A novel IL-10 – DEL-1 axis promotes granulopoiesis and sepsis survival in early life

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Abstract

Newborns' susceptibility to infection is mainly attributed to decreased neutrophil bone marrow reserves and peripheral blood neutropenia. However, the regulation of neonatal neutrophil kinetics in sepsis remains poorly understood. We demonstrate herein that the developmental endothelial locus (DEL-1) is elevated in early life and is integral to a protective host response in neonates, by supporting emergency granulopoiesis. DEL-1-deficient neonate mouse pups subjected to sepsis displayed diminished bone marrow numbers of neutrophils and granulocyte-macrophage progenitors, leading to neutropenia, exaggerated bacteremia, and increased mortality; defects that were rescued by DEL-1 administration. Contrary to adult mice, DEL-1 was not downregulated upon neonatal sepsis. The sustained production of DEL-1 under newborn sepsis was attributed to a high IL-10/IL-17A ratio, as we IL-10 upregulated DEL-1. The expression of DEL-1 and its effect in emergency granulopoiesis in neonates was diminished by anti-IL-10-Receptor blockage. Consistent with the mouse findings, DEL-1 and neutrophil numbers were higher in septic human adult and neonate patients with high IL-10/IL-17A ratio. Furthermore, septic patients with high DEL-1 exhibited lower mortality rates compared to patients with low DEL-1. These findings highlight the role of a hitherto unappreciated IL-10–DEL-1 axis in supporting emergency granulopoiesis, preventing neutropenia and promoting sepsis survival in early life.

Introduction

Sepsis remains a serious threat in early life, leading to significant morbidity and mortality (1–3). Newborn's susceptibility to infection is mainly attributed to immature immune responses (4–7), however, the underlying mechanisms that regulate neonatal immunity remain poorly understood.

Neutrophils are an essential first line of innate immune response against invading pathogens (8, 9). Under resting conditions, neutrophils are retained in the bone marrow and circulation, while, during infection, inflammatory mediators stimulate their release from the bone marrow and their translocation from the circulation to the inflamed tissues (10). In early life, the bone marrow neutrophil storage pool and its ability to increase neutrophil production are limited (11–13). Furthermore, neutrophil recruitment to inflamed tissues is reduced in newborns compared to adults (11–13). In this regard, newborns display both quantitative and qualitative neutrophil defects and, consequently, often exhibit neutrophil bone marrow depletion, peripheral neutropenia and inadequate neutrophil tissue infiltration upon infection (12). Nevertheless, tight regulation of neutrophil kinetics is vital in the neonatal host defense.

The developmental endothelial locus-1 (DEL-1), encoded by the EDIL3 gene, is a soluble endogenous protein secreted by endothelial and other tissue resident cells, which binds to leukocyte $\beta 2$ integrins (such as, the lymphocyte function associated antigen, LFA-1 or CD11a/CD18) and antagonize their interaction with intracellular cell adhesion molecule 1 (ICAM-1) in the endothelium (14–18). By regulating the LFA-1–ICAM-1 interaction, endothelial cell-expressed DEL-1 can control leukocyte recruitment to peripheral tissues and thus the initiation of inflammation. Moreover, macrophage-derived DEL-1 promotes phagocytosis of apoptotic neutrophils (efferocytosis) and reprogramming of macrophages to a pro-

resolving phenotype, thereby contributing to successful resolution of inflammation (19). DEL-1 counteracts IL-17A-dependent inflammation, by restraining the production of IL-17A (20, 21). Consequently, DEL-1 deficiency was associated with increased susceptibility to a variety of inflammatory conditions, especially those driven by IL-17A (22, 23). DEL-1 has been also shown to promote myelopoiesis in the bone marrow by binding to $\beta 3$ integrin (CD61) (24). DEL-1 expression is downregulated in peripheral tissues or organs upon inflammation, for instance by the pro-inflammatory cytokines tumor necrosis factor alpha (TNF α) and IL-17A in adult animal models of inflammatory diseases (25, 26).

Despite the wealth of information on the role of DEL-1 in adult mouse models of inflammation, no information is available on the expression and functional significance of DEL-1 in sepsis in early life. The expression of DEL-1 in neonates and its effect on the outcome of neonatal innate immune responses and neonatal neutrophil kinetics upon sepsis has not been hitherto evaluated. Given the substantial implications of newborn infections, better understanding of the mechanisms regulating inflammation and neutrophil circulation in the neonatal period is pivotal to improve survival in these susceptible young hosts. In this study, we aimed to shed light on the expression and function of DEL-1 in neonatal hosts and evaluate its potential prognostic and/or therapeutic value in the management of neonatal sepsis.

Results

Expression of the homeostatic factor DEL-1 is elevated in the neonatal period

To determine whether tissue expression of DEL-1 differs between adult and neonates, we analyzed different tissues from healthy adult and neonatal mice. We compared the DEL-1 mRNA expression in the lung, kidney, intestine, liver, heart, and brain of neonatal (4 days old) and adult (8-10wks) mice (Figure 1A, B). DEL-1 mRNA was highly expressed in murine neonatal brain and lung tissue, to a lesser degree in intestine and kidney, while very low expression of the DEL-1 transcript was observed in the heart and liver (Figure 1A). DEL-1 mRNA expression was higher in neonate mouse pups compared to adult mice in all tissues studied (lung, kidney, intestine) except for the brain (Figure 1B, C). As postnatal age advanced, DEL-1 mRNA expression was gradually reduced in tissues that were examined (lung and kidney) (Figure 1C). We also measured human DEL-1 protein in cord blood serum from newborns (gestational age 34 to 40wks), peripheral blood serum from children at the age of 4 years (Figure 1D). Median DEL-1 serum concentration was significantly higher in neonates compared to older children (Figure 1D).

