Brief Definitive Report

In Vivo Induction of Interleukin 10 by Anti-CD3 Monoclonal Antibody or Bacterial Lipopolysaccharide: Differential Modulation by Cyclosporin A

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Summary

We investigated the in vivo effects of cyclosporin A (CsA) on the production of interleukin (IL) 10, a cytokine with major immunosuppressive properties. To elicit IL-10 production in vivo, BALB/c mice were injected either with the anti-mouse CD3 145-2C11 monoclonal antibody (mAb) (25 μg) or with bacterial lipopolysaccharide (LPS) (20 μg). A systemic release of IL-10 was observed in both models, IL-10 serum levels reaching 1.60 ± 0.32 U/ml (mean ± SEM) and 0.67 ± 0.09 U/ml 6 h after injection of 145–2C11 mAb and LPS, respectively. Experiments in nude mice indicated that T cells are involved in the induction of IL-10 by anti-CD3 mAb, but not by LPS. Pretreatment with CsA (total dose: 50 mg/kg) before injection of 145-2C11 mAb completely prevented the release of IL-10 in serum as well as IL-10 mRNA accumulation in spleen cells. In contrast, CsA markedly enhanced LPS-induced IL-10 release (IL-10 serum levels at 6 h: 8.31 ± 0.43 vs. 0.71 ± 0.15 U/ml in mice pretreated with CsA vehicle-control, $p < 0.001$), as well as IL-10 mRNA accumulation in spleen. We conclude that CsA differentially affects IL-10 production in vivo depending on the nature of the eliciting agent. This observation might be relevant to clinical settings, especially in organ transplantation.

A major property of IL-10 is to inhibit cell-mediated immunity by blocking several functions of APCs, including the delivery of accessory signals to $CD4^+$ Th cells of the THO or TH1 type (1–3). It is therefore anticipated that IL-10 might play an important regulatory role in the process of allograft rejection. As cyclosporin A (CsA) is currently used in most immunosuppressive protocols in organ transplantation, we were interested in determining the effects of this drug on IL-10 production in vivo.

Since in vitro studies established that in addition to $CD4^+$ cells, macrophages and B cells also represent potential sources of IL-10 (4–6), two different stimuli were used to induce IL-10 production in mice: the 145-2C11 anti-mouse CD3 mAb as a polyclonal T cell activator, and bacterial LPS as an activating agent for B cells and macrophages. In both settings, increased serum levels of IL-10, as well as IL-10 mRNA accumulation in spleen were observed. These two parameters were therefore used to study the in vivo modulation of IL-10 production by CsA.

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Materials and Methods

Mice. 6–8-wk-old BALB/c mice and nude mice were obtained from the Katholieke Universiteit of Leuven (Leuven, Belgium) and from Olac (Bicester, England), respectively.

Agents Injected In Vivo. The hamster mAb 145-2C11 directed against the mouse CD3 complex (7) was prepared from culture supernatants of 145–2C11 hybridoma cells by affinity chromatography over a protein A-Sepharose column (Pharmacia, Uppsala, Sweden), as previously described (8). Control hamster Igs were purified from normal hamster serum by the same procedure. The endotoxin levels of these preparations were <15 pg/ml. CsA and its vehicle for parenteral administration were a kind gift of Sandoz Ltd. (Basel, Switzerland). LPS from *Escherichia coli* was obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental Protocols. Mice received a single intraperitoneal injection of either 145-2C11 mAb (25 μg) or control hamster Igs (25 μg), or LPS (20 μg). Blood samples were obtained by retroorbital puncture at 1.5, 6, 12, and 24 h after injection for measurement of serum IL-10 levels. In separate experiments, mice were killed

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2 h after injection for IL-10 mRNA determination in spleen cells. The effects of CsA on the induction of IL-10 by anti-CD3 mAb or LPS were evaluated by pretreating mice with two 25-mg/kg i.p. injections of CsA (or its vehicle alone as control) given 18 and 3 h before anti-CD3 mAb or LPS challenge. This protocol was found previously to prevent the release of IL-4 induced by the 145-2C11 mAb (9).

