



Vaccine-Induced CD8⁺ T Cell Responses in Children: A Review of Age-Specific Molecular Determinants Contributing to Antigen Cross-Presentation

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Infections are most common and most severe at the extremes of age, the young and the elderly. Vaccination can be a key approach to enhance immunogenicity and protection against pathogens in these vulnerable populations, who have a functionally distinct immune system compared to other age groups. More than 50% of the vaccine market is for pediatric use, yet to date vaccine development is often empiric and not tailored to molecular distinctions in innate and adaptive immune activation in early life. With modern vaccine development shifting from whole-cell based vaccines to subunit vaccines also comes the need for formulations that can elicit a CD8⁺ T cell response when needed, for example, by promoting antigen cross-presentation. While our group and others have identified many cellular and molecular determinants of successful activation of antigen-presenting cells, B cells and CD4⁺ T cells in early life, much less is known about the ontogeny of CD8⁺ T cell induction. In this review, we summarize the literature pertaining to the frequency and phenotype of newborn and infant CD8⁺ T cells, and any evidence of induction of CD8⁺ T cells by currently licensed pediatric vaccine formulations. In addition, we review the molecular determinants of antigen cross-presentation on MHC I and successful CD8⁺ T cell induction and discuss potential distinctions that can be made in children. Finally, we discuss recent advances in development of novel adjuvants and provide future directions for basic and translational research in this area.

Keywords: vaccine, vulnerable population, CD8, children, cross-presentation

INTRODUCTION

British physician Edward Jenner marked the beginning of vaccinology when he developed the world's first vaccine for smallpox in 1796 (1). His invention relied foremostly on the awareness that dairymaids infected with cowpox were immune to outbreaks of smallpox. The next breakthrough occurred in 1880, when the French chemist Louis Pasteur discovered the principle of attenuation

(2). Five years later, Pasteur produced the first laboratory-developed vaccine which tremendously increased the speed of vaccine development.

Most historically developed successful vaccines use weakened or inactivated pathogens. Examples of such vaccines are whole cell pertussis vaccine, which led to large and rapid reductions in pertussis deaths in the United States after its introduction in 1914, (3, 4), or the inactivated polio vaccine which has successfully eradicated poliomyelitis (**Table 1**) (24). More recently, however, technological developments have shifted vaccine development toward the production of formulations that do not contain live material, such as nucleic acid vaccines and subunit vaccines. Subunit vaccines are comprised of purified protein or polysaccharide antigens, often combined with adjuvants, immune potentiators that are capable of stimulating the immune system (24). The first successful example is the hepatitis B subunit vaccine, derived from the hepatitis B surface antigen (HBsAg) (**Table 1**). The development of subunit vaccines has led to improved safety profiles, inclusion of immunostimulants to drive specific types of immune responses, and the opportunity for vaccine component optimization. However, the more defined composition of subunit vaccines can lead to challenges as well, as seen in the case of pertussis vaccination. Replacement of whole-cell pertussis vaccine (wP) by acellular pertussis vaccine (aP), a subunit vaccine, has led to a resurgence of pertussis due to ‘waning immunity’ (25, 26). The efficacy of subunit vaccines often relies on appropriate type and magnitude of immune activation by adjuvants. As the majority of the global vaccine market is for pediatric use, there is an unmet need to critically review the mechanism of action of these adjuvants in a pediatric setting. Studies on adjuvant mechanism of action in early life from our group and others thus far have

focused predominantly on the induction of cytokines, antibodies and CD4⁺ T cells (27–34), but much less is known about the activation of CD8⁺ T cells in early life, and the ability of vaccine formulations or adjuvants to induce these.

In vaccine development, quantitative correlates of protection are often determined by quantification of serum antibody levels or neutralizing ability (35, 36). In antiviral vaccine development, however, absolute correlates of protection are not always defined, and relief of symptoms due to eradication of viral disease is a good indicator of vaccine success. Viruses are intracellular pathogens and use the host cell’s machinery for internalization, translation of viral proteins and viral genome replication (37). Upon viral infection, a cell can use endogenously generated cytosolic viral proteins for antigen presentation *via* major histocompatibility complex (MHC) I molecules on its surface. MHC class I molecules can be found on the cell surface of all nucleated cells (38). CD8⁺ T cells recognize short peptides derived from antigenic proteins presented by these molecules and, hence, play a critical role in the control and elimination of viral infections. MHC class II molecules are expressed on antigen presenting cells (APCs), such as dendritic cells (DCs) (39). CD4⁺ T cells, which recognize peptides presented by MHC class II molecules, promote antibody production which is in many cases sufficient for protection against viruses. While other APCs such as B cells and macrophages are important during different stages of T cell activation, this review will focus on DCs and their role in the instruction of naive T cells.

Activated CD8⁺ T cells can induce apoptotic death of virus-infected cells by the production of Tumor necrosis factor-alpha (TNF- α), Interferon-gamma (IFN- γ) and the release of cytotoxic molecules containing granzymes, perforins, and granulysin (40, 41). These effector functions directly contribute to pathogen clearance. In childhood, when the highest risk for infection

TABLE 1 | Vaccines that are licensed in human newborns and infants in the United States.

Licensed pediatric vaccine	Vaccine type	Antigen(s)	Type(s) of adjuvant (5–7)	Evidence of CD8 ⁺ T cell mediated immunity in control of infection
Hepatitis A virus (HAV)	Inactivated	Inactivated hepatitis A virus (strain HM175)	Virosomes, aluminum hydroxide	Yes (limited data) (8–10)
Trivalent inactivated influenza vaccine (TIV)	Inactivated	Hemagglutinin	Virosomes, MF59, AS03	No (8, 11–14)
Inactivated poliovirus (IPV)	Inactivated	D antigen	None	Yes (15, 16)
Rotavirus (RV)	Live attenuated	Spike protein	No adjuvant used	Unclear (17, 18)
Bacillus Calmette-Guérin (BCG)	Live attenuated	Antigen 85	None	Yes (9, 10, 19)
Measles, mumps, rubella	Live-attenuated	Trivalent antigen	None	Measles: yes (18) Mumps: no (20)
Varicella (VAR)	Live attenuated	Varicella virus live	None	Yes (11–14, 21)
Live attenuated Influenza vaccine (LAIV)	Live attenuated	Hemagglutinin	None	Yes (11–13, 22)
Hepatitis B virus (HBV)	Subunit	HBsAg	Virosomes, AS04	Yes (16, 23)
Diphtheria, Tetanus & acellular Pertussis (DTaP)	Toxoid, subunit	Tetanus toxoid, diphtheria toxoid, detoxified pertussis toxin	Aluminum hydroxide	Yes (acellular Pertussis) (15, 19)
Pneumococcal conjugate (PCV)	Conjugate	Pneumococcal polysaccharides conjugated to a nontoxic form of diphtheria toxin CRM197	Aluminum phosphate	No (limited data) (21, 23)
Haemophilus influenzae type b (Hib)	Polysaccharide conjugate	Polysaccharide conjugated to Hib bacterium	None or with aluminum hydroxide	?

