Diversity of Interferon γ and Granulocyte-Macrophage Colony-Stimulating Factor in Restoring Immune Dysfunction of Dendritic Cells and Macrophages During Polymicrobial Sepsis

Stefanie B Flohé,[1] Hemant Agrawal,[1,2] Sascha Flohé,[1] Meenakshi Rani,[1] Jörg M Bangen,[1] and F Ulrich Schade[1]

[1]Surgical Research, Department of Trauma Surgery, University Hospital Essen, University Duisburg-Essen, Essen, Germany,
[2]Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, United States of America

The development of immunosuppression during polymicrobial sepsis is associated with the failure of dendritic cells (DC) to promote the polarization of T helper (Th) cells toward a protective Th1 type. The aim of the study was to test potential immunomodulatory approaches to restore the capacity of splenic DC to secrete interleukin (IL) 12 that represents the key cytokine in Th1 cell polarization. Murine polymicrobial sepsis was induced by cecal ligation and puncture (CLP). Splenic DC were isolated at different time points after CLP or sham operation, and stimulated with bacterial components in the presence or absence of neutralizing anti-IL-10 antibodies, murine interferon (IFN) γ, and/or granulocyte macrophage colony-stimulating factor (GM-CSF). DC from septic mice showed an impaired capacity to release the pro-inflammatory and Th1-promoting cytokines tumor necrosis factor α, IFN-γ, and IL-12 in response to bacterial stimuli, but secreted IL-10. Endogenous IL-10 was not responsible for the impaired IL-12 secretion. Up to 6 h after CLP, the combined treatment of DC from septic mice with IFN-γ and GM-CSF increased the secretion of IL-12. Later, DC from septic mice responded to IFN-γ and GM-CSF with increased expression of the co-stimulatory molecule CD86, while IL-12 secretion was no more enhanced. In contrast, splenic macrophages from septic mice during late sepsis responded to GM-CSF with increased cytokine release. Thus, therapy of sepsis with IFN-γ/GM-CSF might be sufficient to restore the activity of macrophages, but fails to restore DC function adequate for the development of a protective Th1-like immune response.
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INTRODUCTION

Sepsis is associated with the failure of the host to develop an effective immune response against invading microorganisms due to immunosuppression of unknown genesis. The consequence is unrestricted spreading of bacteria that may lead to multiorgan failure and death (1). During sepsis, monocytes/macrophages fail to secrete tumor-necrosis factor (TNF) α in response to *in vitro* stimulation with lipopolysaccharide (LPS) (2,3). Moreover, the proliferative capacity, as well as the secretion of the T helper (Th) type 1-associated cytokines interleukin (IL) 2 and interferon (IFN) γ from T-lymphocytes, is impaired (2,4–6). Therefore, therapies that modulate this cellular dysfunction might have beneficial effects on the outcome of sepsis.

An effective immune response against bacterial infections requires the development of a Th1 response that is associated with the release of IFN-γ. Antigen-specific T cells are activated by antigen-presenting cells (APC) through the interaction of co-stimulatory molecules such as CD40 and CD86 on the APC, with their respective ligands CD40 ligand (CD40L) and CD28/CTLA-4, respectively, on T cells. Dendritic cells (DC) are the most potent APC due to their high expression of major histocompatibility complex (MHC) and co-stimulatory molecules that are upregulated during DC maturation triggered by microbial agents (7). Stimulated DC secrete a distinct pattern of cytokines that is decisive for the type of subsequent Th cell differentiation. IL-12 is a heterodimeric cytokine and promotes the polarization of naïve Th cells toward Th1. Microbial stimuli such as immunostimulatory oligonucleotides (CpG) or

Note: Stefanie B Flohé and Hemant Agrawal contributed equally to this work.

***Please address correspondence and reprint requests to** Stefanie B Flohé, Surgical Research, Department of Trauma Surgery, University Hospital Essen, University Duisburg-Essen, Virchowstr. 171, D-45147 Essen, Germany. Phone: + 49 201 723 4405; Fax: + 49 201 723 5608; E-mail: stefanie.flohe@uk-essen.de.*

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LPS are recognized by Toll-like receptors (TLR) and are potent inducers of DC-derived IL-12. LPS requires additional ligation of CD40 on the surface of DC for optimal induction of IL-12 synthesis (8,9). In contrast, DC-derived IL-10 favors the development of Th2 cells, but suppresses the development of a Th1 response through inhibition of IL-12 secretion (10). Neutralization of endogenous IL-10 results in increased IL-12 secretion in response to LPS or CpG (11,12).

There is an increasing body of evidence that DC are involved in the pathomechanisms leading to sepsis-associated immune dysfunction. DC are beneficial at least during the early phase of sepsis development because depletion of DC *in vivo* before onset of disease results in increased mortality (13). Later, apoptosis of DC takes place in various lymphoid and non-lymphoid tissues in septic patients (14) as well as in septic mice (15–17), and the extent of DC loss in septic patients correlates with poor outcome (18). We have recently shown that, in mice, splenic DC rapidly increase their expression of CD40 and CD86 after induction of sepsis, but simultaneously develop a dysfunction that is characterized by an impaired capacity to secrete IL-12 in response to bacterial stimuli and to drive Th cell proliferation (19).

The relevance of DC dysfunction in disease development has been clearly shown by Benjamim *et al*. who applied competent DC from naïve mice into post-septic mice and thereby reduced infection-induced mortality (20). IL-12 is essential for survival of sepsis because it promotes the release of IFN-γ that in turn supports the clearance of the infection through enhanced microbicidal activity of the innate immune system (21). The aim of the present study was to find immunomodulatory approaches to restore the suppressed capacity of splenic DC to secrete IL-12 during murine polymicrobial sepsis induced by cecal ligation and puncture (CLP). Selected approaches were the neutralization of endogenous IL-10 and treatment with the immunomodulatory substances IFN-γ and/or GM-CSF.

