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Subcutaneous injection of interleukin 12 induces systemic inflammatory responses in humans: implications for the use of IL-12 as vaccine adjuvant

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Abstract Interleukin 12 (IL-12) is a cytokine with important regulatory functions bridging innate and adaptive immunity. It has been proposed as an immune adjuvant for vaccination therapy of infectious diseases and malignancies. The inflammatory properties of IL-12 play an important role in the adjuvant effect. We studied the effect of s.c. injections of recombinant human IL-12 (rHuIL-12) in 26 patients with renal cell cancer and demonstrated dose-dependent systemic activation of multiple inflammatory mediator systems in humans. rHuIL-12 at a dose of 0.5 µg/kg induced degranulation of neutrophils with a significant increase in the plasma levels of elastase ($p < 0.05$) and lactoferrin ($p = 0.01$) at 24 h. Additionally, rHuIL-12 injection mediated the release of lipid mediators, as demonstrated by a sharp increase in the plasma secretory phospholipase A₂ (sPLA₂) level ($p = 0.003$). rHuIL-12, when administered at a dose of 0.1 µg/kg, showed minimal systemic effects. In conclusion, when IL-12 is used as an adjuvant, doses

should not exceed 0.1 µg/kg, in order to avoid severe systemic inflammatory responses.

Keywords Elastase · Interleukin 12 · Lactoferrin · Neutrophils · sPLA₂

Introduction

Interleukin 12 (IL-12) is a cytokine with important immunoregulatory functions. IL-12 stimulates T cells and NK cells to produce IFN- γ , and increases cytotoxic activity by NK cells. Additionally IL-12 stimulates the helper activity of CD4 T cells toward cellular immune responses and enhances antigen-specific CD8⁺ T-cell responses [49]. As a stimulator of the cellular immune response, IL-12 has potential efficacy in malignant diseases. Studies of systemic cytokine therapy with IL-12 have been performed in different types of cancer [4, 23, 28–30, 37, 43, 54, 55]. However, IL-12, administered either intravenously or subcutaneously, showed disappointing efficacy and, especially with higher dose schedules, substantial toxicity [4, 29, 30, 37].

Recently, it was proposed that IL-12 might be an effective immune adjuvant for vaccination therapy of infectious diseases and malignancies [9, 21, 27, 44]. Also the combination of a monoclonal antibody and IL-12 has been studied [3]. The immunological mechanisms underlying the adjuvant efficacy of IL-12 are not fully defined. Adjuvant substances are strong stimulators of local inflammation, and the proinflammatory characteristics of IL-12 are thought to contribute to its adjuvant effects.

Polymorphonuclear neutrophils (PMNs) are important effectors of the inflammatory response, because they release toxic compounds such as proteases upon activation that damage the microenvironment. Few

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(pre)clinical studies have addressed the effects of IL-12 on PMNs. Following administration of IL-12 to chimpanzees, degranulation of PMNs was observed [26]. In vitro studies have revealed that PMNs express IL-12 β 1 receptors and that binding of IL-12 results in actin polymerization and a concentration-dependent increase in reactive oxygen metabolites in PMNs [13]. Treatment of human neutrophils with IL-12 led to a transient increase in intracellular free calcium [2]. Besides potential direct effects through IL-12 receptor engagement, IL-12 could also exert indirect effects on PMNs through the induction of other proinflammatory cytokines such as tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), or interleukin 8 (IL-8).

The degranulation of PMNs in various physiological situations is appropriately reflected by levels of elastase- α 1-antitrypsin complexes and lactoferrin in the peripheral blood [32, 48, 53]. Elastase is a proteinase released from azurophilic granules of neutrophils that rapidly forms complexes with its natural inhibitor α 1-antitrypsin, while lactoferrin is derived from the specific granules [56]. The increased release of secretory phospholipase A₂ (sPLA₂) from endothelial and other cells is considered another component of the inflammatory cascade [41]. sPLA₂ is a lipolytic enzyme that releases fatty acids, often arachidonic acid, from membrane phospholipids for production of important lipid mediators such as thromboxane A₂, prostaglandins, leukotriens, and platelet-activating factor [15]. sPLA₂ is thought to promote phagocytosis of injured cells and tissue debris, thereby enhancing inflammation [22, 41]. During inflammatory reactions, plasma levels of sPLA₂ may increase markedly up to 100-fold over baseline.