exists, protective antibodies decline rapidly after primary vaccination (42). Newborns and infants are highly susceptible to viral infectious diseases and impaired CD8⁺ T cell responses may lead to progressive or even fatal infection. For example, there is evidence that SARS-CoV-2 virus can infect children (43–47) and can sometimes have severe consequences, such as multisystem inflammatory syndrome in children (MIS-C) (45, 48). SARS-CoV-2-specific CD8⁺ T cells are detectable in infected and convalescent individuals, and potentially correlate with disease outcome (49–52). Vaccine induced CD8⁺ T cell priming may therefore improve the efficacy of immunization in infants against viral pathogens (53, 54).

Nucleic acid-based vaccines and subunit vaccines do not contain a live vector and are therefore generally more safe than inactivated and live attenuated vaccines. However, the high purity of the components can make these vaccines less immunogenic and hence potentially less effective (42), if not adjuvanted properly. Nucleic acid vaccines rely on incorporation of the genetic material into the host antigen-presenting cell genome, potentially resulting in endogenous transcription of viral proteins and therefore effective presentation on MHC class I. Subunit vaccines are composed of only antigenic viral proteins or carbohydrates and therefore the step of genome incorporation into the host is removed. As a consequence, the antigen will not gain access to the cytosol, which is known to be a critical step for MHC class I presentation and subsequent CD8⁺ T cell activation. In general, nucleic acid vaccines are therefore more effective in eliciting CD8⁺ T cell responses (55–57). To improve immunogenicity of subunit vaccines, adjuvants can be added to the formulation. Adjuvants promoting CD8-mediated immunity are therefore a key element for developing effective subunit vaccines against viruses. This can be accomplished by the process of cross-presentation, which enables MHC class I presentation of viral proteins, taken up from extracellular sources. Evidence of adjuvant-induced cross-presentation has been described, often including a proposed mechanism of action (58–68). However, there is to date no published data describing whether and how adjuvants induce cross-presentation in early life. In this review, we address the key concept of how adjuvants can activate CD8⁺ T cell responses and discuss their ability to regulate key molecular pathways relating to antigen cross-presentation in early life (46). Understanding the functionality of CD8⁺ T cells in early life and how they can be effectively induced by adjuvants directly informs the development of subunit vaccines for pediatric use.

CHANGES IN FREQUENCY OF CD8⁺ T CELLS WITH AGE

An important parameter for the induction of an effective antiviral response is that there is a sufficient number of CD8⁺ T cells available to extirpate virus-infected cells. T cell precursors arise from hematopoietic stem cells (HSCs), which are composed of two main cell populations: Sca-1⁻ lymphoid-biased stem cells, and Sca-1⁺ myeloid-biased stem cells. Lymphocytopoiesis in

infants is distinguished by the robust production of T cells, due to a relatively high number of lymphoid-biased HSCs. However, these cells decline with increasing age and as a consequence, the ratio of HSCs in adults shifts toward more myeloid biased HSCs. These cells are less efficient in creating common lymphoid progenitors with high proliferative capacity compared to their counterparts, which directly contributes to the reduction in naive T cell generation in the aged population (69). In addition to a greater influx of HSCs with lymphoid potential into the thymus in children, mouse studies have shown greater efficacy in thymopoiesis in early life (70, 71), resulting in a higher frequency of naive CD8⁺ T cells in the periphery (72, 73). This latter observation is also seen in humans, as both the frequencies of recent thymic emigrants (RTEs) (74, 75) and of naive CD8⁺ T cells (76) decreases with age. Other factors that affect the functioning of HSCs with increasing age are oxidative stress and reduced telomerase activity, which cause the naive CD8⁺ T cell compartment to shrink gradually (77, 78).

In support of the foregoing, experimental data indicate that young infants exhibit higher frequencies of CD8⁺ T cells compared to their adult counterparts. Young adults carry roughly 10¹¹ CD8⁺ T cells (79). Absolute values of neonatal CD8⁺ T cells in human are absent, but limiting dilution studies have shown that the precursor frequency of CD8⁺ cytolytic T cells in neonates is comparable to that in adults (80). In fact, Thome et al. observed that infants (0 – 2 years) express significant higher percentages of CD8⁺ naive T cells compared to young adults (15–25 years) in circulation, lymphoid and mucosal tissues (81).

PHENOTYPIC AND FUNCTIONAL DIFFERENCES OF CD8⁺ T CELLS BETWEEN AGE GROUPS

Phenotypic Differences

In addition to distinctions in frequency of total as well as naive CD8⁺ T cells with age, the expression of certain cell surface receptors can differ between age groups as well, potentially affecting vaccine response to infection or to vaccination (**Table 2**). The main distinctions observed in receptor expression relate to the maturity or activation status of the CD8⁺ T cells. In accordance with findings that newborns and infants have higher levels of naive CD8⁺ T cells, a higher percentage of CD8⁺ T cells express CD28. CD28 serves as a co-stimulator for T-cell activation and survival and is expressed on all naive T cells in newborns (87). In elderly cells, CD28 expression is diminished and sometimes even lost (**Table 2**). This likely contributes to impaired immune responses in elderly. Nevertheless, CD28⁻ T cells express higher levels of effector molecules such as perforin and granzyme B and therefore show improved cytotoxicity (92). This supports the difference in cytotoxicity level between adults and infants, as will be discussed in the next paragraph.

Another activation marker, CD38 is also more frequently expressed on neonatal or infant CD8⁺ T cells compared to adult CD8⁺ T cells (**Table 2**). CD38 is expressed early in ontogeny and

TABLE 2 | Non-exhaustive list of CD8⁺ T cell marker levels in different age groups.

Phenotypic (CD8 ⁺) T cell marker	Level of neonatal versus adult/elderly expression in T cells	Reference
αβ-TCR	Similar	(82, 83)
CD3	Similar	(83–85)
CD5	Similar	(83, 86)
CD8	Similar	(83)
CD28	Lower expression in adults; 40–50% of the elderly (age 80 and above) lack CD28 expression	(87)
CD38	Higher expression in neonates	(83, 88)
KIR	Higher expression in adults and elderly	(89)
CD45RA ^{lo} CD45RO ^{lo}	Expressed by neonates, rare or absent in adult T cells	(83, 90)
CD300a	Higher expression in adults	(91)

is suggested to play an important role during T cell activation (93). In the context of human immunodeficiency virus (HIV) infection, high proportions of CD38⁺CD8⁺ T cells are associated with virologic worsening (88). However, there are studies that have observed opposite findings in children (94, 95). Thus, the significance of CD38 distinctions in the CD8 compartment with age still remains unclear and needs to be further examined in different age groups.

