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NK Cell Tolerance to TLR Agonists Mediated by Regulatory T Cells after Polymicrobial Sepsis

Fernando Souza-Fonseca-Guiamaraes, Marianna Parlato, Catherine Fitting, Jean-Marc Cavaillon, and Minou Adib-Conquy

As sensors of infection, innate immune cells are able to recognize pathogen-associated molecular patterns by receptors such as TLRs. NK cells present in many tissues contribute to inflammatory processes, particularly through the production of IFN-γ. They may display a protective role during infection but also a detrimental role during sterile or infectious systemic inflammatory response syndrome. Nevertheless, the exact status of NK cells during bacterial sepsis and their capacity directly to respond to TLR agonists remain unclear. The expression of TLRs in NK cells has been widely studied by analyzing the mRNA of these receptors. The aim of this study was to gain insight into TLR2/TLR4/TLR9 expression on/in murine NK cells at the protein level and determine if their agonists were able to induce cytokine production. We show, by flow cytometry, a strong intracellular expression of TLR2 and a low of TLR4 in freshly isolated murine spleen NK cells, similar to that of TLR9. In vitro, purified NK cells respond to TLR2, TLR4, and TLR9 agonists, in synergy with activating cytokines (IL-2, IL-15, and/or IL-18), and produce proinflammatory cytokines (IFN-γ and GM-CSF). Finally, we explored the possible tolerance of NK cells to TLR agonists after a polymicrobial sepsis (experimental peritonitis). For the first time, to our knowledge, NK cells are shown to become tolerant in terms of proinflammatory cytokines production after sepsis. We show that this tolerance is associated with a reduction of the CD27⁺ CD11b⁺ subset in the spleen related to the presence of regulatory T cells and mainly mediated by TGF-β. The Journal of Immunology, 2012, 188: 5850–5858.

A mong innate immune cells, NK cells have been known since the 1970s to play a central role during viral infection. It was only in the late 1980s that it appeared evident that NK cells also played a key role during bacterial infection. NK cells are abundant in tissues such as the lungs, the liver, the spleen, and the bloodstream (1). They contribute to the immuno-inflammatory reaction by the production of many cytokines, particularly IFN-γ and GM-CSF, two potent immune-stimulatory cytokines (2). GM-CSF has been shown to contribute to many inflammatory disorders (3). IFN-γ is a well-known proinflammatory cytokine and has been recognized as a factor involved in endotoxin-induced lethality (4, 5). Nevertheless, infectious models have demonstrated its protective role (6, 7). IFN-γ production by NK cells is regulated by cytokines such as IL-2, IL-12, IL-15, and IL-18 released by other cell types (2, 8). As a source of IFN-γ, NK cells can have either a protective role during infection, a deleterious effect during the systemic inflammation and lethality associated with experimental sepsis, or both (9, 10). NK cells have been shown to express TLRs, mainly by measuring mRNA. However, their expressions (mRNA or surface expression) remain controversial (11–14).

A reprogramming of circulating monocytes, neutrophils, and lymphocytes has been regularly reported in sepsis (15–17). Leukocytes of septic patients are poorly responsive to pathogen-associated molecular patterns (PAMPs) compared with cells from healthy subjects, reminiscent of the phenomenon of endotoxin tolerance (18–20). This modified immune status may facilitate the occurrence of secondary infections (20, 21). In addition, apoptosis detected in many organs, including the spleen, probably further contributes to the immunosuppression phase seen in sepsis (22). Despite the demonstration that the cytotoxic activity of NK cells is decreased in sepsis (23), very little is known about their ex vivo capacity to produce inflammatory cytokines.

To examine further the NK cell status during sepsis, we performed a murine polymicrobial sepsis model and investigated the protein expression of TLR2, TLR4, and TLR9 and the capacity of NK cells to respond to a subsequent in vitro challenge with TLR agonists in terms of IFN-γ and GM-CSF production. We further investigated the involvement of regulatory T cells, IL-10, and TGF-β in the modified status of NK cells.

