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Induction of Tolerance to Cardiac Allografts Using Donor Splenocytes Engineered to Display on Their Surface an Exogenous Fas Ligand Protein

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The critical role played by Fas ligand (FasL) in immune homeostasis renders this molecule an attractive target for immunomodulation to achieve tolerance to auto- and transplantation Ags. Immunomodulation with genetically modified cells expressing FasL was shown to induce tolerance to alloantigens. However, genetic modification of primary cells in a rapid, efficient, and clinically applicable manner proved challenging. Therefore, we tested the efficacy of donor splenocytes rapidly and efficiently engineered to display on their surface a chimeric form of FasL protein (SA-FasL) for tolerance induction to cardiac allografts. The i.p. injection of ACI rats with Wistar-Furth rat splenocytes displaying SA-FasL on their surface resulted in tolerance to donor, but not F344 third-party cardiac allografts. Tolerance was associated with apoptosis of donor reactive T effector cells and induction/expansion of CD4+/CD25+FoxP3+ T regulatory (Treg) cells. Treg cells played a critical role in the observed tolerance as adoptive transfer of sorted Treg cells from long-term graft recipients into naive unmanipulated ACI rats resulted in indefinite survival of secondary Wistar-Furth grafts. Immunomodulation with allogeneic cells rapidly and efficiently engineered to display on their surface SA-FasL protein provides an effective and clinically applicable means of cell-based therapy with potential application to regenerative medicine, transplantation, and autoimmunity. The Journal of Immunology, 2008, 181: 931–939.

Transplantation of foreign cellular, tissue, and organ grafts represents an important therapeutic modality for the treatment of various inherited and acquired disorders, such as end-organ failure, autoimmunity, malignancies, and congenital enzyme deficiencies. However, rejection of foreign grafts by immunocompetent recipients presents a major hurdle for the routine application of transplantation in the clinic (1, 2). Although general immunosuppression is presently used to control foreign graft rejection, the chronic use of nonspecific immunosuppression is not only inefficient in preventing graft rejection but also associated with various complications, including but not limited to infections, malignancies, and organ toxicity (1, 2). Induction of specific tolerance to foreign grafts has the potential to overcome these complications, and as such has been the subject of intense studies since the first successful organ transplantation in 1954 (3). Irrespective of extensive efforts, the routine and consistent induction of transplantation tolerance in the clinic remains to be realized (4).

Modulation of immune responses using donor cells genetically modified to express immunological molecules that play key roles in the regulation of the immune system has the potential to induce transplantation tolerance (5). However, using gene therapy to express immunomodulatory molecules has various complications, such as safety, inefficient targeting, and low levels of expression of the therapeutic protein (6). In particular, in settings of organ, tissue, and primary cell transplantation where rapid and robust expression of immunological proteins are a prerequisite for therapeutic efficacy, gene therapy has severe limitations. Inasmuch as cell surface receptor ligand interactions are critical to immune decision making and these interactions do not need to be extensive in duration (7), we sought direct display of exogenous immunological proteins on the cell surface as a practical alternative to gene therapy for immunomodulation and developed the ProtEx technology (8). ProtEx involves: 1) generation of recombinant proteins that contain extracellular domains of immunological ligands fused to a modified form of core streptavidin (SA)4; 2) modification of cell membrane with biotin; and 3) display of chimeric proteins on the modified surfaces (8).

We tested the immunomodulatory potential of our ProtEx technology using Fas ligand (FasL) as an apoptotic molecule to specifically eliminate pathogenic lymphocytes in settings of autoimmunity and allograft transplantation. The choice of FasL as an immunomodulatory molecule is because of its critical role in activation-induced cell death and tolerance to self Ags (9, 10). As

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such, there has been significant interest in using FasL as an immunomodulatory molecule to induce transplantation tolerance with conflicting observations (11). Although some studies reported therapeutic effect of FasL, others demonstrated its contribution to the pathogenesis of the disease (12–20). Mechanisms responsible for these opposing effects are complex and may be regulated by levels of FasL and its receptor, Fas, on target tissues and the sensitivity of these tissues to FasL-mediated apoptosis, the cytokine milieu, the tissue microenvironment, and/or the differential effects of membrane-bound and soluble forms of FasL (reviewed in Ref. 11). FasL is initially expressed on the cell surface as a membrane-bound type II protein that has potent apoptotic activity on Fas-bearing cells (9). However, membrane-bound FasL is cleaved from the cell surface by matrix metalloproteinases into a soluble form (21), which does not have apoptotic activity and may interfere with apoptosis by competing with the membranous form for binding to Fas on target cells (21, 22). Furthermore, soluble FasL has chemotactic activity on neutrophils (23, 24) that may be responsible for the rejection of grafts ectopically expressing Fasl. (13, 19).