The expression of ICAM-1 and the DEL-1 receptor, $\beta 2$ integrin LFA-1 (CD11a/CD18), were also evaluated in neonatal tissues and neutrophils respectively (Supplemental Figure 1). Mean fluorescence intensity of CD11a protein in blood neutrophils did not differ between adult and neonate mice (Figure S1 B, C). There was no difference in ICAM-1 mRNA expression in various tissues from healthy adult and neonatal mice (4 days old) (Figure S1E).

DEL-1 expression is not suppressed upon sepsis in neonates

Earlier studies have shown that DEL-1 is suppressed upon acute inflammation in several animal models of disease and may resurge in the context of resolution of inflammation (15, 19, 25, 27-29). The regulation of DEL-1 expression during neonatal sepsis remains unknown. To address this, we subjected WT adult and neonate mice to the cecal slurry (CS) model of polymicrobial peritonitis, a gold standard model for neonatal sepsis studies (30).

Following intraperitoneal CS administration, mRNA expression of DEL-1 was determined in the lung of neonate pups and adult mice at different time points. DEL-1 mRNA was downregulated six hours after CS-induced sepsis in adults and returned to basal at 20 hours, while it was elevated six hours after CS-induced sepsis in neonate pups (Figure 2A). We then compared changes of DEL-1 expression in lung, kidney, and intestine six hours after CS-induced sepsis. DEL-1 was suppressed in the lung, intestine and kidney, while in neonates it was increased in the lung or remained unchanged in kidney and intestine (Figure 2A, B). A similar pattern was observed in human serum, where DEL-1 was suppressed in septic adults (during the first 24 hours of sepsis) compared to healthy controls, while such a decrease was not evident in neonates with sepsis during the same time interval (Figure 2C). Protein expression of the DEL-1 receptor, CD11a, was upregulated similarly in the lung of both neonatal and adult mice with sepsis (Figure S1 B, D). ICAM-1 mRNA expression was also upregulated in both neonate and adult septic mice, but to a lesser extent in neonates (Figure S1 F, G).

Neonates had increased bacterial load in peritoneal lavage fluid, lung, intestine and kidney tissues compared to adult septic mice (Figure 2D). DEL-1 regulates neutrophil recruitment to the site of inflammation. We, therefore, evaluated potential differences in neutrophil infiltration in the peritoneal cavity of neonatal and adult mice exposed to CS-induced sepsis. Septic neonate pups exhibited lower numbers of neutrophils in peritoneal lavage fluid and reduced MPO activity in the lung and kidney of septic neonatal pups compared to adults (Figure 2E). Thus, higher levels of DEL-1 in neonates, as compared to adults, are associated with reduced neutrophil recruitment and defective bacterial clearance.

DEL-1 controls neonatal tissue neutrophil infiltration and is essential for neonate survival in sepsis

To evaluate the impact of DEL-1 on neonatal neutrophil recruitment, we subjected WT and *Edil3^{-/-}* (hereafter designated *Del1^{-/-}*) neonate mouse pups to CS-induced sepsis and evaluated neutrophil recruitment to the peritoneum and lung. *Del1^{-/-}* septic neonate mice had elevated numbers of neutrophils in peritoneal lavage fluid (Figure 3A) and higher MPO activity in the lung (Figure 3B), compared to WT septic neonate mouse pups. In contrast, septic *Del1^{-/-}* neonate mice that received i.v. recombinant DEL-1 protein fused with human IgG Fc (DEL-1-Fc), had reduced numbers of neutrophils in peritoneal lavage fluid and reduced MPO activity in the lung compared to septic *Del1^{-/-}* neonates that received control IgG-Fc (Figure 3A, B).

Next, we evaluated the effect of DEL-1 deficiency on survival from CS-induced sepsis in neonate pups. Del1^{-/-} neonate pups exhibited reduced survival upon sepsis compared to WT ones when sepsis was mild or moderate, while at severe sepsis the difference was no longer evident (Figure 4A), suggesting that endogenous DEL-1 confers protection against sepsis. Consistent with this notion, DEL-1-Fc administration in the context of severe sepsis significantly improved the survival in Del1^{-/-} septic neonatal pups (Figure 4B). Furthermore, we evaluated the 28-day mortality rate in humans, adult and neonate patients with sepsis that had either low or high serum DEL-1 concentration. Based on the median DEL-1 concentration of healthy sex- and age-matched humans that we included in this study (Figure 2D), the threshold for high/low DEL-1 concentration was determined to 125pg/ml for adult patients and 700pg/ml for neonates. The clinical characteristics, source of sepsis and outcome of the two groups of the neonates and adults are depicted in Supplemental Table 1 and 2 respectively. Neonates with sepsis in the high DEL-1 group appeared to exhibit lower 28-day mortality rate compared to the low DEL-1 group (8.3% vs. 25%, respectively) (Table S1). Furthermore, adult patients with sepsis in the high DEL-1 group exhibited lower 28-day mortality rate compared to the low DEL-1 adult group (13.3% vs 44%, respectively, $p < 0.05$) (Table S2).

