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CD40Ig treatment results in allograft acceptance mediated by CD8+CD45RClow T cells, IFN-γ, and indoleamine 2,3-dioxygenase

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Treatment with CD40Ig results in indefinite allograft survival in a complete MHC-mismatched heart allograft model in the rat. Here we show that serial second, third, and fourth adoptive transfers of total splenocytes from CD40Ig-treated recipients into secondary recipients led to indefinite donor-specific allograft acceptance. Purification of splenocyte subpopulations from CD40Ig-treated recipients demonstrated that only the adoptively transferred CD8+CD45RClow subset resulted in donor-specific long-term survival, whereas CD8+CD45RClow T cells from naive animals did not. Accepted grafts displayed increased indoleamine 2,3-dioxygenase (IDO) expression restricted in the graft to ECs. Coculture of donor ECs with CD8+CD45RClow T cells purified from CD40Ig-treated animals resulted in donor-specific IDO expression dependent on IFN-γ. Neutralization of IFN-γ or IDO triggered acute allograft rejection in both CD40Ig-treated and adoptively transferred recipients. This study demonstrates for what we believe to be the first time that interference in CD40–CD40 ligand (CD40-CD40L) interactions induces allospecific CD8+ Tregs that maintain allograft survival. CD8+CD45RClow T cells act through IFN-γ production, which in turn induces IDO expression by graft ECs. Thus, donor alloantigen-specific CD8+ Tregs may promote local graft immune privilege through IDO expression.

Introduction

Organ transplantation would benefit from immunotherapeutic strategies that enable the development of transplantation tolerance (1). Tolerance of donor antigens would avoid or reduce the administration of immunosuppressors and would simultaneously reduce the incidence of chronic graft dysfunction.

Over the past few years, Tregs of different phenotypes capable of suppressing immune responses have been identified (reviewed in ref. 2). Although several CD4+ populations have been described as being Tregs, CD8+ Tregs have also been identified in different models (3–14). One example of CD8+ Tregs is the non–cytokine-secreting, CD8+CD28− alloantigen-specific Treg, which interacts directly with DCs or ECs and renders them tolerogenic (4, 11). Recently, a new natural regulatory CD8+CD45RClowFoxp3+ subset has also been characterized (3).

In certain cases, allogeneic tolerance can be maintained and perpetuated into subsequent recipients by multiple and successive adoptive transfer of CD4+ Tregs into naive hosts (1). This condition has been termed infectious tolerance and has until now only been described for CD4+ Tregs (1, 15, 16).

Administration of anti-CD40L (17) or anti-CD40 (18) mAbs as well as administration of CD40Ig (19) are among the most efficient treatments available to obtain long-term organ allograft survival in animal models, but the mechanisms are still poorly understood (reviewed in ref. 20). Anti-CD40L mAb has been shown to induce CD4+ Tregs (21) and deplete activated T cells through Fc-dependent mechanisms (22) or apoptosis (23). Interaction of DCs and T cells in the absence of CD40-CD40L interactions results in cross-tolerization of CD8+ T cells against cognate antigens (24–26). Anti-CD40L mAbs have also been shown to induce regulatory cells with a DC and/or NK phenotype in a model of autoimmune diabetes (27). CD40-CD40 ligand (CD40-CD40L) interactions can affect immune responses and inflammation not only through immune cell interactions but also by modulating the function of ECs and platelets (20).

We previously reported that administration of CD40Ig through gene transfer led to indefinite allograft survival in a complete MHC-mismatched heart allograft model in the rat (19). The aim of the present work is to analyze the in vivo mechanisms underlying graft acceptance.

Here we demonstrate that CD40Ig-induced graft acceptance is mediated by alloantigen-specific CD8+CD45RClow Tregs that were able to impose inhibition of acute rejection to naive T cells when they were successively adoptively transferred up to 4 times. This is the first description of manipulation of CD40-CD40L interactions that resulted in the generation of CD8+ Treg cells. We also provide evidence that IFN-γ is produced by CD8+CD45RClow T cells and

Nonstandard abbreviations used: AdCD40Ig, adenovirus encoding CD40Ig; Addl324, adenovirus dl324 (noncoding); BN, Brown-Norway; CD40L, CD40 ligand; CDR3, complementary determining region 3; DST, donor-specific blood transfusion; HO-1, heme oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IP, infectious particles; 1-MT, 1-methyl tryptophan.

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Table 1
Cardiac allograft survival after serial adoptive cell transfers

Group		No. of splenocytes	*n*	Donor strain	Graft survival (d)
Addl324	First adoptive transfer	200×10^6	7	LEW.1W	10 ± 2
AdCD40Ig	First adoptive transfer	200×10^6	3	LEW.1W	>120
		50×10^6	8	LEW.1W	>120
	Second adoptive transfer	200×10^6	1	LEW.1W	>120
		100×10^6	2	LEW.1W	>120
		50×10^6	6	LEW.1W	>120
	Third adoptive transfer	50×10^6	2	LEW.1W	>120
	Fourth adoptive transfer	50×10^6	3	LEW.1W	>120
AdCD40Ig	First adoptive transfer	200×10^6	3	BN	9

LEW.1A recipients were sublethally irradiated (4.5 Gy) at day –1 and received heart allografts and i.v. injections of splenocytes at day 0. Graft survival was monitored by abdominal palpation.

that indoleamine 2,3-dioxygenase (IDO) plays a crucial role in suppression of graft rejection. Finally, we show that these $CD8^+$ Tregs induce ECs from graft origin to produce IDO through IFN-γ.

Results

CD40Ig-induced long-term survival is serially transferable to naive recipients. To assess whether tolerogenic cells were involved in the long-term allograft survival induced by CD40Ig treatment, we performed adoptive cell transfer experiments using splenocytes from CD40Ig-treated recipients. In agreement with our previously published results (19), recipients that received fully MHC-discordant LEW.1W heart allografts transduced with an adenovirus encoding CD40Ig (AdCD40Ig) exhibited long-term allograft survival (>120 days in 93% of recipients), whereas all recipients of grafts transduced with the control noncoding adenovirus dl324 (Addl324), rejected their grafts rapidly (8 ± 0.8 days). The first adoptive transfer of 50×10^6 and 200×10^6 splenocytes from CD40Ig-treated long-term survivor recipients (collected at day 120 after transplantation) to naive syngeneic recipients resulted in long-term acceptance of all LEW.1W cardiac allografts (Table 1). Grafts were rejected (6.5 ± 0.3 days) by recipients that did not receive splenocytes or that received splenocytes from control adenovirus-treated recipients (rejection 10 ± 2 days) (Table 1). Third-party Brown-Norway (BN) hearts transplanted into recipients receiving splenocytes from CD40Ig-treated long-term survivors with a LEW.1W heart were also promptly rejected (9 days) (Table 1). Whereas CD40Ig-treated recipients displayed high levels of circulating CD40Ig at day 10 after transplantation (229 ± 113 μg/ml), recipients of the first adoptive cell transfer had undetectable levels of CD40Ig (<0.02 ng/ml). Recipients with long-surviving grafts after adoptive cell transfer displayed a reduction by more than 90% of serum anti-LEW.1W alloantibodies (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI28801DS1) and a 51% reduction in splenocyte anti-donor CTL activity (Supplemental Figure 1B).

Second adoptive cell transfers of 50×10^6 to 200×10^6 splenocytes from CD40Ig-treated recipients into naive animals also resulted in long-term acceptance of allogeneic hearts in all recipients (Table 1). All subsequent (third and fourth) adoptive cell transfers into naive animals performed with 50×10^6 splenocytes recovered 120 days after transplantation and adoptive cell transfer resulted in long-term cardiac survival (Table 1).

These results demonstrate that CD40Ig-treated recipients contain regulatory cells capable of transferring donor-specific transplantation tolerance, defined as acceptance of a second first-party graft and rejection of a third-party one. This transplantation tolerance was infectious and acted through dominant suppression of antiallogeneic immune responses.