Materials and Methods

Reagents

Abs against the following Ags were used: anti-NK-1.1–PE/Cy5, anti-F4/80–VioBlue, anti-CD3–PE, anti-CD4–A647, anti-CD25 (PC61), anti-CD25–FITC, anti-CD69–VioBlue, anti-Foxp3–Pacific blue, anti–IL-18Rα, anti–CD27–allophycocyanin, and anti–TLR4/CD284–PE from BioLegend (San Diego, CA), anti–NKp46–V450 from BD Horizon (San Diego, CA), anti–CD11b–A488, anti–NKp46–V450 from BD Biosciences (San Diego, CA), anti–NKp36–V450 from BD Biosciences (San Diego, CA), anti–IL-12Rα2–PE/Cy5 from Miltenyi Biotec (Bergisch-Gladbach, Germany), anti–IL-10Rα1–A488 from BioLegend (San Diego, CA), and anti–IL-4Rα–APC from Thermo Scientific. The corresponding isotype and fluorophore controls were obtained from the same companies. The murine recombinant cytokines (IL-2, IL-10, IL-12, IL-15) were from Peprotech (Rocky Hill, NJ) and IL-18 from MBL (Woburn, MA). Recombinant human TGF-β1 (Milenyi Biotec)
was used due to the absence of a commercially available murine cytokine and because of its functional cross-species reactivity in mouse cells (24). ELISA kits to detect GM-CSF, IFN-γ, TGF-β1, and TNF-α were obtained from R&D Systems and used according to the recommendations of the manufacturer. The multiplex combination used to detect eotaxin, KC, IFN-γ, IL-6, IL-10, IL-13, IL-12p40, IL-17, TNF-α, and VEGF was provided by Bio-Rad Laboratories (Hercules, CA). The annexin V/propidium iodide apoptosis detection kit was obtained from BD Pharmingen and used according to the recommendations of the manufacturer. The PAMPs used were LPS TLR grade from *Escherichia coli* serotype 0111:B4 (Alexis, San Diego, CA), Pam3CysSK4 (EMC Microcollections, Tubingen, Germany), and mCpG DNA 5'-TCCATGACGTTCCTGATGCT-3' (Sigma-Genosys, The Woodlands, TX). The TGF-β receptor inhibitor SB431542 was obtained from Invivogen (San Diego, CA) and the IL-10 inhibitor AS101 from Tocris Bioscience (Bristol, U.K.). Heat-killed *Escherichia coli* (strain 082746) was prepared with an exponential growing phase culture in lysogeny broth medium. Bacteria were washed with and resuspended in PBS and killed by incubation in a water bath at 65°C for 15 min.

**Mice**

The studies were performed with 8- to 12 wk-old male BALB/c(J), C57BL/6(J) (Janvier, Le Genest-St.-Isle, France), il-10<sup>−/−</sup> and Rag2<sup>−/−</sup> mice in a C57BL/6 background. Animals were housed in the Institut Pasteur animal facilities under specific pathogen-free conditions. Protocols, performed in compliance with the National Institutes of Health Animal Welfare Insurance No. A5476-01 (issued on February 7, 2007), were approved by the veterinary team of the Institut Pasteur animal facility.

**Preparation of murine NK cells and in vitro culture conditions**

NK cells were enriched from spleen homogenates by negative selection using the NK Cell Isolation Kit on untouched mouse NK Cells (Miltenyi Biotec). NK cell purity was assessed by flow cytometry after CD3<sup>−</sup>NKp46<sup>+</sup> staining and was more than 95%. After purification, cells were plated at 1 × 10<sup>6</sup> cells/well in 96-well plates in RPMI 1640 (Lonza, Rockland, ME) supplemented with 10% FCS (PAA Laboratories, Pasching, Austria), streptomycin at 0.1 mg/ml, and penicillin at 100 U/ml (Sigma-Aldrich, St. Louis, MO) and cultured for 48 h at 37°C and 5% CO<sub>2</sub> in the presence or absence of different cytokines and PAMPs. The cytokines IL-2, IL-12, IL-15, and IL-18 were added in the culture alone or combined in pairs (1 or 10 ng/ml) in the presence or absence of LPS or Pam3CysSK4 (100 ng/ml) or CpG-DNA (1 μM). For the in vitro inhibitory assay for NK cell activation, IL-10 or TGF-β1 (10 or 30 ng/ml) was added in the presence of PAMPs and the accessory cytokines previously mentioned.

In some experiments, peritoneal cells, splenocytes, and bone marrow cells prepared and cultured as previously described were used as controls (25).

