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Is the developmentally immature immune response in paediatric sepsis a recapitulation of immune tolerance?

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Summary

Immunologically immature neonates suffer the highest incidence of paediatric sepsis. Postnatal immunological maturation is characterized by a relatively hypo-inflammatory immune response. The mechanisms that differentiate the mature and immature immune responses resemble those that differentiate the hyper- and hypo-inflammatory responses in severe sepsis. Immunological maturational differences likely affect the neonate's ability to mount an appropriate hyper-inflammatory response, a counteractive hypo-inflammatory response, and subsequent return to immune system homeostasis. To better understand the role of the hypo-inflammatory response in paediatric sepsis, we will explore the maturation of the immune system and the effect it may have on the sepsis-induced hypo-inflammatory response.

Keywords: endotoxin tolerance; immune maturation; immune tolerance; neonatal sepsis; paediatric sepsis

Introduction

Severe sepsis is a systemic mixed, pro- and anti-inflammatory response to an infectious organism and severe tissue injury (Table 1). Commonly, the exaggerated inflammatory response is associated with progression to organ dysfunction and failure.[1] The prevailing model of the severe sepsis response recognizes a pro-inflammatory response and a temporally variable counter-regulatory anti-inflammatory response, characterized by a hypo-inflammatory innate immune response and an impaired adaptive immune response.[1] In clinical studies, the presence and persistence of a hypo-inflammatory state in severe sepsis is associated with an increased risk of secondary infections and death.[2] However, whether the hypo-inflammatory response is mechanistically associated with progression to and persistence of organ failure remains controversial.

Paediatric sepsis in the USA disproportionately affects the neonatal population with a prevalence of 9·7/1000 neonates. This contrasts with 2·25/1000 in non-newborn infants and 0·23–0·52/1000 in older children.[3] Postnatally, the immune system undergoes rapid maturation, approaching adult maturity by 2 years of age, with continued maturation into the second decade of life[4] (Fig. 1). The normal neonatal immune response is relatively hypo-inflammatory compared with the typical adult response.[5] Interestingly, the mechanisms that differentiate the mature adult immune response from the immature neonatal response recapitulate the hyper-inflammatory and hypo-inflammatory states in sepsis (Table 2).

The differing responses based on the immune system's maturational state are likely to play a role in a child's ability to mount an appropriate hyper-inflammatory response, use the hypo-inflammatory response to control and curtail ongoing harmful inflammation, and re-establish immune homeostasis. To more fully understand the potential role of the hypo-inflammatory responses, we explore the developmental differences between the mature and immature immune response to endotoxin and the immune regulatory effects in neonatal and paediatric sepsis. We also explore the role of developmental immune recapitulation in the mature immune responses that characterize sepsis.

Abbreviations: APC, antigen-presenting cell; DAMP, damage-associated molecular pattern; ET, endotoxin tolerance; IL, interleukin; IRAK, interleukin receptor-associated kinase; IT, immune tolerance; NF-κB, nuclear factor-κB; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; ROS, reactive oxygen species; Th1, T helper type 1; TLR, Toll-like receptor; TNF, tumour necrosis factor; Treg, regulatory T

Table 1. Definitions

Hyper-inflammatory response	the net effect of the immunological response is characterized by the production of pro-inflammatory cytokines and activation of leucocytes[1]
Hypo-inflammatory response	the net effect of the immunological response is characterized by the production of anti-inflammatory cytokines, negative regulators of Toll-like receptor signalling, and decreased innate-adaptive immune system communication[1]
Immune tolerance (IT)	a transient state seen in cells of the innate immune system after repeated exposure to low concentrations of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), after which they are unable to respond normally to further PAMP or DAMP exposure[1]
Endotoxin tolerance (ET)	a transient state seen in cells of the innate immune system after repeated exposure to low concentrations of endotoxin after which they are unable to respond normally to endotoxin challenge[34]
Cross-tolerance	the transient state of IT to one PAMP or DAMP induced by low exposure to a different PAMP or DAMP[34]