DEL-1 facilitates sustained neutrophil output in the blood circulation

To delineate the mechanism of DEL-1-mediated protection against neonatal sepsis, we evaluated cytokine responses, neutrophil infiltration and bacteria clearance in WT and Del1^{-/-} neonate mice in the presence or absence of exogenous DEL-1 administration using DEL-1-Fc (26, 27). No difference was observed in serum TNF α and IL-10 protein concentration between WT and Del1^{-/-} neonate pups or between Del1^{-/-} neonate pups treated with either i.v DEL-1-Fc or control IgG 6 hours following CS-induced sepsis (Figure S2A, B). Administration of a broad-spectrum antibiotic (meropenem) significantly reduced the mortality rate of septic Del1^{-/-} neonate mice 3-fold, while to a lesser degree in WT mice (Figure S2C), suggesting that defective bacterial clearance contributes to increased mortality in Del1^{-/-} mice. Indeed, although at six hours following initiation of sepsis Del1^{-/-} mice had less or similar bacterial burden in the peritoneum and blood, respectively, at 12 hours post-sepsis they exhibited higher bacterial load in the blood, as compared to WT neonate mice (Figure 5A). The increased bacterial load associated with DEL-1 deficiency was reversed by DEL-1-Fc administration (Figure 5C, left panel). The time course of neutrophil abundance in the peritoneum and blood followed the reverse pattern of that of the bacterial load. Specifically, at six hours following initiation of sepsis, Del1^{-/-} mice had initially higher or similar neutrophil numbers in the peritoneum and blood respectively, but the number of neutrophils fell significantly at twelve hours post-sepsis and were significantly lower as compared to WT mice (Figure 5B), suggesting bone marrow exhaustion and a failure to replenish neutrophil numbers. Intriguingly, DEL-1-Fc administration prevented the decline in neutrophils numbers in DEL-1 deficient neonate pups (Figure 5C, right panel). Interestingly, neonate patients with sepsis that had high DEL-1 concentration on the first

day of enrollment, exhibited a significant increase in the blood neutrophil count the following 24 hours (2-fold) compared to the neonates of the low DEL-1 group (Figure 5D).

DEL-1 supports emergency granulopoiesis in the bone marrow of neonates

It has been recently shown that DEL-1, derived from endothelial and mesenchymal stromal cells in the bone marrow, promotes myelopoiesis under both steady-state and hematopoietic stress conditions (24). Since $\text{Del1}^{-/-}$ mice failed to sustain circulating neutrophils in the blood early upon sepsis, we evaluated the neutrophil pool and DEL-1 expression in the bone marrow in healthy and septic WT and $\text{Del1}^{-/-}$ mice of neonatal age and compared them to adult WT mice. WT neonate mice had smaller pool of neutrophils in the bone marrow both under steady-state and septic conditions compared to WT adult mice (Figure 6A, B). In comparison to WT neonates, $\text{Del1}^{-/-}$ neonates had similar numbers of neutrophils in the bone marrow at steady-state conditions but significantly lower during sepsis (Figure 6A, B). DEL-1 expression in the bone marrow was not significantly altered upon sepsis in adult mice but was increased 12 hours after sepsis in septic neonate mice compared to healthy ones (Figure 6C), ostensibly to support emergency granulopoiesis. Indeed, compared to WT neonates, $\text{Del1}^{-/-}$ neonates had similar numbers of granulocyte-macrophage progenitors (GMPs) ($\text{Lin}^{-}\text{cKit}^{+}\text{Sca1}^{-}\text{CD16/32}^{+}\text{CD34}^{+}$) in the bone marrow at steady-state conditions but the percentage and the absolute number of GMPs upon sepsis were significantly decreased in $\text{Del1}^{-/-}$ neonatal mice (Figure 6D, E). The expression of DEL-1 receptor, $\alpha\text{v}\beta 3$ integrin (comprising the αv integrin CD51 and the $\beta 3$ integrin CD61) in the bone marrow, did not differ among neonates and adult mice, in either normal or septic conditions (assessed by flow cytometry analysis in bone marrow total cells, supplemental figure 3).

IL-10 expression in neonates promote DEL-1 upregulation in sepsis

DEL-1 is negatively regulated by inflammatory cytokines such as TNF α and IL-17A (26, 31, 32). To identify the key cytokines that control DEL-1 expression in septic neonates, we analyzed serum expression of inflammatory (TNF α , IL-17A, IL-6) and the anti-inflammatory cytokine IL-10 in septic neonatal and adult mice. Induction of TNF α in neonates was similar to adults, induction of IL-17A was less in neonates, while IL-6 and especially IL-10 were higher in septic neonates compared to adult (Figure 7A). Unlike TNF α and IL-17A that are known to downregulate DEL-1 (26), the effect of IL-6 and IL-10 on DEL-1 expression in endothelial cells has not been previously examined. To this end, we treated human endothelial cells (human umbilical vein endothelial cells, HUVECs and Ea.hy926 endothelial cell line) with human recombinant IL-6 or IL-10. IL-6 did not affect DEL-1 expression, while IL-10 promoted a two-fold upregulation of DEL-1 mRNA expression (Figure 7B). Stimulation with IL-10 reversed the suppressive effect of IL-17A on DEL-1 expression (Figure 7C), indicating opposing actions on DEL-1 expression by IL-10 and IL-17A.

To determine whether differences in DEL-1 expression observed in different tissues during neonatal sepsis reflected respective changes in IL-17A and IL-10, we measured IL-17A and IL-10 mRNA expression in tissue extracts from neonates six hours following exposure to CS-induced sepsis. We found that in neonatal lung, intestine and kidney, where DEL-1 was not suppressed in CS sepsis (Figure 2A, B), a higher IL-10 to IL-17A ratio was observed (Figure 7D). Consistent with these findings in mouse tissues, the median human DEL-1 protein concentration was higher in septic adults and septic neonates that exhibited high (>2) serum IL-10 to IL-17A ratio, compared to those with low (<2) IL-10 to IL-17A ratio (Figure 7E), further supporting that the IL-10/IL-17A balance regulates DEL-1 levels in sepsis. Moreover, we showed that IL-10 induced DEL-1 expression in human mesenchymal stromal cells (Figure 7F), suggesting that this cytokine might control DEL-1 expression in the human bone marrow.

