAI

Chronic obesity and expansion of the VAT interfere with β-catenin and PPARγ pathways and abrogate the anti-inflammatory function of cDCs, furthering meta-inflammation (100). Earlier studies in humans and mice demonstrated that obesity is associated with an expansion of VAT DCs, mainly cDCs that accumulate in the VAT in a CCR7-dependent and CCR2-independent manner (101, 102). Another study showed that VAT cDCs have the ability to promote pro-inflammatory Th17 responses (53). pDCs have also been implicated in the pathogenesis of VAT meta-inflammation as they are recruited to the tissue due to elevated levels of the adipokine chemerin, and subsequently activated to promote IFN-1 signals in VAT, resulting in the polarization of ATMs to an M1-like state (103). Furthermore, depletion of IFN signaling by genetic deletion of IFNAR or genetic ablation of pDCs resulted in improved metabolic parameters in HFD-fed mice, strongly indicating the role for this subset in contributing to meta-inflammation (104, 105).

AAI

Current research on peripheral DCs suggests that aging alters DC function in humans, including defective phagocytosis of antigen, migratory capacity, and enhanced secretion of pro-inflammatory cytokines upon stimulation with TLR agonists (106). While this change in function may contribute to DC mediated inflammatory change inside VAT with age, the roles of cDCs and pDCs in aged VAT remains to be determined.

Eosinophils

Homeostasis

Eosinophils are major producers of IL-4 and IL-13 and play a significant role in host defense, notably against helminth infections (107). Under homeostatic conditions, eosinophils are abundant in the adipose tissue and participate in the beiging process through their production of IL-4 and IL-13 and subsequent activation and accumulation of M2-like ATMs. The activation of M2-like ATMs may be one factor that regulates the expression of tyrosine hydroxylase and production of catecholamines inside VAT, enhancing thermogenesis. However, the details surrounding the cells involved, including NAMs, and the mechanisms of action, such as altering catecholamine bioavailability, require further study (59, 108, 109). Furthermore, IL-5 within the VAT is believed to be responsible for recruiting

eosinophils via IL-5 producing cells, which are predominantly ILC-2s (70, 108).

OAI

As a consequence of obesity, there is a decline in eosinophils in the VAT; eosinophil-deficient mice are more susceptible to weight gain and show impaired glucose tolerance, greater insulin resistance, and fewer anti-inflammatory M2-like ATMs (108). Treating obese mice with recombinant IL-5 for 8 weeks successfully restored eosinophil numbers within the VAT but did not reduce weight gain, glucose intolerance, insulin resistance, or alter energy expenditure and beiging capacity, implying that rescuing this immune population is not sufficient in the context of VAT dysfunction; thus, the mechanism of eosinophils in regulating VAT health and function therefore appears to be more complex than initially understood and must be further studied (110).

AAI

Knowledge on the role of VAT eosinophils during aging is very limited and requires additional work. In mice, aging has been associated with only a modest change in VAT eosinophils, in contrast with obesity where eosinophils decrease in numbers (111). It has also been indicated that aged human eosinophils possess altered degranulation abilities though with regular adhesive and chemotactic properties (112). Aging also has been recently described to prevent formation of cold-induced beige adipocytes in mice and humans and it remains to be determined whether eosinophils are implicated in this defect, along with other beiging-inducing immune cells (112, 113).

Mast Cells

Homeostasis

Mast cells play important roles during allergy and inflammation, and are components of the myeloid cell population in mouse VAT under homeostatic conditions. Mast cells have been shown to facilitate the preadipocyte to adipocyte transition in VAT (114, 115).

OAI

The VAT mast cell population increases during obesity, although they initially decrease slightly in the intermediate stages of HFD feeding in WT mice (12 weeks) but rebound in the later stages (114, 116). In humans, mast cell proportions positively correlated with the typical features of expanded obese VAT such as inflammation of endothelium, ATM build-up, and formation of fibrous tissue (117).

Mast cells can contribute to VAT dysfunction through degranulation and release of proteases such as tryptase, chymase, cathepsins, and matrix metalloprotease-9. These enzymes regulate adiponectin action and catabolize ECM collagens and fibronectin to promote adipogenesis and infiltration of pro-inflammatory cells into the tissue (117–120). Furthermore, mast cell-derived IL-6 and IFNγ contribute to adipose tissue cysteine cathepsin expression, which promotes VAT angiogenesis and growth in obese mice (118, 121). Mast cell-derived cathepsins can also degrade adipocyte insulin receptor and the glucose transporter Glut-4, leading to impaired glucose physiology in adipocytes (122).

Earlier reports indicated that mice deficient in mast cells fed a HFD displayed improved metabolic parameters and reduced pro-inflammatory cytokines in the VAT (118). It was further shown that mast cells regulate metabolism through IL-6 and IFNγ (118). Additionally, $Kit^{W-sh/W-sh}$ mice, which are deficient in mast cells, reconstituted with mast cells from *Il6* or *Ifng* knockout mice were protected from metabolic dysregulation (118). However, other models of mast cell deficiency reported no differences in weight gain, glucose physiology, or VAT inflammation (123, 124). The differences observed between studies are likely due to the use of different models of mast cells deficiency such as the $Kit^{W-sh/W-sh}$ mast cell-deficient mice (which are not specific to mast cells), Kit-independent $Cpa3^{Cre/+}$ and *Mcpt5-Cre R-DTA* mice, and mast cell stabilizer disodium cromoglycate treated mice (118, 123, 124).

AAI

To our knowledge, no studies have explored the presence of mast cells in the aged VAT and whether they contribute to tissue specific inflammaging. In mice, aging has been shown to promote the degranulation of mast cells upon exposure to prostaglandin E (PGE), which is not observed in mast cells from young animals (125). Furthermore, aged adipocytes secrete more PGE than young adipocytes and thus this warrants the examination of mast cell degranulation within the adipose tissue and the potential role of various proteases released, in the context of inflammaging and age associated IR (126).

Myeloid Derived Suppressor Cells

Homeostasis

Myeloid derived suppressor cells (MDSCs) are a heterogenous population of immature myeloid cells with anti-inflammatory functions including suppression of adaptive immunity, modulation of macrophage cytokine production, and elevated expression of immunosuppressive factors such as arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS) (127). MDSCs tend to typically develop during a variety of inflammatory conditions such as sepsis, cancer, and instances of autoimmunity but little is known about their presence and/or role in the lean adipose tissue.

OAI

MDSCs have been shown to increase in the adipose tissue of leptin-deficient obese mice, while transferring MDSCs into obese mice improves parameters associated with metabolic disease (128). Accordingly, tumor-bearing mice fed a HFD display an enhanced accumulation of MDSCs. While MDSCs protected against metabolic disease and VAT inflammation, they have a detrimental effect on tumor progression and overall reduced animal survival time (129). Several factors have been implicated in the increased accumulation of MDSCs, including the adipokine leptin, polyunsaturated fats, and exogenous lipids, which could potentially be harnessed to boost MDSC activity and deter inflammation (129–131).

AAI

In mice, MDSCs have been shown to accumulate in the spleen, lymph nodes, and bone marrow of aged mice and also possess greater suppressive activity in T cell proliferation, which is associated with defective PI3K-Akt signaling pathway (132). Furthermore, MDSCs have been linked to limiting B lymphopoiesis during aging, which is driven via their production of IL-1 (133). Whether MDSCs accumulate in the aged VAT to suppress inflammatory cells or to impact insulin resistance remains to be evaluated.

ADAPTIVE IMMUNE CELLS

B Cells

Homeostasis

B cells are a crucial component of the adaptive immune response that achieve their functions via cytokine secretion, antibody secretion, and modulation of the function of other cells (134). The majority of B cells can be categorized into B1 and B2 cells. B1 cells are enriched in the pleural and peritoneal cavities, while B2 cells are found abundantly in secondary lymphoid organs such as the spleen (134). Regulatory B cells (Bregs) are a collection of heterogenous IL-10 producing B cells that can ameliorate inflammation. Inside VAT, a subset of these Bregs are maintained by CXCL12 and free fatty acids and Breg deletion of IL-10 results in aggravated VAT inflammation, insulin resistance, and loss of metabolic homeostasis (135). However, the majority of B cell-derived IL-10 in VAT at steady state is derived from B1 cells, the B cell population that is also predominantly abundant within fat-associated lymphoid clusters in the VAT (136, 137).

OAI

Obesity induces an accumulation of total B cells in the VAT, including the proportion and absolute number of class switched mature IgM– IgD– IgG+ B2 cells (138). IgG isolated from obese mice is capable of driving metabolic disease due to their hyposialylated profile which activates the endothelial IgG receptor FCγRIIB (138, 139). In humans, insulin resistance is associated with a distinct profile of IgG autoantibodies, arguing for (self) antigen-specific targets contributing to B cell-mediated insulin resistance (138). A recent paper extended these findings by characterizing self-antigens targeted by IgG inside human adipose tissue (140). Moreover, B cells isolated from obese mice are responsible for modulating T cells within the VAT in an MHC-I/II-dependent manner by priming both CD4+ and CD8+ T cells. Ablation of the antigen-presenting complex from B cells is sufficient to improve metabolic parameters, indicating a role of B-T cognate interactions in the VAT in modulating metabolic disease (138). Obese B cells also secrete elevated levels of pro-inflammatory cytokines and in line with these various observations, both B cell-deficient μMT mice and anti-CD20-treated mice display improved metabolic and inflammatory parameters (138, 141). Notably, the LTB4/LTB4R1 chemokine/receptor axis has been shown to promote the activation and recruitment of VAT B2 cells, highlighting its potential as a target for insulin sensitizing therapies (142). Unlike B2 cells, tolerogenic B1a cells are reduced in frequency during obesity and produce less IL-10 and transferring B1a cells from lean mice into HFD-fed B null mice can improve metabolic parameters through IL-10- and polyclonal IgM-dependent mechanisms (136). Obesity also induces compromised functionality in natural Bregs (135).

Given the potential pathogenicity of B2 cells and beneficial effects of Bregs and B1 cells, targeting B2-driven VAT inflammation while enhancing the anti-inflammatory function of B1/Breg cells would help alleviate obesity-related adipose pathophysiology. For example, one possible B cell targeting therapy involves the use of B-cell Activating Factor (BAFF) antagonists as BAFF deficiency or inhibition ameliorates obesity-induced inflammation in mice (136, 143). Despite the growing interest in VAT B cells, the role of antibody secreting cells (ASCs) in the VAT is unknown. ASCs are terminally differentiated B cells that secrete antibodies and cytokines and can be further categorized into short-lived plasmablasts and long-lived plasma cells (144, 145). While VAT IgG increase during obesity, it is unclear whether this increase is due to a recruitment of long-lived plasma cells from the bone marrow or from other tissue such as intestines (146).

AAI

Like obesity, aging is characterized by a similar increase in mature B2 cells in the VAT, as well as plasma IgG as early as 12 months in mice (147). Furthermore, aging is associated with an increase in the expression of the B cell-specific nuclear co-factor Oct coactivator from B cells (OcaB) in the VAT. Depletion of OcaB in mice improved metabolic parameters, prevented the accumulation of B2 cells within the VAT, and decreased circulating levels of IgG2c and pro-inflammatory cytokines (147). An increase in the number of B1a and B1b cells has also been observed in the aged VAT. However, this accumulation was much subtler and the role of these innate B cells in aging requires further examination (147). Interestingly, some B1 cells develop pathological features during aging. Indeed, monocytes can convert omental B1a cells into 4-1BBL-expressing B1a cells which promotes immune activation and insulin resistance (148).

Interestingly, aging drives the formation of a unique circulating B cell subset known as age-associated B cells (ABCs), which are defined by their overexpression of the transcription factor T-bet (149–151). ABCs are further characterized by their ability to respond to nucleic acid antigen, and produce pro-inflammatory TNFα and IgG2c, making them prime candidates for mediating VAT inflammaging and age-associated insulin resistance (152, 153). Some evidence suggests that ABCs may represent either a memory B cell population or an ASC population as they express elevated levels of transcription factors involved in ASC formation, namely Prdm1, Irf4, and Xbp1, as well as expressing the ASC surface marker CD138 (151, 153, 154). Moreover, members of the SWEF family of Rho guanine exchange factor (GEF) proteins have been implicated in regulating the expansion of ABCs through the activity of IRF5 (155). Such ABCs have also been implicated in autoimmune disorders such as lupus in young mice, suggesting that aging does not serve as a prerequisite for their induction. This notion warrants the need to determine whether they are present

within the VAT during diet-induced obesity independently of aging (154). Interestingly, ABC expansion is observed more consistently in female mice and it remains to be examined whether sex-specific hormone or other factors are at the root of these differences (149). Recently, another unique population of B cell, aged adipose B cells (AABs), have been shown to accumulate in aged VAT, which are memory-like B cells that expand within the fat-associated lymphoid clusters (FALCs) in aged VAT in an NLRP3 dependent manner; depletion of these cells can improve insulin resistance in mice and reverse age-induced lipolytic dysfunction, and future work will need to assess triggers and targets regulating these cells function during aging (60, 156).

T Cells

Homeostasis

T cells are a major component of the adaptive immune system and can be categorized into various subsets based on their expression of surface markers, the composition of T cell antigen receptors (TCR), and the secretion of different cytokines. T cells with the αβ TCR rearrangement can be categorized according to their expression of CD4 or CD8, with the former being further sub-divided into various subsets based on their effector cytokine profiles including IFNγ-producing T helper 1 (Th1) cells, IL-4-producing Th2 cells, IL-17-producing Th17 cells and IL-10-producing Foxp3+ T regulatory cells (Tregs).

Tregs are highly enriched in the lean adipose tissue, uniquely regulated by PPARγ, and promote skewing of macrophages to an M2-like state (157–160). Furthermore, the accumulation of Tregs depends on antigens presented in an MHC-II manner and the release of soluble mediators, notably IL-33, the majority of which is secreted by mesenchymal stromal cells (161–163). There is a growing appreciation of an evolutionarily conserved requirement for IL-33 in VAT Treg function, as demonstrated by their dependency upon the IL-33 receptor ST2, and downstream transcription factors BATF and IRF4 (164, 165). Interestingly, recent work has shown that sexual dimorphism has also been reported to play a role in VAT Treg function in this axis (166). VAT inflammation is increased with testosterone and limited with estrogen in males. Increased VAT inflammation and male-specific IL-33-producing stromal cells mediate the recruitment and local expansion of Tregs in a BLIMP-1-dependent manner, constituting a male-specific feedback circuit that potentially limits VAT inflammation (166). Research on Th2 cells in the adipose tissue also points toward a protective role, and human VAT is also thought to be enriched in Th2 cells expressing IL-13 under insulin sensitive conditions (157, 167).

OAI

Generally, T cells are known to accumulate within the VAT in obese mice and humans. Depleting T cells with an anti-CD3 antibody improves insulin sensitivity and limits VAT meta-inflammation in obese mice (157, 168). CD4+ cells within the obese VAT produce higher amounts of IFNγ than those from lean VAT, indicating an overall Th1 polarization of CD4+ T cells (157, 169, 170). Indeed, IL-12p35^{null} mice, which are deficient in Th1 cells, show improved insulin sensitivity as the main Th1 effector cytokine, IFNγ, has been shown to be directly responsible for affecting insulin signaling, lipid storage, and differentiation of adipocytes via sustained JAK-STAT1 pathway activation (157, 171).

Obesity results in increased accumulation of VAT Th17 cells in an IL-6-dependent manner, although these cells are present at a lower frequency compared with Th1 cells (157, 170). Mechanistically, extracellular ATP acts through the P2X7 receptor pathway promotes a Th17-polarizing microenvironment (172). This was also accompanied by a greater frequency in Th17 cells and higher expression of Th17 markers such as RORC (RORγt in mice), IL-17, and IL-23R in VAT explants from metabolically unhealthy obese donors compared to metabolically healthy obese and lean donors (172, 173). Despite this research, the role of IL-17 in obesity-induced metabolic disease is unclear. Despite reports of increased IL-17 in obese individuals, mouse models have shown that IL-17 deficiency worsens the effects of diet-induced obesity, accelerates adiposity in mice fed a low-fat diet, and elevates circulating leptin levels (172, 174, 175). However, it is important to note that γδ T cells and MAIT cells are also sources of IL-17 and all studies regarding the impact of IL-17 on metabolic disease cannot be attributed to Th17 cells alone (175). More work is required to distinguish and elucidate the cell specific role of IL-17 in the adipose tissue during metabolic disease.

During obesity, adipose tissue Tregs decrease in number and are outweighed by the function of pro-inflammatory T cells (157, 176). As such, rescuing the Treg population may represent a potential therapeutic strategy in improving metabolic parameters during obesity; treatments with PPARγ agonists such as thiazolidinedione, 5-aminosalicylic acid (5ASA) or IL-33 administration *in vivo* were shown to regulate VAT Treg numbers and improve metabolic parameters in obese mouse models (160, 164, 165, 177).

CD8+ T cells, or cytotoxic T lymphocytes (CTLs), increase in the VAT during obesity and have an enhanced capacity to secrete IFNγ (167, 178). Despite no differences in body weight, obese mice deficient in CD8+ T cells display improved glucose tolerance and insulin sensitivity, suggesting a pathogenic role for CD8+ T cells in impairing metabolic health (105, 178). The cytotoxic activity of CD8+ T cells has been demonstrated to be regulated by perforin, as perforin-deficient CD8+ T cells show an elevated proliferative and inflammatory capacity. Transfer of these perforin-deficient CD8+ T cells into CD8-deficient mice significantly worsened metabolic parameters compared to those transferred with perforin-sufficient CD8+ T cells (75).

Several outstanding questions that remain to be explored include the mechanisms by which co-stimulatory molecules induce T cell pathogenicity during obesity, as well as how T cell metabolism is altered during adipose tissue inflammation. The 4-1BB-4-1BBL checkpoint pathway has been reported to be important in Th1-skewing of CD4+ cells and enhancing CD8+ T cell proliferation (179, 180). In obese mice, there is an increase in the expression of both the receptor and ligand; mice deficient in 4-1BB are reportedly protected from HFD-induced metabolic dysregulation and adipose macrophage and T cell infiltration (181, 182). Thus, it would be possible to target pro-inflammatory

T cell function via blockade of this pathway. Recently, our group has reported that insulin receptor signaling modulates T cell inflammatory function and subset skewing via the control of intrinsic cellular metabolism, though it remains to be determined whether insulin signaling in VAT T cells is impacted during chronic obesity (183). Finally, it will be important to identify which antigens are targeted by T cells within the VAT. Indeed, a large majority of T cells in the VAT are effector memory T cells that exhibit a markedly restricted TCR diversity (157, 184). This, along with the presence of pathogenic IgG autoantibodies produced by B cells, suggests that there may potentially be specific target antigens within the VAT that drives an antigen-specific T cell response (140, 185). Whether these antigens are bystanders or true drivers of disease remain to be seen.

AAI

Within the aged VAT, similar to obesity, there is an expansion of various T cell populations including CD8+ T cells and CD4+ T cells. However, unlike obesity, Treg numbers increase in VAT with age (58, 111). Age-dependent accumulation of CD8+ T cells appears to be dependent on biological sex as aged CD8+ T cells from the VAT of female mice display an elevated activation status and secrete more pro-inflammatory cytokines than their male counterparts (186). Future work needs to explore the distribution of CD4+ T helper subsets within the VAT micro-environment and what signals are responsible for driving the accumulation of T cells within the aged VAT. Interestingly, Tregs within the VAT are enriched in aged male mice and selectively depleting them improves glucose uptake and age-associated insulin resistance (111), though the mechanisms are unclear. It should be noted that aged females have lower levels of VAT Tregs compared to male counterparts and the mechanism driving this difference is poorly understood (186), though recent work in describing sex differences in VAT Tregs may provide insight to these differences (166). Finally, further work should explore the role of the IL-33-Treg axis in the context of healthy aging, as well as determine whether dysfunctional stromal cell activity contributes toward the expansion of VAT Tregs during aging.

Innate Like T Cells

Homeostasis

There is an emerging appreciation for the role of innate-like T (ILT) cells that possess innate mechanisms to respond rapidly to stress and pathogens while concurrently expressing antigen receptors reminiscent of adaptive immunity (187). There are three main subsets of ILT cells that we will be discussing: natural killer T (NKT) cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells.

NKT cells are a group of ILT cells that are activated upon recognizing glycolipid antigens presented on the non-classical MHC-I-like protein, CD1d, and can be further categorized into two subtypes based on their TCR diversity: type 1 or invariant NKT (iNKT) cells, which express an invariant TCRα chain and limited numbers of TCRβ chains, and type 2 or diverse NKT (dNKT) cells, which show more diverse usage of TCRα and β chains (188, 189). The VAT is enriched with iNKT cells which help maintain the adipose tissue microenvironment under homeostatic conditions by promoting IL-4-dependent M2-like macrophage polarization and sustaining Treg activity in an IL-10/IL-2-dependent manner; indeed, depletion of CD1d in adipocytes worsened inflammation and insulin resistance (190–192). Surprisingly, VAT iNKT cells in mice constitute a specialized tissue resident subset that express the transcription factor E4BP4 and lack PLZF expression, unlike circulating iNKT, and produce IL-2 and IL-10 to regulate immune homeostasis within the VAT (193, 194).

γδ T cells also possess innate-like features that allow for their activation upon recognition of conserved stress-induced ligands and subsequent rapid secretion of cytokines such as IFNγ, TNFα, and IL-17A (195). In lean wild type mice, adipose tissue resident PLZF+ γδ T cells were shown to produce TNFα and IL-17A which together promoted IL-33 production by adipose stromal cells (196). IL-33 production induced by γδ T cells was shown to promote thermogenesis as mice lacking γδ T cells or IL-17A showed impaired adipocyte UCP1 expression and thermoregulation (196).

Mucosal associated invariant T (MAIT) cells are found primarily in peripheral blood, the intestinal mucosa, and the liver and are evolutionary restricted by the MHC-related molecule 1 (MR1) (197). Upon stimulation, human peripheral MAIT cells produce IFNγ, TNFα, IL-17, IL-2, and granzyme B, whereas mouse spleen MAIT produce higher levels of IL-17 and lower levels of IL-10, IFNγ, and TNFα (198–202). In humans, adipose tissue MAIT cells but not peripheral blood MAIT cells produce more IL-10 than IL-17, highlighting site-specific differences in MAIT function (202). Although the role of MAIT cells inside VAT is poorly understood, they are potent secretors of IFNγ and IL-17 and warrant further investigation under homeostatic conditions (197).

OAI

During obesity, there is reportedly a decrease in the numbers of iNKT cells; furthermore, $CD1d^{-/-}$ mice deficient in iNKT cells display an insulin resistant phenotype even in the absence of a high fat diet and adipose inflammation, providing evidence for their protective function in the VAT during obesity (190, 203, 204). Conversely, in a Vα14 transgenic mouse model, which has elevated levels of iNKT cells, having increased iNKT cells on a $Ldlr^{-/-}$ background resulted in worsened metabolic parameters when mice were placed on an obesogenic diet. These findings suggest that all iNKT cells might not be outright protective, but rather the reduction in iNKT cells might be a compensatory mechanism in response to obesity (205). This observation could also be explained by the differential roles between peripheral and local iNKT subsets in maintaining tissue homeostasis. Further, most of these studies did not assess the role of type 2 dNKT cells as $CD1d^{-/-}$ mice lack both NKT subsets. dNKT cells have been shown to play a protective role during obesity as adoptive transfer of dNKT cells into obese mice improved weight loss and glucose homeostasis (206). Future studies should aim to use newly developed mouse strains to better address the specific roles of each NKT subsets in obesity (207). The observation that VAT PLZF+ γδ T cells promote the IL-33-Treg axis and thermogenesis under homeostatic conditions warrants the need

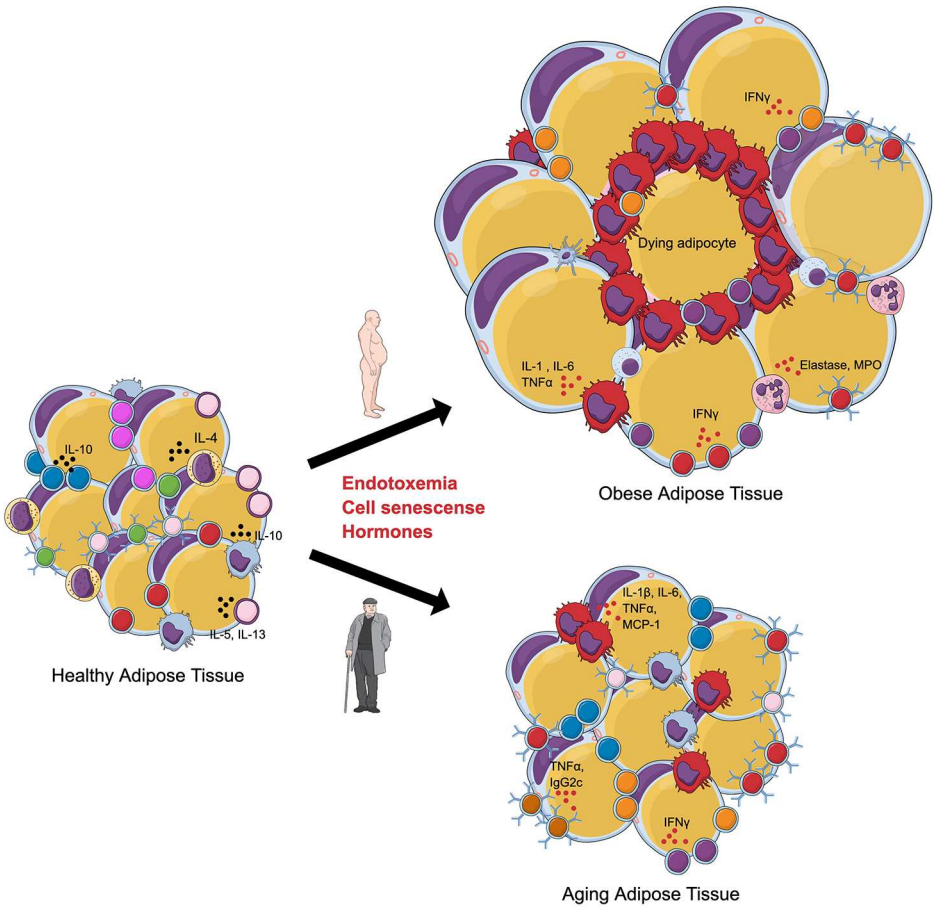

FIGURE 1 | Alterations in the visceral adipose tissue (VAT) immune cells during obesity and aging. In lean VAT, homeostasis is maintained via the secretion of anti-inflammatory cytokines by regulatory T cells (Tregs), T helper 2 (Th2) cells, tolerogenic macrophages, group 1 innate lymphoid cells (ILC1s), ILC2s, regulatory B cells (Bregs), B1 cells, and eosinophils. **(Top)** Obesity induces an expansion in adipocyte size and promotes a shift in the phenotype of local immune cells toward a pro-inflammatory state with increases in pro-inflammatory macrophages, NK Cells, B2 cells, Th1 CD4 cells, CD8+ T cells, and neutrophils. Inflammation of adipose tissue leads to tissue damage, cell death, and metabolic disturbances. **(Bottom)** During aging, the VAT is characterized by alterations in the immune cell environment. Emerging evidence indicates that these changes are associated with a shift in the phenotype of macrophages, expansion of B2 cells, age-associated B cells (AABs), CD8+ T cells and, paradoxically, regulatory T cells (Tregs). Changes in the composition of adipose tissue immune cells during aging may contribute to insulin resistance and ectopic lipid storage. Illustration created in the Mind the Graph platform: www.mindthegraph.com.

for studies to explore the functions of PLZF+ γδ T cells in obese adipose tissue, whether they are lost during obesity, and whether IL-17 secretion by γδ T cells is impaired during obesity (196).

Patients with T2D and/or obesity show decreased frequency of peripheral MAIT cells but increased frequency in adipose tissue MAIT cells (202, 208, 209). Further, MAIT cells in both the adipose tissue and periphery from obese patients produced higher levels of IL-17 and reduced levels of IL-10, different from the lean state, suggesting a potential role for MAIT cells in obesity-associated inflammation (210). The mechanisms underlying the alterations in MAIT cell numbers, activation, and IL-17 production need to be further elucidated, with a need to

TABLE 1 | Comparison between innate immune cells in OAI and AAI.

Innate immune cells	Obesity	Aging
Macrophages	↑ in ATM population [H/M] (33–35, 39–43) ↑ pro-inflammatory cytokine release [H/M] (33–35, 39–43) ↑ inflammatory cross-talk [H/M] (33–35, 39–43) ↑ crown-like structures [H/M] (44–47) ↑ in pro-inflammatory CD9+ macrophages (LAMs) [H/M] (54)	↑ pro-inflammatory phenotype [M] (58) ↓ proportion of M2-like ATMs [M] (58) ↔ proportion of M1-like ATMs [M] (58) ↑ proportion of CD11c-CD206- ATMs [M] (58) ↑ expression of CCR2, IL-6, MCP-1, TNFα [M] (58) ↓ expression of PPARγ [M] (58)
ILCs		
- ILC-1	↑ proportion in early obesity [H/M] (64, 65) ↓ proportion in chronic obesity [H/M] (64, 65) ↑ IFNγ and TNFα secretion [H/M] (65, 74, 76, 77) ↑ mNK recruitment and expansion [H/M] (64, 65) ↓ in NK degranulation and pro-inflammatory cytokine production during chronic obesity [H/M] (79) ↑ myNK expansion in chronic obesity [H/M] (80)	↔ proportion [H] (62)
- ILC-2	↓ frequency in WAT [H/M] (68)	↔ proportion [H] (62)
- ILC-3	x	↔ proportion [H] (62)
Neutrophils	↑ frequency [H/M] (85, 89) ↑ activation [H/M] (85, 89) ↑ production of elastase [M] (85) ↑ release and ↓ clearance of NETs [M] (86)	x
DCs		
- cDCs	↓ anti-inflammatory profile [M] (10) ↑ accumulation of VAT cDCs [H/M] (101, 102) ↑ promotion of Th17 cell responses [H/M] (53)	x
- pDCs	↑ recruitment to VAT [H/M] (103–105) ↑ IFN-1 signaling in VAT and polarization of ATMs to M1-like state [H/M] (103–105)	x
Eosinophils	↓ eosinophils in VAT [M] (108)	↔↓ eosinophils in VAT [M] (111)
Mast Cells	↑ mast cells in VAT [H/M] (114, 116, 117) ↑ inflammation of VAT endothelium, ATM accumulation, formation of fibrous tissue [H] (117)	x
MDSCs	↑ MDSCs in VAT [M] (128) ↑ protection against metabolic disease [M] (129)	x

A table of summary for the changes in innate immune cell populations specifically within the adipose tissue in obesity and aging.
"x" indicates studies are lacking.
↑ indicates increased, ↓ indicates decreased, and ↔ indicates no change or no consensus.
[H/M] indicates data seen in human and/or mouse studies.

TABLE 2 | Comparison between adaptive immune cells in OAI and AAI.

Adaptive immune cells	Obesity	Aging
B cells		
- B2	↑ accumulation of B cells (138) - ↑ class switched mature IgG+ B2 cells [M] (138) - Distinct IgG autoantibodies [H/M] (138, 141) - ↑ antigen presentation to T cells [M] (138) - ↑ pro-inflammatory cytokines [M] (138, 141)	↑ mature B2 in VAT [M] (147) ↑ circulating IgG levels [M] (147) ↑ OcaB expression in VAT [M] (147)
- B1	↓ frequency in B1a cells [M] (136) ↓ IL-10 production [M] (136) ↓ Breg functionality [M] (135)	↔↑ numbers of B1a and B1b [M] (147) ↑ accumulation of 4-1BBL$^+$ B1a cells [H/M] (148)
- ABCs	Unclear if present in obesity independent of aging (154)	↑ in aging [H/M] (149–153) ↑ in females [M] (149) ↑ accumulation of aged adipose B cells (AABs) [M] (60, 156)
T cells	↑ in VAT [H/M] (157, 168–170)	x
CD4+	↑ in VAT [H/M] (157, 168–170)	↑ in VAT [M] (58)
- Th1	↑ Th1 polarization in CD4+ T cells [H/M] (157, 169, 170) ↑ production of IFNγ [H/M] (157, 169, 170)	x
- Th17	↑ accumulation in VAT [H/M] (157, 170, 172, 173) ↑ expression of Th17 markers (RORC/RORγt, IL-17, IL-23R) [H/M] (172, 173)	x
- Treg	↓ population in VAT [H/M] (157, 176)	↑ in VAT [M] (58, 111) ↑ enrichment in males [M] (111, 166, 186)
CD8+	↑ population in VAT [H/M] (167, 178) ↑ IFNγ secretion [H/M] (167, 178)	↑ in VAT [M] (58, 111) ↑ activation and pro-inflammatory cytokine secretion in females [M] (186)
ILTs		
- iNKT	↓ population in VAT [H/M] (190, 203, 204)	x
- dNKT	↑ protection against metabolic disease [M] (206)	x
- γδ T cells	x	x
- MAIT cells	↑ frequency [H] (202, 208, 209) ↑ production of IL-17 [H] (210) ↓ production of IL-10 [H] (210)	x

A table of summary for the changes in adaptive immune cell populations specifically within the adipose tissue in obesity and aging.
"x" indicates studies are lacking.
↑ indicates increased, ↓ indicates decreased, and ↔ indicates no change or no consensus.
[H/M] indicates data seen in human and/or mouse studies.

determine the ontogeny of adipose tissue MAIT cells and their role in the adipose environment during obesity.

AAI

Only a few studies have explored the role of ILT cells within the aged VAT. In mice, NKT cell numbers were increased 2- to 3-fold in the secondary lymphoid organs of aged mice compared to young mice, suggesting that a similar increase or accumulation of peripheral PLZF+ NKT cells might be seen within the VAT (211). However, it remains to be determined how the population of adipose resident E4BP4+ iNKT cells are affected and whether the distribution of peripheral vs. resident NKT cells is shifted (193). Similar to obesity, it has also been suggested that peripheral MAIT cells are reduced in aged individuals and future studies need to determine whether they are being recruited to metabolic tissues such as the liver and/or VAT (212).

CONCLUDING REMARKS

Aging and diet-induced obesity present themselves as metabolic diseases that are characterized by an alteration of the VAT immune landscape and display an activation of chronic inflammatory pathways that contribute to insulin resistance and diabetes (**Figure 1**, **Tables 1**, **2**). There are several overlapping mechanisms of VAT inflammation in obesity and aging that could help direct future research. One common driver of VAT inflammation in both conditions is the altered gut and the resident host microbiota. Obesity is associated with microbial dysbiosis and an overall reduction in bacterial diversity, which imparts features of the metabolic syndrome as the obese phenotype can be transferred from obese humans to mice through transplant of the gut microbiota (213–216). A consequence of microbial dysbiosis is increased intestinal permeability, characterized by leakage of bacterial antigen or their products such as metabolites or pathogen associated molecular patterns including lipopolysaccharide (LPS) across the intestinal epithelial barrier. These products can access metabolic tissues such as the VAT by entering systemic circulation and also being taken up by chylomicrons, which further drives meta-inflammation by activating pro-inflammatory cascades in immune cells via pattern recognition receptor signaling (177, 217–219). Aging is also potentially associated with intestinal microbial dysbiosis that contributes to worsened intestinal permeability, serum endotoxemia and inflammation, which can be recapitulated upon transfer of gut microbiota from aged mice into young germ-free mice, as seen during obesity (219–221). Low grade chronic intestinal inflammation is an early manifestation of obesity which precedes systemic metabolic disease and contributes to worsened barrier function and insulin resistance via secretion of pro-inflammatory cytokines (213). Moreover, an increased flux of bacterial product entering the VAT may be one common mechanism linking aging and obesity related insulin resistance to distinct downstream inflammasome receptors such as the NLRP3 inflammasome, which ultimately represent immune activation in response to gut bacterial products in obesity and aging (60, 156).

