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In Vivo Immunomodulation Following Intradermal Injection with DNA Encoding IL-18

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IL-18, a recently identified cytokine synthesized by different cell types, including Kupffer cells, activated macrophages, and keratinocytes, induces IFN-γ production by T cells and NK cells. The cDNA encoding IL-18 with its natural signal peptide was cloned under control of the CMV promoter and injected into the skin of mice. A single intradermal injection of this construction led to efficient in vivo expression of IL-18 in cutaneous dermal cells and induced IFN-γ mRNA production, indicating that it was produced in a biologically active form. In addition, a massive cellular infiltrate was observed in the skin 2 days after injection. When the mice were subsequently infected with Mycobacterium bovis bacillus Calmette-Guérin (BCG), they produced lower levels of anti-BCG Abs than control animals. However, in contrast to their lowered humoral immune response, the mice produced higher amounts of Ag-specific IFN-γ after in vitro restimulation, as compared with the controls. Therefore, injection of DNA encoding IL-18 into the skin modulates both Ag-specific humoral and T cell responses upon mycobacterial infection. It increases the Th1 type response, which may be particularly useful for the development of new immunotherapeutic or immunoprotective approaches against infections by intracellular parasites, such as mycobacteria. The Journal of Immunology, 1999, 163: 3226–3231.

The Th1 cytokines IL-2 and IFN-γ are associated with the generation of cell-mediated immunity and resistance to intracellular parasites (1), whereas Th2 cytokines favor the induction of humoral immunity and resistance to extracellular parasites (2). IL-18, first designated as IFN-γ-inducing factor, is a newly identified cytokine of the Th1 type (3), and the cDNAs encoding murine and human IL-18 have recently been cloned (4, 5). Murine IL-18 (mIL-18) activates NK cells, induces IFN-γ production by T cells stimulated with Con A, anti-CD3 Abs, or IL-2, and promotes their proliferation (3, 4). IL-18 shares some biological activities with IL-12, although both cytokines have different receptor-binding activities and signal transduction pathways (6). It has been shown that IL-18, but not IL-12, induces IL-2 production by Th1 clones stimulated with immobilized anti-CD3 (6), and that IL-18-induced T cell proliferation is IL-2-dependent (7). However, most biological activities of IL-18 have been studied in vitro, and there is only sparse information on the in vivo relevance of this cytokine in immunological processes (8, 9). Therefore, we investigated the immunomodulatory effects of IL-18 in the context of a mycobacterial infection in vivo.

Protective immunity against mycobacterial infections is mediated by interactions between specifically sensitized T cells and activated macrophages (10). IFN-γ plays a crucial role in antimycobacterial protection, as illustrated by the severe disseminated form of tuberculosis in IFN-γ knockout mice (11, 12). Infection with Mycobacterium tuberculosis also results in secretion of IL-12, which is essential to generate protective immunity through the induction of IFN-γ and the activation of Ag-specific lymphocytes (13). Thus, cytokines prompting the immune response to the development of the Th1 arm, able to induce cell-mediated immunity, are of main interest for potential immunotherapy against mycobacterial infections.

With the aim to study the function(s) and immunomodulatory effect(s) of IL-18 in vivo, we used a DNA-based delivery approach. The mIL-18 cDNA was cloned downstream of a CMV promoter and injected intradermally (i.d.) into mice. The level of IFN-γ mRNA was assessed in the skin following DNA administration, and histological studies revealed a massive cellular infiltrate. Infection with Mycobacterium bovis bacillus Calmette-Guérin (BCG) of mice injected with the IL-18-encoding vector led to a reduction in the humoral immune response against BCG, associated with a marked increase of Ag-specific IFN-γ secretion. In addition to describing the immunological properties of IL-18 in vivo, this study also demonstrates the utility of this cytokine through DNA delivery, to tailor induction of specific immune responses during infection.