Therefore, a chimeric molecule containing the extracellular functional domain of FasL lacking the metalloproteinase cleavage sites fused to streptavidin (SA-FasL) was generated (8). We previously demonstrated that the direct display of SA-FasL, on heart graft vasculature resulted in the prevention of acute rejection after transplantation into allogeneic recipients (25). However, this approach of direct manipulation of the donor graft resulted in moderate prolongation of graft survival without achieving tolerance. The lack of tolerance in this model might have been due to the inability of the transient, local display of SA-FasL on the graft vasculature to deplete a significant pool of alloreactive T effector (Teff) cells. Hence, we herein tested whether recipient immunomodulation by systemic infusion of donor splenocytes engineered to display SA-FasL on their surface induces tolerance to cardiac grafts in a totally allogeneic rat strain combination. Infusion of ACI graft recipients with Wistar-Furth (WF) donor splenocytes displaying SA-FasL on their surface resulted in robust tolerance to cardiac allografts. Tolerance was donor specific and maintained by CD4+CD25+Foxp3+ T regulatory (Treg) cells. The rapid and efficient display of exogenous proteins on the cell membrane, therefore, represents a practical and effective cell-based therapy with potential application to autoimmunity, transplantation, and regenerative medicine.

Materials and Methods

Animals

Male 8- to 12-wk-old WF (RT1a), ACI (RT1a), and Fischer (RT1b) rats were purchased from Harlan Sprague Dawley, and 2C mice were generously provided by Dr. J. Connolly (Washington University School of Medicine, St. Louis, MO). All animals were maintained under specific pathogen-free conditions in our barrier facility and used according to the University of Louisville and National Institutes of Health institutional guidelines.

Construction of SA-FasL and protein expression in Drosophila S2 cells

The construction of the SA-FasL gene was reported previously (8). Briefly, DNA segment encoding core streptavidin was amplified using specific primers (forward 5′-GAACTTCATCATCAGGCTGACATACGACCCACC and reverse 5′-GAAATTCGGAGGCGGACGGCT) and total genomic DNA from Streptomyces avidinii (American Type Culture Collection) as a template for PCR. The 5′-primer was designed to include a BgII restriction site and 6 histidine residues to allow cloning in frame with the Drosophila secretion signal (BiP) in pMT/BiP/V5-His vector for expression in S2 cells as a secreted protein and purification using Chelating Sepharose Fast Flow Columns. The cDNA encoding the extracellular domain of rat FasL, without the metalloproteinase site (nucleotides 428–998) was cloned using total RNA from Con A-activated splenocytes as a template and specific primers (5′-primer, nucleotides 428–453; and 3′-primer, nucleotides 977–998). These primers were engineered to include EcoRI sites for cloning in frame with streptavidin in the pMT/BiP/V5-His vector. The chimeric gene in pMT/BiP/V5-His vector was used to stably transfect S2 cells (Invitrogen). Stable transfectants were induced by 1.0 mM CuSO4 and secreted chimeric protein in culture medium was collected 1–4 days after induction. The supernatant was either used immediately or precipitated with 50% ammonium sulfate, dialyzed against PBS, and purified using Chelating Sepharose Fast Flow Columns (Amersham). The concentration of purified SA-FasL or protein in culture supernatant was determined by Western blot or ELISA using known amounts of commercially available streptavidin as standard.

Engineering donor splenocytes to display SA-FasL on their surface

Spleens were harvested from WF rats and processed into single-cell suspension, and RBC were lysed using buffered ammonium chloride solution. Cells were biotinylated by incubation in 5 μM biotin solution (Pierce) in PBS at room temperature for 30 min. Cells were washed twice with PBS, and then incubated with −200 ng of SA-FasL protein per 1 × 106 cells in PBS for 30 min by constant rocking in a cold room. After two washings, cells were resuspended in PBS and counted as previously described (8). A portion of the cells were stained with streptavidin-allophycocyanin, FITC-labeled anti-FasL, or FITC-labeled anti-streptavidin Abs to assess the level of biotinylation and SA-FasL-decorated, respectively, using flow cytometry.