At baseline, children age 6–15 and age 16–17 have similar levels of central memory CD8⁺ T cells compared to adults, but significantly less effector memory CD8⁺ T cells (96). Upon activation with staphylococcal enterotoxin B (SEB), the increase in expression of activation marker CD69 was significantly reduced in these cells, in particular in the 6–15 age group.

Effector CD8⁺ T cells can be distinguished by Killer cell Immunoglobulin-like Receptors (KIR) expression. KIR⁺ cells are estimated to represent approximately 5% of the CD8⁺ T cells in adults and can increase up to 30% in elderly individuals (89). In contrast, roughly 1.67% of CD8⁺ T cells express KIRs in cord blood (97). CD8⁺ T cells acquire KIRs when differentiating into effector molecules (98). This confirms that neonates have more naive T cells than their adult counterparts. The biological functions of KIRs on T cells remain poorly understood although it has been shown that these receptors enhance the efficiency of HLA class I-mediated CD8⁺ T cell responses (99) and therefore could positively influence the outcome of viral infections.

Upon activation, CD8⁺ T cells can introduce the expression of inhibitory molecules aiming to prevent an immoderate immune response. One of these receptors is CD300a, a transmembrane protein with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) capable of conduct inhibitory signaling (100). In a comparative study exploring the CD300a expression on human neonatal versus adult immune cells, significant differences in presence of CD300 receptors on CD8⁺ T cells derived from cord blood and adult blood were observed. The research group showed that naive and memory CD8⁺ T cells from cord blood exhibited significant lower levels of CD300a when compared to adult T cells (91).

In summary, expression profiles of activation CD8⁺ T cell markers correlate with age, displaying more activated T cells when older, due to repeated antigen exposure.

Functional Differences

Neonatal and adult lymphocytes exhibit differential expression of genes involved in T cell receptor (TCR) signaling. Notably, with

regards to the neonatal TCR pool, it has been proposed that neonatal T cells may be less dependent on TCR recognition than their adult counterparts (101). TCRs are integral membrane proteins, which control T cell activation through recognition of specific peptides presented by MHC molecules (102). Neonatal T cells exhibit a less diverse TCR repertoire than adult T cells due to a lag in expression of the enzyme terminal deoxynucleotidyl transferase (TdT) (101). TdT is responsible for adding nontemplated (N) nucleotides in V, D, and J gene segments of TCRs (103) and hence plays an important role in diversifying these receptors. Diversification in TCR signaling is of essence, because a larger pool of different TCRs increases the possibility of recognizing all kinds of peptide antigens. Interestingly, the diverse TCR repertoire of adult CD8⁺ T cells diminishes with increasing age, which contributes to increased susceptibility to viral infections (104).

Upon TCR stimulation, newborn CD4⁺ T cells favor the secretion of IL-8 but less IFN-γ secreting T-helper 1 cells are observed as compared to adult CD4⁺ T cells (101). This is a result of impaired production of type-1-polarizing cytokines by neonatal DCs in response to stimulation through Toll-like Receptors (29, 105). This also affects the CD8 compartment, resulting in CD8⁺ T cells with a more type-2 phenotype (Tc2), which can exacerbate allergy-type reactions in asthma or infection with respiratory syncytial virus (RSV) (106–109). Thus, the immune response generated by neonatal T cells is more of an innate nature, whereas adults produce cytokines that are typically associated with adaptive immune responses. Furthermore, neonatal T cells are less likely to secrete multiple cytokines simultaneously (110). In other words, neonatal T cells are less polyfunctional, which could subsequently lead to less potent T cell responses (96). In a recent study on HIV-1 responses by CD8⁺ T cells, the results showed that HIV-1 specific adult CD8⁺ T cells with high frequencies of CD300a were more polyfunctional (111). These observations are in line with the difference in CD300a expression levels between adults and neonates, as described in the previous paragraph.

Galindo-Albarrán et al. have observed that neonatal T lymphocytes are less cytotoxic than adult CD8⁺ T cells due to lower expression of IFN-γ, a signature molecule for activating the cytolytic pathway (112). Furthermore, they showed that certain enhancers of cytotoxic genes were only expressed in adults and that neonatal CD8⁺ T cells express only low numbers of granzyme producing cells. Interestingly, expression levels of

granzyme B by neonatal NK cells are found to be similar or even higher than adult NK cells (113). Therefore, it could be postulated that neonatal NK cells are being deployed as a compensation mechanism for having CD8⁺ T cells bearing low cytotoxicity.

In elderly, differentiation of CD8⁺ T cells into effector molecules has shown to be impaired in response to infection due to reduced expression of important cytokines, such as IFN- γ , TNF- α , granzyme B, and IL-2 (114). Another functional decline of the immune response in the elderly is suggested to originate from down regulation of certain genes in CD8⁺ T cells which affect a variety of stages of gene transcription, such as transcription initiation, elongation, RNA stabilization, and protein translation and translocation (115). Certainly, more studies are required to fully understand the primary causes of the impaired gene expression that occurs in CD8⁺ T cells in the older population and their functional consequences.

Another significant discrepancy between newborn and adult CD8⁺ T cells is that neonatal cytotoxic T cells have higher proliferative rates than adult naive CD8⁺ T cells and, subsequently, differentiate more rapidly into effector cells (116). As a consequence, an imbalance in effector and memory CD8⁺ T cell formation emerges in neonatal cells, with a shift toward more CD8⁺ T cell effector cells. Thus, newborn cells are less capable of creating immunological memory which has direct implications for creating adaptive immune responses after re-infection. It has been suggested that differences in microRNA (miRNA) expression profiles are accountable for these findings. miRNAs are non-coding mRNA molecules that modulate different aspects of immune responses, such as T cell differentiation. Wissink et al. observed that age-dependent changes in miR-29 and miR-130 in human CD8⁺ T cells may contribute to the diminished development of neonatal memory cells (117). Further research is required to support this hypothesis.

Age-related changes in CD8⁺ T cell frequency and proliferation rate may also be influenced by the presence of homeostatic cytokines, such as IL-7. IL-7 plays a central role in maintaining T cell homeostasis and serves as a key factor in the proliferation and survival of naive T cells (118, 119). During thymic development, stromal and epithelial cells in the thymus produce IL-7 to promote CD8⁺ T cell differentiation in the thymus (120). Thymic production declines with age and, as a consequence, IL-7 levels may decrease during the aging process (121). This could negatively affect CD8⁺ T cell expansion in response to vaccination and potentially result in failure of immunization.

