Interleukin-10 (IL-10) Augments Allograft Arterial Disease

Paradoxical Effects of IL-10 in Vivo

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Interleukin-10 (IL-10) is an anti-inflammatory helper T cell type 2 (Th2) cytokine that modulates Th1-type cytokine production. Graft arterial disease (GAD) is a vascular obliterative process mediated via the Th1 cytokine interferon- γ (IFN- γ); allografts in IFN- γ -deficient animals do not develop GAD. We investigated the effect of IL-10 and anti-IL-10 on GAD in murine heart transplants and whether anti-IL-10 reestablishes GAD in IFN- γ -deficient hosts. Major histocompatibility complex class II-mismatched hearts were transplanted for 8 weeks into wild-type or IFN- γ -deficient mice. In one set of experiments, wild-type hosts received daily administration of phosphate-buffered saline (PBS) or increasing IL-10; in a subsequent set of experiments, wild-type hosts received weekly PBS, rat IgG, or anti-IL-10 monoclonal antibody; IFN- γ -deficient recipients received weekly PBS or anti-IL-10 monoclonal antibody. Explanted allografts were assessed for parenchymal rejection and GAD, cytokine profiles, and adhesion/costimulatory-molecule expression. Exogenous IL-10 resulted in increased Th2-like cytokine production; nevertheless, it exacerbated parenchymal rejection and GAD and increased CD8⁺ infiltration. Anti-IL-10 did not significantly affect the extent of rejection or GAD, cytokine profiles, or immunohistology of the allografts in wild-type hosts. Adhesion molecule (CD54 and CD106) expression was not diminished by IL-10 treatment, and costimulatory-molecule (CD80 and CD86) expression was augmented by administration of exogenous IL-10. Allografts in IFN- γ -deficient recipients showed mild rejection and no GAD, regardless of anti-IL-10 treatment. IL-10 *in vivo* thus has markedly different effects than predicted from *in vitro* experience. Although allografts develop Th2-like cytokine profiles treatment with IL-10 causes exacerbated rejection and GAD. (*Am J Pathol* 1999, 155:1929–1939)

Interleukin-10 (IL-10) is an 18-kD-homodimeric protein produced in mice by a helper T cell type 2 (Th2) subset of CD4⁺ T cells, by a subpopulation of activated B cells (CD5 and CD11 positive), and by activated macrophages. IL-10 blocks the synthesis of macrophage-derived cytokines including IL-1 β , IL-12, and tumor necrosis factor- α , and it reduces macrophage cytotoxic activity and nitric oxide production.^{1,2} IL-10 thus indirectly reduces helper T cell type 1 (Th1) differentiation by blocking macrophage IL-12 synthesis.^{3,4} In lymphocyte cultures, IL-10 can also directly inhibit T cell proliferation and the production of the Th1-type cytokines, IL-2, and interferon- γ (IFN- γ), mediators that initiate and/or regulate a delayed-type hypersensitivity response.^{5–7} In addition to regulating macrophage cytokine production, IL-10 inhibits T cell activation by decreasing antigen presentation by macrophages via suppression of major histocompatibility complex (MHC) class II molecule (MHC II) expression and by limiting the expression of costimulatory molecules necessary for T cell activation.⁸ Finally, IL-10 blocks the up-regulation of adhesion molecule expression, thereby theoretically reducing mononuclear cell emigration.^{9,10}

In organ transplantation, Th1 cells are purported to promote allograft rejection by inducing the development of alloantigen-specific cytotoxic T lymphocytes and delayed-type hypersensitivity responses.¹¹ In contrast, Th2 lymphocytes may theoretically promote long-term allograft acceptance. In a tolerogenic transplant model with anti-CD4 antibody, mouse hearts showed an increasing frequency of intragraft IL-10 and IL-4 expression, whereas untreated rejecting hearts expressed Th1 cytokines and IL-4, but not IL-10.¹² Similarly, in humans, a study of severe combined immunodeficient patients transplanted with allogeneic stem cells showed that only tolerized patients secreted a high level of IL-10.¹³ In nonvascularized murine heart allografts, viral IL-10, homologous to murine and human IL-10 and sharing their inhibitory effect on cytokine synthesis, prolonged allo-

Supported by National Institutes of Health Grant RO1 HL-43364.

Accepted for publication August 24, 1999.