MATERIALS AND METHODS

Cecal Ligation and Puncture

Female BALB/c mice (Harlan Winkelmann, Borchen, Germany) were 8–10 weeks old and had access to standard rodent food and water *ad libitum*. All animal procedures were carried out following institutional guidelines at the Medical Faculty, University of Duisburg-Essen, Germany. Polymicrobial sepsis was induced by CLP using a 17-gauge needle as described previously (19). Under these conditions, the mortality was 20% within 24 h. Sham animals underwent a laparotomy without ligation and puncture of the cecum.

Culture Medium and Reagents

Very low endotoxin medium VLE RPMI 1640 (Biochrom, Berlin, Germany) containing 10% heat-inactivated FCS (Sigma, Taufkirchen, Germany), 10 mM HEPES (Biochrom), 2 mM L-Glutamine (Biochrom), 0.06 mg/mL Penicillin (Sigma), 0.02 mg/mL Gentamicin G (Sigma), and 0.05 mM 2-ME (Sigma) was used as culture medium throughout all experiments. Murine recombinant GM-CSF, IFN-γ, CD40L, neutralizing anti-IL-10 antibodies (clone JES052A5), and the respective rat IgG1 isotype control were purchased from R&D Systems, Wiesbaden, Germany. Synthetic phosphorothioated CpG 1668 oligonucleotides (22) were purchased from Qiagen, Köln, Germany. All these reagents were free of detectable LPS contaminations as tested using Limulus Amebocyte Assay (Biowhittaker, Walkersville, MD, USA). LPS (*E. coli* 026:B6) was obtained from Sigma.

Preparation and Culture of Total Spleen Cells

At different time points after CLP or sham operation, spleens were removed and single cell suspensions were prepared through collagenase digestion using 0.02 U/mL Blendzyme 2 (Roche, Grenzach-Wyhlen, Germany) at 37°C for 18 min. Spleens were minced through a cell strainer (70 μm diameter) and red blood cells were lysed using ammonium chloride. For flow cytometric analyses of DC, total spleen cells were cultured in 48-well plates (Nunc, Wiesbaden, Germany; 10^6 cells/well in 300 μL culture medium). In case of macrophage analyses, total spleen cells were kept in 24-well "low attachment" plates (Corning, Schiphol-Rijk, The Netherlands; 2.5 × 10^6/well in 500 μL culture medium) to enable detachment of adherent cells. CD86 or CD40 expression on DC was analyzed using total spleen cells cultured in the presence or absence of 10 ng/mL GM-CSF, 10 ng/mL IFN-γ, 5 μg/mL CpG, or 100 ng/mL LPS for 18 h. Stimulation of total spleen cells with 10 ng/mL LPS for 8 h in the absence or presence of 10 ng/mL GM-CSF or with 5 μg/mL CpG for 18 h was used for the examination of intracellular levels of TNF-α and IL-12p40, respectively. Unstimulated cells served as negative controls. All cultures were set up in triplicate and were pooled before flow cytometric analyses.

Purification of Splenic DC

Splenic DC were purified from freshly isolated total spleen cells using CD11c Microbeads (Miltenyi Biotech, Moenchengladbach, Germany) and magnetic cell sorting (MACS, Miltenyi Biotech) and were cultured as described previously (19). Purity was generally 85% to 90% as confirmed by CD11c staining and flow cytometry. Purified splenic DC were cultured with 5 μg/mL CpG or 100 ng/mL LPS + 2.5 μg/mL CD40L. In some experiments, purified splenic DC were stimulated with CpG in combination with medium, GM-CSF (10 ng/mL), IFN-γ (10 ng/mL), IFN-γ + GM-CSF, anti-IL-10 antibodies (10 μg/mL), or the respective rat IgG1 isotype control (10 μg/mL). Previous experiments have shown that 10 μg/mL anti-IL-10 antibodies were sufficient to neutralize the activity of 1 ng/mL IL-10 (data not shown). All cultures were set up in triplicate. After 18 h, supernatants were analyzed for the presence of IL-12p70, TNF-α, IFN-γ, or IL-10 using the cytometric bead array (CBA) Mouse Inflammation Kit

(BD Biosciences, Heidelberg, Germany) or ELISA (ebioscience, NatuTec, Frankfurt, Germany).

Flow Cytometry

Total spleen cells were sequentially incubated with total mouse IgG (100 μg/mL; Sigma) to block unspecific binding, and with an antibody mixture (all antibodies from BD Biosciences) containing anti-CD11c-allophycocyanin (APC; clone HL3) either in combination with biotinylated anti-IFN-γ receptor (clone GR20), with anti-CD86-phycoerythrin (PE; clone GL1), or with anti-CD40-fluorescein isothiocyanate (FITC; clone 3/23). Streptavidin-PE was added in a third step to cells labeled with the biotin-conjugated antibody. Appropriate isotype controls were used for all stainings. For intracellular IL-12p40 or TNF-α staining, monensin (GolgiStop 0.66 μL/mL, BD Biosciences) was added to the cells during the last 6 h of culture. After surface staining using APC-labeled anti-CD11c or anti-F4/80 (clone BM8; ebioscience) antibodies, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) for 20 min at RT. Thereafter, intracellular TNF-α or IL-12p40 were stained using anti-TNF-α-PE (clone MP6-XT22), anti-IL-12p40-PE (clone C15.6) antibodies, or the respective isotype control antibody (all from BD Biosciences). Cells were washed with permeabilization buffer (BD Biosciences) and were resuspended in Cell Wash. All data were acquired using a FACScalibur (BD Biosciences). Living cells were selected according to forward and side scatter properties. DC were gated as CD11c-positive cells, macrophages were gated as F4/80-positive cells. The fluorescence intensity value that was exceeded by less than 2% of the cells upon isotype control staining was defined as the threshold for specific staining. According to this threshold, the percentage of positive cells and the corresponding mean fluorescence intensity (MFI) of the gated cells were determined.