Splenocyte treatment and transplantation

ACI graft recipients received 13 × 106 unmodified WF donor splenocytes by i.p. injection 6 days before heart transplantation. Graft recipients were then treated on days −3, −1, +1, +3, and +5 with 200 μg of anti-CD4, anti-CD8, anti-CD25, anti-CD28, and anti-Fas monoclonal Abs (BD Pharmingen). Chimeric molecule containing the extracellular functional domain of FasL lacking the metalloproteinase cleavage sites fused to streptavidin (SA-FasL) was generated (8). We previously demonstrated that the direct display of SA-FasL, on heart graft vasculature resulted in the prevention of acute rejection after transplantation into allogeneic recipients (25). However, this approach of direct manipulation of the donor graft resulted in moderate prolongation of graft survival without achieving tolerance. The lack of tolerance in this model might have been due to the inability of the transient, local display of SA-FasL on the graft vasculature to deplete a significant pool of alloreactive T effector (Teff) cells. Hence, we herein tested whether recipient immunomodulation by systemic infusion of donor splenocytes engineered to display SA-FasL on their surface induces tolerance to cardiac grafts in a totally allogeneic rat strain combination. Infusion of ACI graft recipients with Wistar-Furth (WF) donor splenocytes displaying SA-FasL on their surface resulted in robust tolerance to cardiac allografts. Tolerance was donor specific and maintained by CD4+CD25+Foxp3+ T regulatory (Treg) cells. The rapid and efficient display of exogenous proteins on the cell membrane, therefore, represents a practical and effective cell-based therapy with potential application to autoimmunity, transplantation, and regenerative medicine.

T cell tracking using 2C model

A total of 20 million 2C spleen cells labeled with CFSE were injected into the tail vein of C57BL/6.JL mice. These animals were then immunized 1 day later by i.v. injection of 20 million allogeneic BALB/c splenocytes either left unmodified or engineered with SA-FasL, or control protein SA. Animals were euthanized 3 days after immunization, and their spleens and mesenteric lymph nodes were harvested for single-cell preparation. Cells were stained with Abs to CD45.2, Vb8.2, and CD8 and analyzed using flow cytometry.

MLR

Spleens were processed into single-cell suspensions, labeled with 2.5 μM CFSE, and resuspended in DMEM; then 50 × 10^6 CFSE labeled splenocytes were plated on a petri dish for 45 min at 37°C to enrich lymphocytes. After 45 min, nonadherent cells were collected, washed, and incubated (1 × 10^6 cells) with irradiated (2000 cGy; 1 × 10^6 cells) naive ACI, WF, or Fischer splenocytes in 96-well U-bottom Titer plates in MLR medium. Irradiated syngeneic cells and F344 third-party splenocytes were used as controls. After 5 days, cells were stained with fluorochrome-labeled Abs against rat CD4 and CD8 and analyzed by flow cytometry. Data were analyzed using FlowJo (Tree Star) software. All proliferation assays were performed in triplicates and are representative of a minimum of three animals per group.

Intracellular cytokine staining

Splenocytes were resuspended in MLR medium at a concentration of 2 × 10^6 cells/ml and stimulated with PMA (5 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) for 2 h at 37°C in a 5% CO2 incubator. Two hours later, cultures were supplemented with Golgi Plug (1 μl/ml; BD Biosciences) and incubated for an additional 2.5 h. Cells were then stained with fluorescent-conjugated Abs against rat CD4, CD8, and IFN-γ and analyzed by flow cytometry as previously described (27).
monitored by abdominal palpation. Cells were transplanted with WF hearts 24 h after cell transfer. Graft survival was assessed using flow cytometry. Extensive washing to remove unbound antibody, cells were incubated with the appropriate amount of anti-PE microbeads (Miltenyi Biotec). Magnetic separation was performed according to the manufacturer's instructions. Positively selected CD25+/FoxP31+ T cells were used for adoptive transfer experiments. Briefly, lymphocytes from rats with long-term graft survival or acutely rejecting animals at rejection and processed into single-cell suspensions and stained with CD4-allophycocyanin, CD8-PerCP, and CD25-PE mAbs or isotype controls at 4°C for 30 min. Cells were then washed twice with FACS buffer and analyzed by flow cytometry. For FoxP3 staining, cells were stained with Abs against CD4, CD8, and CD25; washed twice with FACS buffer; fixed; and then stained for intracellular FoxP3 according to the manufacturer's protocol (eBiosciences).

