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Franco and V Cusumano
F Nicoletti, G Mancuso, F A Ciliberti, C Beninati, M Carbone, S


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Endotoxin-Induced Lethality in Neonatal Mice Is Counteracted by Interleukin-10 (IL-10) and Exacerbated by Anti-IL-10

FERDINANDO NICOLETTI,1 GIUSEPPE MANCUSO,2 FEDERICO ANZANI CILIBERTI,2 CONCETTA BENINATI,2 MARIA CARBONE,2 SABRINA FRANCO,3 AND VITALIANO CUSUMANO2*

Institutes of Microbiology, University of Milan, Milan,1 University of Messina, Messina,2 and University of Catania, Catania,3 Italy

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The lethal effects occurring in neonatal (<24-h-old) BALB/c mice after challenge with 25 mg of lipopolysaccharide (LPS) per kg of body weight were significantly counteracted by pretreatment with recombinant interleukin-10 (rIL-10; 25 or 50 ng/mouse). Concordantly, blockage of endogenous IL-10 with the SXC1 monoclonal antibody increased LPS-induced mortality. Both IL-10 and SXC1 modulated the release of tumor necrosis factor alpha (TNF-α) so that, relative to controls, peak TNF-α values after LPS challenge were decreased by rIL-10 and increased by anti-IL-10.

Interleukin-10 (IL-10) is a cytokine produced by T and B lymphocytes and macrophages that exerts pleiotropic effects on the immune system (19). Because IL-10 down-regulates the production of proinflammatory cytokines such as IL-12, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) (19), much attention has been focused during the last years on the use of this cytokine as an anti-inflammatory agent. IL-10 successfully counteracts the lethal effects of either Staphylococcus aureus enterotoxin B (1, 8) or lipopolysaccharide (LPS) (11) in adult mice, which, conversely, are rendered more sensitive to LPS by anti-IL-10 monoclonal antibody (MAb) (21).

Neonatal sepsis caused by gram-negative bacteria is a frequent cause of lethality and permanent disabilities (14, 15). In the mouse system, neonatal sepsis seems to differ from the adult form both from the pathophysiological and therapeutic points of view (16, 17). For example, IFN-γ is likely to play a more important role in adult than in neonatal rodent models, and we have demonstrated that LPS-induced IFN-γ release is markedly reduced in 24-h-old mice compared to the adult animals (4). Moreover, neonatal murine endotoxemia is more resistant than the adult form to treatment with TNF-α and eicosanoid inhibitors, possibly resulting from derangements in carbohydrate metabolism (9, 17, 23). Finally, neonatal pups produce significantly higher TNF-α levels in response to LPS than adults (4). However, except for our recent observation on the protective action of IL-10 in neonatal mice lethally infected with group B streptococcus (3), little is known about the effects of this cytokine in neonatal models of sepsis. This study was undertaken to examine the effects of IL-10 in a neonatal murine model of shock induced by LPS.

Neonatal (<24-h-old) BALB/c mice of both sexes were used. Parental mice were obtained from Harlan-Nossan (Milan, Italy). In preliminary experiments we observed that LPS-induced lethality was similar in female and male mice in both neonates and adults (data not shown). Pups from each litter were randomly assigned to control or experimental groups, marked, and kept with the mother. Before each experiment, LPS from Salmonella enteritidis (Sigma Chimica, Milan, Italy) was diluted in phosphate-buffered saline (PBS; 0.15 M NaCl-0.01 M phosphate, pH 7.2). The neonatal mice were weighed and injected subcutaneously with 25 μl of LPS at either of two doses (see Table 1).

To investigate the effects of systemic administration of recombinant murine IL-10 (rIL-10; Genzyme, Cinisello Balsamo, Italy), several groups of pups were treated with either PBS or rIL-10 according to the doses and administration schedules shown in Tables 1 and 2. As expected, most of the control mice died within 96 h after LPS injection (Table 1). In contrast, when neonatal mice were treated with either 25 or 50 ng of rIL-10 at 18 h prior to challenge a clear protective effect was observed (Table 1). Although a trend toward a reduced rate of mortality could also be noticed when 10 ng of rIL-10 per pup was administered, this effect was not statistically significant (Table 1). These data indicate that rIL-10 is protective in neonates at doses similar to those previously reported to protect adult mice against endotoxin shock (11). Moreover, the time of administration relative to LPS application was critical, with rIL-10 injection at later time points becoming progressively less effective (Table 2). While substantial protective effects were seen when rIL-10 was administered at 18 or 4 h prior to LPS challenge, simultaneous administration of the cytokine with LPS produced only a significant increase in survival time along with a slight, but not significant, reduction in mortality at the end of the experiment (Table 2). Therapeutic administration at 24 h after challenge was no longer effective (Table 2).