Statistics

Results on cytokine expression are presented as the mean ± SD of triplicate cultures. Statistical differences between selected groups, as indicated in the Figure legends, were tested using one-way analysis of variance (ANOVA, two-tailed) followed by Bonferroni's multiple comparison test. Data on IFN-γ receptor expression on DC from separate mice were normalized to the mean value obtained from sham mice and were tested using two-tailed Mann-Whitney U test (see Figure 1). A $P < 0.05$ was considered to be significant.

RESULTS

Cytokine Profile and CD40 Expression of Splenic DC During Sepsis

To analyze the cytokine expression profile of DC after induction of polymicrobial sepsis, splenic DC were isolated 3, 6, 16, and 24 h after CLP or sham operation and were stimulated with CpG or with LPS + CD40L. The amounts of TNF-α, IL-12, and IFN-γ as pro-inflammatory and Th1-associated cytokines and of IL-10 as the predominant Th2-promoting cytokine were determined in the supernatants. DC from sham mice released IL-12, TNF-α, and IFN-γ in response to CpG or LPS + CD40L (Figure 2A). Low amounts of IL-10 were secreted in response to CpG or LPS + CD40L. In contrast, CpG-stimulated DC from septic animals produced significantly lower amounts of IL-12, IFN-γ, and TNF-α in comparison to the corresponding DC from sham-operated mice as early as 3 h after CLP (Figure 2A). With disease progress, the production of these cytokines from DC stimulated with CpG, as well as with LPS + CD40L, further decreased (Figure 2A). Due to the low number of DC that could be isolated from the spleen, we focused on one stimulus. CpG was a more potent and CD40-independent inducer of IL-12 synthesis by DC in comparison to LPS + CD40L. Therefore, we selected CpG for further studies on the modulation of IL-12 release.

To analyze the cytokine expression of DC that were not separated from other splenic cell populations, total spleen cells were prepared 3 and 24 h after CLP or sham operation, and were stimulated with CpG. DC were stained for intracellular IL-12p40 that represents the regulated subunit of the IL-12 heterodimer in combination with CD11c on the cell surface. At both time points, the percentage of IL-12p40-positive DC from sham mice strongly increased upon stimulation with CpG in comparison to unstimulated cells (Figure 2B). By 3 h after CLP, the percentage of IL-12p40-positive DC increased upon stimulation with CpG,

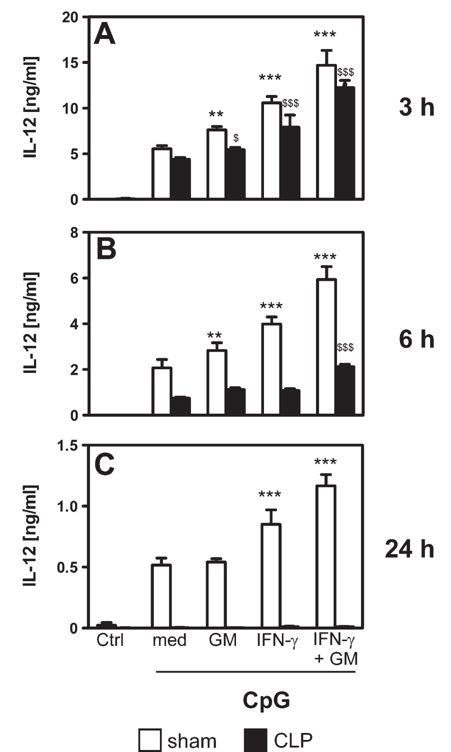

Figure 1. Modulation of CpG-induced IL-12 secretion from DC through IFN-γ and/or GM-CSF. At 3 h (A), 6 h (B), and 24 h (C) after CLP (black bars) or sham operation (white bars), splenic DC were purified and stimulated with CpG in combination with none (med), GM-CSF (GM), IFN-γ, or IFN-γ + GM. Untreated cells served as negative control (Ctrl). After 18 h, the amount of IL-12 was determined in the supernatants. Data show the mean ± SD of triplicate cultures and are representative for at least two experiments. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ versus med sham; \$, $P < 0.05$; \$\$\$, $P < 0.001$ versus med CLP.