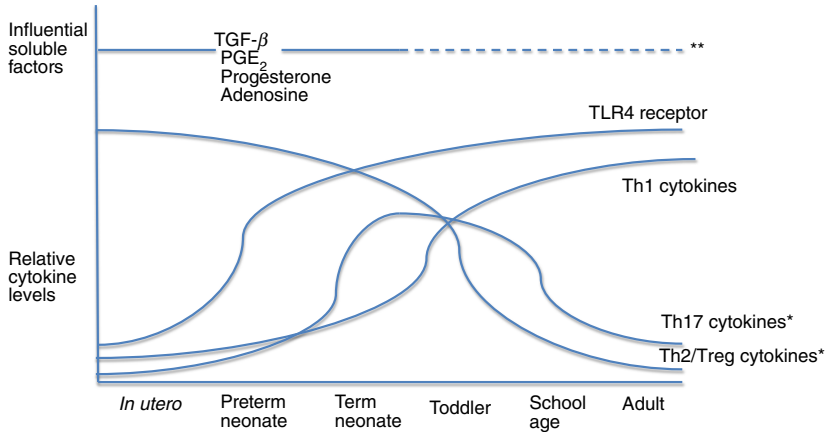

Figure 1. Age-dependent changes in endotoxin-induced T-cell responses. The T-cell response *in utero* and in preterm and term neonates is characterized by predominantly T helper type 2 (Th2) and regulatory T (Treg) responses. As the immune system matures early in life, these responses are replaced by the more mature Th1 response. Toll-like receptor 4 (TLR4) expression is relatively low *in utero* and in very premature neonates, increasing to adult levels later *in utero*. Maternal- and fetal-derived soluble factors [transforming growth factor-β (TGFβ), prostaglandin E_2 (PGE_2), progesterone and adenosine] influence the T-cell response *in utero* and in early postnatal life. *Conflicting data exist regarding the capacity of the neonatal Th17 and Treg responses. **Influences of these soluble factors in adulthood are unclear but may be important.[4,5,9,10,12,13,16,18–20,27,28]

The typical immune response to endotoxin

The canonical immune response to endotoxin has been thoroughly reviewed by Kawai and Akira[6] and Medzhitov and Janeway.[7] In brief, the cells of the innate immune system recognize foreign pathogens (pathogen-associated molecular patterns, PAMPS) and damage-associated molecules (DAMPS) through cell-surface expression of pattern-recognition receptors [PRR, e.g. Toll-like receptors (TLRs)]. The TLRs transduce the extracellular signal to intracellular domains through adapter protein signalling cascades (e.g. TRAM, TRIF, TIRAP, MyD88)[8] (Fig. 2). These signalling cascades form the initial response to endotoxin and create the environment in which T cells of the adaptive immune system are stimulated. Naive $CD4^+$ and $CD8^+$ T cells differentiate into one of several types of effector and memory T helper cells based on co-stimulatory molecules expressed on antigen-presenting cells (APCs) and the cytokine milieu within which they are stimulated[5,9] (Table 3; Fig. 3). The differentiation into particular effector T-cell subtypes defines the quality and effectiveness of the adaptive response to a specific infection. $CD4^+$ T cells include T helper type 1 (Th1) cells that produce a primarily pro-inflammatory response, Th2 cells that stimulate an antibody-mediated response in addition to combating parasitic infections and promoting allergic reactions, regulatory T (Treg) cells that are immune suppressive, and Th17 cells that fight extracellular bacteria[10–17] (Table 3). The interaction between the innate and adaptive responses depends on a coordinated sequence of developmental processes and is fundamental to the maturation-dependent functional difference between neonatal and adult immune responses.[18]

The immature immune response

The immature immune response in preterm and term neonates places them at high risk of developing sepsis.[5]

Table 2. Comparison of the immature, mature and tolerant responses to endotoxin stimulation

Immature immune response relative to mature response	Relative effect of difference	Tolerance recapitulates immature immune response?
Cellular adhesion functions		
↓Leucocyte adhesion and extravasation[5]	Impaired ability to fight infections[5]	Yes[20]
PRR/Signal transduction		
↓TLR4 receptor expression[13,18,20,22]	Minimal effects[13,18,20,22]	Yes[8,21,37]
↓Soluble TLR4 co-receptor expression[18]	Minimal effects[18]	Yes[8,21,37]
↓TLR/MyD88 pathway signalling[14,23,24]	↓ROS production[24]	Yes[2,8,21,37]
Regulation of the adaptive response		
↑Th2-skewing cytokine production[4,12,14,18]	↑Humoral immune response[33]	Yes[2,21]
↓Th1-skewing cytokine production[4,12,14,18]	↓Pro-inflammatory immune response[4,12,14,18]	Yes[2,21]
↓Stimulated T-cell proliferation[4]	↓Cytotoxic T-cell response and ↓Adaptive response[4,24]	Yes[44]
↑Th17/Treg differentiation[a,13,16,20,22,23,27]	↑Anti-inflammatory response[13,16,20,22,23,27]	Unknown
Soluble factors		
↑Maternally produced factors (TGF-β, progesterone, PGE_2)[12]	↑Th2-skewed response[12]	Similar PGE2 (macrophage-produced)[21]
↑Adenosine[12,18–20,31]	↑Th2-skewed response[12,18–20,31]	Unknown
↓Antimicrobial protein peptides[31]	Impaired ability to fight infections[31]	Unknown
Cell–cell interactions		
↓$CD8^+$ T-cell activation[24]	↓Cytotoxic response[24]	Yes[8]
↓$CD4^+$ stimulation by APCs[33]	↑Th2-skewed response[33]	Yes[2]
↑Apoptosis of $CD4^+$ Th1 cells[24]	↑Th2-skewed response[24]	Yes[2]
↓Memory T cells[9]	↓Eradication of viral and intracellular pathogens[5,13]	Unknown

PGE_2, prostaglandin E_2; PRR, pattern recognition receptor; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; Th2, T helper type 2; TLR, Toll-like receptor; Treg, regulatory T.

[a]Conflicting evidence exists regarding the impact of Th17 and Treg differentiation in the immature immune response.

