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Inhibition of neogenin fosters resolution of inflammation and tissue regeneration

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The resolution of inflammation is an active process that is coordinated by endogenous mediators. Previous studies have demonstrated the immunomodulatory properties of the axonal guidance proteins in the initial phase of acute inflammation. We hypothesized that the neuronal guidance protein neogenin (Neo1) modulates mechanisms of inflammation resolution. In murine peritonitis, Neo1 deficiency (Neo1−/−) resulted in higher efficacies in reducing neutrophil migration into injury sites, increasing neutrophil apoptosis, actuating PMN phagocytosis, and increasing the endogenous biosynthesis of specialized proresolving mediators, such as lipoxin A4, maresin-1, and protectin DX. Neo1 expression was limited to Neo1-expressing Ly6C+ monocytes, and Neo1 deficiency induced monocyte polarization toward an antiinflammatory and proresolving phenotype. Signaling network analysis revealed that Neo1−/− monocytes mediate their immunomodulatory effects specifically by activating the PI3K/AKT pathway and suppressing the TGF-β pathway. In a cohort of 59 critically ill, intensive care unit (ICU) pediatric patients, we found a strong correlation between Neo1 blood plasma levels and abdominal compartment syndrome, Pediatric Risk of Mortality III (PRISM-III) score, and ICU length of stay and mortality. Together, these findings identify a crucial role for Neo1 in regulating tissue regeneration and resolution of inflammation, and determined Neo1 to be a predictor of morbidity and mortality in critically ill children affected by clinical inflammation.

Introduction

There are only a few targeting therapies for critically ill patients in the intensive care unit (ICU) who are suffering from complex and potentially life-threatening illnesses, such as acute respiratory distress syndrome and multiple organ failure. Hence, severe inflammation is recognized as a considerable problem in the care of these critically ill patients when resolution of inflammation fails to induce homeostasis (1). It is evident that nonresolving inflammation may lead to the activation of chronic inflammatory processes and ultimately to the development of organ dysfunction and the incurrence of comorbidities (2, 3). The initiation of this pivotal process is guided by diverse classes of mediators including cytokines, chemokines, and lipid mediators (3, 4). These mediators initiate the influx of proinflammatory cells that cause tissue injury. Following the initiation of an inflammatory response, when self-limited, a superfamily of endogenous mediators (SPMs) is generated to activate processes for resolution, indicating that the resolution of inflammation is a process that is distinct from antiinflammatory mechanisms (5). The key steps in this phase include (a) cessation of further PMN influx, (b) normalization of chemokine/cytokine gradients, (c) apoptosis of PMNs, (d) activation of macrophage (MΦ) phagocytosis and efferocytosis, and (e) generation of endogenous proresolving mediators (i.e., SPMs).

A paradigm for neuronal guidance proteins (NGPs) and their target receptors exists in the developing nervous system, where neuronal movement is mediated by the interplay of both attractive and repulsive signals. Analogies with axonal migration have postulated that these NGPs play an important role outside the central nervous system in guiding leukocyte migration (6–11). Neo1, a type I transmembrane protein and receptor for Netrin-1 and the repulsive guidance molecules (RGMs), is recognized to be essential in neurogenic and embryonic processes, in which it contributes to chondrogenesis, myogenesis, organ-specific development of the mammary gland, and neural tube formation (12–14). Recent studies have shown Neo1 to have pivotal nonneuronal functions during the onset of acute inflammation (9, 15, 16). However, the primary issue with inflammation is not the frequency of its initiation, but rather the formation of excessive or unresolved processes (2, 5). This notion, coupled with its immunomodulatory attributes, led us to question whether Neo1 might contribute to local inflammation resolution mechanisms and tissue regeneration processes.

Our studies revealed that functional inhibition of Neo1 induced apoptosis of neutrophils, which is a key feature of the initiation of the inflammation resolution mechanism (17) and ultimately shortened the neutrophil lifespan. Functional inhibition of Neo1 activated eat-me and find-me signals and G pro-
transplant chimeric mouse experiments showed hematopoietic Neo1 repression to be crucial for the reduction of Ly6C hi monocytes, the increase of Ly6C lo monocytes, and finally, the increase in clearance. In our analysis, we found Neo1 –/– monocytes to activate the PI3K/AKT pathway and suppress the TGF-β pathway, both of which are critical in restricting proinflammatory and promoting antiinflammatory responses and activating the monocyte and monocyte-derived MΦ polarization toward the proresolving phenotype. In line with these results, in an observational clinical study that included 59 critically ill ICU pediatric patients suffering from, in part, intraabdominal hypertension (IAH), abdominal compartment syndrome (ACS), internal cardiac and oncolog-

Figure 1. Role of Neo1 on human PMN apoptosis and MΦ efferocytosis. (A) Apoptosis of human PMNs following LPS and/or anti-Neo1 stimulation was determined by flow cytometry and the expression of CX3CL1 mRNA and IL1R2 mRNA was evaluated by RT-PCR. (B) Human MΦ were stimulated with IL-1β and/or anti-Neo1 antibody and the CX3CR1 mRNA and TIM4 mRNA levels were determined by RT-PCR. (C) Neo1 protein expression was assessed by immunofluorescence staining (n = 3/condition, magnification ×630, scale bar 20 μm). (D) The dose-dependent impact of anti-Neo1 treatment on MΦ clearance of the apoptotic PMNs and the corresponding immunofluorescence images (n = 3/condition, magnification ×400, scale bar 20 μm). (E) MΦ efferocytosis of E. coli. (F) mRNA expression of the ALX/FPR2 and GPR32 receptors in human MΦ. Results represent 2 independent experiments and are expressed as median ± 95% CI (n = 6–8 per group). Statistical analysis was done by ANOVA followed by Bonferroni’s post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001.
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1A). In apoptotic PMNs, blockade of Neo1 induced the expression of the decoy receptor IL-1R2, which is known for its strong impact on limiting the proinflammatory effects of IL-1β (Figure 1A) (20).