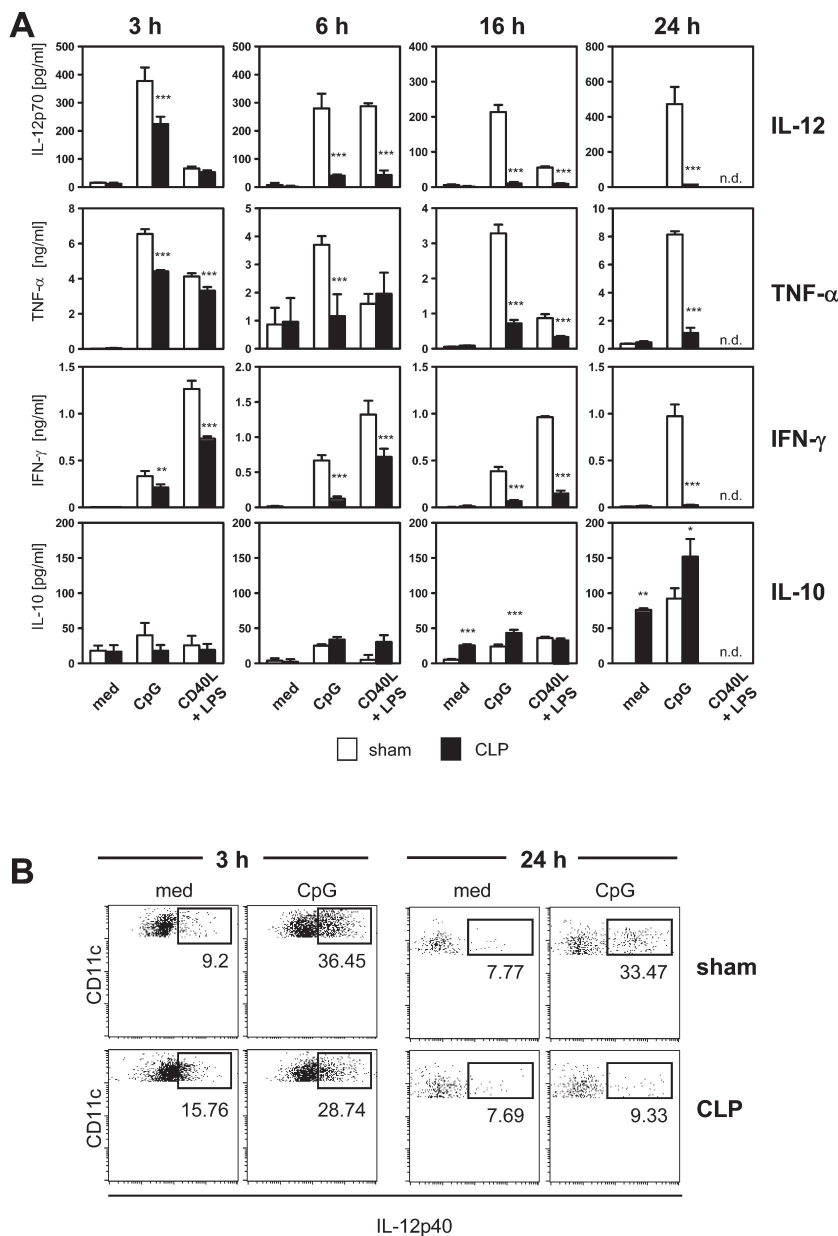

Figure 2. Time course of the cytokine secretion pattern of splenic DC in response to bacterial products. (A) At different time points (3, 6, 16, and 24 h) after CLP (black bars) or sham operation (white bars), splenic DC were purified and cultured with none (med), with CpG, or with CD40L + LPS. After 18 h, the content of different cytokines in the supernatants was determined. Data show the mean ± SD of triplicate cultures. (B) Intracellular IL-12p40 staining of splenic DC upon stimulation with CpG. At 3 and 24 h after CLP or sham operation (*n* = three to four mice per group), total spleen cells were pooled and cultured in medium alone (med), or were stimulated CpG for 18 h. Monensin was present during the last 6 h of culture. After surface staining of CD11c, cells were fixed, permeabilized, and stained for intracellular IL-12p40. CD11c-positive DC were gated and the percentage of IL-12p40–positive cells was determined according to staining with the isotype control antibody. The numbers indicate the percentage of IL-12p40–positive cells. All data are representative for at least two experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ sham versus CLP; n.d., not determined.

but to a lesser extend than it was found for DC from sham mice. In contrast, upon CpG-stimulation of total spleen cells prepared 24 h after CLP, the percentage of IL-12p40-positive DC remained on the level obtained for unstimulated DC (Figure 2B). Thus, the impaired secretion of CpG-induced IL-12 from DC correlates with a strong reduction of the number of DC that express IL-12p40.

The completely contrary effects were seen in terms of IL-10 production. From 16 h after CLP, DC from septic mice released IL-10 even in the absence of any stimulus and further enhanced IL-10 secretion upon stimulation with CpG (Figure 2A). In contrast, the secretion of IL-10 in response to LPS + CD40L did not change significantly within the first 16 h after CLP.

For analyses of CD40 expression on DC after stimulation with bacterial products, total spleen cells were prepared 3, 6, and 24 h after CLP or sham operation and cultured in the absence or presence of CpG or LPS. DC from sham mice responded to CpG or LPS with increased expression of CD40 at each time point (Figure 3). The levels of CD40 on DC from septic mice at 3 h after CLP similarly increased upon challenge with CpG or LPS, but remained below the levels observed on DC from sham mice 6 h after operation (Figure 3). The CpG- or LPS-triggered increase of CD40 expression was less prominent on DC from septic mice 24 h after CLP due to elevated basal levels in comparison to unstimulated DC from sham mice (Figure 3). In summary, early during sepsis, DC selectively lose the capacity to release Th1-associated cytokines, but acquire a bias for increased IL-10 secretion.

Decreased IL-12 Release of DC During Sepsis is Not Caused by Endogenous IL-10 Production

We have recently shown that at later stages during sepsis (24 h after CLP), endogenous IL-10 was not responsible for the failure of DC to release IL-12

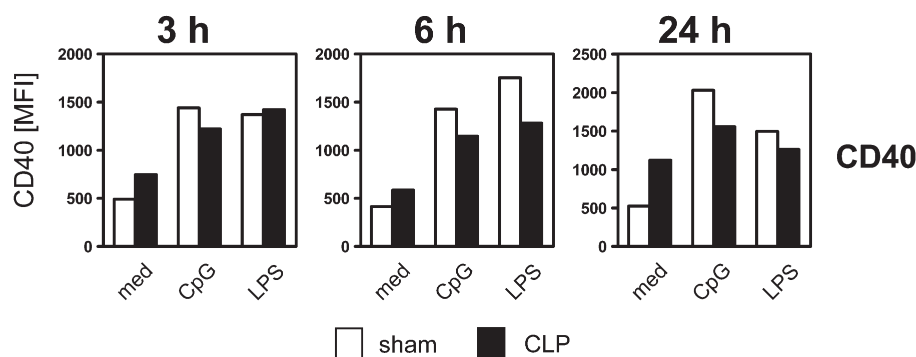

Figure 3. Time course of CD40 expression of splenic DC in response to bacterial products. Three, 6, or 24 h after CLP (black bars) or sham operation (white bars), total spleen cells were stimulated with none (med), CpG, or LPS. The expression of CD40 on DC was examined using flow cytometry. Data are representative for at least two experiments. MFI, mean fluorescence intensity.