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graft survival.¹⁴ However, other groups have reported contradictory results. A long-acting IL-10 fusion protein in pancreatic islet allografts led to accelerated rejection, and, in vascularized heart transplants, grafts were rejected early after administration of a high dose of IL-10.^{15,16} IL-10 transgenic recipients also rejected their grafts earlier than wild-type recipients.¹⁷ Another recent report demonstrated improved heart allograft survival by anti-IL-10 treatment and suggested potential immunostimulatory effects of endogenous IL-10.¹⁸

Overall, the reported effects are contradictory; in some models, IL-10 promotes long-term survival; in others, IL-10 aggravates rejection. Moreover, no study has yet examined the role of IL-10 in graft arterial disease (GAD), the fibroproliferative intimal vascular lesion that is the major long-term limitation to solid-organ allograft survival.^{19,20} Thus, although the *in vivo* data were equivocal regarding parenchymal rejection, the *in vitro* data with IL-10 suggested a number of expected beneficial effects of IL-10 administration potentially resulting in diminished GAD, including a shift to a predominant Th2-type response, and diminished macrophage activation. We and another group had previously demonstrated the critical role of IFN- γ in the pathogenesis of GAD, using IFN- γ -deficient (IFN- γ KO) animals²¹ or neutralizing antibody.²² The corresponding secondary mixed lymphocyte reaction (MLR), using splenocytes from the IFN- γ KO host animals, revealed increased IL-10 production in the supernatants (unpublished data). This result also suggested that exogenous IL-10 might prevent the development of GAD and that, conversely, neutralizing IL-10 activity could potentially exacerbate GAD.

Therefore, to investigate the modulating effects of IL-10 on parenchymal rejection and/or GAD, we administered recombinant murine IL-10 or anti-IL-10 monoclonal antibody (mAb) in a well-characterized vascularized heart transplant model in which both pathologic lesions occur. Moreover, because of the reported effects on T cell cytokine profiles, we analyzed the induced immune response (Th1 versus Th2). Finally, because the absence of IFN- γ has been shown to prevent GAD,²¹ potentially via augmented IL-10 production, we also investigated the effects of anti-IL-10 in transplants in IFN- γ KO recipients.

Materials and Methods

Animals

C57BL/6 (B6, H-2^b) and B6 IFN- γ -deficient mice (IFN- γ KO, H-2^b), 25 to 30 g, were used as allograft recipients; C-H-2^{bm12}KhEg (bm12, H-2^{bm12}) mice were used as heart donors. The original IFN- γ KOs were generated by homologous recombination and provided by Dr. Tim Stewart (Genentech, South San Francisco, CA).²³ All IFN- γ KOs were homozygotes and at least eighth-generation backcrossed into the B6 background.²³ The backcrossed IFN- γ KOs were confirmed as homozygotes by polymerase chain reaction amplification of tail DNA as reported previously.²¹ B6 and bm12 mice aged 8 to 10 weeks were obtained from Taconic Farms (Germantown,

NY) and the Jackson Laboratory (Bar Harbor, ME), respectively. The mice were maintained in the Harvard Medical School animal facilities on acidified water; sentinel animals in the same room that were surveyed serologically were consistently negative for all viral pathogens tested. All experiments conformed to approved animal care protocols.

Heart Transplantation and Immunosuppression

Heterotopic cardiac transplantation was performed using a modification²¹ of the method described by Corry et al.²⁴ Ischemic time was routinely 30 to 35 minutes, with a success rate of approximately 90%. The viability of the cardiac allografts was assessed by daily abdominal palpation.

Immunosuppression consisted of a pretransplant course of anti-CD4 (GK 1.5) and anti-CD8 (2.43) mAbs injected intraperitoneally 6, 3, and 1 days before transplantation.²⁵ Anti-CD4 and anti-CD8 antibodies were prepared from hybridoma clones (American Type Culture Collection, Manassas, VA) and used as ascites preparations or from comparable concentrations of antibody prepared from serum-free supernatants in an artificial capillary system (Cellmax, Celluco, Rockville, MD). In IL-10 treatment experiments, wild-type recipients were injected daily subcutaneously with recombinant murine IL-10 (rmIL-10, a generous gift of Schering-Plough, Kenilworth, NJ) or phosphate-buffered saline (PBS) ($n = 6$ per group). IL-10 doses used were 0.5, 1.0, and 2.5 μ g/day, roughly corresponding to 17, 34, and 85 μ g/kg per day in the 25- to 30-g-recipient animals. The rmIL-10 was a clinical-grade reagent containing no detectable lipopolysaccharide; this was confirmed by *in vitro* culture of adhesive splenocytes with 0 to 0.1 μ g/ml rmIL-10 and measurement of IL-12 gene expression by RNase protection assay. With increasing concentration of IL-10, there was decreasing IL-12 mRNA synthesis; no IL-12 was detected at concentrations of rmIL-10 > 0.01 μ g/ml (data not shown). For anti-IL-10 experiments, purified anti-IL-10 mAb, either SXC.1 (a generous gift of Schering-Plough) or JES5-2A5 (prepared from a hybridoma clone, American Type Culture Collection) was used. Animals were given intraperitoneal injections of 2 mg of purified anti-IL-10 mAb at the time of transplant and then 1 mg weekly, or an equivalent volume of PBS or rat IgG (Sigma Chemical Co., St. Louis, MO). All grafts were explanted 8 weeks after transplantation, a time shown previously to yield GAD lesions.²⁵

