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High expression levels of macrophage migration inhibitory factor sustain the innate immune responses of neonates

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The vulnerability to infection of newborns is associated with a limited ability to mount efficient immune responses. High concentrations of adenosine and prostaglandins in the fetal and neonatal circulation hamper the antimicrobial responses of newborn immune cells. However, the existence of mechanisms counterbalancing neonatal immunosuppression has not been investigated. Remarkably, circulating levels of macrophage migration inhibitory factor (MIF), a proinflammatory immunoregulatory cytokine expressed constitutively, were 10-fold higher in newborns than in children and adults. Newborn monocytes expressed high levels of MIF and released MIF upon stimulation with *Escherichia coli* and group B *Streptococcus*, the leading pathogens of early-onset neonatal sepsis. Inhibition of MIF activity or MIF expression reduced microbial product-induced phosphorylation of p38 and ERK1/2 mitogen-activated protein kinases and secretion of cytokines. Recombinant MIF used at newborn, but not adult, concentrations counterregulated adenosine and prostaglandin E2-mediated inhibition of ERK1/2 activation and TNF production in newborn monocytes exposed to *E. coli*. In agreement with the concept that once infection is established high levels of MIF are detrimental to the host, treatment with a small molecule inhibitor of MIF reduced systemic inflammatory response, bacterial proliferation, and mortality of septic newborn mice. Altogether, these data provide a mechanistic explanation for how newborns may cope with an immunosuppressive environment to maintain a certain threshold of innate defenses. However, the same defense mechanisms may be at the expense of the host in conditions of severe infection, suggesting that MIF could represent a potential attractive target for immune-modulating adjunctive therapies for neonatal sepsis.

newborns | *Escherischia coli* | prostaglandin | adenosine | sepsis

Early-onset neonatal sepsis occurs during the first week of life in 0.7–1.0 per 1,000 infants and is associated with high mortality and morbidity rates (1, 2). Group B *Streptococcus* (GBS) and *Escherichia coli* are the leading pathogens, accounting for over 60% of cases of early-onset sepsis. The high susceptibility to infection has been associated with a limited ability of newborns to mount efficient innate immune responses (3). Indeed, neonatal monocytes and dendritic cells exposed to microbial products release lower levels of the proinflammatory and T_H1-polarizing cytokines TNF, IFNγ, and IL-12p70, whereas the T_H17-polarizing and antiinflammatory cytokines IL-6, IL-10, and IL-23 are produced at similar or even higher levels than in adult cells (4, 5).

Soluble factors in blood influence immune responses (6–8). Notably, adenosine, prostaglandins, cortisol, estradiol, and progesterone are present at high circulating concentrations perinatally and have the ability to reduce the production of proinflammatory cytokines by neonatal innate immune cells exposed to microbial products (9–13). Fetal exposure to antiinflammatory mediators may be advantageous in utero to maintain tolerance to maternal antigens, to avoid powerful proinflammatory responses that can lead to preterm delivery and damage-developing organs, and to establish tolerance to colonizing microorganisms immediately after birth (3, 14). However, an excessive polarization of the neonatal immune system against the development of proinflammatory responses would dramatically increase susceptibility to infection during the neonatal period. Therefore, we hypothesized that newborns have developed counterregulatory mechanisms to balance innate immune responses perinatally and tested whether macrophage migration inhibitory factor (MIF) could perform that function.

MIF is a pleiotropic cytokine constitutively expressed in blood and immune cells and an important regulator of innate immune responses (15). In contrast to most cytokines, MIF exists at homeostasis as a preformed molecule in leukocytes and is present in the plasma in the low ng/mL range in healthy adults. MIF mediates biological activities through binding to a receptor complex made of CD74 with or without CD44, CXCR2, and CXCR4 to initiate intracellular signaling (16, 17). Composition or expression of these receptor proteins can vary in a cell- and microenvironment-specific manner. Alternatively, MIF can act through direct intracellular interaction with p53, c-JUN-activation

Significance

During pregnancy, high circulating levels of adenosine and prostaglandins reduce the ability of fetal immune cells to mount powerful proinflammatory responses. In contrast, newborns express 10-fold higher levels of the proinflammatory immune regulator migration inhibitory factor (MIF) compared with adults. MIF sustains cell activation and cytokine production and counterregulates adenosine and prostaglandin E2-mediated immunosuppression in newborn monocytes. Yet excessive MIF expression during an established infection worsens the outcome of newborn mice. Thus, we identify a unique role for MIF in regulating neonatal innate immune responses and propose that MIF has a protective role to reduce susceptibility to infection during the neonatal period but may favor uncontrolled inflammation during sepsis, leading to adverse outcomes.

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domain-binding protein 1 (JAB-1), or ribosomal protein S19 (RPS19) among others (18–20). Through these interactions, MIF stimulates mitogen-activated protein kinases (MAPKs) and phosphoinositide-3-kinase/Akt signaling pathways, inhibits MAPK phosphatase-1 activation (MKP-1) (21), and modulates AP-1 activity (18, 19), promoting cell survival, cell proliferation, and inflammatory processes.

MIF has been implicated in the pathogenesis of inflammatory diseases, including sepsis (22). Blood concentrations of MIF are elevated in septic patients and correlate with the expression of proinflammatory markers, dysregulated pituitary and adrenal function, severity scores, and disease outcome (23–26). Interestingly, MIF has the unique property to override the antiinflammatory effects of glucocorticoids on immune cells, leading to the concept that MIF is a physiological antagonist of glucocorticoids (21, 27). Experimental studies have identified an important role for MIF in fetal lung development (28). However, the impact of MIF on neonatal immune responses has not been investigated.

Here, we addressed the role of MIF in neonatal immunity by investigating the profile of MIF expression from birth to adulthood and the impact of MIF on the innate immune responses of newborn monocytes. We provide compelling evidence that MIF plays a critical role in the regulation of innate immune responses in newborns and identify MIF as a physiologic antagonist of adenosine and prostaglandin E2 (PGE_2)-mediated inhibition of MAPK signaling and cytokine production. Observations derived from preclinical models further suggest that high expression levels of MIF worsen the outcome of septic neonates. Therefore, as in adults, MIF might represent a target for immune-modulating adjunctive therapies for neonatal sepsis.