Fms-like tyrosine kinase 3 ligand (FL) also functions as an important regulator of hematopoiesis and is widely distributed in both murine and human tissues (122). FL has an important role in regulating immunity, due to its capacity to stimulate the expansion of DCs (123). Its receptor, Fms-like tyrosine kinase 3 (FLT3), is mostly expressed by immature hematopoietic cells and shows similar expression patterns in newborn and adult mice (122). To our knowledge, however, no differences in FL levels among age groups have been reported.

ONTOGENY OF VACCINE INDUCED CD8⁺ T CELL RESPONSES

Table 1 lists the commercially available vaccines for pediatric and adult use in the United States. The majority of the live attenuated or inactivated vaccines do induce protective CD8⁺ T cell mediated immunity, providing empiric evidence that there is at least no impairment in MHC class I loading or CD8⁺ T cell functionality in early life. Empiric evidence of protective CD8⁺ T cell mediated immunity induced by protein-based vaccines is much less substantial (**Table 1**). There are different mechanisms to create CD8⁺ T cell responses after immunization. Modern vaccines may use viral vectors or nucleic acids as a vaccine delivery system (124). These delivery systems are attractive for vaccine therapy because of their capability to provoke potent and sustained CD8⁺ T cell responses (57, 125). However, the kinetics of nucleic acid delivery and expression of the antigen by APCs likely makes adjuvantation very challenging. Enhancement of the immune response to nucleic acid-based vaccines can be achieved by inclusion of plasmids that encode cytokines, costimulatory receptors, or Toll-like receptor (TLR) ligands (126, 127). The ability to instruct appropriate (often Th1-mediated) CD4⁺ T cell responses in newborns and infants is impaired (128, 129) and requires adjuvantation with select molecules or combinations that have shown the ability to overcome this impairment (27, 29, 130).

An alternative method for inducing CD8⁺ T cell responses is through the mechanism of cross-presentation in which MHC class I molecules present exogenous peptides to naive CD8⁺ T cells. This is in contrast to classical MHC class I presentation, in which a foreign peptide will be displayed after it has arrived into the cytosol of the cell *inter alia* after the cell has been infected. Antigen cross-presentation has been studied for decades, since its discovery in 1976 (131, 132), but there are still many aspects of this concept which are controversial and not fully understood. However, it is clear that there are different subcellular pathways involved in cross-presentation, each consisting of crucial steps for MHC class I presentation. In order to evaluate the potential of adjuvants to induce cross-presentation in children, it is important to summarize the components and mechanisms of cross-presentation to the extent that they are currently known and understood. **Figure 1** provides an illustrated summary of the different components, cytokines, receptors, and biological processes contributing to successful vaccine-induced CD8⁺ T cell activation discussed in this review, and the extent to which changes with age have been observed.

HUMAN DENDRITIC CELL SUBSETS AND CROSS-PRESENTATION

Dendritic cells are a class of bone-marrow-derived cells which can be found in blood, tissues and lymphoid organs. They are referred to as 'professional' APCs because of their unique ability to bridge the innate and adaptive immune system *via* the

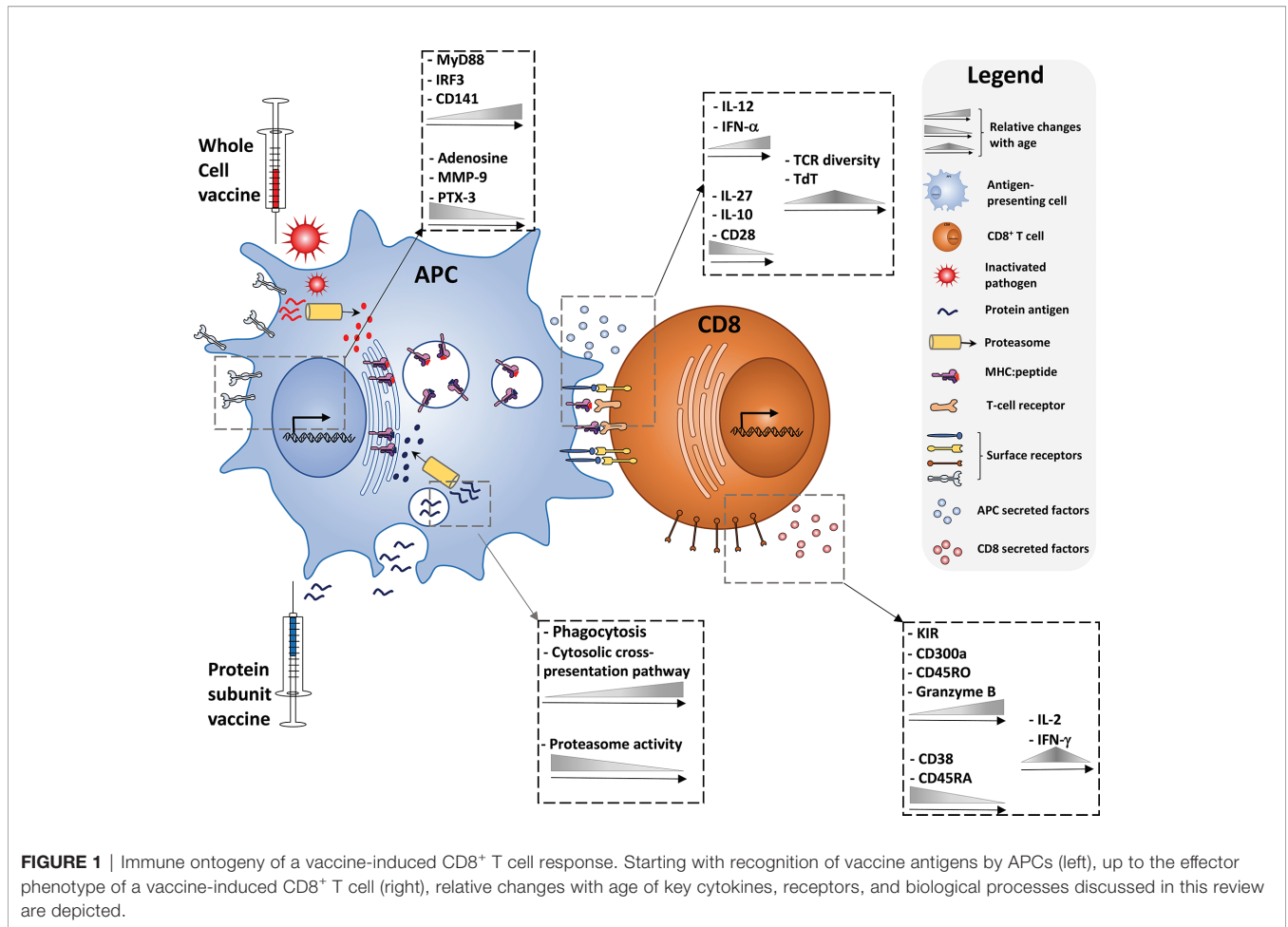


FIGURE 1 | Immune ontogeny of a vaccine-induced CD8⁺ T cell response. Starting with recognition of vaccine antigens by APCs (left), up to the effector phenotype of a vaccine-induced CD8⁺ T cell (right), relative changes with age of key cytokines, receptors, and biological processes discussed in this review are depicted.

