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Chapter 4

Activation of mononuclear cells by interleukin-12: an in vivo study in chimpanzees

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Abstract

Interleukin (IL)-12 is considered a central regulator of host resistance against a variety of pathogens. Therefore, IL-12 has been advocated as a potential therapeutic agent in infections. To determine the in vivo effects of IL-12 on mononuclear cells involved in the host immune response, 4 chimpanzees received an intravenous injection of recombinant IL-12 (1 μg/kg). IL-12 induced a sustained decrease of lymphocyte counts, with decreases in CD3+/CD4+ and CD3+/CD8+ cells, while monocyte counts showed a transient increase. IL-12 injection resulted in a shift towards a Th1 mediated immune response as indicated by increased interferon-γ production during whole blood stimulation, while not influencing IL-4 production. IL-12 induced activation of NK cells and phagocytes, as indicated by increased NK cell cytotoxicity, and increased plasma levels of granzymes A and B, and of chitotriosidase activity. These data support the hypothesis that IL-12 may serve as a useful therapeutic agent in infections where a cell-mediated response is protective.

Introduction

Interleukin-12 (IL-12) is a pro-inflammatory heterodimeric cytokine formed by two covalently linked chains of 35 kD (p35) and 40 kD (p40) (1). These two subunits are encoded by two different and independently regulated genes, and production of both subunits within the same cell is required for the formation of the biologically active p70 heterodimer. IL-12 is produced mainly by activated monocytes and macrophages, and by other antigen-presenting cells (APC). Potent inducers for IL-12 production are bacteria, bacterial products and intracellular parasites. Most biological effects of IL-12 involve activities on natural killer (NK) and T cells. IL-12-induced activation of NK and T cells leads to enhanced cytotoxic lytic activity and the production of cytokines, most importantly of interferon-γ (IFN-γ), which is a potent activator of the antimicrobial activity of phagocytic cells (2). In addition, IL-12 promotes the differentiation of CD4+ naive T cells into T helper-1 (Th1) cells and hereby plays a central role in the regulation of the Th1/Th2 balance (3). A Th1 response is associated with a cell-mediated immune response, and found to be protective against a variety of intracellular pathogens. Thus, by its divergent activities, IL-12 plays an important role in both innate resistance and antigen specific cell-mediated immunity (4).

IL-12 has been shown to augment host defense in animal models of several infections (5, 6). Indeed, in mice endogenous IL-12 was essential for host defense against a number of intracellular pathogens like *Mycobacterium tuberculosis*, *Leishmania major* and *Listeria monocytogenes* (7-9). In addition, exogenously administered IL-12 increased host resistance to *Mycobacterium tuberculosis* (10, 11), and induced a protective immune response to *Leishmania major* and *Listeria monocytogenes* (12-14). In a mouse model of *Klebsiella* pneumonia, endogenous IL-12 was essential for adequate local bacterial clearance and survival, while temporary IL-12 overexpression in the lung protected against mortality (15). Furthermore, administration of IL-12 protected *Plasmodium cynomolgi*-infected rhesus monkeys against malaria (16).

Since IL-12 has been implicated as a central mediator of host resistance against a variety of pathogens, it has been suggested that IL-12 may be used as a potential adjuvant therapeutic agent in infections by intracellular mycobacteria and parasites in humans. However, data on the in vivo effects of IL-12 in primates are limited. Therefore, in the present study we investigated the effects of an intravenous injection of IL-12 in healthy chimpanzees on mononuclear cells involved in the host immune response to infection.

Materials and methods

Study design and sampling

Four healthy adult chimpanzees (Pan troglodytes; 22.4 - 35.4 kg) were studied. They were recruited from the primate colony at the Biomedical Primate Research Center (BPRC), Rijswijk, the Netherlands. None of the animals had abnormalities on routine examination. The present study was performed simultaneously with investigations on the effects of IL-12 on host inflammatory mediator systems like the cytokine network, coagulation and fibrinolysis, the results of which will be reported elsewhere (17). The chimpanzees were sedated with ketamine chloride i.m., and after intubation kept under general anesthesia with nitrous oxide and halothene 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. All animals received an i.v. bolus injection of recombinant human (rh) IL-12 (provided by Genetics Institute, Inc, Andover, MA) at a dose of 1 μg/kg body weight (specific activity $1.1x10^7$ U/mg rhIL12). Previous in vitro work demonstrated that human IL-12 is active on chimpanzee peripheral blood mononuclear cells (PBMC's), as reflected by its capacity to induce the production of IFN-γ (data not shown).