Histological Techniques

Harvested allografts were transversely sectioned in three roughly equal parts. The most basal section was fixed in 10% buffered formalin for morphological examination, the mid-portion was frozen in OCT compound (Ames Co., Elkhart, IN) and stored at -80°C for immunohistochemical staining, and the apical portion was used for intracellular cytokine analysis of mononuclear inflammatory cells by flow cytometry.^{26–28} The formalin-fixed sections were

embedded in paraffin and stained with hematoxylin and eosin or the elastic fiber stain (Weigert's method). For immunohistochemistry, to 4- to 5- μ m frozen heart sections were fixed in acetone for 10 minutes, then incubated with mAbs to Mac-3, CD4, CD8, CD40, CD54 (ICAM-1), CD80 (B7-1), CD86 (B7-2), CD106 (VCAM-1), or MHC II I-A^b (PharMingen, San Diego, CA) for 90 minutes. Control, isotype-matched nonspecific antibodies were used to establish background staining. After appropriate secondary biotin-labeled antibodies against the primary mAbs, sections were stained with avidin-alkaline phosphatase (Vector Laboratories, Burlingame, CA) by a modified avidin-biotin complex method.²⁹ Sections were counterstained with hematoxylin.

Histological Evaluation

The basal third of each heart graft, where coronary arteries generally have largest caliber, was used for histological evaluation. Occasionally, some sections included coronary arteries at their take-off at the coronary sinus. Short axial sections were stained with hematoxylin and eosin (H&E) and elastin staining and were analyzed for severity of parenchymal rejection and GAD as previously reported.²¹ Scores for parenchymal rejection and GAD were blindly graded by three independent observers (Y. F., G. B., R. N. M.). Parenchymal rejection was graded using a scale modified from the International Society for Heart and Lung Transplantation (0, no rejection; 1, focal mononuclear cell infiltrates without necrosis; 2 focal mononuclear cell infiltrates with necrosis; 3, multifocal infiltrates with necrosis; 4, widespread infiltrate with hemorrhage and/or vasculitis),^{21,30} and the GAD score was calculated from the number and severity of involved vessels (0, vascular occlusion <10%; 1, 10–25% occlusion; 2, 25–50% occlusion; 3, 50–75% occlusion; 4, >75% occlusion). Typically, 10 or more vessels were scored for each heart, and the degree of vascular occlusion for each was averaged. Scores for each specimen uniformly fell within a range of one grade for all observers and were averaged among observers. Immunohistochemical analyses (–, absent; +, weak, focal; ++, weak, diffuse; +++, strong, focal; +++++, strong, diffuse) were performed by three independent observers (Y. F., G. B., R. N. M.).

Mixed Lymphocyte Reaction

One-way MLRs were performed using whole-splenocyte or CD4⁺ lymphocyte populations. Spleens were ground through a cytoscreen into RPMI 1640 (Gibco, Gaithersburg, MD). Cells and residue were pelleted at 300 g for 5 minutes and resuspended in 10 ml ammonium chloride buffer (0.83% NH₄Cl, 5 mmol/L Tris, pH 7.2) at 37°C for 7 to 8 minutes to lyse erythrocytes, followed by washing in RPMI 1640. Cells were resuspended in RPMI 1640 supplemented with 1% nonessential amino acids, 1% L-glutamine, 1% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer, 1% sodium pyruvate, 1% penicillin/streptomycin, 0.1% 2-mercaptoethanol, and 10% heat-inacti-

vated fetal calf serum (C/10). In primary MLR, immunobeads (Dynabeads, Dynal, Lake Success, NY) and polyclonal anti-Fab antibodies (Detachabeads, Dynal) were used for CD4⁺ purification after the protocol suggested by the manufacturer. Stimulator cells were irradiated with 30 Gy. Responder cells and irradiated stimulator splenocytes (5×10^5 of each) were cultured in quadruplicate in 96-well plates in a 5% CO₂ humidified atmosphere. In some primary MLR groups, 0.1 μ g/ml IL-10 was also added.