Transfer of graft acceptance is dependent on $CD8^+$ T cells. Whereas transfer of total splenocytes from CD40Ig-treated recipients uniformly transferred tolerance, T cell or CD8 depletion from the splenocytes abrogated the tolerance transfer (rejection at 14.5 ± 3.5 and 11 ± 0.7 days, respectively) (Figure 1A). In contrast, transfer of CD4-depleted or NK-depleted splenocytes resulted in indefinite graft survival (Figure 1A). To confirm these results, we adoptively transferred positively selected CD40Ig T cells, CD40Ig $CD4^+$ T cells, and CD40Ig $CD8^+$ T cells. Adoptive transfer of positively selected T cells and $CD8^+$ T cells resulted in indefinite allograft survival in all recipients, whereas adoptive transfer of $CD4^+$ T cells led to allograft rejection 15 days (Figure 1B). $CD8^+$ T cells were routinely more than 97% $TCR\alpha\beta^+$ and $CD8\alpha^+$ and less than 2% $CD4^+$ or $CD161^+$ (data not shown).

Moreover, in vivo depletion of $CD8^+$ T cells resulted in allograft rejection in 50% of the recipients ($n = 12$, $P < 0.0001$ versus CD40Ig plus control mAb) (Figure 1C), confirming an important role for these cells in the establishment of tolerogenic mechanisms during CD40Ig treatment. This treatment induced a permanent and marked reduction in peripheral blood $CD8\alpha^+$ and $CD8\beta^+$ cells as shown by flow cytometry analyses performed every 2 weeks (Supplemental Figure 2) and by histological analysis of rejected cardiac grafts that lacked infiltrating $CD8\alpha^+$ and $CD8\beta^+$ cells (data not shown). Administration to CD40Ig-treated recipients of an anti-MHC class I monomorphic mAb abolished long-term allograft survival, indicating that recognition of MHC class I antigens by $CD8^+$ cells was implicated in the effect of CD40Ig (Figure 1C). Together, these data demonstrate that CD40Ig-induced donor-specific allograft acceptance is mediated by CD8 T cells.

$CD8^+CD45RC^{low}$ T cells are the key regulators induced by CD40Ig treatment. It has recently been shown that the level of CD45RC expression on rat $CD8^+$ T cells distinguishes 2 $CD8^+$ subsets that differ in their cytokine profiles and function (3). The $CD8^+CD45RC^{low}$ subset has been shown to contain Tregs that inhibit graft versus host disease and mixed lymphocyte reactions. To evaluate the contribution of $CD8^+CD45RC^{low}$ T cells in $CD8^+$-mediated tolerance after CD40Ig treatment, $CD8^+CD45RC^{low}$ T cells and $CD8^+CD45RC^{high}$ T cells were sorted to a purity of greater than 99% (Figure 2A) and adoptively transferred into naive graft recipients (Figure 2B). Adoptive transfer of 2.5×10^6 $CD8^+CD45RC^{low}$ T cells from CD40Ig-treated donors into syngeneic recipients resulted in indefinite allograft survival, whereas transfer of the same number of $CD8^+CD45RC^{high}$ T cells failed to inhibit acute rejection (Figure 2B). Adoptive transfer of $CD8^+CD45RC^{low}$ T cells from naive nongrafted animals in the same conditions did not inhibit acute rejection (Figure 2B).

Thus, CD40Ig treatment functionally modified $CD8^+CD45RC^{low}$ T cells to induce indefinite allograft survival, since $CD8^+CD45RC^{high}$

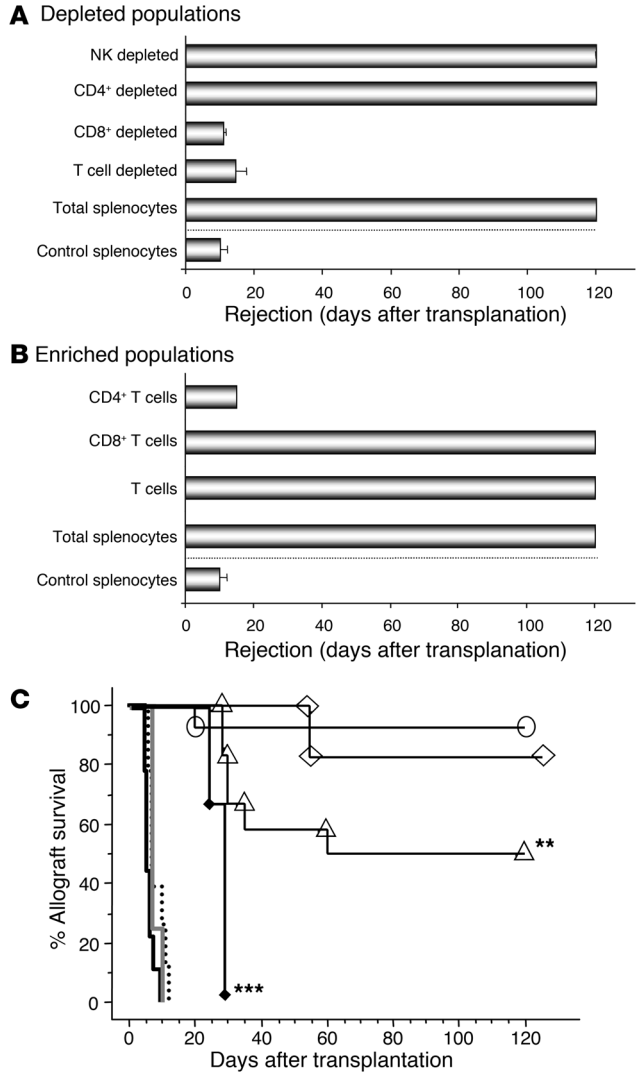

Figure 1
CD8+ T cells mediate transfer of transplantation tolerance after CD40Ig treatment. Cells from control rats that had rejected their grafts or from CD40Ig-treated recipients were injected i.v. the day of transplantation into LEW.1A recipients that received LEW.1W heart transplants (day 0) and that were sublethally irradiated (4.5 Gy, at day –1). (**A**) Total splenocytes (50×10^6; $n = 6$) or splenocytes depleted of T cells ($n = 2$), CD4+ cells ($n = 2$), CD8+ cells ($n = 4$), or NK cells ($n = 2$) were injected. (**B**) Splenocytes (50×10^6; $n = 6$), purified T cells ($n = 3$), CD8+ T cells ($n = 4$), or CD4+ T cells ($n = 2$) were injected. Graft survival was assessed by abdominal palpation of cardiac beating. (**C**) Grafts were either untreated ($n = 9$; thick black line), treated with a control mAb (3G8) ($n = 6$, gray line), transduced with Addl324 (5×10^{10} IP; $n = 9$; dotted line), transduced with AdCD40Ig ($n = 27$, black line with open circles), or transduced with AdCD40Ig and injected with a depleting anti-CD8 mAb (OX8) ($n = 12$; black line with open triangles) or an anti-MHC class I mAb (OX18) ($n = 3$; black line with filled diamonds). A group of control animals received the control mAb (3G8) in addition to CD40Ig ($n = 6$; black line with open diamonds). $^{**}P < 0.01$, $^{***}P < 0.0001$ versus animals injected with AdCD40Ig or AdCD40Ig and 3G8.

T cells from the same animals or CD8+CD45RC^{low} T cells from naive ones did not inhibit acute rejection.

Analysis of CD8+CD45RC^{low} T cell cytokine profile, Foxp3 expression, and phenotype. Analysis of IFN-γ production by intracellular staining of activated splenocytes showed that CD40Ig treatment or adoptive transfer of splenocytes significantly increased the frequency of IFN-γ–producing cells compared with that found in naive rats (14.1% ± 1.7% and 24.9% ± 1.4% versus 5.1% ± 1.2%, $P < 0.05$ and $P < 0.0001$, respectively) and untreated recipients (8.9% ± 1.1%) (Figure 3A). Moreover, a significantly higher percentage of IFN-γ–producing cells was detected in the CD8+CD45RC^{low} T cell population of CD40Ig-treated rats compared with the CD8+CD45RC^{high} population (36.4% ± 11.6% versus 7.1% ± 4.4%, $P = 0.001$). IL-10, TGF-β, IL-13, and IL-4 were either low or barely detectable (data not shown).