**Flow cytometry**

NK cell surface Ags were labeled in staining buffer (PBS, EDTA 2 mM and FCS 0.5%) with Abs at the concentration suggested by the manufacturers. Intracellular staining of TLR2, TLR4, and TLR9 was performed after fixation and permeabilization of the cells by Inside Stain Kit (Miltenyi Biotec) according to the manufacturer’s instructions. All flow cytometry data were acquired and analyzed on a MACSQuant flow cytometer (Miltenyi Biotec). TLR Abs were previously tested on peritoneal macrophages as positive controls (data not shown).
In vivo polymicrobial sepsis and in vitro NK cell challenge with PAMPs

Polymicrobial intra-abdominal sepsis was induced by surgical cecal puncture (CP) at the large part of the ileocecal section (high-grade sepsis). To induce polymicrobial sepsis, a modified protocol was performed as previously described (26). Briefly, animals were anesthetized with an i.p. injection of ketamine (10%) and xylazine (2%) in saline. Then, a 10-mm midline incision was performed and 2 ml of saline at 37˚C was injected inside the cavity, and the cecum was carefully exposed to avoid damaging the blood vessels. Then, the cecal stump was punctured twice with a 26-gauge needle. A small amount of stool was expelled from the punctures to initiate the infectious process. The cecum was placed back into the peritoneal cavity in its normal position, and the abdomen was closed in two layers. Sham-operated mice were included as controls. Twenty-four hours later, spleen cells were harvested, and NK cells were isolated and challenged in vitro with PAMPs and cytokines as described before.

To assess bacterial peritonitis, the peritoneal cavity of each mouse was washed with 2 ml saline after 24 h. Fifty microliters of this solution was plated for 24 h on lysogeny broth agar plates at 37˚C to detect the presence of bacterial CFUs (data not shown).

NK cells/regulatory T cells or CD4+ coculture in the presence or absence of anti–TGF-β neutralizing Ab

The NK cells/regulatory T cells (Tregs) coculture was performed as described by Ghiringhelli et al. (27) with both direct cell contact and indirect contact (using Transwell chambers 0.4-µm pores; Corning, Acton, MA) culture conditions. Briefly, 5 × 10^5 NK cells from sham or CP mice were cultured alone, or with Tregs from CP mice or with CD4+ T cells from naive animals, in a ratio of 1:1 in the presence of IL-15/IL-18 and CpG DNA. Tregs (CD4+CD25+) and naive CD4+ (CD25−) T lymphocytes were isolated from spleen homogenates using the Regulatory T Cell Isolation Kit (Miltenyi Biotec), and the purity were assessed by the Treg Detection Kit (Miltenyi Biotec). In some wells, a neutralizing anti–TGF-β (clone 1D11) Ab or its isotype control at 1 µg/ml was added. Supernatants of these cocultures were harvested after 48 h, and IFN-γ and GM-CSF concentrations were determined by ELISA.

Tregs /TGFB-1,2,3 in vivo depletion

To deplete Tregs in vivo, an anti-CD25 Ab (clone PC61) or the corresponding isotype control Ab were injected i.p. in each mouse at 100 µg in a 200-µl final volume of saline 48 h before and just after CP. The degree of depletion was assessed by analyzing spleen CD4+CD25+Foxp3+ cells by flow cytometry.

To deplete TGFB-1,2,3 in vivo, an anti–TGF-β1,2,3 was used according to a previously described protocol (28). A pharmacological approach to target the TGF-βR signaling disruption was used as described by Chen et al. (29) by i.v. injection (10 mg/kg or vehicle) of SB431542, a specific inhibitor of the activin receptor-like kinase-5, 2 h prior to sepsis induction. The potential of action of the inhibitor was checked by flow cytometry by measuring phospho-Smad2,3 in total splenocytes treated in vitro with recombinant TGFB-1 at 10 ng/ml and SB431542 at 10 µM for 2 or 24 h (data not shown).

Statistical analysis

All experiments were performed at least three times using at least n = 3 mice per assay. One-way ANOVA and Fisher protected least significant difference or unpaired t test were used for statistical analysis. A p value <.05 was considered to be significant.
Results

TLR2, TLR4, and TLR9 expression and cytokine production by NK cells

mRNA expression of TLR2, TLR4, and TLR9 was previously reported in murine NK cells (13), as well as the capacity of these cells to respond to the agonists of these pattern recognition receptors (14). The surface expression of TLR2 and TLR4 remains somewhat controversial. We first analyzed their expression by flow cytometry on murine splenic NK cells. In contrast to peritoneal macrophages used as positive controls (Fig. 1A), we failed to detect cell surface expression of TLR2 and TLR4 on freshly isolated NK cells (Fig. 1B). Nevertheless, a large expression of TLR2 and a low expression of TLR4 were found intracellularly, as expected for TLR9.

