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STEM CELLS: POLICIES FROM THE BENCH TO THE CLINIC (AI CAPLAN AND TL BONFIELD, SECTION EDITORS)

MSCS in Scenarios of Infection and Inflammation: Focus on Neonatal Diseases

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Abstract The imbalance of inflammatory and anti-inflammatory responses in favor of inflammation plays a major role in the pathogenesis of many neonatal diseases. Inflammation in the perinatal period carries important long-term consequences, including neurodevelopmental and respiratory complications. Treatments able to restore immune homeostasis may reduce neonatal mortality and prevent long-term deleterious multi-organ consequences of unchecked inflammation. Mesenchymal stromal cells are a heterogenous group of progenitors cells with potent anti-inflammatory and immunomodulatory potential, among other diverse mechanisms of action. Thus, mesenchymal stromal cells (MSCs) may represent a novel and effective therapy for several neonatal diseases, potentially capable of preventing their long-term complications. This paper reviews the role of inflammation in the perinatal period and the therapeutic role of MSCs, focusing on their anti-inflammatory potential.

This article is part of the Topical Collection on *Stem Cells*: *Policies from the Bench to the Clinic*

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Introduction

Inflammation represents the biological attempt of the body to eliminate harmful stimuli. The inflammatory process triggers a compensatory anti-inflammatory reaction, aimed at counterbalancing the pro-inflammatory cascade to prevent multi-organ dysfunction [1]. The balance between these two antithetical processes is carefully orchestrated in order to eliminate the hazard while limiting the damage to the host.

Neonates face several noxious events with an immature immune system, since the complete maturation of the immune responses occurs gradually after term birth. Neonatal anti-inflammatory response mechanisms are particularly inefficient, resulting in increased morbidity and mortality due to uncontrolled inflammation [2]. Recent studies have suggested that exposure to fetal and neonatal inflammation and infections is associated with debilitating respiratory diseases in childhood such as asthma [3, 4], a variety of neurological long term sequelae including cerebral palsy [5–7], autism spectrum disorders [8] and schizophrenia [9], and increased risk of death prior to 18 months of age [6].

The development of treatments that are able to limit the detrimental consequences of the inflammatory cascade and allow normal organ development is a major goal in perinatal medicine. Mesenchymal stromal cells (MSCs) are multipotent progenitor cells capable of a peculiar interaction with the immune system and a pronounced anti-inflammatory effect in the context of inflammation [10]. MSCs, defined by three minimal criteria based on plastic adherence, surface marker expression and differentiation potential [11], can be easily isolated from a variety of tissue, including bone marrow,

umbilical cord blood, umbilical cord Wharton's jelly, placenta and adipose tissue [12, 13]. Although MSCs, or at least subsets of MSCs, have multipotent differentiation capacity [14], these cells exert their therapeutic potential mainly through the release of bioactive molecules that induce cell proliferation and angiogenesis and modulate immune responses and inflammation [15]. Animal models have proven MSCs as a novel therapeutic strategy for a variety of neonatal diseases, sparking the clinical translation of regenerative medicine (https://clinicaltrials.gov/ct2/results?intr=mesenchymal+stem+cell).

This review will focus on the anti-inflammatory component of the MSCs' mechanisms of action as a potential strategy for treating and preventing neonatal diseases.

Sustained Inflammation in the Perinatal Period Contributes to Neonatal Diseases

Neonates May Experience Extremely High Levels of Inflammation

For many infants, exposure to inflammation begins during fetal life, especially for those born prematurely [16]. Intrauterine inflammation most commonly results from chorioamnionitis, which is defined as inflammation of the fetal membranes (chorion and amnion) and placenta [16]. Microbial invasion of the amniotic cavity and danger signals of non-microbial origin induce a robust local inflammatory response, with high levels of pro-inflammatory cytokines that promote neutrophil recruitment [16]. Microbial invasion and neutrophil migration can progress to cord inflammation (funisitis) and fetal invasion. These events produce a fetal inflammatory response syndrome (FIRS), with potential damage to the fetal brain, lung, gastrointestinal tract and skin [16–18] that can lead to long-term complications after birth and beyond childhood [17]. Funisitis causes a significant increase in pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) in cord blood of premature infants [19]. Interestingly, compared to adult peripheral blood cells, the compensatory anti-inflammatory response of term and preterm cord blood cells after exposure to lipopolysaccharide (LPS) in vitro seems to be immature and reduced [2]. During postnatal life, infections and medical interventions in some instances may magnify inflammation, and thus contribute to the multifactorial origin of neonatal diseases.