For proliferation assay, cells were exposed to [³H]thymidine (New England Nuclear, Boston, MA) for 6 hours on day 3, and incorporated radioactivity was measured in a Betaplate scintillation counter (Wallac, Gaithersburg, MD). Proliferation is reported as counts per minute, and results are expressed as the mean $\pm$ SD. IFN- γ was measured from primary MLR culture supernatants collected on day 3, using a two-site sandwich enzyme-linked immunosorbent assay, following the protocol recommended by the manufacturer.

Intracellular Cytokine Staining and Flow Cytometry

The explanted grafts were minced with a sterile razor blade and placed in 10 ml borate buffered saline with 2% bovine serum albumin and 20 mg collagenase (Sigma). These mixtures were rocked at 37°C for 2 hours and strained through a 70- μ m nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Erythrocytes and dead lymphocytes were removed by centrifugation through Ficoll (Organon Teknica Corp., Durham, NY) for 20 minutes at 200 $\times$ g. Recovered interface cells were washed twice in RPMI 1640 and resuspended in C/10. Splenocytes were prepared as described above.

Extracted cells were stimulated with 25 μ mol/L ionomycin (Sigma) and 10 ng/ml phorbol myristate acetate (Sigma) for 4 hours at 37°C in a 5% CO₂ humidified atmosphere, in the presence of 10 μ g/ml brefeldin A (Sigma) to block cytokine secretion. Cells were then fixed at room temperature for 10 to 15 minutes with 4% paraformaldehyde in PBS and washed twice with PBS. The cells were permeabilized with a saponin buffer (0.5% saponin and 1% bovine serum albumin in PBS) and incubated with CD16/CD32 mAb (PharMingen) to block Fc receptors, thereby reducing background staining. For intracellular cytokine staining, biotin-labeled anti-IL-4, anti-IL-10, and anti-IFN- γ mAbs or isotype-matched control antibody was used in a final concentration of 10 μ g/ml. After 30 minutes staining at room temperature and two washes with saponin buffer, the cells were incubated with allophycocyanin (APC)-conjugated streptavidin for a further 30 minutes. After two washes with saponin buffer, the cells were washed with PBS, allowing the membranes to reseal, and surface staining was performed using anti-CD11b fluorescein isothiocyanate (FITC) (macrophage marker), anti-CD8-phycoerythrin (PE) and anti-CD4- peridinin chlorophyll protein (PerCP).²⁷ Flow cytometry was performed on a four-color FACScan flow cytometer (Becton-Dickinson, Mountain View, CA), using CellQuest

software. For each sample, the threshold was adjusted to 5% for background staining in the isotype-matched control antibody staining of the same sample. The percentage of positively stained population for each cytokine was calculated by subtracting 5 from the percentage of the cells in the positive range.²⁷⁻²⁹

Statistical Analysis

Values for parenchymal rejection and GAD scores, IFN- γ concentration in the supernatants, and proliferative response in MLR are expressed as the mean $\pm$ SD. Statistical analyses of parenchymal rejection and GAD scores and proliferative response in MLR were performed by analysis of variance followed by Fisher's probable least-squares difference post hoc test. Values for IFN- γ concentration in the supernatants were analyzed by Student's *t* test. *P* < 0.05 was considered statistically significant.

Results

Allograft Parenchymal Rejection and GAD

Figure 1 summarizes parenchymal rejection and GAD scores; representative histology is shown in Figure 2. All allografts continued beating until harvest at 8 weeks. Compared with grafts in the control PBS-treated B6 recipients, there was a dose-dependent increase in parenchymal rejection scores in grafts in IL-10-treated hosts, achieving statistical significance at the highest concentration of IL-10 (Figure 1A). Grafts in the IFN- γ KO recipients had significantly lower parenchymal rejection than those in the B6 recipients, and weekly injection of anti-10, using a protocol previously identified by Schering-Plough to block IL-10 activity, did not influence the scores. Similar results were found for GAD; allografts in the highest dose of IL-10-treated recipients showed a significant increase of GAD compared with the PBS-treated recipients. Anti-IL-10 treatment did not attenuate or accentuate GAD in wild-type hosts. As shown previously, grafts in IFN- γ KO recipients had negligible GAD²⁰; treatment with anti-IL-10 mAb did not promote GAD development. Although IL-10 treatment exacerbated parenchymal rejection and GAD in wild-type hosts, no significant difference was observed in transplanted hearts between recipients treated with anti-IL-10 or control rat IgG (Figure 1B).

