Interruption of Macrophage-Derived IL-27(p28) Production by IL-10 during Sepsis Requires STAT3 but Not SOCS3

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Severe sepsis and septic shock are leading causes of morbidity and mortality worldwide. Infection-associated inflammation promotes the development and progression of adverse outcomes in sepsis. The effects of heterodimeric IL-27 (p28/EBI3) have been implicated in the natural course of sepsis, whereas the molecular mechanisms underlying the regulation of gene expression and release of IL-27 in sepsis are poorly understood. We studied the events regulating the p28 subunit of IL-27 in endotoxic shock and polymicrobial sepsis following cecal ligation and puncture. Neutralizing Abs to IL-27(p28) improved survival rates, restricted cytokine release, and reduced bacterial burden in C57BL/6 mice during sepsis. Genetic disruption of IL-27 signaling enhanced the respiratory burst of macrophages. Experiments using splenectomized mice or treatment with clodronate liposomes suggested that macrophages in the spleen may be a significant source of IL-27(p28) during sepsis. In cultures of TLR4-activated macrophages, the frequency of F4/80+CD11b+IL-27(p28)+ cells was reduced by the addition of IL-10. IL-10 antagonized both MyD88-dependent and TRIF-dependent release of IL-27(p28). Genetic deletion of STAT3 in Tie2-Cre/STAT3flox macrophages completely interrupted the inhibition of IL-27(p28) by IL-10 after TLR4 activation. In contrast, IL-10 remained fully active to suppress IL-27(p28) with deletion of SOCS3 in Tie2-Cre/SOCS3flox macrophages. Blockade of IL-10R by Ab or genetic deficiency of IL-10 resulted in 3–5-fold higher concentrations of IL-27(p28) in endotoxic shock and polymicrobial sepsis. Our studies identify IL-10 as a critical suppressing factor for IL-27(p28) production during infection-associated inflammation. These findings may be helpful for a beneficial manipulation of adverse IL-27(p28) release during sepsis.
IL-10 mediates its immunosuppressive effects and macrophage M2 polarization via the IL-10Rα/IL-10Rβ receptor complex (20, 21). Upon binding of IL-10, IL-10Rα associates with Jak1, IL-10Rβ, and Tyk2. Phosphorylation of Jak1 and Tyk2 facilitates direct interaction with STAT3 and subsequent IL-10–induced gene activation (20, 22).

Although the role of IL-27 in the induction of IL-10 by lymphocytes has been well characterized, little is known about IL-10 reciprocally controlling the release of IL-27(p28) from APCs, which, according to in vitro studies, appear to be a predominant cellular source of IL-27. The aim of our current study was to assess the regulatory networks associated with IL-27(p28) production during experimental sepsis. We uncovered that IL-27(p28) is to assess the regulatory networks associated with IL-27(p28) dominant cellular source of IL-27. The activation of Stat3 via interaction with STAT3 and subsequent IL-10–induced gene expression (20, 22).

Materials and Methods

Animals

All procedures with animals were performed in accordance with the National Institutes of Health guidelines, the University Committee on Use and Care of Animals of the University of Michigan, the animal protection act of Germany, the State Investigation Office of Rhineland-Palatinate, and directive 2010/63/UE of the European Parliament and of the Council of the European Union. TRIF−/− mice were bred at the University of Michigan. STAT3-deficient mice (Tie2-Cre/STAT3fl/fl) and SOCS3-deficient mice (Tie2-Cre/SOCS3fl/fl) were generated by breeding Tie2-Cre mice with floxed STAT3 mice or floxed SOCS3 mice at St. Jude Children’s Research Hospital (23). STAT3-deficient mice were phenotyped by Western blotting and demonstrated missing phospho-STAT3 in IL-6–activated macrophages. Male C57BL/6J, IL-10−/− (B6.129P2-Il2rgtm1Jwg/J), MyD88−/− (B6.129P2/SiL-J-Myd88tm1Egr/J), and TLR4−/− (B6.12B6ScN-Tlr4tm1d4/J) JthJ mice were from The Jackson Laboratory (Bar Harbor, ME). IL-10−/− mice were from The Jackson Laboratory (Bar Harbor, ME).

In vivo experiments

Splenectomy was performed in male C57BL/6J mice. A left-sided infracostal incision (1–1.5 cm) was made, and the peritoneum was opened. The spleen was separated using a pair of forceps. The spleen was removed before wound closure with 6-0 silk suture, and the wound was covered with a sterile dressing.