The Neonatal Immune System Is Immature and Incapable of Managing Excessive Inflammation

Intrinsic deficiencies of neonatal immunity lead to inefficient clearance of pathogens [20], making neonates particularly prone to overwhelming infections. At the same time, neonates might suffer from unchecked inflammatory responses due to inefficient counter-regulatory mechanisms of both innate and adaptive immunity [21]. The innate immune system provides immediate defense against infection and dangerous stimuli (e.g., tissue trauma and injury, stress, malignancy) in a non-specific manner. Molecules expressed by pathogens or associated with tissue injury are recognized by Toll-like receptors (TLRs) present on innate effector cells, including phagocytic leukocytes (monocytes and macrophages), dendritic cells and natural killer (NK) cells. TLR activation triggers phagocytosis and the release of inflammatory mediators [22]. Under physiological conditions, apoptosis of activated monocytes and neutrophils [1] and polarization of macrophages from a pro-inflammatory M1 phenotype towards an anti-inflammatory M2 phenotype prevent the hyper-responsiveness of the innate immunity [22]. Cord blood-derived monocytes and neutrophils appear to be more resistant to apoptosis than adult peripheral blood-derived cells [23, 24]. The surviving neutrophils contribute to sustained inflammation through the secretion of particularly high levels of pro-inflammatory cytokines after LPS stimulation in vitro [23]. Moreover, immaturity of Toll-like receptor signaling is associated with a pronounced inflammatory response in neonatal immune cells [25].

In addition, the adaptive immune system in neonates is different at birth compared to later life. Attenuated pro-inflammatory responses due to deficient pro-inflammatory CD4+ T helper (Th) 17 have been reported in neonates [26, 27]. The Th1/Th2 ratio is shifted towards Th2 to prevent fetal immune rejection, leading to inherent bias towards the production of anti-inflammatory cytokines [26, 27]. Neonatal murine CD8+ cytotoxic T cells respond rapidly to infection but are unable to differentiate into memory CD8+ T cells, because they undergo terminal differentiation [28]. Regulatory T cells (Tregs), which constitute the anti-inflammatory T subset designed to limit and suppress innate and adaptive immune responses, are increased in number in preterm cord blood compared to term cord blood and adult peripheral blood. After premature birth until 16 months of age, Treg cell number decreases over time, although it never reaches the level of term infants at birth [29]. On the other hand, neonatal Treg cells present markedly reduced function compared to their adult counterpart in terms of NK suppression of cytotoxicity and cytokine productions [30], as well as inhibition of dendritic cell activation [31]. Tregs from preterm infants show less suppression of T-cell activation than Tregs from adults or term infants [32]. Exposure to prenatal inflammation further reduces Treg activity in preterm infants [32]. Moreover, following intrauterine inflammation, the fetal Th1/Th2 ratio shifts towards Th1 cells, with a corresponding increase in interferon-gamma (IFN-γ) [33]. Interestingly, several neonatal diseases are characterized by a pro-inflammatory state due to excessive pro-inflammatory lymphocyte subsets

(Th1, Th17) and deficient representation in Tregs [34–36] (Table 1).

Taken together, these data suggest that the inappropriate inactivation of the immune system, once activated by multiple inflammatory stimuli typical of the perinatal period, leads to sustained inflammation. This sustained inflammation contributes significantly to several neonatal diseases and their lifelong consequences.