In a dose-dependent fashion, IL-10 caused a relative increase in graft-infiltrating CD8⁺ lymphocytes, from approximately 13% of graft-infiltrating lymphocytes in PBS-treated B6 recipients to approximately 33% of graft-infiltrating cells in B6 recipients treated with 2.5 μ g/day IL-10. Correspondingly, there was a decrease in CD4⁺ lymphocytes from 45% of infiltrating cells in PBS-treated B6 recipients to 34% of cells in hearts in B6 recipients treated with 2.5 μ g/day IL-10 (Figure 3). In the wild-type recipient group treated with anti-IL-10 mAb, rat IgG, or PBS injection, there were no changes in the relative percentages of graft-infiltrating CD4⁺ and CD8⁺ T lymphocytes; CD8⁺ T cells constituted 14 to 16% of all graft-

A

*statistically significant vs. PBS-treated B6 recipients, *p* < 0.05

B

Figure 1. Parenchymal rejection (open bars) and GAD (closed bars) of MHC II-mismatched bm12 allografts 8 weeks after transplantation into B6 wild-type or IFN- γ KO recipients. **A:** Bar graphs showing results from a series of experiments in which B6 wild-type recipients received daily subcutaneous 0.5-, 1.0-, and 2.5- μ g rmlL-10 or PBS treatment, whereas IFN- γ KO recipients received either weekly intraperitoneal anti-IL-10 mAb or PBS treatment. Parenchymal rejection and GAD scores at the highest dosage of IL-10 (2.5 μ g/day) are significantly increased in grafts from IL-10-treated B6 recipients compared with the PBS-treated B6 recipients. Parenchymal rejection and GAD are significantly reduced in IFN- γ KO recipients compared with B6 recipients with or without anti-IL-10 treatment. **B:** Results from anti-IL-10 treatment experiments in B6 wild-type recipients. No significant difference was observed between the anti-IL-10 treatment group and control rat IgG-treated group.

infiltrating lymphocytes in these donor hearts (data not shown). Compared with wild-type B6 recipients, grafts in IFN- γ KO recipients had reduced infiltration of both CD4⁺ and CD8⁺ lymphocytes, and treatment with anti-IL-10 did not influence the relative numbers of CD4⁺ and CD8⁺ lymphocytes.

Because IL-10 *in vitro* normally causes decreased MHC II, costimulatory molecule, and adhesion molecule expression, we examined, as a function of rmlL-10 dosage, the expression of MHC II, CD40, CD80 (B7-1), and CD86 (B7-2), as well as CD54 (ICAM-1) and CD106 (VCAM-1) (Table 1). MHC II expression on graft endothelium and inflammatory cells *in vivo* was slightly enhanced with increasing doses of rmlL-10. CD40 was consistently barely detectable on infiltrating leukocytes or vascular wall cells. Expression of CD80 and CD86 increased in IL-10-treated allografts in a dose-dependent manner. An-

Figure 2. Representative sections of bm12 allografts explanted from either B6 wild-type recipients or IFN- γ KO recipients 8 weeks after transplantation. **A–D:** H&E staining; **E–H:** Elastic tissue staining. Note that bm12 allografts from B6 recipients show multifocal mononuclear infiltrates (**A** and **B**), whereas the graft from IFN- γ KO recipients show reduced parenchymal rejection (**C** and **D**); coronary vessels of bm12 allografts from B6 recipients show well-developed GAD (**E** and **F**), and, in contrast, the vessels of grafts from IFN- γ KO recipients show no sign of GAD (**G** and **H**). Scale bar = 50 μ m.

Figure 3. Representative flow cytometry analysis of extracted intragraft CD4⁺ and CD8⁺ lymphocytes 8 weeks after transplantation. **A:** Grafts from PBS-treated B6 recipients; **B:** Grafts from B6 recipient with 2.5 µg/day IL-10 treatment subcutaneously. IL-10 treatment resulted in a shift to greater relative numbers of CD8⁺ cells.

ti-IL-10 treatment of wild-type recipients did not significantly modify MHC II and costimulatory molecule expression, compared with rat IgG or PBS treatment (data not shown). Costimulatory molecule expression in grafts in IFN-γKO recipients was comparable to that seen in wild-type B6 recipients; anti-IL-10 administration increased the expression of CD86 (compared with PBS-treated IFN-γKO recipients). As seen previously,²¹ allografts in IFN-γKO recipients demonstrated minimal expression of MHC II and adhesion molecules; anti-IL-10 treatment resulted in modest increases in MHC II, CD54, and CD106 expression in the transplanted hearts in these IFN-γKO hosts.

