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Interleukin-12 Induces Sustained Activation of Multiple Host Inflammatory Mediator Systems in Chimpanzees


To determine in vivo effects of interleukin (IL)-12 on host inflammatory mediator systems, 4 healthy chimpanzees received recombinant human IL-12 (1 μg/kg) by intravenous injection. IL-12 induced increases in plasma concentrations of IL-15, IL-18, and interferon-γ (IFN-γ), plus a marked antinflammatory cytokine response (IL-10, soluble tumor necrosis factor [TNF] receptors, IL-1 receptor antagonist) and secretion of α-chemokines (IL-8, IFN-γ-inducible protein 10) and β-chemokines (monocyte chemoattractant protein-1, macrophage inflammatory protein-1β). In addition, IL-12 elicited neutrophilic leukocytosis, neutrophil degranulation (elastase-α1-antitrypsin complexes), coagulation activation (F1 + 2 prothrombin fragment, thrombin–antithrombin III complexes), and fibrinolytic activation (tissue-type plasminogen activator, plasmin–α2-antiplasmin complexes). IL-12–induced activation of multiple host mediator systems was found only after 8–24 h, remained detectable until the end of the 48-h observation period, and occurred in the absence of detectable TNF and IL-1β. These data may contribute to understanding the role of IL-12 in the pathogenesis of sepsis syndrome and the toxicity found after repeated injections of IL-12.

Interleukin (IL)-12 is a heterodimeric proinflammatory cytokine formed by two covalently linked subunits of 35 kDa (p35) and 40 kDa (p40) [1]. Both chains, which are encoded by different and unrelated genes, have to be produced for the formation of the biologically active p70 heterodimer. The main producer cells of IL-12 are monocytes, macrophages, and other antigen-presenting cells. The most potent stimuli for IL-12 production are bacteria, bacterial products (e.g., endotoxin), and intracellular parasites. Most biologic effects of IL-12 involve activities on NK cells and T lymphocytes. IL-12 can induce the production of interferon-γ (IFN-γ) and other cytokines and enhances cytotoxic activities of NK and lymphokine-activated killer cells. Furthermore, IL-12 plays an important role in the pathogenesis of infectious diseases by promoting the differentiation of naive CD4 T cells into Th1 cells [1, 2].

Studies in patients and experimental animals have shown that IL-12 is produced during sepsis and endotoxemia [3–6]. Importantly, in a murine model of endotoxemia, passive immunization against IL-12 offered protection against the lethal effects of endotoxin [7]. Whereas bacille Calmette Guérin–primed mice died after a dose of 10 μg of endotoxin, all mice preinjected with an anti–IL-12 monoclonal antibody (MAb) survived. At higher endotoxin doses, anti–IL-12 still strongly reduced lethality. Therefore, it is likely that endogenously produced IL-12 plays an important role in the toxic sequelae of sepsis and endotoxemia.

During sepsis and endotoxemia, several host inflammatory mediator systems become activated, including the cytokine network, leukocytes, and the hemostatic mechanism [8, 9]. Excessive activation of each of these mediator systems is considered to contribute to the development of tissue injury and mortality during sepsis. The effects of IL-12 on these inflammatory cascades are largely unknown. Such knowledge may also be relevant for the understanding of IL-12 toxicity reported in cancer patients [10]. Therefore, in the present study, we sought to determine the effects of a bolus intravenous (iv) injection of recombinant human (rh) IL-12 on the cytokine network, leukocytes, coagulation, and fibrinolysis in healthy chimpanzees.
Materials and Methods

Study design. Four healthy adult chimpanzees (Pan troglodytes; 22.4–35.4 kg) from the primate colony at the Biomedical Primate Research Center (BPRC, Rijswijk, The Netherlands) were studied. None of the animals had abnormalities on routine laboratory investigation. The chimpanzees were sedated with ketamine chloride intramuscularly and, after intubation, were kept under general anesthesia with nitrous oxide and halothane until 4 h after IL-12 administration. Vital functions were registered continuously during this period. Follow-up sampling after the first 4 h was done in animals that were briefly sedated with ketamine chloride. Each animal received an iv bolus injection of rhIL-12 (provided by Genetics Institute, Andover, MA) at a dose of 1 µg/kg of body weight (specific activity, 1.1 × 10^7 U/mg of rhIL-12). Previous in vitro work demonstrated that human IL-12 is active on chimpanzee peripheral blood mononuclear cells, as reflected by its capacity to induce the production of IFN-γ (data not shown). Control studies have shown that the experimental procedures themselves do not elicit changes in the inflammatory parameters under investigation [11, 12].