To determine whether IL-10 regulates DEL-1 expression in the bone marrow during sepsis, we treated neonatal septic mice with an IL-10 receptor blocking antibody (anti-IL-10R). Administration of anti-IL-10R led to suppression of DEL-1 in the bone marrow of WT neonates (Figure 7G) and reduction of the neutrophil pool in the bone marrow and blood (Figure 7H). Importantly, treatment with anti-IL-10R resulted in higher bacterial load in blood (Figure 7I) and reduced survival in septic neonate mice (Figure 7J). Together, these findings indicate that a novel IL-10–DEL-1 axis promotes neutrophil production and host survival under septic stress conditions in neonates

Discussion

In this study, we demonstrate that the soluble endogenous molecule DEL-1 is elevated in early life under normal and septic conditions in both murine and human neonates. DEL-1 expression is under the control of IL-10 and is essential to promote emergency granulopoiesis in neonatal sepsis, thereby facilitating sustained output of circulating neutrophils, bacteremia control and survival from sepsis. Unlike earlier studies where the protective effect of DEL-1 was dependent predominantly on its anti-inflammatory/anti-leukocyte recruitment and pro-resolution properties (19, 21–23), in the present study the mechanism whereby DEL-1 protects from sepsis appears to depend strongly on its ability to support stress myelopoiesis. Additionally, this is the first study to identify IL-10 as a positive regulator of DEL-1 production. Although other endogenous molecules, such as specialized pro-resolution mediators (D-resolvins) were shown earlier to promote DEL-1 expression during inflammation resolution (31), IL-10 is unique in that it can induce DEL-1 also under inflammatory conditions. Hence, IL-10 has an important role in sustaining DEL-1 production under sepsis.

DEL-1 was elevated in the neonatal period and gradually reached levels similar to adults as postnatal age advanced. The differential expression of DEL-1 in tissues observed here is in accordance with a previous report in adult mouse tissues, indicating that the tissue-related requirements for immune privilege are unaffected by age (15). Contrary to what was observed in adults, DEL-1 was not downregulated upon sepsis in neonates. DEL-1 expression is transiently suppressed locally in tissues in response to pro-inflammatory triggers in adult mouse models of LPS-induced lung injury, cecal ligation and puncture (CLP)-induced sepsis, inflammatory bone loss, experimental autoimmune encephalomyelitis and sepsis-

induced adrenal gland inflammation (8, 15, 25, 27, 28, 33). Our results demonstrate that in septic neonates, DEL-1 expression does not follow the same expression pattern as in adults, which was attributed to the higher IL-10 to IL-17A ratio in neonates compared to adults.

The role of DEL-1 in the outcome of sepsis in neonates has not been previously evaluated. Our findings showed that DEL-1 deficiency had a substantial negative impact on survival of septic neonates. Furthermore, low serum DEL-1 concentration in adult and neonate patients with sepsis were correlated with reduced 28-day survival compared to septic adult and neonate individuals with high serum DEL-1. DEL-1 has been reported to promote homeostasis and improve the outcome of disease in adult experimental models of periodontal inflammation, experimental autoimmune encephalomyelitis and LPS-induced adrenal gland inflammation (8, 15, 25, 27). On the contrary, in a study in CLP-induced sepsis in adult mice and adult human septic patients, high DEL-1 expression was correlated with increased mortality risk (33). This discrepancy supports the complexity of sepsis pathogenesis and may also be attributed to the differences in immune responses and bone marrow reserves between adults and neonates.

Neonatal leukocytes exhibit reduced ability to adhere and transmigrate via the endothelium to the tissues during inflammatory stimuli (34–36) and, based on our results, the increased DEL-1 concentration in the neonatal period could at least contribute to this phenomenon. It has been suggested though, that insufficient leukocyte recruitment in fetal life and in the newborn period may be one of the reasons for the high incidence of severe infections (37–39). Based on our findings, tissue leukocyte infiltration in neonates was negatively associated with sepsis survival, as $\text{Del1}^{-/-}$ mice had increased neutrophilic infiltration in tissues but worse survival and, consistently, DEL-1 administration abrogated tissue neutrophil infiltration but improved survival from sepsis. In accordance to our observation, in a previous study in adult murine model of polymicrobial sepsis, ICAM-1-deficient mice demonstrated a significant reduction of mortality compared to WT mice (40). Therefore, although leukocyte tissue recruitment is essential for pathogen elimination, it may provoke an overwhelming inflammatory response that may lead to microvasculature injury and contribute to organ damage and failure (9, 41–46). Our finding that enhanced leukocyte recruitment was detrimental in septic $\text{Del1}^{-/-}$ neonates, highlights the impact of DEL-1 in controlling exaggerated organ inflammation and damage in young hosts.

In addition to the function of DEL-1 to regulate local tissue inflammation, this report expands our knowledge by demonstrating that DEL-1 is essential to maintain granulopoiesis in the bone marrow of neonate mice and is required to prevent sepsis-induced neutropenia. In the bone marrow, there is a pool of neutrophil precursors that maintain the ability to multiply and rapidly replenish neutrophil numbers in the circulation upon sepsis (47, 48). In term neonates, the bone marrow neutrophil pool is greatly diminished compared to adults and, moreover, have a limited ability to recruit or generate new neutrophils during infections, contributing to the development of neutropenia (49, 50). In accordance with the above, WT neonate mice in our study had significantly lower numbers of neutrophils in the bone marrow at steady-state and in sepsis, compared to adults. In another report, generation of new granulocytes from the bone marrow following CS-induced sepsis was defective and delayed in neonates compared to adults (13). In

addition, $Del1^{-/-}$ mice exhibited excessive depletion of the bone marrow neutrophil and GMPs pool, supporting the importance of DEL-1 in supporting emergency granulopoiesis.

We further explain the mechanistic basis of the discrepancy in DEL-1 expression between neonates and adults in sepsis, by demonstrating that DEL-1 is under the control of IL-10 and particularly of the IL-10 to IL-17A ratio. IL-10 administration promoted DEL-1 expression in both endothelial and mesenchymal stromal cells, two major cell sources of DEL-1. In accordance with our findings, IL-10 production in response to pro-inflammatory stimuli in neonates has been shown to inhibit neutrophil migration to tissues (51, 52). TNF α and IL-17A upregulation, as well as bacterial products, have been previously shown to suppress DEL-1 expression (8, 25, 26). In accordance with this finding, we showed that not only IL-10, but the balance of IL-10 and the pro-inflammatory cytokine IL-17A, determined the expression of DEL-1 in murine tissues and in serum of septic patients, regardless of age. Finally, DEL-1 suppression in the bone marrow of WT septic mice treated with anti-IL-10R antibody also led to diminished neutrophil pool in both the bone marrow and the circulation, and had a negative impact on survival from sepsis, further supporting the role of the IL-10/DEL-1 axis in the regulation of emergency myelopoiesis and sepsis outcome in neonates.