Blood samples were obtained by separate venipunctures directly before IL-12 administration (0 h) and at 4, 8, 24, and 48 h thereafter. Blood for FACScan analysis and NK cell cytotoxicity assay was collected in heparin containing vacutainer tubes, blood for measurement of chitotriosidase levels in tubes containing buffered citrate; all other samples were collected in EDTA (K_3) tubes. Plasma was prepared by centrifugation (1600 x *g* for 20 minutes at 4°C) and stored at-20°C until assays were performed. The protocol was approved by the scientific committee of the Academic Medical Center and the Institutional Animal Care and Use Committee of BPRC.

FACScan analysis

Blood for FACS analysis was immediately placed on ice. Erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) for 10 minutes. Cells were centrifuged at 600 x *g* for 5 minutes at 4°C. The remaining cells were washed twice with cold FACS buffer (phosphate buffered saline (PBS) supplemented with 0.01% NaN_3, 0.5 % BSA, and 0.3 mM EDTA) and resuspended in FACS buffer. For staining, $1x10^6$ cells/ tube were incubated with the following mouse monoclonal antibodies (mAbs): CD2 (Becton Dickinson & Co, Rutherford, NJ), CD 8 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands), CD11a (clone SPV-L7 (18)), CD25 or CD56 (all CLB), MHCII (clone Q5/13 (19)). FITC-conjugated $F(ab)_2$ fragments of goat-anti-mouse immunoglobulins (Zymed, Inc., Camarillo,

CA) were added as a second antibody. In a final incubation step, Cy-Chrome5-labeled anti-CD4 mAb (Dako, Glostrup, Denmark) and/ or phycoerythrin-labeled anti-CD3 (Immunotech, Marseille, France) were added. The appropriate isotype controls were included in all experiments. All FACS reagents were used in concentrations recommended by the manufacturers. Lymphocytes and monocytes were gated by forward and side scatter using a FACScan (Becton Dickinson) and 5,000 cells were counted. Results are expressed as the percentage of gated cells positive for the Abs used, or as the mean cell fluorenscence intensity (MFI) after subtraction of control IgG fluorescence.

Whole blood stimulation

Blood for in vitro stimulation was collected aseptically using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson). Anticoagulation was obtained using endotoxin-free heparin (Leo Pharmaceutical Products B.V., Weesp, the Netherlands; final concentration 10 U/ml blood). Whole blood, diluted 1:5 in sterile RPMI 1640 supplemented with L-glutamine (Bio Whittaker, Verviers, Belgium) was stimulated for 24 h at 37°C in the presence or absence of anti-CD3/anti-CD28 (CLB; final concentration 1:1000 both) or Staphylococcal Enterotoxin B (SEB) (Sigma, St. Louis, MO; 1 μg/ml). After the incubation, supernatant was collected after centrifugation (at 4°C for 12 minutes at 1600 x *g*) and stored at –20°C until assays were performed.

NK cell cytotoxicity assay

NK cell cytotoxicity was measured against K562 target cells in a 4-h ^{51}Chromium (^{51}Cr) - release assay (20). Heparin blood was diluted 1:1 in sterile cold PBS. PBMC's were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll Paque, Pharmacia Biotech, Uppsala, Sweden) at room temperature for 20 minutes at 1000 x *g*. PBMC's were collected in the inter-phase and washed twice with Iscove's modified Dulbecco's medium (IMDM, Bio Whittaker) supplemented with L-glutamine, containing 10% fetal calf serum (FCS), and 1% Antibiotic-Antimycotic (GibcoBRL, Life Technologies, Grand Island, NY). K562 target cells were labeled with ^{51}Cr-labeled sodium chromate (100 μCi/10^6 cells) for 1 h at 37°C. After the incubation, cells were washed twice with IMDM. Target cells were added to wells of a U-bottom 96-well plate (Costar, Cambridge, MA) at a concentration of 1 x 10^4/well and, after centrifugation (at room temperature for 2 minutes at 800 x *g*) incubated for 4h at 37°C with PBMC's at effector to target (E:T) ratios from 80:1 to 5:1. Maximal release (MR) and spontaneous release (SR) were determined by incubating K562 cells with 5% Triton X-100 or medium alone, respectively. All determinations were done in quadruplicate. Radioactivity in supernatant was counted in a gamma counter. Percentage of specific lysis was calculated using the formula: (mean cpm experimental release - mean cpm SR) / (mean cpm MR - mean cpm SR).