Determination of IL-10 Serum Levels by ELISA. Serum samples were assayed for IL-10 by ELISA (10) using the following anti-mouse IL-10 mAbs: SXC1, a rat IgM kindly provided by Dr. T. Mosmann (University of Alberta, Edmonton, Canada), and JES5-2A5, a rat IgG1 obtained from Pharmingen (San Diego, CA). Briefly, 96-well trays were coated with SXC1 mAb (5 μg/ml in PBS) during a 3-h incubation at 37°C. Excess protein binding sites were blocked by a further incubation with 2% BSA in PBS. Serum samples diluted in PBS containing 0.5% BSA were then added and incubated overnight at 4°C. After washing, the JES5-2A5 mAb (50 ng/well) was incubated for 2 h at 37°C. Bound JES5-2A5 mAb was revealed by a biotinylated mouse anti-rat IgG1 mAb (Experimental Immunology Unit, Université Catholique de Louvain) followed by streptavidin-horseradish peroxidase conjugate. Results were expressed in IL-10 U by reference with a standard curve obtained with a preparation of recombinant mouse IL-10 (Pharmingen). The lower limit of detection of IL-10 in this assay was 0.5 U/ml. For calculation of mean ± SEM values, samples below that threshold were arbitrarily assigned a value of 0.4 U/ml.

Reverse PCR for IL-10 mRNA. RNA extraction from mouse spleen cells using the guanidinium thiocyanate method, preparation of cDNA and PCR for IL-10 and hypoxanthine phosphoribosyl transferase (HPRT) were performed by standard procedures (11). Briefly, 1 μg of total RNA was incubated 10 min at 65°C with 1 μg oligo(dT)15 and was further incubated for 60 min at 37°C with 120 U RNasin (Promega Corp., Madison, WI), 1 mM dNTPs, 200 U Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase, 0.01 mg/ml acetylated BSA, and RT buffer (75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM Tris HCl, pH 8.3) in a final volume of 20 μl. PCR was performed using aliquots of the resulting cDNA (equivalent to 50 and 500 ng of total RNA for HPRT and IL-10 assays, respectively). To this was added 0.1 mM dNTPs, 2.5 U Taq DNA polymerase, 1 μg of each sense/antisense primer, and PCR buffer (1.2 mM $MgCl_2$, 50 mM KCl, 0.001% gelatine, 10 mM Tris HCl, pH 8.3) in a total volume of 25 μl. Primers used have been published (2, 12). Reactions were incubated in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 28 cycles (denaturation: 1 min, 93°C; annealing: 2 min, 55°C; extension: 3 min, 72°C). PCR products were run on a 3% agarose gel and stained with ethidium bromide.

Statistical Analysis. Statistical comparisons were made using the unpaired Student's *t* test. In each group, values from all mice were included.

Results

Release of IL-10 in Serum after Injection of Anti-CD3 mAb or LPS into BALB/c Mice. Whereas serum from normal BALB/c mice did not contain detectable IL-10 as assayed by ELISA (lower limit of detection: 0.5 U/ml), the injection of 25 μg of the anti-CD3 mAb 145-2C11 was followed by the appearance of circulating IL-10 (Fig. 1, *top*). Serum IL-10 was already detected at 1.5 h in three out of eight mice (mean ± SEM: 0.70 ± 0.19 U/ml), peaked at 6 h (mean ± SEM: 1.60 ± 0.32 U/ml), and returned to near background values at 24 h. This release of IL-10 was directly related to the anti-