Although some studies conclude that the neonatal inflammatory response is similar to an adult's after a highly toxic exposure, less potent PAMP activation consistently results in an attenuated pro-inflammatory response in neonates compared with an adult exposed to a similar stimulus.[10,13,18–20] These data indicate that neonatal phagocytes are less responsive to PAMP stimulation, resulting in relatively deficient adhesion and extravasation. Sub-maximal cytokine proliferation, preferential Th2-skewed cytokine production, and decreased antigen-presenting capacity result in a globally hypo-inflammatory immune response.[5] A comprehensive understanding of the functional differential endotoxin activation of the immature versus mature immune systems can be explored from four interacting perspectives: PAMP signal transduction, cytokine release, plasma components and cell–cell interactions.

Pathogen recognition and signal transduction

Transduction of the prototypical endotoxin immune signal is initiated by PAMP–PRR binding and is regulated at multiple levels including the monocyte cell surface receptors, intracellular signalling molecules, mRNA transcription, protein translation, cytokine secretion and protein ubiquitination pathways[8,11,21] (Fig. 2).

Signal transduction begins with the TLR4 receptor, which has lower cell surface density expression in very premature infants compared with neonates delivered after 30 weeks of gestation. From that latter time-point, TLR4 expression approximates that of adults.[13,18,20,22] Initiation of the neonatal TLR4 response is impaired by lower soluble co-receptor expression and impaired up-regulation of the CD14 co-receptor expression after stimulation.[18] However, these deficiencies probably play a minor role in attenuating the neonatal pro-inflammatory response.[18,19,23]

Downstream, activation of the neonatal TLR4/MyD88 pathway results in decreased production of reactive oxygen species (ROS) compared with the mature adult response. This difference significantly hinders the neonate's ability to clear infection.[24] The clinical effects of this impairment are best exemplified by impaired ROS production in patients with chronic granulomatous disease who suffer recurrent, severe invasive bacterial and fungal infections.[25]

Further evaluation of the individual steps in the TLR4/MyD88 pathway reveals differential expression of signal transducing proteins based on immune maturation status. Reported in a systematic comparison of the TLR4 signalling pathways of adults and neonates, similar basal cell surface receptor and intracellular signalling protein expression Levy *et al.*[14] Separately, Yan *et al.* demonstrated that differential tumour necrosis factor-α (TNF-α) production is due, in part, to decreased MyD88 expres-

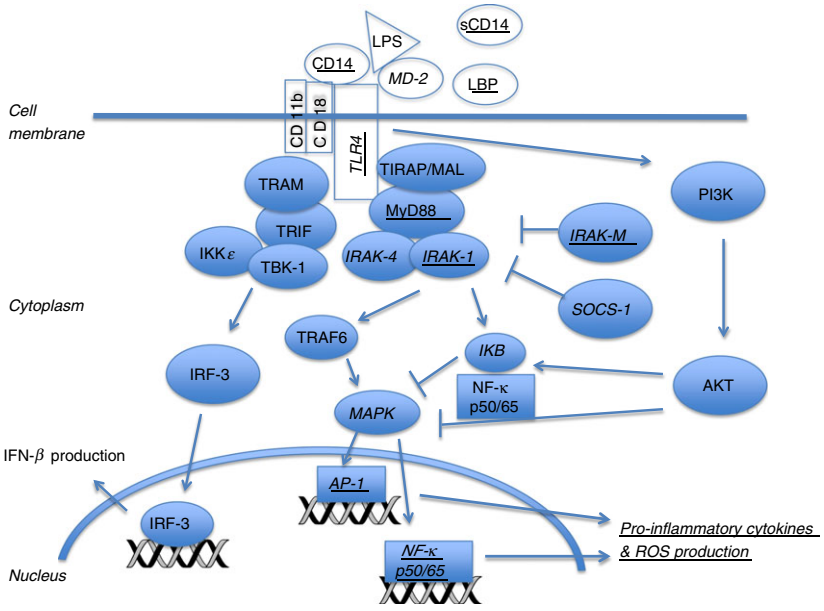

Figure 2. Toll-like receptor 4 (TLR4) signalling. Lipopolysaccharide (LPS) binding to TLR4 with co-stimulatory molecules, CD14, lipopolysaccharide binding protein (LBP), CD11b, CD18 and MD-2, recruits MyD88-dependent and -independent intracellular proteins. MyD88-independent pathway: signalling proteins, TRAM and TRIF, and kinases, IKKε and TBK-1, are recruited to the intracellular domain of TLR4. These are activated and phosphorylate interferon regulatory factor 3 (IRF-3). Phosphorylated IRF-3 translocates into the nucleus for transcription of interferon-β. MyD88-dependent pathway: MyD88 and TIRAP/MAL are recruited to the intracellular domain of the TLR4 receptor. IRAK-4 and IRAK-1 are activated via phosphorylation. IRAK-1 interacts with TRAF6, inducing activation of MAPK and transcription of AP-1 and NF-κB genes to produce pro-inflammatory cytokines and reactive oxygen species (ROS). Activation of the PI3K–AKT pathway results in impaired AP-1/NF-κB cytokine production. IRAK-M negatively regulates MyD88-pathway signalling.[34] Underlined co-stimulatory molecules and signalling proteins represent those that are differentially expressed in the immature immune response. Italicized co-stimulatory molecules and signalling proteins represent those that are differentially expressed in the endotoxin and immune tolerant responses.

sion in neonatal monocytes after low-dose endotoxin stimulation.[23] However, high-dose endotoxin stimulation abolished this differential effect, suggesting a MyD88-independent pathway with receptor-saturating insults.[23]

Hence, the initial immature innate immune response is effectively inhibited at the level of intracellular signal transduction with evidence of some ability to overcome this inhibition with high-dose stimulation.