Based on our results, IL-10-induced DEL-1 is central in promoting emergency granulopoiesis, maintenance of neutrophilia and improving host survival in sepsis. This mechanism may not be crucial in adult sepsis where the bone marrow reserves are adequate, as shown in the present study. Neutropenia in neonates is a known risk factor for bacterial infection and has been linked with adverse outcome in sepsis (53). Together, these data strongly suggest that DEL-1 remains elevated in the neonatal period to compensate for the small size of the neutrophil pool at this young age. Ultimately, neutrophil numbers rise over the first few weeks of life to achieve adult values by 4 weeks of age (54) and this by time also DEL-1 expression decreases and reaches adult levels.

To date, there are no reliable prognostic factors for sepsis outcome in neonates and there is no effective immunomodulatory strategy to simultaneously control bone marrow neutrophil production and tissue recruitment upon infection. Trials investigating the clinical use of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) that solely increase neutrophil numbers in preterm infants have yielded disappointing results (55, 56). The critical functions of DEL-1, its correlation with neutrophilic response and survival from sepsis and the fact that it is a secreted protein that could be administered in vivo in a controlled manner depending on the clinical situation, renders DEL-1 an attractive and promising prognostic and/or therapeutic target in neonatal sepsis.

Materials And Methods

Mice and monitoring

C57BL/6 mice of neonatal (4-6 days old) or adult age (8-10 weeks old) were used. The generation of $Edil3^{-/-}$ mice (hereafter designated $Del1^{-/-}$) in a C57BL/6 background has been previously described

(15). Mice were kept in the animal house of the University of Crete, School of Medicine, in a temperature-controlled room and 12h light/dark cycle, with free access to standard laboratory chow and water. Non-breeding adult mice were maintained on standard rodent food (Mucedola, Italy, 4RF21), and breeding adult mice were on a high-protein diet (Mucedola, Italy, 4RF25). To achieve synchronized births of neonatal mice, paired matings of WT and *Del1*^{-/-} mice were placed weekly; pregnant females were isolated from males and followed closely thereafter three times per week until their date of birth. For all experimental procedures, neonates were removed from their mothers as a group and placed on a warming blanket. After the end of procedure, littermates remained with their mother till the end of the experiment.

Murine polymicrobial sepsis

Polymicrobial sepsis was induced using the fecal peritonitis method as previously described (30, 57, 58). Briefly, the cecum contents of adult C57BL/6 mice (6-8 weeks old) were suspended in 5% dextrose solution at a final concentration of 80 mg/ml and then passed through a 70-nm filter. The suspension was aliquoted and stored in 15% containing glycerol stocks at -80°C for up to three months until challenge. To account for batch and/or donor variation, each CS preparation was first titrated in control adult and neonate mice to reach desired lethal dose. The same batch of CS and challenge dose was kept constant among different mouse genotypes and ages for an entire experiment. An amount of 1.1 to 1.6 mg/per gr of mouse bodyweight of CS (depending on the intended severity of sepsis) was injected intraperitoneally (i.p.) in C57BL/6 wild type (WT) or C57BL/6 *Del1*^{-/-} mice of neonatal or adult age. Age- and gender- matched mice received equal amount of 5% dextrose and served as controls.

In vivo DEL-1 and anti - IL-10 receptor treatments

A group of *Del1*^{-/-} newborn septic mice were treated with recombinant DEL-1 protein fused with human IgG Fc (DEL-1-Fc). The generation of DEL-1-Fc protein has been previously described (26, 27). Five µg DEL-1-Fc per mouse was administrated once intravenously (i.v; orbital vein), 15 minutes prior to the induction of sepsis. Age-matched newborn mice received the same amount of recombinant IgG1- Fc (Fc control, R&D Systems, Minneapolis, USA) and served as controls. Another group of WT newborn septic mice were treated with purified endotoxin-free anti-mouse IL-10R blocking antibody (CD210; Biolegend, San Diego, CA). Five µg of IL-10R blocking antibody per mouse was administrated once i.v (orbital vein), 15 minutes prior to the induction of sepsis. Mice were briefly anesthetized by sevoflurane inhalation prior to orbital vein injections. Age-matched newborn mice received the same amount of recombinant IgG1 (R&D Systems, Minneapolis, USA) and served as controls. In a different set of experiments, the antibiotic meropenem at a concentration of 25µg/gr was administrated i.p 2 hours after CS induced sepsis.

In vivo murine sample collection, analysis, and survival experiments

At specific time points following CS administration, mice were anaesthetized with i.p ketamine (100 mg/kg) and xylazine (8 mg/kg) and peritoneal lavage, whole blood, bone marrow and tissues (liver, small

intestine, kidney, lung, heart, brain) were harvested for analysis. Peritoneal lavage was collected by i.p. injection of 600µl of sterile PBS (Gibco, Invitrogen, Carlsbad, CA) per newborn mouse and 1.5 ml per adult mouse, and then by gently massaging the body cavity 20 times before aseptic aspiration. Blood was drawn via cardiac puncture into a 25-gauge insulin syringe containing 20 µl heparin (1000 U/ml) and placed on ice. Prior to tissue collection, mice were perfused with ice cold PBS via the right ventricle. To isolate bone marrow cells, femoral bones were removed and transferred into sterile petri dishes. Bone marrow was flushed out of femurs, using a 23-gauge needle. In another set of experiments, sepsis survival of WT and *Del1*^{-/-} mice, and WT mice that received either IgG1 or IL-10R blocking antibody or DEL-1-Fc was evaluated.