It is evident that apoptotic neutrophils induce their own clearance by expressing find-me and eat-me signals (17, 21). Therefore, we sought to determine the expression of CX3CL1, a critical protein contributing as a find-me signal in MΦ, and its receptor CX3CR1, which is crucial for sensing chemokines and recruiting monocytes (22). We found that blockade of Neo1 markedly increased both the CX3CL1 mRNA in apoptotic PMNs and the CX3CR1 mRNA in MΦ. These data were substantiated by increased levels of one of the most crucial eat-me receptors, TIM4, which mediates the direct recognition of phosphatidylserine by MΦ (Figure 1B). In addition to neutrophil apoptosis, MΦ efferocytosis is a key feature of resolution programs. We therefore set out to investigate the expression of Neo1 in human MΦ and PMNs (Figure 1C and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI96259DS1). Our data revealed a strong induction of Neo1 expression in MΦ following IL-1β stimulation. We found that the functional inhibition of Neo1 significantly increased the phagocytosis rate in a dose-dependent manner. These results were confirmed by immunofluorescence analysis (Figure 1D).

Results

Impact of Neo1 on human PMN apoptosis and macrophage efferocytosis. It is now evident that the failed clearance of dying cells alters immune tolerance and promotes nonresolving inflammation (2, 17). In the early phase of inflammation, apoptosis of neutrophils induces neutrophil functional shutdown, which is a key feature of the initiation of inflammation resolution mechanisms (18, 19). We therefore sought to investigate whether Neo1 plays a role in the apoptosis of neutrophils. Human PMNs were stimulated with vehicle or LPS (100 ng/ml) and/or anti–Neo1 antibody (Ab) and then allowed to undergo apoptosis for 20 hours. Functional inhibition of Neo1 induced the apoptosis of neutrophils, suggesting that anti-Neo1 treatment shortened the neutrophil lifespan (Figure 1A). In apoptotic PMNs, blockade of Neo1 induced the expression of the decoy receptor IL-1R2, which is known for its strong impact on limiting the proinflammatory effects of IL-1β (Figure 1A) (20).

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Our studies indicate a critical role for Neo1 in controlling processes involved in the inflammation resolution and tissue regeneration phases, and they may represent an advance in our understanding of the pathways that can restrain or promote the resolution of inflammation. In turn, our data may help identify new potential targets in diseases of major global health significance.
apoptosis, as an important control point in resolution processes, was enhanced in Neo1 –/– mice compared with the littermate control animals (Figure 2D). This was accompanied by a significant reduction in the classical Ly6Chi monocytes and an increase in the alternatively activated Ly6Clo monocytes and peritoneal MΦ Neo1–/– mice versus controls (Figure 2E). In this context, the phagocytosis of apoptotic neutrophils was strongly enhanced in Neo1–/– mice, suggesting that Neo1 affects a delayed-resolution phenotype in acute peritonitis (Figure 2F). To underline the influence of Neo1 on the resolution phase, we then measured the levels of IL-6, KC, MIP2, and MCP-1 within the peritonitis lavages collected 12 hours after zymosan A (ZyA) injection and found significantly decreased levels in Neo1-deficient exudates compared with WT controls (Supplemental Figure 2A). To further substantiate the temporal pattern of Neo1-mediated influence on efferocytosis and tissue homeostasis, we collected peritoneal MΦ either from WT or Neo1–/– mice, and the phagocytosis of fluorescent ZyA particles was determined at 2, 4, and 6 hours after injection. Our data demonstrate that depletion of Neo1 markedly increases the phagocytosis rate at the indicated time points (Supplemental Figure 2B). Together, these data point to a role for Neo1 in the initiation and resolution of inflammatory processes, particularly in the removal of apoptotic cells.

Mice deficient in Neo1 display a reduction in PMN recruitment, enhancement of neutrophil apoptosis, and augmentation of efferocytosis. Based on the results described above, we hypothesized that Neo1 is a major player in the active resolution of acute inflammatory responses. As mentioned before, key characteristics of resolution are the cessation of neutrophil migration, the enhancement of uptake and clearance of apoptotic cells and microorganisms in inflamed tissues, and the biosynthesis of proresolving mediators (5). Using mice deficient in Neo1 (Neo1−/−), we modeled a self-limited resolving murine peritonitis and examined the cellular events in both the early phase and the resolution phase. In a time series (4 hours, 12 hours, 24 hours, and 48 hours), wild-type (WT) littermates displayed maximal PMN infiltration at 4 hours in the peritoneal exudates (Figure 2A), followed by a reduction, providing a resolution interval (Ri) of 27 hours. In Neo1 –/– mice, we found a strong reduction in leukocyte recruitment, with a shift of maximal PMNs to 12 hours and a resolution interval of 9 hours (Figure 2B). Additionally, TNF-α and IL-1β, 2 well-known proinflammatory cytokines that mediate the inflammatory response and contribute to apoptotic cell death, were significantly decreased (Figure 2C). Neutrophil apoptosis, as an important control point in resolution processes, was enhanced in Neo1−/− mice compared with the littermate control animals (Figure 2D). This was accompanied by a significant reduction in the classical Ly6C hi monocytes and an increase in the alternatively activated Ly6C lo monocytes and peritoneal MΦ Neo1−/− mice versus controls (Figure 2E). In this context, the phagocytosis of apoptotic neutrophils was strongly enhanced in Neo1−/− mice, suggesting that Neo1 affects a delayed-resolution phenotype in acute peritonitis (Figure 2F). To underline the influence of Neo1 on the resolution phase, we then measured the levels of IL-6, KC, MIP2, and MCP-1 within the peritonitis lavages collected 12 hours after zymosan A (ZyA) injection and found significantly decreased levels in Neo1-deficient exudates compared with WT controls (Supplemental Figure 2A).