Effect of IL-10 or Anti-IL-10 Antibody MLRs

Because administration of IL-10 caused an unexpectedly augmented allograft response with increased parenchy-

mal rejection and GAD, we verified the absence of lipopolysaccharide in this clinical-grade reagent by demonstrating no IL-12 production with increasing doses of rmlIL-10 up to 0.1 µg/ml (data not shown). We also sought to demonstrate that rmlIL-10 exhibited *in vitro* effects comparable with those described previously. Thus, in primary one-way MLRs using purified naive CD4⁺ lymphocytes, IFN-γ production was diminished in the presence of 0.1 µg/ml IL-10 (25 ± 1 versus 123 ± 19 U/ml, mean ± SD; Figure 4A), consistent with the known activity of IL-10¹. Using splenocytes from heart transplant recipients, one-way MLRs showed an IL-10 dose-dependent decrease in proliferation at day 3 (Figure 4B). In the 2.5-µg/day IL-10-treated group, there was a significant decrease of proliferation compared with splenocytes from PBS-treated recipients. In one-way MLRs with responder splenocytes from transplanted hosts, modest increases in proliferation were observed in the wild-type recipient

Table 1. Summary of Immunohistology 8 Weeks after Transplantation

Treatment	Wild-type recipient				IFN-γ (–/–) recipient	
	PBS	0.5 µg/day IL-10	1.0 µg/day IL-10	2.5 µg/day IL-10	PBS	α-IL-10 mAb
CD4	++++	++	++	++	+	+
CD8	++	++	+++	++++	–/+	–/+
Mac-3	+++	++++	++++	++++	++	+++
MHCII	++	++	++	+++	–/+	+
CD54 (ICAM-1)	++++	++++	++++	++++	+	++
CD106 (VCAM-1)	++++	++++	++++	++++	+	+++
CD40	–/+	–	–	–/+	–	–
CD80 (B7-1)	+	++	++	+++	–/+	+
CD86 (B7-2)	+	++	++	+++	–/+	+

–, absent; +, weak, focal; ++, weak, diffuse; +++, strong, focal; +++++, strong, diffuse.

Figure 4. A: Primary one-way MLRs with or without 0.1 µg/ml rIL-10. Irradiated bm12 splenocytes were used as stimulators, and purified CD4⁺ lymphocytes were used as responders (5×10^5 of each). Culture supernatants from quadruplicate wells were collected on day 3. IFN- γ concentration in the culture supernatants was measured by enzyme-linked immunosorbent assay. IL-10 diminished IFN- γ production from 123 ± 19 U/ml to 25 ± 1 U/ml. **B:** One-way MLRs using whole splenocytes from transplanted B6 wild-type recipients as responders. Proliferation decreased with increasing IL-10 dose; in the 2.5-µg/day IL-10-treated group, proliferation was significantly decreased compared with the PBS-treated group. * $P < 0.05$.

group treated with anti-IL-10 mAb, compared with rat IgG- or PBS-treated controls. However, there was no significant difference between any of the groups (data not shown).

Cytokine Expression in Graft-Infiltrating Cells

The cytokine profiles of graft-infiltrating lymphocytes in the presence or absence of rIL-10 were also assessed. As shown in Figure 5 and described previously,²⁸ both CD4⁺ and CD8⁺ populations in grafts from PBS-treated B6 recipients secreted IFN- γ at approximately equal levels, and little IL-4 and IL-10 were detected. Administration of anti-IL-10 mAb did not affect the cytokine-producing capability of graft-infiltrating CD4⁺ or CD8⁺ cells, compared with treatment with rat IgG or PBS (data not shown). Consistent with its known *in vitro* effects, treatment with IL-10 markedly altered the cytokine pattern of the infiltrating cells; strong IL-4 and IL-10 signals appeared, although there was also a persistent and augmented IFN- γ signal (Figure 5, B and D). CD4⁺ lymphocytes were the major source of IL-4 and IL-10 in IL-10-treated B6 recipients, although infiltrating CD8⁺ lymphocytes also secreted IL-4 and IL-10 (Figure 5, B and D).

Discussion

IL-10 is a well-tolerated cytokine, and, because of its inhibitory effect on cytokine synthesis, it may have substantial utility in a variety of clinical settings.³¹ To inves-

tigate the impact of IL-10 in allograft rejection and particularly GAD, we studied vascularized MHC II-mismatched mouse heart allografts, in which the predominant infiltrating T lymphocytes are CD4⁺.³² This MHC II-disparate strain combination has been well-characterized by our group²¹ and others.²⁵ It yields coronary arterial lesions morphologically and histochemically identical to those seen in total allogeneic mismatched murine grafts³³ and in human cardiac transplants.^{20,21,34} By using pretransplant treatment with anti-CD4 and anti-CD8 mAb, allografts survive sufficiently long (typically > 8–12 weeks) to develop GAD.^{21,25,33}