Results

MIF Plasma Concentrations from Birth to Adulthood. In healthy subjects, plasma levels of MIF (median, interquartile range) measured by ELISA were markedly higher at birth (82.6 ng/mL, 66.1–115.4) and on postnatal day 4 (109.5 ng/mL, 79.4–147.2) than in infants (7.4 ng/mL, 6.6–10.8), children (5.2 ng/mL, 3.4–7.7), and adults (5.7 ng/mL, 4.0–8.3) (Fig. 1). Western blot analyses confirmed this observation (Fig. S1). Thus, MIF levels are extremely elevated in newborns and reach the concentrations observed in adults within the first months of life.

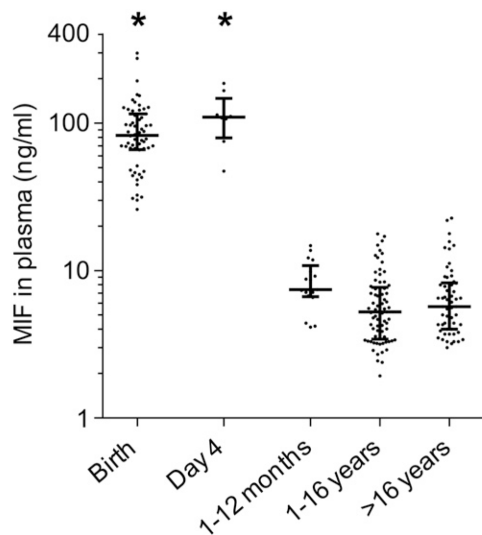

Fig. 1. Circulating MIF concentrations are markedly elevated in newborns. MIF plasma levels were measured by ELISA in umbilical cord blood collected after birth in 60 newborns and in peripheral blood of 10 newborns (on postnatal day 4), 17 infants (1–12 mo), 73 children (1–16 y), and 58 adults (>16 y). The median and the interquartile range are shown. $*P < 0.05$ versus infants, children, and adults.

***E. coli* and GBS Induce MIF Secretion by Newborn Monocytes.** Considering that MIF blood levels are particularly elevated in septic patients (23–25), we evaluated the ability of newborns to release MIF ex vivo upon exposure for 24 h to *E. coli* and GBS, the two leading pathogens involved in early-onset neonatal sepsis. MIF levels in cord blood were higher than in adult blood at baseline and after stimulation with *E. coli* or GBS, both of which dose-dependently increased MIF secretion (Fig. 2 *A* and *B*). MIF levels raised 1.7- and 3.0-fold in cord blood and 2.6- and 3.0-fold in adult blood incubated with *E. coli* and GBS (10^8 bacteria/mL), respectively.

Among white blood cells, monocytes express high levels of MIF that is released upon microbial stimulation (29, 30). Intracellular MIF levels were twofold higher in freshly isolated newborn monocytes than in adult monocytes (Fig. 2 *C* and *D*). Exposure of newborn monocytes to *E. coli* and GBS increased MIF release 2.4- and 2.3-fold at 24 h (Fig. 2*E*). In contrast, *E. coli* leads to the secretion of much higher levels of TNF and IL-6 than GBS (Fig. S2 *A* and *B*) (10), in agreement with the concept that MIF production is regulated differently than the production of classical proinflammatory cytokines (30, 31). Of note, 24-h exposure to LPS increased MIF release 1.3-fold without affecting the intracellular MIF pool in newborn monocytes. Exposure to Pam_3CSK_4 did not impact MIF release but reduced the intracellular pool of MIF by 34% (Fig. S2 *C* and *D*). This suggests that whole microorganisms are more powerful than pure microbial products at inducing MIF release by monocytes. Estradiol, progesterone, and cortisol are present at high concentrations in the fetal and neonatal circulation (10). Exposure of newborn monocytes to estradiol, progesterone, and hydrocortisone at concentrations similar to those observed in the circulation at birth increased MIF secretion, implying that these hormones could participate in maintaining high levels of MIF in newborns (Fig. S2*E*). Overall, steroid hormones and early-onset sepsis pathogens induced MIF secretion by newborn monocytes, suggesting that MIF might be implicated in host defenses against these microorganisms.

MIF Inhibition Reduces Cytokine Production by Newborn Monocytes Exposed to Microbial Products. The impact of MIF on the innate immune response of neonatal monocytes was investigated using three approaches: (*i*) inhibition of MIF activity using the specific small molecule inhibitor (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid, methyl ester (ISO-1) that blocks MIF binding to its receptor CD74 (32), (*ii*) reduction of MIF expression by transfection with MIF small interfering RNA (siRNA), and (*iii*) supplementation with recombinant human MIF (rhMIF). ISO-1 reduced by 50% to >90% the secretion of TNF, IL-6, and IL-8 by newborn monocytes stimulated with *E. coli*, GBS, LPS, or Pam_3CSK_4 (Fig. 3 *A*–*C*). Transfection of newborn monocytes with the MIF siRNA reduced MIF mRNA levels by 80% (Fig. 4*A*) and MIF protein levels by 60% (Fig. 4*B*). The consequences of MIF silencing were first addressed by measuring the production of a panel of proinflammatory (TNF, IL-1β, IL-6, IL-8, IL-12p40, IL-12p70, and IL-23) and antiinflammatory (IL-10, IL-20, and IL-27) cytokines by newborn monocytes stimulated with *E. coli*, GBS, LPS, and Pam_3CSK_4 (Fig. 4 *C*–*F*). MIF silencing reduced microbial product-induced secretion of all cytokines, as illustrated for TNF, IL-6, and IL-8 in response to the microbial panel (Fig. 4 *C*–*E*) and for all other cytokines in response to *E. coli* (Fig. 4*F*). Importantly, in a specificity-confirming rescue experiment, preincubation with rhMIF almost fully restored *E. coli*-induced TNF production in newborn monocytes transfected with the MIF siRNA, whereas rhMIF had no impact on the response of newborn monocytes transfected with the scrambled (Scr) siRNA (Fig. 5). These results demonstrated that inhibition of MIF activity by