At present, data regarding the potential of locally injected IL-12 to induce systemic inflammatory responses in humans are still scarce. We performed a phase I study with IL-12 in patients with advanced renal cell cancer [37]. In the present analysis, we address systemic inflammatory responses in these patients. Our results show that IL-12 dose-dependently triggers these responses. These data may be useful for the design of studies in which IL-12 is used as an adjuvant.

Materials and methods

Patients

We studied 26 patients with advanced renal cell cancer who participated in a phase I dose escalation trial carried out in the Rotterdam and Mainz cancer centers to evaluate the safety and tolerability of recombinant human IL-12 (rHuIL-12). Toxicity analysis of the phase I study indicated that 0.5 μ g/kg was the maximum tolerated dose of the first injection of rHuIL-12 [37]. We studied markers of PMN degranulation in these patients after the first injection of rHuIL-12 at a dose of 0.1 μ g/kg ($n=3$), 0.5 μ g/kg ($n=19$), or 1.0 μ g/kg ($n=4$). Prior to treatment, patients had a World Health Organization

(WHO) performance score of 0–1, and adequate hematological, renal, hepatic, cardiovascular, and pulmonary functions. All former therapies were terminated at least 6 weeks prior to start of treatment with rHuIL-12. The patients did not use systemic corticosteroids. Patients with concurrent systemic disease were excluded. Patients gave informed consent, and the ethics committees approved the protocol.

Recombinant human IL-12 (Ro 24-7472) was supplied by Hoffmann La Roche (Nutley, NJ, USA) and administered by s.c. injection. All injections were given at 8:00 a.m. Acetaminophen was prescribed to alleviate fever, headache, and myalgia. Metoclopramide was prescribed in case of nausea and vomiting. No other medications were given routinely.

Blood sampling and assays

Blood samples for elastase- α 1-antitrypsin complexes, lactoferrin, and sPLA₂ measurement were obtained directly before and 4, 8, 12, 24, 48, and 72 h after the first rHuIL-12 administration. In 12 patients, blood was also obtained after 96 and 168 h. Blood was drawn through an indwelling intravenous infusion needle (Venflon, 16-gauge). Plasma was obtained by centrifugation of blood for 10 min at 1,300 g. Plasma samples were stored at -70°C until tested. All assays were done with EDTA anti-coagulated plasma. Plasma levels of elastase- α 1-antitrypsin complexes and lactoferrin were assayed by RIA as described in detail previously [32]. Briefly, sepharose beads, to which polyclonal antibodies against human elastase or a mAb against human lactoferrin were coupled, were incubated with the samples to be tested. Elastase- α 1-antitrypsin or lactoferrin bound to the beads was quantitated by incubation with ^{125}I -mAb against complexed α 1-antitrypsin (RIA for elastase- α 1-antitrypsin) or polyclonal ^{125}I -antilactoferrin (RIA for lactoferrin). Results were expressed as nanograms of elastase complexes or lactoferrin per milliliter by reference to standard curves. The lower limit of detection of elastase- α 1-antitrypsin complexes is 25 ng/ml; normal values are less than 100 ng/ml. The lower limit of detection of lactoferrin is 100 ng/ml; normal values are less than 400 ng/ml.