To analyze the capacity of NK cells to respond to TLR agonists, we first defined the best culture conditions leading to the strongest activation by TLR2, TLR4, or TLR9 agonists (Pam3CysSK4, LPS, or CpG DNA, respectively) in the presence of different cytokines (IL-2, IL-12, IL-15, IL-18), alone or in pairs at 1 and 10 ng/ml. Among the different cytokine combinations used, only the IL-12/IL-18 pair failed to display a synergy with the different PAMPs, as by itself this combination led to the release of large amounts of IFN-γ and GM-CSF (Fig. 1C). In the other cases, a significant synergy was observed with accessory cytokines and Pam3CysSK4 or CpG. In all cases, the activation by LPS was not significantly higher than that obtained with the cytokines alone, in agreement with the low intracellular expression of TLR4. PAMPs alone and all cytokines added individually to PAMPs did not allow a significant production of IFN-γ and GM-CSF (data not shown).

Additionally, there was no TNF-α production by NK cells in these experimental conditions (data not shown). The two combinations that gave the strongest synergy with Pam3CysSK4 and CpG DNA (IL-15/IL-18 and IL-2/IL-18) were used in the subsequent experiments to stimulate NK cells ex vivo.

Ex vivo tolerance to TLR2, TLR4, and TLR9 agonists after polymicrobial sepsis

We investigated whether polymicrobial sepsis could lead to the tolerization of NK cells to TLRs agonists. Mice underwent a CP model of peritonitis. Sham mice were only subjected to the surgical procedures without CP. Twenty-four hours after surgery and CP, bone marrow cells, splenocytes, and purified spleen NK cells were incubated ex vivo with PAMPs. After polymicrobial sepsis and as expected, bone marrow cells and splenocytes showed an ex vivo tolerance to TLR2, TLR4, and TLR9 agonists as illustrated by a significant reduction of TNF-α production compared with that of sham-operated animals (Fig. 2A).

We then studied the capacity of purified spleen NK cells derived from CP mice to produce cytokines ex vivo in the presence of PAMPs and the IL-15 and IL-18 combination. After CP, NK cells displayed a significant decrease of their capacity to produce IFN-γ and GM-CSF compared with that of sham animals after challenge with LPS, Pam3CysSK4, and CpG (Fig. 2B). There was no production of IFN-γ or GM-CSF in response to non-CpG DNA, suggesting that the DNA recognition in NK cells is specific to CpG motifs. As E. coli is presumably one of the major bacterial agonists after CP, we tested the reactivity of NK cells to heat-killed E. coli. It can be seen that CP also induced a profound tolerance to heat-killed E. coli in terms of IFN-γ and GM-CSF production (Fig. 2B). Similar results were obtained in the presence of IL-2 and IL-18 (Supplemental Fig. 1). In order to know whether the tolerance of NK cells after CP was associated with a modification of surface or intracellular expression of TLRs, we measured their expression by flow cytometry. As shown...
on Fig. 2C, CP induced neither a modification of surface expression of TLR2 or TLR4 nor a modification of the intracellular expression of TLR2, TLR4, or TLR9. In addition, no differential expression of the early activation marker CD69 (Fig. 2C), IL-2Rα, IL-15Rα, or IL-18Rα (data not shown) was found with purified NK cells from CP and sham animals. In a more detailed analysis of this tolerance event, as illustrated in Fig. 3 in IL-15/IL-18 culture condition, all studied cytokines (IL-6, IL-10, IL-12p40, IL-13), chemokines (eotaxin, KC), and growth factor (VEGF) were significantly reduced compared with those of sham mice, suggesting a wide tolerance status of NK cells to the different TLR agonists. Similar results were observed under IL-2/IL-18 supplementation (Supplemental Fig. 2).