Analysis of mRNA expression by CD8+CD45RC^{low} T cells from CD40Ig-treated animals revealed significantly higher amounts of *Foxp3*, *glucocorticoid-induced tumor necrosis factor receptor family–related gene* (*GITR*), *IFN-γ*, *IL-13*, *IL-10*, *TGF-β*, and *IL-2* as compared with amounts found in CD8+CD45RC^{high} T cells, whereas *CTLA4*, *heme oxygenase-1* (*HO-1*), *IL-4*, *CD28*, and *perforin* were expressed at comparable amounts. *IDO* was undetectable in both groups (Figure 3B). CD8+CD45RC^{low} T cells from CD40Ig-treated animals versus naive animals revealed significantly higher amounts of GITR, TGF-β, IL-2, and CD25, whereas CD28 was decreased (Figure 3B).

The majority of CD8+CD45RC^{low} T cells from naive, Addl324-treated, and AdCD40Ig-treated recipients were negative for CD4 (95%), CD28 (90%), OX40 (93%), CD103 (90%), CD161 (80%), CD11b (90%), CD11c (97%), MHC class II (80%), and CD25 (90%), whereas they were positive for CD62L (40%), CD8β (95%), and TCRαβ (96%) (Supplemental Figure 3 and data not shown).

These data demonstrate that CD40Ig-induced allograft acceptance is mediated by a CD8+CD45RC^{low} T cell subset characterized by production of IFN-γ and regulatory cytokines that expresses several markers that characterize regulatory cells.

Selected usage of TCR Vβ11 family in CD8+CD45RC^{low} T cells from CD40Ig-treated animals. The length distribution of the TCR β-chain complementary determining region 3 (CDR3) hypervariable region is gaussian in naive T cells and is altered in activated T cell responses. Qualitative analysis of the TCR Vβ families' repertoire of spleen CD8+CD45RC^{low} T cells from CD40Ig-treated recipients showed a significantly altered distribution of the Vβ11 family (a mean alteration ± SD of 46.2% ± 14.7%), with a predominant peak of the same CDR3 length in all animals (Figure 4) and a normal gaussian-type distribution for all other Vβ families (data not shown). Compared with naive animals, *Vβ11* family transcript accumulation was increased 11-fold in spleen CD8+CD45RC^{low} T cells in CD40Ig-treated animals. As controls, spleen CD8+CD45RC^{low} T cells from naive animals or CD8+CD45RC^{high} T cells from CD40Ig-treated animals showed unaltered gaussian-type Vβ11 family distribution (Figure 4) (9.85% ± 1% and 11.4% ± 1.5%, respectively; $P < 0.01$ compared with CD8+CD45RC^{low} T cells from CD40Ig-treated animals).

That CD8+CD45RC^{low} Tregs from CD40Ig-treated recipients express a selected TCR Vβ11 gene family repertoire demonstrates an induced immune response and suggests public TCR responses to common donor antigen(s).

Following CD40Ig treatment, long-surviving grafts showed strong upregulation of regulatory molecule transcripts and accumulation of CD8+IFN-γ+ cells. Quantitative RT-PCR analysis showed that, com-

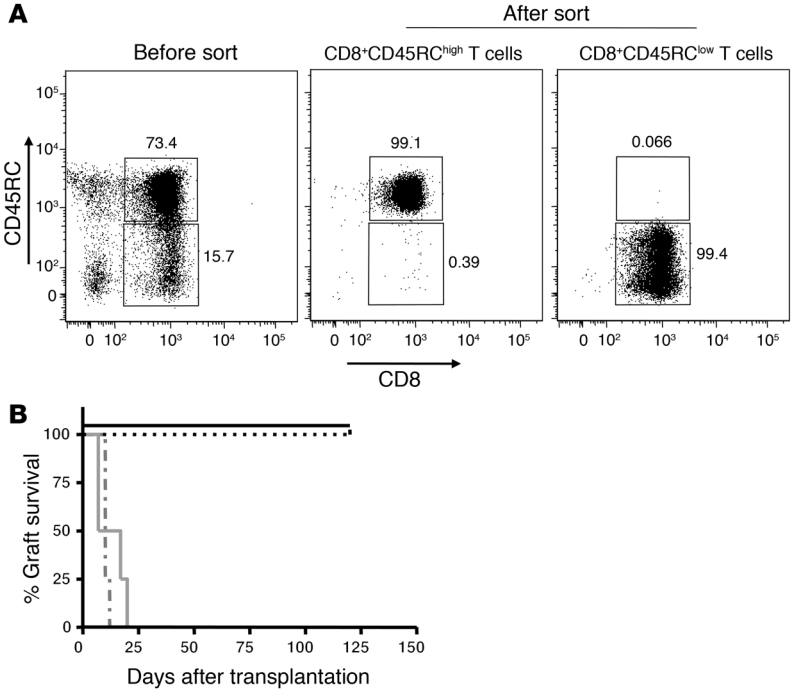

Figure 2
$CD8^+CD45RC^{low}$ T cells mediate transfer of transplantation tolerance after CD40Ig treatment. (**A**) $CD8^+CD45RC^{low}$ and $CD8^+CD45RC^{high}$ T cells were sorted to greater than 99% purity from spleens. (**B**) Cells were purified from naive, CD40Ig-treated, or adoptively transferred recipients 120 days after transplantation. A total of 2.5×10^6 cells of each cell population was injected i.v. the day of transplantation of LEW.1W hearts into naive LEW.1A recipients sublethally irradiated (4.5 Gy) at day –1. Graft survival was assessed by abdominal palpation of cardiac beating. Black line, $CD8^+CD45RC^{low}$ T cells from CD40Ig-treated animals ($n = 4$). Dotted line, $CD8^+CD45RC^{low}$ T cells from adoptively transferred animals ($n = 2$). Gray line, $CD8^+CD45RC^{high}$ T cells from CD40Ig-treated animals ($n = 4$). Dashed gray line, $CD8^+CD45RC^{low}$ T cells from naive animals ($n = 4$). $P < 0.01$ for $CD8^+CD45RC^{low}$ from CD40Ig-treated animals versus $CD8^+CD45RC^{high}$ and $CD8^+CD45RC^{low}$ from naive animals.

pared with syngeneic grafts, long-surviving grafts from CD40Ig-treated recipients displayed a 50- to 100-fold increase in *Foxp3* and *IDO* mRNA, a 5- to 10-fold increase in PIR-B (the rat homolog of human ILT4), *CTLA4*, *HO-1*, a trend toward an increase in IFN-γ ($P = 0.052$), and the *TCR* β-*chain*, *perforin*, and *IL-2*. No significant differences were observed for TGF-β, IL-6, or IL-10 (Figure 5A). Long-surviving grafts from adoptively transferred recipients showed 20- and 100-fold increased accumulation of *IFN-γ* and *IDO*, respectively (Supplemental Figure 4), indicating that mRNA accumulation for regulatory molecules was a specific tolerogenic phenomenon independent of adenoviral gene transfer into the graft.

Immunohistology analysis showed the presence of abundant $CD8^+$ cells expressing IFN-γ in CD40Ig long-surviving grafts but not in syngeneic grafts (Figure 5B). Thus, long-surviving grafts display $CD8^+$ IFN-γ^+ cells and increased expression of molecules with tolerogenic or antiinflammatory activities, such as PIR-B, HO-1 and IDO, that may create local tolerogenic mechanisms.

IFN-γ and IDO mediated the effects of CD40Ig and $CD8^+CD45RC^{low}$ T cells. To analyze the contribution of IFN-γ to CD40Ig-induced donor-specific allograft acceptance mediated by $CD8^+CD45RC^{low}$ T cells, we treated recipients with a neutralizing anti–IFN-γ mAb from the day of transplantation and $CD8^+CD45RC^{low}$ transfer. This treatment abrogated long-term allograft survival in 3 of 4 recipients (Figure 6A). The remaining cardiac graft displayed reduced beating strength (from +++ to +). Administration of anti–IFN-γ alone did not modify the kinetics of acute rejection (data not shown).

Since IFN-γ is a major stimulus for IDO expression (28) and IDO was among the genes with the highest expression in the graft, we evaluated the role of IDO by pharmacologically blocking its activity with 1-methyl tryptophan (1-MT). Administration of 1-MT from the day of transplantation together with CD40Ig treatment at day 0 or 120 after adoptive transfer of $CD8^+CD45RC^{low}$ T cells resulted in rapid graft rejection in the majority of treated animals (Figure 6A).