Another shared factor that could potentially link obesity and age driven VAT inflammation is cellular senescence. Senescence is regarded as a hallmark of aging, and metabolically active senescent cells secrete a variety of cytokines and chemokines, in an NFKB dependent manner, which further drives local inflammation (222, 223). Senescent cells have been demonstrated to also accumulate within the VAT during obesity, and their clearance is associated with improved metabolic parameters and decreased macrophage homing (224). Interestingly, within the VAT, CD4+ T cells adopt a senescent phenotype which drives VAT inflammation and insulin resistance in an osteopontin dependent manner (225). A combination of cell senescence and inflammatory cell death within the fat might represent a source of shared antigenic targets between obesity and aging, which likely underpins a mechanism fueling local immune cells.

Finally, both obesity and aging are associated with changes in the levels of hormones that could impact immune cell function in the VAT. For instance, leptin and insulin typically rise with obesity and aging. Leptin metabolically activates T cells and skews them toward a Th1 cytokine secretion profile, promotes pro-inflammatory cytokine secretion by circulating monocytes, and enhances expression of perforin in NK cells (226–229). Insulin has been shown to facilitate T cell glycolytic programming and IFNγ mediated effector functions (183). The interactions of these hormones and adipokines in an environment of increasing cell senescence and bacterial products likely represents additional common drivers between aging and obesity related VAT inflammation.

Given these potential underlying common drivers of obesity- and aging-related IR, it will be important to understand the mechanistic differences between both conditions. For instance, why do VAT Tregs function differently with age compared to obesity? What are the underlying reasons behind the differences in VAT macrophage numbers? It will also be important to tease out the differences in inflammatory responses between mouse models and humans. In particular, do aged humans show similar changes to VAT immune cells as seen in aged mice? Understanding the differences and similarities between VAT immune populations with age and obesity will help identify unifying pathophysiological root causes of the associated metabolic disease.

Over the past 20 years, our knowledge about the immune landscape of the VAT has grown from the simple identification of the role of cytokines and macrophages, to include adaptive immunity, and lately most known immune cell populations. The level of cellular characterization will continue to improve to further refine these populations using single-cell genomics and advanced cytometry methods. Such analyses will ultimately yield new insights into disease pathogenesis and may lead to new therapies to combat obesity and aging related metabolic disease.

AUTHOR CONTRIBUTIONS

SK, YC, XR, and DW contributed to the design and writing of the manuscript and the generation of the figures.

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Cytokine Output of Adipocyte-iNKT Cell Interplay Is Skewed by a Lipid-Rich Microenvironment

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The complex direct and indirect interplay between adipocytes and various adipose tissue (AT)-resident immune cells plays an important role in maintaining local and whole-body insulin sensitivity. Adipocytes can directly interact with and activate AT-resident invariant natural killer T (iNKT) cells through CD1d-dependent presentation of lipid antigens, which is associated with anti-inflammatory cytokine production in lean AT (IL-4, IL-10). Whether alterations in the microenvironment, i.e., increased free fatty acids concentrations or altered cytokine/adipokine profiles as observed in obesity, directly affect adipocyte-iNKT cell communication and subsequent cytokine output is currently unknown. Here we show that the cytokine output of adipocyte-iNKT cell interplay is skewed by a lipid-rich microenvironment. Incubation of mature 3T3-L1 adipocytes with a mixture of saturated and unsaturated fatty acids specifically reduced insulin sensitivity and increased lipolysis. Reduced activation of the CD1d-invariant T-Cell Receptor (TCR) signaling axis was observed in Jurkat reporter cells expressing the invariant NKT TCR, while co-culture assays with a iNKT hybridoma cell line (DN32.D3) skewed the cytokine output toward reduced IL-4 secretion and increased IFNγ secretion. Importantly, co-culture assays of mature 3T3-L1 adipocytes with primary iNKT cells isolated from visceral AT showed a similar shift in cytokine output. Collectively, these data indicate that iNKT cells display considerable plasticity with respect to their cytokine output, which can be skewed toward a more pro-inflammatory profile *in vitro* by microenvironmental factors like fatty acids.

Keywords: adipocytes, iNKT cell, CD1d, lipolysis, insulin resistance

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INTRODUCTION

Insulin resistance is one of the hallmarks of type II diabetes mellitus (T2DM) pathogenesis, with both overlapping and unique molecular mechanisms affecting different metabolic organs, including muscle, liver and adipose tissue (AT) (1, 2). AT has long been thought of as a simple storage organ, however it has more recently been shown to be an extremely complex tissue which plays a key role in global homeostasis (3, 4). Important and intertwined mechanisms through which AT can communicate with other metabolic organs and cells are facilitated by the production of adipokines (5), which can act locally but also enter into the circulation. From here the AT adipokine action, both locally and systemically, acts to regulate immune behavior in specific cytokine output

(3, 4, 6, 7). While the pathways leading from obesity to whole body insulin resistance and T2DM are complex and multifactorial (2), the increased uptake of nutrients leading to hyperplasia and hypertrophy of adipocytes is clearly an important early event, resulting in an altered adipokine secretion profile (5). In addition, hypertrophic AT displays a shift in AT-resident immune cells and cytokine output, where pro-inflammatory immune cells overwhelm the previously predominant anti-inflammatory immune cell populations (3, 6). The resulting chronic low-grade inflammation causes dysregulation of lipolysis, where adipocytes secrete higher levels of FFA, and glycerol (8, 9), which together with adipokines and cytokines can be viewed as an additional AT output. Combined these factors can have local or systemic effects and contribute to the development of whole-body insulin resistance and T2DM.

One of the AT-resident immune cell types that decrease dramatically with obesity both in mouse models and in humans are the invariant natural killer (iNKT) cells [reviewed in (10, 11)]. iNKT cells serve as a bridge between the innate and the adaptive immune system and are able to produce both pro-inflammatory cytokines, including IFNγ, and anti-inflammatory cytokines like IL-4 and IL-10 (10, 12, 13). IL-4 and IFNγ were initially thought to be on either end of the inflammatory spectrum, however recent research has shown that they also play a role in regulating other immune populations (14). Interestingly, the final steps of maturation of iNKT cells are thought to occur in the tissue where they reside, resulting in various tissue-defined subsets (10, 15). AT-resident iNKT cells for example have a Th2 cell phenotype and mainly produce anti-inflammatory cytokines IL-4 and IL-10 under lean conditions, which help to maintain AT homeostasis (16–20). iNKT cells are activated through a (semi)invariant TCR, that recognizes lipid antigens presented in the context of CD1d, a molecule that is expressed on the cell surface of various APC (13, 21, 22). In AT, iNKT cells can be directly activated by adipocytes, as they express not only CD1d itself but also possess a functional lipid antigen presentation pathway (16, 23–28), as well as a biosynthetic pathway for the production of lipid self-antigens (27, 28). Whilst in other biological settings the nature of lipid antigens, the type of APC and the microenvironment of the tissue have all been recognized as regulators of the secretion of Th1 and/or Th2 cytokines (10, 12, 29, 30), if and how the same parameters help to define AT-resident iNKT cell subsets and their cytokine output is largely unknown.

To study adipocyte-iNKT cell communication directly, i.e., without interference of other cell types, we and others have developed and characterized various co-culture assays, combining mouse or human (pre)adipocyte cell lines or primary adipocytes, with either reporter cells expressing the iNKT TCR (31), iNKT hybridoma cells (16, 24–28, 32) or primary iNKT cells isolated from AT or spleen (16, 24–28, 32). All these different assays strongly support direct adipocyte-iNKT cell communication, as they all show CD1d-dependent activation of the CD1d-iNKT TCR pathway upon co-culture with adipocytes, an activation boosted by the exogenous prototypical lipid antigen α-galactosylceramide (αGalCer), resulting in simultaneous production of both multiple pro- and anti-inflammatory cytokines (16, 24–28, 32).

Here we investigated the effects of FFA, as well as other obesity-associated components of the AT microenvironment, on the direct communication between adipocyte, and iNKT cells. First, we developed and characterized a cell culture model using mature murine 3T3-L1 adipocytes cell line treated with a commercially available and chemically-defined lipid mixture to mimic a high lipid microenvironment. Impaired insulin signaling and increased lipolysis was observed, without overall disruption of adipocyte-specific gene expression. Interestingly, while iNKT hybridoma cells, and primary iNKT cells produced both IL-4 and IFNγ under basal conditions, their cytokine output was skewed when adipocytes were pre-treated with the lipid mixture toward a low IL-4, high IFNγ profile. Taken together, these data indicate that iNKT cells display considerable plasticity with respect to their cytokine output, which can be skewed by microenvironmental factors like FFA.

MATERIALS AND METHODS

Materials

Dexamethasone (Sigma), 3-isobutyl-1-methylxanthine (IBMX)(Sigma), insulin (I9278, Sigma), Lipid mix 1 (sigma L0288), albumin conjugated linoleic (sigma L9530), and oleic acid (sigma O3008), sodium palmitate (sigma P9767), myristic acid (sigma M3128), stearic acid (sigma S4751), cholesterol (MP biochemicals, 219934230), αGalactosylceramide KRN7000 (Avanti, 867000), IFNγ (sigma SRP3058), TNFα (sigma H8916), Pam3Cys (Calbiochem), LPS (sigma L4516), rabbit-anti-AKT (also named PKB) (33), rabbit-anti-pAKT-ser473 (4060S Cell Signaling).

Cell Culture

The murine 3T3-L1 cell line (ZenBio) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum (Invitrogen), penicillin and streptomycin (both 100 μg/ml; Invitrogen). For differentiation of 3T3-L1 cells to adipocytes, the cells were grown to confluence and after 2 days (day 0) stimulated with culture medium containing dexamethasone (250 nM), 3-isobutyl-1-methylxanthine (500 μM), and insulin (170 nM) for 2 days. On day 2, the medium was changed for culture medium containing insulin (170 nM) and maintained for 4–6 days. The murine iNKT cell hybridoma line DN32.D3 was cultured in RPMI-1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, penicillin and streptomycin (both 100 μg/ml), MEM Non-Essential Amino Acids Solution (100x; ThermoFisher Scientific), HEPES (100x; Sigma Aldrich), Glutamine (100x, Sigma Aldrich) and βMeOH (100 μM; Sigma Aldrich). The JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cell lines (31) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin (both 100 μg/ml).

Stimulation of 3T3-L1 adipocytes was done post differentiation for 3 days with lipid mix and individual fatty acids, and for 24 h with the residual obesogenic stimuli. One Millimolar of Palmitic, stearic and myristic acid was conjugated to fatty acid free BSA in a 6:1 ratio. Lipids were dissolved in 150 mM NaCl at 70°C while stirring. Before added to BSA

150 mM NaCl solution at 37°C while stirring. After adjusting pH to 7, 4 aliquots where stored at −20°C before further use (protocol adapted from Seahorse Bioscience).

Western Blot Analysis

3T3-L1 cells were grown in a six well format and differentiated accordingly. After stimulation cells were washed with ice cold PBS, scraped and lysed in 300 μl ice cold RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium DOC, 0.1 % SDS, 25 mM Tris pH 7.4, supplemented with protease inhibitor (Roche) and NaF acting as phosphorylase inhibitor) for 15′ at 4°C. After centrifugation on max speed at 4°C in table top centrifuge, supernatant was collected, supplemented with Laemmli Sample Buffer (LSB). All western blot samples were boiled for 5 min at 95°C before use. Samples were subjected to SDS-PAGE and transferred to PVDF membrane (Milipore). Blocking was done with 5% Skim Milk TBST. Primary antibody staining was performed overnight at 4°C (AKT & pAKT 1:2,000 in 5% BSA TBST). Secondary antibody staining was performed for 1 h at RT (1:1,000 in 5% milk TBST). After staining, membranes were washed for 1 h with TBST before being treated with ECL western blot substrate solution (Pierce). Protein expression was measured with LAS4000 ImageQuant.

Glycerol Measurements

Secreted Glycerol was measured using the Sigma-aldrich Glycerol Assay Kit (#MAK117-1KT) with 50 μl of media.

Triglyceride Measurements

Intracellular Triglycerides were measured using the kit Stanbio™ Triglycerides LiquiColor™ (#SB2200-225). Cells were washed with PBS and lysed in 50 μl cold PBS via syringe pull technique.

Co-culture Assays

3T3-L1 wild type (Zenbio) were plated in a 96-well format and differentiated according to the protocol mentioned above. Mature 3T3-L1 Cells were then treated with lipid mix or individual fatty acids for 4 days, or 24 h with residual obesogenic stimulants. After stimulation, either DN32.D3 iNKT hybridoma cells (50.000 cells/well) or JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells (50.000 cells/well) were added to the 3T3-L1 adipocytes and co-cultured for 24 h. Medium from DN32.D3 co-cultures (200 μl) was used to determine secreted IL-4 and IFNγ via the Invitrogen™ eBioscience™ Mouse IL-4 ELISA Ready-SET-Go!™ Kit (#501128931) and the IFNγ ELISA kit (BD-bioscience).

JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells (31) were collected after co-culture by resuspending and collection in round bottom 96-well plates. Cells were then centrifuged (1,600 RPM, 5′, RT) and resuspended in 200 μl PBS. Cells were analyzed for eGFP expression by flow cytometry with FACSDiva (BD) and FlowJo (Tree Star Inc.) software. Data is presented as geometric Mean Fluorescent Intensity.

RT-qPCR Analysis

RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using the superscript first strand synthesis system (Invitrogen) according to manufacturer's protocol. Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to 36B4 expression. Primers for quantitative RT-PCR are described in **Table S1**.

Single Cell Suspension From Spleen and Adipose Tissue (eWAT)

Tissue collection was performed on animals sacrificed for other purposes and therefore exempted form ethical approval by the local Animal Welfare Body Utrecht, a body of Utrecht University and the University Medical Center Utrecht (https://www.ivd-utrecht.nl/en/). Spleens and epididymal white AT (eWAT) were dissected from male C57BL/6J mice aged between 11 and 14 weeks. Spleens were disintegrated through a 70 μM cell strainer into 50 ml cold PBS and centrifuged for 10 min at 400 g. After discarding the supernatant, the pellet was resuspended in 5 ml of 10x Red Blood Cell (RBC) Lysis Buffer (Abcam; ab204733) for 5 min at room temperature. Forty five milliliter of cold PBS was added and cells centrifuged for 10 min at 400 g. After discarding the supernatant, the pellet was resuspended in 10 ml of cold PBS and filtered through a 70 μM cell strainer. Cells were retained on ice.

Before processing blood vessels and lymph nodes were removed from the eWAT which was then minced in 10 ml cold digestion buffer (Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} supplemented with 0.5% bovine serum albumin). Per 1 g adipose, 1 ml of 10 mg/ml collagenase (Sigma-Aldrich; C6885) was added and incubated at 37°C for 15–20 min, with vigorous shaking every 5 min until AT was digested completely. Digested AT was then washed through a 100-μm cell filter with 20 ml of cold digestion buffer. Following centrifugation (500 g for 10 min) supernatant was removed and the cell pellet was resuspended in cold PBS. Cells were retained on ice.

iNKT Purification

Cells isolated from spleen and adipose mice were pooled, respectively, before purification in order to remove individual variation and to maintain consistent cell numbers during co-culture. iNKT cells were purified from the pooled populations using the NK1.1+ iNKT Cell Isolation Kit, mouse (Miltenyi Biotec; #130-0960513). Purified cells were then used in co-culture as described above.

RESULTS

Lipid Mixture Causes Insulin Insensitivity and Increased Lipolysis in Adipocytes

The increased concentration of circulating FFA observed in obesity contributes to the development of insulin resistance in adipose tissue (1, 2). To mimic this phenomenon in a cell culture model we used a chemically defined, commercially available lipid mixture containing cholesterol plus monounsaturated FFA (oleic acid), polyunsaturated FFA (arachidonic, linolenic, and linoleic acid) and saturated FFA (myristic, palmitic, and stearic acid), as reported previously (34). A significant increase in triglyceride (TG) storage was observed in mature 3T3-L1 adipocytes after

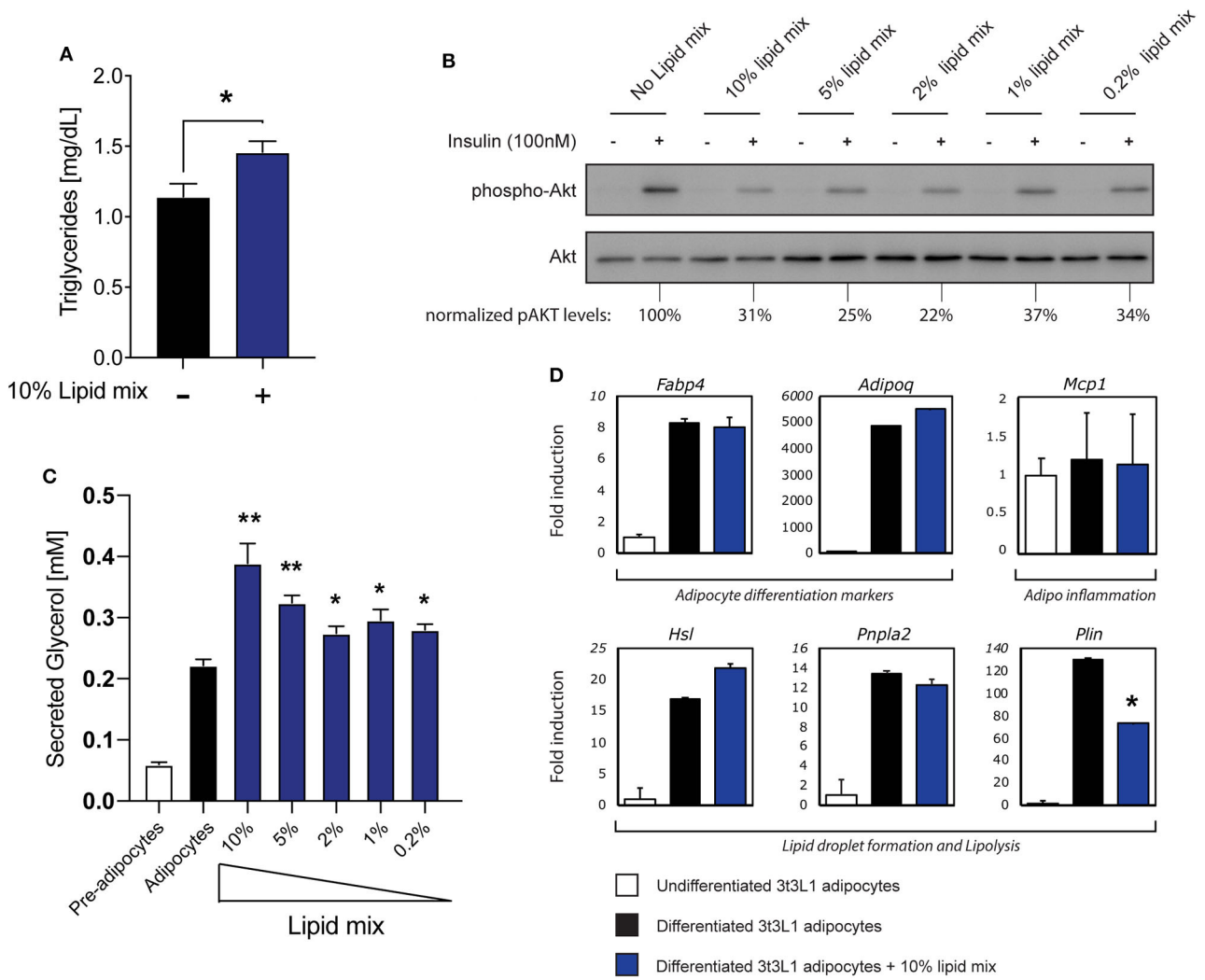

FIGURE 1 | Stimulation with a lipid mixture causes an insulin resistance phenotype in 3T3-L1 adipocytes. **(A)** Mouse 3T3-L1 preadipocytes were differentiated into mature adipocytes and cultured with 10% lipid mixture for 4 days and intracellular triglyceride levels were determined. Statistical analysis via Students *t*-test against adipocytes glycerol secretion (NS $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) ($n = 3$). **(B)** Mature 3T3-L1 adipocytes were treated with different concentrations (10–0.2%) Lipid Mixture for 4 days and deprived of insulin signaling before insulin stimulation (30 min). Lysates subjected to Western blot analysis. Western blot quantification of phospho-AKT (ser473) normalized to total AKT levels. **(C)** Quantification of glycerol secreted by 3T3-L1 adipocytes, and control pre-adipocytes, after 4 days in culture with titrated lipid mix dilutions. Statistical analysis via Students *t*-test against adipocyte glycerol secretion (NS $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$) ($n = 3$). **(D)** Mouse 3T3-L1 preadipocytes were differentiated into mature adipocytes and treated with 10% lipid mix for 4 days before being subjected to RNA isolation and quantitative RT PCR. Transcriptional activity of genes involved in adipogenesis, inflammation and lipolysis are depicted as fold induction relative to undifferentiated 3T3-L1 adipocytes. Data are normalized to housekeeping gene 36B4 and presented as mean +/– SD ($n = 6$).

culturing with 10% lipid mixture (**Figure 1A**), Next, the effects of different concentrations of lipid mixture on insulin sensitivity was assessed by re-stimulating insulin deprived mature 3T3-L1 adipocytes cultured in the absence or presence of various concentrations of lipid mixture. Insulin signaling decreased 3–4-fold compared to untreated adipocytes, with 10% lipid mixture displaying the maximal effect (**Figure 1B**). When tested individually, the inhibitory effect on insulin signaling by the different components of the lipid mixture was not observed when compared to the lipid mixture (**Figure S1A**). In addition, no inhibitory effect was observed after stimulation with cytokines TNFα and IFNγ (**Figure S1A**), which are both elevated in obesity (35, 36). To analyse functional effects of the reduced insulin signaling, we focused on lipolysis, a process that is highly regulated by insulin (2). As shown in **Figure 1C** a significant increase in lipolysis was observed when cells were subjected to the lipid mixture, as assessed by analyzing secreted glycerol concentrations. Again, stimulation with the individual components of the lipid mixture did not increase glycerol secretion significantly (**Figure S1B**). Additionally, stimulation with cytokines TNFα and IFNγ increases glycerol secretion (**Figure S1C**) but similar stimulation showed no effect on insulin

signaling (**Figure S1A**). Also, activation of TLR2 (Pam3Cys) or TLR4 (LPS), which has been shown to occur in obesity (37–39), has no outspoken effect on glycerol secretion (**Figure S1C**).

To characterize this cell model further, we analyzed the effects of the lipid mixture on mRNA expression of genes associated with adipocyte differentiation (adipogenesis), inflammation, and lipid storage by RT-qPCR (**Figure 1D**). Expression of the adipogenesis markers FABP4 and Adipoq (encoding adiponectin) were clearly upregulated during adipogenesis with expression remaining consistent irrespective of Lipid Mix (**Figure 1D**). The inflammation marker gene *Mcp1* was expressed in both undifferentiated and differentiated cells, and unaffected by the lipid mixture (**Figure 1D**). Furthermore, as we observed increased triglyceride storage and glycerol secretion in mature 3T3-L1 adipocytes subjected to the lipid mixture (**Figures 1A,C**), we analyzed the expression of genes involved in lipid storage and lipolysis. *HSL* and *PNPLA2* (encoding the ATGL protein) were more highly expressed in differentiated cells compared to undifferentiated 3T3-L1 cells, but no significant change in expression was observed upon lipid mixture treatment. The *Plin* gene, encoding Perilipin1, was the only gene tested here to show a significant decrease in expression. As also observed for insulin signaling (**Figure S1A**) and lipolysis (**Figure S1B**), none of the lipolysis related genes we tested increased upon stimulation with the main individual components that make up the lipid mix (**Figure S1D**). To verify that expression of the genes analyzed in general was not static but could be modified by other stimuli, we subjected the cells to other obesity-associated stimuli (TNFα, IFNγ) or inflammatory stimuli (Pam3Cys, LPS) and observed various changes in gene expression (**Figure S1E**).

Taken together, these data indicate that treatment of mature 3T3-L1 adipocytes with a chemically-defined lipid mixture results in a robust insulin resistance phenotype with increased lipolysis, without causing an overall disruption of cellular functionality, as the cells were clearly functional in terms of lipid metabolism and no dramatic changes in various key genes were observed. These observed characteristics support this cellular model as suitable for subsequent experimental approaches, including *in vitro* studies on adipocyte-iNKT cell communication.

Lipid Mixture Skews Cytokine Output in Adipocyte-iNKT Interplay

Having established a cellular adipocyte model with a high-lipid microenvironment (**Figure 1**), we next wished to investigate if and how adipocyte-iNKT cell communication is altered under these experimental conditions. For this we used different experimental co-culture approaches. First, we cultured mature 3T3-L1 adipocytes pre-treated with lipid mixture or left untreated together with the recently developed JE6-$1^{REP-iNKT-\beta2M\_KO}$ reporter cells (31). This reporter cell line is based on the Jurkat T cell line, stably transfected with an NFkB-eGFP reporter and the human Vα24-Jα18 TCRα chain and Vβ11 TCRβ chain (31). Co-culture of lipid antigen presenting cells followed by quantification by FACS analysis provides a sensitive fluorescence-based readout of iNKT TCR-lipid antigen interaction (31). In addition, the β2M gene was deleted from these cells using CRISPR/Cas9, to eliminate antigen-selfpresentation and self-activation (31). When JE6-$1^{REP-iNKT-\beta2M\_KO}$ reporters were cultured for 24 h with lipid mixture- treated mature 3T3-L1 adipocytes, an increase in eGFP expression was observed compared to individually cultured reporter cells, which was boosted by the prototypical lipid antigen αGalCer (**Figures 2A,B**). These results expand the use of these reporter cells as a read-out for CD1d-iNKT TCR signaling to adipocytes (31). Furthermore, as similar observations were previously made using iNKT hybridoma cells (16, 24–28, 32), we conclude that these reporter cells provide a robust read-out system for 3T3-L1 adipocyte-iNKT cell interaction. When using this read-out system to investigate the effect of lipid mixture treatment, eGFP expression decreased when adipocytes were stimulated with αGalCer 24 h prior to co-culture (**Figure 2D**), a trend not observed in the absence of αGalCer (**Figure 2C**). Importantly, the lipid mixture or individual components did not alter eGFP expression when the reporter cells were tested in isolation, indicating that CD1d-iNKT TCR signaling is required (**Figure S2B**). Also, individual components of the lipid mix and the inflammatory stimuli IFNγ, Pam3Cys, and LPS did not alter eGFP expression after co-culture significantly (**Figures S2C–F**). It should be noted that TNFα did induce eGFP expression, as it can activate the NFkB reporter present in the JE6-$1^{REP-iNKT-\beta2M\_KO}$ reporter cells (**Figure S2A**).

To investigate potential mechanisms behind this change in adipocyte-iNKT reporter cell communication, we analyzed the expression of several genes that have previously been implicated in lipid antigen presentation in adipocytes (*Cd1d*, *MtpA,* and *MtpB*), or that could potentially play in role in this based on findings in other lipid APC (*Gla, Npc2, Psap*). In addition, we analyzed 3 genes implicated in the biosynthesis of potential endogenous lipid antigens in adipocytes (*Ugcg*) and other lipid APC's (*Glb1, Gba*). In agreement with previous reports (26, 27, 32), the *Cd1d* gene, encoding the actual lipid antigen presenting molecule, was upregulated during 3T3-L1 differentiation, here no effect of the lipid mixture was observed (**Figure 2E**). Also, expression of the 2 isoforms of Microsomal Triglyceride Transfer Protein [*MtpA* and *MtpB;* note that *MtpB* is predominantly expressed in 3T3-L1 adipocytes (27)], a factor we previously implicated in lipid antigen presentation in adipocytes (27), displayed < 2-fold change. The expression of other lipid antigen loading machinery genes which potentially play a role in lipid antigen presentation in adipocytes (27) like pro-saposin (*Psap*), Niemann Pick type C2 (*Npc2*), α-galactosidase (*Gla*), were also not altered dramatically (**Figure 2E**). The *Ugcg* gene, encoding ceramide glucosyltransferase and implicated in the biosynthesis of endogenous lipid antigens in adipocytes and other APC (28, 40), also displayed a limited change upon lipid mixture treatment (**Figure 2E**), as did two other genes potentially involved in the biosynthesis of endogenous lipid antigens, *Glb1*, encoding β-galactosidase, and *Gba*, encoding beta-glucocerebrosidase (**Figure 2E**). Stimulation with individual lipid mix components

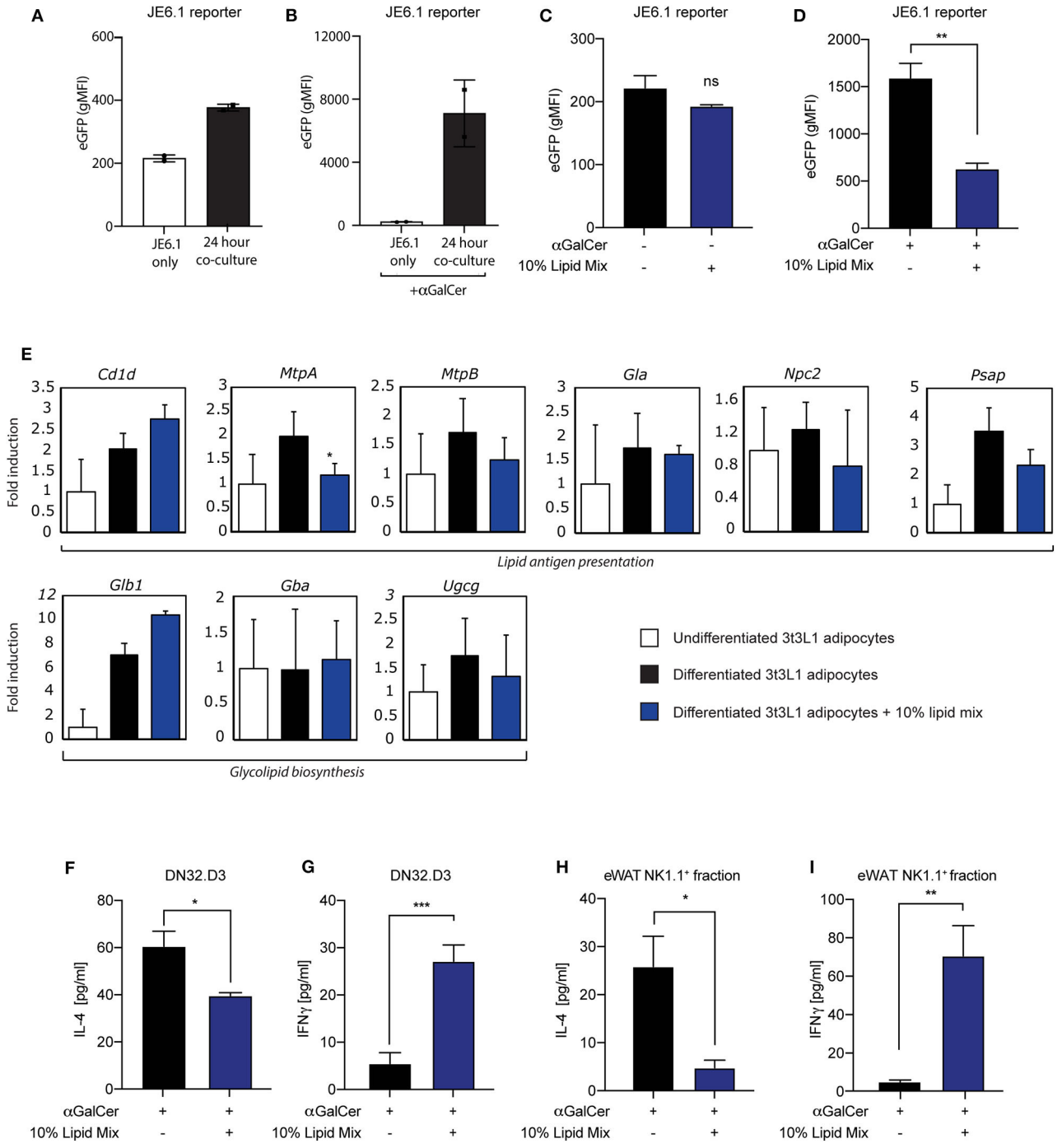

FIGURE 2 | Lipid mixture treatment skews iNKT cell cytokine secretion toward a pro-inflammatory profile after co-culture. **(A,B)** Co-culture between JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells and mature 3T3-L1 adipocytes stimulated with **(B)** or without **(A)** αGalCer (0.5 μg/ml) 24 h prior to co-culture ($n = 2$) Data is presented as geometric Mean Fluorescent Intensity (gMFI). **(C,D)** Co-culture between JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cell (5×10^4 cells per well in a 96 well-format) and mature 3T3-L1 adipocytes treated with or without 10% lipid mix and stimulated with **(D)** or without **(C)** αGalCer (0.5 μg/ml). JE6-1$^{REP-iNKT-\beta2M_KO}$ cells were collected after 24 h co-culture and analyzed for GFP expression by FACS. Data is presented as geometric Mean Fluorescent Intensity (gMFI) +/− SD ($n = 3$, $P = 0.0044$). **(E)** Mouse 3T3-L1 preadipocytes were differentiated into mature adipocytes and treated with 10% lipid mix for 4 days before being subjected to RNA isolations and Quantitative RT PCR. Transcriptional activity of genes involved in adipogenesis, glycolipid biosynthesis, lipid antigen presentation, and lipolysis are depicted as fold induction relative to undifferentiated 3T3-L1 adipocytes. Data is normalized to housekeeping gene 36B4 and presented as mean +/− SD ($n = 6$). **(F,G)** ELISA analysis of IL-4 and IFNγ secreted by DN32.D3 cells after 24 h co-culture with mature 3T3-L1 adipocytes treated with or without 10%

(Continued)

FIGURE 2 | lipid mix ($n = 3$, $p = 0.0319$ and 0.0004 for IL-4 & IFNγ, respectively) Each data point represents 5×10^4 cells. **(H)** IL-4 secretion following 24 h co-culture of NK1.1 positive *ex-vivo* fraction extracted from visceral eWAT, cultured with αGalCer stimulated (0.5 μg/ml) mature 3T3-L1 adipocytes treated with and without 10% lipid mix. Each data point represents 5×10^4 cells taken from a pooled population, statistical analysis via Students *t*-test against (NS $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) ($n = 3$) **(I)** IFNγ secretion following 24 h co-culture of NK1.1 positive *ex-vivo* fraction extracted from eWAT, cultured with αGalCer stimulated (0.5 μg/ml) mature 3T3-L1 adipocytes treated with and without 10% lipid mixture. Each data point represents 5×10^4 cells taken from a pooled population. Statistical analysis via Students *t*-test against (NS $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) ($n = 3$–6).

also shows no dramatic differences in gene expression of lipid antigen presentation and glycolipid biosynthesis genes (**Figure S1D**). Taken together, these data indicate that a lipid-rich microenvironment can alter communication through the CD1d-iNKT TCR axis, but that changes in genes implicated in lipid antigen presentation are not likely to present the underlying mechanism.

The second read-out system used was co-culture of mature 3T3-L1 adipocytes with mouse iNKT hybridoma cells followed by cytokine analysis, a robust system previously used by us and others (26–28, 32). In line with the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter-based system (**Figure 2D**), we saw a significant decrease in secretion of the anti-inflammatory cytokine IL-4 by the iNKT cell hybridoma DN32.D3 when adipocytes were subjected to the lipid mixture (**Figure 2F**). In contrast, a significant increase in secretion of the pro-inflammatory cytokine IFNγ was observed upon lipid mixture treatment (**Figure 2G**), indicating plasticity of the iNKT cell in terms of cytokine output depending on the microenvironment. Again, in line with the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter-based system, individual components of the lipid mix and inflammatory stimuli did not alter cytokine secretion by DN32.D3 iNKT cells in a co-culture setting (**Figures S2G,H**). Also, 3T3-L1 adipocytes treated with 10% lipid mixture did not produce high levels of IFNγ that may conflict with the outcome of 3T3-L1-DN32.D3 co-cultures (**Figure S2I**).