Measurement of myeloperoxidase (MPO) activity

For MPO determination, tissues were homogenized in 50 nmol/l phosphate buffer, pH 6.0, with 0.5% hexadecyltrimethylammonium bromide using mortar and pestle. Samples were frozen/thawed three times, centrifuged for 10 min at 10,000 *g*, and supernatants were stored at -20°C. MPO was determined in 96-well plates using a modification of the method previously described (59). Briefly, 10 µl of sample was added to 190 µl assay buffer containing phosphate buffer 50 mM, pH 6.0 and 0.167 mg/ml o-dianisidine (Sigma Chemical Co) and 0.0005% H₂O₂. Absorbance at 450 nm (A_{450 nm}) was measured in a microplate reader at 15 min. Results were normalized per protein content of tissues and were reported as arbitrary units per mg of tissue.

Determination of leukocyte infiltration and bacteria clearance in vivo

Total white blood cell (WBC) counts of cells isolated from peritoneal lavage, whole blood and bone marrow were estimated by hemocytometer, whereas differential counts were determined by flow cytometry (see below). Peritoneal and whole blood bacterial counts were determined by culturing 100 µl of serially diluted peritoneal lavage or whole blood sample on Luria - Bertani (LB) agar plates at 37°C overnight. Plates were counted after 24h of plating. Bacterial counts in tissues were determined as follows; whole organs were briefly immersed in 70% ethanol and sterile water, and then disrupted with mortar and pestle. The homogenized tissues were suspended in 500 µl of sterile PBS. Suspensions were serially diluted, plated on LB agar plates and incubated at 37°C for 24h. Results are reported in colony-forming units (CFU) per milliliter of blood or CFU per tissues.

Regulation of DEL-1 expression by cytokines in HUVECs and Ea929 cells

HUVEC cells were purchased from Lonza (Basel, Switzerland) and Ea.hy926 endothelial cell line were kindly provided by Prof. Kardasis, University of Crete, School of Medicine. Cells were cultured in Endothelial Cell Growth Medium with Supplement Mix (PromoCell, Heidelberg, Germany). Cells were seeded in 12 well plates (300,000 per well) and on the next day they were treated with 20ng/ml human IL-6, 20ng/ml human IL-10, or 20ng/ml human IL-17A (PerProtTech) for 4, 8 and 24 hours in basal

Endothelial Cell Growth Medium without supplements. Total RNA was isolated and quantitative PCR was performed as described below.

Mesenchymal Stromal Cell isolation and tissue culture

Mesenchymal Stromal Cells (MSCs), isolated from Wharton jelly, were cultured in fresh Dulbecco modified Eagle Medium, (DMEM) low glucose (1g/L) supplemented with 10% fetal bovine serum (FBS) and 1% (100 IU/mL) penicillin-streptomycin at 37 C. Culture medium was replaced twice per week; when MSCs reached 80–90% confluency, they were detached using 0.25% Trypsin–1mM EDTA (Gibco, Invitrogen, Carlsbad, CA). Cells were then reseeded at a concentration of 2000-3000 cells/cm² and further expanded for a total of twelve passages (P12).

RNA isolation and quantitative PCR

Total RNA was extracted from adult and tissues, human Mesenchymal Stromal Cells (hMSCs), human Umbilical Vein Endothelial Cells (HUVEC) and Ea.hy926 human endothelial cells using TRIzol reagent (Life Technologies, Carlsbad, CA) and quantified by spectrometry at 260 and 280nm. One microgram of total DNA-digested RNA was used for cDNA synthesis (Thermoscript RT; Invitrogen, Carlsbad, CA). The SYBR Green method was followed in the PCR reaction.

The murine and human primers were used in the PCR reaction as depicted in a table in supplementary materials and methods. Annealing was carried out at 60°C for 30 sec, extension at 72°C for 30 sec, and denaturation at 95°C for 30 sec for 40 cycles using the ABI 7500 System, according to the manufacturer's protocol (Applied Biosystems). Analysis of the fold change was performed based on the Pfaffl method (60).

Study Subjects

Human serum from the cord blood of 20 healthy newborns and 20 four-year-old healthy children were kindly provided by Prof L. Chatzi from the Mother-Child cohort in Crete (Rhea cohort). Serum from 40 adult septic patients (>18 years-old), and 24 neonate septic patients (0-28 days old), admitted to the neonatal and adult Intensive Care Units of the Heraklion University hospital were obtained. Serum from 40 sex and age-matched healthy adult volunteers, 28 sex and age-matched healthy newborns were used as controls.

Neonatal sepsis is defined as neonates (age < 28 days on admission) with presence of at least one clinical sign (temperature instability, cardiovascular or respiratory instability, skin symptoms, gastrointestinal symptoms) and at least one laboratory result which is suggestive for neonatal sepsis (white blood cell count, platelet count, C- reactive protein, absolute neutrophil count) and elevated interleukin - 6 > 50 pg/dl (55, 56). Adult sepsis was defined as adults (>18 years old) with presence of evidence of infection (possible or confirmed) and at least two out of four SIRS criteria (systemic inflammatory response syndrome) or two-point increase in SOFA (sequential organ failure assessment) score (57). Additional information such as age, sex, gestational age (for neonates), blood neutrophil counts and outcome (length of stay and 28-day mortality) were collected.

Murine and human serum cytokine and DEL-1 measurement

Cytokine concentration of IL-6, TNF, IL-10 and IL-17A in mouse and human serum was determined by enzyme-linked immunoabsorbent assay (ELISA) at the indicated time points using ELISA kits (Biolegend, ELISA Max Deluxe assays), according to manufacturer's instructions. Human serum DEL-1 was measured using a validated commercially available assay (Human EDIL3 DuoSet ELISA, RnD Systems, Minnesota, MN). Triton 0.5% was added to the serum samples prior to analysis.