Neuro1 expression is confined to peritoneal Ly6C hi monocytes. After demonstrating that Neo1 controls apoptosis and phagocytosis programs in vitro and in vivo, we next aimed to investigate more precisely the role of Neo1 in the regulatory mechanisms underlying these processes. It is evident that monocytes derived from precursors in the bone marrow circulate first in the blood, and from there into tissues to mature to macrophages (24). Knowing that Ly6C is mainly expressed on the migrating inflammatory mono-
cyte population with less expression on the alternatively activated monocytes (25), we determined the Ly6C expression in bone marrow monocytes (BMMs) and the peritoneal monocytes in WT and Neo1–/– mice 12 hours after ZyA injection. Interestingly, we found that proinflammatory Ly6C<sup>hi</sup> monocytes had increased Ly6C expression after leaving the bone marrow and migrating into the peritoneal cavity (Figure 3, A and B). This increase in Ly6C expression was significantly reduced in peritoneal Neo1<sup>–/–</sup> monocytes compared with littermate controls. We then examined the Neo1 expression in BMMs and peritoneal monocytes and found Neo1 expression to be specifically restricted to the peritoneal inflammatory Ly6C<sup>hi</sup> monocytes (Figure 3C).

To more precisely determine the migration patterns of Ly6C<sup>hi</sup> monocytes, we generated chimeric animals through bone marrow transplantation between Neo1<sup>+/+</sup> and Neo1<sup>–/–</sup> mice and vice versa, with WT to Neo1<sup>+/+</sup> and Neo1<sup>–/–</sup> to Neo1<sup>–/–</sup> transplanted animals as controls for nonspecific radiation effects. We then exposed the chimeric animals to ZyA peritonitis and analyzed the cellular events in defined time intervals (4 hours and 12 hours). Bone marrow chimeric animals with hematopoietic Neo1 repression demonstrated a strong reduction in the classical Ly6C<sup>hi</sup>, an increase in the nonclassical Ly6C<sup>lo</sup> monocytes, and finally an increase of the MΦ phagocytosis of apoptotic PMNs in both time points (Supplementary Figure 3C). Ly6C<sup>hi</sup>, known to be mainly expressed on migrating proinflammatory monocytes, was significantly reduced in peritoneal Neo1<sup>–/–</sup> monocytes compared with littermate controls (Figure 3B). This effect was also reflected in the bone marrow chimeric animals with hematopoietic Neo repression (Supplementary Figure 3C). We were able to show that Ly6C MFI is strongly decreased in Ly6C<sup>hi</sup> cells in bone marrow chimeric animals with hematopoietic Neo repression, suggesting that Neo1 impacts the proinflammatory Ly6C<sup>hi</sup> monocytes. These findings suggest that upon activation and migration to the site of inflammation, Ly6C<sup>hi</sup> monocytes induce Neo1 expression, enabling them to contribute to the immune response.

**Figure 4. Neo1-dependent monocyte intracellular signaling in the PI3K/AKT pathway.** The PI3K/AKT signaling pathway was assessed in peritoneal monocytes by using a protein microarray. Samples were pooled from 4 mice in each group for each experiment.
expression and/or phosphorylation of enzymes such as AKT1, AKT2, MAPK1, MAPK3, mTOR, PIK3R1, and PIK3R3, which are required for AKT activity, were increased in Neo1−/− monocytes. The TGF-β pathway plays divergent roles in the innate immune system (28). Our microarray data on Neo1−/− monocytes revealed TGF-β signaling to be activated specifically in the context of cell apoptosis, whereas in WT monocytes TGF-β signaling is induced and associated with proinflammatory monocyte migration, fibrosis, chronic inflammation, and cell survival (Figure 5A, Supplemental Figure 4, and Supplemental Table 1). Collectively, these findings provide evidence that deficiency of Neo1 contributes to proresolving and proregenerative actions in monocytes and ultimately in MΦ, and this action is associated with the PI3K/AKT/mTOR and TGF-β signaling pathways.