Assays

Leukocyte counts and differentials were determined by flow cytometry. IFN-γ and IL-4 were measured by ELISA according to the instructions of the manufacturer (both CLB, detection limit 5 pg/ml and 1.2 pg/ml respectively).

Granzyme A and B levels were measured in EDTA plasma by sensitive ELISA's as described previously (21).Nitric oxide (NO) production was assessed by measurement of nitrite and nitrate by a colorimetric assay according to the instructions of the manufacturer (Cayman Chemical Company, Ann Arbor, MI). Total NO production was expressed as the sum of both nitrite and nitrate production. Chitotriosidase activity was measured by incubating 10 μl of citrated plasma for 30 minutes at 37°C with 4 MU-chitotrioside substrate as described previously (22).

Statistics

Either individual data are given, or data are expressed as mean ± SE of the four chimpanzees. Changes in time were analyzed by one-way analysis of variance. Differences from baseline levels were assessed by Dunnett t-test for multiple comparisons where appropriate. $P < 0.05$ was considered to represent a significant difference.

Results

Cell counts and activation markers

IL-12 administration induced a sustained decrease in lymphocyte counts, which was associated with decreases in both CD3+/CD4+ and CD3+/CD8+ cells (Table I). In contrast, IL-12 elicited a transient increase in monocyte counts, peaking after 8 h (Table I). IL-12 also induced up-regulation of the adhesion molecules CD2 and CD11a on CD3+ cells (Table 2), while not influencing the expression of CD25 (data not shown). On monocytes, IL-12 induced upregulation of MHC class II expression (Table II), while the expression on lymphocytes did not alter (data not shown).

In vitro stimulation

In order to determine the effects of IL-12 on the Th1/Th2 balance, cytokine production was assessed during whole blood stimulation with T-cell agonists. IFN-γ was measured as the prototypic Th1 cytokine while IL-4 was measured as a characteristic Th2 cytokine. IL-4 and IFN-γ levels were corrected for the number of CD3+/CD4+ cells at the selected timepoints. IFN-γ was detectable in the unstimulated samples at 24 and 48 h after IL-12 injection; therefore, IFN-γ levels are expressed as the difference between levels in the stimulated samples and levels in the unstimulated samples. Anti-CD3/CD28 and the bacterial superantigen SEB induced the production of IL-4 and IFN-γ in whole

blood. After IL-12 injection, the IFN-γ production in the in vitro stimulations with both stimuli strongly increased, with a maximal IFN-γ response at 48 h (Fig.1). In contrast, IL-4 production remained unaltered.

Table I. Effects of IL-12 on lymphocyte and monocyte counts.

Time (h)	0	4	8	24	48	P-value
lymphocytes (x10^9/L)	2.1 ± 0.4	2.0 ± 0.4	1.0 ± 0.2	0.9 ± 0.2*	1.1 ± 0.2	0.028
CD3+/CD4+ cells (x10^9/L)	0.9 ± 0.1	0.9 ± 0.1	0.3 ± 0.1*	0.3 ± 0.1*	0.4 ± 0.1	0.002
CD3+/CD4+ cells (%)	42.9 ± 7.5	43.9 ± 3.8	25.1 ± 2.3*	38.9 ± 9.1	38.9 ± 9.1	
CD3+/CD8+ cells (x10^9/L)	0.6 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	NS
CD3+/CD8+ cells (%)	26.4 ± 3.7	21.9 ± 4.4	10.8 ± 1.1	27.3 ± 4.5	27.2 ± 4.5	
monocytes (x10^9/L)	0.6 ± 0.2	0.7 ± 0.2	1.8 ± 0.4*	0.9 ± 0.3	0.5 ± 0.2	0.009

Values are mean ± SE of 4 chimpanzees. RhIL-12 (1 μg/kg) was given as an i.v. bolus injection at t= 0 h. Analysis by flow cytometry and FACScan analysis. Data are expressed as cell counts or percentage positive cells within the lymphocyte population. P-value reflects changes in time analyzed by one-way analysis of variance. * indicates P < 0.05 versus baseline by Dunnett t-test. NS= not significant.