Whilst the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter cells and DN32.D3 iNKT cell hybridoma represent useful proxies for iNKT cells they do not fully reflect all iNKT cell characteristics, which are in part tissue-specific (10). Therefore, as a third read-out system we replaced the DN32.D3 hybridoma with primary iNKT cells isolated from either spleen or eWAT, based on the NK1.1 marker (NK1.1.+ iNKT cells) as reported previously (16). Similar to the DN32.D3 based co-culture system (**Figures 2F,G**), NK1.1$^+$ eWAT iNKT cells co-cultured with mature 3T3-L1 adipocytes treated with lipid mixture show a significant reduction ($p = 0.0233$) in IL-4 (**Figure 2H**) and an significant increase ($p = 0.0086$) in IFNγ secretion (**Figure 2I**). On the other hand, the response of NK1.1$^+$ iNKT cells isolated from mouse spleens was far less pronounced for both IL-4 and IFNγ secretion (**Figures S3A,B**). Furthermore, the NK1.1$^-$ fraction from both eWAT and spleen followed the same trend as their positive selection counterparts, but to a lower extent (**Figures S3C,D**). These data indicate that the eWAT iNKT cytokine secretion profile has a specific plasticity and is highly responsive to the adipose environment, with a key role for free fatty acids in this process.

DISCUSSION

Aside from their CD1d restricted TCR, iNKT cells can have divergent functions, surface markers and cytokine secretion preferences, all of which seem to be determined by the nature of lipid antigens, the type of APC and the microenvironment of the tissue (21, 41, 42). Despite their environmental priming, they also appear to retain plasticity, enabling them to respond swiftly and dynamically to changes in their surroundings (10, 12, 29, 43). Given the complex nature of AT and the various interactions between the different cell types present in this tissue, we decided to develop assay systems where it would be possible to study adipose—iNKT crosstalk in isolation. To facilitate this, we optimized a lipid enriched adipose cell line 3T3-L1 which exhibits several obesity-associated characteristics without disturbing functionality (**Figure 1E**). Using two iNKT model cell lines, DN32.D3, and JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter cells, we show that cross-talk with the lipid enriched 3T3-L1 adipocytes influences output following TCR stimulation (**Figure 2**). Interestingly, iNKT cells extracted from eWAT, like DN32.D3 hybridoma cells, display an IFNγ secretion skew over IL-4 in lipid rich environments. On the other hand, splenic iNKT cells do not show the same capacity in this context, highlighting the divergence between the two populations. Based on this rapid response to a lipid rich environment, we conclude that eWAT iNKT cells harbor significant plasticity in terms of their cytokine output, which is pre-primed by their tissue micro-environment. Interestingly, a short term HFD diet intervention in which skewing of cytokine output by AT-resident iNKT cells was reported by Li et al. (20). It should be noted however that while we observed a skew toward IFNγ production upon treatment with a defined lipid mixture, Li et al. (20) reported skewing toward higher IL-4 production in AT upon HFD feeding. Although further research is needed to establish how the *in vivo* and *in vitro* interventions can be translated into each other, both studies support the view that the regulatory function of iNKT cells is dynamic and complex, exemplified by their ability to rapidly switch their cytokine preference based on their micro-environment.

In the present study we compared several co-culture systems to address the delicate cross-talk between adipose and iNKT cells in a lipid-rich environment. Under our experimental conditions, we obtained similar results from both DN32.D3 hybridoma cells and primary iNKT cells form eWAT, suggesting that DN32.D3 hybridoma cells represent an appropriate model for iNKT cells in the context of eWAT (**Figure 2**). In our co-culture assays, the response of the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$

reporter cells (31) reflected the effects observed in the other systems on IL-4 secretion, i.e., reduced output when adipocytes were pre-treated with lipid mixture, but not the increased IFNγ secretion. It should be noted that the NFkB-eGFP reporter in the JE6-$1^{REP-iNKT-\beta2M\_KO}$ reporter cells provides a single read-out (31); our data suggest that the intracellular pathways ultimately leading to IFNγ and IL-4 secretion are wired differently at some level, but the underlying molecular mechanisms remain to be established. The JE6-$1^{REP-iNKT-\beta2M\_KO}$ reporter cells nonetheless clearly present a very valuable tool for identification and verification of (endogenous) lipid antigens (31).

In contrast to iNKT cells from eWAT, we have also shown in the present study that splenic iNKT cells do not exhibit an IL-4/IFNγ preference in co-culture assays upon lipid mixture treatment. Splenic iNKT cells have been previously reported to have an IL-4 and IFNγ cytokine secretion capacity (10, 43), we show that this capacity is not significantly altered by lipid enrichment. As previously stated, we focused on IL-4 and IFNγ because of their duel nature, as both are linked to inflammatory balance as well as their regulatory implications for the surrounding immune populations (44–49). Therefore, the contrast between AT and splenic iNKT populations emphasizes that isolating immune cells from the tissue that is being studied for subsequent analyses is recommendable. This is particularly true for iNKT cells, as iNKT populations for the most remain in one tissue environment with only few cells in circulation, in contrast to for example the much more mobile macrophages (17, 41, 43, 46).

Immune cell plasticity refers to immune cells which have a flexible, context-dependent inflammatory phenotype. This flexibility allows for rapid response to stimulus without being permanently defined by it (41, 43, 48, 50). We can draw parallels with other more defined immune populations. A good example of this is M1/M2 macrophages, where the macrophage population is capable of being on either end of the inflammatory spectrum and anywhere in between (19, 48, 49, 51). Similarly, iNKT cells are able to secrete a multitude of cytokines but in a context dependent pre-primed manner (6, 10, 14, 41, 52–54). Therefore, we propose that iNKT cells have a plasticity in response to their surroundings, and that their response is potentially tailored to the specific requirements of their micro-environment. How the plasticity observed in our proof of principle studies translates into the complex interplay between adipocytes and various AT-resident immune cell types *in vivo*, and ultimately into whole body energy homeostasis will be the topic of future studies. Indeed, very recent *in vivo* studies by LaMarche et al. already underscored the microenvironmental impact on iNKT cell output (55).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because it was exempted by the local animal ethics committee.

AUTHOR CONTRIBUTIONS

RE, IM, AB, AM, and EK designed the experiments. RE, IM, AB, and AM performed experiments and analyzed the data. RE and IM drafted the manuscript. RE, IM, and EK edited and revised the manuscript. 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/fendo.2020.00479/full#supplementary-material

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REVIEW
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Shaping of Dendritic Cell Function by the Metabolic Micro-Environment

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Nutrients are required for growth and survival of all cells, but are also crucially involved in cell fate determination of many cell types, including immune cells. There is a growing appreciation that the metabolic micro-environment also plays a major role in shaping the functional properties of dendritic cells (DCs). Under pathological conditions nutrient availability can range from a very restricted supply, such as seen in a tumor micro-environment, to an overabundance of nutrients found in for example obese adipose tissue. In this review we will discuss what is currently known about the metabolic requirements for DC differentiation and immunogenicity and compare that to how function and fate of DCs under pathological conditions can be affected by alterations in environmental levels of carbohydrates, lipids and amino acids as well as by other metabolic cues, including availability of oxygen, redox homeostasis and lactate levels. Many of these insights have been generated using *in vitro* model systems, which have revealed highly diverse effects of different metabolic cues on DC function. However, they also stress the importance of shifting toward more physiologically relevant experimental settings to be able to fully delineate the role of the metabolic surroundings in its full complexity in shaping the functional properties of DCs in health and disease.

Keywords: dendritic cells, metabolism, nutrient availability, tumor micro-environment, diabetes

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INTRODUCTION

DCs are crucial for the regulation of immunity and tolerance during infections as well as during immune homeostasis in steady state, by governing the activation and maintenance of different $CD4^+$ T helper subsets and $CD8^+$ cytotoxic T cell responses. DCs are a heterogenic population of cells that comprise several lineages, including conventional DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells (LCs), and inflammatory monocyte-derived DCs (infDCs) (1). Two main lineages can be identified within the cDCs, IRF8-dependent cDC1s and IRF4-dependent cDC2s (2). These different DC subsets have specialized largely non-redundant functions in priming and regulation of T cell responses (2). For instance, cDC1s and cDC2s are considered to be the most potent activators of T cells, that are particularly well-equipped to prime $CD8^+$ and $CD4^+$ T cell responses, respectively. pDCs, on the other hand, play an important role in the viral defense by producing large amounts of type I interferons. infDCs develop from monocytes during inflammation and have been described to have a role in innate inflammatory responses as well as in T cell priming (3). Given the scarcity of DCs *in vivo*, several *in vitro* experimental models have been developed to study DC biology. In this respect monocyte-derived DCs (moDCs), obtained from monocyte cultures supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 are a widely used model to study human DC biology and metabolism (4). moDCs share phenotypical and functional similarities with cDCs, but closely resemble infDCs at the

transcriptional level (5, 6). *In vitro* generated murine DCs are commonly derived from GM-CSF-stimulated bone marrow (BMDCs), which bear resemblance to antigen presenting monocyte-derived murine inflammatory DCs. Flt3L-stimulated bone marrow cultures (Flt3L-DCs) give rise to equivalents of multiple steady state splenic DC subsets, including cDC1s, cDC2s, and pDCs (7–9). Regulation of the functional properties of DCs is dictated by their ontogeny, but also strongly influenced by their micro-environment. While the effects of danger signals, cytokines and chemokines are extensively reviewed elsewhere, we will focus on the role of metabolic cues in tuning DC function (10–12).

Over the last decade it has become increasingly clear that immune cell activation, proliferation, fate and function are closely linked to, and dependent on activation of specific metabolic pathways (13). Since these metabolic shifts are largely fueled and dependent on nutrients present in the immune cell niche it is becoming evident that the metabolic micro-environment is a vital factor in shaping the outcome of an immune response (14, 15). This review describes the latest insights into the nutritional requirements for DCs to drive an effective immune response and how altered nutrient availability in diseased states may affect the immunogenic or tolerogenic properties of DCs. In this context we will particularly focus on nutrient-limiting and excessive nutrient conditions as found in cancer and diabetes, respectively. Overall we here aim to provide an overview of how the metabolic micro-environment affects the functional properties of DCs.

METABOLIC DEMANDS OF DCs DURING AN IMMUNE RESPONSE

During homeostasis DCs reside in peripheral tissues in a relatively quiescent state. However, upon sensing of changes in this homeostatic state, either due to invading pathogens or tissue-derived inflammatory signals, DCs undergo a well-defined activation program that involves increased capturing and processing of antigens for presentation in major histocompatibility complex I (MHC-I) and MHC-II and the induction of expression of chemokine receptors, cytokines, and costimulatory molecules. This enables DCs to traffic, via tissue-draining lymphatic vessels, to T cell zones of secondary lymphoid organs to efficiently prime and control effector T cell responses. Here, we discuss how the availability of carbohydrates (glucose), amino acids and lipids influences these functions of DCs during an immune response.

Glucose

A well-described phenomenon in immune cells, including DCs, is the switch from catabolic metabolism, characterized by fatty acid oxidation and mitochondrial respiration (**Figure 1A**) to anabolic metabolism, with enhanced glycolytic rates and lowered oxidative phosphorylation following activation (**Figure 1B**). BMDCs increase glycolytic rates within minutes after stimulation with LPS (TLR4), Poly(I:C) (TLR3), and CPG (TLR9) and also for moDCs a rapid glycolytic increase has been observed upon LPS stimulation (16–20). Likewise, human myeloid CD1c$^+$ DCs and human pDCs show a similar metabolic reprogramming upon pRNA (TLR7/8) stimulation or viral infection (TLR7), respectively (21, 22). Although this points toward increased glucose utilization to fuel glycolysis as a conserved metabolic response to TLR stimulation by DCs, the function of this metabolic reprogramming and fate of glucose-derived carbons can change over time after activation, as discussed below.

For LPS-induced BMDCs the switch to enhanced glycolysis is essential, given that inhibition of glycolysis using 2-deoxyglucose (2-DG) interferes with activation and their capacity to induce T cell proliferation *in vitro* and *in vivo* (16, 23). Correspondingly, changing glucose concentrations in culture media from 10 to 0 mM diminished the upregulation of co-stimulatory markers CD40 and CD86, and production of p40, subunit of IL-12 and IL-23 in LPS-stimulated murine BMDCs (16). In the presence of glucose, LPS stimulation enhances glycolysis via activation of the PI3K/Akt pathway and promotes loss of mitochondrial respiration via stabilization of Hypoxia-inducible factor 1-alpha (HIF1α) and expression of Inducible nitric oxide synthase (iNOS) in BMDCs (16, 17, 24). iNOS is involved in production of nitric oxide (NO) which in an autocrine manner poisons oxidative phosphorylation (17, 24). These TLR-induced events are largely absent in BMDCs activated by LPS in the absence of glucose, due to impaired mTOR activation, thereby further establishing the crucial role for glucose in supporting TLR-induced DC activation (**Figure 1B**). Mechanistically, BMDCs require glucose as a carbon source for fatty acid synthesis (FAS) to support ER and Golgi membrane expansion that is needed to accommodate the increased demands for protein translation during activation (23). To this end, glucose is metabolized in the TCA cycle to citrate, which is used as a carbon substrate for FAS (**Figure 1B**). In contrast to this anabolic role that glucose-derived carbon plays during BMDC activation, once activated, glucose is used in glycolysis by BMDCs purely for bioenergetic purposes (e.g., synthesis of ATP) to compensate for loss of OXPHOS due to the autocrine effects of NO derived from iNOS that poisons the ETC (17). Apart from direct usage of extracellular glucose, BMDCs and moDCs can also utilize intracellular glycogen stores to fuel glycolysis. These glycogen stores are formed prior to TLR stimulation and essential for activation and subsequent T cell stimulation in both moDCs and BMDCs by directly contributing to FAS (**Figures 1A,B**) (19).

Upon TLR-ligation, DCs upregulate CCR7 and are attracted toward lymphatic vessels that secrete CCR7 ligand CCL21 (25). In BMDCs the presence of glucose in the medium and subsequent activation of glycolysis are required for CCR7 oligomerization and cytoskeletal changes that support the mobility of DCs. Correspondingly, glucose depletion reduced migration of splenic DCs toward CCL21 *ex vivo* and 2-DG administration in an experimental model of allergic asthma, reduced migration of CD11c$^+$MHCIIhi DCs to the lung (26). Similarly, BMDCs pulsed *ex vivo* with OVA plus LPS in the presence of 2-DG, displayed impaired migration toward skin-draining lymph nodes following subcutaneous injection. Altogether this points toward an important role for glucose in DC migration *in vivo* (**Figure 1C**) (16).

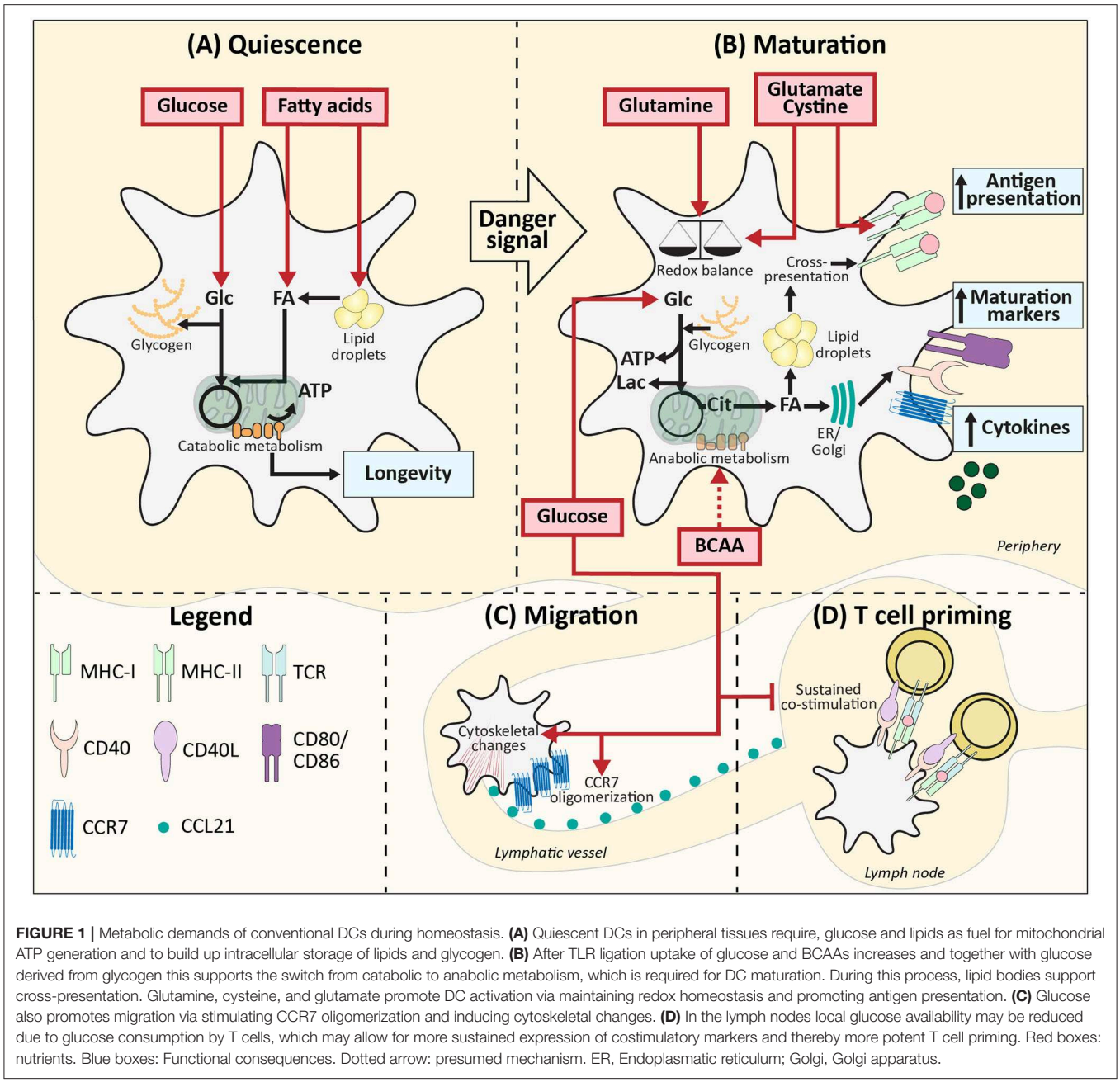

FIGURE 1 | Metabolic demands of conventional DCs during homeostasis. **(A)** Quiescent DCs in peripheral tissues require, glucose and lipids as fuel for mitochondrial ATP generation and to build up intracellular storage of lipids and glycogen. **(B)** After TLR ligation uptake of glucose and BCAAs increases and together with glucose derived from glycogen this supports the switch from catabolic to anabolic metabolism, which is required for DC maturation. During this process, lipid bodies support cross-presentation. Glutamine, cysteine, and glutamate promote DC activation via maintaining redox homeostasis and promoting antigen presentation. **(C)** Glucose also promotes migration via stimulating CCR7 oligomerization and inducing cytoskeletal changes. **(D)** In the lymph nodes local glucose availability may be reduced due to glucose consumption by T cells, which may allow for more sustained expression of costimulatory markers and thereby more potent T cell priming. Red boxes: nutrients. Blue boxes: Functional consequences. Dotted arrow: presumed mechanism. ER, Endoplasmatic reticulum; Golgi, Golgi apparatus.

Interestingly, there are indications that the role of glucose in priming of T cell responses by DCs changes once the cells interact with T cells. It has been shown that the T cell-priming capacity of BMDCs declines after 24 h following TLR stimulation, which was associated with a reduction in expression of costimulatory molecules (27). However, this reduction in expression was prevented when 8 h after stimulation glucose was replaced with galactose, a carbohydrate that cannot be efficiently used in glycolysis. These data indicate that after the initial need for glucose, sustained high glycolytic rates repress BMDC activation (**Figure 1D**). Hence a local glucose-limiting micro-environment when DCs interact with T cells, may actually support T cell priming and an active immune response (24). Interestingly, both *in vitro* and *in vivo* data suggest that reduction of glucose availability may occur naturally during T cell priming in lymph nodes, due to scavenging of glucose by T cells that are being activated by DCs (24).

Many studies addressing the role of glycolysis, use glucose analog 2-DG to mimic glucose starvation. 2-DG can reduce mitochondrial metabolism independently of reducing glycolysis (28). Moreover, treatment of LPS-activated moDCs with 2-DG has been documented to result in ER-induced upregulation of IL-23 expression, while glucose depletion did not. These data indicate that the effects of 2-DG are not always connected to

glycolysis and warrant caution when interpreting data from studies that have used 2-DG to interrogate the role of glycolysis in DC biology (29). In addition, most of the studies described above have been performed in BMDCs, in which iNOS plays a major role in the suppression of mitochondrial respiration in response to TLR stimulation. Most other DC subsets, including moDCs and primary human DCs, do not express iNOS (30). Hence, the role of oxidative phosphorylation during an immune response in human DCs may be underestimated and in physiological settings DCs may rely less on glucose as predicted based on BMDC experiments. Thus far, it has not been addressed whether other nutritional carbohydrates shape DC function, but it is worth investigating, given that an immunosuppressive role for D-mannose was found in T cells (31). In conclusion, there is clear evidence that many aspects of DC activation are dependent on availability of glucose in the micro-environment. Glucose initiates metabolic reprogramming required for activation and boosts DC migration toward lymph nodes, while during T cell interaction glucose may have an immunosuppressive effect on DCs.

Amino Acids

Amino acids are important for fueling mitochondrial respiration, for protein synthesis, as well as acting as a source of carbon and nitrogen for the synthesis of various other macromolecules. There are clear indications that amino acids in the environment of DCs play an important role in regulating their function. For example, lowering the supraphysiological amino acid concentrations commonly found in standard culture media to ones found in human plasma, increased the efficiency of moDC differentiation (32, 33). Conversely, moDCs in media containing imbalanced amino acid concentrations, as found in plasma of liver cirrhosis patients, showed impaired expression of maturation markers, secretion of IL-12 and migratory capacity in response to LPS stimulation, compared to moDCs stimulated in control medium (32, 33). The amino acid imbalance interfered with mitochondrial metabolism of immature DCs, causing a reduction in ATP levels and an increase in glucose consumption, which could not be further increased by LPS stimulation. This together supports the idea that several aspects of DC biology, including differentiation, activation and core metabolism, are sensitive to changes in amino acid concentrations in their environment (33).

In addition to the aforementioned work implicating amino acids in general in regulating DC function, there are several studies that have specifically interrogated the role of individual amino acids in this context. LPS stimulation of moDCs has been shown to enhance the uptake of aspartate, cystine, glutamate, and branched chain amino acids (BCAAs) valine, leucine, and isoleucine (33). Depletion of BCAAs and in particular valine from culture media of moDCs impairs maturation upon LPS stimulation, characterized by lowered CD83 levels and decreased ability to induce T cell proliferation. Additionally, mTOR signaling was impaired, which raises the possibility that BCAAs may affect DC maturation through modulation of metabolism via the mTOR pathway (**Figure 1B**) (34). Of note, as the above mentioned studies were performed in serum-free media with high glucose concentrations (25 mM), the relevance of these results under more physiological settings remains to be established. BCAAs are also important for maturation of moDCs stimulated with TLR7/8 ligand protamine-RNA (pRNA). In contrast to LPS stimulation, pRNA ligation boosts fatty acid oxidation (FAO)-dependent mitochondrial respiration and high intracellular levels of BCAAs are required to induce moDC maturation via FAO (20). BCAA leucine may play a key role in supporting FAO, as leucine can promote mitochondrial biogenesis (35). LPS treatment also increases uptake of glutamate and cystine in moDCs and inhibition of the cystine/glutamate antiporter in these cells reduced glutathione synthesis, but did not change the expression of maturation markers. Nevertheless, treating murine splenic DCs with an cystine/glutamate antiporter inhibitor resulted in lowered antigen presentation to both class I and class II MHC-restricted T cells (**Figure 1B**) (36). Hence, cystine and glutamate may be crucial metabolites for DC maturation via their role in redox homeostasis and antigen presentation.

As mentioned before, FAS is upregulated in BMDCs following LPS-stimulation. In cancer cells glutamine can contribute to lipogenesis via NADPH production that takes place when glutamine is metabolized to lactate or when glutamine is converted to citrate, facilitated by reductive carboxylation (37, 38). However, depleting glutamine (from 2 to 0 mM) from BMDC culture media did not affect CD40 and CD86 levels and inhibition of glutaminolysis had no effect on the metabolic alterations 6 h after LPS stimulation (19, 23). Interestingly, in BMDCs stimulated with TLR7/8 ligand imiquimod, glutamine deprivation, or disruption of glutaminolysis enhanced mitochondrial reactive oxygen species (ROS) production and subsequently IL-23 expression. This may suggest that glutamine, by supporting NADPH production, may contribute to scavenging of ROS in BMDCs, rather than to FAS-dependent activation (**Figure 1B**) (39). In addition, glutamine may fuel the TCA cycle to support oxidative phosphorylation in DCs as human pDCs increased oxidative phosphorylation following pRNA stimulation and inhibition of glutaminolysis in these cells caused a significant decrease in activation, IFNα secretion and mitochondrial respiration (21). Since activation of human $CD1c^+$ myeloid DCs using pRNA resulted in reduced oxidative phosphorylation and as immunogenicity remained unaffected by inhibition of glutaminolysis, this effect of glutamine may be selectively associated with DC subsets that depend on mitochondrial respiration upon activation, such as pDCs (21).

Apart from glutamine, the importance of availability of different amino acids on DC biology are still poorly defined and are mainly addressed in moDCs. Possibly, due to the low proliferative capacity of differentiated DCs and therefore expected relative little dependency on anabolic metabolism, general amino acid availability may be less of critical factor for DC function than for other more proliferating cells. Nonetheless, as studies exploring the role of glutamine on DC function suggest, specific amino acids may be important in regulation of certain metabolic properties of DCs that are essential for their functional output. Hence, single amino acid depletion studies under more

physiological nutrient levels may unravel novel roles of amino acids in DC function.

Lipids

In contrast to activated DCs, in which glycolysis is often the main bioenergetic pathway, immature quiescent BMDCs and Flt3L-induced cDC1s rely on FAO for energy generation, which would support a longer lifespan for these immature cells (**Figure 1A**) (16, 17, 40). Lipids from the local micro-environment may function as important nutrients for FAO in resting DCs. In human Lin-HLA-DR$^+$ and murine CD11c$^+$ hepatic DCs, high lipid content is associated with a stronger immune response (41). These lipids derived from both fatty acid (FA) uptake and synthesis and are stored in lipid bodies. Short term priming with triacyl glycerides of murine DCs containing few lipid bodies did not boost their immunogenic capacities, suggesting that pre-stored lipids rather than direct lipid availability in the micro-environment is important for hepatic DC immunogenicity (41). Mechanistically, lipid bodies in murine BMDCs and splenic CD11c$^+$ DCs have been shown to boost CD8$^+$ T cell priming by supporting cross-presentation, a process by which peptides from exogenous antigens are presented in MHC-I (42). It is therefore tempting to speculate that resting DCs may not only utilize FAs from the extracellular environment to fuel FAO for their bioenergetic homeostasis, but also to form lipid bodies to help prepare them for potent T cell priming after activation (**Figure 1A**).

In contract to conventional DCs, FAO can increase upon TLR7/8/9 stimulation of pDCs (20, 43). Interestingly, FAO in murine bone marrow-derived pDCs is fueled with FAs that are synthesized *de novo* (43). LPS is also known to increase FAS in BMDCs, possibly suggesting that FAO during an immune response predominantly depends on *de novo* synthesis and not on the FA availability in the micro-environment (**Figure 1B**) (18, 23). Correspondingly, in rats it was found that lipid content between different cell types in the same micro-environment was more similar than lipid content between DCs from distinct lymph nodes. Additionally, *in vivo* LPS stimulation diminished the differences observed between distinct DCs, supporting the notion that lipid accumulation during inflammation is independent of FA availability, while lipid storage during homeostasis does appear to be determined by the micro-environment (**Figure 1A**) (44).

In summary, it appears that during homeostasis lipid availability influences the types and amount of lipids stored in DCs and at least in some tissues this is important for their immunogenic potential. During an immune response, both conventional and pDCs accumulate lipids, most likely independent of the FA availability in the local micro-environment, but fueled by FAS. For cDCs the reduced ability of activated DCs to burn FAs by FAO may also contribute to lipid accumulation (16). Cultures in lipid-restricted conditions, ^{13}C-labeled lipid metabolic flux analysis and lipid profiling of DCs during homeostasis and upon activation can further elucidate the role of extracellular lipids on DC function.

Concluding Remarks

The metabolic demands of DCs in non-pathological conditions are dependent on the subset, their location and the maturation stage, as summarized in **Figure 1**. Given that most of these data are obtained from *in vitro* studies it is important to realize that *in vitro* nutrient availability is often not limiting and exceed the levels that occur *in vivo*. Furthermore, nutrient competition with cells in the proximity of DCs and metabolites secreted by these surrounding cells are metabolic settings that are hard to mimic *in vitro* and difficult to measure *in vivo*, but likely to affect the metabolic micro-environment. Nevertheless, it is evident that nutrient availability is of great importance for the functional output by DCs.

EFFECTS OF THE METABOLIC ENVIRONMENT ON DCs IN CANCER

Metabolic Properties of the Tumor Micro-Environment

Disturbance of nutrient homeostasis is a cause and consequence of many pathologies. A well-studied and complex disease is cancer, which is characterized by a wide range of local metabolic alterations, including nutrient deficiency, hypoxia and oxidative stress. Cancer is a heterogenous disease that arises from cells with traits that allow uncontrolled proliferation. One of these traits, or hallmarks, is avoiding immune detection, required to prevent elimination by the immune system (45). Cells within the tumor micro-environment (TME), including tumor cells, fibroblasts, endothelial cells, and immune cells secrete immunomodulatory signals that regulate the anti-tumor immune response (46). Among these factors are cytokines, growth factors and metabolites. During the initial phases of tumor growth, tumor-associated DCs (TADCs) are able to recognize tumor antigens and initiate an anti-tumor T cell response. However, during tumor progression DCs gain tolerogenic rather than pro-inflammatory properties (47–49). A major contributor to immune suppression and another hallmark of cancer is deregulated cellular metabolism (45). The best known metabolic adaptation of cancer cells is the Warburg effect, the conversion of glucose to lactate under aerobic conditions, which allows for rapid production of ATP and biosynthetic precursors (50). In addition, tumors also use amino acids and lipids to fuel the TCA cycle, which promotes ATP generation via oxidative phosphorylation and synthesis of macromolecules to support cell growth and proliferation (51–53). Another cancer-specific metabolic feature is the accumulation of oncometabolites due to mutations in metabolic enzymes. L- or D-2-hydroxyglutarate (L- or D2-HG) is a well-known oncometabolite that promotes tumor growth by regulating DNA and histone modifying enzymes (54). Finally, malignancies are often characterized by unusually high concentrations of extracellular ATP and adenosine, hypoxia and by large quantities of ROS in poorly vascularized regions (52, 55). The above mentioned metabolic changes and stressors do not only affect tumor cells, but also reach stromal cells residing in the TME. Here we will describe how these metabolic cues affect DCs.

Nutrient Starvation

The excessive utilization of carbohydrates, amino acids and lipids by cancer cells results in a limited nutrient supply for cells residing in the TME. Although there are several seminal papers showing how nutrient limitation in the TME impairs $CD8^+$ T cell function, there are few studies that have directly interrogated the contribution of nutrient starvation to the known suppressive effects of the TME on DCs (56, 57). Initially upon entering of DCs into the TME, one could imagine that TADCs may be able to utilize internal stores of glycogen and lipids to support the metabolic demands for their survival and immunogenic activation (19, 41). However, sustained limited access to glucose may impair metabolic rewiring and thereby DC maturation and migration to tumor draining lymph nodes (16, 24, 26). Likewise, based on *in vitro* studies, as discussed in section Amino Acids, it stands to reason that also insufficient access to amino acids may compromise TADC function by affecting mitochondrial respiration, redox homeostasis and antigen presenting capacity (33, 36, 39). However, to date no studies have directly addressed this. On the other hand, the effects of lipids on the function of TADCs have been studied more extensively and hence will be discussed in a separate section.

Tolerogenic properties of DCs have been linked to increased FAO and mitochondrial respiration (18, 58). These processes are both stimulated by activation of AMPK, an energy-sensing enzyme that is activated under nutrient limiting conditions (59). AMPK has been shown to be inactivated in DCs upon LPS-induced activation (16). Conversely, in TADCs of mice inoculated with MC38 colon adenocarcinoma cells, activation of LKB1, one of the main activating kinases of AMPK, was elevated (60). Hence, the nutrient-poor TME may boost AMPK signaling in DCs to induce catabolic metabolism that favors tolerogenic properties. Although no study to date has examined it directly, it is likely that limited nutrient access in the TME contributes to immune suppression of DCs (**Table 1**). Addressing the influence of tumors with different bioenergetic profiles on DC activation *in vivo* and *in situ* will provide more insights into the effects of nutrient deprivation on DC-driven immune suppression.

Lipid Accumulation in Tumor-Associated DCs

Although tumor cells are generally characterized by high lipid uptake, TADCs can also take up large amounts of FAs from the TME (106). Acquisition of lipids by TADCs is facilitated by the upregulation of genes involved in lipid uptake, including lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4) and macrophage scavenger receptor (Msr1). The lipids are stored in large lipid droplets, which are associated with a reduced capacity to activate T cells (72–74). As discussed earlier, high lipid content in hepatic DCs is associated with higher immunogenicity and LPS stimulation of BMDCs stimulates lipid droplet formation (23, 41). However, in contrast to lipid droplets of these DCs, lipid droplets from TADCs contain high levels of oxidized polyunsaturated FAs. These oxidized FAs cause accumulation of peptide-bound MHC-I complexes in late endosomes and lysosomes via capturing of heat shock protein 70, an important mediator of cross-presentation (75, 76). This

TABLE 1 | Metabolic determinants from pathological environments that influence DC function.

Effects associated with the TME	Effects associated with diabetes	References
Glucose		(16, 19, 23, 26, 61–71)
Local depletion	Hyperglycemia	
Impaired anabolic metabolism ≫ ↓ Activation ≫ ↓ Migration *AMPK activation* ≫ ↓ *Activation*	Blood DCs ↓/↑ DC counts = Activation ↑ *Migration to metabolic tissues* *In vitro* DCs ↓/ = Differentiation ↑ Activation ≫ ↑ *Th17 T cell priming*	
Lipids		(39, 72–83)
High intracellular storage	Hyperlipidemia	
Oxidized lipids ↓ Cross-presentation ↑ *Fatty acid oxidation* ≫ ↓ Activation	PA ↑ NFκB signaling Metabolic rearrangements ≫ ↑ Activation SA ↓ Activation OA = Activation	
Oxygen levels		(84–89)
Low	Low	
= Differentiation ↑ Innate immunity (immature DCs) ↑ Migration (immature DCs) = /↑ /↓ Activation ↓ Migration (mature DCs)		
ROS		(90, 91)
High	High	
Short term ROS ↑ Activation Long term ROS *Lipid oxidation* ≫ ↓ *Activation*		
Lactate		(92–96)
High		
Impaired mitochondrial respiration ≫ ↓ Differentiation ↓ Cross-presentation *Epigenetic modifications* ≫ ↓ Activation		
ATP and adenosine		(97–104)
High		
ATP ↑ Migration NLRP3 inflammasome activation ≫ ↑ $CD8^+$ T cell priming ↑ regulatory T cell priming Adenosine ↓ Activation		
2-Hydroxyglutarate		(105)
High		
↓ IL-12 ↑ Catabolic metabolism ≫ = Activation *Epigenetic modifications*		

Overview of extracellular metabolic cues observed in cancer and diabetes that affect DC function. ↑/↓/=: increased, decreased or equal effect compared to control conditions. ≫ Consequence of aforementioned mechanism. Italics: presumed effect.

limits cross-presentation and thereby priming of cytotoxic T cell responses (**Table 1**). Given the importance of FAO in supporting a tolerogenic phenotype by DCs it is tempting to speculate that perhaps oxidation of these lipids in the mitochondria contributes to DC tolerogenicity within the TME (**Table 1**) (18, 58). What signals trigger the initial increase in lipid uptake by DCs in the TME remains to be determined.