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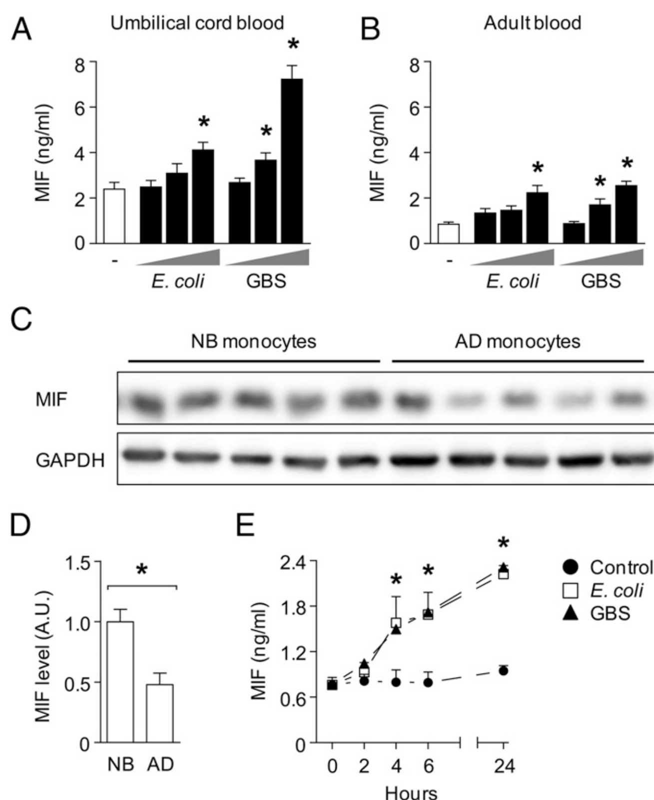

Fig. 2. Higher baseline and *E. coli* and GBS-induced MIF levels in newborns than in adults. (*A* and *B*) Umbilical cord and adult whole blood were diluted fivefold in RPMI medium and stimulated with *E. coli* or GBS (10^6–10^8 bacteria/mL). MIF concentrations were measured by ELISA in supernatant collected after 24 h. Data represent means ± SEMs of 6–7 independent experiments performed in triplicates. *$P < 0.05$ versus controls. (*C* and *D*) Intracellular MIF was detected by Western blot in freshly isolated newborn (NB) and adult (AD) monocytes. Densitometric values (means ± SEMs, $n = 14$) are expressed in A.U. *$P < 0.05$. (*E*) Newborn monocytes cultured in autologous plasma were stimulated with *E. coli* or GBS (10^8 bacteria/mL). MIF concentrations were measured by ELISA in supernatant collected at the indicated time. Data represent means ± SEMs of five independent experiments performed in triplicates. *$P < 0.05$ versus controls.

ISO-1 and reduction of MIF expression levels by siRNA strongly impaired the production of a number of cytokines in newborn monocytes exposed to microbial products. Together with the restoration of TNF secretion by rhMIF in MIF-silenced monocytes, these data advocated for an autocrine mode of action of MIF.

To address whether MIF has a specific impact on newborn monocytes, experiments were carried out in adult monocytes. ISO-1 reduced by up to >90% the secretion of TNF, IL-6, and IL-8 induced by *E. coli*, GBS, LPS, and Pam_3CSK_4 (Fig. S3 *A–C*). Transfection of adult monocytes with the MIF siRNA reduced MIF protein levels by 50% (Fig. S4*A*). MIF silencing in adult monocytes reduced production of TNF, IL-6, and IL-8 induced by LPS and reduced production of IL-6 induced by Pam_3CSK_4 (Fig. S4 *B–D*). However, production of TNF, IL-6, and IL-8 induced by *E. coli* and GBS was not affected by MIF silencing in adult monocytes. Thus, in adult monocytes, exogenous MIF might be more important than endogenous MIF, as MIF silencing did not influence the release of inflammatory cytokines upon exposure to *E. coli* and GBS. Altogether, these data suggest that MIF plays an important role to promote production of inflammatory cytokines by monocytes, both in newborns and adults.

MIF Silencing Inhibits Activation of MAPKs. Innate immune cells detect microbial structures through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). TLR2, TLR6, and CD14 are involved in the sensing of GBS, whereas TLR4, CD14, and MD-2 are essential for sensing of *E. coli* (33, 34). Upon ligand binding, most TLRs recruit MYD88 that initiates intracellular signaling pathways such as the NF-κB and MAPK pathways, which drive the production of cytokines. Considering that MIF was previously reported to up-regulate TLR4 expression in mouse macrophages and to activate MAPK and NF-κB signaling pathways in immortalized cell lines and mouse macrophages (35–37), we investigated the influence of MIF on the expression of PRRs and the activation of downstream signaling pathways in newborn monocytes.