Impact of Neo1 on lipid mediator biosynthesis. The SPMs — namely, lipoxins, resolvins, protectins, and maresins — have been identified as important determinants of inflammation resolution (5). To examine whether Neo1 impacts the generation of SPMs during inflammation resolution, we carried out liquid chromatography–tandem mass spectrometry–based (LC-MS/MS-based) profiling. In inflammatory peritoneal exudates obtained from Neo1−/− mice and their littermate controls, we identified SPMs as well as their precursors and pathway markers. Specifically, we identified arachidonic acid–derived LXA₄ (Figure 6, A and D), docosahexanoic acid–derived (DHA-derived) PDX (also referred to as 10S,17S-diH-DHA), and Mar1 to be increased in Neo1−/− (Figure 6, B and D). It is well appreciated that prostanoids such as PGD₂, PGE₂, and PGI₂ elicit immunomodulatory and antiinflammatory effects (23, 29–31). In particular, PGD₂ and PGE₂, which are known to induce the inflammatory response, subsequently stimulate antiinflammatory effects by activating the 15-LOX in neutrophils to ultimately promote lipid mediator class-switching during the resolution of acute inflammation. Our data demonstrate the enhanced production of PGD₂ and PGE₂ in the initial phase, suggesting that the mediator class switch is implemented in the resolution phase (Figure 6A and Supplemental Table 2) (31). We also found enhanced levels of the arachidonic acid–derived products 5-hydroxyeicosatetraenoic acid (5-HETE) and 15-HETE, and the eicosapentaenoic acid–derived (EPA-derived) 15-hydroxyeicosapentaenoic acid (15-HEPE) and 18-HEPE in Neo1−/− (Figure 6, A and C). Furthermore, metabolites 14,15-diHETE and 19,20-DiHDPA produced by cytochrome P450 epoxygenases, and the actions of soluble epoxidehydrolase (sEH), thus belonging to a different class of antiinflammatory and proresolving lipids, were also significantly increased in Neo1-deficient mice (Figure 6, A and B). Knowing that the enzymes 5-LOX and 12/15-LOX contribute to the generation of proresolving mediators...
Figure 6. Endogenous deficiency of Neo1 activates proresolving lipid mediator biosynthesis. Neo1−/− and WT mice were challenged with ZyA peritonitis. Peritoneal lavages were collected at 4 hours and analyzed using LC-MS/MS. (A) Lipid mediators and precursors derived from arachidonic acid (AA), (B) docosahexaenoic acid (DHA), and (C) eicosapentaenoic acid (EPA). (D) Corresponding MS/MS spectra and the multiple reaction monitoring chromatograms (MRM) for the identified lipid mediators. Results represent 3 independent experiments and are expressed as median ± 95% CI (n = 8–12 mice/group). Statistical analysis was done by unpaired Student’s t test, *P < 0.05, **P < 0.01, ***P < 0.001. All results are reported as ng/10^7 peritoneal cells.
and finally to increased resolution effects, we incubated peritoneal MΦ from WT or 12/15-LOX–deficient mice with Neo1 Ab and found a reduced efferocytosis rate of fluorescence-labeled ZyA particles after stimulation with Neo1 Ab (Supplemental Figure 6D). In a second set of experiments, we incubated peritoneal MΦ from WT or Neo1–/– mice with 5-LOX and 12/15-LOX inhibitors baicalein or cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) and found a reduced ZyA efferocytosis rate in Neo1–/– cells (Supplemental Figure 6E). To substantiate these data we incubated human MΦ with Neo1 Ab and baicalein or CDC. The impact of Neo1 inhibition on MΦ phagocytosis was significantly reduced when costimulated with 5-LOX and 12/15-LOX inhibitors, suggesting that the Neo1 effects on resolution are 5-LOX and 12/15-LOX dependent (Supplemental Figure 6F). This result is consistent with the increased biosynthesis of the 5-LOX- and 12/15-LOX–dependent proresolving mediators, such as LXA₄, Mar1, and PDX in the Neo1–/– mice.

Taken together, these results strongly highlighted that targeted deletion of Neo1 modulates the lipid mediator profile in murine exudates toward an antiinflammatory and proresolving state.

**Genetic deletion of Neo1 contributed to tissue regeneration mechanisms in vivo.** After demonstrating that genetic deletion of Neo1 promoted key resolution features, we turned our attention to the influence of Neo1 on tissue repair/regeneration and found increased levels of IL-10 and TGF-β peritonitis exudate, 2 parameters contributing to peritoneal tissue repair and regeneration in Neo1–/– mice (Figure 7A) (2, 18, 32). This is in line with our cellular data that shows Neo1–/– monocytes activate the PI3K/AKT pathway for the induction of cell polarization toward the M2 phenotype. To substantiate this proregenerative impact, we performed a staining for proliferating cell–nuclear antigen (PCNA) within the peritoneum, which displayed an index increase of approximately 20% in Neo1–/– peritonitis compared with the WT group (Figure 7B). Since our data revealed Neo1 to be a negative regulator in the resolving and regenerative processes, we next elucidated the temporal regulation of Neo1 during the initiation and resolution phase (Figure 7C). Exudate Neo1 was markedly increased between 4 hours and 24 hours and subsequently decreased at the end of the resolution phase, suggesting that Neo1 impacts processes during initiation and resolution/regeneration of acute inflammation. To clarify whether Neo1 expression is cell type–specific and not only related to the cell trafficking events, we determined the Neo1 expression on cellular exudates and found Neo1 to be strongly increased between 4 hours and 12 hours, followed by a decrease at the end of the resolution phase (Supplemental Figure 5).

**Functional inhibition of Neo1 promotes the resolution and regeneration processes.** Having shown that endogenous deletion of Neo1 initiated the resolution of acute inflammation by inducing the apoptosis of PMNs, the cessation of PMN influx, efficient clearance of PMNs, and the biosynthesis of SPMs, we next sought to investigate whether anti-Neo1 has any therapeutic efficacy in acute inflammation (e.g., potentially resolving processes such as peritonitis). When a functional anti-Neo1 Ab was given as a prophylactic treatment (in parallel with ZyA injection) for murine peritonitis, WT mice displayed reduced PMN infiltration and shortened resolution interval, from 26 hours to 7 hours (Figure 8, A and B). Furthermore, administration of an anti-Neo1 Ab decreased classical Ly6C⁺ monocytes and increased nonclassical Ly6C⁻ monocytes and MΦ, which led to strong enhancement of MΦ clearance of apoptotic PMNs (Figure 8C). Also, the inflammation-initiated cytokines, such as TNF-α, IL-1β, IL-6, and keratinocyte chemoattractant (KC, IL-8 in humans), were reduced (Figure 8D). In a second set of experiments, we investigated the therapeutic administration of an anti-Neo1 Ab. The agent was given at the peak of inflammation as monitored by maximal neutrophil recruitment, and peritoneal lavages were collected at 12, 24, and 48 hours. As expected, we found activation of cardinal signs of resolution with a shortening of the resolution interval from 23 hours to only 16 hours (Figure 9, A and B), suggesting a stronger treatment effect.
clearance of apoptotic PMNs (Supplemental Figure 7). Studies have revealed Neo1 to be a specific receptor for 2 ligands, namely Netrin-1 and RGM-A (12, 13). We incubated peritoneal MΦ from WT and Neo1–/– mice with RGM-A or Netrin-1 to determine a possible influence on MΦ efferocytosis of fluorescent ZyA particles. Collected data revealed that RGM-A did not increase MΦ clearance in the Neo1–/– cells. When MΦ were stimulated with Netrin-1, efferocytosis was not significantly affected, suggesting that the actions of Neo1 are RGM-A dependent (Supplemental Figure 8). These results indicate a critical role for the functional inhibition of Neo1 in controlling inflammation processes in the resolution and regeneration phases, and might demonstrate a possible therapeutic approach.