For endotoxemia, randomized and age/sex-matched groups were used, and the body weight of each animal was measured directly before injection. Mice were injected with LPS (10 mg/kg body weight i.p., 0111:B4; Sigma-Aldrich) and sham surgery to exclude cell aggregates and debris from analysis. All Abs used were anti-mouse or mouse monoclonal IgG with matched fluorochrome-labeled isotype controls. PE-STAT3(pY705) (clone 4/P-STAT3) was from BD Pharmingen, and PE-IL-27(p28) (clone MM27-27B1), and anti-CD11b (clone M1/70) were from eBioscience (San Diego, CA). Quantification of oxidative burst activity

Peritoneal elicited cells were harvested 20 h after i.p. injection with 1 ml thioglycollate 2% (w/v). A total of 1 × 106 cells/sample was incubated with 2–4 × 105 opsonized E. coli (for 10 min at 37°C (Phagoburst Kit; ORPEGEN Pharma, Heidelberg, Germany). Dihydrodorhamidine (DHR)123 (10 min incubation) and Abs were added before analysis by flow cytometry, as described above.

Fluorescence microscopy

Cryosections of spleens were fixed with 4% formaldehyde solution (Thermo Scientific, Pittsburgh, PA). Macrophages were grown in Lab-Tek chamber slides (Thermo Scientific) and stimulated in the presence of monensin. Blocking (10% BSA, 10% normal mouse serum) was followed by overnight incubation with primary Abs. After incubation with secondary Abs, the slides were mounted with Poly-Prep Gold with DAPI (Invirotigen, Life Technologies, Carlsbad, CA). Abs used were rat anti-mouse F4/80 (clone B93), rat IgG2a isotype control, mouse isotype IgG2b control (all from eBiosoience), mouse anti-IgM-IL-27(p28) (clone MM27-27B1; a gift from Dr. P. Just, eBioscience), and rabbit anti-mouse AF594 IgG and donkey anti-rabbit AF594 IgG (both from Invitrogen). Microscopic images were acquired using an Olympus BX-51 microscope (40×/0.9, 60×/1.4 oil, 100×/1.4 oil) with an Olympus DP-70 camera and DP Controller Software.

Abs and reagents

LPS was from E. coli (0111:B4; Sigma-Aldrich).Neutralizing polyclonal goat anti-IgM-IL-27(p28) IgG, neutralizing polyclonal goat anti-mouse.
IL-10, total goat IgG, and recombinant mouse IL-10 were all from R&D Systems. Neutralizing anti-mouse IL-10R (clone 1B1.3a) was affinity purified using protein G-Sepharose (GE Healthcare, Munich, Germany), according to a standard protocol, and used together with purified rat IgG1 isotype Ab (BioLegend, San Diego, CA). Multilamellar liposomes were prepared from phosphatidylcholine and cholesterol (Sigma-Aldrich) and filled with clodronate (dichloromethylene bisphosphonate; Roche Diagnostics, Basel, Switzerland) or PBS (25).

Statistical analysis

GraphPad Prism Version 5.04 software was used for statistical analysis. In vitro experiments were performed independently two or three times, and in vivo data were generated with the numbers of mice indicated in the figure legends. All values are expressed as the mean, and error bars represent SEM. Data sets were analyzed by one-way ANOVA and two-tailed Student t test, and survival curves were analyzed by the log-rank (Mantel–Cox) test. We considered differences significant at p < 0.05.

Results

Blockade of IL-27(p28) improves the outcome of sepsis

To confirm the biological relevance of IL-27 during sepsis, we used neutralizing polyclonal Abs directed against the p28 subunit in models of endotoxic shock and polymicrobial sepsis following CLP. Treatment with anti-mouse IL-27(p28) Ab dramatically improved survival during endotoxic shock compared with normal IgG (25 versus 80%, Fig. 1A). Similarly, in polymicrobial sepsis following CLP, neutralization of IL-27(p28) with anti–IL-27(p28) Ab significantly improved mortality (Fig. 1B). Time-course studies revealed that maximum concentrations of IL-27(p28) in plasma were detectable by ELISA at 6 h after LPS injections in vivo (Fig. 1C). Blocking of IL-27(p28) during endotoxic shock attenuated the systemic “cytokine storm,” defined by reduced concentrations of mediators, such as IFN-γ, IL-17, IL-1, IL-1β, and MCP-1, in plasma (Fig. 1D). For IFN-γ and IL-1β, it was established that these mediators contribute to the detrimental pathophysiology of sepsis (3). Furthermore, blocking of IL-27(p28) during CLP improved bacterial clearance in blood and the peritoneal compartment (Fig. 1E, 1F). Counts of CFU in blood and peritoneal lavage fluids were significantly reduced at 20 h following CLP, whereas effects at 10 h were less consistent for peritoneal lavage fluids (Fig. 1E, 1F). When plasma mediators, such as IL-1β, IL-17, and IFN-γ, were quantified at 10 and 20 h following CLP, they were reduced by anti–IL-27(p28) Ab treatment at the 20-h time point compared with mediator concentrations in the control IgG group (Fig. 1G). Differences between the two groups were less prominent at the 10-h time point (Fig. 1G), which may be related to the fact that the release of IL-27(p28) requires several hours to occur (Fig. 1C). Furthermore, blockade of IL-27(p28) during endotoxic shock or polymicrobial sepsis following CLP did not significantly reduce the appearance of IL-10 at any time point studied (Fig. 1D, 1G).

