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*J Immunol* 2000; 164:5739-5745; doi: 10.4049/jimmunol.164.11.5739

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Regulatory Cells Potentiate the Efficacy of IL-4 Gene Transfer by Up-Regulating Th2-Dependent Expression of Protective Molecules in the Infectious Tolerance Pathway in Transplant Recipients

Bibo Ke,* Thomas Ritter,²† Hirohisa Kato,* Yuan Zhai,* Jiye Li,* Manfred Lehmann,³† Ronald W. Busuttil,* Hans-Dieter Volk,²† and Jerzy W. Kupiec-Weglinski⁴*¹

We have previously shown that the tolerant state in allograft recipients can be maintained and perpetuated by an “infectious” T cell-dependent regulatory mechanism. Hence, 1) treatment of LEW rats with RIB-5/2, a CD4 nondepleting mAb, produces indefinite survival of LBNF₁ cardiac allografts; 2) donor-specific tolerance can be then transferred by spleen cells into new cohorts of test allograft recipients; and 3) putative regulatory CD4⁺ Th2-like cells are instrumental in this tolerance model. We now report on studies aimed at exposing mechanisms underlying the infectious tolerance pathway, with emphasis on the interactions between intragraft adenovirus-IL-4 gene transfer and systemic infusion of regulatory cells from tolerant hosts. Unlike individual treatment regimens, adjunctive therapy with adenovirus-IL-4 and suboptimal doses of regulatory spleen cells was strongly synergistic and extended donor-type test cardiac allograft survival to about 2 mo. RT-PCR-based expression of intragraft mRNA coding for IL-2 and IFN-γ remained depressed, whereas that of IL-4 and IL-10 reciprocally increased selectively in the combined treatment group, data supported by ELISA studies. In parallel, only adjunctive treatment triggered intragraft induction of molecules with antioxidant (HO-1) and anti-apoptotic (Bcl-xL /Bag-1) but not with pro-apoptotic (CPP-32) functions, both in the early and late posttransplant phases. Hence, systemic infusion of regulatory cells potentiates the effects of local adenovirus-IL-4 gene transfer in transplant recipients. Th2-driven up-regulation of protective molecule programs at the graft site, such as of anti-oxidant HO-1 and/or anti-apoptotic Bcl-xL and Bag-1, may contribute, at least in part, to the maintenance of the infectious tolerance pathway in transplant recipients. The Journal of Immunology, 2000, 164: 5739–5745.

A number of reports have indicated that the maintenance of tolerance to MHC-incompatible organ allografts may be linked to immune deviation toward Th2-type response (1, 2). Based on our own studies (3–5) and parallel insights from the literature (6–9), differential activation of Th2-like effector cells and a predominance of the Th2 cytokine program at the graft site often correlate with long-term transplant acceptance. In contrast, attempts to significantly prolong graft survival by systemic infusion (10, 11) or selective up-regulation (12, 13) of Th2-type cytokines have failed, suggesting that Th2 overexpression per se does not inevitably lead to tolerance. Indeed, Th2 dominance in parallel with down-regulation of Th1 development and function (14) may promote a Th2-enriched tolerance-permissive environment at the graft site.

A series of elegant studies from Dr. Herman Waldmann and coworkers have shown that short courses of anti-T cell mAbs can reprogram and then guide the immune system of experimental animals to accept a transplant, or to arrest autoimmunity (15). The most prominent feature was the demonstration that once induced, tolerance could be maintained and perpetuated by what has been termed infectious T cell-dependent regulatory mechanism (16). Our own studies have confirmed that features of infectious tolerance as described to minor histocompatibility-mismatched skin grafts in thymectomized mice conditioned with nonlytic CD4 and CD8 mAbs (16) may be applied to euthymic-primed rats rendered tolerant to MHC-incompatible vascularized organ allografts by CD4-targeted monotherapy (3–5). Hence, the tolerant cells in CD4 mAb-treated hosts could disable naive or even alloreactive cells so that they failed to trigger graft rejection (3). Moreover, a donornspecific but organ nonspecific unresponsive state could be transferred by regulatory spleen T cells in a dose-dependent manner into new cohorts of test graft recipients’ cells. The CD4⁺ T cell subset was instrumental in the induction of such adoptively transferred tolerance. Interestingly, unlike in the original CD4 mAb-treated tolerant hosts, selective up-regulation of IL-4 at the graft site was required for tolerance maintenance in test rat recipients conditioned i.v. with regulatory cells (4). Hence, local cytokine expression patterns in the graft itself and systemic infusion of regulatory cells may both influence allograft outcome in the infectious tolerance pathway.