Because sepsis has also been associated with apoptosis, we checked for the occurrence of this phenomenon after CP. Twenty-four hours after CP, the number of NK cells recovered from the spleen and blood of CP mice was significantly decreased (37 and 50% of that of sham-operated animals, respectively), as already reported in the literature. Nevertheless, the percentage of apoptotic NK cells still detectable at that time point was below 3% (measured by flow cytometry after annexin V and propidium iodide stain), both in the blood and the spleen.

Reduction of the CD27⁺ CD11b⁻ subset in the spleen after CP

NK cells can be subdivided into different subsets depending on CD27, CD11b, or CXCR3 expression (30, 31). These subsets define different maturation stages, proliferation capacity, NK effector function, or IFN-γ production. We found that CP was associated with a significant decrease in percentage and absolute number of the CD27⁺ CD11b⁻ population in the spleen, but not of the other subsets (Fig. 4A). In contrast, the percentage and absolute number of CXCR3⁺ NK cells was not different between CP and sham-operated animals (Fig. 4B).

Treg depletion breaks NK cell tolerance to TLR2, TLR4, and TLR9 agonists

To verify the involvement of Tregs and their potential regulatory function on NK cells, a depletion of Tregs was achieved in vivo prior to polymicrobial sepsis. The i.p. injection of PC61 mAb 48 h before sepsis or surgery alone (sham mice) led to an ~90–95% depletion of Tregs (CD4⁺ CD25⁺ Foxp3⁺ cells) in the spleen and blood of both mice (data not shown). Treg depletion before CP led to a complete prevention of the tolerance status of NK cells in terms of IFN-γ and GM-CSF production in response to PAMPs, in the presence of IL-15 and IL-18 (Fig. 5A), or IL-2 and IL-18 (Supplemental Fig. 3). To corroborate the depletion in absence of an external mAb component, we used the same experimental design in a system that naturally lacks T cells (including Tregs), the Rag2²⁻/² mice. The NK cells from Rag2⁻⁻ mice also showed a complete prevention of the tolerance on NK cells under the same circumstances (Fig. 5B).
We observed that the IFN-γ conditions (e.g., CpG DNA in the presence of IL-15 plus IL-18). of cells, the experiment was performed in the best stimulatory analyzed using a neutralizing Ab. Because of the limiting number CSF production by NK cells in coculture experiments and if this Finally, we determined if Tregs were able to inhibit IFN-γ neutralizing Abs

for both IFN-γ and GM-CSF production. Results represent the mean

of values from IL-10 or TGF-β1 treatment versus control cells).

In vitro inhibition of IFN-γ and GM-CSF production by IL-10 or TGF-β1. IFN-γ and GM-CSF production by NK cells in coculture experiments and if this effect needed cell contact. Furthermore, the role of TGF-β was analyzed using a neutralizing Ab. Because of the limiting number of cells, the experiment was performed in the best stimulatory conditions (e.g., CpG DNA in the presence of IL-15 plus IL-18). NK cells were purified from spleen of sham and CP mice and were cultured alone or in the presence of Tregs of CP mice (Fig. 7A). We observed that the IFN-γ and GM-CSF production by NK cells of sham-operated animals was inhibited by Tregs. No significant inhibition was observed when NK cells were in culture with naive CD44 T lymphocytes, used as negative controls. Supporting our results with the pharmacological inhibitor of TGF-β used in vivo, the blockade of TGF-β with an Ab prevented the induction of tolerance by Tregs in vitro, especially for GM-CSF production. For NK cells of CP mice, the levels of IFN-γ and GM-CSF were

Table I. Role of IL-10 in NK cells tolerance

Tolerization on Purified NK Cells After CP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent (%) of Tolerization in Terms of IFN-γ Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>il-10/−/−</td>
</tr>
<tr>
<td>LPS</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>97 ± 6.1 63.1 ± 17.2</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>99.2 ± 3.6 82.6 ± 16.3</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>85.7 ± 11.9 53.6 ± 17.8</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>98.8 ± 5.9 68.9 ± 15.6</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>68.7 ± 7.9 55.1 ± 12.1</td>
</tr>
<tr>
<td>IL-15/18 – CP</td>
<td>73.1 ± 7.1 51.4 ± 20.6</td>
</tr>
<tr>
<td></td>
<td>il-10/−/−</td>
</tr>
<tr>
<td>LPS</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>88.34 ± 10.1 58.6 ± 20.9</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>91.88 ± 15.5 82.6 ± 17.5</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>91.9 ± 7.2 66.4 ± 24.6</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>79.9 ± 13.3 69.3 ± 21.5</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>79.1 ± 7.1 43.5 ± 19.4</td>
</tr>
<tr>
<td>IL-15/18 – CP</td>
<td>87.2 ± 13.1 52.8 ± 18.9</td>
</tr>
</tbody>
</table>

Cells from wild-type (WT) and IL-10–deficient (il-10/−/−) mice were compared in sham and CP groups. Values are represented for CP mice as percent of tolerization compared with sham for IFN-γ and GM-CSF production. Results represent the mean ± SEM of three different experiments, three mice/group each.