Materials and Methods

Bacterial strains, plasmids, and DNA manipulation

All cloning steps were performed in Escherichia coli XL1-Blue (Stratagene, La Jolla, CA). Expression of recombinant proteins was conducted in E. coli M15(pREP4) supplied by Qiagen (Courtaboeuf, France). BCG (vaccine strain 1173P2; World Health Organization, Stockholm, Sweden) was grown in Sauton medium (14) supplemented with 100 µg/ml ampicillin at 37°C using stationary tissue culture flasks. Vectors pQE-30 and pCR3.1-uni were purchased from Qiagen and Invitrogen (San Diego, CA), respectively. Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were purchased from Boehringer.
Plasmid administration and infection protocols

Six-week-old female BALB/c mice (Iffa Credo, l’Arbresle, France) were injected i.d. in the abdomen with 100 μl DNA solutions in a 1:ml syringe and a 27-gauge needle. For histochemical studies and RNA extractions, mice received a single injection of 50 μg of pCR3.1 or pCR3.1::IL-18. Mice were then sacrificed, and the skin from the injection site was carefully removed for histology blotting against the purified protein.

Construction of the IL-18 expression vectors

The cDNA encoding mIL-18 was obtained by RT-PCR on total RNA from J774 cells activated for 6 h with PMA (50 ng/ml), ionomycin (1 μg/ml), and LPS (1 μg/ml). Total RNA was extracted using RNAzol, according to the manufacturer’s recommendations (Bioprobe, Montreuil, France) and then subjected to RT-PCR using the GeneAmp Thermocycle Reverse Transcriptase PCR kit (Perkin-Elmer, Roissy CDG, France) and the primers with the following sequences: 5'-CCCGATCTAATTTGCG CGACTTCAACTG-3’ (P1) and 5'-AAAGTAACACCTAACTTTGATG TAAG-3’ (P2), containing a BglII and an Asp718 sites (underlined). For expression in E. coli, the cDNA encoding the mature portion of mIL-18 was first amplified by PCR and inserted into pQE-30 to produce a mIL-18 fused at its N terminus to a histidine tag. The 491-bp PCR product was cut by BglII and Asp718 and cloned into BsmHI/Asp718-restricted pQE-30, yielding pQE30::IL-18. For expression in eukaryotic cells, the complete IL-18-encoding gene was first amplified by RT-PCR using the primers P2 and P3 with the following sequence: 5’-CCCCAGGAACCATGGCTGC CATGTC-3’, containing a NcoI site (underlined). The resulting 598-bp fragment was inserted downstream of the CMV promoter into pCR3.1- Uni, using the Eukaryotic Transferring Kit-Unidirectional (Invitrogen) to generate pCR3.1::IL-18. pCR3.1 was generated by removing the 3’-protruding T nucleotides of pCR3.1-Uni with T4 DNA polymerase and religating the vector. For injection into mice, pCR3.1 and pCR3.1::IL-18 were prepared by alkaline lysis of the bacterial cultures, purified on CsCl gradient, and resuspended in pyrogenic injectable saline solution (Lavoisier, Paris, France).

Preparation of anti-IL-18 antiserum

The IL-18 expression plasmid, pCR3.1::IL-18, contains the cDNA encoding full-length mIL-18 under the control of the CMV promoter (Fig. 1A). After transient transfection of this plasmid into COS-1 cells, cytokine production was detected using the anti-IL-18 rabbit antiserum. As shown in Fig. 1B, an immunoreactive

Protein purification

Recombinant mIL-18 fused to the histidine tag (His6-IL-18) was produced in E. coli M15(pREP4) and purified from bacterial extracts, recovered 3 h after induction with 1 mM IPTG, using Co2+–based TALON Metal affinity chromatography under denaturing conditions (Clontech Laboratories, Palo Alto, CA). The eluted fractions were analyzed by SDS-PAGE (16) and Coomassie blue staining. Fractions containing His6-IL-18 were pooled and dialyzed against PBS and then subjected to preparative SDS-PAGE. His6-IL-18 was recovered from the gel by electroelution. The protein concentration was determined using the BCA Protein Assay Reagent kit (Pierce, Rockford, IL). Optical densities at 492 nm were determined using a standard curve obtained with known amounts of recombinant purified IFN-γ (Sigma). After washing with TBS, Ab binding was visualized using the New Fuchsin Substrate System (Dako, Carpinteria, CA). The slides were counterstained with hematoxylin and examined by light microscopy.