*Dumont-University of California Los Angeles Transplant Center, Department of Surgery, University of California Los Angeles School of Medicine, Los Angeles, CA 90095; †Department of Medical Immunology, Humboldt University, Berlin, Germany; and ‡Institute of Medical Biochemistry, University of Rostock, Rostock, Germany

Received for publication December 3, 1999. Accepted for publication March 15, 2000.

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1 This work was supported by National Institutes of Health Grants RO1 AI42223 and RO1 AI23847 and by the Dumont Research Foundation. B.K. and H.K. are recipients of the Young Investigator Award from the American Society of Transplant Surgeons.

2 Current address: Institute of Medical Immunology, Humboldt University, Berlin, Germany.

3 Current address: Institute of Medical Immunology, Humboldt University, Berlin, Germany.

4 Current address: Institute of Medical Biochemistry, University of Rostock, Rostock, Germany.

5 Address correspondence and reprint requests to Dr. Jerzy W. Kupiec-Weglinski, Dumont-University of California Los Angeles Transplant Center, Room 77-120 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail address: jkupiec@mednet.ucla.edu.
On the basis of these findings, our present study was designed to test a hypothesis that administration of regulatory cells may potentiate the effects of intragraft adenovirus (Ad)-mediated IL-4 gene transfer in the infectious tolerance pathway. Indeed, our results demonstrate striking synergistic effects, as evidenced by long-term test cardiac allograft survival after intragraft Ad-IL-4 gene therapy combined with i.v. infusion of a suboptimal dose of spleen cells from tolerant hosts. Only such a combined treatment increased intragraft expression of anti-oxidant heme oxygenase-1 (HO-1) as well as anti-apoptotic (Bcl-xL, Bag-1) molecules, in parallel with preferential induction of IL-4 and IL-10. Hence, our results are the first to document the benefits of systemic infusion of regulatory cells upon local Ad-IL-4 gene therapy in transplant recipients. Perhaps, Th2-driven up-regulation of protective molecules at the graft site may contribute, at least in part, to the effects of adenoviral IL-4 and regulatory cells in the infectious tolerance pathway.

Materials and Methods

Animals and grafting techniques

Inbred male adult rats weighing 200–250 g were used (Harlan Sprague-Dawley, Indianapolis, IN). Lewis (LEW, RT1+) served as recipients of cardiac allografts from LEW x BN/BL hybrid (BN, RT1b) rats or BN, and Wistar-Furth (WF, RT1+) served as heart donors for specificity studies. Full-thickness skin was subcutaneously transplanted at the graft site may contribute, at least in part, to the effects of adenoviral IL-4 and regulatory cells in the infectious tolerance pathway.

Ad vectors

The Ad vector encoding for rat IL-4 (Ad-IL-4) was constructed, as described (18). Briefly, the cDNA for IL-4 was subcloned into pACCMV, and the resulting pACCMV-IL-4 was cotransfected with pM17 vector. Homologous recombination resulted in a replication-defective Ad-IL-4.

Histology

Cardiac allografts were sliced into pieces and preserved in 10% neutral-buffered formalin. Tissue samples were embedded in paraffin, cut into 5-μm sections, and then assessed by routine staining with hematoxylin and eosin for myocyte damage and graft infiltrating cells.

Histological analysis

Frozen cardiac allograft samples were homogenized, total RNA was extracted, and RNA concentration was determined by spectrophotometer. A total of 3 μg of RNA was reverse-transcribed using oligo(dT)15 primers and superscript reverse transcriptase according to the manufacturer’s instructions (Life Technologies, Grand Island, NY).