While these data provided evidence for the beneficial effects of exogenously administered rIL-10 in the treatment of neonatal endotoxemia, they did not provide information as to the role of endogenous IL-10 in the pathophysiology of this condition. To address this point, we first examined whether, as in adult murine and human sepsis (5, 18, 21), IL-10 is produced and released in the circulation of neonatal mice in response to LPS. For this purpose, pups were sacrificed prior to and at 4, 8, and 12 h after injection with 25 μg of LPS per kg. Plasma samples from three mice were pooled and used for IL-10 measurements. These were performed by solid-phase enzyme-linked immunosorbent assay (Bender Med System, Vienna, Austria). Samples were diluted 1:4 prior to testing in duplicate. The lower limit of sensitivity of the assay was 100 pg/ml. While IL-10 was below the detection threshold in the circulation before LPS challenge, levels of the cytokine in serum rapidly rose at both 4 and 8 h after LPS challenge, declining at 12 h (Fig. 1). In further experiments we evaluated the effects of
blockage of IL-10 with the neutralizing MAb SXC1, which is a rat anti-mouse IL-10 immunoglobulin M (20). This MAb was purified from hybridoma culture supernatants as described previously (3). A dose of 90 μg of SXC1 per pup, given subcutaneously at 6 h prior to LPS challenge, was used. The decision to use anti-IL-10 MAb at this dose and at this time point was made according to our previous experiments with neonatal mice lethally infected with group B streptococci (3), as well as according to kinetic studies showing that this treatment totally inhibited the LPS-induced release of IL-10 (Fig. 1). Thereafter, neonatal mice received either PBS or SXC1 (90 mg/mouse) at 6 h prior to challenge with a 20 or a 50% lethal dose of LPS. As shown in Table 3, the SXC1 MAb dramatically increased the rate of LPS-induced mortality, indicating that endogenous IL-10 may indeed play an important immunoregulatory and protective role in the development of neonatal sepsis.

Because TNF-α is known to be one, and perhaps the major, mediator of the lethal effects of LPS in both neonatal and adult models of sepsis (2, 6, 22), we next examined if rIL-10 or anti-IL-10 could modulate the TNF-α response in the development of endotoxin shock. Therefore, mice were pretreated with rIL-10, anti-IL-10 MAb, or PBS and sacrificed at different times after LPS challenge (Fig. 2). Plasma from three mice was pooled for TNF-α measurements, using a previously described bioassay (7). Results were expressed as units per milliliter, with 1 U being defined as the amount of cytokine causing 50% lysis of WEHI 164 clone 13 cells. Consistent with our previous study (9), serum TNF-α levels, which were undetectable prior to LPS challenge, reached peak values at 2 h and fell back below the limit of sensitivity of the assay at 4 h after challenge. Compared with values for PBS-treated animals, peak TNF-α values were dramatically increased by SXC1 MAb and reduced by rIL-10 treatment (P < 0.05). The ability of anti-IL-10 to augment TNF-α release is in agreement with previous studies conducted with both normal BALB/c mice (12) and autoimmunity-prone (NZB × NZW)F1 mice (13) where continuous administration of the same MAb led to elevated circulating levels of TNF-α.

| TABLE 1. Effects of various doses of rIL-10 on LPS-induced lethalitya | No. of dead mice (%) at:
<table>
<thead>
<tr>
<th>Expt and dose of IL-10 (ng/mouse)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
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<td></td>
<td></td>
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<tr>
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<td>8 (57)</td>
<td>9 (64)</td>
<td>10 (71)</td>
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<td>2 (14)</td>
<td>7 (50)</td>
<td>10 (71)</td>
<td>10 (71)</td>
</tr>
<tr>
<td>5</td>
<td>3 (21)</td>
<td>9 (64)</td>
<td>9 (64)</td>
<td>11 (79)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
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<td>9 (64)</td>
<td>11 (79)</td>
<td>11 (79)</td>
</tr>
<tr>
<td>10</td>
<td>3 (21)</td>
<td>4 (29)</td>
<td>6 (45)</td>
<td>8 (57)</td>
</tr>
<tr>
<td>25</td>
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<td>1 (7)</td>
<td>2 (14)</td>
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</tr>
<tr>
<td>3</td>
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<tr>
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<td>10 (71)</td>
<td>12 (86)</td>
<td>12 (86)</td>
</tr>
<tr>
<td>25</td>
<td>1 (7)</td>
<td>2 (14)</td>
<td>3 (21)</td>
<td>4 (29)</td>
</tr>
<tr>
<td>50</td>
<td>0 (0)</td>
<td>3 (21)</td>
<td>3 (21)</td>
<td>5 (35)</td>
</tr>
</tbody>
</table>

a Mouse pups (14 per group) were challenged with 25 mg of S. enteritidis LPS per kg.