Next, we sought to study the effects of IL-27 on production of ROS. F4/80+CD11b+ PEMs from IL-27RA−/− mice displayed an increased respiratory burst following activation by opsonized E. coli compared with C57BL/6 (WT) macrophages (Fig. 2). This enhanced production of ROS may explain a higher bactericidal

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**FIGURE 1.** Neutralization of IL-27(p28) is protective during polymicrobial sepsis and endotoxic shock. (A) Survival of WT mice during endotoxic shock (LPS 10 mg/kg body weight i.p.) following treatment with neutralizing anti–IL-27(p28) Ab (40 μg/mouse i.p., n = 10) or control IgG (40 μg/mouse i.p., n = 12). (B) Survival of WT mice after CLP (high-grade) and treatment with either control IgG or neutralizing anti–IL-27(p28) Ab (40 μg/mouse i.p., n = 10 for each group). (C) Time course for appearance of IL-27(p28) in plasma of WT mice, by ELISA, during endotoxic shock (n = 4-6 mice/time point). (D) Reduction of plasma mediators during endotoxic shock in WT mice, using control IgG or anti–IL-27(p28) Ab (n = 5/group), as determined by a 12-h, bead-based assay. (E) Quantification of CFU in peritoneal lavage fluids of WT mice at different time points after CLP with application of control IgG or blocking anti–IL-27(p28) Ab (40 μg/mouse i.p., n = 10 for both groups). (F) Determination of bacteremia (CFU) in blood from the same experiment described in (E). (G) Detection of plasma mediators after 10 and 20 h following CLP in WT mice treated with neutralizing anti–IL-27(p28) Ab or control IgG (n ≥ 7/group). Abs were injected 1 h before CLP or LPS in all experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
activity and improved pathogen clearance following blockade of IL-27 during sepsis. Our findings are in accordance with another report on the targeting of heterodimeric IL-27 with IL-27RA–Fc fusion protein or using EBI3−/− mice in sepsis (12).

**Macrophages in the spleen are a major source of IL-27(p28) during sepsis**

To define the cellular sources of IL-27(p28) during sepsis, various organs were collected from C57BL/6 (WT) mice following endotoxic shock and analyzed for IL-27(p28) mRNA expression (normalized to GAPDH). The greatest increases in IL-27(p28) mRNA were detected in spleens (73-fold) and lungs (9-fold), with the lowest levels in heart (1-fold) (Fig. 3A). The spleen is known to participate in the inflammatory reflex, and splenectomy protects against sepsis-associated lethality (26, 27). When splenectomized mice were subjected to endotoxemia, plasma concentrations of IL-27(p28) were greatly reduced (Fig. 3B). We were not able to visualize IL-27(p28) by immunohistofluorescence in spleen (data not shown), which may be explained by rapid in vivo secretion of IL-27(p28) in the absence of Golgi transport inhibitors. Next, we depleted F4/80+ macrophages in spleen (and liver) by i.p. injection of clodronate liposomes and used PBS-containing liposomes as negative controls (Fig. 3C). Following treatment with clodronate liposomes, mice were deficient in IL-27(p28) production (−86%) during endotox shock (Fig. 3D). Other mediators, including IFN-γ (−31%), MCP-1 (−50%), RANTES (−24%), and IL-1α (−50%), were less affected by macrophage depletion followed by endotox shock (data not shown). As reported before, treatment with clodronate liposomes did not induce cytokine responses or activated macrophages (28–30). Collectively, our data suggested that splenic macrophages are a cellular source of IL-27(p28) during sepsis.

**IL-10 mediates suppression of IL-27(p28) release**

We used cultures of BMDMs to characterize how the production of IL-27(p28) is regulated during inflammation. BMDMs were phenotyped by flow cytometry as >95% F4/80+CD11b+ double-positive and CD11clow (Supplemental Fig. 1A). LPS in a dose range of 1 ng/ml–1000 ng/ml was effective at inducing IL-27(p28) from BMDMs (Supplemental Fig. 1B).

Because IL-27 signaling is known to induce IL-10 secretion by T cells, we sought to investigate whether IL-10 would mediate a negative feedback on the production of IL-27(p28) in macrophages. Indeed, rIL-10 caused long-lasting suppression of IL-27(p28) release in supernatants from LPS-activated BMDMs derived from WT mice (Fig. 4A). Induction of mRNA for IL-27(p28) preceded the appearance of secreted protein, because mRNA for IL-27(p28) already peaked at 3 h following TLR4 activation by LPS in BMDMs (Fig. 4B). The levels of mRNA for IL-27(p28) also were greatly antagonized by rIL-10 (Fig. 4B). IL-10 potently suppressed the production of IL-27(p28) in a dose-dependent manner (IC50: 200–400 pg/ml) in macrophages (Fig. 4C).