Table II. Role of TGF-β in NK cells tolerance

Tolerization on Purified NK Cells After CP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent (%) of Tolerization in Terms of GM-CSF Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>SB43</td>
</tr>
<tr>
<td>LPS</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>90.8 ± 11.4 0 ± 19.5***</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>96.4 ± 20.8 26 ± 23.3*</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>93.1 ± 15.2 16.3 ± 19.1**</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>98.3 ± 13.4 53.8 ± 7.8</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>56.9 ± 12.3 0 ± 4.1**</td>
</tr>
<tr>
<td>IL-15/18 – CP</td>
<td>80.28 ± 11.6 22.4 ± 8.6*</td>
</tr>
<tr>
<td></td>
<td>il-10/−/−</td>
</tr>
<tr>
<td>LPS</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>83.3 ± 12.3 33.9 ± 22.4</td>
</tr>
<tr>
<td>PAM3</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>70.7 ± 15.5 24.1 ± 19.9</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-2/18 – CP</td>
</tr>
<tr>
<td></td>
<td>42.8 ± 14 15.4 ± 4.1*</td>
</tr>
<tr>
<td>CpG</td>
<td>IL-15/18 – CP</td>
</tr>
<tr>
<td></td>
<td>65.3 ± 17.1 51.3 ± 5.9</td>
</tr>
</tbody>
</table>

Cells from vehicle or the TGF-β signaling inhibitor SB431542-treated mice were compared in sham and CP groups. Values are represented for CP mice as percent of tolerization compared with sham for IFN-γ and GM-CSF production. Results represent the mean ± SEM of three different experiments, three mice/group each.

*p < 0.05, **p < 0.01, ***p < 0.001.

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FIGURE 6. In vitro inhibition of IFN-γ and GM-CSF production by IL-10 or TGF-β1. IFN-γ and GM-CSF production by NK cells cultured in the presence TLR2, TLR4, or TLR9 agonists (Pam3CysSK4, LPS, and CpG DNA, respectively) was assessed in the presence of IL-15 plus IL-18 and of IL-10 or TGF-β1 at 10 or 30 ng/ml. Results represent the mean ± SEM of five different experiments performed with three mice/group each. *p < 0.05 (comparison between values from IL-10 or TGF-β1 treatment versus control cells).
NK cells of CP mice was very low, but the presence of Tregs further reduced this production. The addition of the anti–TGF-β neutralizing Ab partially prevented the inhibition induced by Tregs. These results show that NK cells can be tolerated by Tregs of CP mice in vitro, even in the absence of contact, and that TGF-β contributes in part to this inhibitory process.

Discussion

NK cells produce cytokines that contribute to the inflammatory environment during infection. IFN-γ, GM-CSF, and TNF-α are the main cytokines generated by activated NK cells (2). Nevertheless, the exact status of NK cells during bacterial sepsis and their capacity directly to respond to TLR agonists remains unclear. A large part of the knowledge on TLR expression in NK cells, from both human or mouse subjects, has been acquired in studies based on mRNA detection and remains controversial (32). In human NK cells, the expression of most TLR mRNA was found, with some differences depending on the study (11, 12, 33). Regarding murine NK cells, divergences in mRNA expression were also reported. One study showed that all TLR mRNAs were expressed, whereas in the other only TLR2, TLR4, and TLR9 mRNAs were detected (13, 14). However, mRNA detection is not equivalent to protein expression, which depends on translational and posttranslational events that will confer the functionality and the right cellular localization. This is well illustrated for human TLR2, of which the mRNA was found in NK cells (11), but without surface protein expression (34). In the current study, we also failed to demonstrate the expression of TLR2 and TLR4 on the surface of freshly isolated murine spleen NK cells. In contrast, and most interestingly, we are the first to our knowledge to demonstrate that both TLR2 and TLR4 are expressed inside murine NK cells, similarly to TLR9, a well-known intracellular TLR.