Western blot analysis showed higher amounts of IDO in grafts from CD40Ig-treated and adoptively transferred recipients compared with the amounts shown in syngeneic grafts (Figure 6B). Higher amounts of IDO were also found in spleen of transplanted rats treated with adoptively transferred cells compared with spleen of rats with rejected grafts (Supplemental Figure 5). Immunohistologic analysis showed that ECs were the main cellular source of IDO within the grafts (Figure 6C).

To prove that Tregs generated by CD40Ig treatment could induce ECs to produce IDO, we performed in vitro coculture of donor ECs with $CD8^+CD45RC^{low}$ or $CD8^+CD45RC^{high}$ T cells. IDO activity, as measured by kynurenine accumulation in the culture supernatant, was detected when ECs were cocultured with $CD8^+CD45RC^{low}$ but not $CD8^+CD45RC^{high}$ T cells (Figure 6D). Furthermore, induction of IDO expression by ECs was dependent on IFN-γ production by $CD8^+CD45RC^{low}$ T cells, since kynurenine production was abrogated upon neutralization of IFN-γ with an mAb (Figure 6D). Analysis of IDO mRNA accumulation in ECs confirmed induction of IDO expression by recombinant IFN-γ used as a positive control (data not shown) as well as by $CD8^+CD45RC^{low}$ but not $CD8^+CD45RC^{high}$ T cells (Figure 6E). $CD8^+CD45RC^{low}$ T cells from CD40Ig-treated animals only induced IDO expression in ECs from graft donor LEW.1W origin but not from syngeneic LEW.1A ECs (data not shown).

Therefore, IDO is necessary for both the early induction of long-term allograft survival by CD40Ig and for the maintenance phase of tolerance mediated by $CD8^+CD45RC^{low}$ T cells. Infectious tolerance by $CD8^+CD45RC^{low}$ T cells was also dependent on IFN-γ, and $CD8^+CD45RC^{low}$ T cells induced IDO in ECs through IFN-γ production.

IDO overexpression in the graft but not at a distant site inhibits allograft rejection. Since IDO expression in the graft was associated with prolonged survival, we generated a recombinant adenovirus coding for IDO and tested the effect of IDO overexpression in the graft or at a distant site such as the hind limb. When IDO gene transfer

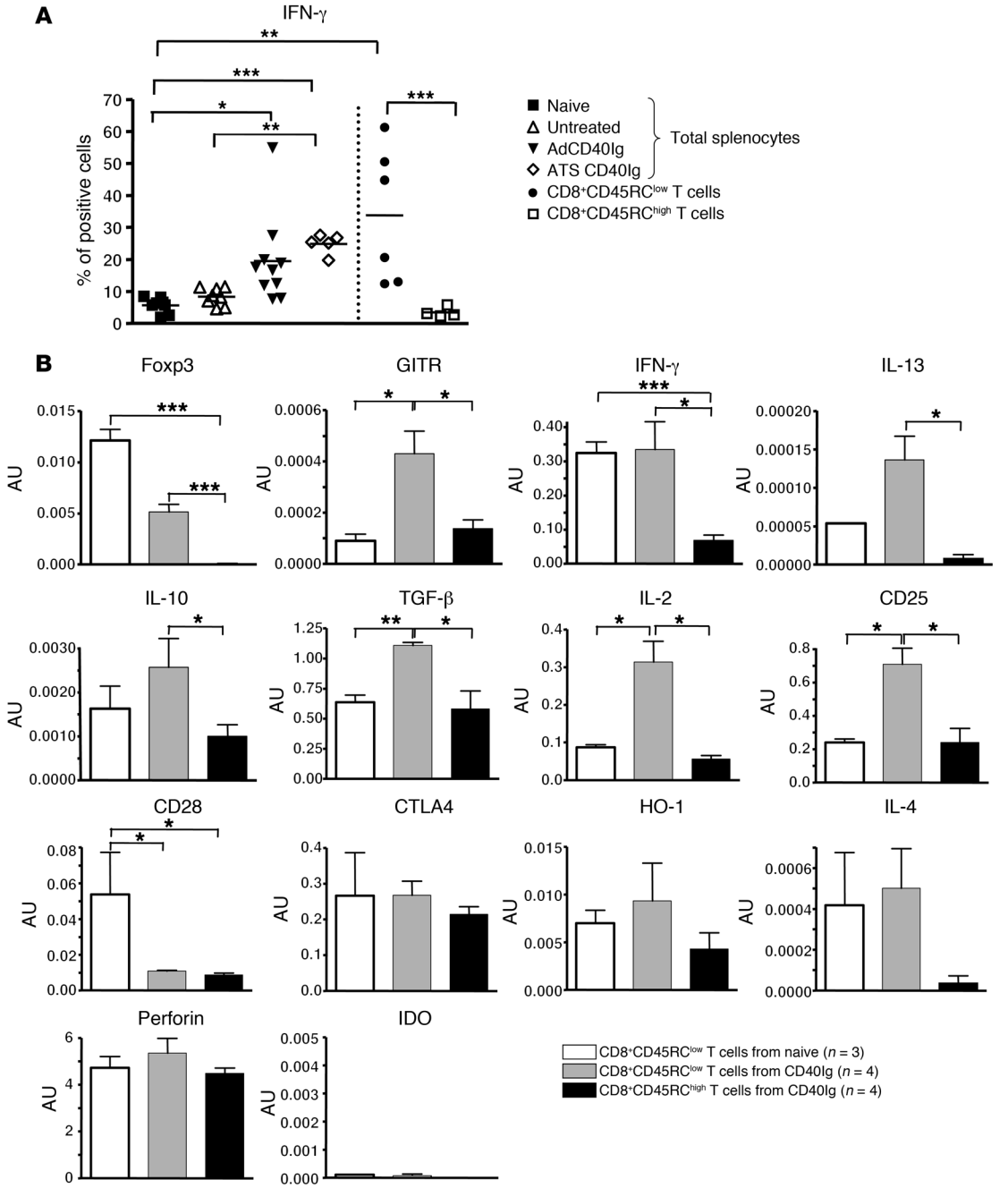

Figure 3

Expression of IFN-γ and regulatory molecules by CD8+CD45RC^{low} T cells. (**A**) Intracytoplasmic analysis of IFN-γ production in total splenocytes and in CD8+CD45RC^{low} and CD8+CD45RC^{high} cells from CD40Ig-treated or adoptively transferred animals. Cells were harvested, stimulated for 7 hours using phorbol myristate acetate and ionomycin, and analyzed with a FITC-labeled anti–IFN-γ mAb. Results are expressed as percent of positive cells. Each symbol indicates a value for an individual animals. Lines indicate mean ± SD. ATS, adoptively transferred. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (**B**) Quantitative analysis of transcript accumulation was performed after isolation in CD8+CD45RC^{low} or CD8+CD45RC^{high} T cells isolated from CD40Ig-treated recipients or in CD8+CD45RC^{low} cells from naive animals. Results are expressed in arbitrary units of molecules normalized to HPRT ± SD. GITR, glucocorticoid-induced tumor necrosis factor receptor family–related gene. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

was performed in the graft, survival was significantly prolonged, with 45% of the grafts surviving more than 90 days, whereas survival was not significantly prolonged when gene transfer was performed in the hind limb (Figure 7). Although 1-MT blocks the effect of CTLA4Ig treatment in a model of islet allotransplantation in mice (29), administration of 1-MT to CTLA4Ig-treated rats did not result in graft rejection (Supplemental Figure 6), indicating that the effects on CD40Ig-treated animals were specific. These results confirmed that IDO overexpression in the graft is sufficient to induce permanent allograft survival, and although