Regulation of the immature adaptive immune response

Cytokine regulation

Discrete cytokine profiles in response to TLR4 stimulation typify the neonatal versus adult responses to monocyte stimulation. While *ex vivo* stimulation of neonatal monocytes in isolation reveals a similar maximal cytokine production capacity to that of adults, endogenous cytokine production appears to be heavily dependent on soluble plasma-derived mediators.[10,12,19,20,23,26] Neonatal monocytes suspended in autologous plasma produce less TNF-α and higher concentrations of cytokines that induce Th2-skewing following low-dose endotoxin stimulation.[4,12,14,18] Furthermore, relatively deficient interferon-γ production results in further skewing towards a Th2 T-cell response[4,12,14,18] (Fig. 3). In association with maturational cytokine changes, the magnitude and nature of the T-cell response is similarly skewed towards a Th2 response and away from a Th1 response in the immature immune system[4,5,9,10,12,13,16,18–20,27,28] (Fig. 3).

Lavoie *et al.* reported clinically relevant Th1 and additional Th17 deficiencies in the most immature immune response of premature neonates. They showed significantly lower post-stimulation production of the p40 subunit common to interleukin-12 (IL-12) and IL-23 in extremely premature neonatal cord blood compared with term cord blood.[29] This difference is observed despite similar numbers of monocyte-derived cells responsible for production of these cytokines. The reduced IL-12 production in these preterm neonates results in deficiency of the Th1 response. Additionally, the deficient IL-23 production results in an impaired Th17 response. More significantly, in a nested case–control cohort study of neonates matched for gestational age and birthweight, Lavoie *et al.*[29] reported that those infants with lower production of the IL-12 and IL-23 p40 subunit had an increased risk of early onset neonatal sepsis despite higher levels of

Table 3. Inducing factors, cytokine production and functions of T-cell subtypes

T-cell subtype	Effects of immune system immaturity on differentiation and/or activity of T-cell subtypes	Inducing factors	Cytokines produced	T-cell functions
$CD8^+$	↓	APC MHC Class I[9]	IFN-γ, TNF-α[11]	**Memory & Effector** Cytolysis, pro-inflammatory, intracellular bacteria clearance[10,11]
$CD4^+$		APC MHC Class II[11]		**Memory & Effector**
Th1	↓	IL-12, HMGB1, IFN-γ[12,15]	IFN-γ, TNF-α, IL-2, IL-1β[11,15]	Pro-inflammatory, intracellular pathogen clearance, induces cell-mediated immune response[15]
Th2	↑	IL-4, IL-33, IL-25, or absence of Th1 promoting cytokines[12,15]	IL-4, IL-10, IL-13, IL-5[11,12,15]	Enhance antibody-mediated responses, induce allergic reactions, fight parasitic infections[11,15]
Treg	↑[1]	IL-10, TGF-β[15]	IL-10, IL-35, TGF-β[15]	Immune suppression, balance Th1/Th2 responses[10,17]
Th17	↑[1]	IL-6, TGF-β[15]	IL-17, IL-21, IL-22, IL-26[11,15]	Neutrophil recruitment and activation, extracellular bacteria clearance, enhance mucosal host defence[15]

APCs, antigen-presenting cells; HMGB1, high mobility group B1; IFN-γ, interferon-γ; IL-2, interleukin-2; TGF-β, transforming growth factor-β; Th2, T helper type 2; TNF-α, tumour necrosis factor-α; Treg, regulatory T.

[1]Conflicting evidence exists regarding the impact of Th17 and Treg cell differentiation in the immature immune response.

Bold indicate the general roles of the CD8+ and CD4+ Tcell subtypes.

other inflammatory cytokines. These findings implicate a clinically relevant deficiency in Th17 and Th1 stimulating cytokines in the most immature immune response of preterm neonates.