Sampling and assays. Venous blood samples were obtained by separate venipunctures directly before IL-12 injection and at 0.5, 1, 2, 3, 4, 8, 24, and 48 h thereafter. It should be noted that IL-12-induced activation of inflammatory cascades occurred relatively late. Therefore, for reasons of clarity, results are presented from time 2 h onward (see Results). For pharmacokinetic studies, additional blood samples were drawn at 1.5, and 15 min after IL-12 injection. All blood samples (except samples for determination of leukocyte counts) were centrifuged at 4°C for 20 min at 1600 g; plasma was collected and stored at −20°C until assays were performed.

Assays of coagulation and fibrinolysis were performed with citrated plasma, all other assays were done with EDTA-anticoagulated plasma. Plasma levels of IL-12 p70 were determined by ELISA using anti–IL-12 p70 MAb 20C2 as coating antibody, biotinylated anti–IL-12 p40 MAb C8.6 as detecting antibody, and rhIL-12 as standard (detection limit, 8 pg/mL). 20C2 was provided by M. K. Gately (Hoffmann–La Roche, Nutley, NJ); C8.6 was donated by G. Trinchieri (Wistar Institute, Philadelphia). For pharmacokinetic analyses, IL-12 concentration data were plotted versus time on a log-scale axis. Terminal elimination phase was determined by inspection (time points from 1 h onward), and the t½ of this phase for each animal was determined by log-linear regression. The following cytokines were measured by ELISA according to the instructions of the manufacturer (with detection limits): IFN-γ (CLB, Amsterdam; 2.5 pg/mL), IL-6 and IL-10 (PharMingen, San Diego; both 8 pg/mL), tumor necrosis factor (TNF) and IL-1β (Medgenix, Fleurus, Belgium; 7 and 8 pg/mL, respectively), and IL-15 (R&D Systems, Abingdon, UK; 4 pg/mL). IL-18 was measured by ELISA exactly as described previously (Fujisaki Institute, Fujisaki, Japan; detection limit, 10 pg/mL) [13].

Plasma concentrations of IL-2 were determined by ELISA using mouse anti–human IL-2 MAb as coating antibody, biotinylated rabbit anti–human IL-2 as detecting antibody, and rhIL-2 (Medgenix) as standard (detection limit, 7 pg/mL). The anti–human IL-2 antibodies were donated by P. H. van der Meide (BPRC). Soluble TNF receptors (sTNFR) types I and II were measured by enzyme-linked immunologic binding assay as described previously [14]. The reagents for sTNFR measurements were donated by Hoffmann–La Roche (both detection limits, 78 pg/mL). IL-1 receptor antagonist (ra) was measured by ELISA using mouse anti–human IL-1ra MAb (Antibody Solutions SARL, Illkirch, France) as coating antibody, biotinylated goat anti–human IL-1ra (R&D Systems) as detecting antibody, and rhIL-1ra (R&D Systems) as standard (detection limit, 41 pg/mL).

Chemokines were measured by ELISA according to the instructions of the manufacturer (with detection limits): IL-8 (CLB; 1 pg/mL), monocyte chemoattractant protein-1 (MCP-1) (PharMingen; 8 pg/mL), macrophage inflammatory protein (MIP)-1β and MIP-1α (R&D Systems; both 15.6 pg/mL). IFN-γ-inducible protein 10 (IP-10) was determined by ELISA using mouse anti–human IP-10 as coating antibody, biotinylated goat anti–human IP-10 as detecting antibody (both R&D Systems), and recombinant human IP-10 as standard (Biosource, Camarillo, CA; detection limit, 20 pg/mL). Leukocyte and differential counts were determined by flow cytometry. Plasma levels of elastase-α, antithrombin complexes were assayed by RIA as described previously [15]. Activation of the coagulation system was determined by measuring the prothrombin fragment F1+2 and thrombin–antithrombin III (TAT) complexes (ELISAs; Behringwerke, Marburg, Germany). Fibrinolytic activation was monitored by measurements of tissue-type plasminogen activator (ELISA; Innogenetics, Nijmegen, The Netherlands), plasmin–α2-antiplasmin complexes (ELISA, Behringwerke), and plasminogen activator inhibitor type 1 (PAI-1) (ELISA; Innogenetics) as described previously [12].