MIF silencing did not modify mRNA expression levels of TLR2, TLR4, CD14, and MYD88 in newborn monocytes (Fig. 6*A*). Moreover, MIF silencing led to a 40% reduction in TNF production induced by phorbol-12-myristate-13-acetate (PMA) plus ionomycin, which stimulates cytokine production independently of TLRs and MYD88 (Fig. 6*B*). Interestingly, MIF silencing did not significantly impact on *E. coli* and GBS-induced nuclear translocation of NF-κB p65 but significantly reduced

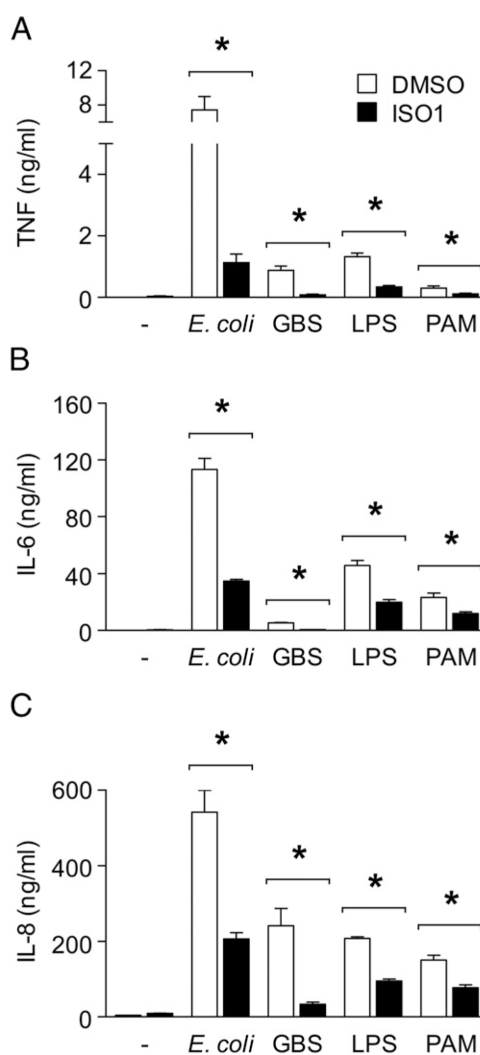

Fig. 3. The MIF inhibitor ISO-1 reduces cytokine production by newborn monocytes. Newborn monocytes were incubated for 2 h with 100 μM ISO-1 (black bars) or solvent (DMSO, white bars) before stimulation with *E. coli* (10^7 bacteria/mL), GBS (10^7 bacteria/mL), LPS (100 ng/mL), or Pam_3CSK_4 (PAM, 1 μg/mL). TNF (*A*), IL-6 (*B*), and IL-8 (*C*) concentrations were measured by ELISA in cell culture supernatant collected after 18 h. Data represent means ± SEMs of five independent experiments performed in triplicates. *$P < 0.05$.

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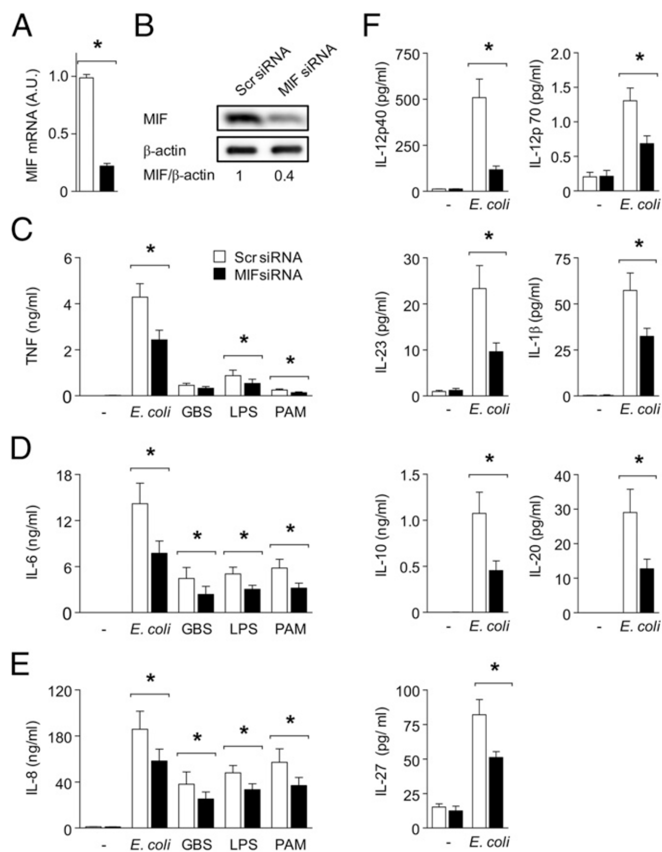

Fig. 4. MIF silencing reduces cytokine production by newborn monocytes. Newborn monocytes were transfected with Scr (white bars) or MIF (black bars) siRNAs. Transfected cells were cultured for 48 h in RPMI medium supplemented with 10% autologous plasma before measurement of MIF mRNA or protein levels or stimulation with microbial products. MIF mRNA levels are expressed in A.U. relative to the expression of HPRT (*A*). Intracellular MIF levels were analyzed by Western blot. Signals were quantified by densitometric measurement (*B*). Cytokine concentrations in cell culture supernatants collected 18 h after stimulation with *E. coli* (10^7 bacteria/mL), GBS (10^7 bacteria/mL), LPS (100 ng/mL), or Pam_3CSK_4 (1 μg/mL) were measured by ELISA (*C*–*E*) and Luminex (*F*). Data represent means ± SEMs of 5–6 independent experiments performed in triplicates. *$P < 0.05$.

phosphorylation of p38 and extracellular signal-regulated kinase (ERK)1/2 MAPKs in newborn monocytes (Fig. 6*C* and Fig. S5). The MEK inhibitor PD 98059 reduced *E. coli*-induced TNF production by 51%, an effect that could not be compensated by rhMIF (Fig. S6). This indicates that the response to *E. coli* is ERK1/2 dependent in newborn monocytes and is in line with the notion that MIF promotes ERK1/2 activation by reducing the expression of MKP-1, a phosphatase that inactivates ERK downstream of MEK (21, 38). Hence, our results suggested that MIF stimulates MAPK activation—at least in part—independently of an effect on PRR and MYD88 mRNA expression.