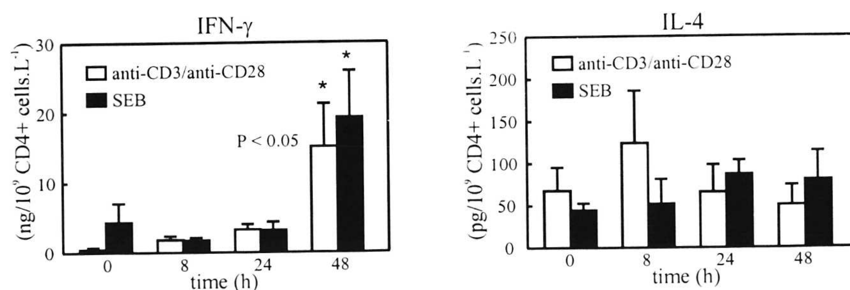

Figure 1. Effects of IL-12 on the Th1/Th2 balance. RhIL-12 (1 μg/kg) was given as an i.v. bolus injection at t= 0 h. Whole blood, diluted 1:5 in RPMI was stimulated for 24 h at 37°C in the presence or absence of anti-CD3/anti-CD28 (final concentration 1:1000 both) or Staphylococcal Enterotoxin B (SEB) (1 μg/ml). IFN-γ, as the prototypic Th1 cytokine and IL-4, a Th2 cytokine, were measured in supernatant. Data are expressed as cytokine levels after subtraction of levels in unstimulated samples. Data are mean ± SE of 4 chimpanzees. P-value reflects changes in time analyzed by one-way analysis of variance. * indicates P < 0.05 versus baseline by Dunnett t-test.

NK cell responses

NK cell cytotoxicity against K562 cells was assessed in 2 chimpanzees. NK cytotoxicity strongly increased after IL-12 injection at E:T ratio's from 80:1 to 20:1, reaching a peak at 8 h after injection (54.5 % specific lysis at E:T ratio 80:1) in 1 chimpanzee, and a peak at 24 h (25.3 %) in the other chimpanzee (Fig. 2). During the experiment, the percentage of $CD3^-$/$CD56^+$ NK cells within the lymphocyte population did not change compared to baseline for the 2 animals in which NK cell cytotoxicity was assessed (data not shown).

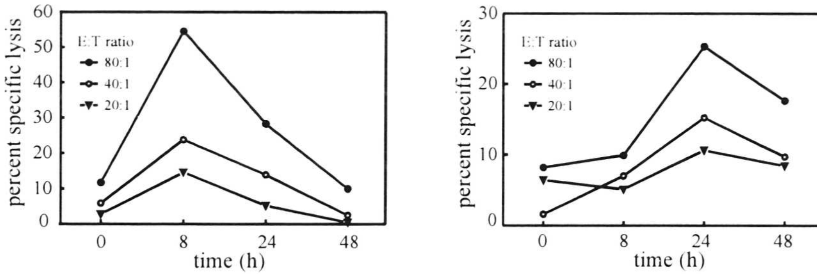

Figure 2. Enhanced NK cell cytotoxicity after an i.v. bolus injection of rhIL-12 (1 μg/kg). NK cell cytotoxicity was measured against K562 cells in a 4-h ^{51}Cr-release assay. PBMC's were incubated for 4 h at 37°C with ^{51}Cr-labeled K562 cells at different effector to target (E:T) ratio's. Results are data from 2 individual chimpanzees.

Soluble products

To determine whether IL-12 administration results in activation of cytotoxic lymphocytes and phagocytes, the effector cells of innate immunity, we measured the secretion of cell activation products in plasma. Both granzyme A and B plasma levels showed a remarkable increase late after IL-12 administration, both reaching peak levels at the end of the study period (9.77 ± 2.70 ng/ml and 0.27 ± 0.07 ng/ml respectively; $P < 0.05$ both) (Fig.3). Plasma chitotriosidase activity modestly increased after IL-12 injection, peaking at 48 h (221.3 ± 20.4 nmol/ml.h; $P < 0.05$). NO plasma levels remained unaltered during the study period (data not shown).