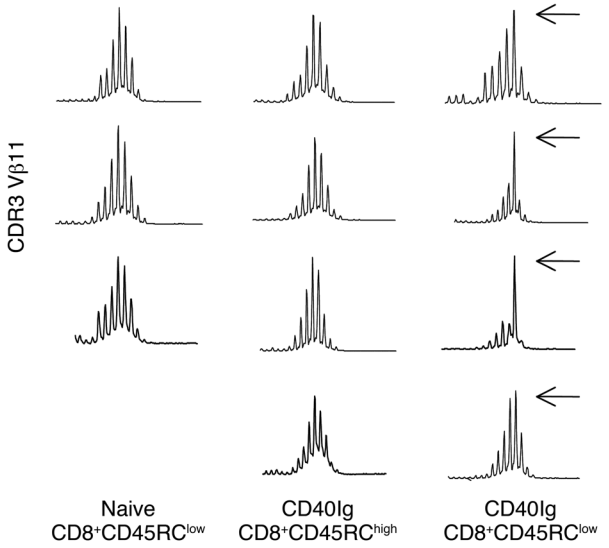

Figure 4
Analysis of the TCR repertoire in $CD8^+CD45RC^{low}$ T cells. $CD8^+CD45RC^{low}$ or $CD8^+CD45RC^{high}$ T cells were purified from spleen of CD40Ig-treated or naive animals. A qualitative and quantitative analysis of the TCR Vβ transcriptome was performed as described in Methods. Each histogram represents a qualitative immunoscope analysis of the Vβ11 family repertoire of 1 animal. The histograms display the intensity of fluorescence in arbitrary units as a function of runoff CDR3 Vβ11 length in nucleotides. The CDR3 length distribution was unaltered (gaussian) in $CD8^+CD45RC^{low}$ from all naive animals or $CD8^+CD45RC^{high}$ from CD40Ig-treated recipients and altered (non-gaussian) in spleen $CD8^+CD45RC^{low}$ T cells from all CD40Ig-treated recipients with a predominant CDR3 of always the same length (27 nucleotides, arrows).

IDO gene transfer in the graft was less efficient than transfer of $CD8^+CD45RC^{low}$ T cells, this can be explained by a transient expression of IDO when using adenoviruses.

Discussion

The major findings of the current study were that long-term allograft survival induced by CD40Ig was dependent on $CD8^+CD45RC^{low}$ Tregs, that IFN-γ and IDO were necessary for long-term allograft survival, and that interaction between $CD8^+CD45RC^{low}$ T cells and graft ECs possibly underlines these mechanisms. The allograft acceptance induced by CD40Ig treatment was obtained in a major MHC-mismatched cardiac allograft model, was able to be adoptively transferred by $CD8^+CD45RC^{low}$ T cells into euthymic transplanted recipients, and was donor specific.

Infectious allogeneic tolerance was first demonstrated in thymectomized mice treated with anti-CD4 and anti-CD8 mAbs, and tolerance was serially transferred using splenocytes or $CD4^+$ but not $CD8^+$ cells (1). The same group showed that anti-CD40L and anti-CD8 mAb treatment also resulted in infectious tolerance mediated by $CD4^+$ T cells (30). Infectious tolerance by $CD4^+$ T cells has also been described in rats following donor-specific blood transfusion (DST) (16) and anti-CD4 mAb treatment (15). In all these models, only $CD4^+$ T cells transferred tolerance or were much more potent than $CD8^+$ T cells (16). Thus, our model of CD40Ig treatment is the first in which infectious tolerance is mediated by $CD8^+$ T cells. Furthermore, the potency of these cells is high, since as few as 50×10^6 splenocytes or 2.5×10^6 $CD8^+CD45RC^{low}$ T cells were able to inhibit acute rejection in an MHC-incompatible strain combination for at least 4 generations without loss of efficacy. It is unclear why $CD4^+$ Tregs were absent in our model but predominate in mouse models using anti-CD40L mAbs. Some differences in these models, such as different molecules (CD40Ig versus anti-CD40L), the continuous presence of CD40Ig versus only inductive treatment with anti-CD40L, and the species used may explain this discrepancy. CD40Ig also induced indefinite survival of allogeneic livers (31), but the role of $CD4^+$ or $CD8^+$ Tregs was not analyzed. Anti-CD40L mAbs have been proposed to act at least in part through depletion of activated $CD4^+$ T cells (22, 23). Absolute numbers of $CD4^+$ T cells were decreased in CD40Ig-treated recipients compared with numbers of $CD4^+$ T cells in grafted untreated or adoptively transferred recipients but were comparable to those of naive animals (Supplemental Figure 7A). $CD8^+$, $CD8^+CD45RC^{low}$, and $CD4^+CD25^{high}$ T cells were not modified in CD40Ig-treated animals versus naive or control Addl324-treated recipients, whereas $CD8^+CD45RC^{low}$ T cells were increased in adoptively transferred recipients versus naive or CD40Ig-treated recipients. CD40Ig also had a differential effect in vitro in $CD4^+$ and $CD8^+$ T cells, since it showed higher inhibition of MLR responses by $CD4^+CD25^-$ naive T cells than $CD8^+CD45RC^{high}$ or $CD8^+CD45RC^{low}$ cells (Supplemental Figure 7B). These results suggest a specific differential effect of CD40Ig on $CD4^+$ T cells compared with $CD8^+$ T cells in grafted animals. This could be related to higher levels of CD40L expression on rat $CD4^+$ versus $CD8^+$ T cells (data not shown). These results also suggest that defective alloantigen presentation due to blockade of CD40-CD40L interactions and decrease of $CD4^+$ T cells may participate in the generation of $CD8^+CD45RC^{low}$ cells.

$CD8^+$ Tregs have been described in several models (3–14). In allotransplantation models, $CD8^+CD28^-$ Tregs were induced by in vitro allogeneic stimulation of human cells (11) or by DST treatment in rats (4). Induction of the immunoreceptor tyrosine-based inhibitory motifs–containing motifs ILT3 and ILT4 on human DCs (11) or of PIR-B on graft rat ECs (4) have been described. The $CD8^+Foxp3^+$ cells induced by DST did not produce IFN-γ and were able to transfer tolerance in only 60% of primary recipients (4). Thus, the $CD8^+$ Tregs described in the present study could be a counterpart to the human $CD8^+CD28^-$ cells but are different from the ones described in DST-treated rats. $CD8^+$ T cells tolerogenic for alloantigens have also been described following oral exposure to alloantigens (5) or to DST treatment (13, 14, 16), but their phenotype or cytokine production was not analyzed further. Administration of agonistic anti-CD137 mAbs to mice to suppress autoimmune arthritis through the generation $CD8^+CD11c^+$ Tregs capable of producing IFN-γ was recently described (8). DCs from these mice expressed IDO, and the tolerogenic effect of anti-CD137 mAb was abolished by treatment with anti–IFN-γ or 1-MT. Thus, agonistic anti-CD137 and CD40Ig seem to generate similar mechanisms of tolerance acting through $CD8^+$ T cells, IFN-γ, and IDO. It remains to be determined which of the 2 costimulatory pathways is upstream of the