Secretory phospholipase A₂ concentrations in plasma were determined with an ELISA that was modified from that reported by Smith [47]. MAbs against human sPLA₂ type II (provided by Dr F.B. Taylor Jr, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) were used as the coating and catching antibodies, respectively. Results are expressed by reference to a standard curve consisting of a dilution of culture supernatant of HepG2 cells stimulated with IL-6, in which the amount of sPLA₂ was assessed by comparison with recombinant human secretory-type PLA₂ (sPLA₂; courtesy of Prof. H.M. Verheij, Department of Enzymology and Protein Engineering, University of Utrecht, Utrecht, The Netherlands). The lower limit of detection

of this assay is 0.2 ng/ml; normal values are less than 5 ng/ml.

Blood samples for determinations of absolute numbers of PMNs were available from 20 patients and collected directly before and 1–4 and 7 days after the first administration of rHuIL-12. PMN counts were determined in EDTA anti-coagulated blood samples using a Technicon H1 automated cell counter (Technicon, Tarrytown, NY, USA).

Pharmacologic data analyses

Individual plasma concentration-time data of cytokines were analyzed by noncompartmental methods using the Siphar software package (version 4.0; Inna Phase, Philadelphia, PA, USA). Pharmacodynamic analysis of the modulation of leukocyte counts, PMN degranulation products, and sPLA₂ induction by rHuIL-12 was also performed using the Siphar software. The total area under the effect curve (AUEC_{total}) for elastase- α 1-antitrypsin complexes, lactoferrin, and sPLA₂ was calculated for each patient using the trapezoidal rule. The AUEC_{net}, the area under the effect curve above baseline values, was calculated by AUEC_{total} minus AUEC_{baseline}. The baseline levels of elastase- α 1-antitrypsin complexes, lactoferrin, and sPLA₂, and the baseline leukocyte counts were obtained from measured pre-dose levels and counts, assuming that baseline values would have been maintained for the duration of the study in the absence of rHuIL-12 administration.

Relationships between the various AUEC_{net} values and between concentration-time profiles of elastase- α 1-antitrypsin complexes, lactoferrin, and sPLA₂ were evaluated by multiple regression analysis. To test parameter differences for statistical significance in the paired samples, a two-tailed paired Student's *t* test was performed. To test parameter differences for statistical significance in the unpaired samples, a two-tailed unpaired Student's *t* test was performed. Probability values of less than 0.05 were regarded as statistically significant. All calculations were performed using Number Cruncher Statistical System (NCSS, version 5.X; Dr Jerry Hintze, Kayesville, UT, USA).

Results

Neutrophil numbers

Recombinant human IL-12 induced depression of the number of peripheral blood PMNs in all patients. After injection of 0.1 μ g/kg rHuIL-12, PMNs decreased from $4.0 \times 10^9/l$ to $0.94 \times 10^9/l$ ($n=1$). Figure 1 shows that after injection of 0.5 μ g/kg rHuIL-12 ($n=14$), PMNs decreased from $4.55 \pm 1.32 \times 10^9/l$ (mean \pm SD) to $1.74 \pm 0.84 \times 10^9/l$ ($p < 0.001$). Finally, after injection of 1.0 μ g/kg rHuIL-12 ($n=4$), PMNs decreased from $3.9 \pm 1.14 \times 10^9/l$ to $1.45 \pm 0.33 \times 10^9/l$. After 0.5 μ g/kg