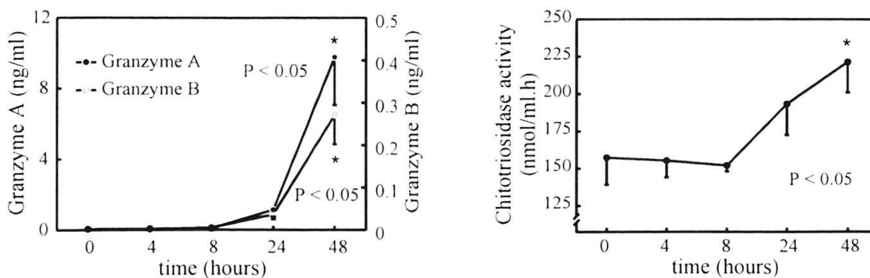

Figure 3. IL-12 injection results in increased plasma levels of granzyme A and B, and of chitotriosidase activity. RhIL-12 (1 μg/kg) was given as an i.v. bolus injection at t= 0 h. Data are mean ± SE of 4 chimpanzees. P-value reflects changes in time analyzed by one-way analysis of variance. * indicates $P < 0.05$ versus baseline by Dunnett t-test.

Discussion

IL-12 is considered to be a key mediator in the regulation of the immune response by its capacity to activate both innate and antigen specific immunity (1, 4). In several animal models endogenous IL-12 was shown to be essential for the host response against a variety of pathogens, while IL-12 administration resulted in increased host resistance and protection against mortality (6-16). Therefore, IL-12 is a potential therapeutic agent in human infectious diseases. In the present study we investigated the in vivo effects of IL-12 in chimpanzees. IL-12 administration resulted in the activation of NK and T cells, indicated by enhanced NK cytotoxicity against K562 cells, and in upregulation of cellular adhesion (CD2 and CD11a) molecules on T cells. IL-12 injection was associated with increased plasma levels of granzyme A and B, and enhanced chitotriosidase activity. In addition, IL-12 induced a shift in the Th1/Th2 balance towards a Th1 immune response as measured by increased IFN-γ production after in vitro stimulation of whole blood with anti-CD3/CD28 or SEB.

Knowledge of the effects of IL-12 in primates is highly limited. A previous study documented hematologic changes in cynomolgus monkeys after repeated daily injections of rhIL-12 (23). In accordance with the present results, decreases in lymphocyte counts and the number of CD4+ and CD8+ cells were found on day 2. Remarkably, in HIV-infected patients IL-12 administration was associated with a reduction in NK cell cytotoxicity as determined by an assay in which HIV-infected target cells were used as target cells (24). In the present study, IL-12 enhanced NK cell cytotoxicity towards the widely used K562 target cells, a finding that is in line with previously reported in vivo effects of IL-12 on NK cell activity in mice (1, 4). IL-12 has been used in phase 1 and 2 trials in cancer patients (25, 26). Daily administration of rhIL-12 was associated with serious toxicity (26). A single injection of IL-12 2 weeks before the start of a cycle of IL-12 administrations and less frequent dosing of IL-12 have been shown to prevent the toxic effects. After repeated IL-12 injections, a selective expansion of $CD8+CD18^{bright}$ T cells was found, with features of both memory and effector cells (27).

It could be argued that the experimental procedures per se may influence the immune system and that the observed effects were not completely due to IL-12 administration. However, previous studies in chimpanzees have shown that sedation with ketamine does not induce changes in inflammatory parameters like the cytokine network, leukocyte counts and degranulation, coagulation or fibrinolysis (28, 29). Furthermore, surgical stress induces immunological changes in an opposite direction when compared with the changes in the present study. Major stress is associated with a shift of the Th1/Th2 balance towards a Th2 immune response (30), while the number of T cells remained unaltered postoperatively. Also, impaired T cell functions and decreased expression of MHC class II on monocytes

were observed after surgery (31). In the present study, we found a shift towards a Th1 immune response and upregulation of MHC class II expression on monocytes, while the number of both CD4+ and CD8+ T cells in plasma decreased. Hence, the effects observed were likely induced by IL-12, not by the experimental procedures.