MSCs as a Possible Regulator of Neonatal Sustained Inflammation

MSCs interact with the effectors of the immune system and regulate their function [43]. In the context of inflammation, MSCs are able to mitigate hyper-activation of the innate immune system to favor tissue regeneration [44]. In animal and in vitro studies, MSCs recruit monocytes into inflamed tissues [45], promote the differentiation of macrophages towards an M2 anti-inflammatory profile [46] and inhibit NK activity and IFN-γ production [47]. MSCs interfere with the cell cycle of human dendritic cells, inhibiting their activation [48] and suppress NOX-1 activity in both adult and neonatal human neutrophils, modulating their oxidative activity [49], (Fig. 1). However, the effects of MSCs on neonatal neutrophils are milder than on adult neutrophils [49]. A possible explanation is the immaturity of neonatal neutrophils and their reduced ability to initiate an inflammatory response compared to adult neutrophils [49, 50].

With regard to the adaptive immune system, MSCs are able to inhibit the proliferation of murine CD4+ and CD8+ T cells [51, 52], although they exert opposite effects on different subsets of T cells. In animal models and clinical studies in adult patients, human MSCs were able to inhibit the cytotoxic T cells and the pro-inflammatory Th1/Th17 subsets. MSC administration may favor the anti-inflammatory Th2 and Treg subsets [53–55], polarizing the lymphocytic cells towards a regulatory phenotype [56] and enhancing the immunosuppressive properties of Treg cells [57•] (Fig. 1). Some studies have suggested that MSCs can also inhibit B cell activation, proliferation, differentiation and chemotactic responses [58], although knowledge regarding MSC-mediated modulation of B cells is still limited (Fig. 1).

In summary, MSCs interact with the innate and adaptive immune system, arresting the hyper-responsiveness of innate effector cells and modulating adaptive immunity to favor tissue repair and homeostasis.

Inflammation and MSCs: Their Strength Grows Out of our Weaknesses

Interestingly, MSCs are not constitutively anti-inflammatory, and require an inflammatory context in order to express their anti-inflammatory potential [59••]. MSCs are immunosuppressive when exposed to sufficiently high levels of pro-inflammatory cytokines such as IFN-γ, tumor necrosis factor alpha (TNF-α) and interleukin-17 (IL-17); otherwise, they promote lymphocyte proliferation and immune responses [59••, 60]. Accordingly, pretreatment of MSCs with pro-inflammatory cytokines to boost their immunosuppressive activity results in better control of inflammation in animal models of graft-versus-host disease (GvHD) and colitis [61, 62]. Conversely, inhibition of the inflammatory environment through the blockage of IFN-γ [51] or through the addition of dexamethasone to the culture condition [63•] abrogates the inhibitory effects of MSCs on T cells. In a mouse GvHD model, MSC administration on the day of bone marrow transplantation did not prevent GvHD [64]. Conversely, administration of MSCs after established GvHD suppressed the progression of GvHD [51]. This confirms the notion that MSCs are most effective when administered in the context of inflammation. Accordingly, the combined administration of both

Table 1 Modifications of the immune system in neonatal diseases and their models

	Phagocytic system	Natural killer	Lymphocytes T
Perinatal brain injury (PVL, HIE)	↑ activated M1 microglia in murine HI cerebral tissue [37] ↑ activated neutrophils in murine HI cerebral tissue [37]	↑ in murine HI cerebral tissue [37]	↑ Th1 and Th17 in murine HI cerebral tissue [34] ↓ Treg in murine HI cerebral tissue [34]
BPD	↓ M1 macrophages in murine lung [38] ↓M2 macrophages in murine lung [38] ↓ activated DCs in murine and human lung [39, 40]	Unchanged in murine lung [38]	↓ Th1 and Th17 in peripheral blood during the first week of life in BPD patients [41] ↓ Treg in cord blood in BPD patients [36]
NEC	↓ M1 macrophages in murine NEC tissue [42]	Unknown	↓ Th17 in human NEC tissue [35] ↓Treg in human NEC tissue [35]