Immunohistochemical analysis of the transfected skin

The skin samples were fixed in 4% paraformaldehyde before being dehydrated and embedded in paraffin. Tissue sections (4-μm thick) were cut, rehydrated and equilibrated with PBS (10 mM Tris-HCl (pH 7.4), 154 mM NaCl). For immunohistochemical staining, slides were incubated overnight at 4°C with rabbit anti-IL-18 serum or preimmune serum, diluted 1/1000 in TBS, washed extensively with TBS, and incubated for 2 h at room temperature with 1/200 goat alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). After washing with TBS, Ab binding was visualized using the New Fuchsin Substrate System (Dako, Carpinteria, CA). The slides were counterstained with hematoxylin and examined by light microscopy.

Assay for IFN-γ production

In vitro expression of mIL-18

COS-1 cells were grown in DMEM (Life Technologies, Courbevoie, France) supplemented with 10% heat-inactivated FCS (Boehringer Mannheim), 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). For transfection, cells were seeded in 6-well Falcon culture plates (Becton Dickinson, Meylan, France) at a concentration of 1–3 × 105 cells/well in 2 ml medium and grown at 37°C in a CO2 incubator until reaching 50–80% confluence. Medium was then removed and replaced by 1 ml of OPTI-MEM serum-free medium (Life Technologies). Cells were incubated for 6 h at 37°C in the presence of 1 μg of plasmid complexed to 3 μl of Exgen500 (Euromedex, Soufflenheim, France). The medium was then removed, and the transfected cells were incubated in complete medium containing PBS at 37°C for 48 h. The cells were then trypsinized and harvested by centrifugation at 1500 rpm for 10 min at room temperature. Lysates were obtained by resuspending the cell pellets in loading buffer (16). Expression of IL-18 was visualized by immunoblotting of culture lysates using the His6-IL-18 rabbit antiserum diluted 1/2000, and goat anti-rabbit alkaline phosphatase-conjugated Abs (Promega, Madison, WI) diluted 1/7000 in PBS containing Tween 20.
protein was present in the lysate of cells transfected with pCR3.1::IL-18, but not in that of cells transfected with pCR3.1, or of untransfected cells. However, the immunoreactive protein migrated slower than the control His6-IL-18 produced in E. coli. Recently, it has been shown that the processing of IL-18 requires the IL-1β converting enzyme (ICE), and that IL-18 could only be processed and activated in COS cells cotransfected with an ICE-expressing vector (18, 19). Therefore, it is likely that the slower electrophoretic mobility of IL-18 produced in COS-1 cells is due to a lack of signal peptide cleavage, rather than posttranslational modification. Similar results were obtained when Chinese hamster ovary and HeLa cells were transiently transfected with pCR3.1::IL-18 (data not shown). Since processing of IL-18 by ICE is necessary for its IFN-γ-inducing activity (18), it is likely that the cytokine produced in these transfected cells is inactive.

In vivo expression of mIL-18

To investigate whether injection of pCR3.1::IL-18 can result in in vivo synthesis of mIL-18, BALB/c mice were i.d. injected with 50 μg of either pCR3.1::IL-18 (A and C) or pCR3.1 (B and D). Expression of IL-18 was analyzed on histological sections after staining using an anti-IL-18 rabbit antiserum (A and B) or the rabbit preimmune serum (C and D). The arrows point at the area of IL-18 production. Bar, 10 μm.
the skin of mice injected with pCR3.1 (Fig. 2B), or with preimmune serum on the pCR3.1::IL-18-transfected skin (Fig. 2C), or on skin of mice injected with pCR3.1 (Fig. 2D). This indicates that cutaneous transfection by pCR3.1::IL-18 led to the production of IL-18 in the skin.

Cell infiltration of skin injected with pCR3.1::IL-18

Histological examination of the pCR3.1::IL-18-injected skin revealed massive infiltration of cells in the dermis early after DNA injection (Fig. 2, A and C). This was not seen in the dermis of mice injected with pCR3.1 (Fig. 2, B and D). The cell infiltration was maximum 2 days postinjection, disappearing almost completely after 10 days (data not shown). This suggests that IL-18 was produced in its active form within the skin, and therefore most likely processed by ICE in vivo. Morphological analysis of infiltrating cells revealed that they mainly consist of mononuclear cells, but we also detected granulocytes (data not shown). The cellular infiltration was observed in three independent experiments with three different DNA preparations.