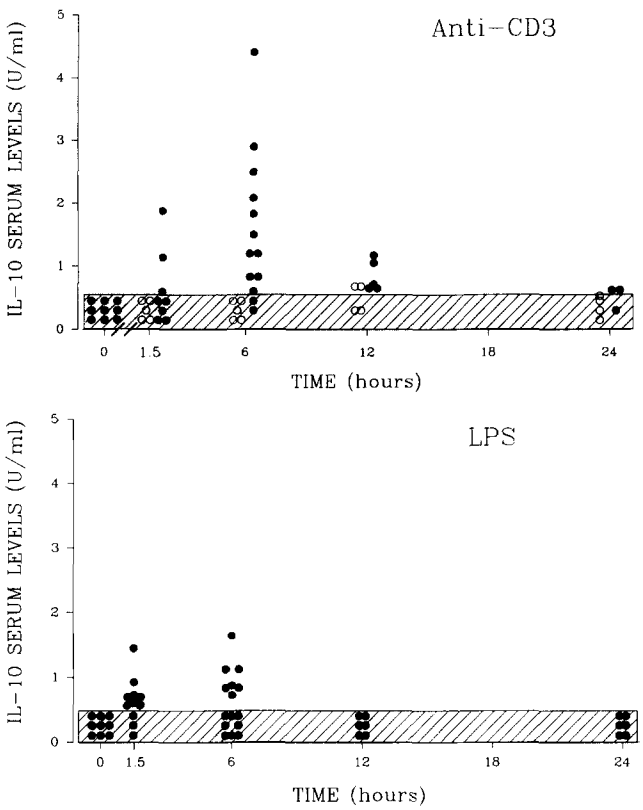

Figure 1. Release of IL-10 in serum after a single injection of 145-2C11 anti-mouse CD3 mAb or bacterial LPS in BALB/c mice. (*Top*): (●) mice injected with 25 μg 145-2C11 mAb; (○) controls injected with 25 μg of control hamster Igs. (*Bottom*) Mice injected with 25 μg LPS.

body specificity of the 145-2C11 mAb as it was not observed in mice injected with control hamster Igs (Fig. 1, *top*).

The injection of LPS (25 μg) into BALB/c animals was also followed by the systemic release of IL-10 (Fig. 1, *bottom*), although IL-10 serum levels were lower than after injection of the 145-2C11 mAb. IL-10 was detectable in serum 1.5 h after LPS injection in 8 out of 11 mice (mean ± SEM: 0.68 ± 0.09 U/ml), and at 6 h in 7 out of 15 mice (mean ± SEM: 0.67 ± 0.9 U/ml). No serum IL-10 was found at later time points.

CsA Prevents Anti-CD3 mAb-induced Production but Enhances LPS-induced IL-10 Production. The in vivo effects of CsA on the systemic release of IL-10 were investigated in animals pretreated with two 25-mg/kg i.p. injections of CsA given 18 and 3 h before challenge with anti-CD3 mAb or LPS (Fig. 2). CsA completely prevented the systemic release of IL-10 induced by anti-CD3 mAb as indicated by the lack of detectable serum IL-10 at all time points studied. As control, mice were pretreated with CsA vehicle alone before anti-CD3 mAb injection. The IL-10 serum levels in these animals were similar to those measured in the absence of pretreatment (mean ± SEM at 6 h: 1.69 ± 0.46 U/ml). In contrast with its inhibitory effect on anti-CD3 mAb-induced IL-10 production, CsA pretreatment led to a major increase in the IL-10 serum levels measured 6 h after LPS challenge (mean ± SEM: 8.31 ±