Periventricular leukomalacia (PVL), Hypoxic ischemic encephalopathy (HIE), Bronchopulmonary dysplasia (BPD), Necrotising enterocolitis (NEC), Lymphocytes T helper (Th), cerebrospinal fluid (CSF), Denditric cells (DCs), Hypoxia-ischemia (HI)

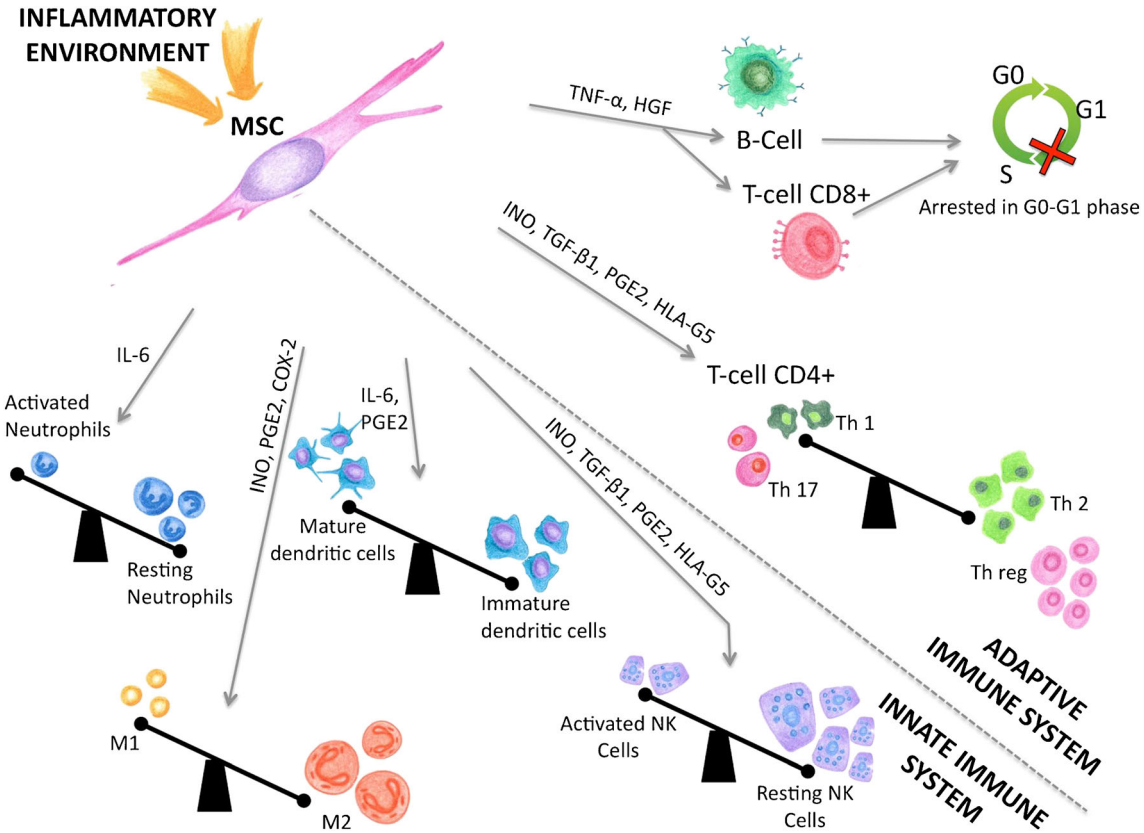

Fig. 1 Role of MSC in modulating innate and adaptive immunity during inflammation. MSCs suppress activation of neutrophils, inhibit NK cell activity, interfere with the maturation of dendritic cells, and target differentiation of monocytes from a pro-inflammatory (M1) towards an anti-inflammatory (M2). MSCs inhibit the cell cycle of cytotoxic CD8+ T cells and B lymphocytes, promote the anti-inflammatory CD4+ T helper Th2 and Treg subsets, and inhibit the pro-inflammatory CD4+ T helper Th1 and Th17 subsets

MSCs and cyclosporine A, a potent immunosuppressant, accelerated graft rejection in experimental GvHD instead of reinforcing MSC immunosuppression [65].