PCR primers/competitive template RT-PCR

Oligonucleotide primer pairs for the 5′ and 3′ rat cytokine regions were selected based on published sequences (20). To compare the relative level of each cytokine in different samples, competitors for IL-2, IFN-γ, IL-4, IL-10, and β-actin were constructed, and the competitive template RT-PCR amplification was performed, as described (4, 20). According to the varying contents of specific cDNA and varying amplification efficiencies, the standards were subjected to different cycle numbers at the annealing temperature that was optimized empirically for each primer pair: 40, 60°C (IL-2), 45, 60°C (IL-4), 40, 55°C (IL-10), 35, 60°C (IFN-γ), and 32, 63°C (β-actin), respectively. PCR products were analyzed in ethidium bromide-stained 2% agarose gel and photographed with Polaroid film (Cambridge, MA) under UV light. The PCR results were scanned, and the density of wild-type (WT) cDNA and gene-specific competitor bands were analyzed using Kodak Digital Science 1D Analysis Software (Version 2.0; Kodak, Rochester, NY). All samples were normalized against the respective β-actin WT cDNA/ACT DNA ratio.

IL-4 ELISA

Sample supernatants were obtained from homogenized cardiac allografts with PBSTDF buffers (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100, pH 7.2). Serum samples were collected from recipients’ peripheral blood. Rat IL-4 was quantitated by using Pharsight technical protocols (San Diego, CA). Briefly, flat-bottom 96-well microtiter plates (Costar, Corning, NY) were coated with 50 μl/well of diluted polyclonal rabbit anti-IL-4 Ab (BioSource International, Camarillo, CA) overnight at 4°C. Nonspecific binding sites were blocked with Blocking Buffer (10% FBS, 1% newborn calf serum, or 1% BSA in PBS) and incubated for 90 min at room temperature (RT). Plates were rinsed with PBSTDF, and then sample supernatant diluted standard and sample supernatant (100 μl) in triplicate were added, followed by incubation for 4 h at RT. Plates were washed, followed by the addition of 100 μl/well biotinylated rabbit anti-IL-4 Ab (2 μg/ml in Blocking Buffer), and incubation for 1 h at RT.
FIGURE 1. The survival of LBNF₁ test cardiac allografts in gamma-
irradiated (450 rad) syngeneic secondary LEW rats after adoptive transfer of regulatory cells from tolerant hosts ± intragraft Ad-IL-4 gene transfer. ○, Spleen cells from tolerant hosts alone (5 × 10⁶) (MST = >100 days; n = 5); ▲, 1:10 mixture of spleen cells from tolerant hosts (5 × 10⁶) and normal naive rats (45 × 10⁶) (MST = 12.2 days; n = 4); ■, intragraft Ad-IL-4 gene transfer alone (MST = 16.8 days; n = 4); △, 1:10 mixture of tolerant and normal spleen cells plus intragraft Ad-IL-4 gene transfer (MST = >60 days; n = 5); ●, 1:10 mixture of tolerant and normal spleen cells plus Ad-IL-4 gene transfer into third-party WF cardiac allograft (MST = 10.0 days; n = 3).

After washing, streptavidin-peroxidase conjugate (PharMingen) was added, and the plates were incubated for 30 min at RT. Plates were washed again, and 2.2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate solution (PharMingen) was added. The plates were incubated at RT for color development, and the reaction was terminated with 50 μl of stopping solution (20% SDS, 50% dimethylformamide). Plates were read at 405 nm in an ELISA reader. The linear region of IL-4 standard curves were obtained in a series of eight 2-fold dilutions of IL-4 standard, from 2000 pg/ml to 15 pg/ml.

**Western blot analysis**

Protein was extracted from heart tissue samples with PBSTDS buffer. Proteins (30 μg/sample) in SDS-loading buffer (50 mM Tris, pH 7.6, 10% glycerol, 1% SDS) were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The gel was then stained with Coomassie blue to document protein loading. The membrane was blocked with 3% dry milk and 0.1% Tween 20 (United States Biochemical, Cleveland, OH) in PBS. Polyclonal rabbit anti-rat HO-1 Ab was kindly provided by Dr. R. Buelow (SangStat, Fremont, CA). Polyclonal Abs against rat anti-apoptotic Bcl-xL and Bag-1, as well as pro-apoptotic CPP-32, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were incubated with Abs, and then developed according to the Amersham enhanced chemiluminescence protocol. Relative quantities of HO-1, Bcl-xL, Bag-1, and CPP-32 proteins were determined by densitometry (Kodak Digital Science 1D Analysis Software).