Based on the recognized *in vitro* inhibitory effects of IL-10 and in particular the blockade of proinflammatory cytokine production by activated macrophages, we expected IL-10 treatment to reduce both allograft parenchymal rejection and GAD. Indeed, in this study, IL-10 showed a dose-dependent *in vitro* inhibition of proliferation in one-way MLRs, using responder splenocytes from B6 recipients; IL-10 also reduced production of IFN- γ in primary MLRs with naive CD4⁺ lymphocytes. In MHC II-mismatched MLRs, CD4⁺ cells are the predominant responding cell population; the *in vitro* data thus confirm the inhibitory effect of IL-10 on CD4⁺ lymphocyte responses. Nevertheless, in contrast to the *in vitro* results, *in vivo* administration of IL-10 enhanced the development of parenchymal rejection as well as GAD.

In vitro, IL-10 inhibits the expression of MHC II and adhesion molecules.^{8–10} Both types of molecules are necessary for emigration and activation of immunocompetent cells in the graft and are regulated by proinflam-

A CD4⁺ cells, PBS control

B CD4⁺ cells, 1.0 μ g/day rmIL-10

C CD8⁺ cells, PBS control

D CD8⁺ cells, 1.0 μ g/day rmIL-10

Figure 5. Representative flow cytometry analysis of graft-infiltrating cells gated on CD4⁺ lymphocytes (**A** and **B**) or CD8⁺ lymphocytes (**C** and **D**) in transplanted hearts. Graft-infiltrating cells were recovered from the allografts and stained using anti-CD8-PE, anti-CD4-PerCP, and either biotinylated anti-IL-4, anti-IL-10, anti-IFN- γ , or isotype-matched control antibody and APC-conjugated streptavidin. The thresholds were adjusted to 5% for background staining in the isotype-matched control antibody staining. **A:** PBS-treated B6 recipients. 51% of the CD4⁺ lymphocytes were IFN- γ -positive above background, and no positive staining was detected for IL-4 or IL-10. **B:** B6 recipients receiving 1.0 μ g/day of IL-10. CD4⁺ lymphocytes secreted Th1 cytokine IFN- γ as well as Th2 cytokines IL-4 and IL-10. **C:** PBS-treated B6 recipients. Of the CD8⁺ lymphocytes, 65% were IFN- γ -positive above background, and no positive staining was detected for IL-4 or IL-10. **D:** B6 recipients receiving 1.0 μ g/day IL-10. CD8⁺ lymphocytes also secreted Th1 cytokine IFN- γ , as well as Th2 cytokines IL-4 and IL-10.

matory cytokines.^{35–37} In particular, the proinflammatory cytokine IFN- γ strongly stimulates increased MHC II and adhesion molecule synthesis and cell surface expression.^{35,38,39} Thus, the absence of IFN- γ leads to reduced MHC II and leukocyte adhesion molecule expression and a concomitant decrease in GAD.²¹ However, in contrast to the *in vitro* data, immunohistochemical analysis of the allografts revealed that administration of IL-10 did not inhibit MHC II or adhesion molecule expression (CD54 or CD106), thus permitting lymphocyte recruitment and leaving antigen presentation in the allografts largely intact. Similarly, in wild-type recipients, anti-IL-10 treatment did not affect the expression of these molecules. In comparison, anti-IL-10 treatment of IFN- γ KO recipients did lead to mild increases in MHC II and adhesion molecule expression in the allografts. Thus, presence of the dominant immunostimulatory Th1 cytokine IFN- γ may overwhelm the immunomodulatory effects of low-level endogenous IL-10; only indirectly does the absence of IFN- γ and concurrent anti-IL-10 treatment uncover the inhibi-

tory effect of IL-10 on surface expression of these molecules. Immunostimulatory effects of endogenous IL-10 could become apparent in a different setting of alloimmune response.¹⁸

In addition to antigen recognition, costimulation is required as a second signal for optimal T cell activation and is necessary for allograft rejection.⁴⁰ IL-10 has been reported *in vitro* to reduce expression of costimulatory molecules on activated macrophages.^{9,41,42} Nevertheless, in this allograft model, immunohistochemistry revealed a dose-dependent increase by IL-10 of costimulatory molecule CD80 (B7-1) and CD86 (B7-2) expression in wild-type recipients. Anti-IL-10 in wild-type recipients did not alter costimulatory-molecule expression, and only by blocking IL-10 in IFN- γ KO recipients was the inhibitory effect of IL-10 indirectly revealed. It therefore appears that in wild-type hosts the intragraft cytokine microenvironment *in vivo* overwhelms any inhibitory effect of endogenous or exogenous IL-10 and results in high expression of costimulatory molecules.⁴³