A leukocyte reprogramming involving TLR signaling, initially known as endotoxin tolerance, has been characterized as a reduced inflammatory responsiveness to a subsequent challenge by LPS or other PAMPs (20) after a first encounter with these microbial products or after different types of insults. Whereas this phenomenon has been widely described for monocytes and macrophages, we report for the first time our knowledge that spleen NK cells can display a similar type of tolerance to TLR agonists or heat-killed E. coli after polymicrobial sepsis. We also made a similar observation in mice injected with LPS (data not shown). We found that similarly to monocytes/macrophages (36, 37), this tolerance was not due to a decreased expression of the TLRs, involving probably more complex mechanisms. During sepsis, the “compensatory anti-inflammatory response syndrome” is illustrated by a reprogramming of immune cells (38). Immune status of NK cells is also affected during sepsis. NK cells’ cytotoxic activity in adult and newborn sepsis patients is decreased (23, 39, 40). Similar observations were reproduced in animal models after surgery, peritonitis, or injection of LPS or other TLR agonists (41–43). Sepsis is also associated with massive apoptosis (44). Indeed, similar to the literature (45), we found that the number of spleen...

**FIGURE 7.** In vitro inhibition of IFN-γ and GM-CSF production by Tregs of CP mice and effect of anti–TGF-β1 neutralizing Ab. **(A)** Coculture in the same well or **(B)** in Transwells. Full bars, NK from sham; hatched bars, NK from CP mice. Results represent the mean ± SEM of three different experiments. *p < 0.05, **p < 0.01 (NK + Treg versus NK cells alone), ***p < 0.05 (NK from sham + Tregs + anti–TGF-β Ab versus the same NK + Tregs without Ab), ****p < 0.05 (NK from CP + Tregs + anti–TGF-β Ab versus the same NK + Tregs without Ab).
NK cells was decreased after peritonitis, but in contrast to this study, IL-15 did not revert the sepsis-induced defect in IFN-γ production. However, the experimental conditions are very different, as IL-15 was given in vivo shortly (30 min) after cecal ligation and puncture by Inoue et al. (45), whereas in our case it was in vitro and 24 h post-CP. In addition, we stimulated purified NK cells, whereas Inoue et al. (45) performed ex vivo culture with total splenocytes and with a T cell-specific stimulus (anti-CD3/anti-CD28 Abs). In addition, sepsis was associated with a reduction of the NK CD27+CD11b− subset.

Tregs have been shown to contribute to the sepsis process. They are increased in percentage in the peripheral blood of sepsis patients compared with that of healthy controls (46), and their number is increased in the spleen of septic mice (47). Whereas their deletion does modify the outcome of septic mice (47), their transfer improved survival in the polymicrobial model of sepsis (54) and because the Treg population can be variable in terms of Foxp3 expression (55), a direct contribution of Tregs on leukocyte tolerance of NK cells. This is the first time, to our knowledge, that a direct contribution of Tregs on leukocyte tolerance of NK cells in vivo TLR tolerance of NK cells. Because the use of mice deficient for TGF-β is inappropriate in our model (54) and because the use of anti-TGF-β (28) was not satisfactory in our hands, we used a pharmacological specific inhibitor of TGF-β signaling (29). Its injection prior to CP led to a decreased tolerance of NK cells in terms of IFN-γ and GM-CSF production, suggesting a link between the TGF-β and NK cells in sepsis. The role of TGF-β in NK tolorization was confirmed in NK/Tregs coculture and in Transwell experiments, using a neutralizing anti–TGF-β Ab. In accordance with previous observations, we showed that the tolerance of NK cells was mostly dependent on TGF-β, corroborating a previous study showing that Tregs are able to inhibit NK cells (53).

Taken together, our data show that naive spleen NK cells express TLR2 and TLR4 intracellularly, as is the case for TLR9, and that a synergy with activating cytokines is required for cytokine production by murine NK cells in response to PAMPs. We also demonstrated that in polymicrobial sepsis, NK cells can undergo tolerance, and that this tolerance is mainly mediated by Tregs and the anti-inflammatory cytokine TGF-β.

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Disclosures
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