Plasma Neo1 is increased in critically ill pediatric patients with IAH or ACS and it is associated with clinical outcome. To translate our preclinical findings to humans, we investigated the association between Neo1 blood plasma levels and IAH grade, ACS, severity of illness, pediatric ICU (PICU) length of stay, and survival in a cohort of 59 critically ill pediatric patients partly suffering from abdominal compartment syndrome (ACS). In brief, we prospectively enrolled medical and surgical patients ranging in age from newborn to 17 years old with cardiac or oncological diseases, or after surgical interventions. In all enrolled subjects, intensive care monitoring was urgently indicated (i.e., admission when anti-Neo1 Ab was given at the onset of inflammation. To further validate the proresolving attributes of the functional inhibition of Neo1, we examined the exudate IL-10 and TGF-β levels, which contribute to resolution and regenerative programs (18) (Figure 9C). Here, we found increased levels of both cytokines following anti-Neo1 Ab administration. To corroborate these results, we performed immunohistochemical characterization of PCNA, and found improved responses in tissue repair (Figure 9D). Finally, to clarify whether the loss of Neo1 with genetic deletion or with anti-Neo1 Ab treatment may blunt the initial inflammatory response, giving the false appearance of improved resolution, we first examined the biosynthesis of the lipid mediators specific to the resolution processes at a later time point (12 hours after ZyA injection), and found increased levels of specifically arachidonic acid–derived LXA4 and DHA-derived PDX in Neo1–/– (Supplemental Figure 6, A–C). Then we exposed WT mice to ZyA peritonitis and this time the anti-Neo1 Ab was given in the resolution phase 6 hours after ZyA injection (e.g., regression of the neutrophil infiltration). The samples were collected 12 hours after ZyA injection. As expected, the collected data demonstrate that supplementation of anti-Neo1 6 hours after ZyA injection promotes the resolution/regeneration mechanism by decreasing the classical Ly6Chi monocytes and increasing the nonclassical (M2) Ly6Clo monocytes and macrophages that indicate a strong enhancement of macrophage clearance of apoptotic PMNs (Supplemental Figure 7). Studies have revealed Neo1 to be a specific receptor for 2 ligands, namely Netrin-1 and RGM-A (12, 13). We incubated peritoneal MΦ from WT and Neo1–/– mice with RGM-A or Netrin-1 to determine a possible influence on MΦ efferocytosis of fluorescent ZyA particles. Collected data revealed that RGM-A did not increase MΦ clearance in the Neo1–/– cells. When MΦ were stimulated with Netrin-1, efferocytosis was not significantly affected, suggesting that the actions of Neo1 are RGM-A dependent (Supplemental Figure 8).

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These results indicate a critical role for the functional inhibition of Neo1 in controlling inflammation processes in the resolution and regeneration phases, and might demonstrate a possible therapeutic approach.
in affected children correlated with PRISM-III score, IAH grade, and clinically established laboratory parameters such as serum C-reactive protein (CRP), lactate, creatinine, and bilirubin (Figure 10C). We also compared conventional laboratory inflammatory parameters with the above-mentioned organ and outcome parameters. Only CRP showed correlations comparable to those of Neo1 (related to Rho and \( P \) values). In contrast to Neo1, to our knowledge CRP has never shown any prognostic value with regard to the development of IAH or ACS. Only D-lactate was identified in the context of 2 animal studies as a biomarker for the development of ACS in the past (34, 35). In the pediatric literature, there is no study that would have identified a biomarker for the development of an AKS. Procalcitonin (PCT; \( n = 6 \)) did show higher correlation coefficients (especially for creatinine, PICU length of stay, and PRISM-III scores); however, these results did not reach statistical significance, probably because PCT was only determined in 6 children on admission to our PICU (Supplemental Table 4). Due to the different severities of the patients’ illnesses, a selective division into 3 test groups was possible. The criteria were Pediatric Risk of Mortality III score (PRISM-III score), organ dysfunction, and intraabdominal pressure (IAP) level. Patient demographic and clinical data are shown in Figure 10, A and B, Table 1, Table 2, Supplemental Table 3, and Supplemental Table 4. The severity of illness was assessed by the PRISM-III score. The vital signs, other cardiorespiratory parameters, drug administration, IAP, and fluid balances were recorded continuously. With regard to ACS associated with substantial morbidity, such as renal failure and multorgan dysfunction syndrome (MODS), and mortality in critically ill patients (33), our data revealed 1.8-fold higher Neo1 plasma concentrations in children with ACS versus those without ACS, and 1.7-fold higher plasma levels in children with ACS versus control ICU patients (Figure 10B). When comparing the Neo1 plasma levels with the severity of illness, we found that significantly increased levels of Neo1 to PICU).
Since plasma levels of Neo1 were significantly higher in the CI+ACS group than in the CI-ACS group, the results of LDH analysis thus indicate that the main source of Neo1 most likely does not arise from cell lysis (Supplemental Table 6). Moreover, the PICU length of stay was also correlated with increased levels of Neo1 (Figure 10C). Since mortality is one of the most reliable endpoints of clinical management in the ICU, we investigated whether plasma Neo1 could be a mortality predictor in critically ill children. We found Neo1 to be 5.0-fold higher in nonsurvivors compared with survivors and 5.7-fold higher in nonsurvivors (Table 5). To clarify whether increased cell lysis might have caused an increase in membrane-bound Neo1 entering the plasma, we subsequently analyzed the serum concentrations of lactate dehydrogenase (LDH). All children enrolled into our study had a mild (non–critically ill [NCI] PICU controls) to marked increase (critically ill [CI] groups) in LDH serum concentration (normal reference values age-dependent, approximately <344 U/l). The difference in circulating LDH between controls and test groups was significant (P = 0.03). On the other hand, there was no significant difference between CI-ACS and CI+ACS (P = 0.69). Since plasma levels of Neo1 were significantly higher in the CI+ACS group than in the CI-ACS group, the results of LDH analysis thus indicate that the main source of Neo1 most likely does not arise from cell lysis (Supplemental Table 6). Moreover, the PICU length of stay was also correlated with increased levels of Neo1 (Figure 10C). Since mortality is one of the most reliable endpoints of clinical management in the ICU, we investigated whether plasma Neo1 could be a mortality predictor in critically ill children. We found Neo1 to be 5.0-fold higher in nonsurvivors compared with survivors and 5.7-fold higher in nonsurvivors.
Table 1. Correlation between Neo1 and ACS in PICU patients with ACS