In our cultures of BMDMs, the addition of LPS triggered a release of endogenous IL-10 (100–300 pg/ml) after 24 h (data not shown). Compared with BMDMs from WT mice, IL-10+− BMDMs showed nearly 4-fold increased levels of IL-27(p28) secretion following TLR4 activation (Fig. 4D). In addition, blocking of endogenous IL-10 with neutralizing Ab in cultures of WT BMDMs enhanced the release of IL-27(p28) (Fig. 4E). This suggested that, in cultures of macrophages, low levels of endogenous IL-10 are sufficient to limit the production of IL-27(p28) in an autocrine and/or paracrine fashion. Variations in the absolute amounts of IL-27(p28) from individual experiments with WT BMDMs (e.g., in Fig. 4D, 4E) are explained by different incubation times and day-to-day variations. For the experiments shown in Fig. 4E, we used longer incubation times (24 h) compared with the ones shown in Fig. 4D, because 10-h incubation periods did not reliably result in increased IL-27(p28) concentrations. This is most likely the result of the limited efficacy of IL-10–blocking Abs and the delayed release of IL-10 during in vitro cultures of LPS-activated macrophages (data not shown). Next, we used flow cytometry to assess intracellular IL-27(p28); we found profound reductions in the frequencies of F4/80+CD11b+IL-27(p28)+ BMDMs in the presence of exogenous IL-10 when added to LPS-activated BMDMs compared with LPS treatment alone (Fig. 4F). Optimal staining for intracellular IL-27(p28) required a methodology including monensin (Golgi transport inhibitor), whereas only few BMDMs stained positive with the matched isotype control Ab (Supplemental Fig. 1C). The suppression of IL-27(p28) synthesis by IL-10 was visualized using immunocytofluorescence with staining for intracellular IL-27(p28) following stimulation of BMDMs with LPS and IL-10, alone or together (Fig. 4G).

Finally, we wanted to study whether the curtailment of IL-27(p28) by IL-10 is a phenomenon restricted to BMDMs. All tested cell types of the monocytic-macrophage cell lineage, including PEMs, RAW 264.7 macrophages, and MH-S macrophages (SV40 transformed mouse alveolar macrophage cell line), produced abundant IL-27(p28) in response to LPS. Recombinant IL-10 antagonized IL-27(p28) levels in all monocytic-macrophage cell types tested, as observed for PEMs, RAW 264.7 macrophages, and MH-S macrophages (Fig. 4H). LPS-induced IL-27(p28) release also was suppressed by IL-10 in cultures of splenocytes (Fig. 4H). When C57BL/6 mice were treated with clodronate liposomes before isolation of splenocytes, the amounts of IL-27(p28) released were greatly reduced, suggesting that...
macrophages/phagocytic cells are the source of IL-27(p28) in cultures of splenocytes (data not shown).

In summary, these data (obtained using multiple methodologies) suggest that IL-10 may negatively affect the synthesis of the IL-10–inducing cytokine IL-27 in cultures of phagocytic cells.

IL-10 inhibition of MyD88/TRIF-dependent IL-27(p28) is mediated via STAT3 but not SOCS3

We sought to characterize the intracellular mechanisms underlying regulation of IL-27(p28) gene expression by IL-10. LPS ligation with TLR4 recruits both the MyD88 and TRIF pathways to induce cytokine IL-27 (31, 32). We confirmed these previous findings by intracellular staining for IL-27(p28) using macrophages from WT, MyD88<sup>−/−</sup> and TRIF<sup>−/−</sup> mice. After 10 h of incubation with LPS, 45.6% of WT macrophages had converted to an F4/80+IL-27(p28)+ phenotype, whereas only 8.6% of MyD88<sup>−/−</sup> macrophages and virtually no TRIF<sup>−/−</sup> macrophages were F4/80+IL-27(p28)+ (Fig. 5A). We quantified the reduction in IL-27(p28) concentrations with the genetic absence of MyD88 (∼65–75%) or TRIF (∼90%) by ELISA (Fig. 5B). More importantly, IL-10

![Graph A](image1.png)

**Figure 3.** Macrophages localized in the spleen are a major cellular source of IL-27(p28) in vivo. (A) RT-PCR of mRNA for IL-27(p28) in organ homogenates from WT mice 6 h after endotoxic shock (LPS 10 mg/kg body weight i.p., n = 5). Relative IL-27(p28) expression levels in the heart were used as 1-fold after normalization to GAPDH expression. (B) IL-27(p28) in plasma 6 h after endotoxic shock, as determined by ELISA. WT mice underwent sham surgery (n = 10) or splenectomy (n = 10) 7 d earlier. (C) Depletion of F4/80<sup>+</sup> macrophages (stained in red) in spleens (nuclei stained in blue) following treatment with PBS liposomes (controls) or clodronate liposomes (300 μl injected i.p. at −72 h and 150 μl injected i.p at −24 h before LPS); n = 4/group. Original magnification ×1000. (D) IL-27(p28) in plasma 6 h after endotoxic shock following pretreatment with clodronate liposomes (n = 10) or PBS liposomes (n = 10), as described above. **p < 0.01, ***p < 0.001.