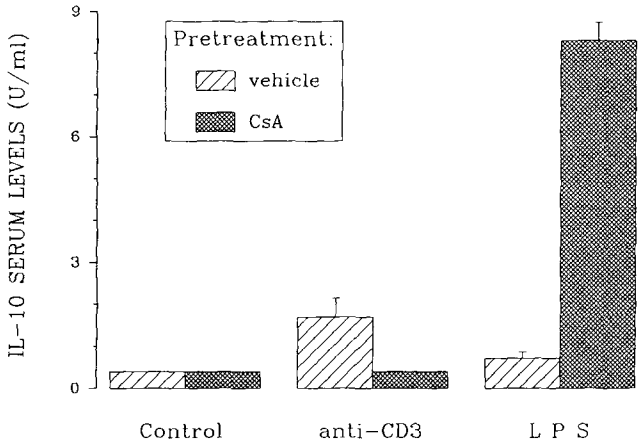

Figure 2. Effects of CsA pretreatment on the systemic release of IL-10 induced by anti-CD3 mAb or LPS in BALB/c mice. Mice (n = 5 in each group) were pretreated with two 25 mg/kg-i.p. injections of CsA or CsA vehicle before injection of 25 μg 145-2C11 mAb or 20 μg LPS. IL-10 serum levels were measured 6 h after challenge, and data of all mice are presented as mean ± SEM.

0.43 U/ml vs. 0.71 ± 0.15 in mice pretreated with CsA vehicle alone, $p < 0.001$) (Fig. 2).

We analyzed whether the effects of CsA on IL-10 release were related to modulation of IL-10 gene expression. No or minimal expression of IL-10 mRNA was found in spleens of untreated mice or of mice injected with CsA alone (Fig. 3). Anti-CD3 mAb injection led to a marked accumulation of IL-10 mRNA which was almost completely prevented by CsA pretreatment (Fig. 3). LPS also led to IL-10 gene induction and in this setting CsA pretreatment resulted in a clear increase in IL-10 mRNA accumulation (Fig. 3).

LPS but Not Anti-CD3 mAb Induces IL-10 Production in Nude Mice. The role of T cells in IL-10 production induced by anti-CD3 mAb or LPS was investigated by challenging nude mice with these stimuli. Neither unmanipulated nude mice

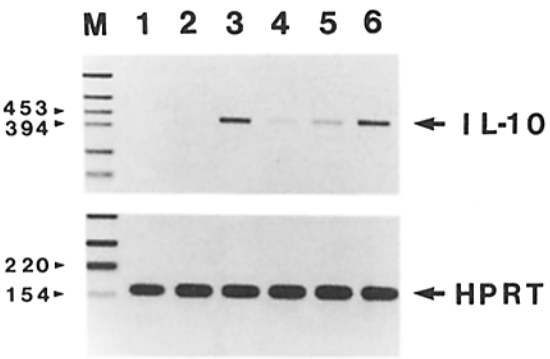

Figure 3. Effects of CsA pretreatment on IL-10 mRNA expression in spleen after injection of 145-2C11 anti-CD3 mAb or LPS in BALB/c mice (same protocol as in Fig. 2). Spleens were removed 2 h after anti-CD3 mAb or LPS challenge and analyzed by reverse PCR for IL-10 mRNA expression. Amplification of the housekeeping gene HPRT was used as control. (Lane *1*) Control mice injected with saline; (lane *2*) CsA alone; (lane *3*) anti-CD3 mAb; (lane *4*) anti-CD3 mAb after CsA pretreatment; (lane *5*) LPS; lane *6*: LPS after CsA pretreatment.

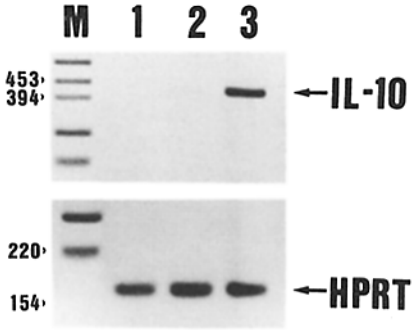

Figure 4. IL-10 mRNA expression in spleens of nude mice after injection of 145-2C11 anti-CD3 mAb or LPS. Spleens were removed 2 h after anti-CD3 mAb or LPS challenge and analyzed by reverse PCR for IL-10 and HPRT mRNA expression. (Lane *1*) Control mice injected with saline; (lane *2*) anti-CD3 mAb; (lane *3*) LPS.

nor nude mice injected with anti-CD3 mAb displayed significant IL-10 gene transcription (Fig. 4). IL-10 also remained undetectable in their serum at all time points. On the other hand, as in BALB/c mice, LPS injection led to IL-10 mRNA accumulation (Fig. 4) and induced a systemic release of IL-10 (mean ± SEM at 6 h: 0.63 ± 0.10 U/ml, n = 5), which was further increased by CsA pretreatment (mean ± SEM at 6 h: 4.82 ± 1.35 U/ml, n = 5, $p < 0.05$ as compared with LPS alone).