<table>
<thead>
<tr>
<th></th>
<th>Number of patients</th>
<th>Male sex, n (%)</th>
<th>Prism-III score, median (min–max)</th>
<th>Age in months, median (min–max)</th>
<th>Days of PICU stay, median (min–max)</th>
<th>Primary reason for PICU admission</th>
</tr>
</thead>
<tbody>
<tr>
<td>PICU control patients</td>
<td>25</td>
<td>9 (36)</td>
<td>5.0 (0–7)</td>
<td>15.0 (0–199)</td>
<td>1.0 (0–20)</td>
<td>7</td>
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<tr>
<td>Critically ill without ACS</td>
<td>20</td>
<td>10 (50)</td>
<td>16.0 (8–31)</td>
<td>31.5 (0–199)</td>
<td>4.0 (1–18)</td>
<td>6</td>
</tr>
<tr>
<td>Critically ill with ACS</td>
<td>14</td>
<td>7 (50)</td>
<td>18.0 (2–35)</td>
<td>28.0 (0–190)</td>
<td>19.5 (1–332)</td>
<td>8</td>
</tr>
</tbody>
</table>

Overview of PICU patient characteristics for survivors and nonsurvivors.

Table 2. Correlation between Neo1 and survival in PICU patients with ACS

<table>
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<th></th>
<th>Critically ill survivors</th>
<th>Critically ill nonsurvivors</th>
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<tr>
<td>Number of patients</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>13 (36)</td>
<td>4 (60)</td>
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<tr>
<td>Prism-III score, median (min–max)</td>
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<td>27.5 (15–35)</td>
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<tr>
<td>Age in months, median (min–max)</td>
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<td>35.5 (0–190)</td>
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<tr>
<td>Days of PICU stay, median (min–max)</td>
<td>7.5 (1–332)</td>
<td>8.5 (1–30)</td>
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Primary reason for admission to PICU

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<th>Cardiology</th>
<th>Sepsis</th>
<th>Postneurosurgery</th>
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<td>Postneurosurgery</td>
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</tr>
</tbody>
</table>

Overview of PICU patient characteristics for survivors and nonsurvivors.
enhanced efferocytosis of apoptotic PMNs. When investigating the role of Neo1 more precisely, we found that in bone marrow and peritoneal monocytes there was a strong reduction of the migratory classical Ly6C hi cells in the peritoneal cavity of Neo1−/− mice. Neo1 expression was restricted to the peritoneal inflammatory Ly6C hi cells, suggesting that the lack of Neo1 induced a phenotypic shift toward the antiinflammatory and proresolving M2 type. To show in more detail that Neo1 has a direct influence on classical and nonclassical monocytes and efferocytosis, we carried out additional experiments. We exposed bone marrow chimeric mice to ZyA peritonitis and analyzed the leukocytes and clearance at 4 hours and 12 hours after ZyA injection. As expected, bone marrow chimeric animals with hematopoietic Neo1 repression demonstrated a reduction in classical Ly6C hi cells, an increase in nonclassical Ly6C lo monocytes, and finally a strong enhancement of the efferocytic capacity at both time points. As demonstrated before, Ly6C, which is known to be mainly expressed on the migrating proinflammatory monocytes, was markedly decreased in peritoneal Neo1−/− monocytes compared with littermate controls. This influence was also noted in the bone marrow chimeric animals with hematopoietic Neo repression. Ly6C MFI was markedly decreased in Ly6C hi cells in bone marrow chimeric animals with hematopoietic Neo repression, implying that Neo1 may impact the proinflammatory Ly6C hi monocytes.

At the signaling level, we were able to demonstrate the activation of the PI3K/AKT pathway and the suppression of the TGF-β pathway in Neo1−/− monocytes. Specifically, the activation of the PI3K/AKT pathway has been reported to be a crucial step toward the proresolving M2 phenotype (26). Since the resolution of inflammation is induced to a large extent by SPMs, the generation of several of these endogenous lipid mediators and their pathway markers (i.e., LXA 4, Mar1, and FLIP) have been reported to be a crucial step toward the resolution, giving the false appearance of improved resolution. In conclusion, our study reveals a key role for Neo1 in controlling inflammation resolution and regeneration programs. Our findings demonstrate that deficiency of Neo1 directly promotes antiinflammatory, proresolving effects (i.e., shortening of resolution phase, activating SPM generation, reducing PMN influx, activating PMN apoptosis, and increasing MΦ phagocytosis of apoptotic PMNs). Moreover, our data revealed that Neo1 correlates with ACS, PRISM-III score, ICU length of stay, and survival in critically ill children, and might therefore evolve as a new clinical marker and therapeutic target in inflammatory conditions.