![Graph B](image2.png)

**Figure 4.** Suppression of IL-27(p28) production by IL-10 in macrophages. (A) Time course of IL-27(p28) release from BMDMs (WT) after incubation with LPS (1 μg/ml), with or without IL-10 (10 ng/ml), as assessed by ELISA. (B) RT-PCR of IL-27(p28) mRNA levels in BMDMs (WT) with LPS or LPS plus IL-10 (10 ng/ml). (C) Dose-response study of IL-27(p28) release from LPS-activated BMDMs (WT) using several IL-10 concentrations for 10 h. Ctrl indicates resting BMDMs. (D) Release of IL-27(p28) by LPS-activated BMDMs from WT or IL-10<sup>−/−</sup> mice after incubation for 10 h. (E) Release of IL-27 (p28) by BMDMs (WT) after 24 h incubation with LPS with addition of control IgG or neutralizing anti–IL-10 Ab (10 μg/ml). (F) Flow cytometry of BMDMs (WT) after LPS incubation, with or without IL-10 for 10 h. (G) Immunocytofluorescence of BMDMs (WT) with red staining for IL-27(p28) after 14 h incubation (original magnification ×1000). (H) Relative inhibition of IL-27(p28) by IL-10 in BMDMs, PEMs, RAW 264.7 macrophages, MH-S macrophages (SV40 transformed mouse alveolar macrophage cell line), and splenocytes from C57BL/6J mice, all after incubation for 10 h. Data are representative of three independent experiments, each performed in duplicates. *p < 0.05, **p < 0.01, ***p < 0.001. **
suppressed the residual IL-27(p28) release in both MyD88−/− and TRIF−/− BMDMs (Fig. 5B). This suggests that IL-10 non-selectively inhibits both the MyD88- and TRIF-dependent pathways during IL-27(p28) production.

To further investigate the mechanism of how IL-10 suppresses IL-27(p28), we first studied NF-κB and IRF-3, because both factors regulate gene expression of IL-27(p28) (31–33). However, neither NF-κB nor IRF-3 was affected by IL-10 in WT BMDMs, as assessed by DNA-binding immunoassays (data not shown).

The role of STAT3 also was studied. As expected, rIL-10 strongly induced STAT3 phosphorylation in F4/80+ BMDMs derived from WT mice (Fig. 6A). IL-10 was equally effective when used alone or in combination with LPS (Fig. 6A). Time-course studies using bead-based immunoassays specific for phospho-STAT3 showed maximal phosphorylation 20 min following IL-10 treatment in WT BMDMs (Fig. 6B). This is in accordance with previous observations (20). Treatment of BMDMs with IL-10 did not affect the patterns of phosphorylation in several other signaling pathways, such as Akt, c-Jun, CREB, ERK1/2, JNK, and p38MAPK (data not shown).

To generate macrophages devoid of STAT3, STAT3fl/fl mice were crossed with a Tie2-Cre strain, resulting in tissue-specific deletion of STAT3 in hematopoietic cells. We used macrophages isolated from neonatal STAT3-deficient mice to bypass the extreme organ inflammation that arises in surviving mice (34). STAT3-deficient mice were phenotyped by Western blotting, which demonstrated missing phospho-STAT3 in activated macrophages (data not shown). IL-10 completely lost its ability to suppress IL-27(p28) in cultures of BMDMs with genetic deficiency of STAT3 (Fig. 6C).

Kinetics with BMDMs derived from WT mice and Tie2-Cre/STAT3fl/fl mice revealed that Tie2-Cre/STAT3fl/fl BMDMs were unresponsive to IL-10 at all time points studied (Fig. 6D).

mRNA for SOCS3 was upregulated by LPS or IL-10 in macrophages, with the combination of LPS and IL-10 being the most effective (Fig. 7A). To investigate the relevance of SOCS3 for inhibition of IL-27(p28) by IL-10, we generated macrophages with genetic deficiency of SOCS3 in cells of the hematopoietic lineage by breeding SOCS3fl/fl mice with the Tie2-Cre strain. In fact, the relative production of IL-27(p28) by Tie2-Cre/SOCS3fl/fl macrophages remained equally sensitive to the inhibitory effects of IL-10 compared with WT macrophages (Fig. 7B). Similar suppression patterns of IL-27(p28) by IL-10 were observed at all studied time points in macrophages from both WT mice and Tie2-Cre/SOCS3fl/fl mice (Fig. 7C). IL-27(p28) release approached a plateau in BMDMs from WT and Tie2-Cre/SOCS3fl/fl mice, whereas a more linear increase was observed for BMDMs from Tie2-Cre/STAT3fl/fl mice (Figs. 6D, 7C), which was most likely related to the loss of endogenous IL-10 effects in the Tie2-Cre/STAT3fl/fl strain. Collectively, these data suggested that IL-10 antagonizes IL-27(p28) production via STAT3, without a requirement for SOCS3.