These data suggest that the setting of sustained inflammation, typical of several neonatal diseases, may represent an optimal environment for MSCs to exert their therapeutic effect.

MSCs and Target Cells: A Long-Distance Relationship

Although MSCs can modulate the environment by cell-to-cell contact [66], a broad spectrum of growth factors, chemokines and cytokines released by MSCs are likely the pivotal effectors of their immunomodulatory mechanism of action [15]. The inhibition of T-lymphocyte proliferation and function is obtained by the secretion of soluble factors such as transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), IL-10, nitric oxide (NO), heme oxygenase (HO) and indoleamine 2,3-dioxygenase (IDO) [67–69]. The secretion of human leukocyte antigen-G5 (HLA-G5), TGF-β1 and PGE2 contribute to the expansion of Treg [70, 71]. IDO, PGE2 and TGF-β1 are also mediators of MSC inhibition of NK functions [48, 71]. IL-6, which is involved in the reversion of the maturation of dendritic cells to an immature phenotype, mediates MSC interference with the maturation of dendritic cells [50]. MSCs were found to inhibit B-cell proliferation by releasing soluble factors, especially B lymphocyte-induced maturation protein-1(Blimp-1), which is necessary for immunoglobulin production [72].

United We Stand, Divided We Fall: The Role of MSC Extracellular Vesicles in Delivering Healing Factors

Despite the identification of numerous factors involved in MSC function, none can individually account for their therapeutic benefit [73]. MSC therapeutic potential, including immunomodulatory activity, is most likely achieved through the synergism of several of these molecules. Recent studies indicated that MSCs, as well as many other cell types, secrete various extracellular vesicles which are involved in cell-to-cell communication [73, 74]. Cell-derived extracellular vesicles are generally classified according to their size and intracellular origin. Exosomes are vesicles 40–100 nm in size that derive from the endosomal compartment and are secreted into the extracellular space through fusion with the plasma

membrane [75]. Microvesicles are a heterogeneous population of vesicles directly derived from budding of the cell membrane, and are generally larger (up to <1000 nm in diameter) [74]. MSC-derived extracellular vesicles mimic the pro-regenerative and immunomodulatory effects of cellular administration in different animal models [76•]. MSC-derived extracellular vesicles are able to inhibit proliferation and induce apoptosis of activated T cells while promoting secretion of anti-inflammatory cytokines [77]. In animal and in vitro studies, MSC-derived extracellular vesicles restored Th1/Th2 balance and induced generation of Tregs, implementing the regulatory component of the adaptive immune system [77, 78]. A dose-dependent inhibitory effect of MSC-derived extracellular vesicles on B-cell proliferation, differentiation and Ig production has been reported [58]. In a mouse model of severe refractory asthma, MSC-derived extracellular vesicles were as potent as the MSCs themselves in mitigating allergic airway inflammation [79]. In a model of hypoxia-induced pulmonary hypertension, treatment with MSC-derived exosomes ameliorated pulmonary hypertension and suppressed inflammation and alternative macrophage activation [80]. A preliminary clinical study [81•] demonstrated that MSC-derived extracellular vesicles containing high quantities of anti-inflammatory factors IL-10, TGF-β1 and HLA-G5 were able to alleviate the symptoms of resistant grade IV acute GvHD in a patient, who remained stable for five months after MSC-derived extracellular vesicles therapy, with no side effects [81•].

MSCs for the Treatment of Neonatal Diseases

MSCs have been tested in preclinical models of several neonatal diseases, ameliorating diverse aspects of disease pathogenesis. In this section we will focus on the inflammatory component of each disease and the therapeutic potential of MSCs in mitigating inflammation and improving outcomes.