**Statistical analysis**

Statistical comparisons between groups were analyzed by Student’s t test. The differences were considered significant at p < 0.05.

**Results**

**Allograft survival**

First, we confirmed our previous findings (3–5) by demonstrating that LBNF₁, cardiac allografts survived >100 days following treatment of LEW rats with a nondepleting CD4 (RIB-5/2) mAb (n = 12); 2) adoptive transfer of spleen cells from tolerant RIB-5/2 mAb-treated hosts (50 × 10⁶) prolonged survival of test LBNF₁ cardiac allografts to >100 days in lightly gamma-irradiated (450 rad) secondary rat recipients (Fig. 1); and 3) cardiac allografts were rejected within 12 days in gamma-irradiated otherwise untreated rats (n = 4). Interestingly, as shown in Fig. 1, infusion of a suboptimal mixture of tolerant and normal spleen cells in a 1:10 ratio failed to significantly affect test cardiac allograft survival (mean survival time (MST) ± SD = 12.2 ± 3.5 days). Similarly, LBNF₁ hearts transfected with Ad-IL-4 alone were rejected within 3 wk (16.8 ± 5.8 days) following transplantation into gamma-
irradiated and otherwise untreated LEW rats. However, combination of adoptively transferred tolerant and normal cells (at 1:10 ratio) plus Ad-IL-4 gene transfer extended test cardiac allograft survival to about 2 mo, with some surviving >100 days (MST ± SD = >60 ± 38 days, p < 0.05). This effect was 1) Ad-IL-4 specific, because test cardiac allografts were rejected in <14 days in animals undergoing combined local transfer of Ad vector expressing nonimmunoregulatory molecule (βGal) plus suboptimal dose of regulatory cells (n = 3; not shown); and 2) donor specific, because Ad-IL-4-transfected third party (WF) hearts were rejected promptly (10.0 ± 1.7 days) in rats infused with spleen cells from tolerant CD4 mAb-treated syngeneic recipients of LBNF₁ grafts (Fig. 1). Similarly, WF hearts transfected with Ad-IL-4 were rejected within 12 days (n = 3) in gamma-irradiated otherwise untreated LEW rats (not shown). Thus, systemic infusion of the sub-

**Histopathology**

Test cardiac allografts were harvested at day 8 for histopathological evaluation. Allografts from animals treated with suboptimal dose of regulatory cells showed dense areas of mononuclear cell (MNC) infiltration, myocyte necrosis, severe interstitial edema, and lymphocytic vasculitis (Fig. 2A). Cardiac allografts transfected with Ad-IL-4 were characterized by diffuse MNC infiltration, moderate to severe interstitial edema, and vasculitis (Fig. 2B). In contrast, combined systemic treatment with regulatory cells and local Ad-IL-4 gene transfer largely preserved cardiac architecture and arteries and markedly depressed MNC infiltration (Fig. 2C).

**Intragraft expression of βGal**

To analyze putative mechanisms of in vivo synergy between reg-
ulatory cells and intragraft Ad-IL-4 gene transfer, we analyzed cardiac allografts histologically for X-Gal staining. By day 7, the mean percentage of βGal⁺ cells in cardiac allografts was 1–5% after Ad-βGal gene transfer alone (Fig. 3A); it increased to 10–15% after adjunctive infusion of regulatory cells (Fig. 3B). Thus, the infusion of regulatory cells from tolerant hosts increased the expression of the reporter gene at the graft site.

**Intragraft cytokine gene expression**

Because of our previous findings on the role of Th2-type cytokines in the infectious tolerance pathway (3–5), we then used competitive template RT-PCR to analyze intragraft Th1 and Th2 cytokine gene programs in our transplantation models. As shown in Fig. 4, by day 8, the expression of both IL-2 and IFN-γ remained consistently increased after infusion of cells or Ad-IL-4 gene transfer alone, as compared with combined treatment regimen (p < 0.05). In contrast, the level of transcripts coding for IL-4 and IL-10 re-