Adoptive transfer studies

Spleens were harvested from long-term (>90 days) graft survivors or acutely rejecting animals at rejection and processed into single-cell suspensions and stained with CD4-allophycocyanin, CD8-PerCP, and CD25-PE mAbs or isotype controls at 4°C for 30 min. Cells were then washed twice with FACS buffer and analyzed by flow cytometry. For FoxP3 staining, cells were stained with Abs against CD4, CD8, and CD25; washed twice with FACS buffer; fixed; and then stained for intracellular FoxP3 according to the manufacturer's instructions. Positively selected CD25+ and lymphocytes depleted of this population were stained with fluorochrome-labeled Abs to FoxP3 and CD4 and analyzed using multiparameter flow cytometry. CD4+ FoxP3+ and CD4+ FoxP3- populations were found to be >90% pure.

Histology

Cardiac graft samples fixed in 10% formalin were embedded in paraffin, sectioned, and stained with H&E for evaluation of cellular infiltration. Elastin staining was performed using a kit from Sigma-Aldrich according to the manufacturer's instructions to demonstrate the vascular lesions and myocardial fibrosis. Briefly, tissue sections were stained in hematoxylin-chloride solution. Van Gieson solution was used as a counterstain to stain collagen fibers red and myocardium yellow. Chronic rejection was assessed by light microscopy. The severity of chronic rejection was graded according to the percentage luminal occlusion by intimal thickening as previously described (28).

Statistics

Data were analyzed using Student's t test, the nonparametric Mann-Whitney U test, Kaplan-Meier’s log-rank test, and Tarone-Ware tests as appropriate. A p value of <0.05 was considered significant. Statistical analysis was performed using SPSS 13.0 software.

Results

The display of SA-FasL on the surface of splenocytes does not alter the distribution of endogenous cell surface proteins.

The transplant experiments were preceded by a series of preliminary studies to test whether the display of SA-FasL on the surface of splenocytes interferes with the normal distribution of endogenous cell surface proteins, including molecules involved in Ag presentation. WF rat splenocytes were labeled with biotin (5 μM) and engineered with SA-FasL (~200 ng/10^6 cells). The levels of biotin and SA-FasL on the cell surface were assessed by fluorochrome-conjugated mAbs and an Ab against SA or FasL, respectively, using flow cytometry. Almost all targeted cells were positive for the cell surface biotin and SA-FasL (Fig. 1A). Biotinylated and SA-FasL-engineered cells were then analyzed for the presence of a series of endogenous cell surface molecules, such as CD3, CD4, CD8, class I MHC, and CD80 molecules, involved in the immune response using their respective Abs in flow cytometry. As shown in Fig. 1B, neither biotinylation nor engineering with SA-FasL significantly altered the cell surface expression patterns of these molecules. Taken together, these data demonstrate that primary cells, such as splenocytes, can be engineered to display on their surface SA-FasL, in a rapid and efficient manner without significantly altering the cell surface distribution of endogenous proteins.

Systemic immunomodulation with SA-FasL-engineered donor splenocytes induces allograft tolerance