Further evaluations in term neonates have yielded conflicting evidence regarding their ability to mount appropriate Th17 and Treg responses.[13,22,23] Schaub *et al.*[16] describe decreased Treg proliferation and less effective Treg anti-inflammatory effects. In contrast, Dijksta *et al.* demonstrate preferential Treg cell differentiation until 1 year of age.[27] Further evidence describes differential Treg cell activity based on atopic phenotype wherein atopic children display delayed Treg cell maturation.[30] Dijksta *et al.* provide further evidence that neonates have an inability for Th17 differentiation until 3 months of age after which Th17 differentiation is no longer inhibited.[27] This contrasts with evidence suggesting that the Th17 response predominates at birth and decreases over the first year of life.[20] While incompletely understood, the balance of Th1 and Th2 phenotypes and regulation of the pro-inflammatory and anti-inflammatory responses to pathogens that are dependent on the proliferation of Th17 and Treg cell types is an area that warrants further study.[16,31]

In addition to differential stimulation by the innate immune system, impaired T-cell cytokine production also attenuates the neonatal hyper-inflammatory response through limitation of T-cell proliferation. T-cell levels are high at birth, increase throughout the first year of life, and subsequently stabilize to adult levels by school age. However, relatively impaired IL-2 production results in sub-optimal T-cell function and proliferative capacity after stimulation.[4]

Over the first few years of life, the neonatal-predominant Th2 response shifts to a more mature Th1 response.[20] Although incompletely elucidated, evidence suggests that soluble plasma factors, in addition to the cytokines described above, play a dominant role in directing the immature to mature transition.[14]

Other soluble factors

Neonatal haemocytes suspended in adult plasma demonstrate a significantly greater capacity to produce TNF-α compared with suspension in autologous plasma. Conversely, adult cells suspended in neonatal plasma yield reduced TNF-α production capacity.[26] These observations suggest the presence of an agonist in adult plasma and/or an inhibitor in neonatal plasma. Further, both neonatal and adult monocytes display enhanced responses to low-dose endotoxin stimulation after adult plasma incubation compared with stimulation in media alone.[23] Addition-

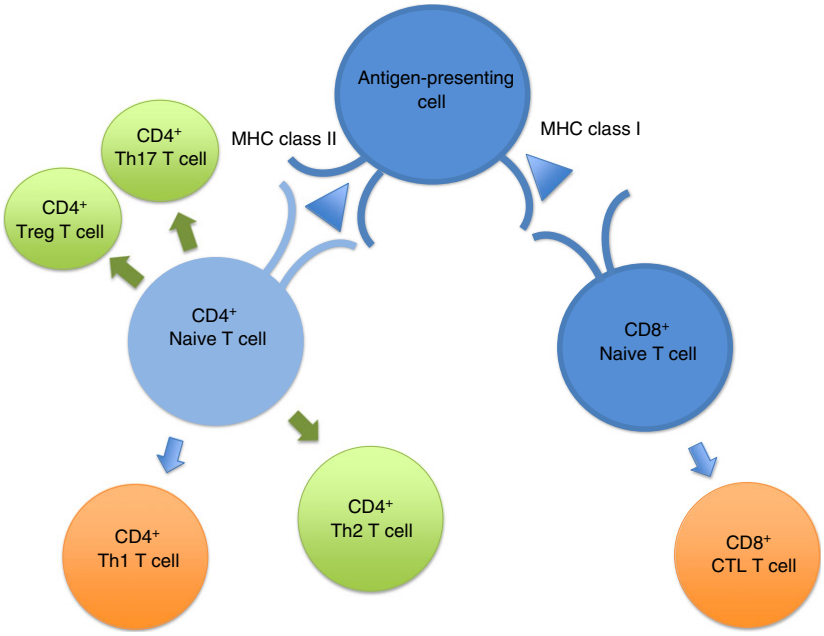

Figure 3. The innate immune response directs differentiation of T-cell subsets and the adaptive immune response: predominant immature versus mature responses. The antigen-presenting cells of the innate immune system stimulate $CD4^+$ and $CD8^+$ naive T cells via antigen (triangle) MHC class II and I interactions, respectively. The differentiated green T-cell subsets represent the predominant immature T-cell subtypes produced in response to stimulation [$CD4^+$ T helper type 17 (Th17), regulatory T (Treg) and Th2 cells]. The differentiated orange T-cell subsets represent the predominant mature T-cell subtypes produced in response to stimulation [$CD4^+$ Th1 and $CD8^+$ cytotoxic T lymphocyte (CTL) T cells].[4,5,9,10,12,13,16,18–20,27,28]

ally, Kollmann *et al.*[19] report greater maturity-based differences in TNF-α production after endotoxin stimulation of whole blood compared with monocytes suspended in medium alone. Of note, neonatal monocytes, particularly when stimulated in whole blood, elaborate a more constricted cytokine profile compared with adult cells.[19] Taken together, these studies strongly suggest that soluble factors are a significant maturation-associated and independent determinant of differential monocyte-derived pro-inflammatory cytokine production after endotoxin stimulation.

Of the many putative soluble factors, extracellular adenosine, a purine metabolite, is established as a key factor in directing the character of the neonatal immune response. Adenosine's disruption of the cAMP–mitogen-activated protein kinase pathway impairs production of Th1 cytokines while enhancing production of Th2 and Th17 skewing cytokines.[12,18–20] Additionally, adenosine inhibits neutrophil–endothelial adhesion and attenuates T-cell proliferation and effector functions.[31] Neonatal blood has a fourfold higher concentration of adenosine and a 20-fold higher concentration of cAMP when compared with adult levels.[18,20] Furthermore, neonatal monocytes are more sensitive to the inhibitory effects of adenosine.[20,23]

Additional, potentially influential factors that impair effective resistance to infection are antimicrobial protein peptides such as cathelicidin, lactoferrin, bactericidal/permeability-increasing protein and α- and β-defensins.[31] Likewise, maternally produced factors such as transforming growth factor-β, prostaglandin E_2 and progesterone further skew lymphocytes towards a Th2 response *in utero* and postnatally.[12] The differential concentrations of these soluble factors probably play a major role in the differential immature and mature immune response.