**IL-10-mediated suppression of IL-27(p28) during sepsis**

We determined whether the mechanism of IL-10-mediated reduction in IL-27(p28) production was functional in two models of sepsis. Time-course studies of plasma concentrations of IL-10 during endotoxic shock revealed a very early surge of IL-10 (maximum concentrations after 1 h, Fig. 8A), which preceded the appearance of IL-27(p28) (maximum concentrations after 6 h, Fig. 1C). Next, C57BL/6J (WT) mice were treated with neutralizing anti–IL-10R Ab to interrupt IL-10 signaling during endotoxic shock (Fig. 8B). IL-27(p28) plasma concentrations were increased 5-fold in mice receiving anti–IL-10R (Fig. 8B). Likewise, in IL-10−/− mice (≤8-wk old), the amounts of IL-27(p28) were increased 6.5-fold during endotoxic shock (Fig. 8C). In the setting of polymicrobial sepsis following CLP, the amounts of IL-27(p28) in plasma were much higher in WT mice pretreated with anti–IL-10R Ab (Fig. 8D). Moreover, IL-27(p28) was released in IL-10−/− mice compared with WT mice during polymicrobial sepsis (Fig. 8E). This is in accordance with findings that the genetic absence of IL-10 is associated with hyperproduction of many proinflammatory mediators (20). As expected for IL-10’s broad anti-inflammatory properties, IL-10−/− mice had lower survival rates compared with WT mice (0 versus 58%, Fig. 8F), even when CLP was performed with less severity (mid-grade) (24). Moreover, neutralization of IL-27(p28) was partially effective in rescuing IL-10−/− mice from CLP-associated mortality (Fig. 8F). These findings demonstrated that IL-10 acted as an early natural antagonist of IL-27(p28) in vivo, thereby conferring protection against the adverse outcomes of sepsis.

**Discussion**

The classification of IL-27 as a negative or positive regulator of inflammation is a controversial issue. The key to understanding the functions of IL-27 as a promoter or silencer of inflammatory responses may be to distinguish between acute and chronic states of inflammation. We present data suggesting that, in the early phases of severe sepsis, the IL-27(p28) subunit mediates sepsis-associated mortality and cytokine release and regulates production of ROS. At the same time, IL-27(p28) expression is effectively silenced by anti-inflammatory IL-10–dependent mechanisms. This would be consistent with the idea that IL-27(p28) is not a universal anti-inflammatory mediator. Moreover, this may suggest the existence of a negative-regulatory loop, with IL-27 mediating the appearance of severe sepsis, the IL-27(p28) subunit mediates sepsis-associated mortality and cytokine release and regulates production of ROS. By contrast, in CLP, the amounts of IL-27(p28) were much higher in WT mice pretreated with anti–IL-10R Ab (Fig. 8D). Moreover, IL-27(p28) was released in IL-10−/− mice compared with WT mice during polymicrobial sepsis (Fig. 8E). This is in accordance with findings that the genetic absence of IL-10 is associated with hyperproduction of many proinflammatory mediators (20). As expected for IL-10’s broad anti-inflammatory properties, IL-10−/− mice had lower survival rates compared with WT mice (0 versus 58%, Fig. 8F), even when CLP was performed with less severity (mid-grade) (24). Moreover, neutralization of IL-27(p28) was partially effective in rescuing IL-10−/− mice from CLP-associated mortality (Fig. 8F). These findings demonstrated that IL-10 acted as an early natural antagonist of IL-27(p28) in vivo, thereby conferring protection against the adverse outcomes of sepsis.
by IL-10 are LPS-activated macrophages and dendritic cells (20). Interestingly, IL-10 only suppresses subsets of genes induced by TLRs but does not antagonize all functions elicited by pattern-recognition receptors (20). According to our data, the group of IL-10–regulated cytokines includes IL-27(p28), and IL-10 limits both MyD88- and TRIF-dependent production of IL-27(p28). Other studies established the requirement of NF-κB and IRF-3 for activation of IL-27(p28) transcription (31–33). However, IL-10 neither inhibited NF-κB activity, which is consistent with previous findings (35), nor suppressed IRF-3 activation (data not shown).