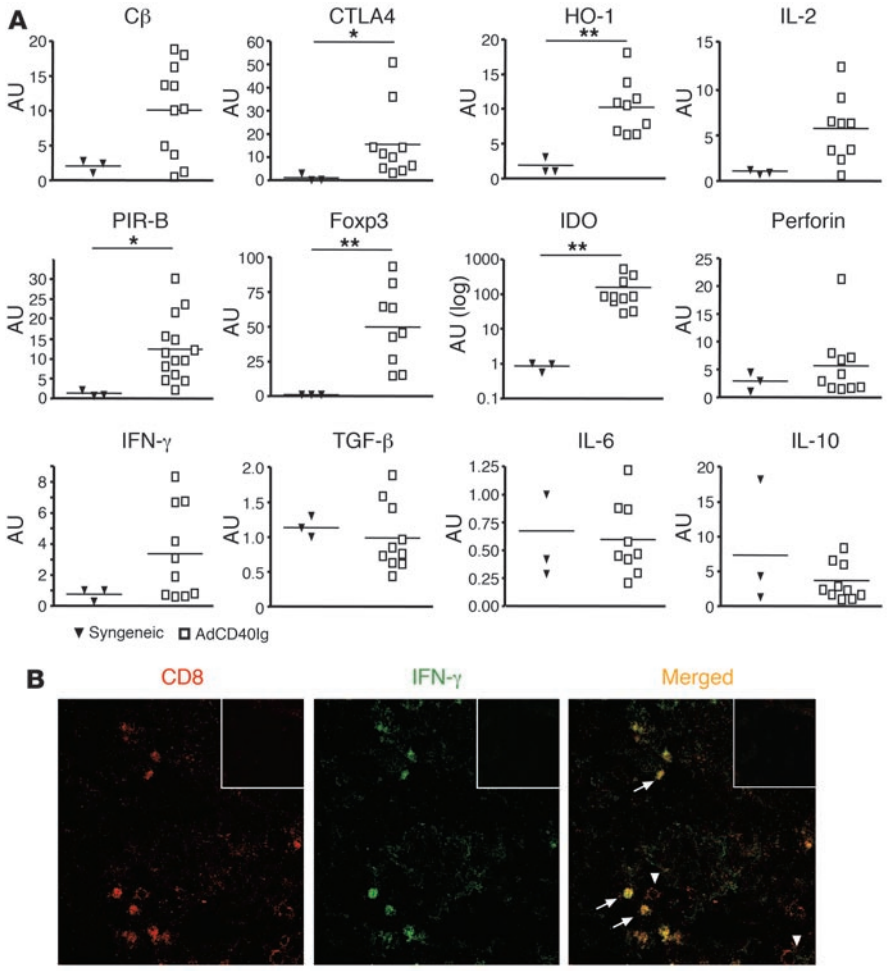

Figure 5
Grafts accepted long term after CD40Ig treatment showed strong accumulation of transcripts for regulatory molecules. Heart grafts from syngeneic donors or from allogeneic donors were harvested 120 days after transplantation. Recipients from allogeneic donors were treated with CD40Ig. (**A**) Heart total RNA was analyzed using real-time quantitative RT-PCR. Symbols indicate values for individual animals in arbitrary units. *$P < 0.05$, and **$P < 0.01$ versus syngeneic animals. (**B**) Confocal analysis of allogeneic or syngeneic (inserts) grafts using anti-CD8α (red) or anti–IFN-γ (green) antibodies. Merge analysis shows IFN-γ expression almost exclusively restricted to $CD8^+$ cells (arrows), whereas some cells are IFN-γ^- (arrowheads). Identical results were obtained with 3 grafts from CD40Ig-treated animals and from adoptively transferred grafts. Original magnification, ×400.

other in each model and whether certain phenotypic differences truly distinguish the different cell populations ($CD8^+CD45RC^{low}$ T cells are $CD11c^-$). Finally, a population of naturally occurring $CD8^+CD45RC^{low}$ Tregs was recently described in the rat (3). In contrast to the lack of effect of these natural $CD8^+CD45RC^{low}$ cells in our cardiac transplantation model, CD40Ig-treated recipients showed $CD8^+CD45RC^{low}$ cells able to suppress graft rejection. This indicated that blockade of CD40 signals may have enabled an increase in suppressive function and/or expansion of alloantigen-specific T cells within naturally occurring $CD8^+CD45RC^{low}$ T cells, as described for natural versus induced $CD4^+$ Tregs in the absence of CD40 signals (26) or following other treatments (32, 33). The presence of a restricted TCR Vβ11 usage by $CD8^+CD45RC^{low}$ T cells from CD40Ig-treated recipients and not naive animals suggests the expansion of an antigen-specific population of cells. As previously reported for natural and induced $CD4^+$ Tregs (33), transcript levels of several cytokines and markers (IL-10, IFN-γ, and Foxp3) were not significantly different in $CD8^+CD45RC^{low}$ T cells from CD40Ig-treated compared with naive animals. Nevertheless, for other molecules analyzed (GITR, TGF-β, IL-2, CD25, and CD28), there were significant differences, and a DNA microarray analysis has shown 127 genes differentially regulated between the 2 populations (B. Le Mauff and I. Anegon, unpublished observations).

Although induction of tolerance to alloantigens in vivo using anti-CD40L (34), or $CD25^+CD4^+$ Tregs (35) has also been shown to require IFN-γ, the role in tolerance of downstream molecules regulated by IFN-γ, such as IDO, was not demonstrated. IFN-γ may act as a double-edged sword, since it may not only inhibit acute rejection but also promote chronic rejection (36). Thus, the production of IFN-γ might explain why most graft tolerance–promoting strategies, including those resulting in infectious tolerance, such as administration of anti-CD40L, result in simultaneous efficient inhibition of acute rejection and development of chronic rejection lesions (37).

Long-surviving grafts showed increased expression of mRNA for *PIR-B*, *HO-1*, and *IDO*. PIR-B is homologous to human ILT4, which is expressed by DCs and ECs and inhibits alloreactive T cell activation (4). HO-1 is a tissue-protective gene which was shown to be necessary for tolerance induction in anti-CD40L and DST treatment (38), but its inhibition in our model by in vivo administration of tin-protoporphyrin did not abrogate CD40Ig-induced tolerance (data not shown).

IDO degrades tryptophan, which can be further degraded through the kynurenine pathway (28), and its activity can induce T cell apoptosis or anergy (28, 39). IDO can be produced by several cell types including DCs and ECs, and IFN-γ is a major stimulus of its functional expression (28, 40). Specific inhibition of IDO using 1-MT has been shown to abrogate tolerance in a number of models (8, 28, 29, 41). In all of these studies, IDO production was analyzed in DCs but not in other cell types, despite the fact that