Discussion

The first observation of this study is that both anti-CD3 mAb and LPS induce the release of immunoreactive IL-10 in the bloodstream of normal mice. The range of IL-10 serum levels in both settings was quite large, with some samples remaining below the detection limit. Such wide dispersion of cytokine serum levels has been previously observed in similar models (13, 14). It is likely that the cell types involved in IL-10 production after injection of anti-CD3 mAb or LPS are different. Indeed, experiments in nude mice established that T cells are required for anti-CD3 mAb–induced but not for LPS-induced IL-10 production. As previously demonstrated for IL-2, IFN-γ, and IL-4 mRNAs (15), we might thus assume that T cells are the major source of IL-10 after injection of anti-CD3 mAb. Along this line, it was recently shown that the CD4$^+$ cell population was the cell type in which IL-10 mRNA accumulates after injection of anti–mouse IgD mAb (16). As far as LPS-induced IL-10 production is concerned, both macrophages and B cells, especially Ly-1$^+$ B cells, might be involved (5, 6). Whatever its precise cell source, IL-10 might be responsible for some of the immune disturbances observed after injection of anti-CD3 mAb or LPS. First, the long-lasting immunosuppressive effects of anti-CD3 mAbs which have been observed both in experimental and clinical transplantation (17, 18) could be related, at least in part, to the production of IL-10. The immunosuppression observed after LPS injection (19, 20) could also represent a consequence of IL-10 production. Experiments using neutralizing anti–IL-10 mAb or mice made IL-10–deficient by gene

targeting should help to define the exact role of IL-10 in the immunosuppression induced by anti-CD3 mAb and LPS.

The main findings reported in this paper concern the differential effects of CsA on the production of IL-10 induced by anti-CD3 mAb or LPS. Although it has been well established that CsA inhibits in vitro and in vivo the transcription of several cytokine genes in mouse T cells including IL-2, IFN-γ, and IL-4 genes (9, 21, 22), it was recently reported that the drug does not affect the production of IL-10 by a TH2 clone (23). The experiments described herein clearly establish that CsA blocks the systemic release of IL-10, as well as IL-10 gene expression induced by anti-CD3 mAb in vivo. In contrast, CsA pretreatment in the LPS model resulted in the superinduction of IL-10 gene expression and in a dramatic enhancement in the systemic release of IL-10. Similar gene superinduction by CsA has been previously reported for TGF-β in human T cells (24), for IL-6 in human PBMCs (25), and for the Ly-6E surface antigen in the T cell lymphoma–derived YAC-1 cell line (26). Mechanisms proposed to explain CsA-mediated cytokine gene superinduction include the inhibition of the production of nuclear factors binding to negative regulatory sequences of corresponding gene or the lack of inhibition of regulatory proteins that promote cytokine gene expression (24, 26). Our experiments in nude mice indicate that the enhancement by CsA of LPS-induced IL-10 gene expression and IL-10 production does not depend on the action of the drug on T cells and could therefore be related to a direct effect on IL-10–producing cells.

The in vivo effects of CsA on IL-10 production might be relevant to clinical settings where CsA is used to induce immunosuppression, such as in organ transplantation or in autoimmune diseases. Administration of CsA in transplant recipients receiving the OKT3 mAb has been recommended to reduce the antibody response to the mAb. However, by blocking OKT3-induced IL-10 production, CsA might also inhibit an important pathway of immunosuppression. In patients developing endotoxemia under CsA therapy, the enhanced IL-10 production might be beneficial by reducing the release of TNF-α and IL-1 (27), but detrimental by accentuating the Th cell defects induced by LPS.

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