Statistical analysis. All values are given as mean ± SE. Changes in time were analyzed by one-way analysis of variance. Differences from baseline levels were assessed by the Dunnett t test for multiple comparisons where appropriate. P < .05 was considered to represent a significant difference.

Results

Plasma IL-12 levels. Baseline levels of the biologically active IL-12 p70 heterodimer were below the detection limit. After IL-12 administration, plasma levels peaked at 24.57 ± 1.48 ng/mL 1 min after injection and remained detectable at high concentrations long thereafter (8 h: 11.86 ± 0.38 ng/mL (figure 1). Plasma t½ of IL-12 during the elimination phase was 10.6 ± 0.5 h.

Clinical features. IL-12 administration was not associated with a febrile response or hemodynamic changes during the first 4 h of the experiment (data not shown). After discontinuation of the anesthesia, all chimpanzees appeared healthy and behaved normally. None of the animals had clinical complications due to IL-12 administration.

Cytokines. IL-12 injection induced the production of both pro- and antiinflammatory cytokines. IL-12 elicited increases in plasma concentrations of IL-15, IL-18, and IFN-γ, starting after 4 h and peaking after 24 h (IL-15: 21.5 ± 2.3 pg/mL; IFN-γ: 1170 ± 438 pg/mL) or 48 h (IL-18: 114 ± 24 pg/mL) (figure 2; all P < .05). In contrast, neither TNF nor IL-1β became
detectable at any time point. Also, IL-2 remained undetectable, whereas IL-6 showed a variable, nonsignificant increase (8 h: 129 ± 104 pg/mL). IL-12 elicited a strong anti-inflammatory cytokine response, as reflected by late increases in the plasma concentrations of IL-10, sTNFR-I and -II, and IL-1ra, all peaking at the end of the 48-h study period (table 1; all P < .05).

Chemokines. IL-12 injection resulted in the production of both α- and β-chemokines (table 2). Whereas IL-8, the prototype of C-X-C chemokines, showed a slight, nonsignificant rise, IP-10 levels increased strongly. Also, C-C chemokines MCP-1 and MIP-1β reached high levels after 48 h, although this increase was significant for MCP-1 only. In contrast, MIP-1α levels remained undetectable.

Leukocytes. IL-12 injection resulted in a marked leukocytosis (peak at 8 h: 23.4 ± 3.7 × 10⁹/L), mainly caused by neutrophilia (peak at 8 h: 20.2 ± 3.6 × 10⁹/L; both P < .05) (figure 3). Monocyte counts increased modestly, whereas lymphocyte counts decreased (data not shown). IL-12 was also capable of inducing neutrophil degranulation, as reflected by increased plasma levels of elastase–α₁-antitrypsin complexes (peak at 48 h: 148 ± 26 ng/mL; P < .05) (figure 3).

Coagulation and fibrinolysis. IL-12 injection resulted in activation of the common pathway of the coagulation system, as indicated by increases in prothrombin fragment F1 + 2 levels and TAT complexes in the circulation, both reaching a plateau between 8 and 48 h (F1 + 2: 1.78 ± 0.14 nmol/L at 24 h; TAT complexes: 31.73 ± 4.91 μg/L at 48 h; both P < .05) (figure 4A). Activation of the coagulation system was followed by activation of the fibrinolytic system, as reflected by increased plasma levels of tissue-type plasminogen activator and plasma–α₂-antiplasmin complexes, both peaking after 48 h (16.3 ± 1.7 ng/mL and 1520 ± 340 μg/L, respectively) (figure 4B). Plasma levels of plasminogen activator inhibitor type 1 did not change after IL-12 injection (data not shown).