presentation of antigens to naive T cells. In human, dendritic cells are divided between two major lineages: conventional DCs (sometimes called myeloid DCs) and non-classical DCs. Based on their phenotypic and functional characteristics, these populations are further compartmentalized into several subtypes (Table 3). Each subset is specialized to react to particular pathogens and to interact with specific T cell subsets. In this manner, the immune system can act upon a broad spectrum of several pathogens and danger signals.

Current vaccination strategies take into account the functional specialization of different DC subsets. For example, both the CD1c⁺ subset (also known as cDC2 DCs) and the CD141⁺ subset (also known as cDC1 DCs) have potent capacity to induce T cell responses. Where cDC2 cells are predominantly inducers of CD4⁺ T cell responses, cDC1 cells are uniquely able to cross-present exogenous antigens on MHC I. Interestingly, neonatal cDC1⁺ DCs reach adult-like levels by mid-gestation (160), and therefore, this subpopulation may be a desirable tool for vaccine development to empower antiviral immunity in early life.

In literature, the chemokine receptor XCR1 is presented as a universal surface marker on cross-presenting DCs (161) in mice as well as humans. This marker is also present on cDC1⁺ DCs

and, therefore, it is thought that XCR1⁺ DCs are crucial in creating successful adaptive immune responses against viruses (162). In addition, pDCs, which do not express XCR1, are considered to cross-present in humans (144–146). However, the exact role of pDCs in cross-presentation remains controversial (163, 164).

Full-term newborns and adult pDCs display similar frequencies in whole blood, although subset composition between these age groups may differ (165). However, Zhang et al. observed that these differences do not affect the potency of neonatal antiviral responses (166). In contrast, pDCs from preterm newborns have shown an immature morphology and an impaired capacity to produce IFN- α (165).

It should be noted that it is difficult to determine the functional distinctions with age of DC subsets in humans. To study the characteristics of DC types *in vitro*, studies are mainly carried out with moDCs. For neonates, moDCs are generated from umbilical cord blood. One of the limitations thereof is the presence of maternal factors in the content of the blood, which may influence the characterization of neonatal DCs (167). However, due to the convenience of this method, moDCs are the main subset for studying the phenotype and function of DCs.

TABLE 3 | DC subsets functions and distinctive markers.

Subset	Cross-presents?	Function(s)	Distinctive markers
cDC1	Yes (133)	cross-presentation (134) Necrotic cells uptake (135) Alloactivation (136) Promote Th1 polarization (137)	CD141, XCRI, CLEC9A, CADM1
cDC2-A	Yes (133, 138–140)	Promote Th1/Th17 polarization (141)	CD11c, CD1c, CD32*
cDC2-B	Yes (133, 138, 142)	Promote Th1/Th17 polarization (141)	CD36, CD1c, CD163
Dermal cDC1	?	?	CD141, CD11c
Dermal Langerin ⁺ cDC2	?	?	CD1a, CD11c
Dermal Langerin ⁺ cDC2	?	Promote Th1 polarization, inhibit Th17 cell differentiation (murine model) (143)	Langerin, CD1a, CD11c
pDC	Yes (144–146)	Promote antiviral immune responses (type I IFN production) (147, 148) Th2 polarization (149) Pathogenic functions in autoimmunity (148) Tolerogenic functions: can induce suppressive responses by inducing Tregs through IDO expression (147)	CD123, BDCA2, BDCA4
CD14 ⁺ DC	No (134, 144, 150, 151)	Tolerogenic functions: Treg induction (152) Th2 polarization (153)	CD209 (154)
SLAN DC	?	Produce Th17-programming cytokines and induce Th17/Th1 cells (155) Promote proliferation, cytotoxicity and IFN- α production by NK cells (156)	SLAN, CD16
IDEC	?	Th1 polarization, recruitment of inflammatory cells, amplification of allergic-inflammatory reactions (149)	CD1a, CD11c
Tip DC	?	Might be important for immunoglobulin A production (157, 158) Th1 polarization <i>in vitro</i> (159) Can stimulate the differentiation and activation of Th17 cells, may participate in tumor rejection (158)	iNos, TNF

THE MECHANISM OF ANTIGEN CROSS-PRESENTATION

The Role of Endocytosis: Soluble Versus Particulate Antigens

Cross-presentation of soluble and particulate antigens is regulated by distinct methods of internalization. Particulate antigens are selectively internalized by APCs through phagocytosis. Subsequently, the antigen can be presented through both MHC class I and II molecules, a time-dependent process in which the NADPH oxidase 2 (NOX2) plays a crucial part. This enzyme is found in professional phagocytes and DCs and contributes to the alkalization of phagosomes by ROS production. NOX2 is recruited to phagosomes with the help of Rab27a and Rac2 (168, 169). Thus, NOX2 prevents phagosome acidification and, consequently, abolishes lysosomal antigen degradation which then allows for cross-presentation (170). This means that when ROS production ceases and the phagosomal pH gets more acidic, the particulate antigen will be preferentially loaded onto MHC class II molecules (171).

In contrast to cell-associated antigens, soluble antigens intended for cross-presentation are internalized by endocytic receptors. Burgdorf et al. describe two different endocytic compartments for antigen processing: early endosomes and lysosomes (172). If a soluble antigen is routed into a lysosome, classical MHC II presentation will take place, whereas antigens in endosomes are targeted for presentation on MHC class I molecules. Depending on the type of endocytic receptor the antigen interacts with upon internalization, the antigen will be

sorted into one of the compartments, a process taking place at the plasma membrane (172, 173). Receptors used by DCs to take up extracellular antigens and route these into endosomal compartments include the C-type lectin receptors CLEC9a, DC-SIGN, Mannose Receptor, and DEC-205 (172, 174, 175). Furthermore, molecular chaperones such as heat shock proteins (HSP) can also bind exogenous antigens for MHC class I presentation, through the scavenger receptors LOX1 and SCARF1 (176).