**FIGURE 6.** IL-10 antagonizes secretion of IL-27(p28) via activation of STAT3. (A) Flow cytometry of phospho-STAT3 (pY705) in F4/80+ BMDMs (WT). Macrophages were left untreated (Ctrl) or were incubated with IL-10 (10 ng/ml) or LPS (1 μg/ml) alone or in combination for 1 h. (B) Time course of phosphorylation of STAT3 in response to IL-10 (10 ng/ml) in BMDMs, as assessed by a bead-based assay. (C) Suppression of LPS-induced IL-27(p28) by IL-10 in BMDMs from WT or Tie2-Cre/STAT3 fl/fl mice after incubation for 24 h. (D) Time course of IL-27(p28) release following incubation with LPS alone or in combination with IL-10, using BMDMs derived from WT mice (left panel) or Tie2-Cre/STAT3 fl/fl mice (right panel). Data are representative of at least two independent experiments, each in duplicates (A and B), or were done with n = 4 mice/group (C and D). **p < 0.01, ***p < 0.001.

**FIGURE 7.** Inhibition of IL-27(p28) production by IL-10 is independent of SOCS3. (A) RT-PCR of C57BL/6J (WT) macrophages for mRNA of SOCS3 after a 6-h incubation with LPS (1 μg/ml) or IL-10 (10 ng/ml), alone or in combination. (B) Relative inhibition of LPS-induced IL-27(p28) by IL-10 in macrophages derived from WT mice or Tie2-Cre/SOCS3 fl/fl mice after incubation for 24 h. (C) Time course of the appearance of IL-27(p28) in cell culture supernatants of LPS-activated macrophages from WT mice or Tie2-Cre/SOCS3 fl/fl mice in the absence or presence of IL-10. Data are representative of at least two independent experiments, each in triplicates (A), or were done with n = 4 mice/group (B and C). *p < 0.05, ***p < 0.001.
FIGURE 8. IL-10 limits IL-27(p28) production during polymicrobial sepsis and endotoxic shock. (A) IL-10, as assessed by ELISA, in plasma of WT mice during endotoxic shock (LPS 10 mg/kg body weight i.p.; n = 4–6 mice/time point). (B) WT mice were injected with neutralizing anti–IL-10R Ab (200 μg i.p.; n = 7) or isotype control IgG1κ (200 μg i.p.; n = 8), followed by endotoxemia and detection of circulating IL-27(p28) after 8 h by ELISA. (C) IL-27(p28) in plasma 10 h after endotoxic shock in WT mice (n = 9) compared with IL-10−/− mice (n = 6). (D) Detection of IL-27(p28) in plasma of WT mice injected with neutralizing anti–IL-10R Ab (n = 8) or control IgG1κ (n = 7), followed by polymicrobial sepsis induced by CLP. Plasma samples were collected 10 h after CLP. (E) IL-27(p28) in plasma of WT mice after sham surgery (n = 6) or CLP (n = 9) compared with IL-10−/− mice after CLP (n = 8), 10 h. (F) Survival after CLP (mid-grade) in WT mice treated with control IgG and IL-27(p28)−/− mice treated with control IgG or neutralizing anti–IL-27(p28) Ab (40 μg/mouse i.p.; n = 12/group). Abs were injected 1 h before LPS or CLP in all experiments. *p < 0.05, **p < 0.01, ***p < 0.001. In (F), *p < 0.05, IL-10−/− + Control IgG versus IL-10−/− + anti–IL-27(p28), #p < 0.05, WT + Control IgG versus IL-10−/− + control IgG.

shown). Instead, IL-10 completely lost its ability to antagonize IL-27(p28) in the absence of STAT3. STAT3 activation subsequently induces SOCS3 expression, although we found that SOCS3 is not required for regulation of IL-27(p28) production. It was shown that SOCS3 predominantly inhibits the gp130 subunit of IL-6R (36, 37). According to our data, the high susceptibility of IL-10−/− mice to polymicrobial sepsis and endotoxemia is caused, in part, by IL-27(p28), because blockade of IL-27(p28) resulted in improved survival. Hyperproduction of many proinflammatory cytokines (IL-1β, IL-6, TNF-α) occurs in IL-10−/− mice, in addition to the enhanced secretion of IL-27p28; both may contribute to the high lethality of IL-10−/− mice in models of sepsis. Our data that splenic macrophages are a major source of IL-27(p28) during sepsis are consistent with other reports that macrophages and dendritic cells are the predominant IL-27–producing cell types in other diseases and in vitro (38).

Several reports described IL-27 in promoting autoimmune diseases, including inflammatory bowel disease (11) and lupus nephritis (39). In contrast, IL-27 limits the severity of experimental autoimmune encephalomyelitis (17), whereas there are conflicting data on IL-27’s effects during rheumatoid arthritis (13). During malaria infection, IL-27RA signaling prevents Th1-mediated tissue destruction independent of parasite clearance (40). The effects of IL-27 on Th1 responses are intricate because IL-27 can induce IFN-γ (the classical Th1 signature cytokine), as well as IL-10 (known to suppress Th1 cells). IL-27 and IFN-γ promote distinct populations of Tregs during chronic parasitic disease (41).