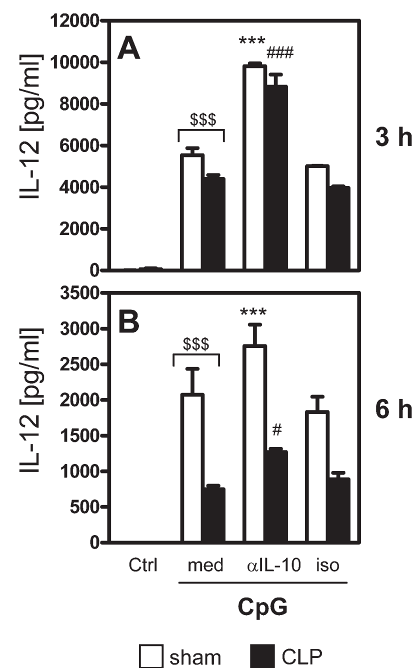

Figure 4. Effect of neutralization of endogenous IL-10 on CpG-induced IL-12 release. At 3 h (A) and 6 h (B) after CLP (black bars) or sham operation (white bars), splenic DC were purified and stimulated with CpG in combination with none (med), anti-IL-10 antibody (αIL-10), or isotype control antibody (iso). Untreated cells served as negative control (Ctrl). After 18 h, the amount of IL-12 was determined in the supernatants. Data show the mean ± SD of triplicate cultures. $$$, $P < 0.001$ med sham versus med CLP; ***, $P < 0.001$ αIL-10 sham versus iso sham; #, $P < 0.05$; ###, $P < 0.001$ αIL-10 CLP versus iso CLP.

(19). At that time point, IL-12 synthesis of DC from septic mice was nearly undetectable (Figure 2A). We addressed the question whether IL-10 might be involved in the suppression of IL-12 synthesis at earlier time points during sepsis when DC dysfunction was less prominent. Therefore, DC were purified and stimulated with CpG in the absence or presence of neutralizing anti-IL-10 or isotype control antibodies at different time points (3 and 6 h) after CLP or sham operation. As described above, DC from septic mice released less IL-12 at both time points than DC from sham mice (Figure 4). The presence of the isotype control antibody during stimulation with CpG did not affect the IL-12 secretion of DC from either group (Figure 4). Increased secretion of IL-12 was observed when DC from sham mice were stimulated in the presence of the anti-IL-10 antibody at both time points (Figure 4). The presence of the anti-IL-10-antibody comparably increased the IL-12 release of DC from septic mice 3 h after CLP (Figure 4A). In contrast, 6 h after CLP, when the suppressed IL-12 production was clearly visible, neutralization of IL-10 only marginally increased the amount of IL-12 secreted by DC (Figure 4B). Thus, endogenous IL-10 does not seem to be responsible for the failure of DC to release IL-12 early during sepsis.

Influence of IFN-γ and GM-CSF on DC-Derived IL-12 Secretion

To investigate whether the suppressed capacity of DC to secrete IL-12 during sepsis can be restored, splenic DC were isolated 3, 6, and 24 h after CLP or sham operation, and were stimulated with CpG either in the absence or presence of GM-CSF, IFN-γ, or a combination of both. GM-CSF, IFN-γ, and IFN-γ + GM-CSF enhanced the CpG-induced secretion of IL-12 from DC of sham mice with IFN-γ + GM-CSF being superior to the individual cytokines (Figure 1). DC from septic mice isolated 3 h after CLP, showed a slightly reduced IL-12 secretion upon stimulation with CpG alone. GM-CSF marginally increased the IL-12 secretion of CpG-stimulated DC from septic mice (Figure 1A). IL-12 levels of DC stimulated in the presence of IFN-γ or of the combination of IFN-γ and GM-CSF, exceeded even the IL-12 level of CpG-stimulated DC from sham mice (Figure 1A). However, DC from septic mice 6 h after CLP had lost the responsiveness to IFN-γ and to GM-CSF. At this time point, the IL-12 production of DC from septic mice was enhanced only through a combined treatment with IFN-γ + GM-CSF, however it did not reach IL-12 levels released by CpG-stimulated DC from sham mice (Figure 1B). At 24 h after CLP, DC from septic mice completely failed to secrete IL-12 and additionally were absolutely unresponsive to any immunomodulator (Figure 1C). IFN-γ or GM-CSF alone did not induce the release of IL-12 from DC of sham or septic mice (data not shown). Thus, during sepsis, DC responded to IFN-γ and/or GM-CSF with increased IL-12 secretion only during a short time frame early after induction of sepsis.

Decreased IFN-γ Receptor Expression on Splenic DC after CLP

The reduced responsiveness of DC from septic mice to IFN-γ might be associated with a decreased expression of the IFN-γ receptor. Therefore, the expression

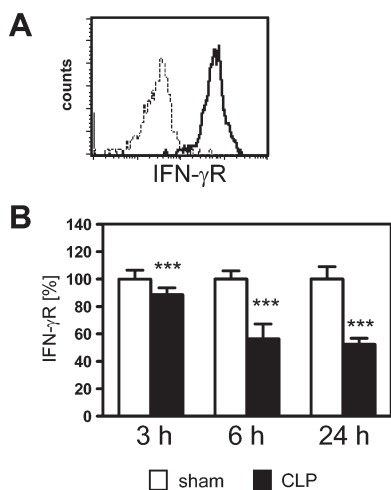

Figure 5. Kinetics of the IFN-γ receptor expression on DC during sepsis. At 3 h, 6 h, and 24 h after CLP or sham operation, total spleen cells were prepared and the expression of the IFN-γ receptor (IFN-γR) on DC was analyzed through flow cytometry. The mean fluorescence intensity (MFI) for IFN-γR expression was determined on gated CD11c-positive DC. (A) Representative histogram for staining of DC from a sham-treated mouse with the anti-IFN-γR antibody (bold line) or with the isotype control antibody (dashed line). (B) For each experiment, the mean value of the MFI for IFN-γR expression on DC from sham-treated mice was set as 100% and was used to normalize the MFI of sham mice (white bars) and CLP mice (black bars). Data show the mean ± SD (n = three experiments each with three to four mice per group). ***, $P < 0.001$ sham versus CLP.

of the IFN-γ receptor on splenic DC was determined at 3, 6, and 24 h after CLP or sham operation by means of flow cytometry. DC from sham mice all expressed high levels of the IFN-γ receptor on the cell surface (Figure 5A, B). The expression of the IFN-γ receptor on DC from septic mice was only slightly lower than on the corresponding cells of sham-operated animals 3 h after CLP (Figure 5B). However, the expression of the IFN-γ receptor on DC decreased during ongoing sepsis to 56% and 52% of the levels on DC from sham animals at 6 h and 24 h, respectively (Figure 5B). Thus, a decrease of the IFN-γ receptor expression on splenic DC takes place during sepsis and at least partly parallels the loss of IFN-γ responsiveness of DC in terms of IL-12 secretion.