Interestingly, in newborns, monocytes and neutrophils exhibit a reduced ability to bind and ingest particles. This impairment is transient as neonatal phagocytic ability has shown to reach adult-like levels after a few days after birth (177). There are many factors that potentially account for this phenomenon. For example, the chemotaxis of cord blood phagocytes is decreased and Fc γ receptor expression is diminished in early life. Furthermore, newborns show reduced numbers of neutrophils with phagocytic capacity and display poor complement activity (178). Notably, it has been observed that preterm infants with low numbers of neutrophils contain higher phagocytic ability compared to term infants (179).

The Cytosolic Pathway: Critical Steps in Antigen Cross-Presentation

The cytosolic pathway is characterized by translocation of internalized soluble or particulate antigens to the cytoplasm where they go through degradation by large protein complexes, referred to as proteasomes (59). The way antigens translocate across the endocytic membrane into the cytoplasm is still

debated. It has been suggested that proteins require an unfolding step before translocation (180). However, experimental studies observed enzymatically active proteins in cytosolic extracts, proposing that these proteins do not get unfolded (150).

A common theory is that antigens are transported into the cytosol by sec61, a member of the endoplasmic reticulum associated degradation (ERAD) machinery (181). However, there are papers that have suggested that cytosol export can be independent of sec61 (182, 183). Sec61 has additional functions relating to protein transport across ER and plasma membranes, making it challenging to explore its exact contribution to antigen cross-presentation.

As mentioned previously, low phagosomal pH prevents cross-presentation of particulate antigens. However, it should be emphasized that a slightly acidic environment in the phagosome is required for transportation into the cytosol (180). Particulate antigens can form aggregates and therefore should be processed before transportation. This means that the phagosomal pH should be strictly regulated to prevent antigens from excessive degradation but still be able to deliver them into the cytosol (184, 185).

The involvement of the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) sec22b, located in the ER-Golgi intermediate compartment (ERGIC), as a mediator of antigen export to the cytosol has been described in many papers. However, in recent literature, the role of sec22b in cross-presentation has been questioned (186, 187). Overall, whether sec22b is critical for antigen cross-presentation remains under investigation.

After antigens undergo protein degradation in the cytosol, the proteasome-generated peptides subsequently follow two possible routes: the antigens are transported back into the endosome [1] or into the ER lumen [2], of which the latter only applies to cell-associated antigens (188). The import of peptide fragments into the ER is suggested to occur *via* the transporter associated with antigen processing (TAP). This protein was also found in antigen-containing lysosomes, supporting the hypothesis that peptide loading could also occur inside the lysosomal compartment (189). Indeed, it has been observed that selective TAP deficiency in endosomes strongly impaired the ability for cross-presentation (190). However, TAP-independent pathways also have been described (191, 192). It has been observed that the majority of cytosolic peptides that are being processed TAP-independently are derived from C terminal ends of proteins or N-terminal signal sequences (193). Many proteases are thought to be involved in this process. It should be noted, however, that direct evidence for ER peptide loading is missing. This means that the exact site of peptide loading has not been clarified yet.

If peptides are routed back into the endosomal compartment, efficient cross-presentation requires the translocation of ER proteins to the endosome. ER protein trafficking takes place with the help of sec22b and syntaxin 4, a transmembrane SNARE member present on phagosomes. In this manner, ERGIC molecules such as sec61 and TAP are recruited to phagosomes and endosomes (184). Furthermore, the ER-associated aminopeptidase 1 (ERAP) and the endosomal insulin-

responsive aminopeptidase (IRAP) are recruited to trim the antigens to obtain the right size for efficient MHC class I complexing (194).

With regards to newborns, Kollman et al. studied the efficacy of cross-presentation in murine neonatal dendritic cells using soluble ovalbumin (OVA) (195). Their results showed a clear reduction in neonatal MHC class I presentation of the soluble antigen, while antigen uptake in neonates and adults were similar. As OVA cross-presentation is dependent on the cytosolic pathway (180, 196), this evidence implies that the cytosolic pathway may be impaired in early life.

The Vacuolar Pathway

Unlike the cytosolic pathway, internalized antigens that follow the vacuolar route do not reach the cytosol. Instead, the antigens are thought to be both degraded and loaded onto MHC class I molecules inside the phagosome or endosome. In literature, TAP (in)dependency is mainly used as a determining criterion to distinguish between the cytosolic and vacuolar pathway. However, as mentioned, the cytosolic pathway could also occur without the involvement of the TAP transporter. Besides, research has indicated that cross-presentation of long peptides through the vacuolar pathway can be TAP dependent (197). Therefore, it seems that this distinction does no longer holds ground.

It has been postulated that active proteases, such as the cysteine protease cathepsin S, can enter the endosome or phagosome to process internalized antigens into smaller peptides (198). However, it has been argued that the variety of hydrolases within phagosomes is too harsh for the production of 8-16 amino acid peptides, required for MHC class I loading (199). This argument might not provide sufficient grounds against the fact that there are approximately 15 degradative peptidases and over 50 acid hydrolases localized in the cytosol available for antigen processing *via* the cytosolic pathway (200).

It is not known whether the vacuolar pathway in newborns and children is fully competent. Human neonatal APCs show distinct features in terms of expression of costimulatory molecules, and therefore it has been proposed that these cells require a higher level of activation than their adult counterparts in order to create similar CD8⁺ T cell responses (201). Considering these data, once a human neonatal APC is activated, it could still be entirely competent to induce an adaptive effector response. In support of this notion, Gold et al. found no defect in human neonatal DCs to process and present particulate antigen and concluded that cross-presentation is fully functional in human newborn DCs. However, as previously described, Kollman et al. observed otherwise (195). It could be proposed that differences between these studies might be due to dissimilarities in engagement of the vacuolar pathway. Another possibility is that these different findings are partially caused by the type of antigenic form used in the experiments. It is known that particulate and soluble antigens have distinct immunologic properties. For example, particulation ensures targeted delivery of antigens to APCs in a more concentrated form and, subsequently, results into an

adjuvant effect (202). Furthermore, the antigen within the particle is exhibited in multiple copies, leading to more robust and persisting cellular responses. In light of the foregoing, it could be possible that the intrinsic properties of particulate antigens offset the mediocre costimulatory support displayed by human neonatal APCs.

There are many other facets of cross-presentation still to be elucidated. For example, it is unknown whether the role of the TAP-transporter and sec22b are age-dependent. Furthermore, animal experiments suggest that proteasome function might be elevated in early life and decline with age (203, 204). In brief, there is an unmet need to conduct research on the MHC I pathway in early life and the age-dependent aspects of this process.