MIF Counterregulates Adenosine and PGE_2 Inhibition of TNF Production by Newborn Monocytes. Newborns are exposed to high levels of adenosine, prostaglandins, and steroid hormones produced by the placenta and the fetal adrenal gland (9–12). Incubation with physiologically relevant concentrations of adenosine (10^{-7} to 10^{-5} M), PGE_2 (10^{-10} to 10^{-8} M), and hydrocortisone (10^{-8} to 10^{-6} M) dose-dependently reduced *E. coli*-induced TNF production by newborn monocytes (47%, 58%, and 72% reduction for 10^{-5} M adenosine, 10^{-8} M PGE_2, and 10^{-6} M hydrocortisone, respectively; Fig. S7). Treatment with rhMIF (100 ng/mL) overcame adenosine and PGE_2-mediated inhibition of *E. coli*-induced TNF production but had no impact on hydrocortisone-mediated inhibition of TNF production in newborn monocytes (Fig. 7*A*). Adenosine is known to selectively impair TNF production in newborn mononuclear cells through adenosine A3 receptor-mediated accumulation of the second messenger cAMP (9). Incubation of newborn monocytes with rhMIF dose-dependently antagonized IB-MECA (a specific agonist of the A3 adenosine receptor) and PGE_2-mediated inhibition of *E. coli*-induced TNF production (Fig. 7*B*). Thus, MIF at concentrations observed in the neonatal circulation is a physiologic antagonist of the antiinflammatory mediators adenosine and PGE_2.

Adenosine A3 Receptor and PGE_2-Mediated Inhibition of ERK1/2 MAPK Activation Is Counterbalanced by MIF. Considering that adenosine A3 receptor activation inhibits LPS-induced activation of ERK1/2 MAPK in mouse macrophages (39), we then examined whether MIF antagonized adenosine and PGE_2-mediated inhibition of ERK1/2, p38, and c-Jun N-terminal kinase (JNK) activation in newborn monocytes exposed to *E. coli*. IB-MECA (10^{-6} M) reduced ERK1/2 phosphorylation but had no impact on p38 and JNK phosphorylation (Fig. 7*C* and Fig. S8*A*). PGE_2 (10^{-8} M) reduced ERK1/2 and, to a lower extent, JNK phosphorylation but had no effect on p38 phosphorylation (Fig. 7*D* and Fig. S8*B*). Recombinant hMIF overcame the inhibition of ERK1/2 phosphorylation induced by both IB-MECA and PGE_2 (Fig. 7*E*). Therefore, MIF antagonized adenosine A3 receptor activation and PGE_2 through modulation of ERK1/2 MAPK signaling.

Blocking MIF with ISO-1 Protects Newborn Mice from Sepsis. In mice, MIF plasma levels (median, interquartile range) were markedly higher on postnatal day 0–1 (45.9 ng/mL, 38.9–55) and 1–2 (41.8 ng/mL, 29.8–66.8) than on postnatal day 5–6 (4.9 ng/mL, 3.2–7.4) and in adults (2.2 ng/mL, 1.3–4.8; Fig. S9*A*). Thus, as in humans (Fig. 1), circulating levels of MIF are markedly higher in newborn compared with adult mice.

High levels of MIF correlate with morbidity and mortality in adults and children with sepsis or septic shock (23, 26, 40), and MIF inhibition protects adult mice from lethal sepsis (23, 41–45). To evaluate the impact of MIF during neonatal sepsis, we developed preclinical models of sepsis using 1–2-d-old newborn mice. In mice infected intraperitoneally with *E. coli*, ISO-1 treatment (100 mg/kg) reduced bacterial dissemination in blood, liver, and spleen (2.4-, 3.0-, and 4.2-fold reduction, $P \leq 0.01$; Fig. S9*B*); decreased TNF, IL-6, IL-12p70, and RANTES plasma levels (3.9-, 2.3-, 1.6-, and 3.2-fold reduction, $P \leq 0.01$; Fig. S9*C*); and increased

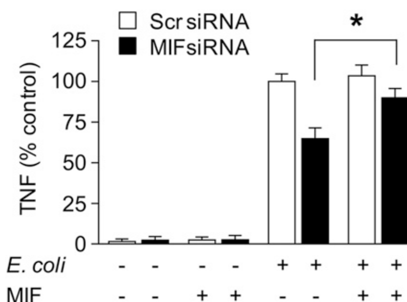

Fig. 5. rhMIF restores *E. coli*-induced TNF production in MIF-silenced monocytes. Newborn monocytes were transfected with Scr (white bars) or MIF (black bars) siRNAs. Transfected cells were incubated for 1 h with or without 100 ng/mL rhMIF before stimulation for 18 h with *E. coli*. TNF concentrations in cell culture supernatants were measured by ELISA. Results are expressed as percent of *E. coli*-induced TNF release in newborn monocytes transfected with the Scr siRNA (control). Data represent means ± SEMs of three independent experiments performed in triplicates. *$P < 0.05$.

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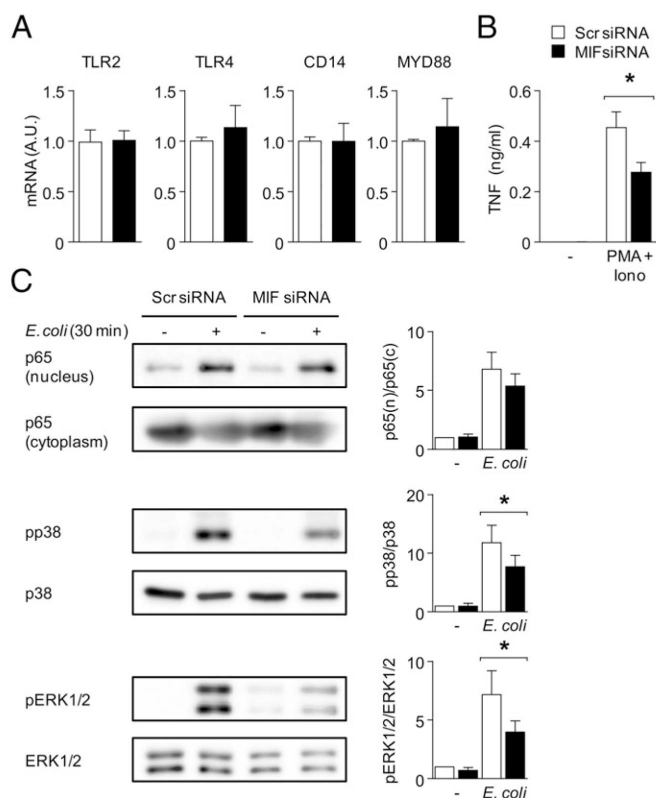