Methods

Animals. This project was approved by the institutional review board and the Regierungspräsidium Tübingen. WT (C57BL/6N), Neo1−/− (C57BL/6N-Neo1Gt(KST265)Byg), and littermate control mice (C57BL/6N) were bred and genotyped as previously described (8). At 8 to 10 weeks old, mice of either sex were assigned to the respective study time points and/or experimental interventions at random.

Murine peritonitis. All animal protocols were performed in accordance with the regulations of the Regierungspräsidium Tübingen and the local ethics committee. All trials took into account Directive 2010/63/EU adopted by the European Parliament and Council. The mice were intraperitoneally injected with 1 ml zymosan A (ZyA; 1 mg/ml; Sigma-Aldrich, catalog Z4250) and subsequently intravenously with either IgG control (Santa Cruz Biotechnology, catalog sc-2028) or 2 μg Neo1 blocking antibody (R&D Systems, catalog AF-1079) in a total volume of 150 μl. Peritoneal fluids and tissues were obtained at 4, 12, 24, and 48 hours and prepared as previously described (37). The collected exudates were washed, suspended in PBS (MilliporeSigma), and counted.

Differential leukocyte counts, FACS analysis, and cytokines. Exudate cells from the murine peritonitis models were prepared to determine their cellular composition. The cells were blocked with mouse anti-CD16/CD32 (Biolegend, catalog 101320) antibodies for 10 minutes at room temperature and then stained with anti-mouse APC-Ly6G (BioLegend, catalog 127614), e45-0-F4/80 (eBioscience, catalog 48-4801-82), and FITC-Ly6C (BioLegend, catalog 128006) antibodies for 30 minutes at 4°C. To analyze the MΦ phagocytosis of apoptotic PMNs in vivo, the cells were permeabilized using a fixation and permeabilization kit (BD Biosciences, catalog 554714) prior to staining with PerCP-Cy5.5–conjugated anti-Ly6G (BioLegend, catalog 127616) for 30 minutes at 4°C. The cells were acquired on a FACSCanto II (BD Biosciences) and analyzed with Flowjo (TreeStar). Cytokines were measured in the murine peritoneal exudates using standard ELISA (R&D Systems).

Lipid mediator lipidomics. LC-MS/MS analysis was carried out as previously described with some modifications (40, 41). Peritoneal lavage samples were thawed, and internal standards were added and subsequently extracted twice using methanol. The combined organ-
ic extracts were cleaned up using solid-phase extraction according to published protocols (41). LC-MS/MS analysis was carried out using a 6500 QTrap LC-MS/MS system as previously described (40). For a detailed description of the analytical procedure please refer to the Supplemental Material.

**Antibody array for protein expression.** Peritoneal monocytes/macrophages from WT and Neo−/− mice were used following 12 hours of Zymosan-induced peritonitis. Protein and phosphorylation (TGF-β Phospho Antibody Array, Full Moon BioSystems, catalog PTG176) profiling of peritoneal monocytes (pooled lavages from 4 mice/condition) was carried out according to the manufacturer’s instructions. The images were acquired by the manufacturer. For each antibody, the average signal intensity of 6 replicates was normalized to the median signal of all antibodies on the array. The presented fold change represents the ratio of the normalized signal from Neo1−/− mice compared with WT littermate controls. GAPDH and beta-actin were used as housekeeping proteins. Data analysis was performed with IPA software (Qiagen). Pathways were substantiated and updated with recent literature, the KEGG database (HSA 04350, HAS 04151; https://www.genome.jp/kegg/), and the Reactome database (R-HSA-198203, R-HSA-2173789; https://reactome.org/). Data were deposited in the NCBI’s Gene Expression Omnibus database (GEO GSE117373) (42).

**Human PMN apoptosis and MΦ efferocytosis.** PMNs and human peripheral blood monocytes were isolated from healthy volunteers or human leukapheresis collars from the Blood Bank of Eberhard Karls University of Tübingen by gradient centrifugation using Histopaque-1077 (MilliporeSigma). Monocytes were cultured in RPMI 1640 medium (MilliporeSigma) with 10 ng/ml human recombinant GM-CSF (Milteny Biotec, catalog 130-093-866) at 37°C in 5% CO2 for 7 days. Human PMNs were labeled with carboxyfluorescein diacetate (10 μM, 30 minutes at 37°C; Molecular Probes) and allowed to undergo apoptosis in serum-free RPMI 1640 medium (Gibco) for 16 to 18 hours. GM-CSF–differentiated MΦ (0.1×10^6 cells/well) were then incubated with human Neol Ab (R&D Systems, catalog AF1079) or IgG control (Santa Cruz Biotechnology, catalog sc-2028) for 15 minutes. Apoptotic PMNs were added in a 1:3 ratio (MΦ/PMNs) and incubated at 37°C for 60 minutes to allow phagocytosis. In a separate experiment, GM-CSF–differentiated MΦ were incubated with Neol Ab (R&D Systems, catalog AF1079) or IgG control (Santa Cruz Biotechnology, catalog sc-2028) for 15 minutes at 37°C and then incubated with fluorescently labeled (BacLight; Thermofisher Scientific, catalog B35000) *E. coli* at a 1:50 ratio for 60 minutes. Efferocytosis was determined using a fluorescent plate reader (Tecan).

To evaluate PMN apoptosis, PMNs were incubated in RPMI 1640 (Gibco) plus 10% FCS in the presence or absence of LPS (MilliporeSigma, catalog LA4391) and/or Neol-antibody (R&D Systems, catalog AF1079) for 20 hours at 37°C in 5% CO2. Apoptosis was measured by FACS analysis using an Annexin V PE apoptosis detection kit with 7-AAD (BioLegend, catalog 640934) according to the manufacturer’s instructions, and transcriptional analysis was performed.