Discussion
Sepsis is associated with the excessive activation of a number of inflammatory cascades, ultimately leading to tissue injury and organ failure [8, 9]. Several lines of evidence support the existence of an important role of IL-12 in the pathogenesis of sepsis syndrome. First, the production of IL-12 is enhanced during sepsis and endotoxemia [3–6]. Second, and of more importance, neutralization of endogenous IL-12 activity confers protection against lethality induced by endotoxin in mice [7]. Importantly, administration of IL-12 to cancer patients has been associated with systemic toxicity. These data prompted us to study the effects of circulating IL-12 on host mediator systems that are known to become activated during severe bacterial infection. It was found that a bolus iv injection of IL-12 induced (besides a marked rise in IFN-γ levels) a strong antiinflammatory cytokine and chemokine response in conjunction with activation of neutrophilic granulocytes, coagulation, and fibrinolysis.

IL-12 injection induced qualitatively similar inflammatory responses as previously reported after administration of endotoxin or live bacteria to humans and/or nonhuman primates [4, 11, 12, 16–20]. There are, however, important differences between systemic inflammatory effects induced by IL-12 and...
those elicited by endotoxin or bacteria. Indeed, IL-12-induced effects occurred relatively late, characteristically peaking after 24–48 h. In contrast, iv injection of endotoxin results in a rapid activation of inflammatory pathways, which usually is detectable within 1–3 h and is offset after 12 h [11, 12, 16–18]. Infusion of a lethal dose of *Escherichia coli* in baboons is also associated with a brisk stimulation of multiple inflammatory cascades within the first hour, although in this situation, at least part of the responses may remain detectable until death [4, 19].

In both sublethal endotoxemia and lethal bacteremia, an early transient rise in the plasma concentrations of TNF can be detected. Neutralization of this endogenous TNF activity abrogates many of the subsequent inflammatory responses and prevents death [16, 18, 21, 22]. Endogenous IL-1 activity also plays an important role in the inflammatory sequelae of lethal bacteremia, since treatment with recombinant IL-1ra strongly attenuates activation of neutrophilic granulocytes and coagulation and improves survival in septic baboons [19, 23]. It is therefore of considerable interest that IL-12–induced effects occurred in the absence of detectable plasma levels of TNF or IL-1β, suggesting that such effects are mediated via pathways that are independent of these two potent proinflammatory cytokines.

Previous studies in humans and nonhuman primates have established that, as with IL-12, administration of recombinant TNF or IL-1 can reproduce observable systemic inflammatory responses in patients or experimental animals with sepsis [19, 24–26]. However, significant differences exist between the kinetics of IL-12–induced effects and those triggered by TNF or IL-1. Whereas IL-12 effects are delayed, the responses to iv TNF and IL-1 are rapid and in part occur even faster than responses induced by endotoxin or live bacteria. Further, whereas IL-12 effects are sustained, TNF and IL-1 effects are transient. Conceivably, the latter difference is related to the long circulating half-life of IL-12. Indeed, whereas cytokines characteristically have a plasma half-life of <30 min, the IL-12 heterodimer was found to have a t1/2 of 10.6 h in the circulation.

Remarkably, IL-12 was able to induce a strong and sustained activation of the coagulation system, which was followed by activation of the fibrinolytic system. IL-12 has been shown to play an important role in the pathogenesis of the generalized Shwartzman reaction in mice, a systemic inflammatory reaction characterized by thrombosis of renal glomeruli and other organs, and disseminated intravascular coagulation [27]. Together, these data suggest that IL-12 may play a role in the hemostatic disorders frequently observed during sepsis [9]. It should be noted that iv administration of endotoxin, bacteria, TNF, or IL-1 to humans and/or nonhuman primates is associated with a rapid activation of fibrinolysis, followed by a more delayed activation of coagulation [9, 12, 16, 18, 19, 24, 25]. In these models, activation of fibrinolysis proceeds via a route that is independent from activation of coagulation, and vice versa [9, 12, 16, 18]. To our knowledge, IL-12 is the first inflammatory mediator that causes a late fibrinolytic response after initial activation of coagulation.