Important Cytokines

In order to obtain a functional cytotoxic T cell response, the sole presence of antigens is not adequate. Instead, pro-inflammatory cytokines and costimulatory molecules are required to create an inflammatory environment that will activate naive CD8⁺ T cells. Several cytokine receptors, such as IL-12R and the type I interferon receptor, are essential to activate key transcription factors that support cellular immunity. However, as mentioned earlier, the neonatal immune system demonstrates a characteristic impairment in the production of Th1 polarizing cytokines, such as IFN- α and IL-12p70, which imposes challenges on creating robust and sustained CD8⁺ T cell responses (29, 205–213). Although cell-intrinsic components contribute to this distinct functionality of newborn DCs, elevated plasma levels of extrinsic factors such as IL-10, adenosine, MMP-9, and PTX-3 (214–216) can also play a role.

IFN- α is a type I interferon (IFN), which is predominantly produced by pDCs *in vivo*. When PRRs such as TLRs and cytosolic RIG-I-like receptors recognize viral proteins, early type I IFN production is initiated. Type I IFNs play a major role in antiviral immunity, as they are capable of upregulating MHC and costimulatory molecules on DCs (205). Besides, through direct CD8⁺ T cell contact, type I IFNs significantly improve clonal expansion of CD8⁺ T cells *in vivo* (206). It is well known that type I IFN levels, such as IFN- α , correlate with age. Indeed, newborns infected with RSV show a significant decline in IFN- α production compared to adults (207). It has been postulated that pDC functionality is impaired in newborns and, therefore, shows poor IFN- α induction (208).

Production levels of IL-12 are notably lower in newborns and infants compared to adults (209). Recent work showed that TCR/IL-12 stimulation can enhance expression of genes in newborns that are associated with T cell functions, including cytotoxicity genes and cell signaling genes (210). The ability of newborn dendritic cells to produce IL-12p70 in response to TLR agonists proposedly can be overcome by combined stimulation through TLR4 and Dectin-1 (213). In this study, however, dendritic cells were generated from cord blood monocytes (moDCs) in the presence bovine serum before activation. We have previously demonstrated that the ability of newborn moDCs to produce IL-12p70 is highly reduced by soluble factors present in cord

plasma, and impaired Th1 induction was instead overcome independently of IL-12p70 production (29).

Another member of the IL-12 family, IL-27, is a cytokine which consists of both inflammatory and immunosuppressive capabilities. One of its functions is to promote the survival and differentiation of CD8⁺ T cells, thereby contributing to their effector functions (211). IL-27 secretion by dendritic cells is highest in childhood, while adults' levels are low (212). Interestingly, IL-27 helps drive T helper 1 (Th1) cell differentiation, while newborns are impaired in inducing this type of immune response. The pleiotropic nature of IL-27 could make it difficult to determine its contribution to the impaired Th1 response observed in newborns.

ADJUVANT-INDUCED CROSS-PRESENTATION

Several studies have described potential mechanisms of cross-presentation induced by clinically relevant adjuvants, such as aluminum, saponin and toll like receptor agonists. The next paragraphs elaborate on the molecular pathways of these adjuvants. However, very little is known about these mechanisms in newborns, and therefore, more research is required in order to comment on potential age-dependent differences between these adjuvants.

Aluminum-Based Adjuvants

Insoluble aluminum (alum) salts are the most broadly used classical adjuvants in human vaccines (217). Alum is known for its ability to provoke strong T helper 2 (Th2) responses but does not typically enhance CD8⁺ T cell-mediated immunity.

Alum salts are particulate adjuvants comprised of crystalline structures, which are thought to be central to their adjuvanticity. It has been shown that alum induces the production of uric acid (218). Uric acid can precipitate into crystals of monosodium urate (MSU), which can be phagocytosed by APCs. Phagocytosis of particulate matter, such as alum or MSU, can trigger disruption of the phagosomal membrane, resulting into the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome. In addition, alum has also been shown to induce cell death, leading into the release of danger signals like DNA and uric acid. These components are also able to activate the NLRP3 inflammasome (218). However, the role of NLRP3 in cross-presentation is likely to be limited since NLRP3 is a transcriptional regulator of Th2 differentiation (219). In support of this notion, alum has shown to be capable of inducing a CD8⁺ T cell response without the involvement of the inflammasome (217).

Interestingly, alum-based nanoparticles in combination with the TLR ligand cpG showed enhanced cross-presentation by DCs (220). With the use of endocytic pathway inhibitors, it was observed that the scavenger receptor A was responsible for internalization of the alum-polymer particles. The nanoparticles were both found in the lysosome and cytosol, indicating lysosomal escape. In addition, both brefeldin A, which

inhibits ER transport to the Golgi apparatus, and MG-132, a proteasome inhibitor, reduced alum-induced cross-presentation in DCs. A potential reason for this enhancement in response could be the involvement of both the cytosolic and vacuolar pathway. This is, because it has been speculated that TLR ligands potentially use the vacuolar pathway (58), while alum-based adjuvants seem to follow the cytosolic pathway. Activating both routes of cross-presentation may enhance MHC class I restricted presentation and, thus, promote CD8⁺ T cell mediated immunity. There are many other factors that could play a role, such as particle size and manufacturing conditions.

Saponin-Based Adjuvants

Saponins are triterpene plant glycosides that exhibit different biological and pharmacological properties. There are several saponins that can stimulate the immune system which has led to significant interest in their potential as vaccine adjuvants (221). The most extensively investigated saponin adjuvant is QS-21, a purified fraction from the soap bark tree (*Quillaja Saponaria*) (222).

The molecular composition of QS-21 revealed that its aldehyde group is key in inducing cellular immunity. This is, because it was observed that after reduction of the aldehyde moiety into a secondary amine, adjuvanticity was lost (223). The immune stimulating role of aldehyde-containing adjuvants has been previously described, such as in case of lipidated tucaresol (224). QS-21 is thought to provide a costimulatory signal to the T cell through imine formation from its aldehyde and the primary amine on the T cell, most likely CD2 (221). However, the aldehyde group is not likely to play a role in cross-presentation because tucaresol is not able to induce CD8⁺ T cell immunity by itself. Furthermore, there are also existing triterpene saponins that lack imine-forming structural groups but still induce cytotoxic T cells against exogenous antigens (223).

Saponin-antigen complexes enter the APC by endocytosis in a cholesterol-dependent way (221). Den Brok et al. proposed that, once the antigen-saponin complex is engulfed by the membrane, MHC class I presentation is induced through lipid body formation (225). As previously described, LBs potentially facilitate antigen export to the cytosol and would therefore play an important role in inducing CD8⁺ T cell responses. LB formation destabilizes the membrane and, therefore, allows the antigen to escape the endosome early (221). Thus, antigen translocation into the cytosol occurs in a proteasome-independent matter. Indeed, saponin-induced cross-presentation was not compromised by different NADPH oxidases and several ROS scavengers.