Hypoxia

Rapid tumor growth results in poorly vascularized regions where oxygen supply is limited. The main metabolic response to hypoxia is stabilization of HIF1α and subsequent activation of glycolysis, which can also occur independently from HIF1α activation during DC activation (16, 17, 107). This may explain why some studies did not find changes in expression of maturation markers or T cell priming capacity when moDCs were stimulated with LPS after differentiation in a 1% O_2 hypoxic chamber (**Table 1**) (84, 89). Also differentiation itself of moDCs is mostly unaffected by hypoxic conditions (84–86, 89). Nonetheless there is also some evidence that 1% oxygen can impair LPS-induced moDC maturation and T cell priming potential (**Table 1**) (85, 87). In addition, hypoxia impaired migration of *in vitro* cultured LPS-treated moDCs and human primary myeloid DCs. Mouse $CD34^+$-derived myeloid DCs injected in the footpad of mice after they were treated with LPS and deferoxamine (DFX), a drug that mimics hypoxia, showed reduced migration to the draining lymph node compared to untreated cells, indicating that hypoxia reduces migration both *in vitro* and *in vivo* (87, 89). Interestingly, enhanced expression of migratory genes was found in immature moDCs cultured under low oxygen conditions (86). This could indicate that the hypoxic TME has immunosuppressive effects via capturing mature DCs and elimination of immature DCs. Interestingly, immature moDCs cultured under hypoxic conditions had increased expression of genes involved in sensing chemotactic signals from pro-inflammatory sites and induced secretion of chemotactic factors that attract neutrophils (84, 85, 87). Additionally, murine myeloid DCs treated with DFX increased local leukocyte infiltration *in vivo* (87). Altogether these data obtained from moDCs, may indicate that an oxygen-poor environment triggers DCs to boost innate rather than adaptive immune responses. However, contrary to human moDCs, murine BMDCs cultured in hypoxic conditions enhanced expression of costimulatory molecules, pro-inflammatory cytokine secretion and T cell proliferation upon LPS stimulation (**Table 1**) (88). Additional studies are needed to determine whether these discrepancies are due to inherent differences between human and murine DCs in their response to hypoxia or caused by differences in experimental setup. In addition, the metabolic context in which DCs are exposed to hypoxia may also affect how DCs respond. For instance, under nutrient replete conditions hypoxia may not compromise DC function, such as in lymph nodes in which hypoxic region have been described (108). In contrast, under pathological conditions, such as in the TME where hypoxia may be also accompanied by nutrient restriction, hypoxia may have anti-inflammatory effects on DCs. Studies addressing the effects of hypoxia on DC biology particularly *in vivo* during homeostasis as well as in pathological settings are needed to fully understand the role of oxygen availability on DC function *in situ*.

Oxidative Stress

The main sources of ROS in tumor cells are dysfunctional mitochondria and NADPH oxidases. This is further enhanced by intracellular ROS production of stromal cells, as a consequence of the metabolic alterations within the TME (109, 110). Intracellular ROS production in DCs during an immune response can have both pro-inflammatory and anti-inflammatory effects, via modulation of cross-presentation and of signaling pathways (39, 61, 77, 110–112). In general, extracellular ROS seems to have a pro-inflammatory effect, although data is limited (**Table 1**). Treatment of immature moDCs with hydrogen peroxide enhanced maturation and their capacity to induce T cell proliferation upon LPS stimulation (90). The inflammatory response of primary DCs to the malaria parasite *Plasmodium falciparum* also increased upon exposure to cells treated with xanthine oxidase, a malaria-induced enzyme that increases extracellular ROS levels (91). However, while transient ROS exposure following DC activation may have pro-inflammatory effects, what the functional consequences are of chronic ROS exposure, a situation DCs presumably have to deal within the TME, remains unclear. Possibly, the highly oxidized lipids that are found in TADCs, are one of the byproducts of chronic ROS exposure (113). Through this mechanism long-term ROS exposure in the TME could lead to impaired DC immunogenicity. Studies addressing the functional consequences of transient vs. chronic ROS exposure as well as of the different types of ROS on DCs, will be required to better define what role tumor-associated ROS and oxidative stress play in DC function in the TME.

Lactate

Tumor cells are known for the Warburg effect, which goes along with secretion of high levels of lactate. Lactate has a major influence on the immune-priming efficiency of DCs. Lactate derived from tumor spheroids, mesenchymal stromal cells or endogenously produced, affects the differentiation and maturation of moDCs. High concentrations of lactate reduce the differentiation capacity of moDCs, as higher numbers of monocyte like $CD14^+/CD1a^-$ cells were detected at the end of the cultures (92–94). This was accompanied by a lactate-dependent reduction of oxidative phosphorylation, but enhanced respiratory capacity in immature moDCs (94). Also maturation of DCs is affected by high lactate levels, reflected by lower levels of maturation markers, an increase in immunosuppressive cytokine secretion, a decrease in pro-inflammatory cytokine secretion and reduced ability to induce T cell proliferation (92, 93, 95). The latter can be caused by detrimental effects of lactate on cross presentation. Using tumor conditioned Flt3L-DCs stimulated with CpG/PolyI:C and OVA-peptide, it was found that high lactate concentrations accelerate antigen processing via lowering the endosomal pH, resulting in impaired preservation of MHC-I epitopes. Thus, high concentrations of lactate in the local environment of differentiating or maturing DCs induces tolerance in DCs, via altering metabolism and antigen processing

(**Table 1**). Extracellular lactate can mediate its anti-inflammatory function via binding to lactate receptor Gi-protein–coupled receptor 81 (GPR81) as was recently shown in DCs derived from murine mammary gland tumors (114). Alternatively, lactate can enter the cells via monocarboxylate transporter 1 (MCT1) as was shown in moDCs (92). Intracellular lactate may also hamper the immune response via a recently discovered epigenetic modification termed lactylation. In M1 macrophages endogenously produced lactate promoted lactylation of lysine residues, thereby promoting M2-like gene expression (115). Whether histone lactylation is another immunosuppressive feature of lactate in DCs is an interesting question that warrants further study.

ATP and Adenosine

Whereas during homeostasis extracellular ATP levels are negligible, ATP is highly abundant in the TME where it functions as a signaling molecule that provokes inflammation (55, 116). It has been proposed that diffusion of ATP out of the TME recruits DCs to the TME, given that BDMCs treated with 500 uM ATP increased migratory speed (**Table 1**) (97, 98). Moreover, extracellular ATP released by tumor cells after chemotherapy can promote anti-tumor immunity via signaling through ATP-receptors P2RX7 on DCs, thereby activating the NLRP3 inflammasome, enhancing IL-1β secretion and boosting $CD8^+$ T cell priming (99, 100). In contrast, moDCs co-cultured with acute myeloid leukemia cells treated with chemotherapy drugs displayed increased potency to expand regulatory T cells in an extracellular ATP-dependent manner (**Table 1**) (101). Hence, there is great value in understanding what factors determine the balance between the pro- and anti-inflammatory effects by extracellular ATP after chemotherapy, as it may be an important predictor for treatment efficacy.

Paradoxically, immunosuppressive nucleoside adenosine, derived from conversion of ATP by membrane-bound ectonucleosides CD39 and CD73, is also abundantly present in the TME (117, 118). Adenosine interacts with four different receptors, of which A2AR and A2BR are most highly expressed on immune cells (119). Irradiation of mouse breast tumors caused upregulation of CD73 expression in tumor cells and increased local adenosine concentrations. Anti-CD73 treatment enhanced cDC1 tumor infiltration, increased the antitumor T cell response and reduced tumor growth (102). Additionally, in mice in which adenosine receptor A2BR was selectively knocked out in $CD11c^+$ DCs, the growth of B16-melanoma was delayed, supporting a role for adenosine signaling in rendering DCs immunosuppressive (103). Furthermore, LPS-stimulated BMDCs treated with adenosine analog NECA increased intracellular cAMP levels, which lowered secretion of IL-12 and TNF-α secretion and enhanced IL-10 release via protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) signaling (104). Overall most studies indicate that extracellular ATP enhances immunogenicity of DCs and anti-tumor immune responses, while adenosine does the opposite (**Table 1**) (98, 120). Shifting the balance in favor of ATP by blocking CD73, CD39 or adenosine receptors is therefore a promising immunotherapy.

2-Hydroxyglutarate

In various tumors the oncometabolite 2-HG accumulates, which in non-malignant tissues is found at low concentrations (54). 2-HG has been shown to contribute to immune suppression in the TME via anti-inflammatory effects on T cells (121, 122). Immature moDCs cultured for 24 h with LPS and 2-HG secreted reduced levels of IL-12, enhanced mitochondrial respiration and lowered lactate secretion, indicating that accumulation of 2-HG affects moDCs via metabolic reprogramming. However, the ability of DCs to induce T cell proliferation remained the same, suggesting that the 2-HG-induced metabolic rearrangement in DCs does not affect their T cell priming capacity (**Table 1**) (105). However, in this context 2-HG was added simultaneously with the TLR ligand, while in the TME immature DCs may reside in a 2-HG-rich environment without immediate activation. Long-term exposure to 2-HG may have a stronger effect on the immunogenic capacities of DCs, potentially via the changes in gene expression, given that 2-HG affects activity of DNA and histone modifying enzymes, but this remains to be determined (54).

Concluding Remarks

Thus far the effects of metabolic perturbations characteristic of the TME on DC biology have been primarily studied in *in vitro* systems using moDCs. However, we have still limited knowledge about the real contribution of those metabolic changes on the functional properties of conventional as well as inflammatory DCs residing in the TME *in situ*. Likewise, if and to what extent these different metabolic perturbations interact and synergize to affect the functional properties of DCs remains to be determined. For successful activation of the immune system via DC-based therapy it is important to know how DCs deal with these metabolic rearrangements in the TME. For instance, how do DCs respond to adjuvants in the metabolic context of the TME? Is there a way to make these cells less vulnerable to potential immunosuppressive metabolic cues from the TME? And once out of the immunosuppressive metabolic TME, how quickly can DCs regain immunogenic function, if at all possible? To answer these questions and to gain better understanding of the immunosuppressive effects of the metabolic TME on DCs, in depth characterization of the metabolic TME and DC phenotype in primary tumors will be key.

EFFECTS OF THE METABOLIC ENVIRONMENT ON DCs IN DIABETES

Interplay Between Metabolic Disturbances and Inflammation Leading to Diabetes

Not only nutrient deprivation, but also excessive amounts of nutrients can disturb immune homeostasis and DC function. A well-known example of a disease that is characterized by elevated concentrations of glucose and lipids is diabetes. The two main types of diabetes are type I and type II Diabetes Mellitus (T1D/T2D), both characterized by dysfunctional insulin regulation and subsequent hyperglycemia. While T1D develops

as a consequence of an auto-immune reaction against beta-cells, common causes for T2D are aging and obesity. Obesity causes hyperglycemia, elevated levels of free fatty acids, hypoxia, oxidative stress and an imbalance in many other metabolites, hormones and cytokines (123–126). This causes a switch in the composition and phenotype of immune cells in metabolic tissues from an anti-inflammatory to a more pro-inflammatory profile and thereby induces chronic low-grade inflammation, which ultimately drives insulin resistance (127–129). cDCs and pDCs are among the immune cells present in adipose tissue and there is a clear correlation between insulin resistance and number of $CD11c^+$ DCs present in adipose tissue (62, 130–132). Moreover, several studies have shown that in response to high-fat diet DCs present in murine adipose tissue transition from Th1- to Th17-priming cells, an inflammatory profile linked to the pathogenesis of diabetes (62, 63, 133).

We will here describe how DCs are affected by the metabolic changes in their environment and focus on hyperglycemia and elevated levels of free fatty acids. Oxidative stress and hypoxia are also major metabolic players in diabetes, but to our knowledge there is no data available looking at the effects of these conditions on DC function in diabetic context (125, 126). Hence, we refer to the previous section for the effects of oxygen deprivation and excessive ROS levels on DC function.

Hyperglycemia

Decreased insulin secretion by beta-cells and lowered sensitivity to insulin signaling reduces the uptake of glucose by cells, which subsequently results in elevated blood glucose levels. As glucose availability plays a major role in DC activation it is conceivable that this glucose imbalance affects DC function. Several studies addressed the effects of hyperglycemia on primary dendritic cells from blood. Both a reduction and an increase in myeloid and pDC counts in blood of patients with T1D and T2D have been reported (**Table 1**) (64–67, 71). Reduced counts seems to be stronger in patients with poor glycemic control (66). Pro- and anti-inflammatory cytokine secretion by DCs from diabetic patients was not altered following *ex vivo* TLR stimulation, indicating that high blood glucose levels do not directly affect the function of circulating DCs, but primarily their numbers (65, 68, 71). It should be noted that hyperglycemia is not the only (metabolic) difference in blood from diabetic patients and other factors may also influence DC frequencies and function. Since a study in mice showed that hyperglycemia does not influence $CD11c^+$ DC differentiation in the bone marrow, it is unlikely that the decrease in circulating DCs is a consequence of impaired DC generation (69). Instead, lower numbers of circulating DCs are possibly a reflection of enhanced migration of DCs to metabolic tissues, where DCs are known to accumulate and contribute to the low-grade inflammation observed in metabolic tissues of T2D patients. As previously mentioned DCs in obese adipose tissue drive a Th17 inflammatory response (62, 63). Interestingly, moDCs exposed to 5.5, 15, and 30 mM glucose for 24 h increase CD83 and CD86 expression and secretion of IL-6 and IL-12 in a dose-dependent manner (61). IL-6 is involved in Th17 differentiation of naïve T cells and was also found to be highly secreted by $CD11c^+$ DCs from obese adipose tissue, suggesting that high glucose levels in adipose tissue may contribute to conditioning DCs for Th17 priming (**Table 1**) (63, 134). However, *in vivo* data connecting glucose levels to DC function in metabolic tissues is currently lacking. *In vitro* generation of tolerogenic moDCs was less efficient with monocytes derived from T1D patients with poor glycemic control in comparison to patients who maintained glycemic control, supporting the hypothesis that a hyperglycemic environment promotes a more pro-inflammatory profile (135). On the other hand, moDCs derived from T2D donors compared to healthy control donors expressed lower levels of maturation markers (64). Moreover, moDC differentiation in high-glucose medium (25 mM) or media supplemented with sera from hyperglycemic T2D patients reduced the number of moDCs, expression of maturation markers and the capacity to induce T cell proliferation after LPS stimulation. In addition, glucose-rich micro-environments increase ROS production and promote activation of the p38 MAPK and Wnt/b-catenin pathways, which are associated with tolerogenic properties of DCs (70, 136–138). Together these *in vitro* studies may indicate that over-abundance of glucose drives monocyte differentiation toward less-proinflammatory DCs, while differentiated moDCs and potentially $CD11c^+$ DCs residing in adipose tissue may become more immunogenic in a hyperglycemic environment (**Table 1**).

Free Fatty Acids

A cause and consequence of obesity and insulin resistance in T2D is the release of free fatty acids by expanding fat mass (124, 139). FAs are well-known regulators of the immune response. Polyunsaturated FAs (PUFAs) often have anti-inflammatory effects while many saturated fatty acids (SFAs) serve as pro-inflammatory molecules (140, 141). Examples of the latter are palmitic acid (PA) and stearic acid (SA), which together with unsaturated oleic acid (OA) are among the most abundant dysregulated FFAs in obese and T2D patients (123, 142). Especially PA is known for its pro-inflammatory effects and detrimental role in T2D pathogenesis (143). This is partly caused via its effects on DCs. PA in combination with LPS can enhance Th1-associated inflammation, which is driven by TLR4-dependent activation of the NFκB pathway and ROS in moDCs (77, 78, 144). PA also boosts inflammatory properties of activated BMDCs in a TLR4-independent manner, via inhibition of hexokinase (HK) during the late stages of metabolic reprogramming. This inhibition of HK and thereby glycolysis resulted in enhanced mitochondrial respiration, increased mitochondrial ROS levels, elevated activation of the unfolded protein response (UPR) and subsequent induction of IL-23 expression. UPR-dependent IL-23 expression was also confirmed in mice fed a high fat diet (39). BMDCs derived from obese mice additionally increased IL-1β secretion in a NLRP3 inflammasome-dependent manner following stimulation with PA (79). IL-1β and IL-23 are key cytokines involved in promoting Th17 responses and hence PA is a potential driver of insulin resistance (133). However, a direct causal link between enhanced pro-inflammatory cytokine secretion by DCs and Th17 induction in settings of FA exposure still needs to be established as for instance DCs isolated from human blood

displayed a reduced capacity to prime T cell responses upon stimulation with PA, despite having increased IL-1β and TNF secretion (80). In contrast to PA, SA does not seem to affect DC function. SA treatment of LPS-stimulated moDCs did not affect expression of maturation markers nor the capacity to induce T cell proliferation (81). Although data on the effects of SA treatment of DCs is still limited, this appears to be different from what is known for macrophages, where SA and PA have been reported to have a similar pro-inflammatory effect (145, 146). OA is a mono-unsaturated FA that has beneficial effects on insulin resistance. In macrophages, this is partly mediated by counteracting the pro-inflammatory effects of SFA (143). Thus far, there is no data available indicating an anti-inflammatory role for OA in DCs. OA treatment had either no effect, or boosted a pro-inflammatory immune response, but has never been tested in combination with SFA stimulation (78, 80, 82, 83).

The balance in dietary intake of SFAs and PUFAs can have great influence on the clinical outcome of diabetes. A comparison of 6, 12, and 24% of SFA in the diet of mice without changing the total dietary fat contribution had a profound effect on macrophage function and insulin resistance, with 12% SFA as the greatest contributor to inflammation and insulin resistance (147). Human data is however thus far inconclusive about the beneficial effects of PUFA-rich and SFA-poor diets on glycemic control of T2D patients (148). Therefore, studies in humans and mice with a focus on the composition in dietary fat and profiling of DCs in metabolic tissues may tell us whether DCs contribute to PUFA-mediated protection against diabetes and/or SFA-mediated development of diabetes, potentially via a PA-induced Th17 response.

In conclusion, compared to other immune cells, there is still little known about the effects on DCs of the FFAs that are most abundant in obesity and T2D patients. While PA stimulation of DCs seems to have the expected pro-inflammatory effects, it is remains unclear if SA and OA influence insulin resistance via DCs (**Table 1**).

Concluding Remarks

T1D is characterized by an active immune response against beta-cells, while T2D is associated with chronic low-grade inflammation. Hence, it is perhaps somewhat surprising that *ex vivo* data indicate that hyperglycemia has minimal effect on the function of DCs, that most *in vitro* studies describe a tolerogenic effect of excessive glucose levels on DC differentiation, and that only *in vitro* mature DCs are likely to become more pro-inflammatory. Although currently it cannot be excluded that the latter observations may be due the use of *in vitro* model systems, such as *in vitro* generated moDCs that possibly cannot sufficiently mimic the metabolic alterations and glucose-rich environment that cDCs and pDCs are exposed to *in vivo*, it may in fact indicate that hyperglycemia is not a major driver of the pro-inflammatory profiles of DCs observed in diabetes and that other metabolic and/or immunological cues are more important (62, 130–132). This idea would be consistent with the fact that hyperglycemia is generally associated with impaired immune response against for example infections and tumors (149, 150). A better understanding of how overabundance of various nutrients may act in concert to modulate DC function and to thereby contribute to local inflammation in the context of diabetes will be important for identification of the pathways that lead to inflammation-driven insulin resistance that could be targeted to control diabetes.

PERSPECTIVES AND OUTLOOK

It is becoming evident that the metabolic micro-environment has a major influence on DC function and that disturbance of metabolic homeostasis can impact immune responses. We aimed to provide an overview of key metabolites that influence DC phenotype and function. Cancer and diabetes are examples of highly prevalent disorders in which metabolic homeostasis is disturbed, but many more pathologies are associated with dysregulated metabolism. Eating disorders alter nutrient availability, organ-specific pathologies, such as hepatic steatosis impair systemic metabolism and oxidative stress is associated with many diseases including atherosclerosis, cardiovascular diseases and neurodegenerative disorders. Hence, understanding the impact of nutrient availability on the function of immune cells, including DCs, is relevant for a broad range of diseases. Reprogramming the metabolic state of DCs by intervening with nutrient availability can be an effective way to control inflammation. This could be achieved by systemic approaches, including nutritional interventions which are commonly used to control inflammation (151). For instance, lowering caloric intake, by reducing fat and glucose content, can improve glycemic control and subsequently reduce diabetes-associated low-grade inflammation (152–154). Given the pro-inflammatory effects of high glucose and SFA concentration on tissue-associated DCs, it is reasonable to assume that dietary interventions that lead to normalization of glucose and SFA concentrations in the tissue that these cells reside in, will render them less pro-inflammatory, thereby contributing to reduction of local tissue inflammation and eventually improvement of metabolic homeostasis. Alternatively, molecular approaches that directly target cellular nutrient uptake or bioenergetic pathways can make DCs potentially less vulnerable to extracellular nutritional changes and interventions that target energy-sensing enzymes like AMPK can also control inflammation (155). In addition, targeting metabolism of non-immune cells can also have a beneficial effect on the metabolic micro-environment of DCs. For example, therapies that interfere with cancer metabolism to directly impair tumor growth could also have indirect anti-tumor effects by potentially creating a TME with higher nutrient availability that would be more permissive to effective anti-tumor immune responses (156).

Current studies addressing the effects of the metabolic micro-environment on DCs are mostly performed *in vitro* using human moDCs or murine BMDCs. While these studies have provided us important new insights into how nutrient availability can shape DC function, *in vitro* culture conditions often do not fully mimic the complexity and concentrations of various nutrients and metabolites these cells are exposed to *in situ*. For instance, *In vitro*-generated DCs are commonly

cultured in media supplemented with 10% fetal calf serum (FCS) and glutamine. Serum is a source for lipids, vitamins, hormones, growth factors, and other compounds, but the exact amounts of these compounds are unknown, differ per batch and may not correspond with concentrations found in tissues that DCs reside in Yao and Asayama (157). Moreover, commonly used culture media, such as RPMI 1640 and DMEM contain supraphysiological levels of glucose (11 and 25 mM, respectively, vs. ∼5.5 mM *in situ*) and lower levels of electrolytes including calcium and magnesium (158). The effects of nutrient availability on the function of DC subsets in a more physiological environment, with other metabolic and non-metabolic immunomodulatory signals around, need to be further evaluated, to be able to better assess what the *in vivo* contribution of the metabolic micro-environment is on the functional properties of DCs. To tackle this issue, there have been recent efforts to develop human plasma-like, physiological medium, which contains components, such as amino acids, metabolites, salt ions, and vitamins that are absent from standard media and holds physiologically relevant concentrations of common media components. To minimize the effects of FCS-derived components, medium can be supplemented with either a low percentage (2.5%) of FCS or dialyzed FCS (159–161). Studies using these media found enhanced *in vitro* T cell activation and increased biological similarity between cultured breast cancer cells and primary mammary tumors, providing promising first evidence that these types of media could be used to better mimic physiological setting *in vitro* than classically used culture media (160, 161). These tools will likely also be key to further the field of DC metabolism and to better delineate the interplay between DC function and extra- or intracellular metabolism. In addition, various novel mass-spectrometry, high dimensional flow cytometry and transcriptomics platforms have been developed in recent years that enable one to assess metabolic profiles in tissues at high spatial resolution as well as to characterize metabolic and immunological phenotypes of immune cells present in those tissues at the single cell level. These novel techniques will no doubt greatly improve our understanding of how nutrients shape DC function *in situ*.

Even though many open questions remain, recent work has revealed profound effects of the metabolic micro-environment on DC function in health and disease, which may pave the way for developing DC metabolism-based approaches to treat metabolic and inflammatory disorders.

AUTHOR CONTRIBUTIONS

EB wrote the manuscript and prepared the figure. BE supervised and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Gastrointestinal Helminth Infection Improves Insulin Sensitivity, Decreases Systemic Inflammation, and Alters the Composition of Gut Microbiota in Distinct Mouse Models of Type 2 Diabetes

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Type 2 diabetes (T2D) is a major health problem and is considered one of the top 10 diseases leading to death globally. T2D has been widely associated with systemic and local inflammatory responses and with alterations in the gut microbiota. Microorganisms, including parasitic worms and gut microbes have exquisitely co-evolved with their hosts to establish an immunological interaction that is essential for the formation and maintenance of a balanced immune system, including suppression of excessive inflammation. Herein we show that both prophylactic and therapeutic infection of mice with the parasitic hookworm-like nematode, *Nippostrongylus brasiliensis*, significantly reduced fasting blood glucose, oral glucose tolerance and body weight gain in two different diet-induced mouse models of T2D. Helminth infection was associated with elevated type 2 immune responses including increased eosinophil numbers in the mesenteric lymph nodes, liver and adipose tissues, as well as increased expression of *IL-4* and alternatively activated macrophage marker genes in adipose tissue, liver and gut. *N. brasiliensis* infection was also associated with significant compositional changes in the gut microbiota at both the phylum and order levels. Our findings show that *N. brasiliensis* infection drives changes in local and systemic immune cell populations, and that these changes are associated with a reduction in systemic and local inflammation and compositional changes in the gut microbiota which cumulatively might be responsible for the improved insulin sensitivity observed in infected mice. Our findings indicate that

carefully controlled therapeutic hookworm infection in humans could be a novel approach for treating metabolic syndrome and thereby preventing T2D.

Keywords: type 2 diabetes, *Nippostrongylus brasiliensis*, helminth, eosinophils, M2 macrophages, high glycemic index diet, high fat diet, microbiota

INTRODUCTION

Diabetes is a metabolic disease resulting from the absence of, or deficiency in, insulin secretion, insulin action or both, leading to an abnormal metabolism of carbohydrates and elevated levels of glucose in the blood (1). The main types of diabetes are type 1 (T1D), which represents around 10% of all diabetes cases, and type 2 (T2D), which represents around 90% of all diabetes globally. Diabetes is a fast-growing health problem worldwide. According to the International Diabetes Federation there were 424.9 million people living with diabetes, and a further 352.1 million with impaired glucose tolerance in 2017 (1). Diabetes caused 4 million deaths and accounted for 10.7% of global all-cause mortality and cost USD 727 billion in healthcare spending in 2017 alone (1).

Cumulative evidence suggests that T2D is associated with inflammation. Induction of T helper 1 (Th1) immune responses, in particular activation of M1 macrophages (MACs) and increased production of pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α, and IL-6 play a crucial role in the destruction of pancreatic β-cells, and insulin resistance in adipose tissue (AT), liver and muscle (2). In contrast, cells such as type 2 innate lymphoid cells (ILC2s), eosinophils, and M2 MACs, as well as increased levels of Th2 cytokines such as IL-5, IL-4, and IL-13 have been found to regulate adipose tissue homeostasis (3, 4), liver regeneration (5), and gastrointestinal homeostasis (6), leading to whole body metabolic homeostasis. Moreover, regulation of metabolic homeostasis and inflammation in obesity, metabolic syndrome and T2D has been increasingly connected with the gut microbiota (7). Disturbance of the intestinal microbial community leads to altered immune responses that can result in various inflammatory disorders (8).

Environmental changes such as altered dietary habits, improved sanitation, vaccination and excessive use of antibiotics has reduced our exposure to various infectious agents and symbiotic microorganisms that had a co-evolutionary relationship with humans (9). This relationship has established an immunological interaction with highly developed regulatory pathways that serve to dampen inappropriate immune responses, which are considered the key drivers in many immune-mediated disorders, including T1D (10). Helminth infections induce Th2 immune responses by expansion of innate immune cells such as eosinophils, M2 MACs, ILCs, and upregulation of cytokines such as IL-4, IL-5, and IL-13. Furthermore, it has been widely shown that helminth infections promote expansion and/or recruitment of regulatory T cells (Tregs) that play an important role in regulating inflammation (11). Recent experimental evidence in animal models has highlighted the therapeutic role of helminth-mediated induction of Th2- and Treg-mediated immune responses in many inflammatory diseases such as inflammatory bowel disease (IBD), multiple sclerosis (MS), rheumatoid arthritis, asthma and T1D (12). Likewise, helminth infections have shown promising results as a therapeutic strategy in human subjects with IBD, celiac disease and MS (13–16).

In the context of diabetes, epidemiological studies from helminth-endemic areas such as Indonesia, rural China, India and Aboriginal communities from North-West Australia found an inverse relationship between helminth infection and incidence of T2D (17–20). Additionally, it has been shown that infections of mice with different species of parasitic helminths are associated with significant increases in ILC2s, eosinophils, M2 MACs, and Th2 cytokines that result in restoration of glucose levels and improved insulin sensitivity in mouse models of obesity (3, 4, 21–24).

Diabetes has been found to associate with alterations in the composition of the gut microbiota. Helminth infection in humans has also been shown to modulate the composition of the gut microbiota (25–27). For example, celiac disease patients infected with the hookworm *Necator americanus* and challenged with gluten showed improved oral tolerance to gluten and displayed an increased species richness in intestinal microbial species, notably the Bacteroidetes (27–29). A protective role for helminth-microbiota interaction in mice has been demonstrated against many inflammatory diseases such as allergy (30), IBD (31) and obesity (32). Human studies as well as studies in animal models of obesity and T2D revealed a shift in the abundance of the dominant gut phyla Bacteroidetes and Firmicutes (7). Shifts in the abundance of these phyla has also been observed after infection with the gastrointestinal nematodes *Nippostrongylus brasiliensis*, *Trichuris muris*, and *Heligmosomoides polygyrus* (33–36), suggesting that helminth infections might have a positive role in maintaining gut homeostasis and preventing the development of T2D *via* modulation of the gut microbiota and short chain fatty acids (SCFAs) (32, 37).

Previous studies have focused on the prophylactic effects of gastrointestinal nematode infection on high-fat diet-induced metabolic syndrome. To better reflect the current pandemic of human T2D, we infected mice fed on both high-fat (HF) and high-glycaemic index (HGI) diets before and after the onset of metabolic syndrome. We showed that infection with *N. brasiliensis* maintains glucose homeostasis both prophylactically and therapeutically, probably *via* induction of Th2 immune responses in lymphoid and non-lymphoid tissues in mice. Infection with *N. brasiliensis* was also associated with changes in some phyla and orders of the gut microbiota.

MATERIALS AND METHODS

Ethics Statement

All procedures were approved by the James Cook University Animal Ethics Committee, ethics application number A2244. The study protocols were in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act. *N. brasiliensis* was maintained in Sprague–Dawley rats (Animal Resources Centre, Perth, WA, Australia) as described elsewhere (38) (Ethics application number A2300).

Animals and Diet

Male C57BL/6 wild-type (WT) (JCU Townsville) mice were used for all experiments (10 mice per group). At 5 weeks of age mice were divided into three groups: (i) normal chow (NC); High Glycaemic Index (HGI) diet with a glycaemic index of close to 100 (SF03-30; Speciality Feeds, Western Australia); High Fat (HF) diet where 61% of total energy is from lipids (SF07-066; Speciality Feeds, Western Australia). **Table S1** describes the composition of each diet.

Helminth Infection

N. brasiliensis life cycle was maintained in our laboratory at James Cook University in a specified pathogen free environment. Briefly, feces from *N. brasiliensis*-infected rats were collected from days 5–9 post-infection. Egg-containing feces were mixed with an equal amount of water and charcoal, distributed into Petri dish plates and incubated at 26°C. One week after incubation, L3 were collected from the fecal/charcoal culture plate, washed three times with PBS, then all infections with *N. brasiliensis* were performed by inoculating subcutaneously 500 third-stage larvae of *N. brasiliensis* (*Nb*L3) into the skin over the interscapular region. *N. brasiliensis* is immunologically cleared from mice within a few weeks, so we reinoculated mice once every month with 500 *Nb*L3 starting at 6 weeks of age for mice receiving prophylactic infections or 24 weeks of age for mice receiving therapeutic infections.

Fasting Blood Glucose and Oral Glucose Tolerance Test

Food was withdrawn for 6 h then fasting blood glucose (FBG) was measured in the unfed mice. Blood sampling was performed by tail bleeding. Mice were screened for blood glucose levels every 2 weeks using Accu-Check® Performa (Roche). Mice were considered diabetic when glucose levels reached >12.0 mmol/L. For the oral glucose tolerance test (OGTT), after initial blood collection (time 0) in the 6-h unfed mice, mice were administered D-glucose orally (2 g/kg body weight) by gavage. Blood sampling was performed by tail bleeding at 15, 30, 60, 90, and 120 min after administration of glucose.

Isolation of Mesenteric Lymph Nodes, Adipose Tissue, and Liver

In brief, epididymal fat pads or liver from male mice fed with NC, HGI, or HF diets were removed and minced into small pieces. Minced tissues were then transferred to a 50 ml conical tube containing 1 ml DPBS (0.5% BSA) (Sigma) and 3 ml collagenase type II (Life Technologies), and incubated in a rotating shaker (200 rpm) at 37°C for 35 min. The homogenates were filtered through a 70 μm tissue strainer into a new tube and centrifuged at 500 g for 10 min. at 4°C. Following centrifugation, the supernatant was discarded and the pellet was resuspended in 1× red blood cell lysis buffer (Sigma) followed by a washing step with 5 ml FACS buffer, and a final centrifugation at 500 g for 10 min at 4°C.

Mesenteric lymph nodes (MLN) were collected and transferred to a 5 ml tube containing 1 ml of RPMI media (Gibco), then filtered through a 70 μm tissue strainer. Cell viability was assessed by Trypan Blue and cells were blocked using FcR blocking reagent (BD biosciences) for FACS analysis.

Flow Cytometry

Cell surface marker analysis was performed using flow cytometry. Single-cell suspensions prepared from MLN, adipose tissue (AT) and liver were collected from mice at the times indicated. Cell surface markers were stained for 30 min at 4°C with rat anti-mouse CD3/CD19-CF594 (Clone:145-2C11,1D3) F4/80-APC (Clone: T45-2342), CD11c-FITC (Clone: HL3), CD301-pecy7 (Clone: LOM-14), CD64-PerCp-Cy5.5 (Clone: X45-5/7.1), CD11b-BV650 (Clone: M1/70), Ly6G-efluor700 (Clone: 1A8) and Siglec-F-PE (Clone: E50-2440) (BD Bioscience). All antibody incubations were performed at 4°C for 30 min (isotype controls were included). Data were acquired using a BD FACS Aria and analyzed using FlowJo software (Tree Star, Inc).

Quantitative Real-Time PCR

A small piece (<0.5 cm) of AT, liver and small intestine (SI) (jejunum) was collected in a 2 ml Eppendorf tube containing 1 ml TRIzol-reagent (Sigma) and homogenized using a TissueLyzer (QIAGEN). Tissues were homogenized and RNA was extracted using TRIzol-reagent (Sigma) following the manufacturer's protocol. RNA samples were reverse transcribed to cDNA as follows. After RNA quantification, 50–70 ng of each sample was transferred to a 0.2-ml tube and 1 μl of each of oligo(dT) (Qiagen) and 10 mM dNTPs were added, followed by incubation at 65°C for 5 min in a Veriti 96-well thermal cycler (Applied Biosystems) followed by incubation on ice for 2 min. Four (4) μl of first strand buffer (Thermo Fischer), 1 μl of each of 0.1 M DTT (Thermo Fischer), RNAse out and 0.5 μl of Superscript III (Thermo Fischer) were added to the sample. The sample was incubated for 60 min at 55°C, then 15 min at 70°C. Finally, cDNA was quantified on a Nanodrop 2000 (Thermo Scientific).

For qPCR reactions, 100 ng of cDNA was mixed with 12.5 μl of SYBR Green and 2.5 μl of each primer of the selected genes in a total volume of 25 μl per sample. A Rotor-Gene Q (QIAGEN) was used for real time thermal cycling. All genes were normalized for levels of transcription relative to the housekeeping gene β-actin.