b Time after LPS administration.

p < 0.05 by Fisher’s exact test compared with the respective vehicle control value.

| TABLE 2. Effect of time of administration of rIL-10 on LPS-induced lethalitya | No. of dead mice (%) at:
<table>
<thead>
<tr>
<th>Expt and rIL-10 administration time (h)b</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
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<td>10 (71)</td>
<td>12 (86)</td>
<td>13 (93)</td>
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<tr>
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</tr>
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<td>11 (79)</td>
<td>11 (79)</td>
<td>12 (86)</td>
</tr>
<tr>
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<td>5 (35)</td>
<td>10 (71)</td>
<td>10 (71)</td>
<td>13 (93)</td>
</tr>
</tbody>
</table>

a Mouse pups (14 per group) were challenged with 25 mg of S. enteritidis LPS per kg.

b Relative to time of challenge.

c Time after LPS administration.

p < 0.05 by Fisher’s exact test compared with the respective vehicle control value.

| TABLE 3. Effect of pretreatment with anti-IL-10 antibodies on lethality induced by S. enteritidis LPSa | No. of dead mice (%) at:
<table>
<thead>
<tr>
<th>Expt and pretreatment</th>
<th>LPS dose (mg/kg)</th>
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<th>72 h</th>
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</tr>
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<td>4 (28)</td>
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<tr>
<td>Anti-IL-10</td>
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<td>5 (35)</td>
<td>11 (79)</td>
<td>13 (93)</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>0 (0)</td>
<td>2 (14)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Anti-IL-10</td>
<td>5</td>
<td>1 (7)</td>
<td>8 (57)</td>
<td>10 (71)</td>
</tr>
</tbody>
</table>

a Mouse pups (14 per group) were pretreated with 90 μg of neutralizing rat anti-mouse IL-10 (SXC1) or PBS vehicle at 6 h before challenge with various doses of S. enteritidis LPS.

b Time after LPS administration.

c P < 0.05 by Fisher’s exact test compared with the respective vehicle control value.
The beneficial effects of exogenously administered IL-10 and the important immunoregulatory role of endogenous IL-10 shown here extend to neonatal murine endotoxemia the preventive efficacy of rIL-10 observed in adult mice (11, 21). However, the contemporaneous administration of IL-10 with LPS counteracted lethal effects in the adult but not in the neonatal model. With the neonates, an 18-h pretreatment was required to achieve optimal protection. It is possible that these differences reflect neonatal age-related changes in TNF-α production. Optimal reduction of TNF-α production was achieved in the present study only with relatively long pretreatment times (data not shown). Previous studies indicated that neonates produce significantly more TNF-α in response to LPS than adults (4). Indeed, in the present study rIL-10- and anti-IL-10-induced changes in lethality closely paralleled variations in TNF-α levels. Taken together these data indicate that the need for longer rIL-10 pretreatment times to achieve protective effects in mouse pups, relative to adult animals, may reflect differences in TNF-α responses. Anti-TNF-α antibodies totally prevented lethality in neonatal pups challenged with LPS (4) while only partial, albeit significant, protection was observed here after rIL-10 administration. This difference may be explained by the total inhibition of plasma TNF-α bioactivity by anti-TNF-α (4), while low levels of TNF-α persisted in rIL-10-treated animals.

Nonetheless, the facts that both neonatal and adult forms of murine endotoxemia respond favorably to IL-10 prophylaxis and that in both cases endogenous IL-10 plays an important immunoregulatory role show that similar IL-10-dependent defensive mechanisms operate in these two conditions after LPS challenge. This is in close agreement with the elevation of circulating IL-10 in human sepsis (5, 18) and further underscores the importance of endogenous anti-inflammatory mechanisms in the net outcome of sepsis. Taken together, these data indicate that approaches aimed at augmenting IL-10 bioactivity with either rIL-10 or specific agonists may be useful for the treatment of endotoxemia in both neonatal and adult patients.

Although rIL-10 was effective in the present study only when given prophylactically, it cannot be excluded that the cytokine is also effective therapeutically when given in conjunction with antibiotics and/or supportive measures. For example, therapeutic administration of anti-TNF-α, albeit ineffective alone, greatly increased the efficacy of antibiotics in a neonatal model of group B streptococcal infection (10). Further studies, however, are needed to better analyze the therapeutic potential of rIL-10 in neonatal sepsis.

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