Fig. 6. MIF silencing reduces *E. coli*-induced p38 and ERK1/2 MAPK activation in newborn monocytes. Newborn monocytes were transfected with Scr (white bars) or MIF (black bars) siRNAs. Transfected cells were cultured for 48 h before extraction of RNA (*A*) or stimulation with either PMA (10 ng/mL) plus ionomycin (Iono; 100 ng/mL) for 18 h (*B*) or *E. coli* (10^7/mL) for 30 min (*C*). (*A*) Gene-specific expression was normalized to the expression of HPRT, and data (means ± SEMs, $n = 6$) are expressed in A.U. (*B*) TNF concentrations in culture supernatants were measured by ELISA. Data represent means ± SEMs of four independent experiments performed in triplicates. *$P < 0.05$. (*C*) NF-κB p65 in nuclear and cytosolic extracts, phosphorylated (p) and total p38, and ERK1/2 in cytosolic extracts were detected by Western blot. Densitometric values (means ± SEMs) are expressed relative to expression in unstimulated monocytes transfected with Scr siRNA. Data represent means ± SEMs of five independent experiments. *$P < 0.05$.

survival from 0% (0/9) to 56% (5/9, $P = 0.01$; Fig. S9*D*). Similarly, in mice infected intranasally with *E. coli*, ISO-1 treatment reduced, albeit only as a trend, bacterial burden in lungs by 7.2-fold ($P = 0.08$) and bacterial dissemination to the blood by more than 200-fold ($P = 0.003$; Fig. S9*E*).

Discussion

Newborns lack a fully developed adaptive immune system and rely on innate immunity to fight infections (3). However, innate immune responses must be tightly regulated to clear pathogens without triggering an uncontrolled state of overactivation that can lead to adverse outcomes. The intrauterine environment is characterized by high circulating levels of mediators that polarize the fetal and neonatal immune system against development of powerful proinflammatory responses (13). At birth, when newborns encounter potentially pathogenic microorganisms, an excessive polarization of the immune system against development of proinflammatory responses would dramatically increase susceptibility to infection. Here we identify MIF as an intracellular and circulating mediator that promotes the innate immune responses of newborn monocytes and antagonizes adenosine and PGE_2-mediated inhibition of TNF production (Fig. S10). These observations support the concept that counterregulatory mechanisms balance neonatal immune responses.

Circulating MIF levels are strikingly elevated at birth, remain high at least until postnatal day 4, and decrease to reach adult levels during the first months of life. This parallels the postnatal fall in circulating concentrations of cortisol, placental steroids, prostaglandins, and adenosine, which are potent inhibitors of neonatal innate immune responses (9–12, 46). The expression pattern of MIF in newborns is unique in the sense that MIF reaches blood levels that are at least 10-fold higher than those measured in healthy children and adults, an observation that has no antecedent for a proinflammatory cytokine or mediator (7, 13). Circulating MIF levels in healthy-term newborns are in fact comparable to those of children and adults with septic shock (23, 26). Exposure to *E. coli* and GBS leads to a further increase in MIF secretion in umbilical cord blood or purified newborn monocytes. The magnitude of the difference in MIF levels between newborns and adults varies according to sample processing, with a 10–15-fold difference in fresh blood and a 1.8–2.8-fold difference in blood incubated for 24 h. We speculate that MIF-inducing mediators present at higher concentrations in neonatal blood are vanishing during the in vitro incubation step. Attractive candidates are steroid hormones. Indeed, estradiol, progesterone, and glucocorticoids are expressed at particularly high levels during the neonatal period; have a short half-life; and stimulate MIF production by immune cells in vitro and in vivo (27, 47, 48). Because newborn whole blood and monocytes show somewhat different MIF secretion profiles in response to *E. coli* and GBS, T cells, B cells, neutrophils, eosinophils, and platelets may participate to release MIF in response to microbial stimulation (49–52). Although the main source of circulating MIF is still unknown, our data indicate that production of MIF by newborn monocytes may play an important role in the control of inflammatory responses, as MIF acts in an autocrine fashion to stimulate the production of inflammatory cytokines by newborn monocytes.

Interestingly, in newborn monocytes, MIF silencing reduces the production of proinflammatory cytokines but also reduces the production of cytokines with antiinflammatory and tissue repair activities such as IL-10 (53), IL-20 (54), and IL-27 (55). This suggests an important role of MIF in initiation, amplification, and termination of inflammatory responses triggered by microbial products, and more globally in fine-tuning of immune responses.

The reduction of cytokine production in MIF-silenced monocytes is not influenced by the type of microbial stimulus and is likely independent of an effect on TLR2, TLR4, CD14, and MYD88 mRNA expression. Still, it remains to be established whether MIF influences expression, modification, localization, or trafficking of the corresponding proteins. Alternative mechanisms through which exogenous and endogenous MIF may influence cell responses comprise its binding to the cell surface receptors CD74, CXCR2, and CXCR4 to initiate intracellular signaling (16, 17); intracellular interaction with p53, JAB-1/CSN5, or RPS19 (18–20); and inhibition of MKP-1 (21). In primary newborn monocytes, MIF sustains MAPK activation by microbial products, in line with previous observations in mice and immortalized cell lines showing that MIF activates the p38, ERK1/2, and JNK pathways to mediate prosurvival, proproliferative, and proinflammatory activities (35, 36, 56–58).