Staining and Quantification of Eosinophils

A 1-cm piece of small intestine (SI) (jejunum) was fixed in 4% paraformaldehyde. The samples were processed in a Histocore

Pearl automatic tissue processer, embedded in paraffin and cut in 5 µm sections with a rotary microtome. The slides were first dewaxed with Xylene (2–6) min, absolute ethanol for 2–6 min, 70% ethanol for 1 min and DI water for 1 min. Slides were then stained with Congo Red solution (Sigma) for 1 h as per the manufacturer's instructions, followed by DI water for 2 min, Harris hematoxylin for 30 s, DI water for 2 min, Scotts tap water for 1 min, DI water for 1 min, 95% ethanol for 1–2 min, absolute ethanol for 1–2 min and xylene for 1–2 min. Cover slips were then placed on slides before scanning with an Aperio CS2 scanner (Leica). Quantification of eosinophils was performed by counting the eosinophils in 15 fields of view (magnification x40) in 2 sections per group.

Data Analysis

Data were tested for statistical significance using GraphPad Prism software (version 8). A Mann-Whitney U test was applied to test statistically significant differences between two unpaired groups with non-parametric distribution. Data that were normally distributed were tested for statistical significance using the unpaired t test for comparisons of two groups or the ANOVA test followed by the Holm-Sidack multiple-comparison test to compare more than two groups. Values of $p < 0.05$ were considered statistically significant. Results are expressed as SEM or means ± SD. Significance values are indicated as $^{*}p < 0.05$; $^{**}p < 0.01$.

DNA Extraction and Bacterial 16S rRNA Illumina Sequencing

After mice were sacrificed, jejunum samples were collected and stored immediately at −80°C for further analysis. DNA extraction and 16s rRNA sequencing were performed by the Australian Centre for Ecogenomics, University of Queensland, Brisbane. In brief, a total of 50 to 100 mg of tissue sample was disrupted mechanically using a Powerlyzer 24 at 2,000 *g* for 5 min. A QIAamp 96 PowerFaecal QIAcube HT Kit (Qiagen) was used to process the resulting lysate as per the manufacturer's instructions, and a Qubit assay (Life Technologies) was used for measuring DNA concentration, which was then adjusted to a concentration of 5 ng/µl. The 16S rRNA gene was targeted, using the 803 forward primer (5′-TTAGAKACCCB NGTAGTC-3′) and 1392 reverse primer (5′- ACGGGCGGTGWGTRC-3′) to cover the V6-V8 regions. Preparation of the 16S library was performed following the protocol outlined in the Illumina guide. In the first stage, 466 bp of the PCR products were amplified. The resulting PCR amplicons were then purified using Agencourt AMPure XP beads (Beckman Coulter). The purified DNA was indexed with unique 8cbp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002). The QIAquick Gel Extraction Kit (Qiagen) was used for the isolation of the indexed amplicons as per the manufacturer's instructions for the specific band at 450 bp (running at 610 bp with the adaptor sequence). Then, the resulting purified indexed amplicons were pooled together in equimolar concentrations and sequenced on a MiSeq Sequencing System (Illumina) using paired end (2 x 300 bp) sequencing with V3 chemistry in the Australian Centre for Ecogenomics according to the manufacturer's protocol. Passing quality control of resulting sequence was determined as 10,000 raw reads per sample prior to data processing and passing quality control metrics in line with Illumina supplied reagent metrics of overall Q30 for 600cbp reads of >70%.

Bioinformatics and Statistical Analysis

Sequence data were analyzed using a modified version of MetaGalaxyDE (39). Briefly, raw reads were run through fastqc for quality control, Trimmomatic (40) for adapter trimming and low quality base removal, QIIME (41) for Operational Taxonomic Units (OTUs) generation, and BLAST (42) for OTU identification. Within QIIME, low-quality reads are filtered with all remaining sequences de-multiplexed and chimeric sequences removed using UCHIME (43). Sequences were subsequently clustered into OTUs on the basis of similarity to known bacterial sequences in the Greengenes database (44) (cut-off: 97% sequence similarity) using the UCLUST software (45).

For each biom file, the taxonomic observation and metadata was added using biom API (46) which was next loaded into the R package phyloseq (47). Within phyloseq, the DESeq2 (48) API was called and a list of most differentially expressed bacteria generated for all possible pairings of conditions (NC and NC infected with *N. brasiliensis*, T2D mice fed HGI and T2D mice fed HGI infected with *N. brasiliensis* or T2D mice fed HF and T2D mice fed HF infected with *N. brasiliensis*). All subsequent plots were generated using ggplot2 and Calypso online software (version 8.84) (http://cgenome.net/calypso/) (49). Within Calypso, data were normalized by total sum normalization (TSS) combined with square root transformation. Multivariate redundancy analysis to overall differences in the microbial profile between groups and Adonis based on the Bray-Curtis dissimilarity and spearman' index was used. Differences in bacterial alpha diversity (Shannon diversity) and richness between groups were used. Values of $p < 0.05$ were considered statistically significant following false discovery rate (FDR) correction. Differences in the bacterial taxa abundance between groups were assessed using ANOVA-like differential expression analysis (ALDEx2) and quantitative visualization of phyla abundance.

Short Chain Fatty Acid Analysis by NMR

Fresh fecal pellets were collected at different time points of the experiment and stored at −80°C for metabolite extraction and analysis. Once thawed, fecal pellets were mixed with 600 µl of PBS and stored at room temperature for 20 min before manual disruption with a pipette tip and vortexing and centrifugation at 15,000 *g* at 4°C for 10 min. The supernatant was then transferred into a new microfuge tube and 50 µl of deuterated water (Cambridge Isotope Laboratories, Inc., USA) and 10 µl of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were added, with the latter being a chemical shift reference. The samples were centrifuged again at 15,000 *g* at 4°C for 10 min and 550 µl of each sample was transferred into a 5 mm NMR tube. NMR spectra were acquired on a Bruker Avance III 600 MHz spectrometer

(Bruker, Karlsruhe, Germany) and recorded at 600 MHz at 298 K equipped with a cryoprobe, using automated data collection *via* IconNMR software and the standard Bruker ^{1}H cpmgpr1d pulse sequence (128 scans) to collect one-dimensional spectra. The NMR spectra were referenced to DSS. The software Metaboanalyst (v 4.0) was used for metabolites analysis and we focused specifically on detecting and comparing fecal SCFAs. The SCFA peaks were identified based on standard samples. The chemical shift region containing the water resonance (δ 4.68–4.89) was removed from the analysis. The calculated regions were normalized to the total sum and Pareto scaling for overall concentration differences prior to statistical data analysis with t-tests.

RESULTS

Infection With *N. brasiliensis* Maintained Glucose Homeostasis and Reduced Body Weight Gain

In order to address the prophylactic and therapeutic effects of infection with *N. brasiliensis* on the outcome of T2D, we used two different models of diet (HGI and HF) to induce T2D in C57BL/6 mice. At 6 weeks of age, male C57BL/6 mice were either kept on a normal control diet (NC) or fed either HGI or HF diets for up to 31 weeks to induce T2D (**Figure 1A**). To ascertain the prophylactic effect of the infection on T2D, mice were infected at week 6 and re-infected once every month until the end of the experiment (week 31) to ensure continuous parasite infection. To ascertain the therapeutic effect, infection with *N. brasiliensis* started at week 24 and continued once every 3 weeks for a total of three infections until the end of the experiment (week 31) (**Figure 1B**).

As predicted, mice on either HGI or HF diets had a significant increase in the level of FBG compared to those on NC diet (**Figures 2A, B**). Prophylactic infection as well as therapeutic infection with *N. brasiliensis* significantly decreased the FBG levels in the diabetic groups fed on HGI and HF diets, compared to their respective uninfected groups fed on othose same diets (**Figures 2A, B**). A significant reduction in FBG was also noted in the NC group infected with *N. brasiliensis* when compared with their uninfected littermates, both prophylactically and therapeutically. A similar result was also observed for the OGTT test. HGI and HF diet infected mice had significantly lower levels of blood glucose than their respective control (uninfected) groups at all time points, both prophylactically and therapeutically (**Figures 2C–F**). Moreover, the NC group infected with *N. brasiliensis* had significantly lower levels of

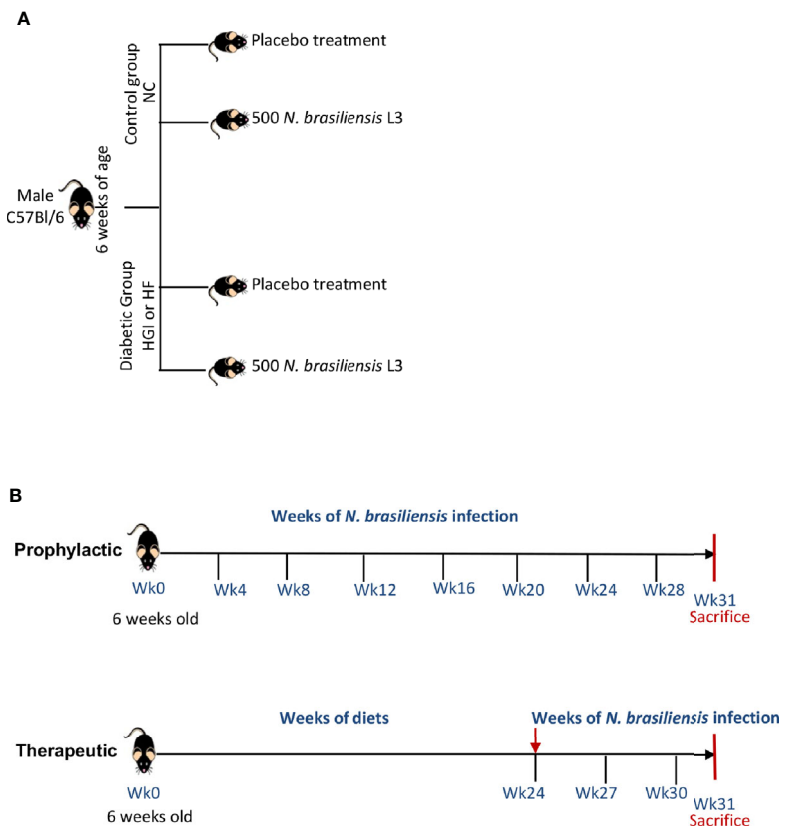

FIGURE 1 | Experimental design **(A)** and timeline for infection of mice with *Nippostrongylus brasiliensis* third stage larvae (L3) **(B)**. NC, Normal Control diet; HGI, High Glycemic Index diet; HF, High Fat diet.

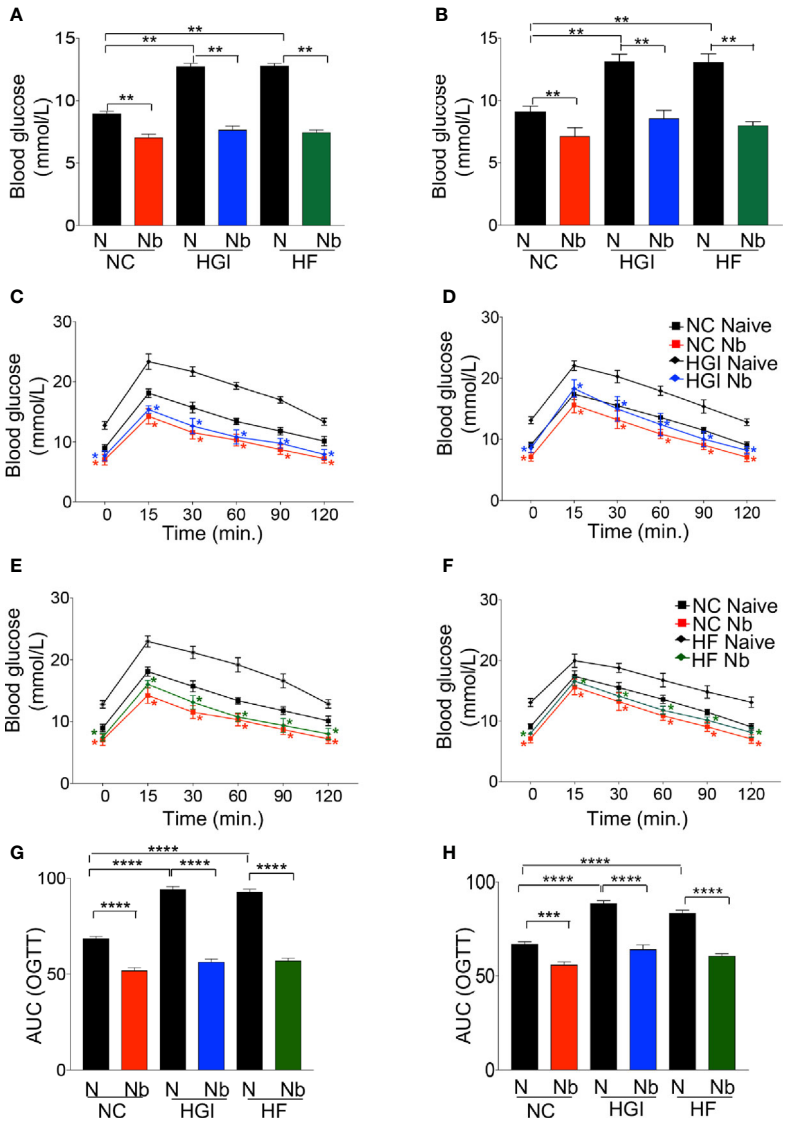

FIGURE 2 | *Nippostrongylus brasiliensis* infection decreased fasting blood glucose (FBG) and improved glucose metabolism in high glycaemic index (HGI) and high fat (HF) diet models of type 2 diabetes. C57BL/6 mice were fed normal control (NC), HF or HGI diet and infected once monthly with 500 infective larvae of *N. brasiliensis* commencing at 6 weeks of age for prophylactic infections and 24 weeks of age for therapeutic infections. **(A)** FBG in mice fed on different diets and administered prophylactic infection with *N. brasiliensis*. **(B)** FBG in mice fed on different diets and administered therapeutic infection with *N. brasiliensis*. Oral glucose tolerance test (OGTT) in mice fed on NC or HGI diets and administered prophylactic **(C)** or therapeutic **(D)** infection with *N. brasiliensis*. Oral glucose tolerance test (OGTT) in mice fed on NC or HF diets and administered prophylactic **(E)** or therapeutic **(F)** infection with *N. brasiliensis*. Area under the curve (AUC) in mice fed on different diets and administered prophylactic **(G)** or therapeutic **(H)** infection with *N. brasiliensis*. Statistical significance was determined with Student's t test or Two-way analysis of variance (ANOVA). Data are expressed as means ± SEM or means ± SD are representative of two experiments where n = 5/group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

blood glucose than their respective control at all time points. Of note, the blood glucose levels of the HGI and HF diet infected groups were also comparable to those mice on a NC diet that were infected with *N. brasiliensis* (**Figures 2C–F**).

N. brasiliensis Infection Slowed Weight Gain in HGI and HF Diet Models of T2D

Reduction in the rate of body weight gain was also observed as a result of infection. As expected, mice on either HGI or HF diets gained significantly more weight compared to mice on a NC diet (**Figure 3**); however, either prophylactic (**Figures 3A, C**) or therapeutic (**Figures 3B, D**) infection with *N. brasiliensis* significantly reduced the body weight gain in the diabetic groups fed on HGI and HF diets compared to their uninfected counterparts. Of note, significant reduction in body weight was also observed in the NC diet group infected with *N. brasiliensis* compared with the uninfected NC group, both prophylactically and therapeutically. When food weight was calculated on a

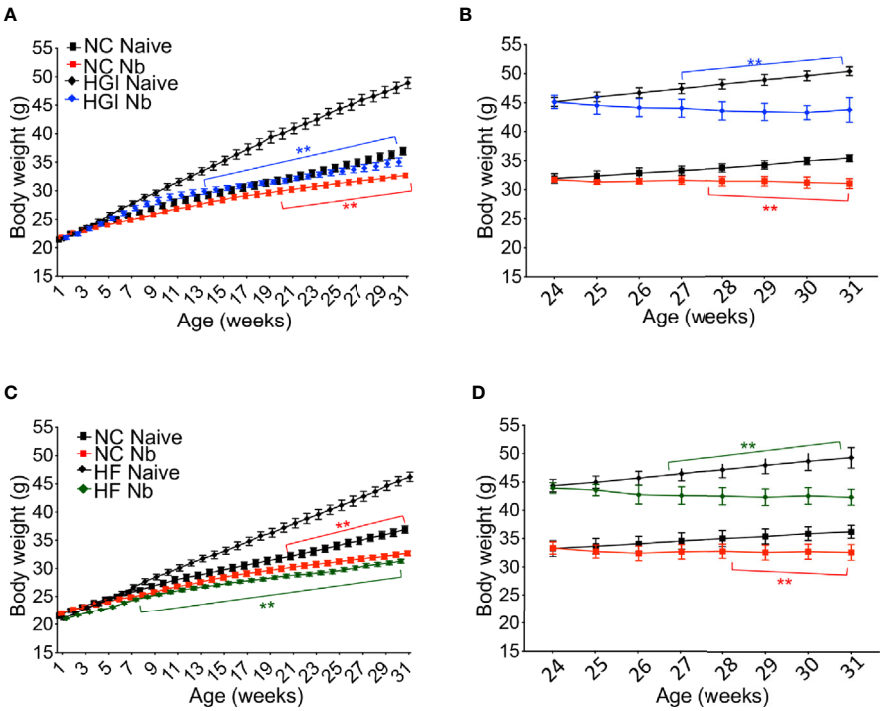

FIGURE 3 | *Nippostrongylus brasiliensis* infection reduced weight gain in high glycemic index (HGI) and high fat (HF) diet models of T2D. C57BL/6 mice were fed normal control (NC), HF, or HGI diet and infected once monthly with 500 infective larvae of *N. brasiliensis* commencing at 6 weeks of age for prophylactic infections and 24 weeks of age for therapeutic infections. **(A)** Body weight of mice fed on HGI diet and administered prophylactic infection with *N. brasiliensis* (Nb). **(B)** Body weight of mice fed on HGI diet and administered therapeutic infection with Nb. **(C)** Body weight of mice fed on HF diet and administered prophylactic infection with Nb. **(D)** Body weight of mice fed on HF diet and administered therapeutic infection with Nb. Statistical significance was determined with Two-way analysis of variance (ANOVA). Data are expressed as mean ± SD and are representative of two experiments where n = 5/group. *$p < 0.05$; **$p < 0.01$.

weekly basis for each cage, no differences in food consumption were detected at any time between these two groups (data not shown).

These data indicate that in response to *N. brasiliensis* infection, HGI and HF diet groups maintained low levels of FBG and displayed improved glucose metabolism compared to uninfected controls.

N. brasiliensis Infection Induces Local Eosinophilia and Th2 Immune Responses

To determine whether *N. brasiliensis* infection induced a potent Th2 cytokine response accompanied by eosinophilia and alternative activation of MACs, mice fed a NC, HGI or HF diet were infected with 500 N. *brasiliensis* L3 and sacrificed at week 31 of the experiment (**Figure 1B**). qPCR analysis was performed on AT, liver and SI to assess M2 MAC expression markers. In response to both prophylactic and therapeutic *N. brasiliensis* infection, there was a significant increase in the total number of eosinophils in the MLN, AT and liver of the diabetic groups as well as the NC group compared to their uninfected littermates (**Figures 4A, B**). Moreover, quantification of eosinophil numbers in the gut revealed a significant increase of these cells in the NC, HGI and HF diet groups infected with *N. brasiliensis* compared to their respective uninfected groups (**Figures 4C, D**). Both prophylactic and therapeutic *N. brasiliensis* infection significantly upregulated expression of genes encoding major Th2 cytokines and associated MAC proteins in all tissues assessed (**Figure 5**). In response to infection, elevated expression of *IL-4* was detected in the AT, liver and SI of mice on all three diets compared to their respective uninfected groups (**Figures 5A, B**). Infection also resulted in increased expression of *Retnla* (encoding the resistin-like alpha protein) and *Chil3* (chitinase-like protein 3) markers of M2 macrophages and a Th2 environment in these same tissues (**Figures 6A, B**).

Infection With *N. brasiliensis* Resulted in Altered Alpha Diversity and Microbial Richness in Mice Fed on NC, HGI, and HF Diets

We wanted to address the effect of infection with *N. brasiliensis* on the composition of the gut microbiota in mice fed NC, HGI and HF diets. SI samples were collected at termination (week 31) to determine the differences in the composition of gut microbiota between infected and uninfected groups. Multivariate redundancy analysis on OTU level showed a different clustering in the microbial profiles of the infected groups compared to the uninfected control groups for all diets (NC, HGI and HF) (**Figure 7A**). Adonis analysis revealed significant differences between infected and uninfected groups on all diets

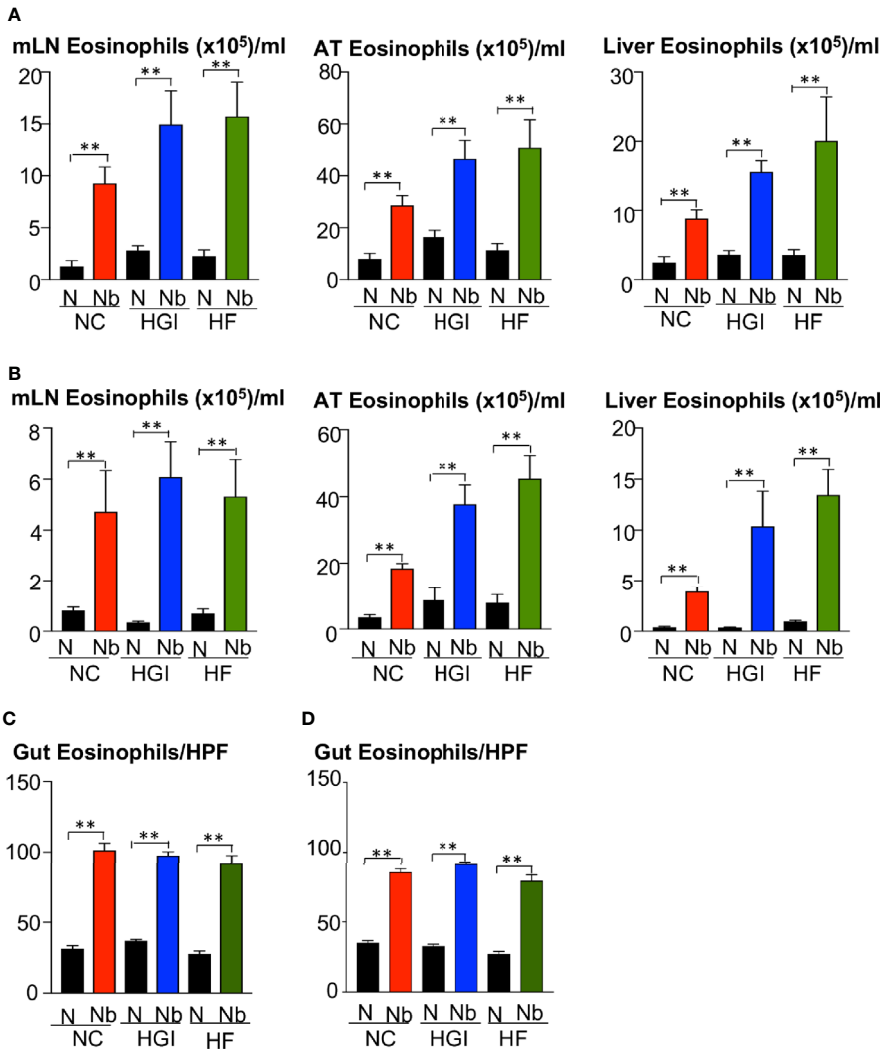

FIGURE 4 | Increase in the frequency of eosinophils in the mesenteric lymph nodes (MLN), adipose tissue (AT), liver, and duodenum in mice fed on different diets and infected or not with *Nippostrongylus brasiliensis*. C57BL/6 mice were fed normal control (NC), high fat (HF), or high glycemic index (HGI) diet and infected once monthly with 500 N. *brasiliensis* infective larvae from 6 weeks of age [prophylactic, panel **(A)**] or 24 weeks of age [therapeutic, panel **(B)**]. Eosinophil frequency and total numbers in MLN, AT, and liver are shown. Eosinophil numbers per high power field (HPF) (magnification x40) in the gut are shown in panel **(C)** (prophylactic) and panel **(D)** (therapeutic). Statistical significance was determined with Student's t test. Data are expressed as mean ± SEM and are representative of two experiments where n = 5/group. **$p < 0.01$.

using at least one of the two indices (Bray-Curtis or spearman) (**Supplementary Table 2**). No significant differences in the Shannon index and species richness were observed in *N. brasiliensis* infected groups on all three diets compared to their respective uninfected groups (**Figure S1**).

In general differences were detected in the abundance of some bacterial taxa between uninfected groups fed on diabetic diets (HF or HGI) and those fed NC diet, as well as between infected and uninfected groups fed on all diets used in this study (**Figures 7B–E**). At the phylum level, mice fed either HF or HGI diets had a significant decrease in the abundance of Bacteroidetes and significant increase in Firmicutes compared to the NC group (**Figure 7B**). However, infection status had no impact on the abundance of these phyla (**Figure 7B**). Moreover, Actinobacteria was significantly decreased in the uninfected HGI group compared to the uninfected NC group, however no such changes were detected between the HF and NC groups (**Figure 7B**). On the other hand, the NC group infected with *N. brasiliensis* had a significant decrease in the abundance of Actinobacteria compared to their uninfected littermates (**Figure 7D**). Also the abundance of Actinobacteria was not different between HF infected and uninfected groups (**Figure 7B**). The uninfected HF diet group displayed decreased abundance of TM7 and Verrucomicrobia compared to the uninfected NC group, whereas the uninfected HGI group displayed increased abundance of both phyla when compared to the uninfected NC group (**Figure 7B**). On the other hand, a significant increase in the abundance of Proteobacteria and a

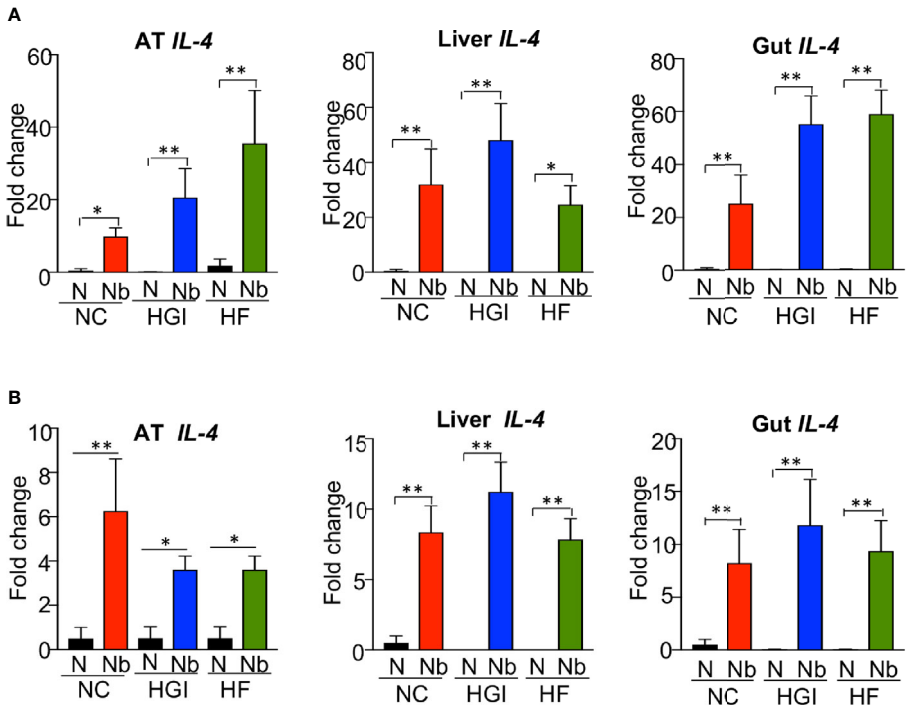

FIGURE 5 | Increased expression of *Il4* in adipose tissue (AT), liver and gut of mice fed on different diets and infected with *Nippostrongylus brasiliensis* compared to uninfected mice. C57BL/6 mice were fed normal control (NC), high fat (HF), or high glycemic index (HGI) diet and infected once monthly with 500 N. *brasiliensis* infective larvae from 6 weeks of age [prophylactic, panel **(A)**] or 24 weeks of age [therapeutic, panel **(B)**]. Statistical significance was determined with Student's t test. Data are expressed as mean ± SEM and are representative of two experiments where n = 5/group. *p < 0.05; **p < 0.01.

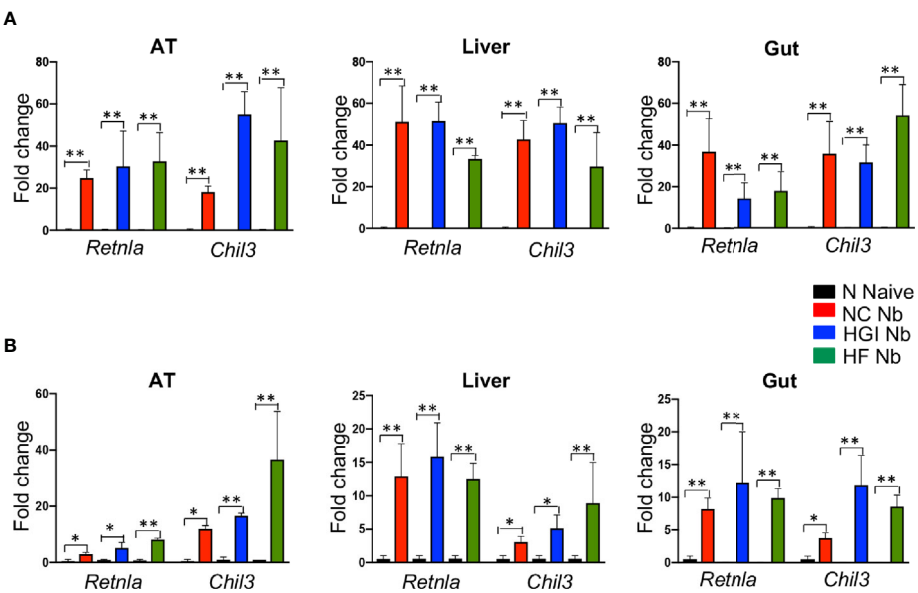

FIGURE 6 | Increased expression of *Retnla* and *Chil3* genes in adipose tissue (AT), liver, and duodenum of mice fed on different diets and infected with *Nippostrongylus brasiliensis* compared to uninfected mice. C57BL/6 mice were fed normal control (NC), high fat (HF), or high glycemic index (HGI) diet and infected once monthly with 500 N. *brasiliensis* infective larvae from 6 weeks of age [prophylactic, panel **(A)**] or 24 weeks of age [therapeutic, panel **(B)**]. Data are expressed as mean ± SEM and are representative of two experiments where n = 5/group. *p < 0.05; **p < 0.01.

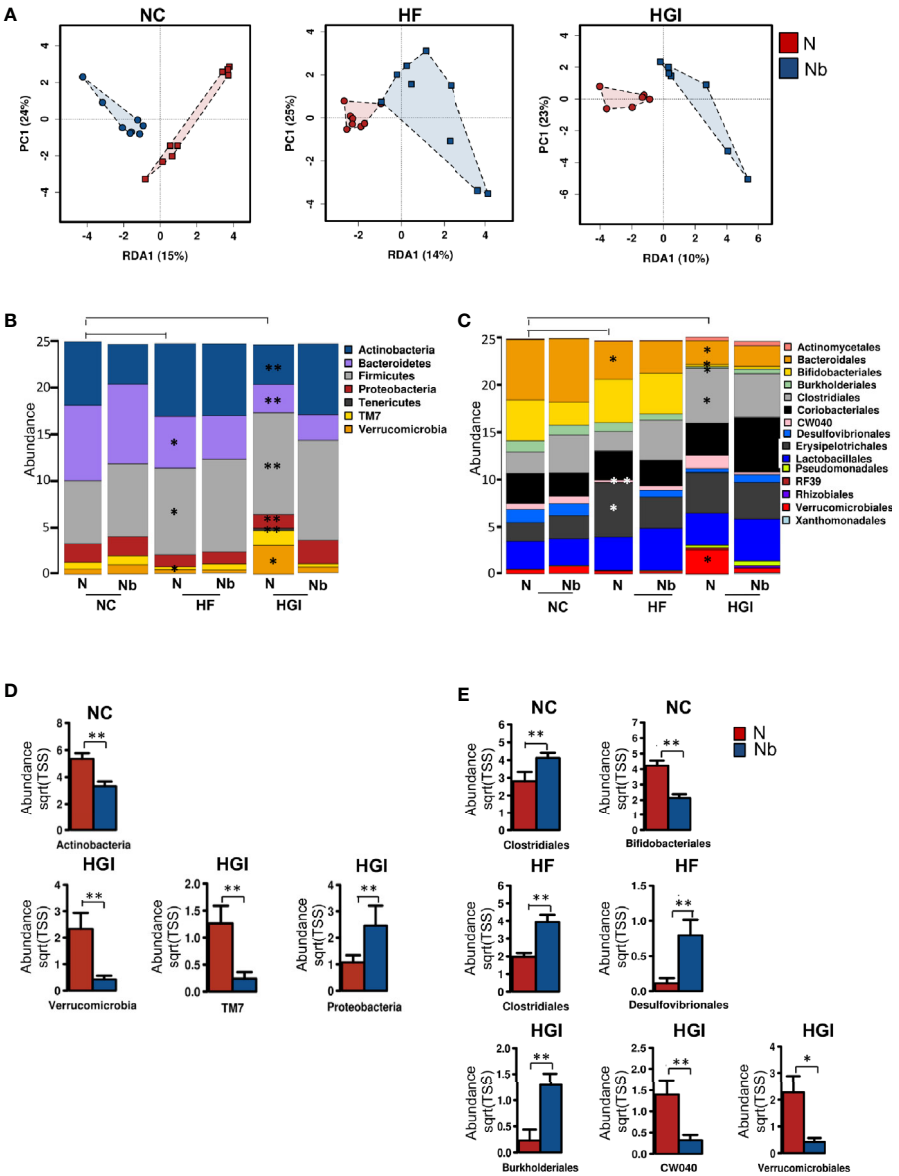

FIGURE 7 | Multivariate analysis of differences in the microbial profiles in the small intestine of *Nippostrongylus brasiliensis* (Nb) infected and uninfected (naïve, N) C57BL/6 mice fed on normal control (NC), high fat (HF), or high glycaemic index (HGI) diet **(A)**. Relative abundance of bacterial phyla in the small intestine of Nb and N mice fed on NC, HF, or HGI diet **(B)** Relative abundance of bacterial orders in the small intestine of Nb and N mice fed on NC, HF, or HGI diets **(C)**, and abundance of defined taxa where significant differences between infected and uninfected groups were detected **(D, E)**. Mice were infected once monthly from 6 weeks of age with Nb infective larvae. P values are based on ANOVA-like differential expression analysis and are representative of two experiments where n = 5/group. *$p < 0.05$; **$p < 0.01$.

significant decrease in the abundance Verrucomicrobia and TM7 were detected in the infected HGI group compared to the uninfected HGI group (**Figure 7D**). However these changes were not detected between infected and uninfected NC or HF groups (**Figure 7B**). No significant differences were detected in any phyla between infected and uninfected groups fed HF diet (**Figure 7B**).