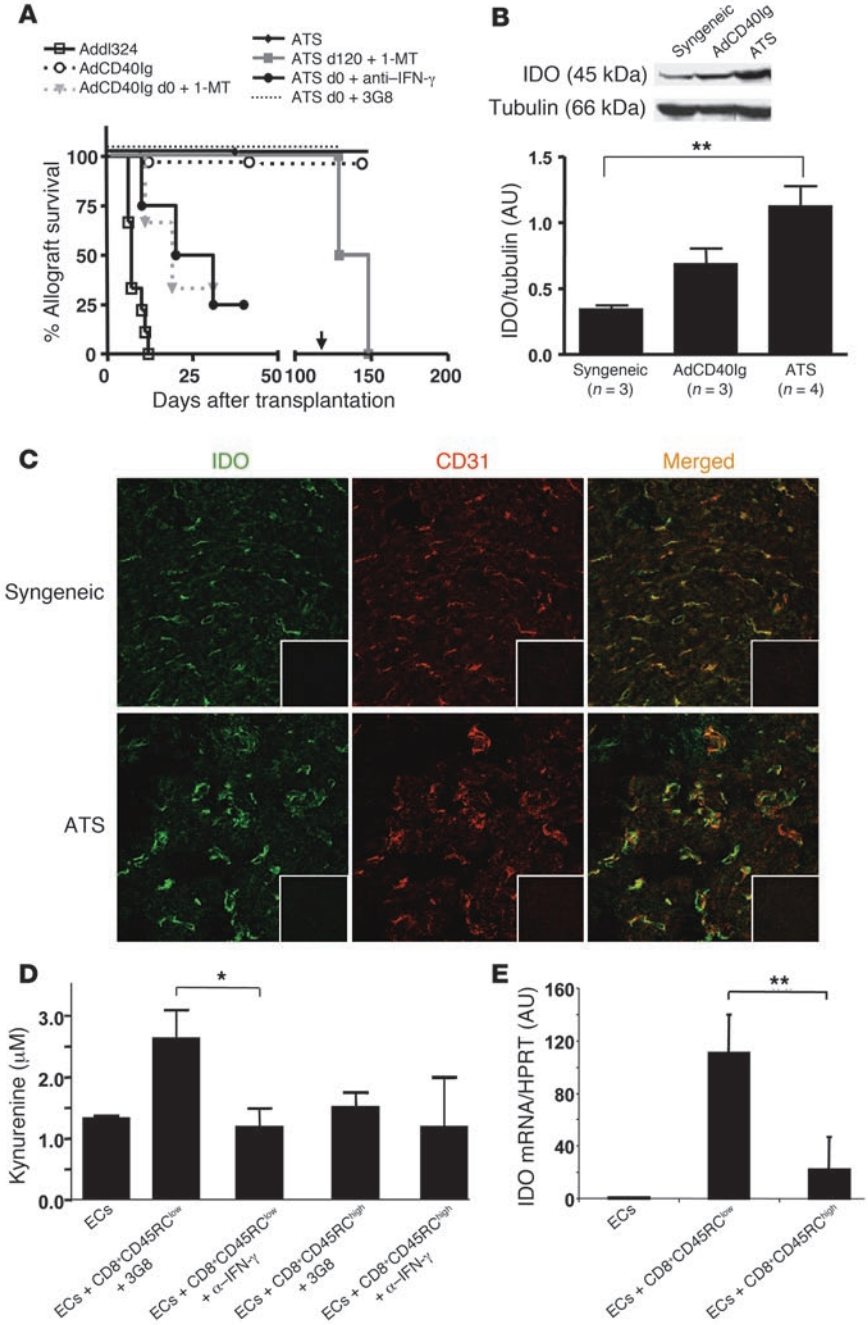

Figure 6
IFN-γ and IDO mediate the effect of CD40Ig and of $CD8^+CD45RC^{low}$ T cells. (**A**) Graft survival after 1-MT or anti–IFN-γ mAb administration beginning the day of transplantation (d0) to CD40Ig-treated animals or to animals receiving adoptive transfers at 120 days after transplantation (d120). Heartbeat of nonrejected grafts after 1-MT or anti–IFN-γ administration was + compared with +++ in CD40Ig-treated or adoptively transferred recipients. Arrow indicates adoptive transfer and beginning of treatment with 1-MT. (**B**) Representative Western blot analysis of IDO expression in cardiac grafts from syngeneic, CD40Ig-treated, and adoptively transferred recipients at day 120. Histograms show quantification of IDO normalized to tubulin in 3–4 samples per group. $^{**}P < 0.01$, syngeneic versus adoptively transferred grafts. (**C**) Confocal analysis of grafts from adoptively transferred or syngeneic recipients using anti-IDO (green) and anti-CD31 (red) antibodies. Merge analysis shows IDO expression almost exclusively restricted to ECs. The inserts represent staining using an irrelevant mAb (3G8) or rabbit serum. Identical results were obtained with 2 other grafts from adoptively transferred animals and 2 grafts from CD40Ig-treated animals. Original magnification, ×400. (**D**) Kynurenine in cultured supernatants. $CD8^+CD45RC^{low}$ or $CD8^+CD45RC^{high}$ T cells from CD40Ig-treated recipients ($n = 2$ in each group) were cultured for 2 days with ECs from LEW.1W animals in the presence of anti–IFN-γ or control mAb (3G8) at 20 μg/ml. Levels are expressed as μM ± SD. $^*P < 0.05$. (**E**) Quantitative RT-PCR analysis of IDO mRNA in ECs cultured for 2 days with $CD8^+CD45RC^{low}$ or $CD8^+CD45RC^{high}$ T cells from CD40Ig-treated recipients ($n = 3$ in each group). $^{**}P < 0.01$ for $CD8^+CD45RC^{low}$ versus $CD8^+CD45RC^{high}$.

ECs also express IDO (40). Our results demonstrate, for what we believe to be the first time, that IDO has a pivotal role in long-term allograft survival following manipulation of CD40-CD40L interactions. The present results also demonstrate for what we believe to be the first time that IDO can be produced by graft ECs and that Tregs can induce the expression of IDO in ECs. Nevertheless, since we have also documented increased IDO expression in spleens of CD40Ig-treated animals, the relative role of graft ECs versus spleen cells producing IDO requires further study.

The activation of $CD8^+CD45RC^{low}$ T cells in CD40Ig-treated animals is donor alloantigen–specific, since BN third-party grafts but not first-donor LEW.1W grafts were rejected upon adoptive transfer. Furthermore, Vβ TCR repertoire alterations also indicate that $CD8^+CD45RC^{low}$ T cell responses were selected and likely directed against donor MHC class I antigens, since anti-MHC class I mAb administration abrogated long-term graft survival. Further experiments are needed to define whether $CD8^+CD45RC^{low}$ T cells recognize MHC class Ia antigens or Ib molecules, as previously described for $CD8^+$ Tregs in mice models of autoimmunity (6). Although triggering of $CD8^+CD45RC^{low}$ T cells was donor alloantigen specific, production of IFN-γ and IDO could theoretically result in the nonspecific inhibition of other concomitant immune responses. Nevertheless, we have previously shown that CD40Ig-treated recipients show normal anticognate immune responses after CD40Ig gene transfer at early and late time points after gene transfer (19). It is possible that activation of $CD8^+CD45RC^{low}$ T cells by

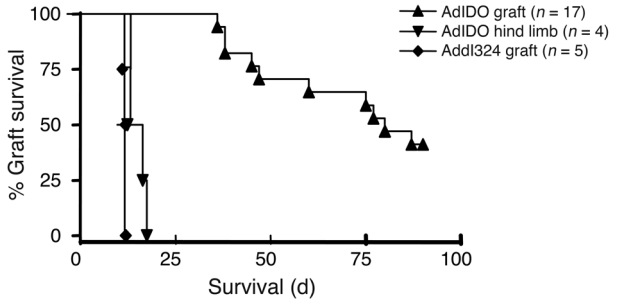

Figure 7
IDO gene transfer into the graft but not at a distant site results in long-term allograft survival. AdIDO or Addl324 (2×10^{10} IP) were injected into the cardiac grafts or intramuscularly into the hind limb the day of transplantation. Rejection was defined when beating of the transplanted heart could no longer be detected. $P < 0.001$, AdIDO graft versus Addl324 and AdIDO i.m.

graft ECs localizes the effects of IFN-γ and IDO to the graft. The localization and/or activation of Tregs in target tissues has been previously described in transplant (42) and autoimmune diabetes (43) models. As recently speculated, Tregs may act by inducing an immune privileged site through mechanisms similar to those occurring in natural immune privileged sites such as for IDO in the placenta (44). Alternatively, IFN-γ is directionally secreted into the immunological synapse, whereas other cytokines show multidirectional secretion (45). This could provide another mechanism for a limited effect even in lymphoid secondary organs.

DC–T cell interaction in the absence of CD40L-CD40 results in cross-tolerization of $CD8^+$ T cells, whereas DC stimulation through CD40 results in T cell priming (24–26). When the role of IDO was analyzed, it was shown to be essential for cross-tolerization of $CD8^+$ T cells by DCs (25). Thus, it is possible that in our model, CD40 blockade resulted in alloantigen presentation by DCs and/or ECs to $CD8^+$ T cells in a tolerogenic manner through IDO expression.

In summary, we have provided in vivo evidence of a role for a $CD8^+CD45RC^{low}$ T cell population with tolerogenic properties following CD40Ig treatment. We have demonstrated that $CD8^+CD45RC^{low}$ T cells are capable of infectious allogeneic tolerance through dominant suppression by expression of IFN-γ, which in turn induces IDO expression.

Methods

Animals and cardiac allograft models. Eight- to 12-week-old male LEW.1W (haplotype $RT1^u$) and LEW. 1A (haplotype $RT1^a$) (Centre d'Elevage Janvier) differ in their entire MHC region. Heterotopic abdominal LEW.1W heart survival in LEW.1A recipients was evaluated by palpation through the abdominal wall and heart beating was graded from +++ to –. The experiments in animals were approved by the official French veterinarian authorities (Services Vétérinaires de la Loire Atlantique, La Chapelle-sur-Erdre, France).

CD40Ig or IDO gene transfer and mAb administration. The adenovirus AdCD40Ig coding for the extracellular portion of mouse CD40 fused to the coding sequences of the constant domains of human IgG1 and the noncoding vector Addl324 have been described previously (19). An adenovirus coding for IDO was constructed as previously described (46) and was confirmed to express enzymatically active IDO (data not shown). A total of 2×10^{10} adenovirus infectious particles (IP) was slowly injected at 3 points into the graft ventricular walls.

$CD8^+$ cells were depleted by injecting 3 mg/kg of an anti-CD8α mAb (OX8, IgG1) i.p. twice a week from day 0 until sacrifice (day 120).

A neutralizing mouse anti-rat IFN-γ mAb (hybridoma DB1, IgG1, kindly provided by P.H. Van der Meisle, Rijswik, The Netherlands) or an anti-MHC class Ia and Ib mAb (OX18) (3 mg/kg) was injected i.p. twice a week from day 0 until rejection or day 40. An irrelevant isotype-matched mAb (anti-human CD16, clone 3G8, IgG1) was used as control.