Periventricular Leukomalacia (PVL)

PVL, a specific form of cerebral white matter injury associated with an increased risk of neurodevelopmental impairment, is the most common form of brain injury in preterm infants [82]. Cerebral ischemia appears to be the principal pathogenetic factor in PVL [82]. Innate and adaptive immune responses are activated after hypoxic ischemic injury, leading to brain inflammation, perpetuated by eventual perinatal infection and inflammation [82]. A robust cerebral immune response, characterized by an imbalance between the pro-inflammatory Th1/Th17- and the anti-inflammatory Th2/Treg-type responses, has been documented in animal models of white matter injury [35] (Table 1).

In animal models of preterm brain injury, systemic administration of human bone marrow-derived and cord-derived MSCs prevented the loss of oligodendrocyte progenitors and improved histological white matter injury [83, 84] by reducing the cerebral inflammatory response and T-cell invasion [83] (Table 2).

Hypoxic Ischemic Encephalopathy (HIE)

Hypoxic ischemic injury has deleterious consequences on the human brain at term gestation as well. Hypoxic ischemic encephalopathy (HIE) is a major cause of newborn death and permanent neurological disability [87]. The incidence of HIE in developed countries is approximately 1 to 3 per 1,000 live births, and is responsible for approximately 30 % of the cases of cerebral palsy in childhood [88]. The intrapartum hypoxic ischemic insult initiates an energy depletion with subsequent reperfusion-induced cell death cascades [37]. In experimental HIE, the inflammatory response to hypoxic ischemic injury caused an increased influx of neutrophils to the brain [37]. Microglia, the brain resident macrophages which can either have a pro-inflammatory phenotype (M1) or promote tissue repair and suppress inflammation (M2) [89], are polarized towards an M1 phenotype after hypoxic ischemic injury [89] (Table 1). High expression of pro-inflammatory cytokines has been found in the cerebrospinal fluid of neonates with HIE [90].

MSC administration through intracranial, intranasal or intravenous injection in experimental HIE has improved functional outcomes, reduced lesion size, induced cellular differentiation towards neurons and oligodendrocytes, decreased gliosis [85, 91, 92] and stimulated microglia polarization towards an M2 phenotype [85] (Table 2). Case reports of intrathecal and intravenous administration of autologous bone marrow-derived and allogeneic cord-derived MSCs in children with cerebral palsy following perinatal injury seem to suggest a partial improvement in gross motor function [93–95] A phase 1 study to evaluate the safety of MSCs in the treatment of HIE is currently recruiting patients (NCT01962233).

Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity, is a leading cause of death in the neonatal period [96]. The incidence of BPD is inversely proportional to gestational age, reaching 60–90 % in extremely preterm infants (22–25 weeks gestation) [97]. In survivors, the diagnosis of BPD increases the risk of respiratory illness in childhood and adulthood [96]. The pathogenesis of BPD is multifactorial and leads to impaired alveolar and lung vascular development, exacerbated by prenatal and postnatal inflammatory stimuli [96]. Increased protein levels and high mRNA

Table 2 MSC immunomodulation in neonatal diseases

	Phagocytic system	Natural killer	Lymphocytes T
Perinatal brain injury (PVL, HIE)	↓ activated M1 microglia in murine HI cerebral tissue [85]	Unknown	↓ *T cells* in murine HI cerebral tissue [83]
	↑ M2 microglia in murine HI cerebral tissue [85]		
BPD	↓ activated macrophages in murine BPD lung [86]	Unknown	Unknown
NEC	Unknown	Unknown	Unknown

Periventricular leukomalacia (PVL), Hypoxic ischemic encephalopathy (HIE), Bronchopulmonary dysplasia (BPD), Necrotising enterocolitis (NEC), Hypoxia-ischemia (HI)

expression of pro-inflammatory cytokines (TNF- α, IL-1, IL-6, IL-8) have been demonstrated in airway secretions of infants with developing BPD [98]. Decreased number of Tregs in the cord blood [36] and higher proportions of activated T cells in the peripheral blood during the first week of life [41] can predict the development of BPD. In experimental BPD, macrophages were polarized towards the M1 phenotype, while the M2 phenotype was inhibited [38] (Table 1).