At the order level, both diabetic groups showed significantly lower abundance of Bacteroidales (Bacteroidetes phylum) compared to the NC group (**Figure 7C**). However, no differences were detected in any of the three diet groups infected with *N. brasiliensis* compared to their uninfected naïve groups (**Figure 7C**). As a result of the diets, significant decrease in the abundance of Bifidobacteriales (Actinobacteria phylum) and Burkholderiales (Proteobacteria phylum) were detected in the uninfected HGI group, but not in the uninfected HF group when compared with the NC group (**Figure 7C**). On the other hand, the NC group infected with *N. brasiliensis* showed a

significant decrease in the abundance of Bifidobacteriales (but not Burkholderiales) compared to the uninfected naïve group (**Figures 7C, E**). However, HGI mice infected with *N. brasiliensis* had significantly higher abundance of Burkholderiales and showed no changes in the abundance of Bifidobacteriales when compared to their uninfected naïve group (**Figures 7C, E**). HF mice that were infected with *N. brasiliensis* showed no differences in the abundance of both Bifidobacteriales and Burkholderiales compared to their uninfected group (**Figure 7C**). Moreover, the uninfected HGI group had significantly higher abundance of Clostridiales (Firmicutes phylum) compared to the NC group, but no changes were detected between HF and NC groups (**Figure 7C**). *N. brasiliensis* infection significantly increased the abundance of Clostridiales in both the NC and HF groups infected with *N. brasiliensis* compared to their respective uninfected diet-matched groups (**Figure 7E**). Furthermore, the abundance of both the CW040 order (TM7 phylum) and Desulfovibrionales (Proteobacteria phylum) was significantly decreased in the HF diet group compared to the NC diet group. While those on the HGI diet had increase in the abundance of CW040 and decrease in the abundance of Desulfovibrionales compared to the NC diet, but this was not significant (**Figure 7C**). *N. brasiliensis* infection caused a significant elevation in the abundance of Desulfovibrionales and trend toward increased abundance of CW040 in the HF diet group infected with *N. brasiliensis* compared to their uninfected group (**Figure 7E**). *N. brasiliensis* infection significantly decreased the abundance of CW040 and showed a trend toward increased abundance of Desulfovibrionales in the infected HGI group compared to their uninfected diet-matched group (**Figures 7C, E**). As a result of the diets, the abundance of Verrucomicrobiales order (Verrucomicrobia phylum) was significantly increased in the HGI but not HF groups compared to the NC group (**Figure 7C**). On the other hand, the abundance of the Verrucomicrobiales was significantly decreased in the infected HGI group compared to the uninfected group (**Figure 7E**).

To summarize, the impact of *N. brasiliensis* infection on the microbiome at the phylum level was most notable in mice on the HGI diet, with reduced abundance of Verrucomicrobia and TM7 phyla in infected mice and increased abundance of Proteobacteria in HGI infected mice. At the order level, infection with *N. brasiliensis* resulted in increased abundance of Clostridiales and Desufovibrionales in mice on the HF diet and Burkholderiales in mice on the HGI diet.

Infection With *N. brasiliensis* Alters Fecal Short Chain Fatty Acid Content in Mice Fed on Different Diets

Metabolic profiling of fecal extracts from infected and uninfected mice fed on the three different diets was carried out using NMR spectroscopy. We compared the SCFAs acetate, butyrate and propionate between the uninfected and *N. brasiliensis* infected groups by t-test. For each diet at least one of the SCFAs was present in higher quantities ($p < 0.05$) in infected versus uninfected mice (**Table 1**; **Figure S2**).

TABLE 1 | Effect of *Nippostrongylus brasiliensis* on fecal short chain fatty acid levels in mice fed different diets.

Diet	SCFA	p-value	Change upon infection
Normal			
	Acetate	ns	
	Butyrate	0.04	increase
	Propionate	0.03	increase
High glycemic index			
	Acetate	ns	
	Butyrate	0.007	increase
	Propionate	ns	
High fat			
	Acetate	ns	
	Butyrate	ns	
	Propionate	0.046	increase

DISCUSSION

Diabetes is recognized as the world's fastest growing chronic condition (1). Helminth infections have been associated with a lower prevalence of T2D due to their ability to induce type 2 immune responses (17–20). We therefore set out to investigate the role of helminth-induced type 2 immunity and the potential mechanisms underlying protection against the development of T2D-induced insulin resistance. C57BL/6 mice were fed a HGI or HF diet and infected frequently with *N. brasiliensis*. This strain of mice is genetically susceptible to obesity, glucose intolerance, hyperglycaemia and T2D when fed a HF or HGI diet (50, 51). We demonstrated that infection with *N. brasiliensis* had a beneficial effect, both prophylactically and therapeutically against T2D in two different diabetes-inducing diets. Our findings are consistent with a role for helminth infection in promoting type 2 immune responses by eliciting eosinophil accumulation in MLN, AT, liver and SI, with increased expression of genes encoding for key Th2 cytokines and M2 MACs in AT, liver and SI. We did not quantify levels of regulatory cells and cytokines, such as regulatory T cells, IL-10 and TGF-β, but a role for such a response in protecting against T2D in this model is plausible and should be addressed in the future.

In line with our results, a number of studies have shown improved glucose tolerance in mouse models of diabetes induced by a range of parasitic nematodes with distinct tissue niches. For example, studies utilizing the HF diet model of obesity showed improvements in glucose tolerance of obese mice after infection with the filarial nematode *Litomosoides sigmodontis* or administration of soluble adult worm extract (21). Infection with *H. polygyrus* also resulted in decreased body weight gain and improved glucose and lipid metabolism, and an associated increase in Th2/Treg immune responses in the MLNs, AT and SI. Moreover, infected mice on a HF diet displayed dysregulated expression of genes and proteins involved in energy expenditure and lipid metabolism in AT and liver (23, 24). Infection with parasitic platyhelminth flatworms (distinct phylum from the Nematoda) has also been shown to protect against metabolic syndrome. Chronic *Schistosoma mansoni* infection and administration of schistosome soluble egg antigens resulted in increased numbers of AT eosinophils, M2 MACs and Th2

cytokines, and a corresponding decrease in body weight gain and improved insulin sensitivity in obese mice (52).

Recently, eosinophils in particular have been implicated in glucose homeostasis and energy expenditure. These cells play an unexpected role in metabolic homeostasis through maintenance of adipose M2 MACs. Absence of eosinophils resulted in increased body weight gain and impaired glucose tolerance in mice (3, 4, 53). Moreover, in the absence of eosinophils, mice exhibit a defect in lipid metabolism in the liver and SI, an increase in the expression of pro-inflammatory IFN-γ and a decrease in the expression of IL-4 and IL-13 in AT (54).

In our work, *N. brasiliensis* induced MLN, AT, liver and SI eosinophilia, and increased gene expression of M2 MAC markers in AT, liver and SI. This was consistent with other studies which showed that infection with *N. brasiliensis* induced adipose eosinophilia and M2 MACs, enhanced glucose tolerance and lipid metabolism and ameliorated body weight gain in different mouse models of obesity (3, 4, 22). These studies have proposed mechanisms by which these cells might influence AT homeostasis. Eosinophils in bone marrow and their recruitment into white AT are largely controlled by IL-5. Mechanistically, the increase in eosinophil numbers in our mice fed a HGI or HF diet following *N. brasiliensis* infection may be the result of local and systemic increases in eosinophils and Th2 cytokines, as well as increases in M2 MAC numbers that regulate many key events involved in the control of metabolic homeostasis. It is not yet clear whether the eosinophil-mediated regulation of obesity-induced insulin resistance and AT inflammation is due to the direct and primary effects of eosinophils on insulin resistance or due to secondary effects of eosinophils on changes in body weight and adiposity. Further studies are required to elucidate the functions of helminth-induced eosinophils in terms of their beneficial and detrimental effects in driving metabolic reprogramming, and the therapeutic utility of this phenomenon for treating the global epidemic of metabolic disorders. On the other hand, changes in environmental and behavioral factors, such as diet, can modulate bacterial composition and metabolic activity (55), which can trigger an inflammatory immune response leading to the development of T2D (7, 55). Indeed, there is growing evidence that helminth infection alters the composition of the gut microbial community, conferring protection against immune mediated diseases such as allergic inflammation (30), IBD (31), and obesity (32). We therefore aimed to address the effect of infection with *N. brasiliensis* on the composition of the gut microbiota in mice fed on different diets. No significant changes were observed in α-diversity in response to *N. brasiliensis* infection in all diets studied (NC, HF and HGI); however, we still found a significant shift in the microbiota composition at the community level. This was in agreement with other studies with *N. brasiliensis* and *Hymenolepis diminuta* that also found no significant differences in α-diversity between infected and uninfected groups (33, 56), and suggests that inter-individual variation occurs in the microbiota composition as a result of infection (33).

In our study, *N. brasiliensis* infection resulted in a decrease in the abundance of Bifidobacteriales on the NC diet. In agreement with our findings, in human studies individuals with different helminth infections (i.e. *Trichuris* spp., *Ascaris* spp. and hookworm) had lower abundance of the Bifidobacteriales compared to uninfected individuals (26). Many linked the latter group with health benefits in T2D (57); however, others have reported them to cause infections (58). In HF diet induced obesity in rats, administration of four *Bifidobacteria* strains had different responses on energy and fat metabolism and showed no differences on serum insulin and glucose levels (59). Moreover, administration of *Bifidobacterium breve* to preterm infants, increased weight gain (60).

We found an increase in the abundance of Clostridiales in the NC and HF diet groups infected with *N. brasiliensis*. Infection with *H. polygyrus* attenuated allergic airway inflammation in mice inoculated with house dust mite allergen, which was associated with an increase in the abundance of Clostridiales (30). An increase in the abundance of Clostridiales was also reported after infection with *T. muris* which protected against colitis in *NOD2*$^{-/-}$ deficient mice *via* a mechanism involving type 2 immunity (31). Moreover, oral administration of a mixture of Clostridia strains known to induce $CD4^+$ $Foxp3^+$Tregs cells attenuated experimental colitis and allergic diarrhea (61, 62). It has also been reported that reduction in the abundance of Clostridia was associated with T2D in humans (63–66), and an increase in this group was associated with an improvement in glucose and lipid metabolism (67). Moreover, in two separate studies, oral administration of probiotic *Clostridium butyricum* also improved diabetic markers (fasting glucose, glucose tolerance, insulin tolerance, glucagonlike peptide and insulin secretion), decreased blood and liver lipids and restored colonic homeostasis of treated groups in two different models of T2D in mice (HF diet and leptin$^{db/db}$) (68, 69). Of note, Clostridiales are abundant producers of the SCFAs that regulate colonic Treg cell homeostasis and strongly involved in the maintenance of overall gut function (70, 71).

In response to *N. brasiliensis* infection we also found a significant increase in the abundance of Desulfovibrionales and Burkholderiales (Proteobacteria) in the HF and HGI diet groups, respectively. In one study, the abundance of Proteobacteria also increased in *H. polygyrus*-infected mice fed a NC or a HF diet compared to naïve littermates (72). This phylum is, in part, responsible for regulating weight gain in HF diet fed mice (72). Abundance of the Desulfovibrionales was increased in mouse fecal samples as a result of infection with *Schistosoma haematobium* (73), *Ascaris lumbricoides*, and *Trichuris trichiura* (74). Moreover, the abundance of Desulfovibrionales and Burkholderiales was lower in obese mice (75). Cold exposure attenuated diet-induced obesity in mice, which was associated with an increase in the abundance of *Desulfovibrionaceae* (76). Desulfovibrionales are sulfate-reducing bacteria that use hydrogen or other compounds such as lactate, pyruvate and ethanol as electron donors to produce hydrogen sulfide (H_2S) (77). H_2S has been found to improve insulin secretion, improve glucose tolerance and reduce food intake *via* direct stimulation of glucagon-like peptide-1 (GLP-1) secretion in gut L-cells and indirectly *via* treatment with prebiotic chondroitin sulfate that

enhanced the level of *Desulfovibrio piger* in the feces and colon of the treated group (78).

Levels of Verrucomicrobiales (Verrucomicrobia phylum) and CW040 (TM7 phylum) were significantly lower in the HGI group infected with *N. brasiliensis* in comparison with HGI-fed uninfected littermates. In agreement with our data, Verrucomicrobia were enriched in mice fed both HF and high sugar diets, but was not detected in mice fed NC diet (79). In another study, the abundance of Verrucomicrobia was significantly elevated when mice switched from NC to high sugar diet (80). Moreover, mice with leptin deficiency (db/db)-induced T2D showed an increase in the abundance of Verrucomicrobia (81). However, these mice exhibited a decrease in the abundance of Verrucomicrobia when subjected to an intermittent fasting regime which protected against diabetic retinopathy (82). The TM7 phylum is a recently identified bacterial group, composed of uncultivable and highly ubiquitous bacteria (83) and has been associated with inflammatory mucosal diseases, periodontitis, IBD and vaginosis in humans (84–86).

Many factors may play a role in modulating the abundance of microbial species in the gut. The variability in the microbiota community composition among different diets and as a result of infection might be due to differences in dietary substrates. Different microbial species might drive different effects on energy recruitment pathways. We also should consider the pathways for the metabolism of these substrates as well as the inter-individual variation in metabolism and its implications on the abundance of different microbial species in the gut (87).

Acetate, propionate and butyrate are the major SCFAs known to play important roles in gastrointestinal physiology and maintenance of gut integrity, metabolism and immune homeostasis (88). A complex interplay of multiple factors including, diet, gut microbiota, gut environment (eg. pH, and gas concentrations) can affect the formation of SCFAs and determine the amounts and types of SCFA that are produced (89). Changes in the concentrations of SCFAs have been implicated in modulating inflammatory pathology in distinct tissues in diseases such as IBD, cancer and T2D (88).

Interestingly, in addition to the impact of helminth infection in modifying host microbiome, several studies have also reported shifts in metabolites during helminth infection (90). We found that fecal SCFA levels were significantly elevated in *N. brasiliensis*-infected mice compared to uninfected mice for all diets tested, and this may have had a therapeutic benefit in modulating inflammation and suppressing insulin resistance.

Many studies have highlighted the roles of SCFAs in the regulation of appetite, weight gain, glucose and lipid metabolism (91). For instance, in overweight and obese individuals, colonic administration of SCFAs increased fat oxidation, energy expenditure and circulating levels of the satiety-stimulating hormones peptide YY (PYY) and GLP-1 concentration (92). Propionate administration stimulated the release of PYY and GLP-1 from colonic cells and increased their concentration in the circulation, reduced energy intake, intra-abdominal adipose tissue distribution, intrahepatocellular lipid content and prevented weight gain (93, 94). In mouse studies, administration of butyrate improved lipid and glucose metabolism which prevented HFD-induced obesity, insulin resistance, hypertriglyceridemia and hepatic steatosis. The effect was due to an increase in peroxisome proliferator–activated receptor-γ coactivator-1α expression that increased mitochondrial function and biogenesis in skeletal muscle and AT (95). The role of butyrate in regulation of the immune response has also been highlighted. Butyrate can regulate intestinal macrophage function to decrease the production of proinflammatory mediators such as nitric oxide (NO), IL-6 and IL-12 (96). Butyrate and propionate attenuated the activation of nuclear factor κB by LPS- stimulated neutrophils and inhibited the production of proinflammatory cytokines and NO (97). Butyrate and propionate have also bene shown to increase the numbers of Treg cells expressing Foxp3 in the colon, spleen and lymph nodes (98, 99).

Correlations between helminth presence and changes in the microbial composition have already been mentioned elsewhere throughout, but it is pertinent to note that many studies reported changes in the composition of the gut microbiota as a result of helminth infection and subsequent improvement in the outcome of immune mediated diseases. Whether the microbial composition changes we found in our study are a direct effect of helminth infection or a consequence of the host's immune response to the infection, and whether these changes are essential to confer protection against T2D are yet to be investigated. A deeper understanding of the interplay between the host-microbiota-helminth triad and other variables may represent a new therapeutic strategy to prevent or even reverse the pathological effects of T2D.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, BioProject https://www.ncbi.nlm.nih.gov/bioproject/PRJNA676176.

ETHICS STATEMENT

The animal study was reviewed and approved by James Cook University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

ZK, PG, JS, and AL conceived the project and designed the experiments. ZK performed data analysis and wrote original drafts of the manuscript. ZK and RA performed the experiments. RE provided material. AK provided advice with flow cytometry.

MF and LK provided bioinformatics assistance and support. JW prepared histological sections. DW and ND provided assistance and support with NMR metabolomic analysis. ZK drafted the manuscript and all authors provided intellectual and editorial feedback. 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/fendo.2020.606530/full#supplementary-material

Supplementary Figure 1 | Shannon index and richness in the small intestine of Nippostrongylus brasiliensis (Nb) infected and uninfected (naïve, N) C57BL/6 mice fed on normal control (NC), high fat (HF) or high glycaemic index (HGI) diet. Mice were infected once monthly from 6 weeks of age with Nb infective larvae. P values are based on multiple linear regression and are representative of 2 experiments where n = 5/group.

Supplementary Figure 2 | Example 1H NMR spectra of acetate, propionate and butyrate faecal extract used in this study.

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Signs of Deregulated Gene Expression Are Present in Both CD14$^+$ and CD14$^-$ PBMC From Non-Obese Men With Family History of T2DM

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Aim: Development of type 2 diabetes (T2DM) is associated with disturbances in immune and metabolic status that may be reflected by an altered gene expression profile of peripheral blood mononuclear cells (PBMC). To reveal a potential family predisposition to these alterations, we investigated the regulation of gene expression profiles in circulating CD14$^+$ and CD14$^-$ PBMC in fasting conditions and in response to oral glucose tolerance test (OGTT) in glucose tolerant first-degree relatives (FDR) of T2DM patients and in control subjects.

Materials and Methods: This work is based on the clinical study LIMEX (NCT03155412). Non-obese 12 non-diabetic (FDR), and 12 control men without family history of diabetes matched for age and BMI underwent OGTT. Blood samples taken before and at the end of OGTT were used for isolation of circulating CD14$^+$ and CD14$^-$ PBMC. In these cells, mRNA levels of 94 genes related to lipid and carbohydrate metabolism, immunity, and inflammation were assessed by qPCR.

Results: Irrespectively of the group, the majority of analyzed genes had different mRNA expression in CD14$^+$ PBMC compared to CD14$^-$ PBMC in the basal (fasting) condition. Seven genes (IRS1, TLR2, TNFα in CD14$^+$ PBMC; ABCA1, ACOX1, ATGL, IL6 in CD14$^-$ PBMC) had different expression in control vs. FDR groups. OGTT regulated mRNA levels of nine genes selectively in CD14$^+$ PBMC and of two genes (ABCA1, PFKL) selectively in CD14$^-$PBMC. Differences in OGTT-induced response between FDR and controls were observed for EGR2, CCL2 in CD14$^+$ PBMC and for ABCA1, ACOX1, DGAT2, MLCYD, and PTGS2 in CD14$^-$ PBMC.

Conclusion: This study revealed a different impact of glucose challenge on gene expression in CD14$^+$ when compared with CD14$^-$ PBMC fractions and suggested

possible impact of family predisposition to T2DM on basal and OGTT-induced gene expression in these PBMC fractions. Future studies on these putative alterations of inflammation and lipid metabolism in fractionated PBMC in larger groups of subjects are warranted.

Keywords: oral glucose tolerance test, first-degree relatives, gene expression, peripheral blood mononuclear cells, type 2 diabetes mellitus, CD14⁻ cells, CD14⁺ cells

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is associated with a number of disturbances of immune status and metabolic pathways (1) that can be detected not only in insulin sensitive tissue but also in easily accessible peripheral blood mononuclear cells (PBMC). PBMC from patients with T2DM have shown alterations in mRNA levels of genes involved in the regulation of inflammation, lipid and glucose metabolism, and several signaling pathways (2–5). Development of T2DM is based on both environmental and genetic factors. While the obesogenic environment and the subsequent development of obesity is one of the most important factors contributing to the development of T2DM (6), the heritability of T2DM reaches 20%–80%, when first-degree relatives (FDR) of T2DM patients are about 3 times more likely to develop the disease than individuals without a positive family history of the disease (7). Thus, it might be hypothesized that alterations in gene expression are present already in non-obese individuals with genetic predisposition to T2DM, such as FDR. Indeed, a number of genes with dysregulated expression was found in FDR when compared with subjects without T2DM in antecedence, including adiponectin in adipose tissue (8) and genes involved in insulin signaling and fatty acid metabolism in skeletal muscle (9, 10). Although PBMC proved to be a good surrogate marker of systemic and adipose tissue metabolic state (11, 12), they represent a mixture of cell types with considerably different roles, behavior and metabolism, i.e. monocytes, dendritic cells and lymphocytes (13) and analysis of PBMC population in whole may limit the interpretation of results.

Therefore, the aim of our study was to examine the gene expression profile in two major fractions of PBMC: CD14 positive (i.e. mostly monocytes, representing 10%–20% of PBMC) and CD14 negative (i.e. mostly lymphocytes and natural killer cells, representing 70%–90% and 5%–10% of PBMC, resp.) in FDR in comparison with healthy controls without diabetic relatives so that the differential behavior of innate and acquired immunity systems could be revealed. The selected genes covered immunity and inflammation pathways and pathways of lipid and carbohydrate metabolism. Since we hypothesized that the nutrient-induced response of the genes might have a higher discriminative power than baseline values when comparing FDR to the control group, we analyzed the possible alteration of mRNA expression in these two PBMC fractions also in response to the nutritional challenge represented by OGTT.

MATERIAL AND METHODS

Subjects Characteristics

This work is based on the clinical study LIMEX (NCT03155412) including 51 healthy non-obese men. The two groups of men - 1) non-diabetic first-degree relatives of T2DM patients (FDR); 2) control group - subjects without any family history of diabetes (CON) - were matched for age and BMI. Family history of diabetes was considered as follows: two first-degree relatives (parents, siblings) or one first-degree and one or more second-degree relatives (grandparents, uncle, aunt) that were diagnosed with T2DM. Exclusion criteria for both groups were: body weight change more than 3 kg within 3 months preceding the study, smoking, any medication, hypertension, hyperlipidaemia, and drug or alcohol abuse.

Subjects were examined at 8.00 h in the fasting state. Body weight, waist, and hip circumferences were measured. Body composition was assessed by bioimpedance (QuadScan 4000, Bodystat, Douglas, British Isles). All men underwent an initial examination consisting of OGTT. Based on OGTT results eight subjects with impaired glucose tolerance were excluded. PBMC were isolated from a subgroup of 37 men (control, n=18; FDR, n=19). 13 subjects (control, n =6; FDR, n=7) were excluded because of the insufficient amount of isolated RNA from at least one fraction of PBMC in either of the time points of OGTT. Gene expression analysis in PBMC was performed in 12 subjects from the control group and 12 subjects from FDR group.

Oral Glucose Tolerance Test

Seventy-five grams of glucose was administered orally and blood samples for routine analysis were taken at the time points 0, 30, 60, 90, and 120 min. PBMC were isolated from 9 ml of full blood in time points 0 and 120 min and immunoseparated into CD14 positive and negative subpopulations using Dynabeads CD14 (Thermo Fisher, MA USA) as described before (14).

Plasma Analysis

Plasma samples were prepared from uncoagulated peripheral blood by centrifugation. Plasma glucose was determined with a glucose oxidase technique (Beckman Instruments, Fullerton, CA). Plasma insulin was measured using an Insulin Irma kit (Immunotech, Prague, Czech Republic). Lipid concentrations were determined using standard biochemical methods in certified laboratories.

Gene Expression Analysis

Total RNA was isolated from $CD14^+$ and $CD14^-$ subpopulation using an RNeasy Mini kit (Qiagen, Germany). RNA concentration was

measured using Nanodrop1000 (Thermo Fisher Scientific, USA). To remove genomic DNA, DNAse I (Invitrogen, USA) treatment was applied. 300 ng of total RNA was reversely transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). For microfluidics, 4 ng of cDNA were preamplified within 18 cycles to improve the detection of target genes during subsequent Real Time qPCR (TaqMan Pre Amp Master Mix Kit, Applied Biosystems, USA). For the preamplification, TaqMan gene expression assays of all target genes (**Supplemental Table 1**) were pooled together and diluted with water to the final concentration 0.2× for each probe. Samples with preamplified cDNAs were diluted 20 times, qPCR was then performed on Biomark Real Time qPCR system using a 96 × 96 array (Fluidigm, USA). Expression of seven genes (AKT1, APOE, CXCL1, GZMB, MMP3, SLC2A4, MLXIPL) was either below the detection limit or their amplification curves did not pass the initial quality test (**Supplementary Table 2**). Data were normalized to geomean of two reference genes (RPS13, TBP), $2^{-\Delta Ct}$ was calculated and used for statistical analysis. Genes with median of $2^{-\Delta Ct}$ below 0.0001 were considered as genes with insignificant level of expression and excluded from the analysis (CLIC3, IL2, IL6, KIR2DL4, MMP2, SLC27A2, VCAM1 in $CD14^+$ PBMC; CCL2, FOS, IL10, IL2, MMP2, PPARγ, SLC27A2, VCAM1 in $CD14^-$ PBMC). Outliers were identified by ROUT method, with Q value set to 0.5%. Genes with more than three missing values per group were excluded from analysis (ACOX1, COX6C, and DGAT1 in $CD14^+$ PBMC). The effect of OGTT was in figures expressed as the median of fold change – OGTT vs. basal- of each subject in the control and FDR group.

Statistical Analysis

Data are presented as means ± SEM (anthropometric and biochemical variables) or median with 25^{th} and 75^{th} percentile. Statistical analysis was performed using GraphPad Prism 9.0 for Windows (LaJolla, USA). Differences in the baseline values of the measured anthropometric, and biochemical variables were evaluated by Multiple Unpaired t test with multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli) set to 5%. Differences in mRNA levels ($2^{-\Delta Ct}$) and responses to OGTT (mRNA fold changes) between groups (FDR vs. controls) were evaluated by Multiple Mann-Whitney tests, with multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli) set to 5%. Evolution of log2 transformed glucose and insulin levels during OGTT was evaluated by Two Way ANOVA with Sidak multiple comparison test. Effects of OGTT on mRNA levels within each of the two groups were evaluated by Multiple Wilcoxon matched pairs signed rank tests, with multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli) set to 5%. The Spearman rank-order correlation coefficient was calculated in correlation analysis. The level of significance was set at $p < 0.05$ and q value <0.05.

RESULTS

Subjects Characteristics

Anthropometric and laboratory characteristics of both groups of subjects are presented in **Table 1**. The groups were not different in respect to age, body weight, waist circumference, BMI, relative fat mass, fat free mass, plasma triacylglycerol (TAG), LDL, cholesterol and uric acid. Plasma HDL was higher in the control group. Fasting plasma glucose and insulin were higher in FDR. Similarly, plasma glucose and insulin were higher at 120 min of OGTT. Time evolution of glucose and insulin levels

TABLE 1 | Anthropometric and biochemical characteristics of the subjects.

	Controls (n = 12)	FDR (n=12)	p value	q value
Age (years)	34.3± 3.5	37.0 ± 3.8	NS	NS
Body weight (kg)	82.6 ± 2.1	86.2 ± 1.2	NS	NS
BMI (kg/m^2)	24.7 ± 0.5	26.0 ± 0.5	NS	NS
Waist circumference (cm)	84.0 ± 1.2	88.0 ± 1.6	NS	NS
Fat Mass (%)	17.8 ± 3.4	17.8 ± 5.4	NS	NS
Fat Mass (kg)	13.6 ± 1.0	16.9 ± 1.0	NS	NS
Fat Free Mass (kg)	68.8 ± 1.2	68.6 ± 1.0	NS	NS
Cholesterol (mmol/l)	4.34 ± 0.2	5.05 ± 0.27	NS	NS
HDL cholesterol (mmol/l)	1.43 ± 0.05	1.09 ± 0.06	0.005	0.021
LDL cholesterol (mmol/l)	2.41 ± 0.16	3.05 ± 0.2	NS	NS
TAG (mmol/l)	1.17 ± 0.27	1.89 ± 0.43	NS	NS
Uric Acid (μmol/l)	342 ± 19	336 ± 13	NS	NS
Fasting glucose (mmol/l)	5.10 ± 0.07	5.61 ± 0.10	0.006	0.021
Fasting insulin (mU/l)	5.30 ± 0.67	10.93 ± 1.10	0.002	0.016
Glucose at 120 min of OGTT (mmol/l)	4.80 ± 0.20	6.17 ± 0.26	0.006	0.021
Insulin at 120 min of OGTT (mU/l)	17.20 ± 5.90	43.01 ± 6.94	0.021	NS
Glucose AUC OGTT (mmol-h/l)	770 ± 24	860 ± 29	NS	NS
Insulin AUC OGTT(mU-h/l)	4381 ± 333	7212 ± 883	0.05	NS
HOMA-IR	1.21 ± 0.16	2.79 ± 0.32	0.002	0.016
Matsuda index	9.91 ± 1.83	5.71 ± 0.97	0.023	NS

Data are shown as mean ± SEM. Statistical difference between the groups evaluated by Multiple Unpaired t tests, with multiple comparisons by Benjamini, Krieger, and Yekutieli method of False Discovery Rate. Level of significance for both p and q value was set to <0.05. AUC, area under the curve; BMI, Body mass index; FDR, first degree relatives of T2DM patients; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low density lipoproteins; NS, not significant; TAG, triglycerides.

during OGTT are shown in **Supplemental Figure 1**. Calculated indices of insulin resistance, HOMA-IR and Matsuda index (15) suggested lower whole body insulin sensitivity in FDR.

Basal Expression in PBMC Subpopulations

Comparison CD14$^+$ vs. CD14$^-$ PBMC. When gene expression was analyzed in all samples, irrespectively of the group, the majority of genes were differentially expressed in CD14$^+$ vs. CD14$^-$ PBMC (only four genes had a similar expression level in both PBMC fractions) (**Supplemental Table 1**). Four genes (CCL2, FOS, IL10, and PPARγ) were detected only in CD14$^+$ PBMC and six genes (CD36, EGR1, EGR2, IL1β, TLR2, TLR4) had more than 20 times higher level of expression in CD14$^+$ vs. CD14$^-$ PBMC. Six genes (ACOX1, CLIC3, COX6C, DGAT1, IL6, KIR2DL4) were detected only in CD14$^-$ PBMC and three genes (CCL5, PRF1, and SLC2A1) had more than 20 times higher level of expression in CD14$^-$ vs. CD14$^+$ PBMC (**Supplemental Table 1**).

Comparison FDR vs. Controls. In the basal (fasting) state, majority of the analyzed genes in PBMC subpopulations had similar mRNA levels in control and FDR groups, except for seven genes. CD14$^+$ PBMC exhibited lower basal mRNA expression of TLR2 and TNFα genes and higher expression of IRS1 gene in FDR group (**Figure 1A**). Expression of neither of these genes correlated with HOMA-IR or Matsuda index (not shown). CD14$^-$ PBMC isolated from FDR exhibited lower basal expression of ABCA1, ACOX1, and ATGL and higher expression of IL6 when compared to controls (**Figure 1B**). Expression of ATGL (in CD14$^-$ PBMC from all subjects) correlated with the expression of markers of *de novo* lipogenesis (FASN, ACACA) and β oxidation (ACOX1) (**Figure 1C**). However, when applying correction for multiple testing we did not confirm these seven genes as significantly different between the groups.

Effect of OGTT on Gene Expression in PBMC Subpopulations

mRNA expression of pyruvate dehydrogenase kinase isozyme 4 (PDK4) was used as a positive control for the OGTT response since glucose inhibits PDK4 expression (12, 16). A significant down-regulation of PDK4 mRNA expression in response to OGTT was found in both experimental groups as well as in both PBMC fractions.

In CD14$^+$ PBMC, 21 genes were regulated in response to OGTT in at least one group of subjects (**Table 2**). Among them, 11 genes were significantly changed in both groups. In nine genes (CD36, DUSP1, GOT2, HIF1α, ICAM1, IRS1, PCK2, TCF7L2, and TGFβ1) the response to OGTT was specific for CD14$^+$ fraction (i.e. the change was not present in CD14$^-$ PBMC). Only EGR2 and CCL2 genes were differentially regulated in FDR vs. controls in response to OGTT, i.e. fold changes of mRNA were higher in FDR compared to controls (**Figure 2A**). In the case of EGR2, mRNA expression was significantly upregulated in FDR but not control group (**Table 2**). OGTT induced changes of

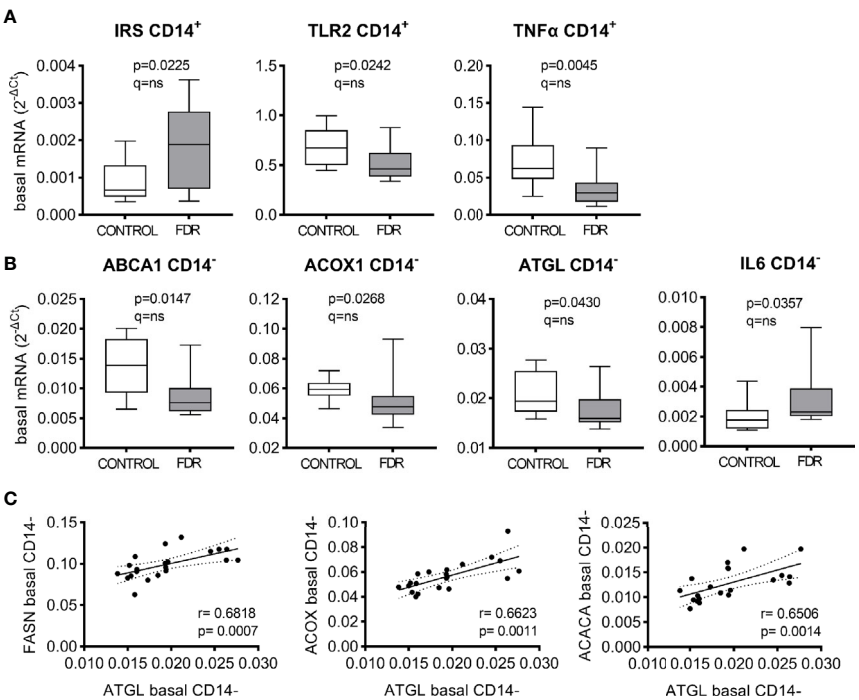

FIGURE 1 | Effect of group (FDR vs. controls) on gene expression in CD14$^+$ **(A)** and CD14$^-$ **(B)** peripheral blood mononuclear cells (PBMC) in the fasting (basal) conditions. Gene expression was normalized to the geomean of reference genes RPS13 and TBP and evaluated by Multiple Mann-Whitney tests, with multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli). Level of significance for both p and q value was set to <0.05. Box plots show the medians with 25th and 75th percentiles, whiskers show min and max values. **(C)** Correlations of basal gene expressions ($2^{-\Delta Ct}$) of ATGL and other genes involved in lipid handling in CD14$^-$ PBMC. The Spearman rank-order correlation coefficient is shown.

TABLE 2 | Effect of oral glucose tolerance test (OGTT) on gene expression.