Cell–cell interactions

The regulatory effects of soluble factors on the adaptive immune response are strongly augmented by immune cell–cell interactions.[10,12,19] The APCs are the cellular bridge between the innate and adaptive immune systems and regulate lymphocyte responses through direct interaction with naive T cells and via cytokine production. Relative to adults, neonatal $CD8^+$ T cells respond more rapidly to infection but are unable to differentiate into memory $CD8^+$ T cells because they undergo terminal differentiation.[32] Additionally, the neonatal cytokine environment is characterized by lower IL-12 and higher IL-10, resulting in suboptimal activation of $CD8^+$ T cells and a weakened cytotoxic response.[24] Clinically, the high prevalence of severe viral infections in children younger than 2 years is likely to be a manifestation of the deficient cytotoxic lymphocyte response.[5] Conversely, neonatal $CD8^+$ T cells are

capable of producing strong cytotoxic responses when stimulated in the presence of Th1-promoting agents.[10] Hence, when stimulated in the appropriate environment, neonatal $CD8^+$ T cells can produce mature cytotoxic responses, again suggesting a significant influence of cytokines and other soluble factors.

In addition, there is a maturation-dependent skewing in HLA class-restricted $CD4^+$ T-cell subsets. Neonatal and adult immature APCs have similar basal expression of MHC Class II receptors and CD80 and CD28 co-stimulatory molecules[12] (Fig. 3). However, after endotoxin stimulation, adult APCs demonstrate a more pronounced up-regulation of receptors and co-stimulatory molecules compared with neonatal APCs.[33] This relatively ineffective response in neonatal cells produces a weakened stimulus of $CD4^+$ T cells to produce interferon-γ, resulting in the Th2-skewed response previously described[33] (Table 3). Additional evidence supports a Th2-skewed response due to apoptosis of $CD4^+$ Th1 cells, but not Th2 cells.[24] Furthermore, lack of antigen-driven memory T cells further impairs the neonatal Th1 response. Naive T cells have a higher stimulation threshold, are more reliant on co-stimulatory molecules, produce less IL-2, and proliferate poorly compared with memory T cells.[2,9] This results in a deficient Th1, pro-inflammatory, cell-mediated response and a relatively impaired ability to eradicate viral infections and intracellular pathogens.[5,13] Instead, the Th2-skewed response is functionally effective in developing immunological memory[33] (Table 3). As the neonate gains memory T cells over the first year of life, their lymphocyte activation threshold decreases and pro-inflammatory cytokine production increases.[9]

Perinatal immune response as an adaptation

The neonate's hypo-inflammatory response may appear teleologically counterproductive; however, a blunted pro-inflammatory response may be functionally protective.[10,14,18,20,29] The dampened immune response *in utero* is thought to decrease the risk of alloimmune reactions between the mother and fetus.[18] Furthermore, in the intrauterine environment, a hyper-inflammatory response is repressed unless a stimulus, such as a significant infection, is of considerable force to activate the fetus to speed lung maturity and remove itself from the infected environment by stimulating preterm labor.[29] Unnecessarily eliciting this type of response would likely prove maladaptive.

After delivery, the neonate transitions from the sterile intrauterine environment to the microbe-rich world, newly exposed to a massive number of antigens, both intrinsic and extrinsic. It is suspected that the hypo-inflammatory response is beneficial as a full Th1 and/or Th17 response could induce detrimental hyper-inflammatory and/or autoimmune reactions.[10,29] For example, neonatal intestinal epithelial cells have been shown to be particularly hypo-inflammatory after endotoxin stimulation. Mechanistically, these cells down-regulate the expression of interleukin receptor-associated kinase 1 (IRAK 1), a key mediator in TLR4 signalling, and increase expression of IRAK-M, a negative regulator of this pathway.[14,20] This response may be protective in that it allows the neonate to tolerate colonization as it forms an intestinal microbiome. Further supporting this theory, the development of necrotizing enterocolitis in preterm neonates is characterized by a dysregulated, hyper-inflammatory response.[18,20] Although both the fetus and neonate maintain a hypo-inflammatory environment with low-level stimuli, their respective immune systems are able to produce a more mature hyper-inflammatory response when stimulated aggressively.[10] This maturational difference favours a safe perinatal transition but feasibly increases the risk that amplified immune activation or infection could have paradoxical pathological effects to a greater extent in neonates than adults.