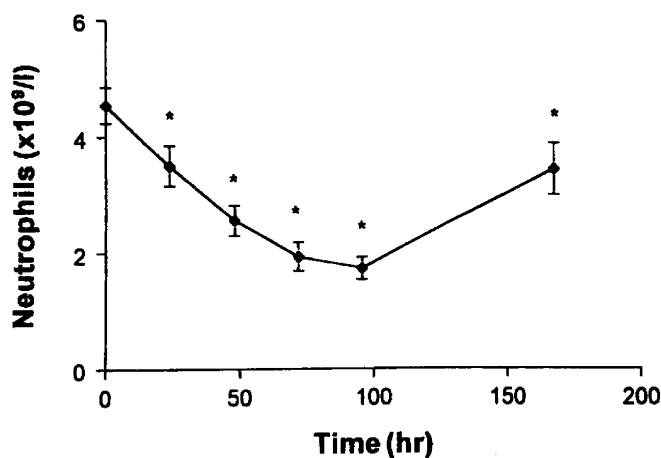


Fig. 1 Neutrophil counts after subcutaneous rHuIL-12. Mean \pm SE neutrophil counts during a period of 168 hours after a single subcutaneous injection of 0.5 μ g/kg rHuIL-12 in 14 patients with advanced renal cell carcinoma. Significant differences between baseline concentrations and concentrations after rHuIL-12 injection are indicated by an asterisk ($p < 0.05$)

rHuIL-12, PMN nadir occurred after a median of 3 days (range, 2–4 days) while after 1.0 μ g/kg rHuIL-12, PMN nadir occurred after a median of 4 days (range, 4–7 days). At dose levels 0.5 and 1.0 μ g/kg rHuIL-12, the number of PMNs remained significantly below baseline values ($p < 0.001$) during the whole 7-day observation period.

PMN degranulation

Recombinant human IL-12 (rHuIL-12) administration resulted in degranulation of PMNs, reflected by (1) increased plasma levels of elastase- α 1-antitrypsin complexes, which reflect the degranulation of azurophilic granules, and (2) increased plasma levels of lactoferrin, which reflect the degranulation of specific granules (Fig. 2).

Elastase- α 1-antitrypsin complexes increased in all patients, and increases were rHuIL-12 dose dependent (Table 1). At doses of 0.1 and 0.5 μ g/kg rHuIL-12, elastase- α 1-antitrypsin complexes peaked after a median of 48 h (range, 8–72 h), whereas at doses of 1.0 μ g/kg rHuIL-12, maximum concentrations were reached after a median of 60 h (range, 48–72 h). Plasma concentrations of lactoferrin increased in 23 out of 26 patients (89%). Individual maximum plasma concentrations of lactoferrin varied considerably among patients, and patients with low baseline levels tended to have relatively low peak levels as well. At doses of 0.1 and 0.5 μ g/kg rHuIL-12, lactoferrin peaked after a median of 24 h (range, 4–96 h), whereas at doses of 1.0 μ g/kg rHuIL-12, maximum concentrations of lactoferrin were reached after a median of 60 h (range, 48–72 h). Elevation of elastase- α 1-antitrypsin complexes after rHuIL-12 injection correlated significantly with elevation of lactoferrin levels ($r^2 = 0.24$, $p = 0.03$).