IFN-γ response in the skin following injection of pCR3.1::IL-18

To test whether injection of pCR3.1::IL-18 is able to induce IFN-γ in vivo, RT-PCR was performed on RNA isolated from skin of mice injected with pCR3.1::IL-18 or pCR3.1 1, 3, and 10 days after DNA inoculation. As shown in Fig. 3, high levels of IFN-γ-specific mRNA were present in the skin 1, 3, and 10 days after injection with pCR3.1::IL-18. In contrast, in skin injected with pCR3.1, IFN-γ-specific mRNA were detectable only 1 day after DNA inoculation and at lower amounts. These results confirm that the IL-18 produced by cutaneous-transfected cells is biologically functional and able to activate cells for the expression of cytokines. The low nonspecific, transient IFN-γ production in the skin of mice injected with pCR3.1 is probably due to DNA immunostimulatory motifs present in the plasmid backbone (20–22).

Cellular and humoral immune responses directed against mycobacterial Ags

A Th1 response is known to be pivotal for controlling mycobacterial infections (11–13). IL-18 has been shown to synergize with IL-12 for the induction of IFN-γ in mice (23), which may modulate both the humoral and cellular immune responses to infections or administered Ags. To examine the effect of IL-18 on the cellular immune response against BCG, mice were infected i. p. with two doses of live BCG (5 × 10⁵ or 5 × 10⁶) after injection of pCR3.1::IL-18 or pCR3.1. Skin-draining LN cells were then re-
in the T cell activation cascade. It induces IFN-γ, especially in synergy with IL-12. The production of IFN-γ and the subsequent effect on T cell activation is particularly important for the protection against intracellular parasites, such as mycobacteria. In most cases, *M. tuberculosis* infections are well controlled by the cell-mediated host immune system. However, in ~10% of the cases, disease develops, probably reflecting an insufficient T cell response. Tuberculosis often occurs after reactivation of dormant bacilli, suggesting that immunomodulation of infected subjects may help preventing reactivation disease. Infection with mycobacteria usually results in the secretion of large amounts of IL-12 (28), leading to IFN-γ production and T cell activation. Therefore, we investigated whether the injection of an IL-18-encoding plasmid could enhance the T cell response to mycobacterial Ags in mice infected with BCG, as used in a model system.

In this study, we show that the injection of an IL-18-encoding plasmid into the skin of mice results in local IL-18 production and induces a prominent and disseminated cell infiltrate at the injection site. The induction of massive infiltration of inflammatory cells in mice injected with purified recombinant IL-18 has been described as one of the hallmarks of IL-18 activities (8), confirming its role in inflammatory processes (3, 4, 29). These observations therefore indicate that the injection of the IL-18-encoding plasmid results in the production of biologically active IL-18. Biological activity of this cytokine is only expressed when the preprotein is processed into the mature form. This maturation requires ICE (18, 19). Production of biologically active IL-18 requires ICE and the subsequent processing did not occur in transiently transfected COS, Chinese hamster ovary, or HeLa cells, most likely because ICE is not coproduced in these cells. Since IL-18 appeared to be biologically active in the skin injected with the IL-18-encoding plasmid, it was most likely produced in cells that also produce ICE. We have indeed found ICE mRNA present in the skin of injected animals (data not shown). Ariizumi et al. (30) have shown that murine epidermal-derived dendritic cell lines express ICE in a biologically active form. Furthermore, dendritic cells, as well as keratinocytes and fibroblasts, are readily transfected upon i.d. injection of plasmid DNA (31). We can therefore hypothesize that dendritic cells may be at least one of the cell types that expresses the IL-18 cDNA upon injection of the IL-18-encoding plasmid into the skin. The coexpression of biologically active ICE within these cells may then result in the production of IL-18 in its mature form.