Endotoxin tolerance as a recapitulation of immature immune function

Endotoxin tolerance (ET) in the mature immune system shares several features with the immature neonatal immune response (Table 2). Endotoxin tolerance, the reduced pro-inflammatory response observed in monocytes after repeated endotoxin exposure, is a transient component of the normal immune response seen in humans and animals.[34] Additionally, the phenomenon of cross-tolerance, the hypo-inflammatory response of endotoxin tolerized monocytes upon re-stimulation with Gram-positive PAMPs, demonstrates that this response is not specific to endotoxin exposure.[34] As such, the concept of immune tolerance (IT) is defined even more broadly as a transient state seen in cells of the innate immune system after repeated exposure to low concentrations of PAMPs and/or DAMPs after which they are unable to respond normally to further PAMP and/or DAMP exposure[1] (Table 1). Endotoxin tolerance is a marker of innate immune exhaustion and is mechanistically discrete from adaptive T-cell tolerance characterized as a lack of reactivity to self-antigens or foreign tissue antigens.[35]

Endotoxin tolerance is highly recapitulative of the normal neonatal immune condition; tolerized phagocytes such as neutrophils, monocytes and dendritic cells in adults exhibit decreased adhesion and extravasation, produce fewer pro-inflammatory cytokines and have impaired antigen-presenting capacity.[20] Additionally, the ET phenotype is associated with decreased neutrophil recruitment and reduced efficacy of endotoxin-induced ROS production similar to what is observed in neonates.[2,36] Furthermore, tolerized innate immune cells produce an altered cytokine profile, shifting the T-cell response from a Th1 to a Th2 response.[2] In the following sections, we will compare the

mechanisms that induce the hypo-inflammatory state in neonates with that of experimental ET. Their similarities could provide insights regarding regulatory mechanisms and potential therapeutic targets.

PAMP recognition and signal transduction alterations in endotoxin tolerance

Similar to the immature immune response, decreased expression of TLR4 and co-receptors (MD-2) have been described in ET, however, these alterations do not appear to be solely responsible for the induction of ET.[8,21,37] Instead, downstream alterations in the MyD88/TLR4 signalling cascade described in tolerized murine models and humans are probably more significant.[8] In human and mouse cell lines, several steps in the TLR4/MyD88 pathway are shown to be inhibited in ET. Examples include decreased IRAK adapter protein levels, impaired p38 MAPK activation, suppressed degradation of inhibitor of κB (IκB), and increased presence of p50 nuclear factor-κB (NF-κB) homodimers, all resulting in impaired transcription of pro-inflammatory cytokines in the NF-κB and activator protein-1 pathways[37] (Fig. 2). Additionally, IRAK-M, a negative regulator of the MyD88/TLR4 pathway, is expressed only after the initial endotoxin exposure.[2] IRAK-M inhibits signal propagation down the MyD88/TLR4 pathway by impairing degradation of IκB, an enzyme complex that binds NF-κB and inhibits intranuclear translocation, resulting in decreased production of pro-inflammatory cytokines and ROS[21] (Fig. 2). The inability of IRAK-M-deficient mice to display ET further supports its role as a primary mediator of ET.[2] Similarly, murine evidence suggests that SOCS-1 acts as a negative regulator of lipopolysaccharide signalling through interaction with the adapter protein, IRAK.[21]

Alterations in cytokines and other soluble factors in endotoxin tolerance

Similar to the neonatal immune response, soluble factors play a significant role in the induction of ET. Stimulated tolerized monocytes preferentially produce Th2 skewing cytokines and fail to produce pro-inflammatory cytokines.[2] As discussed previously, this affects the innate immune response as well as the quality of the adaptive immune response, creating weakened cytotoxic and pro-inflammatory responses and skewing towards a more humoral and relatively anti-inflammatory adaptive response. One particular cytokine, IL-10, has been of particular interest in ET because of its anti-inflammatory properties. It has been evaluated as a sole inducer of the ET response. However, despite its capacity to induce down-regulation of pro-inflammatory cytokines, IL-10-deficient mice can develop ET, suggesting that IL-10 is not the sole mediator of the ET response.[21]

Prostaglandin E_2, an arachidonic acid metabolite, is produced by macrophages after re-stimulation with endotoxin and suppresses pro-inflammatory cytokine production in macrophages and lymphocytes.[21] Similar to the perinatal response, prostaglandin E_2 appears to play a role in induction of the ET response. Further evaluation of soluble factors such as adenosine may reveal more similarities between the neonatal and ET responses that have not yet been studied.

Cell–cell interactions

A well-characterized phenomenon in studies of human tolerized monocytes is the reduced levels of MHC class II expression, functionally impairing their capacity for antigen presentation.[8] This impaired ability for antigen presentation hinders the ability of the innate immune response to activate the adaptive response.

Clinical relevance: immune tolerance in sepsis

The regulation of the TLR4/MyD88 signalling cascade, impaired antigen presentation, and differential production of cytokines attenuate the pro-inflammatory Th1 response in the innate and adaptive immune systems similarly in neonates and ET models. In well-controlled murine models, induction of an ET response yields a survival advantage.[2,36] This is attributed to significant repression of pro-inflammatory cytokine release that is causatively associated with organ injury. However, clinical evidence in humans points to an increased mortality and secondary infections in patients with evidence of the more generalized phenomenon, IT. This association has been seen in patients suffering from acute inflammatory illnesses such as myocardial infarctions, trauma, sepsis and cardiac surgery.[38–43] Taken together, currently available evidence describing the adaptive or maladaptive nature of IT in neonatal and adult humans remains inconclusive. The aggregate biological effect appears to depend on the degree, timing and duration. However, the bulk of the evidence points to the tolerance response as being an essential adaptive component in early immune maturation.