Influence of IFN-γ and GM-CSF on the Expression of CD86 on DC

GM-CSF and IFN-γ alone induced an increased expression of the co-stimulatory molecule CD86 on DC from sham mice (Figure 6A). To further investigate the unresponsiveness of DC from septic mice to GM-CSF or IFN-γ, the expression of CD86 was determined as an additional parameter. Therefore, total spleen cells were isolated 3, 6, and 24 h after CLP or sham operation and cultured with or without IFN-γ or GM-CSF. After 18 h, the expression of CD86 was determined on DC by means of flow cytometry. Stimulation with IFN-γ or GM-CSF increased the levels of CD86 on the cell surface of DC from sham and septic mice at any time point, although to a different extent (Figure 6). DC from septic mice expressed higher basal levels of CD86 as soon as 3 h after CLP (Figure 6B), and these levels further increased during later time points (Figure 6C, D). Even 24 h after CLP, IFN-γ or GM-CSF enhanced the CD86 expression on the surface of DC, although to a minor degree (Figure 6D). Thus, in contrast to the effect on the CpG-induced IL-12 secretion, DC from septic mice responded to both IFN-γ and GM-CSF with increased expression of CD86.

Diverse Responsiveness of Macrophages and DC to GM-CSF During Sepsis

Macrophages from septic mice are impaired in their capacity to release TNF-α upon *ex vivo* stimulation with LPS (3,23). We addressed the question whether GM-CSF was able to restore the depressed TNF-α secretion from macrophages of septic mice in comparison to splenic DC. Because TNF-α production is strongly induced by LPS, it can be detected through intracellular staining and flow cytometry. Total spleen cells were prepared 24 h

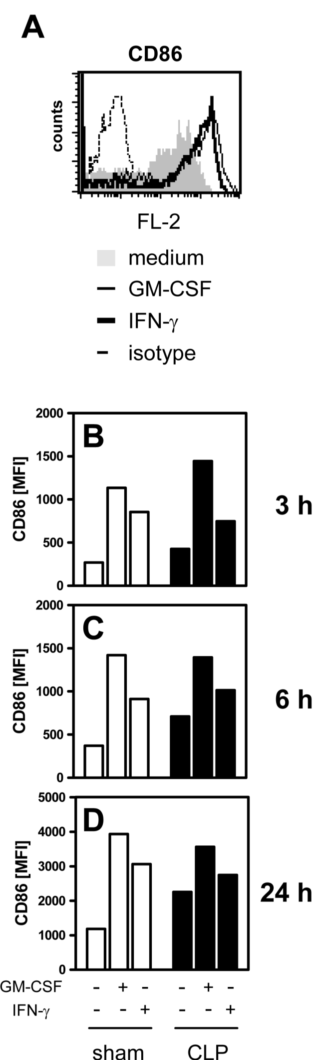

Figure 6. Expression of CD86 on DC during sepsis and its modulation through GM-CSF or IFN-γ. After CLP or sham operation (n = three to four mice per group), total spleen cells were pooled and cultured in the presence or absence of GM-CSF or IFN-γ. After 18 h, the expression of CD86 on DC was determined by means of flow cytometry. (A) Representative histogram of DC from a sham-treated mouse showing the expression of CD86 after culture with none (gray shaded area), GM-CSF (thin line), or IFN-γ (bold line). The dashed line shows the staining with the isotype control antibody. (B-D), mean fluorescence intensity (MFI) values for CD86 on DC within pooled total spleen cells at 3 h (B), 6 h (C), and 24 h (D) after CLP (black bars) or sham operation (white bars). Data are representative for two experiments.

after CLP or sham operation and were stimulated with LPS or with LPS + GM-CSF. Untreated cells served as control. TNF-α production was determined by intracellular staining and flow cytometry. More than 20% of splenic DC from sham mice were positive for TNF-α after stimulation with LPS. The presence of GM-CSF during LPS stimulation clearly increased the percentage of TNF-α-positive DC, as well as the amount of TNF-α per cell as indicated by the elevated MFI value (Figure 7A). In contrast, less than 5% LPS-stimulated splenic DC from septic mice produced TNF-α, and GM-CSF increased the percentage of TNF-α-positive DC only marginally (Figure 7A). Nearly 30% of macrophages in LPS-stimulated total spleen cells from sham mice were positive for TNF-α, and GM-CSF further increased the number of TNF-α-producing cells (Figure 7B). About 10% of splenic macrophages from septic mice spontaneously produced TNF-α. The percentage of TNF-α-positive macrophages further increased upon stimulation with LPS, but fell notably short of reaching the level of LPS-stimulated macrophages from sham mice (Figure 7B). The presence of GM-CSF during LPS stimulation increased the percentage of TNF-α-positive macrophages as well as the MFI value of positive cells from septic mice (Figure 7B). Thus, in contrast to DC, splenic macrophages from septic mice respond to GM-CSF with increased production of TNF-α and reverse, in part, their sepsis-associated immune dysfunction.