In our transplant model, IL-10 treatment induced the development of T cells secreting characteristic Th2 cytokines, IL-4, and IL-10. It is interesting that, despite this augmented Th2-like alloimmune response, the IFN- γ -producing T cell population also increased, and both parenchymal rejection and GAD were aggravated. Nevertheless, anti-IL-10 did not modify the intra-graft cytokine profile, and no effects on allograft parenchymal rejection or GAD scores in wild-type hosts were seen. Although it is believed that a strong Th1 immune response normally occurs in allografts and initiates rejection by promoting the development of alloantigen-specific cytotoxic T lymphocytes and delayed-type hypersensitivity responses, the relative contribution of Th2 lymphocytes is still controversial.⁴⁴ Other investigators have induced a shift toward a Th2 response by blocking IL-12; however, IL-12 antagonism did not inhibit IFN- γ expression nor did it ablate the *in vivo* sensitization of IFN- γ -secreting cells.⁴⁵ Analogous to our results, IL-12 antagonism of recipients by exogenous IL-10 resulted in accelerated graft rejection despite a shift to Th2-like response.⁴⁶

Further analysis of the graft-infiltrating lymphocytes by immunohistochemistry and flow cytometry revealed that IL-10 produced a dose-dependent rise in the number of CD8⁺ lymphocytes with a corresponding decrease of CD4⁺ lymphocytes. It is interesting that IL-10 promotes a relative increase in CD8⁺ lymphocytes, especially because the allograft model we have used has only an MHC II difference. CD8⁺ lymphocyte activation primarily requires antigen presentation on MHC I; therefore, in our MHC II-mismatched allografts, cross-presentation of the foreign MHC II must occur.^{47,48} In addition, IL-10 has chemotactic and stimulatory properties resulting in augmented recruitment of CD8⁺ lymphocytes,⁴⁹ as well as differentiation to a cytotoxic T cell phenotype.⁵⁰ Besides causing cytotoxicity, CD8⁺ lymphocytes will also secrete a variety of cytokines that promote macrophage infiltration and induce delayed-type hypersensitivity.^{27,51,52}

Another possible mechanism for exacerbation of parenchymal rejection and GAD by exogenous IL-10 administration may be its stimulatory effects on humoral alloimmunity. IL-10 promotes antibody production by regulating B cell growth and plasma cell differentiation^{53,54} and enhances antigen-driven antibody responses by regulating helper T cell subset participation.⁵⁵ Alloreactive antibody production and complement activation could play an important role in acute allograft rejection^{56,57} and in the progression of GAD,⁵⁸ although the significance of humoral immunity in chronic vascular rejection is still controversial.⁵⁹

IFN- γ is a cytokine that is critical in the development of GAD^{21,22}; it is produced in roughly equal portions by both graft-infiltrating CD4⁺ and CD8⁺ T cells.²⁸ Although IL-10 diminished the number of infiltrating CD4⁺ lymphocytes, CD8⁺ lymphocytes would likely continue to provide a rich alternative source of IFN- γ in wild-type recipients. Grafts in PBS-treated IFN- γ KO recipients showed no signs of GAD despite myocardial mononuclear cell infiltration, as described previously.²¹ Blockade of IL-10 in IFN- γ KO recipients, using anti-IL-10 mAb, however, did not lead to reappearance of GAD, suggesting that the lack of GAD in

allografts in IFN- γ KO recipients is not attributable to increased IL-10 production.

In conclusion, although there was an overall shift to a more Th2-like response, allografts in animals treated with IL-10 showed augmented rejection as well as exacerbated GAD, with elevated expression of CD80 (B7-1) and CD86 (B7-2) costimulatory molecules and no inhibition of MHC II and adhesion molecules. Thus, exogenous IL-10 administration *in vivo* led to markedly different results than those anticipated based on the results of *in vitro* experiments. Moreover, IL-10 blockade did not have any significant effect on parenchymal rejection or GAD, attributable to a predominating effect of IFN- γ . The results thus far clearly highlight the hazards of extrapolating *in vitro* studies to *in vivo* situations and the importance of experiments in intact animals to sort out the net effects of perturbing complex integrative cytokine networks.

Acknowledgments

We thank Ms. Eugenia Shvarts, Ms. Krista Condon, and Mr. Gregory Russo, Brigham and Women's Hospital, for their excellent technical assistance, and Dr. Satwant Narula, Director, Immunology Division, Schering-Plough Research Institute, Kenilworth, NJ, for her generous gift of rml-IL-10 and anti-IL-10 mAb.

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