Surprisingly, pharmacological inhibition of LB induction did not reduce antigen export to the cytosol. However, pharmacological and genetic interference with lipid body formation did abrogate saponin-induced cross-presentation. Thus, LBs might contribute to saponin-mediated CD8⁺ T cell immunity in a different yet undefined matter.

TLR-Based Adjuvants

DCs express different subtypes of TLRs on their surface. TLRs recognize various PAMPs and therefore play an important role

in immunosurveillance. Increasing evidence shows that TLR signaling is involved in multiple steps in cross-presentation. It was found that TLR activation controls several aspects of phagocytosis like internalization and phagosome maturation. For example, TLR signals accelerate both phagocytosis and phagolysosomal fusion (226). DC activation status plays a critical role in this process. Indeed, it was shown that activation of DCs with TLR3 and TLR4 ligands significantly reduced the uptake and subsequent cross-presentation of particulate antigen compared with immature DCs (227). This phenomenon was not observed with TLR2 and TLR7 ligands. Another potential explanation for this difference is that TLR3 and TLR4 signaling require Trif as essential adapter, whereas the other TLRs operate Trif independent (228).

TLRs may also contribute to cross-presentation *via* MHC I enrichment, a process which is suggested to occur in a phagosome-autonomous way (59). Gupta et al. observed that TLR4 stimulation in murine BMDCs enhanced the recruitment of MHC class I molecules to phagosomes (229). In their work, they showed that these molecules were not derived from the ERGIC machinery, since recruitment of ERGIC components to phagosomes happened in a TLR-independent matter. This also suggests that TLRs are not involved in TAP recruitment, as proposed in literature (190). Instead, they suggested that MHC I molecules are recruited from the endosomal recycling compartment (ERC), regulated by the activity of rab11a. TLRs would manage this process through TLR-MyD88-IKK2-dependent phosphorylation of phagosomal SNAP-23.

Cross-presentation may be further enhanced through TLR mediated antigen export. Antigen transport from the phagosome to the cytosol was increased after TLR4 stimulation with LPS (227). This would suggest that TLR adjuvanticity favors the cytosolic pathway. However, this would not explain the previous described enhancement in MHC class I molecules in the phagosome, which suggests phagosomal loading instead of ER loading. Furthermore, TLRs accelerate phagosome maturation in the first hours after antigen uptake (230). Phagosome maturation in DCs allows antigens to be processed for antigen presentation. In this way, antigen degradation would not include the proteasome and, therefore, it could be argued that TLR ligands follow the vacuolar pathway. However, evidence points to the contrary, as many papers observed that the cytosolic pathway is ruling in TLR-mediated cross-presentation (68, 231, 232). Very little is known about the underlying molecular mechanism of adjuvant-induced cross-presentation in newborns. However, the type and magnitude of CD4 T cell activation by licensed adjuvants often differs, due to distinct signaling requirements in newborn antigen-presenting cells (233–237). To induce cross-presentation in neonates, TLR-adjuvants are interesting candidates for adjuvant application. TLR expression and downstream signaling have been well studied in newborns and although age distinctions have been observed, specific TLR agonists or combinations have been identified that can induce adult-like levels of pro-inflammatory cytokines such as type I IFNs and IL-12, which are important for cross-presentation and are generally not highly produced in

newborn cells. Furthermore, TLR ligands appear to induce a similar degree of polyfunctionality compared to adults (110). However, IRF3 activation by TLR3 and TLR4 is reduced in newborns (238). This process is Trif-dependent and, as described in the previous chapter, TLR3 and TLR4 ligands showed reduced antigen uptake and cross-presentation, indicating that adjuvants stimulating these receptors will not induce cross-presentation in newborns as effectively.

Most TLR signaling is dependent on the adaptor protein myeloid differentiation primary response 88 (MyD88). It has been suggested that MyD88 functioning in neonatal DCs is impaired (239). As described above, MHC class I upregulation may take place in a MyD88 dependent way and, therefore, it could be postulated that TLR-mediated MHC I enrichment in newborns is reduced, possibly resulting in impaired cross-presentation. However, it has been shown that newborn cells can increase MyD88 mRNA expression after bacterial infection (240), and potent nuclear translocation of NF- κ B can be achieved using TLR7/8 agonists rather than TLR3 or TLR4 agonists. Whether this would also happen upon viral infection is unknown. Even though alum-adjuvants are probably less suitable candidates in early life, because of their propensity to be Th2 skewing, combinations of alum with TLR adjuvants have shown promise, as described above.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This paper highlights key differences between the neonatal, infant, and adult immune system and aims to underline that our understanding of vaccine mediated CD8⁺ induction in early life requires further investigation.

Most commercially available vaccines for pediatric use consist of attenuated or inactivated pathogens. While these vaccines are mostly competent in stimulating CD8⁺ T cell immunity, modern vaccine development is shifting toward subunit and nucleic acid vaccines and, consequently, has imposed major challenges on inducing adequate cellular immunity. Therefore, subunit vaccines often depend on immune activation by adjuvants. Little is known about CD8⁺ T cell induction by adjuvants, for example, *via* cross-presentation, in newborns and infants. Adding to the complexity, in early life, many aspects of the immune system correlate with age. Even though neonates and infants have enough naive CD8⁺ T cells to create a robust antiviral response, they exhibit several functional differences compared to adults that may have direct

implications for their ability to cross-present antigens. As a result, their CD8⁺ T cells have reduced cytotoxicity and are biased toward type 2 immunity. And neonatal APCs receive weak costimulatory stimulation. Altogether, this means that a vaccinated child will produce less pro-inflammatory cytokines important for cross-presentation, does not receive the same stimulation as an adult and shows poor CD8⁺ T cell effector properties. To overcome these hurdles in the pediatric population, adjuvants should be tailored to their distinct immune system.

Future research should examine whether cross-presentation mechanisms in neonates and infants are fully operational, and aim to identify adjuvants that can induce potent CD8⁺ T cell responses. For example, using adjuvant combinations that employ both the vacuolar and cytosolic pathway or use different mechanisms for antigen export to the cytosol may enhance MHC class I presentation. Furthermore, antigen particulation can boost the adjuvant effect and outbalance poor neonatal APC costimulation. Besides, extra stimulation of cytokines such as IL-12 may enhance neonatal cytotoxicity and, thus, improve the antiviral response.

To date, however, it is unknown how adjuvants contribute to cross-presentation in neonates. For example, do TLR adjuvants also enhance antigen uptake and phagolysosomal fusion in newborns or is this an age-dependent process? Do adjuvants use similar cross-presentation pathways in newborns as they do in adults? Refining our understanding of adjuvant-induced CD8⁺ T cell immunity will further improve vaccine formulations in the pediatric setting and, hopefully, create more robust and sustained responses to protect this vulnerable population.

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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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