Neutrophil and GMP analysis by flow cytometry

Total white blood cell (WBC) counts of peritoneal lavage isolated cells, whole blood and bone marrow cells were assessed by hemocytometer counting using acetic acid 3% treatment. For differential WBC analysis, cells were first incubated with Fc-block (BD Pharmigen, San Diego, CA) and then staining was performed using FITC-anti mouse CD45 antibody (BD Pharmigen, San Diego, CA), PE-anti-mouse Ly-6G (Gr-1) (E-bioscience, San Diego, CA), APC anti mouse CD11b (Biolegend, San Diego, CA), PE anti-mouse CD51 (Biolegend, San Diego, CA) and FITC anti-mouse CD61 (Biolegend, San Diego, CA). The proper isotype controls were used in each case.

For GMP progenitor cell analysis, bone marrow total cells were collected as described above. Erythrocytes were removed via incubation with ACK lysing buffer (Thermofisher, Invitrogen, Carlsbad, CA). Cell suspension was then counted as described above and were kept frozen in FBS supplemented with 10% DMSO at -80°C and then in liquid nitrogen for up to 1 month prior to use. For flow cytometry analysis, bone marrow cells were washed and were stained with PE-Cy7-anti mouse Sca-1 antibody (Biolegend, San Diego, CA), PE-anti-mouse CD34 (Biolegend, San Diego, CA), APC anti mouse Lineage antibody cocktail (BD Pharmigen, San Diego, CA), BV480 anti-mouse CD117 (c-Kit) (BD Pharmigen San Diego, CA) and BV421 anti-mouse CD16/32 (Biolegend, San Diego, CA) for 4 hours in 4°C. The proper isotype controls were used in each case. GMP progenitor cells were identified as live cells, Lineage neg, CD117 (c-Kit) positive, Sca-1 negative and CD16/32 and CD34 positive. The flow cytometry events were acquired in a FACS Canto II (BD Biosciences, San Jose, CA) and analyzed with the use of FlowJo v10.7.1 Software.

Statistical analysis

All numeric data were evaluated for normality using the Kolmogorov–Smirnov test. The numerical data that passed the normality test and the PCR results were analyzed using two-sided Student's t-test or one-way ANOVA with the Bonferroni multiple-comparison post-test and were expressed as mean +/- standard deviation (SD). Comparison of measurements that failed normality tests was performed using the Mann–Whitney U test or the Kruskal–Wallis test with the Dunn multiple-comparison post-test and were expressed as boxplots with median and min to max range. Categorical data were analyzed by Fisher exact test. Kaplan-Meier curves were performed for survival experiments, and survival curves were compared between groups using a log-rank test. The GraphPad InStat software (GraphPad 8.0, San Diego, CA) was used for analysis. P values < 0.05 were considered significant. Results are representative of at least three independent experiments.

Statistical approval

All animal experimentation is in adherence to the “NIH Guide for the Care and Use of Laboratory Animals” and all animal procedures were in accordance with institutional guidelines and were approved by the University of Crete’s Animal Care and Use Committee and the Veterinary Department of the Heraklion Prefecture (license number 150760/20-07-2017). For all included human subjects, informed consent was obtained at the time of the recruitment, and the studies were conducted in accordance with the Helsinki Declaration ethical standards. All procedures were conducted upon approval of the Institutional Review Board of the University General Hospital of Heraklion (approval number 2418).

Declarations

Data availability statement: All data generated or analysed during this study are included in this published article (and its supplementary information files).

Author contributions: **EV**: conceived and designed the project, performed experiments, analyzed and interpreted data, and wrote the manuscript., **OK, KL, EI, IL, VLA**, performed experiments, analyzed and interpreted data; **ED, KV, EH, HP** obtained human samples and interpreted data, **EG** supervised research, and revised the manuscript, **GH, TC**, provided critical resources, interpreted data and revised the manuscript, and **CT** supervised research, interpreted data and revised the manuscript. All authors contributed to the writing of the manuscript.

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Figures

Figure 1

DEL-1 expression is elevated in neonatal age. A, DEL-1 relative mRNA expression among different tissues from healthy mice of neonatal age (4 days old). B, C, DEL-1 relative mRNA expression in tissues from healthy mice of adult (8-10wks) or neonatal age (4 days old). C, DEL-1 relative mRNA expression in lung and kidney from healthy mice of adult mice and neonate mice of 1, 7, 14 or 21days of postnatal age. D, Median human DEL-1 protein concentration in cord blood serum from healthy newborns (gestational age 34-40 wks old) and from the healthy children at the age of 4 years old. Mean $\pm$ SD (A, B, C) and median $\pm$ min-max (D) is depicted. Statistical analysis by one way ANOVA with Bonferroni's multiple comparison

post-test (A and C); unpaired two-sided t test (B), and Mann-Whitney test (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

DEL-1 expression is not suppressed upon sepsis in neonates. A, DEL-1 relative mRNA expression in lung tissue from mice of adult (8-10wks) or neonatal age (4days old) upon polymicrobial sepsis (6, 12 and 20 hours after cecal slurry administration). B, DEL-1 relative mRNA expression in kidney and intestine tissue from mice of adult (8-10wks) or neonatal age (4days old) upon polymicrobial sepsis, 6 hours after cecal slurry (CS), administration. C, Median human DEL-1 protein serum from healthy and septic adults and neonates (within <24h and >24 hours of sepsis onset). D, Bacterial counts (cfu) in total lung, kidney, and peritoneum in WT adult and neonatal mice 6 hours after CS-induced sepsis. E, MPO activity in total lung and kidney and total neutrophils in the peritoneum in WT adult and neonatal mice 6 hours after CS-induced sepsis. Mean $\pm$ SD (A, B, C, E) and median $\pm$ min-max (C) is depicted. Statistical analysis by one-

way ANOVA with Bonferroni's multiple comparison post-test (A, B and E); unpaired two-sided Student's t test (D), Kruskal-Wallis test among various adult groups (D) and Mann-Whitney test between group-matched adult and neonatal groups (D, comparisons are indicated with #). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ### $p<0.001$.