The biological activity of IL-18 in the plasmid-injected skin was confirmed by the induction of IFN-γ mRNA within the skin. Substantial amounts of IFN-γ mRNA were produced in the skin of mice injected with the IL-18-encoding plasmid. Interestingly, small, transient amounts of IFN-γ mRNA were also produced in the skin of mice injected with the control plasmid, suggesting that the plasmid itself may have some IFN-γ-inducing activity. Recent studies have shown that plasmid DNA from bacterial origin may contain immunostimulatory sequences (ISS), characterized by a CpG motif, that exert immunomodulatory effects on B cells, T cells, or macrophages (21, 22). These effects are likely to be mediated by the production of cytokines, including IFN-γ, IL-12, and TNF-α (20). Inspection of the sequences of the IL-18-encoding plasmid and the control plasmid indicated that both contain six ISS (data not shown). Among these six CpG-containing hexamers, four have the sequence 5′-GACGTC-3′ and two the sequence 5′-AAGCTT-3′. Both are sequences known to induce IFN-γ secretion (20). These observations suggest that ISS may contribute to the IFN-γ production in the skin of the injected mice. However, the IFN-γ production in the skin of mice injected with the control plasmid was only weak and transient, whereas IFN-γ production in the skin of the mice injected with the IL-18-encoding plasmid was much stronger and lasted for at least 10 days, indicating that it was induced by active IL-18 expressed by transfected cutaneous cells.

Most importantly, the injection of the IL-18-encoding plasmid affected Ab and cellular responses to mycobacterial Ags after infection with BCG. Administration of the IL-18-encoding plasmid led to increased in vitro PPD-dependent IFN-γ production, whereas no IL-4 production could be detected. On the other hand, the level of total soluble antimycobacterial IgG was decreased in the presence of the IL-18 plasmid. Thus, IFN-γ produced by IL-18-activated Th1 cells may have selectively inhibited Th2 cells, thereby contributing to the absence of IL-4 production and decreasing the Ab response against BCG. This latter observation is in line with previous studies showing that injection of Th1 cytokine-expressing plasmids may result in the reduction of a specific Ab response (24, 27). Flow cytometry analysis using Abs specific for B220+, CD4+, and CD8+ cells indicated that the proportion of these different cell types was similar, regardless whether the mice were injected with the IL-18-encoding plasmid or with the control plasmid (data not shown). This suggests that the impaired humoral and the enhanced T cell responses against mycobacteria were not caused by a dysregulation of the B cell/T cell balance or of the CD4+/CD8+ ratio, but rather by the capacity of IL-18 to induce T cell activation, especially of Th1 cells.

It remains to be investigated, whether IL-18 exerts its inflammatory effect directly or via the induction of proinflammatory cytokines, such as TNF-α, IL-1β, or IL-8. Human IL-18 has been found to enhance GM-CSF production in Con A-stimulated PBMC and to inhibit the release of IL-10 (5). It might thereby enhance the accessory functions of dendritic cells, such as Langerhans cells, since GM-CSF is known to be involved in the growth and differentiation of Langerhans cells (32, 33), whereas IL-10 inhibits the maturation of these cells (34). Thus, IL-18 might indirectly enhance the maturation and activation of Langerhans cells, which, in turn, may contribute to the inflammatory reactions observed.

Regardless of the mechanism, the results reported in this study indicate that, delivery of IL-18 through DNA administration can modulate a specific immune response toward a Th1 response in the
course of a bacterial infection. This may be particularly useful for the control or prevention of infection by intracellular parasites, such as mycobacteria, or perhaps HIV. With the emergence of tuberculosis in HIV-infected individuals and of multidrug-resistant strains of *M. tuberculosis*, immunotherapy with recombinant cytokines in adjunct to chemotherapy is currently being considered for treatment of certain cases of tuberculosis (35, 36). In vivo expression of cytokines using DNA delivery systems may help to define the cytokines that are most useful for immunotherapy or to induce protective immunity against tuberculosis. Of course, before IL-18-encoding plasmid DNA can be used in clinical settings, a number of points need to be considered, especially with respect to the safety of this approach. In addition to the concerns regarding DNA vaccination in general (reviewed in Ref. 37), the effects of the important cellular infiltrates after IL-18-DNA injection will have to be addressed. However, we observed that these infiltrates were localized to the site of injection and transient, with a maximum 2 days after injection, and a complete disappearance 10 days after injection. In addition, by using different DNA delivery methods and by using different doses, we hope that optimized conditions can be found to induce maximal immunomodulatory effects with minimal inflammatory effects. If safety issues can be satisfactorily resolved, the use of DNA encoding cytokines, such as IL-18, has the advantage of a more sustained, albeit transient, presence of the cytokine at the site of injection, as compared with the injection of the purified protein. This would circumvent the short half-life of recombinant IL-18 and the side effects due to the administration of repetitive, high doses.

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**References**