**Transcriptional analysis of human MΦ and PMNs.** Human GM-CSF–differentiated MΦ were incubated in RPMI 1640 (Gibco) in the presence or absence of IL-1β (Promokine, catalog C-61120) and/or anti-Neol antibody (R&D Systems, catalog AF1079) for 4 hours prior to transcriptional analysis. Human IBS expression as a housekeeping gene was evaluated with the sense primer 5′-GTA-ACCCCGTGAACCCCGCATTT-3′ and antisense primer 5′-CCATC-

CAATCGGTAGTAGGC-3′. The following primers were used: Cx3cl1: 5′-CGTGTTGCAATGAATCA-3′, 5′-CTCCAAGAT-GATTGGCGGT-3′; Ilr2: 5′-GTGAGCAAAAGC-3′, 5′-TACCAACAGTACAAGCGCA-3′; Cx3cl1: 5′-GAGGCGTT-TAAGTGGGACA-3′, 5′-ATGGTGAAGGCCCCACT-3′; Tim4: 5′-ACAGGACATGTGATGAAATCC-3′, 5′-ACGCTTGT-GTTTCTCGC-3′; Gpr32: 5′-GGGCTCGAATACTCACA-3′, 5′-GGAGGCAGTATTCTGGCAA-3′; Alx/Fpr: 5′-TGTTCTGCG-GATCCCTCATTT-3′, 5′-CTCCCATGCGCATGAGACA-3′.

**Cytology, immunofluorescence, and immunohistochemistry staining.** GM-CSF–differentiated human MΦ were stimulated for 4 hours with IL-1β (Promokine, catalog C-61120) prior to labeling with rabbit anti-Neol (Santa Cruz Biotechnology, catalog sc-15337) and rhodamine phalloidin (Invitrogen, catalog R415). An IgG isotype control antibody (Santa Cruz Biotechnology, catalog sc-2027) was used as a negative control. Alexa Fluor 488–conjugated goat anti-rabbit (Life Technologies, catalog A27034) was used as the secondary antibody. DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, catalog P36931) was employed for nuclear counterstaining. For immunofluorescence analysis of human MΦ efferocytosis of fluorescently labeled PMNs, MΦs were stained with mouse-anti-CDC14 (Santa Cruz Biotechnology, catalog sc-58951) and A594 goat-anti-mouse secondary antibody (Thermo Fisher Scientific, catalog A-11005). DAPI (Invitrogen, catalog P36931) was employed for nuclear counterstaining. Immunofluorescence images were acquired using a confocal microscope (LSM 510 Meta fluorescence microscope, Carl Zeiss) and ZEN software (Carl Zeiss). To perform immunohistochemical staining for PCNA, paraffin-embedded peritoneal tissues were stained with an anti-PCNA antibody (Santa Cruz Biotechnology, catalog sc-56) using a Vectastain ABC Kit (Vector Labs, catalog PK-4004) and DAB peroxidase substrate (Sigma-Aldrich, catalog E109) according to the manufacturers’ instructions. As the secondary antibody, a biotin-conjugated horse-anti-mouse antibody (Vector Labs, catalog BA-2000) was used. The sections were then counterstained with hematoxylin. Light microscopy images were acquired with a DM IRM microscope (Leica) equipped with an AxioCam MRC (Carl Zeiss) using AxioVision software (Carl Zeiss).

**Pediatric ICU patient samples with and without ACS.** A total of 59 plasma samples were taken from patients from the PICU of Hannover Medical School (MIHH) within 24 hours after admission. The 2013 WSACS definitions (43) (with respect to IAP and ACS; www.wsacs.org) were used to define the ACS. Severity of illness in the ICU children was measured using PRISM-III scoring (44).

Vital and cardiorespiratory parameters (including ventilation parameters), drug administration, intraabdominal pressure (measured via gastric Spiegelberg monitoring system) (45), and fluid balances were recorded continuously via the digital patient data management system (mlife, mediside). A Neo1 ELISA (Cusabio, catalog CSB-EL015712HU) was performed according to manufacturer’s instructions. For a detailed description of the criteria for patient selection and monitoring please refer to the Supplemental Material.

**Statistics.** Statistical analysis of murine and in vitro data was performed using ANOVA with Bonferroni’s multiple comparisons test. An unpaired 2-tailed Student’s t test was used to compare 2 independent groups. Experimental data are reported as mean ± SEM. Statistical analyses of data from PICU patients were performed using the non-parametric Kruskal-Wallis test followed by Dunn’s multiple compar-

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sons test. Data are reported as median ± 95% CI. Correlation of clinical data was tested using Spearman’s rank correlation test. For all tests, a *P* value less than 0.05 was considered statistically significant. Analyses were performed using GraphPad Prism5 (GraphPad Software) and JMP 13 (SAS).

**Study approval.** Critically ill children newborn to age 17 years were enrolled between January and August 2015 after informed written consent was obtained from the parents or guardians of each child. The study was approved by the local ethics committee (Ethikkommission der MHH, Hannover, Germany) and Hannover Medical School (MHH 6677) and internationally registered (WHO-ICTRP DRKS00006556). Animal experiments were approved by the institutional review board and the Regierungspräsidium Tübingen (Tübingen, Germany).

**Author contributions**

MS, AK, CG, and UK performed the experiments, and collected and analyzed the data. TK and GH performed the clinical experiments in patients. HJS and MG performed the targeted lipidomic and lipid mediator analysis studies. All authors contributed to manuscript preparation and figure preparation. VM carried out overall experimental design, conceived of the overall research, and wrote the manuscript.

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