In animal models of BPD—mostly neonatal rodents exposed to hyperoxia—airway delivery of cord- and cord blood-derived MSCs improved alveolarization, vascular development and lung function up to the adult age [99]. MSCs dramatically decreased lung influx of neutrophils and macrophages [86] and reduced the levels of pro-inflammatory cytokines in a dose-dependent manner [100] in these animal models, especially when the cells were administered locally to the lung [101].

These promising data have led to a phase I trial with human cord blood-derived MSCs administered to preterm infants at high risk for BPD. The treatment is apparently safe and feasible [102•]. The trial was designed to test safety and was not randomized or powered to study efficacy. This is currently being investigated in two phase II trials (NCT02381366, NCT01828957).

Necrotizing Enterocolitis

Necrotising enterocolitis (NEC) is the most common acquired gastrointestinal emergency in premature infants [103]. Despite prompt medical treatment, NEC can progress towards intestinal perforation and peritonitis, requiring neonatal surgery. Surgical NEC has a mortality rate of up to 30 % [103]. The pathogenesis of NEC is poorly understood, but may result from a combination of intestinal immaturity, a compromised epithelial barrier, increased propensity for hypoxic damage, and microbial translocation [103]. The neonatal murine intestine seems to be more susceptible to an exaggerated inflammatory response after ischemic damage compared to that of adult mice [104]. NEC patients have high levels of plasma pro-inflammatory cytokines compared to neonates with spontaneous intestinal perforation without NEC [105]. A robust presence of CD4+ and CD8+ T effector cells was found in human NEC tissue, with a significant decrease in the functional Treg proportions. This cellular phenotype in NEC tissue was associated with a tissue-specific inflammatory gene expression profile known to inhibit Treg development and induce conversion of Treg into Th17 cells [35]. In experimental NEC, an increased number of intestinal macrophages, mostly M1 macrophages, were found. M1 macrophages promote NEC by increasing intestinal epithelial apoptosis, whereas M2 polarization protects the intestine from NEC [42] (Table 1).

Systemic (intraperitoneal and intravenous) administration of MSC in newborn rat pups subjected to experimental NEC reduced clinical illness and bowel damage, although results on survival were inconsistent. These studies have not investigated the immunomodulatory role of MSCs in experimental NEC [106, 107] (Table 2). Considering that the role of inflammation is paramount in the pathogenesis of NEC, it seems possible that the beneficial effects of MSCs in NEC models are mediated through an immunomodulatory role. However, this conclusion requires further investigation.

The imbalance between the innate and adaptive immune system contributes to the pathogenesis of neonatal diseases (Table 1). MSCs seem to improve the outcome of experimental neonatal diseases. The effect of MSCs in modulating the activation of the innate immune system is well-documented (Table 2), while the impact on the adaptive compartments of neonatal immunity is largely under-investigated in these diseases (Table 2).

Conclusions

Newborn infants experience sustained inflammation. This can lead to various neonatal complications involving the brain, lung and gut. The ability of MSCs to modulate the innate and adaptive immune systems provides a strong rationale for testing the therapeutic potential of these cells in alleviating these neonatal diseases. Preclinical studies indicate that

MSCs attenuate inflammation and prevent brain, lung and gut injury in neonatal rodents modeling PVL, HIE, BPD and NEC. However, MSCs are not constitutively anti-inflammatory. The microenvironment plays a crucial role in determining the preferential switch towards either anti-inflammatory or pro-inflammatory competence of MSCs and, consequently, their therapeutic efficacy. These considerations, together with concomitant treatments, should be taken into account when translating MSC therapy into clinical practice. Cell preconditioning may further influence the success of MSCs in regenerative medicine.

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Compliance with Ethical Standards

Conflict of Interest Maria Pierro and Bernard Thébaud declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All procedures performed in animal studies conducted by the authors were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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- •• Of major importance

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