Previous studies have focused on soluble or cellular factors accounting for the limited ability of newborn immune cells to respond to microbial pathogens (9, 10, 59). During pregnancy, the placenta and the fetal adrenal gland produce mediators that influence fetal and neonatal immune responses. Adenosine plasma concentrations at birth range between 0.02 and 2 μM, which is threefold higher than levels measured in adults (9, 46). Concentrations of PGE_2 range between 0.4 and 10 nM at birth and decrease by fourfold during the first week of life (12, 59). Circulating

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Fig. 7. MIF overrides the antiinflammatory effects of adenosine and PGE_2. Newborn monocytes were incubated for 1 h with or without adenosine (10^{-6} M), PGE_2 (10^{-8} M), hydrocortisone (HCZ, 10^{-7} M), or IB-MECA (a selective agonist of the adenosine A3 receptor; 10^{-6} M) and rhMIF (rhMIF 100 ng/mL unless specified otherwise). Cells were stimulated with *E. coli* for 18 h (*A* and *B*), 15–60 min (*C* and *D*), or 30 min (*E*). TNF concentrations were measured by ELISA in cell culture supernatants (*A* and *B*). Results are expressed in ng/mL (*A*) or as percent of *E. coli*-induced TNF release (*B*). Data represent means ± SEMs of 4–5 independent experiments performed in triplicates. $^{*}P < 0.05$ (*A*); $^{*}P < 0.05$ versus monocytes incubated with IB-MECA but not rhMIF (*B*); $^{\dagger}P < 0.05$ versus monocytes incubated with PGE_2 but not rhMIF (*B*). Phosphorylated (p) and total ERK1/2 in cytosolic extracts were analyzed by Western blot (*C*–*E*). Densitometric values (means ± SEMs of four independent experiments) are expressed relative to expression in unstimulated monocytes. $^{*}P < 0.05$.

levels of cortisol at birth range between 0.07 and 0.8 μM and decrease by 10-fold during the first week of life (11, 60). Adenosine and prostaglandins are well-known modulators of immune responses and share some similarities: production by the placenta, different cellular effects depending on the cell type and receptor expression, and the ability to increase intracellular levels of the second messenger cAMP, leading to specific inhibition of LPS-induced TNF production (9, 61–65). Although a reduction of LPS-induced ERK1/2 and NF-κB activation by adenosine A3 receptor activation has been described in mouse macrophages (39), the pathways modulated by adenosine and PGE_2 have not been investigated downstream of cAMP in human cells (9). Here, we show that both adenosine A3 receptor activation and PGE_2 reduce *E. coli*-induced ERK1/2 activation in primary human monocytes. Furthermore, we identify MIF as a natural antagonist of adenosine and PGE_2. MIF regulates the sensitivity of newborn monocytes to adenosine and PGE_2 by promoting the activation of ERK1/2 MAPK. MIF is able to override adenosine and PGE_2 at the concentrations present in the neonatal circulation (≥50 ng/mL) but not at the concentrations observed in healthy adults (<10 ng/mL), suggesting a specific role for MIF during the neonatal period. Interestingly, and in opposition to previous studies performed with mouse macrophages and peripheral blood mononuclear cells from adults (21, 27), MIF does not function as an antagonist of glucocorticoids in newborn monocytes. A possible explanation is that glucocorticoids and placental steroids reduce neonatal innate immune responses through inhibition of the NF-κB pathway but do not inhibit the MAPK pathway in newborn monocytes (10).

Clinical studies support the theory that a limited ability to mount powerful proinflammatory responses plays a major role in the pathogenesis of sepsis in newborns. Low expression of MHC-II on the surface of cord blood monocytes, lower levels of IL-12p40 in cord blood, and decreased ex vivo production of TNF by whole blood exposed to LPS were observed in newborns who subsequently developed sepsis (66, 67). However, following infection, newborn infants can develop a widespread state of innate immune overactivation leading to septic shock, organ damage, and death (68). Interestingly, MIF inhibition in infected newborn mice reduces systemic inflammatory response, bacterial proliferation, and mortality. These observations are in fact in agreement with the concept that although MIF is required for host defenses, once infection is established high levels of MIF are detrimental to the host (15). Indeed, high levels of MIF correlate with morbidity and mortality in children and adults with sepsis (23, 26, 40), and anti-MIF antibodies or MIF inhibitors protect adult mice from lethal sepsis (23, 41–45). Moreover, in a model of endotoxemia, an uncontrolled proinflammatory innate immune response was associated with higher mortality in newborn than adult mice (69).

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In a clinical study, MIF levels measured before the start of antibiotic treatment were not different between newborns with neonatal infection compared with those who were not infected (70). However, most patients had a mild clinical course and none died. Large clinical studies testing associations between functional *MIF* polymorphisms and MIF expression levels with susceptibility to or severity of neonatal sepsis are now required to substantiate whether MIF represents a potential attractive target for immune-modulating adjunctive therapies for neonatal sepsis. Adding to the recent studies challenging the idea that the neonatal immune system is purely immature (71–75), the recognition of a unique role for MIF in regulating innate immunity supports the concept that neonatal immune responses are tightly regulated by a balance of pro- and antiinflammatory mediators.

Materials and Methods

Subjects and Plasma Samples. Plasma samples were obtained from 200 subjects from three different groups. The first group comprised 60 healthy-term neonates. Cord blood was collected from the umbilical vein after delivery of the placenta. Peripheral blood was collected on postnatal day 4 in 10 healthy-term newborns. Clinical characteristics of the healthy-term neonates are presented in Table S1. The second group comprised 17 infants (aged between 1 and 12 mo) and 73 children (aged between 1 and 16 y) admitted for elective surgery at the Department of Pediatrics, Lausanne University Hospital, Switzerland, between April 26, 2012 and December 20, 2012. Newborns, infants, and children with acute or chronic infection, severe underlying disease, or chromosomal abnormality were excluded from the study. The third group included 58 healthy adult volunteers. Heparinized blood samples were collected, and plasma was stored at –80 °C until analysis. Our study was approved by the Ethics Committee of the University of Lausanne (Switzerland). Informed consent was obtained from the subjects or their parents.