**IL-4 ELISA**

To validate RT-PCR-based data, we then performed serial ELISAs to assess IL-4 levels in groups of engrafted hosts. As shown in Fig.
5, intragraft expression of IL-4 protein (ng/ml) at day 8 in the Ad-IL-4 gene therapy group was significantly higher ($p < 0.05$), as compared with corresponding graft samples following regulatory cell treatment (mean $\pm$ SD $= 0.025 \pm 0.007$ and $0.14 \pm 0.03$, respectively). The combined treatment regimen further increased IL-4 levels ($p < 0.05$) both in the early (day 8, $0.043 \pm 0.003$) and late (day 114, $0.24 \pm 0.04$; $p < 0.005$ as compared with day 8 samples) posttransplant periods. Unlike in the graft itself, little if any IL-4 could be detected in the serum ($<0.03$ ng/ml) regardless of the treatment regimen (data not shown). Hence, combined regulatory cells and Ad-IL-4 gene transfer synergistically enhanced intragraft but not systemic IL-4 protein levels.

**Intragraft expression of protective molecules**

Finally, because of the emerging role of “protective” genes in long-term allograft maintenance in transplant recipients (21, 22), we used Western blots to analyze the expression of anti-oxidant (HO-1), anti-apoptotic (Bcl-xL, Bag-1), and pro-apoptotic (CPP-32) gene products in our model. The protein was extracted from cardiac allografts harvested at day 8 or $>100$ in separate groups of rats that received suboptimal dose of regulatory cells and/or Ad-IL-4 gene transfer. The relative intragraft expression levels of individual protective molecules was determined by densitometry and expressed in absorbance units (AU). As shown in Fig. 6, the expression of anti-oxidant HO-1 and anti-apoptotic Bcl-xL and Bag-1 was strongly up-regulated after combined (regulatory cells plus Ad-IL-4 gene transfer) therapy both in the early and late phases after transplantation ($1.7–2.0$ AU, $1.8 –2.2$ AU, and $1.6 –2.1$ AU, respectively), as compared with Ad-IL-4 (0.5 AU, 0.6 AU, and 0.1 AU, respectively) or infusion of regulatory cells (0.1 AU, 0.3 AU, and 0.7 AU, respectively) alone. In contrast, the expression of pro-apoptotic CPP32 was decreased after combined regulatory cells plus Ad-IL-4 gene transfer (0.2–0.3 AU), as compared with Ad-IL-4 (1.8 AU) or cells (1.9 AU) alone groups. Hence, test allograft survival after adjunctive treatment with regulatory cells and Ad-IL-4 correlated with the selective induction of molecules with anti-oxidant (HO-1) and anti-apoptotic (Bcl-xL, Bag-1) functions.

**Discussion**

This is the first report, to our knowledge, which demonstrates that systemic infusion of regulatory cells potentiates the effects of local Ad-IL-4 gene transfer in transplant recipients. Unlike individual treatment regimens, adjunctive suboptimal regulatory cells plus intragraft Ad-IL-4 gene transfer protocol markedly prolonged donor-specific test cardiac allograft survival. Selective up-regulation of Th2-type cytokines as well as anti-oxidant (HO-1) and anti-apoptotic (Bcl-xL, Bag-1) molecules at the graft site accompanied this therapeutic effect. Hence, by synergistically inducing intragraft expression of protective molecules under the cover of a Th2-type cytokine program, both intragraft IL-4 and regulatory cells contribute to the infectious tolerance pathway in this transplantation model.

That IL-4 might play a role in the acquisition of infectious tolerance has been shown by the ability of neutralizing IL-4 mAb to partially antagonize the induction of donor-specific unresponsiveness in an adoptive transfer system (23). Moreover, in our own
ongoing studies, spleen cells from tolerant animals tend to produce a higher level of IL-4 (but not IL-10 or TGF-β) upon in vitro stimulation with donor cells or Con A than those from naive animals (Y. Z. and J. W. K.-W., unpublished data). Direct evidence for an IL-4 requirement in the induction of regulatory mechanism was demonstrated in a murine cardiac transplant model in which CD2- plus CD3-targeted therapy failed to induce tolerance in IL-4-deficient recipients, and infusion of IL-4 mAb abrogated tolerance in normal hosts (24). Although neutralizing IL-4 at the time of donor-specific blood transfusion plus CD4 mAb-induced tolerance had no effect on allograft outcome in primary recipients, neutralizing IL-4 in adoptively transferred donors prevented long-term engraftment in the majority of secondary hosts. Moreover, although in our recent study, treatment with IL-4 mAb did not prevent tolerance induction in primary hosts, this tolerant state was not “infectious,” as further transfers of splenocytes failed to prevent rejection in new cohorts of engrafted rats (25). Our present results show that selective induction of IL-4 after Ad-based gene therapy alone failed to significantly affect cardiac allograft survival in two different (LBNF1→LEW and WF→LEW) rat strain combinations. This may have resulted from limitations of the system in which the actual Ad-bGal transfection rate in rat myocytes was very low (1–5% at day 7), consistent with our previous report (18).