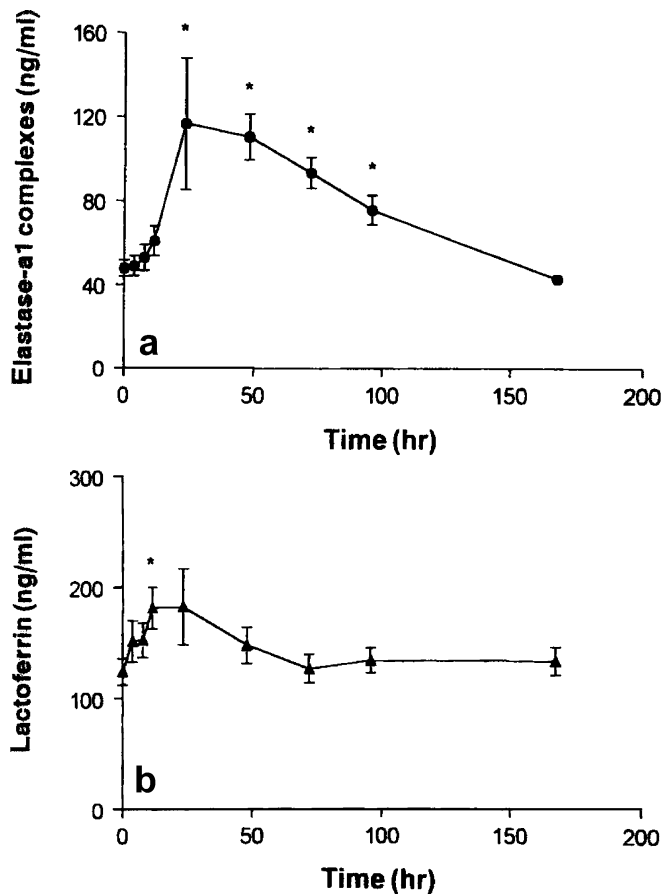


Fig. 2 Neutrophil degranulation after subcutaneous rHuIL-12. Mean \pm SE plasma concentrations of elastase- α 1-antitrypsin complexes (a) and lactoferrin (b) during a period of 168 h after a single subcutaneous injection of 0.5 μ g/kg rHuIL-12 in 19 patients with advanced renal cell carcinoma. Significant differences between baseline concentrations and concentrations after rHuIL-12 injection are indicated by an asterisk ($p < 0.05$)

Plasma concentrations of sPLA₂

Levels of circulating sPLA₂ were measured as an indirect parameter for the formation of lipid mediators such as thromboxane A₂, prostaglandins, leukotriens, and platelet-activating factor. rHuIL-12 induced an increase of circulating sPLA₂ in 25 of 26 patients and elevation was IL-12 dose dependent (Table 1). Nine patients had elevated sPLA₂ concentrations before rHuIL-12 administration, with values ranging from 6.9 to 63 ng/ml.

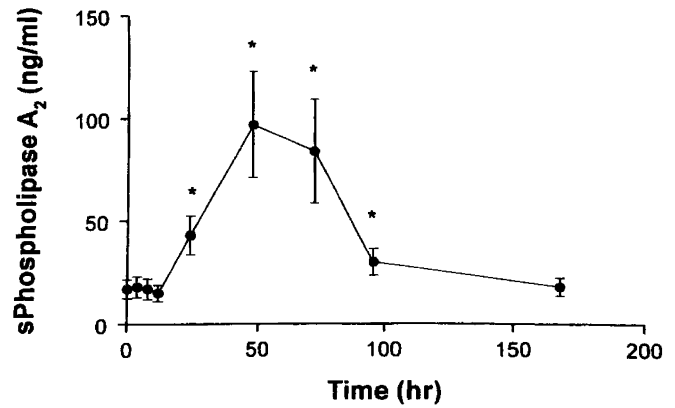


Fig. 3 Plasma concentrations of sPLA₂ after subcutaneous rHuIL-12. Mean \pm SE plasma concentrations of sPLA₂ during a period of 168 h after a single subcutaneous injection of 0.5 μ g/kg rHuIL-12 in 19 patients with advanced renal cell carcinoma. Significant differences between baseline concentrations and concentrations after rHuIL-12 injection are indicated by an asterisk ($p < 0.05$)

Patients with elevated baseline concentrations had significantly higher peak levels than patients with normal baseline levels (205 ± 149 ng/ml [mean \pm SD] vs 31 ± 17 ng/ml; $p < 0.01$). Figure 3 shows the mean concentration of sPLA₂ complexes after 0.5 μ g/kg rHuIL-12.

The median time to peak concentration was 48 h after a dose of 0.1 and 0.5 μ g/kg rHuIL-12, and 72 h after a dose of 1.0 μ g/kg. Individual peak concentrations were reached between 48 and 168 h. Levels slowly declined to baseline within the 7 days of observation.

Discussion

The present results demonstrate that s.c. IL-12 induces a dose-dependent systemic activation of multiple inflammatory mediator systems in humans. IL-12 induced activation and degranulation of PMNs in a dose-dependent way. The activation of PMNs was sustained, with ultimate normalization of plasma levels of degranulation products after 7 days. The effect of IL-12 on PMN degranulation is consistent with a study in chimpanzees that described PMN activation with maximum elastase- α 1-antitrypsin complex concentrations at the last plasma sampling time-point of 48 h [26].