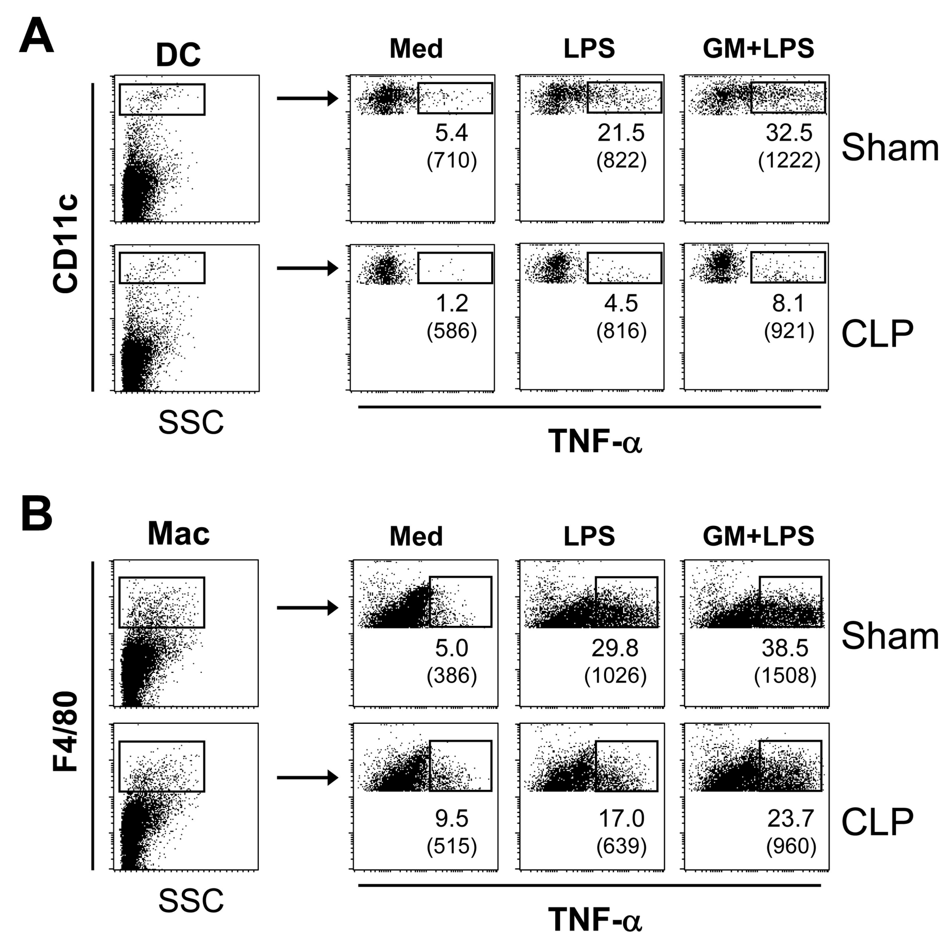

Figure 7. Modulation of the expression of TNF-α through GM-CSF in splenic DC and macrophages. At 24 h after CLP or sham operation (*n* = three to four mice per group), total spleen cells were pooled and cultured in medium alone (Med), or were stimulated with LPS or with GM-CSF (GM) + LPS for 8 h. Monensin was present during the last 6 h of culture. After surface staining of CD11c or F4/80, cells were fixed, permeabilized, and stained for intracellular TNF-α. CD11c-positive DC (A) and F4/80-positive macrophages (B) were gated and the percentage of TNF-α-positive cells was determined according to staining with the isotype control antibody. The numbers indicate the percentage of TNF-α-positive cells and the respective mean fluorescence intensity of TNF-α staining (in brackets). Data are representative for two experiments. SSC, side scatter; DC, dendritic cells; Mac, macrophages.

DISCUSSION

The present study shows that during murine polymicrobial sepsis, splenic DC rapidly lose their capacity to respond to bacterial components with the release of pro-inflammatory and Th1-promoting cytokines. The impaired capacity of DC to secrete IL-12 was not mediated by the parallel rise of endogenous IL-10 production. Moreover, attempts to restore the suppressed release of IL-12 through treatment with the immunomodulatory cytokines IFN-γ and GM-CSF showed that DC responded to these mediators only during the very early phase after induction of sepsis. Downregulation of cytokine receptors does not seem to be responsible for this insensitivity of DC to IFN-γ and GM-CSF because DC still responded to these cytokines with increased expression of CD86 even at later time points during sepsis. In contrast, macrophages that similarly showed a sepsis-associated reduced responsiveness to bacterial products could be reactivated through GM-CSF.

DC play a decisive role in the interaction between the innate and the adaptive immune system due to their cytokine secretion pattern. We show here that DC dysfunction characterized by the reduced secretion of IL-12, IFN-γ, and TNF-α in response to bacterial stimuli becomes visible as soon as 3 h after induction of sepsis (Figure 2A). Among these three cytokines, IL-12 represents the most relevant one with regard to its contribution to the development of Th1 responses.

This state of abnormal responsiveness of DC to bacterial stimuli is not restricted to the acute phase of sepsis as we describe here, but has also been found even after resolution of the disease—at which time it contributes to an enhanced susceptibility to secondary infections (20). The mechanisms that underlie the impaired capacity of DC from septic mice to secrete Th1-promoting cytokines are not clear so far. The finding that DC from septic mice increase the expression of CD40 on the surface at time points when IL-12 secretion is already reduced (for example, 3 and 6 h after CLP; Figure 3), argues against a defect in the recognition of CpG or LPS through their receptors. We can exclude that endogenous IL-10 is involved neither at early time points when IL-12 secretion is not yet completely blocked (Figure 4), nor at later time points during sepsis when the DC-derived IL-10 secretion increases (19). Sepsis is not associated with a general dysfunction of DC. The finding that DC in the peritoneal cavity that represents the site of sepsis initiation are able to secrete IL-12 (24) suggests that DC in the spleen are modulated through factors, so far unknown, prior or in parallel to their contact with spreading bacteria. Such potential mediators are transforming growth factor β prostaglandin E2, IL-10, but also catecholamines, such as norepinephrine, that are produced in the peritoneal cavity, in the gut, or in the liver, and might reach the DC in the spleen via circulation (3,25,26). All these factors are known to suppress DC-derived IL-12 production (27–30).