FIGURE 4. Competitive template RT-PCR-assisted expression of mRNA coding for Th1 (IL-2 and IFN-γ)- and Th2 (IL-4 and IL-10)-type cytokines in cardiac allografts at 8 days (□) and 114 days (●). Note that the expression of both IL-2 and IFN-γ remained consistently increased after infusion of a suboptimal dose of regulatory cells (A) or Ad-IL-4 gene transfer (B) alone, as compared with combined treatment (C) (A or B vs C, p < 0.05). In contrast, the level of transcripts coding for IL-4 and IL-10 remained significantly elevated after adjunctive treatment with regulatory cells and Ad-IL-4, as compared with individual treatment regimens (C vs A or B, p < 0.01). Each column represents the mean ratio ± SD of WT cDNA/CT DNA for the cytokine normalized against the ratio of WT cDNA/CT DNA for β-actin (n = 2–3 samples/group).

FIGURE 5. ELISA-assisted expression of IL-4 in cardiac allografts at 8 days (□) and 114 days (●). Note that intragraft IL-4 protein levels (ng/ml) after individual cell (A) or Ad-IL-4 (B) treatment regimens were consistently and significantly lower as compared with corresponding graft samples following combined therapy at day 8 (C) or day 114 (D) (A vs B or C, p < 0.05; B vs C, p < 0.05; C vs D, p < 0.005). Each column represents the mean ± SD (n = 3 samples/group).

FIGURE 6. Western blot analysis of anti-oxidative (HO-1), anti-apoptotic (Bcl-xL and Bag-1), and pro-apoptotic (CPP-32) gene products in rat cardiac allografts at day 8 and >100 posttransplant. The expression of HO-1, Bcl-xL, Bag-1, and CPP-32 was probed using Abs against rat HO-1 (A), Bcl-xL (B), Bag-1 (C-16) (C), and CPP-32 Ab (D). Lane 1, Day 8 cardiac allografts treated with suboptimal dose of regulatory cells; lane 2, day 8 cardiac allografts treated with Ad-IL-4; lane 3, day 8 cardiac allografts treated with suboptimal dose of regulatory spleen cells plus Ad-IL-4; lane 4, day 114 cardiac allografts treated with suboptimal dose of regulatory spleen cells and Ad-IL-4. Note that combined treatment increased intragraft expression of HO-1, Bcl-xL, and Bag-1 but decreased CPP-32 expression. The data shown are representative of two to three separate experiments.
Interestingly, we now show that an adjunctive suboptimal dose of regulatory cells markedly increased transgene expression by recombinant Ad in cardiac allografts. Consistent with our present RT-PCR data, this may have resulted from depression of an intense proinflammatory Th1-type response that otherwise down-regulates the transgene expression-driven CMV promoter (26). Interestingly, mouse cardiac isografts transplanted with βGal were spared from inflammatory response, despite the presence of an immune response to the vector (27). We have recently reported that treatment with RIB-5/2, a mAb used in our present tolerogenic regimen, prolonged lung-directed Ad-mediated gene expression (28). As our present RT-PCR assay has not been constructed to differentiate between Ad- and endogenously derived IL-4, enhanced intragraft expression of this Th2 cytokine at both mRNA (RT-PCR) and protein (ELISA) levels in the combined treatment group could come from Ad vector, host lymphocytes, and/or adoptively transferred lymphocytes. Hence, our results support the idea that ex vivo-generated regulatory cells might be considered as an adjunctive measure to enhance the efficacy of Ad-mediated cytokine gene therapy in transplant recipients.