Since PMNs express functional IL-12 receptors, IL-12 may directly induce PMN degranulation. This is

Table 1 Circulating inflammatory parameters after subcutaneous administration of rHuIL-12

	Baseline (all)		0.1 μ g/kg ($n=3$)		0.5 μ g/kg ($n=19$)		1.0 μ g/kg ($n=4$)	
	Mean	SD	Mean ^a	SD	Mean ^a	SD	Mean ^a	SD
Elastase- α 1-ATc (< 100 ng/ml)	46	16	87	35	117*	136	202*	79
Lactoferrin (< 400 ng/ml)	122	45	197	85	181*	82	248	112
sPLA ₂ (< 5 ng/ml)	14	18	23	17	97*	113	144	99

* $p < 0.05$ vs baseline

^aMaximum mean values are shown

supported by *in vitro* experiments showing that IL-12 increases intracellular free calcium and induces actin polymerization, tyrosine phosphorylation, and production of oxygen radicals and platelet-activating factor in PMNs [2, 10, 13]. In the present study, maximal concentrations of degranulation products coincided with peak levels of IL-12, *i.e.*, at 24 h postinjection. A detailed pharmacokinetic analysis of IL-12 administration in our phase I study has been published earlier [37]. In addition, IL-12 may activate PMNs indirectly by other cytokines. We have observed a dose-dependent induction of IL-6, IL-8, and TNF- α after IL-12 administration [39]. These cytokines have known PMN-activating effects. However, our results suggest that TNF- α or IL-8 are less likely to be responsible for PMN degranulation after IL-12 administration. In humans, TNF- α induces a very rapid degranulation of PMNs, with maximum levels of elastase- α 1-antitrypsin complexes and lactoferrin 3 h after TNF- α administration [52]. In the present study, maximum levels of elastase- α 1-antitrypsin complexes and lactoferrin were, however, reached at 24 h, while TNF- α peak level was reached at 48 h [39]. Similarly, IL-8 is a strong PMN activator [18, 34, 46], but again, peak plasma concentrations of IL-8 were observed 24 h after levels of elastase- α 1-antitrypsin complexes and lactoferrin reached their maximum [39]. The assumption that IL-8 did not contribute to PMN degranulation is further supported by the observation that administration of interleukin 1 β to humans induced elevation of IL-8 to levels similar to those observed in the present study; however, degranulation of PMNs did not occur [33]. IL-6 is another potential mediator of PMN activation after IL-12. PMNs express IL-6 receptors [24], and IL-6 exposure has been shown to induce elastase and lactoferrin release and production of platelet-activating factor and oxygen-free radicals by PMNs *in vitro* [7, 8]. In our earlier report, peak levels of IL-6 were reached at 12 h, preceding peak levels of lactoferrin and elastase- α 1-antitrypsin complexes at 24 h post IL-12 injection as observed now. This result is consistent with the possibility that IL-6, formed in response to IL-12 injection, contributes to the release of elastase- α 1-antitrypsin complexes and lactoferrin. However, in a chimpanzee model of endotoxemia, in which IL-12 is an important mediator of the inflammatory response, PMN degranulation occurs independently of TNF- α , IL-8, and IL-6 synthesis [25]. These results indicate that IL-12 may activate PMNs directly as well as indirectly.

Interleukin 12 was also a powerful stimulus for the synthesis of sPLA₂, the enzyme that generates arachidonic acid and catalyzes the rate-limiting step in the formation of lipid mediators. Endothelial cells, an important source of sPLA₂ in the peripheral blood, have not been shown to express IL-12 receptors. IL-6 and TNF- α are possible mediators of sPLA₂ synthesis after IL-12 injection, since either cytokine stimulates the production and release of sPLA₂ *in vitro* by various cell types, including liver cells, endothelial cells, and macrophages [35, 42]. In healthy volunteers, TNF- α

resulted in increased sPLA₂ levels, with a maximum after 6 h [51]. The antitumor effect of TNF- α in a variety of tumor cell models depends on cytolysis that requires the activation of sPLA₂ by TNF- α [31]. Furthermore, increased sPLA₂ synthesis after IL-12 may play an important role in inflammatory colitis, a severe adverse effect observed with systemic IL-12 administration [28], as suggested by the observation that IL-12 does not cause gastrointestinal toxicity in an sPLA₂-deficient strain of mice [11].