Lymphocyte preparation, isolation or depletion of subpopulations, and adoptive transfer. Spleens were harvested 120 days after transplantation from recipients that had accepted their grafts long-term and from untreated recipients that had rejected their allografts at around day 7.

Cell transfers were performed by i.v. injection of total splenocytes or purified splenocyte subpopulations on the day of transplantation into LEW.1A recipients sublethally irradiated (4.5 Gy whole-body irradiation) 1 day earlier.

For adoptive cell transfer of splenocytes depleted of different leukocyte subpopulations, 50×10^6 splenocytes were depleted of T cells, $CD4^+$ cells, $CD8^+$ cells, MHC class II^+ cells, and NK^+ cells using negative selection with anti-TCRαβ (R.7.3), anti-CD4 (W3.25), anti-CD8α, anti–MHC class II (OX6), and anti-CD161 (NKR-P1A, 3.2.3), followed by incubation with anti-mouse IgG–coated magnetic beads (Dynal). Cells remaining after depletion of the different cell populations contained less than 1% of contaminating cells and were injected i.v.

For adoptive cell transfer of purified splenocyte subpopulations, cells were isolated by negative selection. T cells were isolated by nylon wool adherence, and depletion of non–T cells was performed with a mixture of anti-CD161, anti-CD11b/c (OX42), anti-CD45R (His 24), and anti–Igκ chain (OX12) and incubation with anti-mouse IgG-coated beads. $CD8^+$ or $CD4^+$ T cells were purified using this cocktail of mAbs including an anti-CD4 mAb or an anti-CD8α mAb, respectively. Purity of all these subpopulations was routinely greater than 95%. The number of cells of each population injected, either depleted or purified, was calculated to be equivalent to that contained in 50×10^6 total splenocytes after analysis of their frequency in the following splenocytes: 25×10^6 (T cells), 18×10^6 ($CD8^+$ T cells), and 12×10^6 ($CD4^+$ T cells).

$CD45RC^{high}$ and $CD45RC^{low}$ $CD8^+$ T cells were first purified from total splenocytes after nylon wool adherence and depletion with a cocktail of anti-CD4, anti-CD45R, and anti-CD11b/c reactive mAbs using magnetic beads. Purified $CD8^+$ T cells were then incubated with FITC-labeled anti-CD45RC and PE-labeled anti-CD8α mAbs and sorted using FACSAria (BD). Purity of sorted populations was systematically greater than 99%.

Flow cytometry. Cells were stained with the following mAbs: anti-TCRαβ (R73, Alexa Fluor 647–conjugated), anti-CD8α (OX8, PE-conjugated), anti-CD4 (W3.25, PE-conjugated), anti-CD45RC (OX22, FITC-conjugated), anti-CD28 (JJ319, biotin-labeled), anti-CD103 (OX62, biotin-labeled), anti-CD62L (OX85, biotin-labeled), anti-OX40 (OX40, biotin-labeled), anti-MHC II (OX6, biotin-labeled), and anti-CD25 (OX39, biotin-labeled). All biotin-labeled mAbs were visualized using avidin-PerCP.Cy5.5. A FACSCalibur cytofluorimeter (BD Biosciences) was used to measure fluorescence, and data were analyzed using CellQuest software (version 4.0.2; BD Biosciences).

Immunofluorescence. For immunofluorescence studies of IDO expression, cryostat sections from grafts were thawed, fixed in acetone (10 minutes at room temperature), and incubated (overnight at 4°C, 1.5 μg/ml) with a polyclonal rabbit anti-IPO Ab produced by us against rat IDO peptides (47), followed by a FITC-conjugated anti-rabbit Ab. An anti-CD31 mAb (an endothelial cell marker; Serotec) was used at 5 μg/ml, followed by an Alexa Fluor 568–conjugated anti-mouse Ab. IFN-γ was detected using a FITC-conjugated mAb (DB1, 5 mg/ml), and $CD8^+$ cells were detected using a biotin-conjugated anti-CD8α mAb (5 mg/ml) and Alexa Fluor 568–conjugated avidin. Sections were mounted in ProLong AntiFade Kit

(Molecular Probes) and analyzed by confocal microscopy (Leica TCS-SP1). The green and red emissions were collected using 2 photomultiplier tubes. Grayscale digital images were visualized with a 24-bit imaging system using Leica's TCS-NT software.

Intracellular cytokine staining. Splenocytes were harvested and immediately restimulated for 7 hours using phorbol 12-myristate 13-acetate (50 ng/ml, Sigma-Aldrich) and ionomycin (1 μg/ml, Sigma-Aldrich) in the presence of brefeldin A (5 μg/ml) for the last 4 hours. Cells were then fixed in 2% paraformaldehyde and incubated with PBS/0.2% FCS/0.5% saponin for 10 minutes. After 2 washes in PBS/0.2% FCS/0.1% saponin, cells were incubated with isotype controls or FITC-labeled anti-rat IFN-γ mAb, anti–IL-10 (clone A5-4; BD Pharmingen), anti–IL-13 (clone 12G5-IE4; Biosource International), anti–IL-4 (clone OX-81; BD Biosciences — Pharmingen), and anti–TGF-β1 (clone 2G7, kindly provided by K. Melief, Academisch Ziekenhuis, Leiden, The Netherlands, at 5 μg/ml for 30 minutes. After 2 washes with PBS/0.2% FCS/0.1% saponin and 1 wash with PBS/2% FCS/0.2% azide, cells were fixed with 2% paraformaldehyde.

Quantitative RT-PCR. Messenger RNA transcript analysis was performed by quantitative RT-PCR. Total RNA was isolated using TRIzol (Invitrogen). Ten micrograms of RNA were treated with DNase and reverse transcribed using an MMLV Reverse-Transcriptase kit (Invitrogen). Quantitative RT-PCR was performed with a ABI PRISM 7700 Sequence Detection System using Complete Power SYBR Green master mix 2 (Applied Biosystems). The sequences of primer pairs for HPRT, TGF-β1, IFN-γ, IL-10 (48), and HO-1 (46) have been described previously, and the other primer sequences used are listed in Supplemental Table 1. The PCR and quantification methods, which have been previously described (46), were used after normalization of sequences to HPRT values.

TCR repertoire analysis. For quantitative analysis, cDNA were amplified by PCR using a Cβ primer and the 21 specific primers described previously (13) by quantitative RT-PCR. Data were expressed as the ratio of Vβ transcripts levels to HPRT. For the TCR Vβ CDR3 length distribution qualitative analysis, amplification products were used for an elongation reaction using a dye-labeled Cβ primer, then heat denatured, loaded onto a 6% acrylamide-8M urea gel, and electrophoresed using an Applied Biosystems 377A DNA sequencer. The Immunoscope software (version 3.03; Institut Pasteur) resolved the raw data of DNA sequencing gel into sets of peaks separated by 3 nucleotides, including size, area of peaks representing the TCR-CD3 length distribution profiles, and alteration of distribution.

Treatment with 1-MT and analysis of IDO expression. 1-MT (Sigma-Aldrich) was prepared daily and administered by gavage at 0.2 g/kg twice daily as previously described (49). IDO expression was analyzed by Western blot using an anti-rat IDO rabbit antibody and by measuring kynurenine in culture medium (47).

Culture of ECs and $CD8^+CD45RC^{low}$ T cells. ECs of LEW.1W or LEW.1A origin were isolated and plated into 12-well plates (5×10^5 cells/well), and after overnight culture, $CD8^+CD45RC^{low}$ T cells from CD40Ig-treated or adoptively transferred LEW.1A recipients were added (4×10^6 cells/well). After 2 days of culture, supernatants were harvested, lymphocytes were discarded, and kynurenines were analyzed. Adherent ECs were washed several times and lysed for RT-PCR analysis of IDO mRNA.

Statistics. Statistical significance was evaluated using the 2-tailed Student's *t* test for 2 groups and the Kruskal-Wallis test followed by a Dunn's post-hoc test for more than 2 groups. Graft survival was analyzed by Kaplan-Meier log-rank test. *P* values less than or equal to 0.05 were considered significant.

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