It is becoming increasingly appreciated that transplantation tolerance exists at multiple levels (29), and its maintenance may require utilization of several immune mechanisms, including deletion, anergy, and the emergence of regulatory T cells. These mechanisms may not only overlap, but their individual contributions may vary depending on MHC barrier, the type of organ graft, therapeutic strategy applied, and species being studied. Our present findings complement recent reports that provide compelling evidence for the role of regulatory T cells in host unresponsiveness, especially in the infectious tolerance (30). We have previously shown that at least $5 \times 10^6$ spleen cells were required to confer operational tolerance from tolerant hosts to new cohorts of test rat recipients (3, 4). By choosing the suboptimal regimen, we have now elected to use 1/10 of the effective dose ($5 \times 10^5$), and to keep with the total cellular load these regulatory cells were mixed with $45 \times 10^6$ of naive splenocytes. Such a mixture alone produced marginal prolongation of test graft survival after adoptive transfer, consistent with our previous dose-response trials (3, 4). However, if combined with Ad-IL-4 gene therapy, a significant prolongation of donor-type (LBNF1) but not third party (WF) test graft survival was observed. Hence, our results demonstrate that 1) Ad-IL-4 gene transfer potentiates the operational activity of regulatory cells, and 2) regulatory cells maintain MHC specificity of the infectious tolerance pathway. CD4+ T cells are instrumental in that pathway, as their selective depletion in the original tolerant animals or after transfer into new cohorts of test recipients prevents induction of tolerance (3, 31, 32). In all animal models tested to date, the maintenance of regulatory T cells was shown to depend on the presence of donor Ag (30). However, unlike in mice (16), the induction of regulatory T cells in rats (5) or pigs (33) was thymus dependent, suggesting that both central and peripheral immune mechanisms are required for the acquisition of infectious tolerance.

Prolonged allograft survival after adjunctive Ad-IL-4 and regulatory cell treatment in this study was accompanied by overexpression of protective molecules at the graft site, including anti-oxidant HO-1 and anti-apoptotic Bcl-2, which has been implicated in T cell activation-induced apoptosis (40). Hence, as combined Ad-IL-4 gene transfer and infusion of regulatory cells were required to achieve therapeutic effect, both graft and host factors might play a role in the induction of protective genes in the infectious tolerance pathway.

Putative mechanisms that may trigger intragraft expression of protective genes are ill defined. Interestingly, as in xenograft “accommodation” (21), the induction of protective molecules in the infectious tolerance pathway correlate with preferential infiltration of cardiac allografts by Th2-like IL-4 and IL-10-producing cells. Indeed, the Th2 cytokine program has been shown to trigger the expression of protective genes in MNC (41) and endothelial cells (39). This is consistent with our findings on the role of Th2-like cells and local secretion of Th2-type cytokines in animals rendered tolerant by transfer of regulatory cells (3, 4, 25). We favor the notion that the expression of protective molecules is Th2-type dependent, because treatment of rats with neutralizing IL-4 mAb depressed intragraft expression of HO-1 and Bcl-2, and prevented the acquisition of tolerance in cohorts of adoptively transferred rats (J. W. Kupiec-Weglinski, unpublished data). Perhaps future studies in knockout mice will determine whether the host Th2 environment might promote graft survival by influencing natural mechanisms of protection.

In conclusion, our present study demonstrates that systemic infusion of regulatory cells from tolerant hosts potentiates the effects of local Ad-IL-4 gene transfer in transplant recipients. Th2-driven up-regulation of protective molecule programs at the graft site, such as anti-oxidant HO-1 and/or anti-apoptotic Bcl-2 and Bag-1, may contribute, at least in part, to the maintenance of the infectious tolerance pathway. This pathway is important, and defining the underlying mechanisms may lead to new insights into the complexities of establishing immune tolerance to solid organ transplants. Clearly, tolerogenic strategies that trigger generation and then support the maintenance of regulatory cells in rodent transplantation models warrant more attention in future primate and human studies.

Acknowledgments

We thank Drs. Herman Waldmann and Wayne Hancock for discussion and for critical review of this manuscript.

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