Figure 3

DEL-1 controls tissue neutrophil infiltration in neonatal sepsis. A, Total neutrophil count in peritoneal lavage and B, myeloperoxidase (MPO) activity in lung in WT and *Del1*^{-/-} neonatal mice as well as in *Del1*^{-/-} neonatal mice that received either DEL-1-Fc or IgG-Fc i.v 15 minutes prior to 6h of cecal slurry (CS) sepsis. Mean ± SD is depicted. Statistical analysis by one-way ANOVA with Bonferroni's multiple comparison post-test (A, B). * $p<0.05$, ** $p<0.01$.

Figure 4

The impact of DEL-1 on neonatal sepsis survival. A, Survival rates of WT and *Del1*^{-/-} neonatal mice after cecal slurry (CS) induced sepsis of either severe, moderate or mild severity. B, Survival rates of *Del1*^{-/-} neonatal mice treated with i.v DEL-1-Fc or IgG-Fc 15 minutes prior to the induction of CS sepsis. N= 16-25 mice per group. Statistical analysis by long-rank test (A, B). * $p<0.05$. Ctl: control.

Figure 5

Regulation of circulating neutrophil pool by DEL-1. A, Bacterial counts (cfu) in peritoneum and blood in WT and *Del1*^{-/-} neonatal mice 6 hours and 12 hours after cecal slurry (CS) induced sepsis B, Neutrophil counts (cfu) in peritoneum and blood in WT and *Del1*^{-/-} neonatal mice 6 hours and 12 hours after CS induced sepsis C, Bacterial counts (cfu) and neutrophils in blood in *Del1*^{-/-} neonate pups treated with either i.v DEL-1-Fc or IgG-Fc 6 hours and 12 hours after CS induced sepsis. D, Neutrophil numbers in blood

in neonates with sepsis the first day of sample collection (day 0) and 24 hours later (day 1), and fold increase in neutrophil numbers within 24 hours in neonates of either low DEL-1 (<700pg/ml) or high DEL-1 (> 700pg/ml) group. N= 6-22 mice per group in A, B, C and n= 12 human subjects are depicted per group in D. Mean $\pm$ SD is depicted (A-D). Statistical analysis by one way ANOVA with Bonferroni's multiple comparison post-test (A, B and C) (*indicates comparison of septic animals to the control animals of the same group, unless otherwise indicated and # indicates comparison of *Del1*^{-/-} to WT mice of the same time point), unpaired two-sided Student's t test (D). **p<0.01, ***p<0.001, #p<0.05, ##p<0.01, ###p<0.001.

Figure 6

DEL-1 regulates bone marrow granulopoiesis in neonatal sepsis. A, Total neutrophil numbers (per femur/per mouse gr) in bone marrow of WT adult, WT and *Del1*^{-/-} neonate pups under basal conditions and upon 6 and 12 hours of cecal slurry (CS) induced sepsis (n=5-24 mice per group, per time point), B,

representative images of flow cytometry analysis of CD45+CD11b+Gr-1+ positive cells in bone marrow cell suspension of WT adult, WT and Del1^{-/-} neonate pups under basal conditions and upon 12 hours CS induced sepsis. C, DEL-1 mRNA relative expression in bone marrow upon basal conditions and 6- and 12-hours of CS induced sepsis. D, representative flow cytometry plots of granulocyte-macrophage progenitors (GMPs) (Lin⁻, c-Kit⁺, Sca-1⁻, CD16/32⁺ and CD34⁺) in WT and Del1^{-/-} neonate pups upon 12 hours of CS induced sepsis. E, percentage (lower panel) and absolute numbers (per femur) of GMPs in WT and Del1^{-/-} neonate pups under basal conditions and upon 12 hours of CS - induced sepsis. Mean $\pm$ SD is depicted. Statistical analysis by one-way ANOVA with Bonferroni's multiple comparison post-test (A, C, E). * p <0.05, ** p <0.01, *** p <0.001. Ctl: control, Ad: adults, Neo: neonate.

Figure 7

IL-10 promotes DEL-1 expression in neonatal sepsis. A, Serum TNF, IL-17A, IL-6 and IL-10 protein concentration in neonate pups and adult mice 6 hours after exposure to cecal slurry (CS) – induced sepsis. B, DEL-1 mRNA relative expression in HUVECs at several time points upon stimulation with human recombinant IL-10 or IL-6 protein. C, DEL-1 mRNA relative expression in Ea.hy926 endothelial cells upon stimulation with human recombinant IL-17, IL-10 or both, for 4 hours. D, Tissue (lung, kidney and intestine) IL-10 to IL-17A mRNA ratio in neonatal and adult mice exposed to CS sepsis (6 hours). E, Median human DEL-1 protein serum from septic adults and neonates that exhibited either low (<2) or high (≥ 2) serum IL-10 to IL-17 ratio. F, DEL-1 mRNA relative expression in human mesenchymal stem

cells upon 4h of IL-10 stimulation. G, DEL-1 mRNA expression in bone marrow (BM), H, Total neutrophils in bone marrow and I, Bacterial colony forming units (cfu) in the blood of WT neonatal septic mice that received either i.v anti-IL-10R or IgG and J, survival in WT neonatal septic mice that received either i.v. anti-IL-10R or IgG cecal slurry (CS) - induced sepsis. Mean $\pm$ SD (A, B, C, D, F, G, H), median $\pm$ min-max (E, I) and frequency % (J) is depicted. Statistical analysis by one way ANOVA with Bonferroni's multiple comparison post-test (A, B, C, D and G), Mann-Whitney test (E) unpaired two-sided t test (F, H, I) and Fisher's exact test (J). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Ctl: control, Ad: adults, Neo: neonates.

Figure 8

Mechanisms of IL-10-DEL-1 mediated protection in sepsis in early life

Supplementary Files

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