$CD14^+$ PBMC	Control				FDR				Group difference	
Gene	**Fold change**	**75^{th} - 25^{th} percentiles**	**p value**	**q value**	**Fold change**	**75^{th} - 25^{th} percentiles**	**p value**	**q value**	**p value**	**q value**
ACACA	0.873	0.993-0.691	0.0269	NS	1.497	2.997-0.541	0.0015	0.0104	NS	NS
CCL2	0.820	0.908-0.639	NS	NS	1.046	1.412-0.877	NS	NS	0.0205	NS
CD36	1.344	1.595-1.076	0.0034	0.0230	1.226	1.423-1.072	0.001	0.0078	NS	NS
CPT1α	0.682	1.131-0.548	0.0210	NS	0.696	0.906-0.600	0.0034	0.0182	NS	NS
DUSP1	1.148	1.941-1.079	0.0093	0.0445	1.296	1.432-1.049	0.0034	0.0182	NS	NS
EGR2	0.931	1.203-0.444	NS	NS	2.060	2.352-0.991	0.0137	0.0486	0.0045	NS
GOT2	1.078	1.132-1.028	0.0034	0.0230	1.130	1.269-1.073	0.001	0.0078	NS	NS
HIF1α	0.905	0.928-0.732	0.0068	0.0353	0.785	0.853-0.652	0.0137	0.0486	NS	NS
ICAM1	1.215	1.437-1.083	0.0020	0.0230	1.156	1.329-1.084	0.0093	0.0371	NS	NS
IL10	1.874	2.543-1.065	0.0420	NS	2.693	3.668-1.132	0.0068	0.0313	NS	NS
IRS1	1.462	1.715-1.387	0.0024	0.0230	1.367	1.598-1.277	0.001	0.0078	NS	NS
JUN	1.237	1.547-1.187	0.0068	0.0353	1.188	1.556-0.968	NS	NS	NS	NS
MMP9	1.285	2.046-0.892	NS	NS	1.509	2.154-1.342	0.001	0.0078	NS	NS
p53	1.116	1.149-1.043	0.0029	0.0230	1.039	1.143-0.935	NS	NS	NS	NS
PCK2	1.244	1.299-1.161	0.0005	0.0164	1.193	1.331-1.129	0.0005	0.0078	NS	NS
PDK4	0.201	0.264-0.079	0.005	0.0164	0.143	0.250-0.088	0.0005	0.0078	NS	NS
PLIN2	0.809	0.887-0.617	0.001	0.0219	0.700	0.818-0.595	0.0005	0.0078	NS	NS
PPARα	1.088	1.158-1.028	0.0244	NS	1.169	1.243-1.083	0.0034	0.0182	NS	NS
SLC27A1	1.147	1.288-1.088	0.0068	0.0353	1.065	1.262-.966	NS	NS	NS	NS
SLC2A1	1.217	1.333-0.995	0.0210	NS	1.281	1.723-1.050	0.0068	0.0313	NS	NS
TCF7L2	1.453	1.631-1.207	0.0015	0.0230	1.511	1.585-1.224	0.001	0.0078	NS	NS
TGF-β1	1.108	1.164-1.040	0.0034	0.0230	1.069	1.164-1.000	0.0093	0.0371	NS	NS
$CD14^+$ PBMC	Control				FDR				Group difference	
Gene	**Fold change**	**75^{th} - 25^{th} percentiles**	**p value**	**q value**	**Fold change**	**75^{th} - 25^{th} percentiles**	**p value**	**q value**	**p value**	**q value**
ABCA1	0.448	0.544-0.320	0.002	0.0167	0.546	0.718-0.474	0.002	0.0379	0.0433	NS
ACOX1	0.899	1.002-0.558	0.0342	NS	1.080	1.217-0.946	NS	NS	0.0106	NS
ATF4	1.125	1.200-1.034	0.0024	0.0167	1.231	1.402-1.031	0.0098	NS	NS	NS
CPT1α	0.739	0.885-0.485	0.0024	0.0167	0.723	0.985-0.653	0.0322	NS	NS	NS
DGAT2	1.070	1.356-0.660	NS	NS	1.511	2.118-1.323	0.001	0.0379	0.0045	NS
EGR2	1.723	2.231-1.212	0.0015	0.0167	1.338	2.193-0.924	NS	NS	NS	NS
H6PD	1.261	1.572-1.072	0.0029	0.0182	1.285	1.350-0.941	NS	NS	NS	NS
IL8	1.070	3.691-0.629	NS	NS	2.927	4.349-1.767	0.0029	0.0455	NS	NS
JUN	1.230	1.387-1.136	0.0049	0.0256	1.137	1.385-0.856	NS	NS	NS	NS
KCNQ1	1.127	1.337-0.993	0.0068	0.03333	1.259	1.354-1.017	NS	NS	NS	NS
KIR2DL4	0.534	0.790-0.321	0.0039	0.0222	0.686	1.412-0.494	NS	NS	NS	NS
MLCYD	0.910	1.123-0.808	NS	NS	1.136	1.527-0.952	NS	NS	0.0439	NS
PDK4	0.285	0.488-0.187	0.0005	0.0111	0.245	0.412-0.170	0.001	0.0379	NS	NS
PFKL	0.765	0.794-0.695	0.001	0.0133	0.674	0.791-0.579	0.0039	NS	NS	NS
PLIN2	0.793	0.897-0.724	0.001	0.0133	0.758	0.971-0.711	0.0068	NS	NS	NS
PTGS2	0.989	1.618-0.583	NS	NS	2.402	2.862-1.273	0.002	0.0379	0.0295	NS
SREBP1	1.313	1.486-1.153	0.0005	0.0111	1.219	1.390-1.106	0.0322	NS	NS	NS
STAT1	1.170	1.250-1.127	0.0005	0.0111	1.216	1.318-1.144	NS	NS	NS	NS
TNFα	1.922	3.145-1.136	0.0024	0.0167	1.614	2.227-0.615	NS	NS	NS	NS

OGTT – induced regulation of gene expression is expressed as a fold change in circulating $CD14^+$ PBMC and $CD14^-$ PBMC in first degree relatives of T2DM diabetic patients (FDR) and in controls. Data are presented as median and 75^{th}–25^{th} percentile range of fold change (2h OGTT vs. basal gene expression). Effects of OGTT on mRNA levels within individual groups were evaluated by paired Multiple Wilcoxon matched pairs signed rank tests, Multiple Mann-Whitney tests were used to analyze difference in OGTT-induced fold change between the groups. Both tests were corrected to multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli). Level of significance for both p and q value was set to <0.05. FDR, first-degree relatives of T2DM patients; NS, not significant; PBMC, peripheral blood mononuclear cells. Genes found to have a differential response to OGTT in FDR vs. controls are in bold.

mRNA levels of these two genes were positively correlated with each other and the change of EGR2 was correlated with 2h OGTT glucose levels (**Figure 2A**). However, it should be noted that the significant differences in the OGTT-induced response between the groups (FDR vs. control) were not confirmed after correction for multiple testing.

In $CD14^-$ PBMC, 17 genes were regulated in response to OGTT in at least one group of subjects (**Table 2**), when two genes (ABCA1, PDK4) were significantly changed in both groups. This change was specific for $CD14^-$ PBMC fraction in the case of ABCA1. There were five genes for which the OGTT-induced change in expression was different in the FDR group when compared with controls (**Figure 2B**). Out of these genes, fold changes of ACOX1, DGAT2, MLCYD, and PTGS2 mRNA were higher in FDR compared to controls. In the case of DGAT2 and PTGS2 mRNA expression was significantly upregulated in FDR but not control group (**Table 2**). ABCA1 was down-regulated in both groups, the downregulation being less pronounced in FDR (**Figure 2B**,

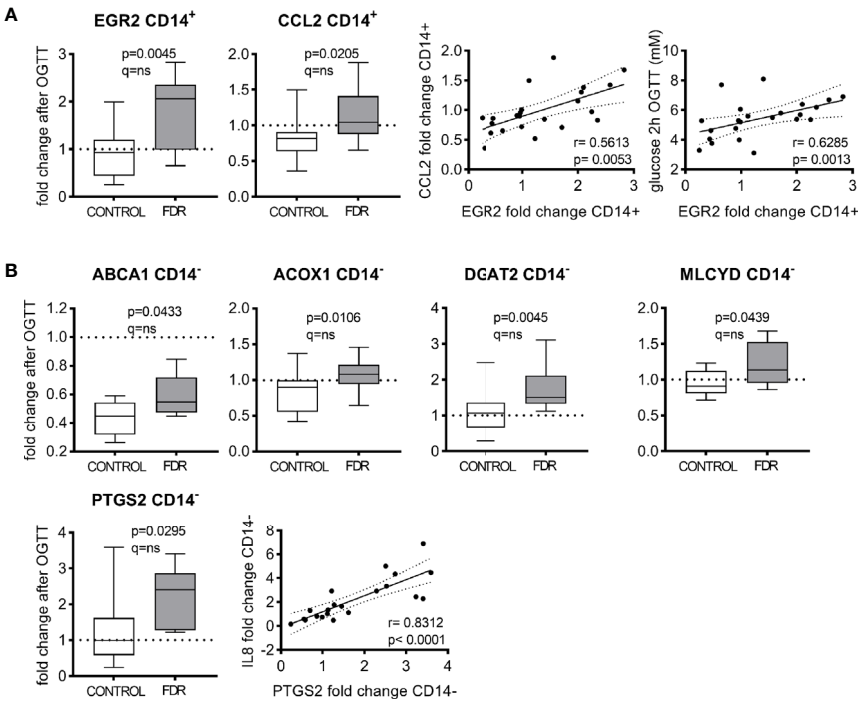

FIGURE 2 | Effect of group (FDR vs controls) on oral glucose tolerance test (OGTT) –induced gene expression changes in circulating $CD14^+$ **(A)** and $CD14^-$ PBMC **(B)**. The effect is presented as fold change (OGTT vs. basal) in each group. Gene expression was normalized to the geomean of reference genes RPS13 and TBP. Box plots show the medians with 25^{th} and 75^{th} percentiles, whiskers show min and max values. Effects of OGTT on mRNA levels within individual groups were evaluated by Multiple Mann-Whitney tests, with multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli). Correlations of changes in gene expressions (fold change) and glucose levels at the end of OGTT (Spearman rank-order correlation coefficient) are shown. Level of significance for both p and q value was set to <0.05.

Table 2). OGTT induced changes of PTGS2 mRNA were positively correlated with the changes of another pro-inflammatory gene IL8 (**Figure 2B**). However the correction for multiple testing did not confirm that the change of mRNA expression of above described genes in response to OGTT was significantly different between FDR and control subjects.

DISCUSSION

In this study we investigated the impact of family history of T2DM on mRNA gene expression in circulating $CD14^+$ and $CD14^-$ PBMC in the fasting conditions and in response to OGTT. FDR subjects included in our study were non-obese and with normal glucose tolerance, but they already showed markers of metabolic impairment: they had higher fasting and post-OGTT glucose and insulin levels and higher HOMA-IR index. Thus, blood cells of these FDR are chronically exposed to mildly higher insulin and glucose levels and, consequently, may react differently to glucose challenge. Indeed, our study suggested that several genes could have different basal expression and/or could be differentially regulated in response to OGTT in FDR when compared to controls, in agreement with several previous studies showing that the changes of gene expression in PBMC induced by glucose intake reflect the metabolic status of the individual (12, 16, 17).

Importantly, we demonstrated that the detected alterations in basal gene expression and its OGTT-induced regulation were different in respect to the type of immune cell population, i.e. $CD14^+$ and $CD14^-$ PBMC. Among the genes that could be considered as markers of one of the PBMC fractions (i.e., they were expressed only in one fraction or had more than 20 times higher/lower level of expression in $CD14^+$ vs. $CD14^-$ PBMC), we identified genes already recognized for its predominant expression in $CD14^+$ PBMC (CCL2, TLR2/4) or $CD14^-$ PBMC (CCL5, PRF1) (18) but also genes that were not previously recognized as markers of circulating $CD14^+$ PBMC, namely two members of EGR family of transcription factors (EGR1, EGR2) and CD36, a transporter of fatty acids. These findings are generally in accordance with the recently published dataset by Monaco et al. who performed RNASeq in 29 subpopulations of PBMC (19).

Although correction for multiple testing of mRNA levels in the basal state revealed no differences between experimental groups in either PBMC fraction, according to individual Mann Whitney comparison, we found rather contra intuitive alterations of IRS1, TLR2 and TNFα expression in $CD14^+$ PBMC from FDR when compared with cells from controls.

Higher mRNA levels of IRS-1, which we found in $CD14^+$ PBMC from FDR, are linked to higher insulin sensitivity and fewer vascular complications in adipose tissue (20), i.e. to better metabolic and cardiovascular health. Nevertheless overall levels of IRS1 in monocytes were very low and together with undetectable levels of GLUT4, it is unlikely that this difference in IRS1 expression in $CD14^+$ PBMC from FDR could have a major impact on glucose metabolism in FDR. Similarly, lower expression of pro-inflammatory TNFα and TLR2 detected in monocytes from FDR could be considered as a sign of lower inflammation but lower expression of these genes was not associated with increased insulin resistance as evaluated by HOMA-IR and Matsuda index (not shown).

FDR $CD14^-$ PBMC exhibited also lower expression of ATGL, a lipase that was found to regulate phagocytosis by macrophages (21) and the production of arachidonic acid in neutrophils and mast cells (22). Although the role of ATGL in $CD14^-$ PBMC remains unknown, ATGL mRNA levels in $CD14^-$ PBMC correlated strongly with mRNA for genes involved in lipogenesis (FASN, ACACA) as well as genes implicated with β oxidation (ACOX1, **Figure 1C**). This nicely supports the existence of a newly discovered metabolic interplay among lipogenesis, β oxidation, and lipolysis in lymphocytes (abundant in $CD14^-$ fraction of PBMC), which appears to be necessary for their activation, proliferation, and differentiation, as fatty acids serve not only as a source of energy but are also important building blocks for membrane synthesis required for lymphocyte function and survival (23).

Results of our study suggest that FDR could differ from controls by the response of gene expression in circulating $CD14^+$ and $CD14^-$ PBMC to OGTT. Although the alteration of the dynamic response to glucose challenge in FDR was more pronounced in $CD14^-$ PBMC, two genes expressed predominantly in $CD14^+$ PBMC, i.e. CCL2 and EGR2, were dysregulated in FDR vs. controls in response to OGTT. EGR2 belongs to a zing-finger containing family of transcription factors and plays a role in the activation of monocytes and polarization of macrophages (24, 25). CCL2 is a well–known chemoattractant for $CD14^+$ PBMC, triggering their infiltration to tissues and differentiation into macrophages (26). Thus, the dysregulation of CCL2 expression in FDR could contribute to the increased infiltration of CD206 negative monocytes/macrophages into adipose tissue, which we observed previously in response to experimental hyperglycaemia in obese women (27). In fact, regulation of both, CCL2 and EGR2 mRNA expression, by higher concentrations of glucose was previously demonstrated on the model of THP1 macrophages (28) and on PBMC in newly diagnosed pediatric patients with T1DM and T2DM suffering from hyperglycaemia (3). Thus, the higher expression of EGR2 in response to OGTT in FDR could lead to an aberrant immune activation of monocytes after meals rich in simple carbohydrates.

In $CD14^-$ PBMC, glucose challenge uncovered an immunity-related gene PTGS2 with potentially differential behavior in FDR compared to controls. PTGS2 is involved in the conversion of arachidonic acid to prostaglandins regulating the immune response to inflammation (29). In T lymphocytes, PTGS2 expression is induced upon T cell receptor activation by nuclear factor of activated T-cells (NFAT) C2 (30, 31). Since NFAT transcription factors were shown to be glucose responsive in various cells, including β cells producing insulin (32), PTGS2 upregulation could be dependent on NFAT glucose sensing. Interestingly, the changes induced by OGTT in PGST2 were correlated with those of IL8, a well-known pro-inflammatory cytokine supporting the recruitment and infiltration of neutrophils and T cells into local inflammatory sites (33) and with glucose levels in 2 h OGTT (**Figure 1C**). This suggests that not only $CD14^+$ but also $CD14^-$ PBMC of FDR could be predisposed to pro-inflammatory activation by glucose challenge.

Nevertheless, majority of genes with differential behavior in $CD14^-$ PBMC in response to glucose challenge in FDR vs. control were associated with lipid metabolism. Despite the fact that the regulation of lipid metabolism appears to be crucial for the activation of lymphocytes (34), not much is known about the possible link between glucose challenge, T2DM development and lymphocyte activation. First, we observed an alteration of OGTT-induced response of DGAT2, a gene involved in lipogenesis, in FDR. Lipogenesis has been recently demonstrated to be required for the survival, proliferation, and differentiation of various types of lymphocytes (23, 34). Expression of DGAT2, the enzyme catalyzing the terminal step in triglyceride synthesis, was shown to be higher in leucocytes from T2DM patients and to correlate with plasma glucose levels (35). Thus, it could be envisioned that the higher glucose and insulin levels seen in FDR (vs. controls) at the end of OGTT might stimulate lipogenesis in lymphocytes through activation of SREBPs. These transcription factors are, similarly to NFAT, expressed in lymphocytes and function as sensitive glucose sensors (36).

Next, we found that not only lipogenesis but also lipid efflux could be dysregulated in FDR lymphocytes since the expression of ABCA1, a transporter enabling cholesterol export from the cells to HDL particle (34), was lower in FDR lymphocytes in basal conditions, and it was less suppressed in response to glucose challenge. These results are in line with similar findings described previously in macrophages - the deficit of ABCA1 was detected in macrophage-derived foam cells and peritoneal macrophages of diabetic mice (37) and human macrophages in response to glucose (38). Although the data on the role of ABCA1 or cholesterol efflux in lymphocytes are scarce, the impairment of cholesterol efflux has been shown to stimulate the proliferation of $CD4^+$ T lymphocytes, which are implicated in the development of atherosclerosis (39).

The last two genes with higher OGTT-induced response in $CD14^-$ PBMC from FDR were ACOX1 and MLCYD, i.e. genes implicated in β oxidation of fatty acids. ACOX1 is the first enzyme of β-oxidation while MLCYD reduces levels of malonyl CoA, a potent allosteric inhibitor of CPT1 activity, and thus indirectly stimulates β oxidation. Both enzymes are present prevalently in peroxisomes, which are involved in the metabolism of long-chain and very-long-chain fatty acids (40). Interestingly, peroxisomal oxidation has been suggested as a compensatory mechanism to metabolize straight medium- and long-chain fatty acids in cases of mitochondrial fatty acid β-oxidation defects (41), which were observed in cells from T2DM patients (42). Although peroxisomes have been identified as

pivotal regulators of immune function and inflammation (40), the detailed consequences of altered peroxisomal β oxidation in lymphocytes have not been thoroughly investigated yet. Together, as lipid synthesis and accumulation tend to drive a pro-inflammatory phenotype, while pathways augmenting β-oxidation and lipid efflux support an anti-inflammatory phenotype of immune cells (34), the assessment of lipid content and analysis of lipid turnover in FDR $CD14^-$ PBMC in further studies is of great interest.

Although this is one of the rare studies enabling the assessment of the nutrient challenge in circulating $CD14^+$ and $CD14^-$ PBMC in human subjects with or without T2DM family background, it is, at the same time, subject to several limitations. Namely, it is a low number of subjects (given by the thorough matching of the two groups and insufficient RNA amount isolated from $CD14^+$ PBMC). Indeed, correction for multiple testing revealed the lower statistical power of the results (higher risk of false discovery), which is probably due to the combination of low number of subjects and large number of analyzed genes. Another limitation is the fact that only one sex (men) was included in the study despite evidences that OGTT- induced effects may be sex-dependent (43). In addition, the splitting of PBMC into only two populations of cells limited the interpretations as possible variations in the gene expression of individual lymphocyte subtypes, dendritic and NK cells, which could not be detected by this study, might have important functional consequences on immunity but also the development of T2DM. Nevertheless, the separation of PBMC into two major subpopulations represents a simple strategy to obtain cells of innate vs. adaptive immunity. Finally, predisposition to T2DM was based only on the family history of T2DM and was not assessed by the analysis of risk variants of alleles known to contribute to T2DM development. Thus, we cannot exclude that these subjects could be predisposed by the exposition to the same environmental factors as their family members with T2DM. In every way, FDR included in the study exerted signs of worsened glucose metabolism showing that the increased risk of T2DM in these subjects is biologically relevant.

In conclusion, although limited by a lower number of examined subjects, our data suggest an altered basal and glucose-intake-induced response of expression of genes involved in the control of inflammation and lipid metabolism in both $CD14^+$ (mostly monocytes) and $CD14^-$ (mostly lymphocytes) PBMC in FDR of T2DM patients when compared with controls. Importantly, the gene regulation appeared to be PBMC-fraction dependent: it was different in $CD14^+$ PBMC when compared with $CD14^-$ PBMC. This suggests essential differences in metabolism of $CD14^+$ PBMC when compared with $CD14^-$ PBMC and points at pathways sensitive to the nutritional challenge represented by glucose intake. Future studies on cell-specific gene regulation of PBMC in larger groups of subjects are warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are presented in **Supplementary Table 2**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of Third Faculty of Medicine, Charles University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MK performed isolation of PBMC, gene expression analysis, and contributed to writing of the manuscript. MŠi prepared the concept of the study and contributed to data analysis. VeŠ contributed to gene expression analysis. MŠt recruited volunteers and performed OGTT. EK recruited volunteers and performed OGTT. VlŠ prepared the concept of the study and contributed to the writing of the manuscript. LR contributed to data analysis, interpretation of results, and writing of manuscript. LR is a guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. 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/fendo.2020.582732/full#supplementary-material

Supplementary Table 1 | List of analysed genes. For all analysed genes gene symbols, name of genes, TaqMan assay ID, median level and 75^{th}–25^{th} percentile range of expression ($2^{-\Delta Ct}$), and the ratio of expression between $CD14^+$ and $CD14^-$ PBMC are shown. Differences in expression between $CD14^+$ and $CD14^-$ PBMC (joint data of control and FDR subjects) were evaluated by paired Multiple Wilcoxon matched pairs signed rank tests, with multiple comparisons by False Discovery Rate (method by Benjamini, Krieger, and Yekutieli). ND, not detected. Level of significance for both p and q value was set to <0.05.

Supplementary Table 2 | mRNA levels-raw data. The output from Fluidigm Biomark instrument is shown, i.e. the values represent Ct values. FDR, first degree relatives of T2DM patients; NTC, non template control.

Supplementary Figure 1 | Evolution of glucose and insulin levels during OGTT. Data show means and SEM. Log2 transformed data were analysed by Two Way ANOVA with Sidak multiple comparison test. G-effect of group, T-effect of time, GxT-interaction, posthoc tests * p<0.05.

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MINI REVIEW
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Healthy Gut, Healthy Bones: Targeting the Gut Microbiome to Promote Bone Health

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Over the past decade, the use of probiotics to modify the gut microbiome has become a public spotlight in reducing the severity of a number of chronic diseases such as autoimmune disease, diabetes, cancer and cardiovascular disease. Recently, the gut microbiome has been shown to play an important role in regulating bone mass. Therefore, targeting the gut microbiome may be a potential alternative avenue for those with osteopenia or osteoporosis. In this mini-review, we take the opportunity to delve into how the different components of the gut work together and how the gut-related diseases impact on bone health.

Keywords: gut, microbiome, bone, Th17 & Tregs cells, short chain fatty acid

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INTRODUCTION

Over a century ago, Metchnikoff had discovered that complex living organisms, now known as microbes, were living symbiotically within the human gut (1). However, their contribution to human health and disease remained understudied. Fast-forward to the past decade and we have seen the gut microbiome taking center stage in various diseases. This has been due to the advance in cutting-edge technologies such as 16S ribosomal RNA sequencing and shotgun metagenomics. Numerous pre-clinical studies now demonstrate that the diversity of the gut microbiome influences a wide range of diseases including autoimmune disorders (2–4), diabetes (5, 6), obesity (7, 8), cardiovascular disease (9, 10), and cancer (11, 12). Interestingly, a growing body of evidence suggests that reintroducing 'good' bacteria to the microbiome in the form of probiotics can dampen the severity of disease (13, 14). Recently, a new interdisciplinary field bridging the study of gut microbiome and bone biology, known as 'osteomicrobiology' has emerged. Over the past couple of decades, various groups have documented the influence of the gut microbiome on bone health and disease.

The skeletal bone is an essential organ that provides the human body with both structural (mobility and support) and a reservoir (storage for minerals such as calcium and phosphorus) function. Bones are composed of minerals deposited around protein, which allows the bone to absorb without breaking (15). The minerals within the bone consist mostly of calcium and phosphorus, important for providing 'hardness' to the bone. On the other hand, proteins, which are made up of a dense network of collagen are important for adding 'softness' to the bone.

Together, they form a scaffold allowing the bone to sustain some degree of mechanical pressure without easily breaking. During childhood to adolescence, the bone predominantly undergoes a process known as 'modeling' where new bones are formed at one site of the bone while on the other side old bones are removed, allowing the bone to change its size/shape. Modeling in humans typically reaches its peak by 20-30 years of age, after which, a different process known as 'remodeling' occurs (16). This is when old bones are removed and replaced with new bones, which is important especially when bone fractures occur or when old bones become brittle and needs replacing. In addition, remodeling can also be activated when the body is deficient or in need of calcium and phosphorus for other cellular or tissue functions.

Bones are formed by non-hematopoietic cells known as osteoblasts (OBs), which are derived from stromal cells within the bone marrow (BM). OBs produce collagen that forms a scaffold for calcium and phosphorus to be deposited into, thus laying down new bone (17). On the other hand, osteoclasts (OCs) resorb bone and are derived from hematopoietic stem cells also located within the BM. OCs adhere and secrete hydrogen ions to the surface of the bone, which dissolves and releases the mineral deposits from the bone. Consequently, constant interaction between OBs and OCs are essential during bone remodeling to maintain bone homeostasis (18). Unfortunately, when bone homeostasis is not maintained, osteopenia or debilitating bone diseases such as osteoporosis can occur. This is mainly due to an over activation of OC activity, which degrades more bone than OBs can form new bones, or, when OB activity is inhibited. Osteoporosis is a global health crisis and primarily occurs within the aging population worldwide (19). As such, the need for deeper understanding on how to maintain bone health and how to treat those with osteopenia is vital as the human lifespan continues to increase thanks to modern medicine.

While the lion's share of the research on bone biology has focused on therapeutics that can directly target the bone to prevent or treat osteoporosis, such as bisphosphonates, it is becoming apparent that the gut may have indirect effects in maintaining bone health. Thus, targeting the gut may be an attractive alternative therapy. In this brief review, we briefly discuss the importance of the gut in maintaining body homeostasis. Next, we discuss in more detail the interactions between the gut and the bone by exploring emerging mechanisms that have come to light in recent years.

THE GUT MICROBIOTA CAN INFLUENCE INTESTINAL PERMEABILITY IN GUT-RELATED DISEASES

The gut is one of the largest organs in the body. As part of the digestive tract, its main goal is to absorb vital nutrients into the bloodstream in order to maintain energy for cellular and body functions. However, in addition to resident gut microbiota (GM), the food and drinks we consume consist of many foreign pathogens/toxins; therefore, without a defense barrier within the gut, critical and complex functions in the body becomes compromised. Thus, to protect the body, there are multiple defense barriers inside the gut. One of the most important barriers of the gut is a single layer of tightly-packed intestinal epithelial cells (IECs), which act as a physical barrier that separates the outside environment from the inside 'sterile' organs of the body (20, 21). These IECs are connected *via* intercellular junctions composed of three junctions; tight junctions (TJs), adherens junctions (AJs), and desmosomes. These junctions work together to assist with selective transport of ions across the epithelial layer and to also protect the integrity of the epithelial layer. To further aid the integrity of IECs, specialized cells among the epithelial layer known as goblet cells secrete mucin to add and maintain a layer of mucus (22). This acts as a biochemical barrier preventing GM and pathogens from directly interacting with the epithelium. The mucus layer is subdivided into an inner and outer mucus layer, in which the GM exist in the outer layer consisting of ~10^4 bacterium from over 100 species resides (23). Despite the detrimental effects the GM potentially poses to body, when kept at homeostasis, the GM plays an essential role in gut functionality by aiding the digestion of foods that the gut itself cannot process (23). Finally, below the epithelium is a host of immune cells, which act as a third line of defense to prevent the GM from entering the systemic circulation. These gut immune cells, including macrophages, dendritic cells, T cells and B cells, are housed within gut associated lymphoid tissues such as Peyers Patches or scattered throughout the lamina propria (24, 25) (**Figure 1**).

During inflammatory disease states, the intestinal barrier can become compromised, increasing intestinal permeability. This makes the body susceptible to foreign particles or GM entering into circulation thereby exacerbating inflammation (26–28). This term is also commonly known as a 'leaky gut'. It was recently demonstrated in a pre-clinical model of RA that the TJ protein, zonula occludens (ZO)-1, which is known to stimulate the opening of TJs, was significantly increased, leading to inflammation. When ZO-1 was inhibited using a zonulin antagonist, inflammation in RA was markedly dampened (29). In addition, increased intestinal permeability has also been associated with inflammatory bowel disease (IBD) due to dysregulation of the pre-dominant AJ protein, E-cadherin (30). Metabolic diseases such as diabetes and obesity have also been linked to increased intestinal permeability, which again is involved in further increasing inflammation (31, 32). Besides direct dysregulation of intercellular junctions, a reduction in the protective mucus layer secreted by goblet cells is also known to cause a breakdown of the barrier integrity allowing for GM to interact and pass through the IECs, which is one of the hallmarks of IBD (33). Interestingly, it was recently shown that the diversity of GM can also affect the integrity of the intestinal barrier and inflammation. With accumulating evidence over the years, restoring or balancing the GM has become an attractive therapeutic avenue in preventing increased intestinal permeability and dampening inflammation. Reintroducing 'good' bacteria by supplementing diets with probiotics has been heavily investigated in both mice and human, in which the supplementation of probiotics has been shown to restore the

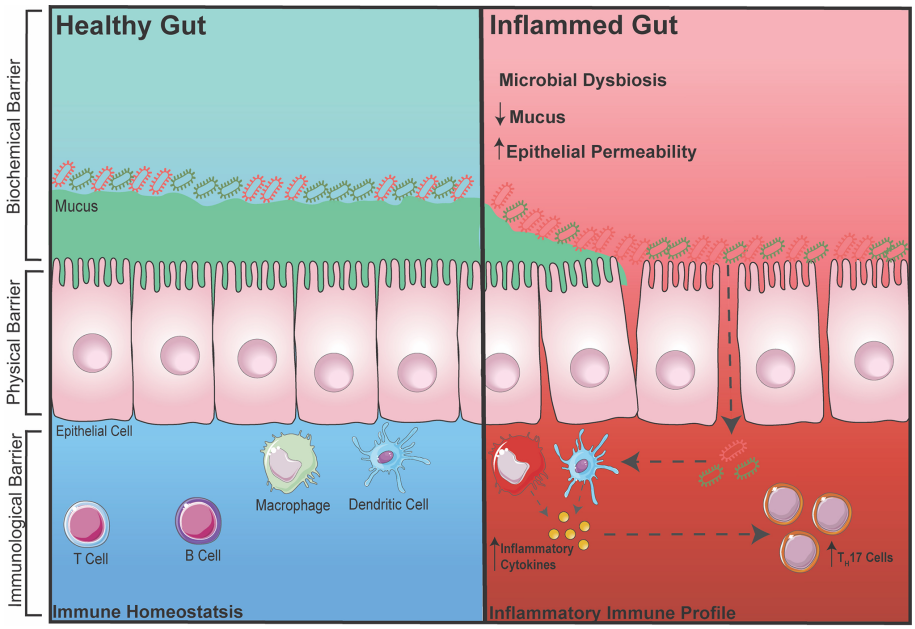

FIGURE 1 | Healthy vs. Inflamed Gut During intestinal homeostasis (BLUE) the biochemical barrier contains a mucus layer that helps to prevent GM from contacting the epithelial layer. The physical barrier consists of tightly-packed single layer of epithelial cells connected by intercellular junctions. The immunological barrier consists of innate and adaptive immune cells that help to surveillance the gut for any foreign entry of GM (gram positive/negative bacteria). During gut-related inflammation (RED), the physical barrier can become compromised, increasing intestinal permeability, making it susceptible for unwanted GM to pass through and activating an immune response.

GM and reduce disease severity. For example, increased dietary choline (9) (obtained from foods such as eggs) and carnitine (34) (from red meats) can cause atherosclerotic-cardiovascular disease *via* the gut microbiota-derived metabolite TMA which converts into TMAO in the liver. By administrating *Lactobacillus*, TMAO levels were reduced, thereby reducing the development of atherosclerosis (35). Furthermore, diabetes has been shown to shift the abundance of two dominating bacterial phyla, Firmicutes and Bacteroidetes that leads to increased disease severity. Supplementation with *Lactobacillus* was shown to reduce inflammation (36, 37). However, with a biased focus now on the effects of probiotics and how it may reduce inflammation, many studies now fail to also investigate whether administering probiotics reduces inflammation due to a restoration in intestinal permeability and immune cells in the gut. Thus, it would be beneficial to measure all three components (GM, immune cell, and intestinal permeability) when intervening with probiotics in gut-related diseases.

POTENTIAL MECHANISMS OF HOW THE GUT MICROBIOTA INFLUENCES BONE MASS

In the past two decades, gut-related inflammatory diseases have been linked to a decrease in bone mass, suggesting that the gut may be interlinked with the bone. Recently, it was shown that people with osteoporosis had significantly higher microbiome diversity compared to healthy individuals, specifically in the abundance of Firmicutes (38). In line with this, another clinical study comparing healthy individuals, people with osteopenia and patients with osteoporosis, showed that the severity of bone loss was correlated to the diversity of the gut microbiome (39). In addition, a clinical study conducted in elderly women with low bone mineral density proposed that restoring the microbiome with supplementation of probiotics reduced bone loss (40). Although, the use of probiotics may be a viable therapeutic option, more investigation on how they influence bone biology is warranted for any major changes for treating bone defects. For example, in a recent clinical trial, it was shown that probiotics had no effect on hip bone mineral density, but rather showed a reduction in femoral neck bone mineral density (41). Therefore, studies in pre-clinical mouse models are critically important to directly test how changes in the GM mechanistically impacts the bone.

The Ohloson group was the first to discover in mouse models that germ-free (GF) mice had significantly increased trabecular bone volume compared to conventionally raised (CONV-R) mice (42). In support of this, depletion of the gut microbiota, through antibiotic administration, has also been shown to restore bone mass (43, 44). Despite these findings, it is still unknown exactly why total deletion of GM would be beneficial for bone health. One reason may be that there is a slow or oscillating penetrance of microbial products through the gut that through any number of pathways result in the activation of OCs or suppress OBs. Whether this occurs during development when

the gut is not fully formed to prevent unwanted bacterial translocation into bloodstream of the body is also a potential mechanism. However, once the body (and gut) is developed, the GM is clearly beneficial for a number of biological functions central to human health. The symbiotic relationship between the gut and the microbiome is important in the absorption of nutrients that the gut itself cannot process. The GM is also a source for vitamin K2, which is required for the function of osteocalcin and can influence bone formation by stimulating OBs (45, 46). Moreover, studies have shown that decreased levels of vitamin K2 due to antibiotic-induced microbiome depletion is associated with a reduction in osteocalcin and bone strength in mice (47). Based on the relationship between the gut and bone, re-introducing beneficial strains of bacteria in the form of probiotics has recently garnered interest in the bone field. Pre-clinical mouse studies have found that administering probiotics such as VSL#3 and bacterial strain *Lactobacillus rhamnosus* GG can be beneficial for bone health *via* restoring GM and intestinal permeability (48). In estrogen deficient mice, in which estrogen dampens cytokines involved in stimulating osteoclastogenesis and bone loss (49, 50), it was found that the administration of *Lactobacillus reuteri* protected against bone loss. Moreover, other studies have demonstrated that in mice with glucocorticoid-induced microbial dysbiosis or post-antibiotic-induced gut dysbiosis, supplementation of *Lactobacillus reuteri* could dampen trabecular bone loss by reducing gut dysbiosis and intestinal barrier dysfunction (51–53).

Despite the potential therapeutic approach in using probiotics to reduce intestinal permeability and bone loss, the question still remains as to how exactly changes in the GM influences bone homeostasis. Obviously, increased intestinal permeability could result in the translocation of bacteria or its microbial products to the bone, increasing and prolonging osteoclastogenesis. In addition, to prevent systemic infection, immune cells within the bone and in other organs express toll-like receptors (TLRs) which recognize pathogen associated molecular patterns. TLR4, one of the most well studied TLRs, is highly expressed on immune cells and is activated by lipopolysaccharides (LPS) and damage-associated molecular pattern ligands. Once activated, TLR4 promotes innate immune responses and the production of inflammatory cytokines. In addition, an increase in inflammatory cytokines, particularly IL-23 can promote the maturation of pathogenic T_H17 cells in the BM, which in turn can stimulate osteoclastogenesis, leading to increased bone loss (54). TLR4 has also been shown to be expressed on mesenchymal stromal cells (MSCs) which plays a critical role in bone formation (55–57). The activation of TLR4 on MSCs has been shown to promote the differentiation of osteoblasts through Wnt3a and Wnt5a signaling (55). While osteoblasts promote bone formation, some studies have suggested that when stimulated by LPS they can promote the differentiation of OCs and bone degradation (**Figure 2A**) (56, 57).