The mechanisms by which IL-12 elicited a large variety of systemic inflammatory responses are not elucidated by our study. Little is known about possible direct effects of IL-12 on the mediator systems that were monitored. IL-12 injection resulted in a marked increase in the plasma concentrations of IFN-γ, which is considered to be an important mediator of many IL-12 activities. In vitro studies and studies in mice have shown that IL-12 can induce optimal IFN-γ by T lymphocytes and NK cells only in the presence of other lymphocyte- or monocyte-derived cytokines, including TNF, IL-2, and IL-15 [28–30]. In addition, IL-12 synergizes with IL-18, also known as IFN-γ–inducing factor, for optimal IFN-γ production by T lymphocytes and NK cells [31, 32]. An important mechanism for this synergy is that IL-12 increases the responsiveness of cells to IL-18, likely by up-regulation of IL-18 receptors [33].

The importance of the synergistic effect of IL-18 on IFN-γ production has been demonstrated in IL-18–deficient mice, which produce little IFN-γ despite normal IL-12 levels [34]. In addition, splenocytes of mice lacking IL-1β–converting enzyme, which converts pro–IL-18 to bioactive IL-18, produce reduced

<p>| Table 1. The anti-inflammatory cytokine response to an intravenous injection of IL-12. |
|----------------------------------|-----|-----|-----|-----|</p>
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IL-10 (pg/mL)</th>
<th>sTNFR-I (ng/mL)</th>
<th>sTNFR-II (ng/mL)</th>
<th>IL-1ra (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;8</td>
<td>1.34 ± 0.16</td>
<td>2.49 ± 0.57</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>2</td>
<td>47 ± 27</td>
<td>1.33 ± 0.10</td>
<td>2.50 ± 0.46</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>4</td>
<td>60 ± 25</td>
<td>1.27 ± 0.11</td>
<td>2.55 ± 0.54</td>
<td>0.26 ± 0.26</td>
</tr>
<tr>
<td>8</td>
<td>159 ± 45</td>
<td>1.80 ± 0.32</td>
<td>3.63 ± 0.85</td>
<td>2.02 ± 1.30</td>
</tr>
<tr>
<td>24</td>
<td>349 ± 85</td>
<td>2.02 ± 0.11a</td>
<td>5.27 ± 0.79</td>
<td>9.26 ± 2.10a</td>
</tr>
<tr>
<td>48</td>
<td>1569 ± 652a</td>
<td>2.71 ± 0.18a</td>
<td>8.67 ± 1.23a</td>
<td>14.96 ± 1.62a</td>
</tr>
<tr>
<td><em>P</em></td>
<td>&lt;0.5 NS</td>
<td>&lt;0.5 &lt;0.5</td>
<td>&lt;0.5 &lt;0.5</td>
<td>&lt;0.5 &lt;0.5</td>
</tr>
</tbody>
</table>

NOTE. Values are mean ± SE of 4 chimpanzees. Recombinant human IL-12 (1 μg/kg) was given as intravenous bolus injection at time 0. *P* reflects changes in time analyzed by 1-way analysis of variance. sTNFR, soluble tumor necrosis factor receptor; ra, receptor antagonist.

* P < 0.05 vs. baseline by Dunnett t test.