CD4+ T helper cells can be divided into Th1 and Th2 cells, that can be distinguished by the pattern of cytokine production upon ex vivo stimulation (32). Th1 cells produce IFN-γ, IL-2 and lymphotoxin, while Th2 cells produce IL-4, IL-5, IL-6 and IL-10. The Th1/Th2 balance plays a critical role in the outcome of several infectious and autoimmune diseases (33). A Th1 mediated response is known to enhance cell-mediated immunity and to protect against intracellular pathogens like *Mycobacterium tuberculosis* and *Listeria monocytogenes*, while a Th2 type response in associated with humoral immunity (34). IL-12 promotes a Th1 immune response in vitro and in rodents. We demonstrate here that IL-12 also enhances a Th1 type response in primates. Indeed, IL-12 injection resulted in increased IFN-γ production during whole blood stimulation with specific T cell stimuli, while not influencing the production of IL-4. We used the whole blood assay to study the Th1/Th2 balance since this method is thought to mimic in vivo conditions best (35). Unmanipulated blood contains circulating cytokines and hormones that can influence T cell function, while isolation of PBMC's may importantly influence their function (36).

The central role of NK cells in the innate resistance to infection has been demonstrated in models of infectious disease in severe combined immune deficiency (SCID) mice, mice which lack B and T cells but have a normal NK cell population (37). NK cells are a subpopulation of lymphocytes which are characterized by their MHC-independent antigen recognition. NK cells together with cytotoxic T lymphocytes are often referred to as cytotoxic lymphocytes (CL). Cytotoxicity of these CL on their target cells is mediated either by Fas ligation or by the secretion of cytoplasmic granules (38). Granule exocytosis leads to the release of a number of proteins into the intracellular space, including the pore-forming protein perforin and a family of serine proteinases called granzymes, which in a concerted action trigger pathways of apoptosis (39). We found that IL-12 injection induced an increase in the plasma levels of both granzyme A and granzyme B, together with increased cytotoxicity in a NK cytotoxicity assay. Previous in vitro studies have indicated that IL-12 presumably stimulates NK cell cytotoxicity through the induction of effector molecules such as perforin and granzymes (40, 41). Indeed, in response to IL-12 increased mRNA levels of both perforin and granzyme A and B were found together with increased NK cell cytotoxicity. It should be noted that in the present in vivo study maximal specific release in the NK assay was found at 8 h or 24 h after IL-12 injection, while maximal granzyme levels were measured at 48 h. NK cell derived cytokine production, most importantly of IFN-γ, has been proven to stimulate early innate resistance by increasing the antimicrobial activity of phagocytes. We found previously that IL-12 injection resulted in

high plasma levels of IFN-γ from 24 h and onward, which conceivably contributed to IL-12 effects on monocytes/macrophages (17).

It has been established that IL-12 requires co-stimuli for optimal activities on NK and T cells. Other monocyte/macrophage-derived cytokines like TNF, IL-15 and IL-18 have been reported to be important co-stimulatory mediators for IL-12-induced IFN-γ production by both NK and T cells (42-44). In addition, the response of T cells to IL-12 can be augmented by the interaction of co-stimulatory molecules between T cells and APC's. Both B7/CD28 and CD2/CD58 interaction can facilitate adhesion between T cells and APC's and importantly enhance the responsiveness of T cells to IL-12 (45-47). We found earlier that IL-12 injection triggers the production of co-stimulatory cytokines for IFN-γ production. i.e. IL-15 and IL-18 (17). We here show that IL-12 administration also caused an upregulation of co-stimulatory molecules (i.e. CD2). Together, these data suggest that IL-12 can stimulate various pathways by which it can augment its own effects in vivo.
Chitotriosidase is the human chitinase and a secretory protein of activated macrophages and neutrophils (22). A marked elevation of plasma chitotriosidase activity is found in patients with Gaucher disease, which is considered to reflect enhanced secretion of chitotriosidase by macrophages (48). In addition, small elevations can be found in some patients with other lysosomal storage disorders (49). Plasma levels of chitotriosidase activity found after IL-12 injection were much lower than levels found in patients with Gaucher disease. Therefore, our data can not discriminate between release of chitotriosidase from specific neutrophilic granules or from activated macrophages.

In conclusion, IL-12 injection resulted in the activation of both NK cells and phagocytes, the effector cells of innate immunity, and a shift towards a Th1 mediated immune response in vivo. Hence, important in vitro IL-12 effects could be reproduced in chimpanzees in vivo. These results support the hypothesis that IL-12 may be a useful therapeutic agent to enhance host resistance against infections in which a cell-mediated response is considered to be protective.

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