In this study, we used polyclonal-neutralizing Abs directed against the p28 subunit of IL-27. Earlier studies did not specifically investigate p28, but rather assessed EBI3 or heterodimeric IL-27 (12). One has to keep in mind that EBI3 is also a subunit of IL-35, whereas, all data obtained in EBI3−/− mice may not exclusively reflect the biology of IL-27. The subunit p28 appears to be much more specific for IL-27, although other binding partners of p28, such as CLF-1, have been suggested (42). The existence of p28 homodimers is still speculative. However, transgenic mice with overexpression of p28 showed that it can act as a natural antagonist of IL-27/gp130 signaling (9).

The murine models of endotoxemia and polymicrobial sepsis after CLP are characterized by detrimental acute inflammatory responses similar to clinical diseases, such as systemic inflammatory response syndrome and human sepsis. Our study focused on the early hyperinflammatory phases of sepsis rather than later-occurring immunosuppression of the disease (4, 43, 44). The CLP model is considered by many investigators to have the best correlation with human sepsis, although this view was challenged recently using gene expression profiling (45). Severe sepsis is among the leading causes of death in developed countries. Despite tremendous scientific efforts, specific treatment options targeting the pathophysiologic mechanisms of sepsis beyond the use of antibiotics are not available (3).

Our data on the role of IL-27(p28) during sepsis are supported by the previous work of Wirtz et al. (12), who reported on the protective effects of a soluble IL-27RA–Fc fusion protein during polymicrobial sepsis. We showed recently that IL-27RA−/− mice have improved survival rates during endotoxic shock (46). IL-27(p28) production during endotoxic shock and polymicrobial sepsis induced by CLP is partially dependent on Tyk2 via an auto-
crine type I IFN loop (46). Our finding that F4/80^+CD11b^+ macrophages derived from IL-27RA^-/^- mice displayed increased ROS production after stimulation with opsonized E. coli is consistent with findings in LPS-stimulated phagocytes derived from EB13^-/- mice (12). In EB13^-/- mice, the release of cytokines (IL-1, IL-6) is reduced following CLP (12). In contrast, the production of IL-10 is not significantly impaired in EB13^-/- mice during polymicrobial sepsis (12), which is in line with the data presented in this article using neutralizing anti-IL-27(p28) Abs during endotoxic shock or polymicrobial sepsis (Fig. 1). Treatment with rIL-27 impaired survival following i.p. challenge with microbial sepsis (Fig. 1). Treatment with rIL-27 impaired survival neutralizing anti–IL-27(p28) Abs during endotoxic shock or polymicrobial sepsis (12). In contrast, the production of IL-10 is not significantly impaired in EB13^-/- mice during polymicrobial sepsis (12), which is in line with the data presented in this article using neutralizing anti–IL-27(p28) Abs during endotoxic shock or polymicrobial sepsis (Fig. 1). Treatment with rIL-27 impaired survival following i.p. challenge with Pseudomonas aeruginosa (47). IL-27RA^-/- mice were protected when low-grade CLP (= 100% survival) was followed 24 h later by intratracheal infection with P. aeruginosa (47).

In critically ill children the quantification of plasma IL-27 concentrations was useful as a biomarker for discriminating between patients suffering from systemic inflammatory response syndrome (n = 101) or sepsis (n = 130) (48). In contrast, in adult patients with sepsis, the detection of IL-27 was only helpful for diagnosis when applied in combination with procalcitonin (49). The concentrations of IL-27 are higher in children compared with adults, further arguing for the usefulness of IL-27 in pediatric cases of sepsis (50). It appears that, in human sepsis, plasma levels of heterodimeric IL-27 may be substantially higher than IL-27(p28) concentrations was useful as a biomarker for discriminating between patients suffering from systemic inflammatory response syndrome (n = 101) or sepsis (n = 130) (48). In contrast, in adult patients with sepsis, the detection of IL-27 was only helpful for diagnosis when applied in combination with procalcitonin (49). The concentrations of IL-27 are higher in children compared with adults, further arguing for the usefulness of IL-27 in pediatric cases of sepsis (50). It appears that, in human sepsis, plasma levels of heterodimeric IL-27 may be substantially higher than IL-27(p28) detected in our CLP model using time points < 24 h (47).

Given the data presented in this article, directly limiting IL-27(p28) activity with either neutralizing Abs or administration of rIL-10, or indirectly by modulation of STAT3 activity, may be considered a future treatment strategy for sepsis. However, such endeavors will undoubtedly require further experimental studies to provide a more complete understanding of the double-edged role played by IL-27(p28) during acute and chronic settings of unbalanced inflammation.

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References