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<p>| Table 2. Chemokine production after an intravenous injection of IL-12. |
|----------------------------------|-----|-----|-----|-----|</p>
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IL-8 (pg/mL)</th>
<th>IP-10 (ng/mL)</th>
<th>MCP-1 (pg/mL)</th>
<th>MIP-1α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;1</td>
<td>0.26 ± 0.05</td>
<td>160 ± 18</td>
<td>222 ± 125</td>
</tr>
<tr>
<td>2</td>
<td>2.38 ± 2.38</td>
<td>0.24 ± 0.04</td>
<td>181 ± 22</td>
<td>202 ± 112</td>
</tr>
<tr>
<td>4</td>
<td>8.60 ± 4.98</td>
<td>0.32 ± 0.04</td>
<td>257 ± 39</td>
<td>236 ± 120</td>
</tr>
<tr>
<td>8</td>
<td>7.28 ± 4.75</td>
<td>1.66 ± 0.30</td>
<td>397 ± 51</td>
<td>545 ± 156</td>
</tr>
<tr>
<td>24</td>
<td>6.25 ± 0.36</td>
<td>19.15 ± 7.61a</td>
<td>1,554 ± 353a</td>
<td>669 ± 195</td>
</tr>
<tr>
<td>48</td>
<td>11.18 ± 1.62</td>
<td>34.84 ± 7.55a</td>
<td>1,830 ± 458a</td>
<td>726 ± 279</td>
</tr>
</tbody>
</table>

NOTE. Values are mean ± SE of 4 chimpanzees. Recombinant human (rh) IL-12 (1 μg/kg) was given as intravenous bolus injection at time 0. *P* reflects changes in time analyzed by 1-way analysis of variance. IP, interferon-γ–inducible protein; MCP, monocyte chemotactant protein; MIP, macrophage inflammatory protein; NS, not significant. MIP-1α remained <15.6 pg/mL at all time points.

* P < 0.05 vs. baseline by Dunnett t test.
IFN-γ levels during in vitro stimulation [35]. IL-18 can induce a number of effects without any cofactor, for example, production of several cytokines and chemokines (TNF, IL-1β, IL-8, MIP-1α), activation of nuclear factor κB, enhancement of Fas ligand expression, and induction of apoptosis [36, 37, 38]. Therefore, IL-18 is a potent cytokine itself and plays an essential synergistic role with IL-12 in the production of IFN-γ. Furthermore, C-C chemokine receptor 2, the primary receptor for MCP-1, is required for maximal IFN-γ production during a Th1 response in mice, suggesting that MCP-1 is involved in optimal IFN-γ production [39]. In the present study, IL-12-induced IL-15, IL-18, and MCP-1 may therefore have contributed to the strong increase in IFN-γ levels.

It can be argued that the effects seen after IL-12 injection are mediated by IFN-γ and not by IL-12 itself. For instance, IFN-γ has been reported to induce the production of MCP-1 and IP-10 in vitro [40, 41]. However, many changes (anti-inflammatory cytokine response, neutrophilia, and activation of coagulation) were already going on while levels of IFN-γ were either undetectable or very low. In addition, studies in IFN-γ receptor knockout mice have shown that they remain capable of mounting an inflammatory response after administration of IL-12 [42].

It should be noted that, in our study, the plasma concentrations of IL-12 p70 were at least 1 log higher than those commonly found in severe sepsis. It is important to realize, however, that during sepsis, plasma IL-12 levels are a reflection of tissue IL-12 concentrations, which are conceivably much higher. Nonetheless, our findings should be interpreted with caution in the context of the role of IL-12 in the pathophysiology of sepsis.

Daily iv administration of rhIL-12 at a dose of 0.5 µg/kg (i.e., half the dose used in the present study) to cancer patients has been associated with serious toxicity and two deaths that were considered to be related to the use of IL-12 [10]. The toxicity found in that phase 2 trial was unexpected, since in an earlier phase 1 study, in which the first IL-12 injection was given 13 days before the start of a 5-day cycle of daily iv IL-12 injections, the maximum tolerated dose was established at 0.5 µg/kg [43]. Additional studies in mice and monkeys, performed after this calamity, have revealed that a single first IL-12 dose 2 weeks before a cycle of daily IL-12 administrations has an unexpected abrogating effect on IFN-γ production and toxicity [10]. Our present findings indicate that careful attention is required when IL-12 is given on consecutive days, considering the risk of a cumulative effect of repeated IL-12 injections on systemic inflammatory responses.

A single injection of IL-12 caused a delayed and sustained activation of multiple inflammatory pathways in the absence of detectable plasma concentrations of TNF and IL-1α. These findings may be relevant for the understanding of the role of IL-12 in the pathogenesis of sepsis syndrome and the toxicity
found in cancer patients after repeated injections of recombinant IL-12.

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References