Measurement of MIF Levels in Plasma. MIF levels were measured by enzyme-linked immunosorbent assay (ELISA), using the four-span approach, as previously described (70, 76). Briefly, microtiter plates were coated with a duck anti-chicken antibody. Anti-human MIF polyclonal antibodies raised in chicken and rabbit were used as capture and trapping antibodies. A horseradish peroxidase-labeled goat anti-rabbit antibody was used for detection, and rhMIF was used as a standard (70). The analytic sensitivity of the human MIF ELISA was 39 pg/mL intrarun, and interrun coefficients of variation were 6% and 12%, respectively.

Cells and Reagents. Umbilical cord blood from healthy-term newborns and peripheral blood from healthy adult volunteers was collected according to a protocol approved by the Ethics Committee of the University of Lausanne. None of the subjects included in the study had any sign of infection or history of intra-amniotic infection. Blood was anticoagulated with heparin (10 U/mL). Mononuclear cells were extracted by Ficoll Hypaque (GE Healthcare) gradient density centrifugation. Monocytes were isolated from blood mononuclear cells by positive selection using magnetic microbeads coupled to an anti-CD14 antibody (Miltenyi Biotec). Purity assessed by flow cytometry was >95%. Viability determined by trypan blue exclusion and staining with acridine orange and propidium iodide, using the Cellometer Vision system (Nexcelom Bioscience), was >98%. Whole blood was diluted fivefold with RPMI medium 1640 (Invitrogen) for cell culture. Monocytes were cultured in RPMI medium 1640 supplemented with 10% (vol/vol) FCS (GE Healthcare) unless indicated otherwise. Reagents were obtained from Sigma-Aldrich unless indicated otherwise. *E. coli* O18 and GBS were obtained from blood cultures of septic newborns (10). Ultrapure *E. coli* O111:B4 LPS was purchased form List Biological Laboratories. Pam_3CSK_4 was purchased from EMC microcollections. The MIF antagonist ISO-1 was purchased from Calbiochem. For experiments investigating the ability of MIF to override the antiinflammatory activity of specific mediators, newborn monocytes were incubated for 1 h with adenosine, 1-deoxy-1-[6-[[(3-iodophenyl) methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA; Tocris), PGE_2 (Cayman Chemical), or hydrocortisone, with or without rhMIF (77) before stimulation with *E. coli*.

Measurements of Cytokine Concentrations. Cytokine concentrations in cell-culture supernatants were measured by ELISA (R&D Systems for MIF; BD Biosciences for TNF, IL-6, and IL-8) and Luminex technology (Affymetrix for IL-1β, IL-10, IL-12p40, IL-12p70, IL-20, IL-23, and IL-27). Cytokine concentrations in mouse blood were measured by Luminex technology (Affymetrix).

siRNA. Newborn monocytes were obtained from cord blood mononuclear cells by negative selection using the Pan Monocyte Isolation Kit (Miltenyi Biotec). Purity assessed by flow cytometry was >90%. Monocytes were transfected using the Human Monocyte Nucleofector Kit (Lonza). Briefly, 5×10^6 monocytes were resuspended into 100 μL nucleofector solution with 5 μM of either MIF siRNA (CAACUCCACCUUCGCCUAAtt) or Scr siRNA (AGGUAGUGUAAUCGCCUUGtt; Microsynth, Switzerland) before electroporation. Monocytes were used for functional studies 48 h after transfection, and analysis of the efficiency of silencing was performed at the same time.

RNA Analyses by Quantitative Real-Time PCR. Total RNA was isolated from monocytes and reverse-transcribed (Qiagen). Real-time PCR was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) (78). The following sense and antisense primers (5′–3′ sequences) were used for amplification: MIF, TCCTTCTGCCATCATGCCGA and TCTTAGGCGAAGGTGGAGTT; TLR2, GCCTCTCCAAGGAAGAATCC and TCCTGTTGTTGGACAGGTCA; TLR4, ACCTGGCTGGTTTACACGTC and CTGCCAGAGACATTGCAGAA; CD14, CTGCAACTTCTCCGAACCTC and CCAGTAGCTGAGCAGGAACC; MYD88, GGATGGTGGTGGTTGTCTCT and AGGATGCTGGGGAACTCTTT; and hypoxanthine phosphoribosyltransferase (HPRT), GAACGTCTTGCTCGAGATGTG and CCAGCAGGTCAGCAAAGAATT. Gene-specific expression was normalized to the expression of HPRT and was expressed in arbitrary units (A.U.).

Western Blot Analysis. Whole cellular extracts and cytoplasmic and nuclear extracts were prepared as described previously (10). An equal amount of protein extract was loaded on 12% or 15% gels. After SDS/PAGE, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell). Membranes were incubated with antibodies directed against MIF (79), NF-κB p65, total and phosphorylated p38, ERK1/2, and JNK MAPKs, GAPDH and β-actin (Cell Signaling Technology). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology Inc.). Signals were revealed using enhanced chemiluminescence detection (GE Healthcare).

Animal Experimentation. Animal experiments were approved by the Office Vétérinaire du Canton de Vaud (authorization 877.8) and performed according to the guidelines of the University of Lausanne, Swiss National guidelines, and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (www.nc3rs.org.uk/arrive-guidelines). Male and female C57BL6/N mice (8 wk old; Charles River Laboratories) were housed under specific pathogen-free conditions. Mating was for 2 d. Newborns were killed to collect blood or injected intraperitoneally with ISO-1 (100 mg/kg) or DMSO before i.p. or intranasal challenge with *E. coli* O18:K1:H7. Sixteen hours later, mice were killed. Bacterial burden was assessed by platting 10-fold dilutions of blood or homogenized organs onto blood agar plates.

Statistical Analyses. Statistical analyses were performed using PRISM (GraphPad Software Inc.). Data are expressed as means ± SEs of the means (SEMs). Comparisons between the different groups were performed by two-tailed *t* tests or analysis of variance followed by the Turkey's multiple comparison test when appropriate. Findings were considered statistically significant when $P < 0.05$.

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