In accordance with previous clinical results [4, 5], we observed a rapid IL-12 dose-dependent decrease of PMNs in the peripheral circulation, with protracted depressed cell counts during the whole 7-day period of observation. In mice also, IL-12 administration caused decreased numbers of circulating leukocytes and neutrophils [19, 45]. The decreased PMN counts are thought to occur due to compartmental cellular shift with accumulation of cells in liver, spleen, and tumor sites. *In vitro*, IL-12 serves as a chemotactic stimulus for human PMNs [1], and IL-12-induced platelet-activating factor is thought to play a critical role [10]. IL-12 potentially mediates PMN chemotaxis through the induction of TNF- α and IL-8. TNF- α up-regulates adhesion molecules on endothelial cells and can mediate the migration of PMNs [6, 12, 36]. In healthy individuals, intravenous TNF- α administration results in short-lived neutropenia followed by a 24-h neutrophilia [52]. IL-8 is a member of the α -chemokine family and an important PMN chemotactic factor [46, 50]. Intravenous injection of IL-8 in mice, resulted in an instant neutropenia, followed by profound neutrophilia for several hours [40]. The protracted elevated IL-8 and TNF- α levels after IL-12 injection—*i.e.*, lasting for more than 2 and 7 days, respectively—may well be responsible for the protracted neutropenia observed in our patients.

Clinical studies have addressed the systemic administration of IL-12, but associated toxicity combined with a lack of encouraging results hamper further development of IL-12 [4, 23, 30, 37]. Recently, the application of IL-12 as an immune adjuvant has received attention. Preclinical results have demonstrated that IL-12 provides a critical third signal along with antigen and IL-2 to activate CD8⁺ T cells [16]. In mice, IL-12 has an adjuvant effect in the activation of CD8⁺ T-cell responses to antigenic peptides [20]. The mechanisms underlying the adjuvant efficacy of IL-12 are incompletely understood. We showed that IL-12 can activate PMNs, which are thought to be engaged in a complex cross-talk with immune and endothelial cells that bridges innate and adaptive immunity [14, 17]. IL-12 has strong inflammatory effects *in vivo* and may be a potent adjuvant by providing inflammatory signals, which may optimize adequate antigen presentation. Indeed, most, if not all, classical adjuvant substances promote local inflammation. However, the stimulation of excessive systemic inflammatory responses seems undesirable for an adjuvant. Although the number of patients in our study treated at doses other than 0.5 μ g/kg of IL-12 is

small, our results give an indication of the dose to be used as adjuvant. At doses of 0.5 and 1.0 $\mu\text{g}/\text{kg}$, IL-12 turned out to be a strong stimulator of systemic inflammatory mediator systems. However, after a dose of 0.1 $\mu\text{g}/\text{kg}$, IL-12, the systemic inflammatory responses were limited. Previously, we showed that at a dose of 0.5 $\mu\text{g}/\text{kg}$, IL-12 induces activation of fibrinolysis and coagulation [38]. These side effects may have contributed to hemorrhagic events, observed in other IL-12 studies [4, 28, 29]. Based on our findings that at a dose of 0.1 $\mu\text{g}/\text{kg}$ IL-12, PMNs are activated, while only small amounts of sPLA₂ are formed, activation of fibrinolysis is minimal, and activation of coagulation is absent [38], we suggest that IL-12 as an adjuvant should not be used at doses higher than 0.1 $\mu\text{g}/\text{kg}$ in order to prevent serious systemic inflammatory responses.

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