Metabolites, in particular short chain fatty acids (SCFA), produced by GM can play essential roles in regulating immune responses (58, 59). Interestingly SCFA have been linked to improving bone health (60–62). A series of studies by the Pacifici group explored the cross talk between the gut and the bone. Firstly, they demonstrated that promoting the production of butyrate with the administration of lactobacillus or directly supplementing mice with butyrate promoted bone formation *via* an increase in the expression of osteogenic Wnt ligand Wnt10b from Treg cells in the BM. This activates the Wnt signaling pathway in osteoblasts to increase bone formation (61). In a separate study, to further elucidate how the gut communicates with the bone, the Pacifici group investigated how butyrate influences bone formation in response to parathyroid hormone treatment. To determine whether butyrate signals *via* T cells in the BM, they adoptively transfer splenic T cells from $Gpr43^{+/+}$ and $Gpr43^{-/-}$ mice into $Tcb^{-/-}$ mice, a mouse model that lacks T cells (62). After donor T cell reconstitution, they supplemented mice with butyrate in model that recapitulates bone loss *via* administering parathyroid hormone inhibitors (iPTHx) and confirm that butyrate does not directly signal through GPR43 on T cells to increase bone formation. Next, they hypothesized that butyrate may be acting on GPR43 on myeloid cells particularly dendritic cells. To do this, they co-cultured BM isolated dendritic cells from $Gpr43^{+/+}$ or $Gpr43^{-/-}$ mice with WT T cells and discovered that GPR43 on dendritic cells was required to differentiate T cells into Treg cells, which then go on to expressing Wnt10b to stimulate bone formation (**Figure 2B**).

The dysregulation of gut homeostasis can result in an inflammatory immune phenotype. This includes an increase in interleukin IL-17 producing T_H17 cells. Studies have shown that an increase in T_H17 cells and IL-17 in the BM promotes bone degradation by stimulating the differentiation of OCs in the BM (63, 64). The Pacific group investigating the role intestinal immune cells play in bone remodeling in the setting of hyperparathyroidism (65). They demonstrated that in mice with microbiomes enriched with segmented filamentous bacteria, parathyroid hormones expanded the population of gut T_H17 cells which egressed out of the gut, into circulation, migrating into the BM to cause bone degradation (65). To show egress of intestinal T_H17 cells, they inhibited sphingosine 1 phosphate (S1P) receptor-1 with an FTY720 antagonist, which prevents the egress of lymphocytes from the mesenteric lymph nodes and showed a decrease in BM T_H17 cells and bone degradation. Furthermore, to show specifically the importance of T_H17 cell migration into the BM, they showed that the chemoattractant CCL20 was upregulated in the BM, which acts to guide T_H17 cells. When they administered neutralizing anti-CCL20 antibody, the number of intestinal T_H17 cells were unaltered but it prevented the increase in T_H17 cells in the BM as well as a reduction in bone loss. Furthermore, they also differentiated T_H17 cells from isolated splenic T cells from IL-17A-eGFP reporter mice, transplanted into recipient mice and counted GFP+T_H17 cells after inducing bone loss with infusion of parathyroid hormones (**Figure 2C**).

CONCLUDING REMARKS

The inter-disciplinary roles between gut and bone has increasingly garnered attention in those in the field of bone

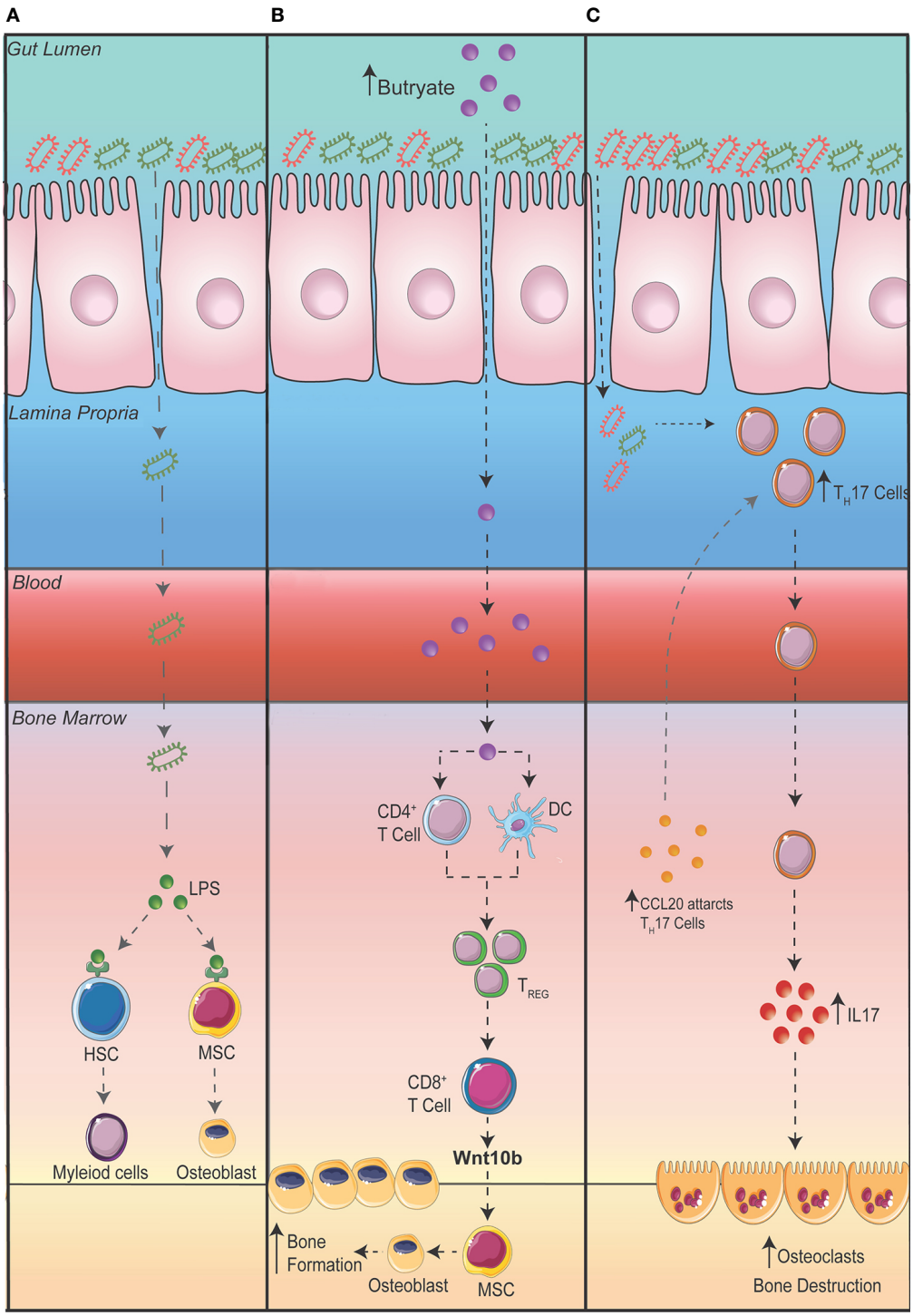

FIGURE 2 | Potential mechanisms connecting the gut to the bone. **(A)** A leaky gut can result in bacteria translocating to other organs such as the bone where lipopolysaccharides (LPS) are recognized by toll-like receptor (TLR) 4 on hematopoietic stem cells (HSC) and mesenchymal stromal cells (MSC). **(B)** Increasing the production of butyrate in the intestine promotes bone formation via an increase in the differentiation of regulatory T cells (Treg). Tregs stimulate $CD8^+$ T cells to secrete Wnt10b promoting the differentiation of osteoblasts and bone formation. **(C)** An expansion of T Helper (T_H) 17 cells in the gut can result in the migration of T_H17 cells to the bone. Additionally, the upregulation of the chemoattractant CCL20 in the bone marrow can aid the migration of intestinal T_H17 cells to the bone where the production of interleukin (IL) 17 can promote the differentiation of osteoclasts thereby promoting bone destruction.

biology. From knowledge gathered so far, it seems that restoring the balancing in GM using probiotics or prebiotics may be beneficial in restoring bone health. However, there are still many questions to be answered before the use of probiotics should be recommended to the aging community who are more susceptible to osteopenia. This will require a multi-disciplinary approach where microbiologists, immunologists, gastroenterologists, computational scientists, and the respective disease experts to work together to find the best approach to cure gut-related diseases.

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

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Impact of Sodium Butyrate Treatment in LPS-Stimulated Peripheral Blood Mononuclear Cells of Poorly Controlled Type 2 DM

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Type 2 diabetes mellitus (T2DM) is associated with chronic low-grade inflammation, which is marked by the dysregulation of innate and adaptive immune responses. Therefore, reducing inflammation, possibly through an immunoregulatory agent, may play a role in T2DM treatment. Butyrate is the most potent short-chain fatty acid (SCFA), and it exerts anti-inflammatory properties by inhibiting histone deacetylase activity. As an immunoregulatory agent, sodium butyrate can inhibit nuclear factor kB (NF-kB) activation and reduce the production of pro-inflammatory cytokines in immune cells. The aim of the study was to measure the level of plasma butyrate in poorly controlled T2DM and normoglycemic participants and to compare the response of peripheral blood mononuclear cells (PBMCs) to sodium butyrate treatment between the groups by measuring production of the following cytokines: tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, IL-13, and IL-10. The *in vitro* study examined the PBMCs of 15 participants with poorly controlled T2DM and 15 normoglycemic participants. PBMCs were cultured with the following stimulations for two days at a temperature of 37°C and 5% CO_2: 100 ng/mL lipopolysaccharide (LPS), 1 mM sodium butyrate, or a combination of 100 ng/mL LPS and 1 mM sodium butyrate. Plasma butyrate was measured using gas chromatography-mass spectrometry, and cytokines from culture supernatant were analyzed using magnetic beads multiplex assay. Plasma butyrate levels in participants with poorly controlled T2DM did not significantly differ from those in normoglycemic participants ($p = 0.105$). Compared to treatment with an LPS-stimulated PBMC culture, treatment with 1 mM sodium butyrate reduced the levels of TNF-α ($p < 0.039$) and IFN-γ ($p < 0.038$) in normoglycemic participants. The same general trend was seen in PBMC from participants with poorly controlled T2DM, but higher variability appeared to preclude statistical significance. These data suggest that butyrate

may modulate inflammatory cytokine production in human PBMCs, but more research is needed to determine if butyrate is anti-inflammatory in poorly controlled T2DM.

Keywords: poorly controlled type 2 diabetes mellitus, inflammatory response, peripheral blood mononuclear cells, butyrate, lipopolysaccharide (LPS)

INTRODUCTION

Gut microbiota have an essential function in maintaining intestinal homeostasis and human health by regulating the immune system, maintaining epithelial barriers, and protecting against several diseases (1). Among the numerous metabolites produced by commensal gut bacteria, short-chain fatty acids (SCFAs) have received the most attention in the context of alleviating host health conditions. SCFAs are the end products of fermentation contained carbolic acids with aliphatic tails. SCFAs are found in high concentrations in the gut lumen, and they can be absorbed *via* non-ionic diffusion across colonic epithelial cells (2).

In addition to being the major energy source for colonocytes (3), SCFAs have long been known to modulate the immune response. Among SCFAs, butyrate is the main type that exerts anti-inflammatory properties (4). Butyrate affects neutrophil function and migration (5) and inhibits nuclear factor kappa B (NF-κB), tumor necrosis factor (TNF)-α, and interleukin (IL)-1β (6). In addition, butyrate reduces the adherence of monocytes or lymphocytes to cytokine-stimulated endothelial cells (7) and inhibits interferon-γ signaling (8). Butyrate probably regulates the immune system by modifying cellular processes, such as promoting the activation of G-protein coupled receptors (GPCRs), inhibiting histone deacetylase (HDAC), and stimulating histone acetyltransferase (4). Correa-Oliveira et al. (4) demonstrated that butyrate could hinder the development of murine bone marrow-derived macrophages, suppress the activation of T cells, and induce apoptosis. In Crohn's disease, butyrate has been known to reduce TNF production and inhibit NF-κB activation in lamina propria cells and peripheral blood mononuclear cells (PBMCs) (9).

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by hyperglycemia and insulin resistance (10, 11) and is often associated with low-grade inflammatory conditions (12). In addition, T2DM is correlated with adaptive immune dysfunction, which is assessed by T helper (Th)-1/Th-2 imbalance (12). Our previous study showed that PBMCs from T2DM patients demonstrated an enhanced cellular responsiveness to phytohemagglutinin (PHA), marked by lower interferon (IFN)-γ production, lower indoleamine 2,3 dioxygenase production, and higher TNF-α/IFN-γ and IL-6/IFN-γ ratios compared to normoglycemic participants (13, 14).

Treatment with SCFAs, particularly butyrate, is thought to be beneficial for patients with low-grade inflammatory conditions such as T2DM, but data concerning the effect of butyrate on reducing cytokine production from PBMC in T2DM patients are limited. Sodium butyrate was found to ameliorate insulin resistance and decrease plasma glucose levels in diabetic rats (15). In addition, Larasari et al. (16) showed that the short-term butyrate treatment reduced the accumulated distance of monocyte migration and decreased the inflammatory potential released by monocytes in healthy subjects and in T2DM patients. In the current study, participants' levels of plasma butyrate were measured, and the responses of peripheral blood mononuclear cells (PBMCs) to sodium butyrate were compared between poorly controlled T2DM patients and normoglycemic participants in the presence of lipopolysaccharide; this comparison was made by measuring cytokine production from innate immunity (TNF-α and IL-6) and Th1/Th2 cytokine balance (IFN-γ, IL-13, and IL-10).

MATERIALS AND METHODS

Participants

The *in vitro* study was performed on PBMCs collected from 15 normoglycemic participants and 15 participants with poorly controlled T2DM. In our former study of T2DM, the sample size of participants with T2DM was 21 people. In this study, we excluded six of those participants, whose T2DM was controlled. Therefore, only 15 participants were included in this analysis.

The normoglycemic participants were 30–55-year-olds with fasting blood glucose (FBG) < 100 mg/dL and glycated hemoglobin (HbA1c) < 5.7% who had never been diagnosed with T2DM by a physician. The criteria for poorly controlled T2DM patients, according to a glycemic target from the American Diabetes Association (17), are people 30–55 years of age, with FBG > 130 mg/dL, and/or HbA1c > 7%. The exclusion criteria for both groups were current pregnancy, steroid use, use of non-steroidal anti-inflammatory drugs or antibiotics within the two weeks preceding the study, and infection at the time of blood collection, including symptoms of fever, sore throat, respiratory tract infection, or urinary tract infection.

The study was approved by the ethical committee of the Faculty of Medicine of Universitas Indonesia, reference number 18-03-0236. All participants provided their written informed consent prior to taking part in this research.

Plasma Butyrate Analysis

Plasma butyrate was analyzed using Agilent 5973 mass spectrometry with 6890 Plus gas chromatography system (Agilent Technologies, Santa Clara, CA, USA). In brief, 1 mL of plasma was added to 3 mL of 5% formic acid and 2.5 mL of ethyl acetate. The suspension was homogenized for 30 minutes and centrifuged at 15000 rpm for 5 minutes. Six mL of supernatant was isolated, and 0.1 g sodium sulfate anhydrous was added. Then, the solution was injected into the gas chromatography–mass spectrometry machine.

Peripheral Blood Mononuclear Cells (PBMC) Isolation and Culture

PBMCs were isolated by centrifugation from Roswell Park Memorial Institute (RPMI) 1640-diluted blood through Ficoll-Paque Plus (GE Healthcare Life Sciences, Chicago, IL, USA). Then, PBMCs were resuspended in a cell culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at a concentration of 10^6 cells/mL. A 500 µL cell suspension was incubated in a 24-well tissue culture plate for one day at a temperature of 37°C with 5% CO2. Then, either 500 µL of 200 ng/mL LPS (Sigma Aldrich, St. Louis, MO, USA), 500 µL of 2 mM sodium butyrate (Sigma Aldrich), 500 µL of 200 ng/mL LPS and 2 mM sodium butyrate, or 500 µL of cell culture medium (the unstimulated group) were added to the wells. Then, the culture plates were incubated for 48 h at a temperature of 37°C with 5% CO2. On day three, cell culture supernatants were harvested and stored at a temperature of –80°C.

Cytokines Assay

Cell culture supernatants were measured for *in vitro* cytokine production. TNF-α, IL-6, IFN-γ, IL-13, and IL-10 were measured using bead-based multiplex assay (R&D Systems, Minneapolis, MN, USA) as described in a previous paper (13). The detection limits were 0.6 pg/mL for TNF-α; 1.11 pg/mL for interleukin-6 (IL-6); 1.27 pg/mL for interferon-γ (IFN- γ); 2.01 pg/mL for interleukin-13 (IL-13); and 0.3 pg/mL for interleukin-10 (IL-10).

Data Analysis

Data were analyzed using IBM SPSS Statistics, Version 23.0 (IBM, Armonk, NY, USA). A Shapiro–Wilk test was used to assess normality between groups. Since the baseline characteristics were normally distributed, unpaired Student's *t*-tests were used to compare the baseline characteristics of the two groups. Plasma butyrate concentration and cytokine production were not normally distributed. Therefore, the Mann–Whitney *U*-test was used to compare the plasma butyrate concentrations of the two groups, and the Kruskal–Wallis test followed by Bonferroni adjustment were used to conduct six comparisons of the differences in cytokine production among the four stimulation groups: LPS, LPS and butyrate, butyrate, and unstimulated culture. Because they were not normally distributed, data of plasma butyrate concentration and cytokines' production were presented as medians (minimum-maximum). GraphPad Prism 8 was used to create graphs and data visualization. The data were considered statistically significant if the *p*-value was < 0.05.

RESULTS

Subject Characteristics

The baseline characteristics of the 15 normoglycemic participants and 15 participants with poorly controlled T2DM enrolled in this study are presented in **Table 1**. As expected, the poorly controlled T2DM group had higher FBG and HbA1c with a mean ± standard deviation of 205.53 ± 99.59 mg/dL and 9.38 ± 2.95%, respectively. Since this study did not match the age and body mass index (BMI) of participants, the normoglycemic group was younger and leaner than the T2DM group, which introduced a high chance of confounding factors; thus, we included age and BMI as covariates for further analysis.

TABLE 1 | Baseline subject characteristics.

Baseline subject characteristics	Normoglycemic group (*n* = 15)	Poorly controlled T2DM group (*n* = 15)	*p*-value
Female sex (*n*, %)	10 (66.7)	8 (55.6)	0.355
Male sex (*n*, %)	5 (33.3)	7 (44.4)	
Age (years)	39.73 ± 4.52	47.33 ± 6.55	0.001**
BMI (kg/m^2)	22.81 ± 2.78	26.80 ± 2.81	0.001**
FBG (mg/dL)	89.51 ± 11.84	205.53 ± 99.59	<0.001***
HbA1c (%)	4.87 ± 0.52	9.38 ± 2.95	<0.001***

*T2DM, type 2 diabetes mellitus; BMI, body mass index; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin; **p < 0.01, ***p < 0.001. Age, BMI, FBG, and HbA1c were presented as mean ± standard deviation, and statistical significances were calculated using unpaired Student's t-tests. The percentage of participants of each sex was calculated using Fisher's exact test.*

Plasma Butyrate in T2DM Patients

As shown in **Figure 1**, even after age and BMI adjustment, plasma butyrate levels of participants with poorly controlled T2DM did not significantly differ from normoglycemic participants [median 0.24 (0.14 – 0.78) *vs* 0.18 (0.12 – 0.69) mM, $p = 0.105$].

Cytokine Concentration in PBMC Culture Supernatants

According to the Kruskal–Wallis test (**Table 2**), the production of TNF-α, IL-6, IFN-γ, and IL-10 were different in participants with poorly controlled T2DM and normoglycemic participants. However, there was no difference in the cytokine production of PBMC cultures between poorly controlled T2DM and normoglycemic participants (**Supplemental Table 1**). *Post hoc* testing found that TNF-α, IL-6, IFN-γ, and IL-10 were more greatly increased with LPS stimulation alone compared to butyrate stimulation and no stimulation in both normoglycemic and poorly controlled T2DM participants (**Figure 2**).

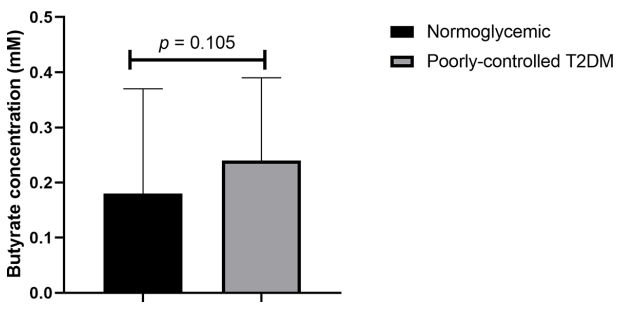

FIGURE 1 | Comparison of plasma butyrate concentration of poorly controlled T2DM patients and normoglycemic participants. T2DM, type 2 diabetes mellitus. The comparison was assessed using the Mann–Whitney test.

TABLE 2 | Kruskal–Wallis test to compare the cytokine production of various stimulated PBMC cultures in the study groups.

Cytokines	Stimulants	n	Median (minimum-maximum)	H statistics	df	*p*-value
Normoglycemic subject						
TNF-α	LPS	15	707 (73 – 4071)	45.832	3	<0.001***
	LPS + Butyrate	15	104 (2 – 743)			
	Butyrate	15	4 (0.6 – 15)			
	Unstimulated	15	2 (1 – 8)			
IL-6	LPS	15	2285.32 (373.66 – 8034.47)	42.013	3	<0.001***
	LPS + Butyrate	15	1618 (8.85 – 6044.9)			
	Butyrate	15	49 (7 – 184.15)			
	Unstimulated	15	20.724 (3.55 – 79.38)			
Interferon-γ	LPS	15	31.52 (6.12 – 584.63)	20.018	3	<0.001***
	LPS + Butyrate	15	13.93 (6.12 – 29.57)			
	Butyrate	15	8.07 (1.27 – 25.66)			
	Unstimulated	15	10.02 (1.27 – 19.80)			
IL-13	LPS	15	254.381 (12.44 – 625.13)	4.511	3	0.211
	LPS + Butyrate	15	66.286 (8.26 – 328.18)			
	Butyrate	15	61.194 (8.25 – 454.08)			
	Unstimulated	15	61.194 (12.44 – 259.42)			
IL-10	LPS	15	50.909 (15.85 – 365.6)	42.247	3	<0.001***
	LPS + Butyrate	15	19 (4.46 – 59.49)			
	Butyrate	15	3 (0.3 – 4.38)			
	Unstimulated	15	3.62 (0.3 – 24.09)			
Poorly Controlled Type 2 Diabetes Mellitus						
TNF-α	LPS	15	1030 (301 – 4235)	47.725	3	<0.001***
	LPS + Butyrate	15	208 (27 – 2461)			
	Butyrate	15	3 (1 – 34)			
	Unstimulated	15	3 (1 – 7)			
IL-6	LPS	15	3185.405 (1215.6 – 7591.23)	47.010	3	<0.001***
	LPS + Butyrate	15	1299.974 (189.2 – 16554.46)			
	Butyrate	15	36.752 (10.44 – 351.70)			
	Unstimulated	15	21.737 (3.73 – 110.93)			
Interferon-γ	LPS	15	33.48 (8.07 – 178.11)	19.716	3	<0.001***
	LPS + Butyrate	15	14.19 (1.27 – 295.37)			
	Butyrate	15	8.07 (1.27 – 19.80)			
	Unstimulated	15	11 (1.27 – 28.89)			
IL-13	LPS	15	329.319 (24.78 – 731.42)	7.287	3	0.063
	LPS + Butyrate	15	121.426 (16.52 – 517.07)			
	Butyrate	15	121.426 (8.26 – 675)			
	Unstimulated	15	97.505 (8.26 – 315.86)			
IL-10	LPS	15	60.931 (14.55 – 258.00)	45.945	3	<0.001***
	LPS + Butyrate	15	22.437 (8.45 – 67.89)			
	Butyrate	15	2.838 (0.3 – 5.62)			
	Unstimulated	15	2.208 (0.3 – 16.75)			

*TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-13, interleukin-13; IL-10, interleukin 10; LPS, lipopolysaccharide at total concentration of 100 ng/mL; LPS + butyrate, lipopolysaccharide at total concentration of 100 ng/mL and sodium butyrate at total concentration of 1 mM; and butyrate, sodium butyrate stimulation at total concentration of 1 mM; df, degree of freedom. The comparisons were assessed using the Kruskal–Wallis test. The data are presented as median (minimum–maximum) pg/mL. ***p < 0.001.*

Compared to PBMC culture stimulated with LPS alone, treatment with sodium butyrate in LPS-stimulated culture suppressed only pro-inflammatory cytokines—TNF-α (p = 0.039) and IFN-γ (p = 0.038)—in normoglycemic participants. Interestingly, although cytokine production from LPS stimulated culture in participants with poorly controlled T2DM was similar to that of normoglycemic participants, treatment with 1 mM sodium butyrate did not significantly reduce TNF-α (p = 0.062) or IFN-γ (p = 0.065) production.

Without LPS stimulation, treatment with sodium butyrate was unable to stimulate or suppress PBMC culture, which was shown by the similar amount of cytokine production in the unstimulated PBMC culture and the culture stimulated with sodium butyrate alone (**Figure 2**). Moreover, sodium butyrate treatment did not affect the production of IL-6, IL-13, and IL-10 by LPS-stimulated culture either in the normoglycemic or poorly controlled T2DM group (**Figures 2B, D, E**).

DISCUSSION

T2DM is classified as a low-grade inflammatory condition (12). Low-grade inflammatory condition is closely related to altered intestinal microbiota or dysbiosis (18, 19). Dysbiosis alters SCFA production in T2DM, thus making the body vulnerable to inflammation (20). Moreover, several studies have revealed that translocation of gut microbiota, especially endotoxins and LPS, worsens inflammation in T2DM (21, 22).

Butyrate is the most potent SCFA that exerts anti-inflammatory properties. About 95% of butyrate is absorbed

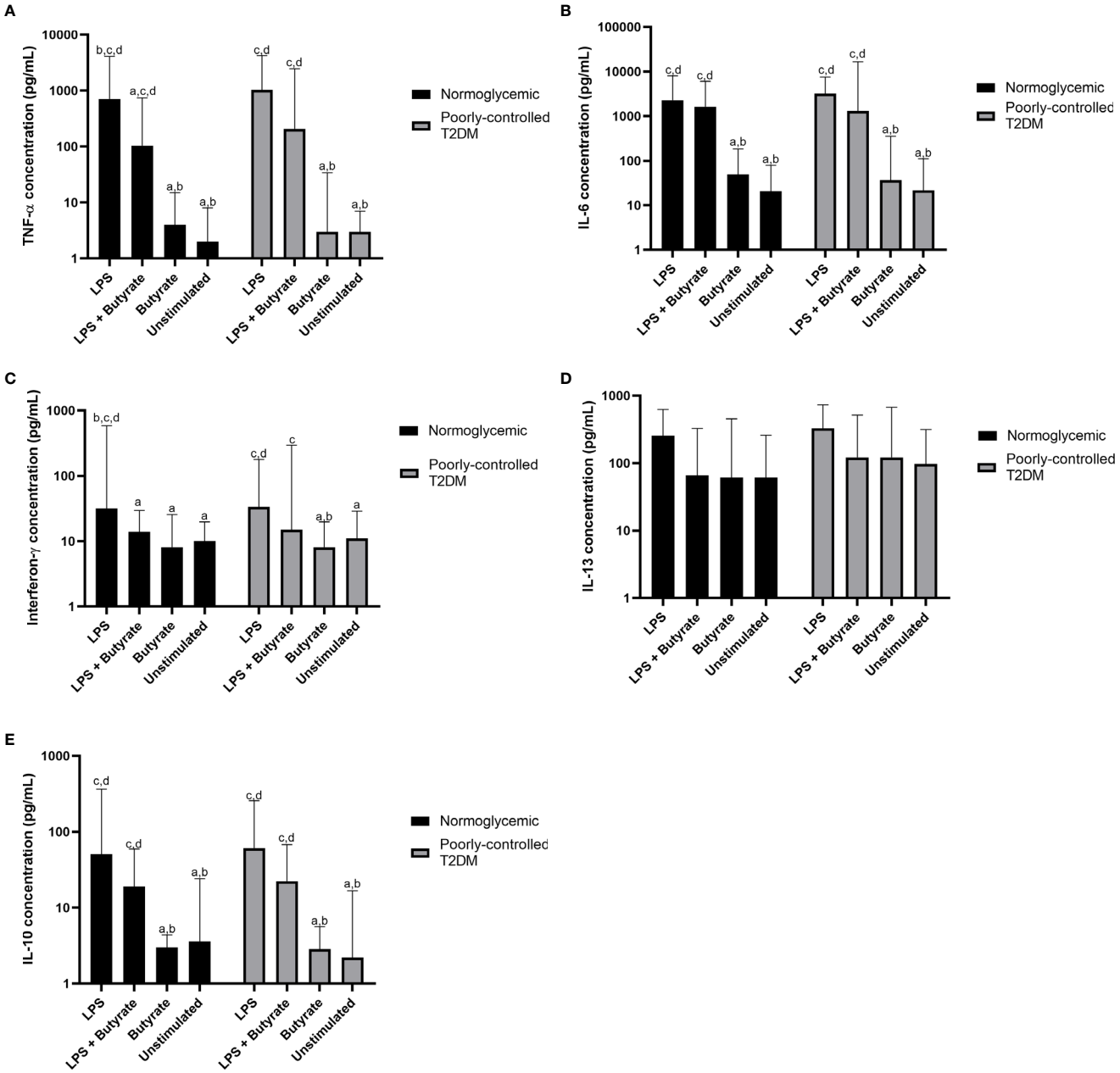

FIGURE 2 | Comparisons of the production of **(A)** TNF-α, **(B)** IL-6, **(C)** interferon-γ, **(D)** IL-13, and **(E)** IL-10 among LPS, LPS + butyrate, butyrate stimulated PBMC cultures, and unstimulated PBMC cultures. T2DM, type 2 DM; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-13, interleukin-13; IL-10, interleukin 10; LPS, lipopolysaccharide at total concentration of 100 ng/mL; LPS + butyrate, lipopolysaccharide at total concentration of 100 ng/mL and sodium butyrate at total concentration of 1 mM; and butyrate, sodium butyrate stimulation at total concentration of 1 mM. The comparisons were assessed using the Kruskal–Wallis test with a *post hoc* pairwise comparisons. The data were presented as median ± range. a: $p < 0.05$ *vs* LPS; b: $p < 0.05$ *vs* LPS + butyrate; c: $p < 0.05$ *vs* butyrate; d: $p < 0.05$ *vs* unstimulated.

from the intestinal lumen (23). However, the plasma butyrate level is far lower than the intracolonic butyrate level because colonocytes use butyrate as an energy source. In our study, the plasma butyrate concentration was in the range of 0.1–0.8 mM in participants with poorly controlled T2DM and 0.1–0.7 mM in normoglycemic participants. These concentrations are higher than that of healthy normoglycemic western population, which has a concentration in the range of 0.01–0.1 mM (24, 25). This finding might be influenced by the Asian diet, which is relatively low in meat, dairy products, and sugar but high in vegetables and resistant starches. We also found that plasma butyrate did not differ between poorly controlled T2DM and normoglycemic

participants. Contrary to our finding, Muller et al. (26) showed that fasting circulating butyrate was negatively associated with fasting glucose and free fatty acid levels but was not correlated with inflammatory markers in obese, non-diabetes participants. Nishitsuji et al. (20) also found that although total SCFA levels were lower in obese diabetic mice than in non-obese mice, the plasma butyrate levels of obese diabetic mice were higher, which was correlated with an increased ratio of Gram-positive to Gram-negative gut microbiota.

Nancey et al. (27) reported that the anti-inflammatory effect of butyrate was achieved when butyrate was given in concentrations higher than the physiological level. In our study, we cultured PBMC in the presence of 1 mM sodium butyrate. In normoglycemic participants, sodium butyrate significantly reduced the LPS-stimulated release of TNF-α but not IL-6. Segain et al. (9) reported that the LPS-induced translocation of NF-kB was inhibited by sodium butyrate. Therefore, butyrate treatment downregulated the LPS-stimulated mRNA expression of TNF-α, TNF-β, IL-6, and IL-1β. Similarly to our result, Nancey et al. (27) demonstrated that although TNF-α and IL-6 were encoded by the same transcription factor, NF-kB, sodium butyrate (0.0625–2 mM) was found to inhibit the LPS + PHA–stimulated release of TNF-α but not IL-6. Moreover, butyrate did not affect IL-6 production induced by *Mycobacterium tuberculosis* (28). Butyrate also had an inhibitory effect on Th-1 cell activity, marked by a reduced release of IFN-γ in normoglycemic subjects, but sodium butyrate did not affect the anti-inflammatory activity of Th-2 cytokines (IL-13 and IL-10) in either group. In line with this result, and despite the possibility that IL-10 would act as a mediator of butyrate's anti-inflammatory effect, Lachmandas et al. (28) reported that SCFAs, including butyrate, also failed to affect LPS-induced IL-10 production. The presence of such conflicting results even in normoglycemic or healthy participants suggests that the mechanism by which sodium butyrate affects cytokine production is complex and warrants further study.

Interestingly, in the PBMC of participants with poorly controlled T2DM, 1 mM butyrate treatment did not affect LPS-stimulated cytokine release, either innate or adaptive, in contrast to findings in normoglycemic participants. Cleophas et al. (29) found that four-week oral sodium butyrate supplementation in participants with metabolic syndrome showed that oral sodium butyrate did not influence the cytokine-producing capacity of PBMC assessed by measuring IL-1β, IL-1Ra, IL-6, and IL-10 upon bacterial stimulation. We can only speculate that our sodium butyrate concentration was inadequate to inhibit HDAC activity or that PBMCs from participants with poorly controlled T2DM have an impaired response to sodium butyrate treatment. The failure of sodium butyrate treatment was also seen in ulcerative colitis patients: Magnusson et al. (30) reported that sodium butyrate was more potent in down-regulating inflammatory gene expression in non-inflamed healthy tissue than in the inflamed tissue of ulcerative colitis patients.

Some limitations of our study need to be addressed. First, we studied the effects of a single concentration of sodium butyrate on LPS-stimulated cytokine release in PBMCs. As the 1 mM sodium butyrate only partly affected cytokine release in normoglycemic and poorly controlled T2DM participants, gene expression and the dose-effect relationship should be studied to elaborate our findings. Second, we did not compare the plasma butyrate and cytokine production of participants with well-controlled T2DM to those with metabolic syndrome to investigate the time when the cytokine-producing capacity of PBMC is impaired. Third, these results do not reflect the *in vivo* condition in participants with poorly controlled T2DM because the duration of diabetes, micro- and macrovascular complications, and various diabetes medications could interfere with the intestinal microbiota and immune responses. However, these findings may at least partly explain why high plasma butyrate levels are not adequate to maintain an anti-inflammatory response in T2DM, particularly in poorly controlled T2DM.

CONCLUSION

Plasma butyrate levels in participants with poorly controlled T2DM were not significantly different from those in normoglycemic participants. Compared to treatment with an LPS-stimulated PBMC culture, treatment with 1 mM sodium butyrate reduced the levels of TNF-α ($p < 0.039$) and IFN-γ ($p < 0.038$) in normoglycemic participants. The same general trend was seen in PBMC from participants with poorly controlled T2DM, but higher variability appeared to preclude statistical significance. These data suggest that sodium butyrate may modulate inflammatory cytokine production in human PBMCs but more research is needed to determine if butyrate is anti-inflammatory in poorly controlled T2DM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committee of the Faculty of Medicine from Universitas Indonesia with reference number 18-03-0236. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HW and DS contributed to conception, design, and acquisition; critically drafted the manuscript; revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. DT and RK contributed to acquisition, critically drafted the manuscript, revised the

manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. SP and RL contributed to interpretation, critically revised the manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. 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/fendo.2021.652942/full#supplementary-material

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