The IT phenomenon has been described extensively in patients with sepsis.[1,2,39–41,43,44] The prevailing model posits that the initial hyper-inflammatory response is temporally related with a counter-regulatory hypo-inflammatory IT response. Hence, the patient's immunological state is determined by the phenotype of the *net* response.[1] The IT response is thought to be teleologically adaptive, resulting in abrogated propagation of the injurious hyper-inflammatory response. However, just as the hypo-inflammatory neonatal response although adaptive, may, in some circumstances, place the neonate at greater risk of severe infection, the IT response in severe sepsis may have a threshold above which it also becomes maladaptive.[5]

Described mechanisms of IT induction in sepsis patients include alterations in inflammatory signal transduction, cytokine production, and apoptosis of lymphocytes.[2,44]

In sepsis, the IT phenotype is pleiotropic and efforts to evaluate immune capacity rely on surrogate markers including monocyte HLA-DR expression and exogenous cytokine responses to PAMP stimulation such as quantification of TNF-α production after *ex vivo* endotoxin stimulation.[39] These factors are fundamental for cell–cell communication and down-regulated expression represents attenuation of the hyper-inflammatory response, making them commonly used surrogate markers in clinical studies.

A translational study evaluated the signalling apparatus of APCs in post-mortem sepsis patients and revealed decreased expression of T-cell-activating co-stimulatory molecules, CD80 and CD86. Further hampering the communication between the innate to adaptive immune systems is the lack of T-cell expression of the co-stimulatory molecule, CD28, described in post-mortem sepsis patients compared with controls.[44] The consequent altered signalling results in decreased IL-2 production and increased T-cell anergy and apoptosis.[44] Furthermore, *ex vivo* studies have demonstrated significant inflammation-induced apoptosis of $CD4^+$ and $CD8^+$ T cells in sepsis patients.[44] Additionally, IL-10 production suppresses cytotoxic $CD8^+$ T cells and the Th1 hyper-inflammatory responses, favouring a Th2 skewed response.[2,44] However, similar to murine ET models, the IL-10 response in humans with sepsis is variable and probably insufficient to induce IT.[34]

An important limitation of using *ex vivo* TNF-α production as a surrogate for integrated immune responses is highlighted by a study of 148 patients with severe sepsis in which 28-day mortality was not associated with *ex vivo*-stimulated TNF-α production.[45] These findings contrast many studies that describe an association between worse outcomes including higher mortality, increased incidence of secondary infections and increased length of stay in patients who display a lower stimulated TNF-α production capacity.[39–41] This is feasibly explained by the isolated cell-stimulation method used in this study, which contrasts with the whole blood endotoxin stimulation method used in the other studies.[39–41] Given the influence of soluble factors and cell–cell interactions on the character of the inflammatory response, it is imperative that *ex vivo* immune stimulation closely approximates the *in vivo* environment for complete and valid interpretation.

Other studies in adults consistently show an association between a prolonged IT response and an increased risk of death and secondary infections.[39,46,47]

Immune maturation as a regulator of immune tolerance in severe sepsis

Similar to the neonatal immune response, IT in adult sepsis is Th2-skewed.[34] Evaluations of IT in paediatric sepsis patients have shown an association between a severely suppressed pro-inflammatory response at admission and an increased risk of mortality and longer hospital stays.[40,41,43] It is unknown, however, if the effects of a persistent IT response during the duration of a sepsis episode causatively affects outcomes. Additionally, it remains unknown if this association is present in neonates, the most immunologically immature demographic. Nor is it established if low-dose endotoxin exposure, shown to have tolerizing effects in adults, has similar, dampening or amplifying effects in neonates and whether the maturational-based immunological differences could alter the amplitude and/or direction of the immune response to an infection and clinical course of severe sepsis. Future research in neonatal and paediatric sepsis should focus on evaluation for a causative association between the inherently tolerant, immature immune response and poor outcomes. Further testing should focus on determining the specific cell–cell interactions and/or soluble factors most appropriate for trials of therapeutic modulation in the septic child. The care of profoundly immune-immature patients would be enriched by the ability to characterize and beneficially enhance their immunological response.

Conclusions

The overall characteristics of the immature immune response differ from the mature immune response in ways similar to the differences between the ET and normal endotoxin responses. A complete understanding of the specific mechanisms underlying these differences remains to be elucidated. To improve the care of adult, paediatric and neonatal sepsis patients, better characterization of the initial and sustained immune responses in neonatal versus paediatric and adult sepsis is imperative. More importantly, determining correlations between alterations in immune responses with intermediate and definitive outcomes from severe sepsis in neonates, children and adults will provide great insight into their specific immunological adaptations and deficiencies. A better understanding of the mechanisms that mediate these immune response impairments will allow targeting of future therapies to improve patient outcomes.

Disclosures

The authors listed on this manuscript do not have any conflicts of interest to report.

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