During acute sepsis, the presence of IL-12 is required for the polarization of Th cells toward Th1 and for the release of IFN-γ that stimulate the bactericidal activity of phagocytes (21). Whether DC are the cellular source of the indispensable IL-12 has not been determined in that report. Strategies that increase the levels of IL-12 at the site of infection, either through application of DC from naïve mice or through adenoviral transfection, lead to an improved immune response against microorganisms (20,31). Therefore, it is assumed that treatment regimens increasing the DC-derived levels of IL-12 at the site of DC/T cell interaction during sepsis result in an improved bacterial clearance and, possibly, outcome.

As possible candidates to restore the capacity of DC from septic mice to release IL-12, we analyzed the immunomodulatory cytokines GM-CSF and IFN-γ. DC from sham mice responded to both agents with increased expression of the co-stimulatory molecule CD86 and enhanced secretion of IL-12 and TNF-α upon challenge with bacterial stimuli (Figures 1,6,7). GM-CSF and IFN-γ and, most effectively, a combination of both substances, increased the release of IL-12 from DC of septic mice only within the first 6 h after induction of sepsis (Figure 1). At 24 h after sepsis, DC were irreversibly changed and became refractory to both immunostimulatory substances in terms of IL-12 secretion (Figure 1). A downregulation of the IFN-γ-receptor might contribute, at least in part, to the unresponsiveness of DC to IFN-γ during the later phase of sepsis (Figure 5B). However, the missing responsiveness of DC to GM-CSF and IFN-γ during sepsis is restricted to the modulation of cytokine secretion, because the expression of the co-stimulatory molecule CD86 was enhanced upon treatment with either or both substances even 24 h after sepsis (Figure 6). This fact argues against a receptor-dependent mechanism for the failure of GM-CSF and IFN-γ to restore DC-derived IL-12 secretion, but rather indicates changes in the IFN-γ and GM-CSF receptors downstream signaling pathways in DC during sepsis.

IFN-γ signals via Janus kinase (JAK)-mediated phosphorylation of transcription factors termed signal transducer and activation of transcription (STAT) 1. Additional STAT1 activation can occur through mitogen-activated protein kinases (MAPK) that are also involved in TLR signaling (32). This cross-signaling between STAT1 and MAPK is supposed to account for the amplifying effect of IFN-γ on TLR ligand-induced gene activation. Similarly, increased MAPK activation is involved in the GM-CSF-mediated increase of TNF-α secretion upon stimulation with LPS (33,34). The majority of reports on GM-CSF- or IFN-γ-mediated signaling pathways has been performed with macrophages. Whether IFN-γ and GM-CSF-induced signaling pathways in splenic DC equal those of macrophages is not clear. The finding that macrophages maintain their responsiveness to GM-CSF during sepsis (Figure 7) argues against identical signaling events in macrophages and DC. This assumption is supported by a previous report that GM-CSF induces a diverse pattern of activated STAT molecules in DC and macrophages (35). Thus, a disturbance of the JAK/STAT and/or MAPK pathway might be responsible for the impaired sensitivity of DC to IFN-γ and GM-CSF during sepsis.

The present study shows that LPS-stimulated macrophages from septic mice increased the secretion of TNF-α upon exposure to GM-CSF and, thus, behaved like monocytes from septic patients (2). *Ex vivo* studies showing that GM-CSF and IFN-γ stimulated increased HLA-DR expression on monocytes from septic patients and enhanced LPS-induced TNF-α secretion prompted several clinical trials using GM-CSF or IFN-γ in sepsis therapy (36–39). However, treatment of trauma patients with IFN-γ showed only minor effects on the rate of infections and did not reduce mortality significantly, despite an IFN-γ-mediated increased HLA-DR expression on monocytes in all studies (40,41). In other trials, GM-CSF did not show any benefit in terms of postoperative septic complications (42) or survival, nevertheless it led to a faster clearance of the infection (43). Additionally, GM-CSF given 3 h after CLP had no protective effect on septic rats (44). The reasons why such immunomodulatory therapies did not accomplish previous expectations are not clear. Considering the requirement of intact DC function in the development of a protective immune response against the infection, the unresponsiveness of DC to GM-CSF and IFN-γ during

sepsis might explain the partial failure of IFN-γ and GM-CSF in sepsis therapy. To verify this hypothesis, further studies that correlate the responsiveness of DC to GM-CSF and IFN-γ with disease development after treatment with these cytokines must be performed in the same animal model.

A novel approach for immunotherapy of pancreatitis associated with the development of immunosuppression similar to sepsis is the application of a combination of GM-CSF and IFN-γ. *Ex vivo* treatment of monocytes from patients with acute pancreatitis with GM-CSF plus IFN-γ normalized both the suppressed HLA-DR expression and the impaired capacity to secrete TNF-α up to normal levels while each cytokine alone failed to elevate these immune functions (45). Similarly, the combination of GM-CSF and IFN-γ was the most effective in restoring IL-12 production of DC from septic mice in our model (Figure 1). The combination was still effective when the single substances failed to exert any effects (Figure 1B). Under this view, we expect that an efficient treatment of sepsis with the combination of GM-CSF and IFN-γ must be initiated within the first 6 h after the appearance of bacteria. The mechanisms that underlie the synergism of GM-CSF and IFN-γ have not yet been elucidated. Signaling molecules of the MAPK pathway are activated by both cytokines and therefore might amplify each other (32–34). Further studies are required to address this issue.

In summary, the development of immunosuppression during sepsis is associated with an impaired capacity of splenic DC and macrophages to release Th1-promoting cytokines upon stimulation with bacterial products. GM-CSF can restore the impaired cytokine secretion of macrophages, whereas DC are insensitive to GM-CSF and IFN-γ in this regard. Future studies should aim to understand the cause of DC dysfunction during sepsis and to find strategies that restore the unique features of DC as major players in the induction of protective Th1 cell responses.

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