T cells become sensitive to Fas/FasL-mediated apoptosis following activation, several rounds of proliferation, and re-encounter with the Ag (10). We therefore pretreated ACI recipients of WF donor heart allografts with 13×10^6 unmodified donor splenocytes i.p. to mobilize the alloreactive T cell pool and sensitize them to Fas/FasL-mediated apoptosis. These animals were then injected...
with several doses of SA-FasL-engineered donor splenocytes pre- and post-cardiac allograft transplantation as shown in Fig. 2A. Seventy percent of ACI rats treated with SA-FasL-modified donor splenocytes accepted WF heart grafts over the 100-day observation period, whereas the remaining 30% rejected their allografts in a median survival time (MST) of 12 ± 1.2 (Fig. 2B). In contrast, all untreated animals (n = 10) rejected their grafts in a MST of 9 ± 0.4 days (Fig. 2B). Treatment with splenocytes engineered with control SA protein (n = 20) resulted in only ~25% graft survival, which was similar to that achieved using unmodified splenocytes (n = 8). The induced tolerance was donor specific given that ACI rats (n = 4) treated with SA-FasL-engineered WF splenocytes rejected F344 third-party heart allografts in a normal tempo (MST of 11 ± 1.2 days. Taken together, these data demonstrate that systemic immunomodulation with SA-FasL-engineered splenocytes was effective in inducing tolerance to cardiac allografts without the use of immunosuppression.

**Systemic immunomodulation with SA-FasL-engineered allogeneic splenocytes results in reduced proliferation and accumulation of alloreactive T cells**

To determine whether systemic infusion of SA-FasL-engineered splenocytes physically eliminates alloreactive cells in the host, we took advantage of the TCR-transgenic 2C mouse model the CD8+ T cells of which are specific for H-2Ld class I alloantigen (31). C57BL/6 (CD45.2) mice were adoptively transferred with congenic CFSE-labeled 2C T cells (C57BL/6; CD45.2) followed by immunization with BALB/c (H-2d) splenocytes engineered with SA-FasL or SA control protein. Three days later, splenocytes and lymph node cells from the immunized animals were harvested and analyzed using flow cytometry for the proliferation of 2C cells. Immunization with SA-engineered BALB/c spleenocytes resulted in robust proliferation (more than eight cycles) of 2C cells and accumulation of alloreactive T cells (Fig. 3A). In marked contrast, mice immunized with SA-FasL-engineered BALB/c spleenocytes had moderate proliferation (~4 cycles) of 2C cells. Importantly, there was a steady decrease in the number of daughter cells per generation plausibly due to apoptosis. This notion is consistent with published literature that naive T cells require few cycles of proliferation in response to Ag before becoming sensitive to Fas/FasL-mediated apoptosis (10).

To provide direct evidence that alloreactive T cells undergo apoptosis following Ag recognition in the context of FasL on donor cells, we performed in vitro stimulation assays where BALB/c
spleenocytes engineered with SA-FasL or SA control protein were used as stimulators for 2C cells. There was a significant reduction in the number of proliferating 2C cells responding to SA-FasL-engineered donor cells as compared with control (Fig. 3B, top). The observed reduction in proliferation was due to significant apoptosis of responding 2C T cells (Fig. 3B, bottom).

Allograft tolerance is associated with donor-specific T cell hypoproliferation and reduced expression of IFN-γ

To further elucidate the mechanisms responsible for the observed tolerance to WF hearts, ACI graft recipients were evaluated for evidence of donor-specific proliferation using CFSE-based MLRs (32). As shown in Fig. 4A, lymphocytes from SA-FasL-engineered cells treated long-term graft recipients (SA-FasL LT) responded poorly to donor cells but showed a normal response to third-party cells, indicating Ag specificity. In marked contrast, lymphocytes from SA-engineered cell-treated animals with acute graft rejection (SA Rej) and naive control animals proliferated vigorously to both donor and third-party cells. Moreover, tolerant rats had lower percentages of CD8+ T cells expressing IFN-γ, a signature cytokine for graft rejection (Refs. 33 and 34) and Fig. 4B) as compared with SA-engineered-cell-treated rats with acute graft rejection (7.4 ± 3.6% vs 28.5 ± 3.0% in CD8+ T cell gate; Fig. 4C). In marked contrast to CD8+ T cells, only a small percentage of CD4+ T cells expressed IFN-γ in all groups irrespective to the treatment regimen (Fig. 4C; gated on CD4+ cells). There was a slight increase in the percentage of CD4+ T cells expressing IFN-γ in FasL-treated animals, which was not statistically significant.

Tolerance is associated with increased percentage of peripheral CD4+CD25+FoxP3+ Treg cells

Although we demonstrated that systemic immunomodulation with SA-FasL-engineered splenocytes induces apoptosis in activated alloreactive T cells (Fig. 3), this effect may be short lived due to the transient present of SA-FasL on the cell surface. Therefore, physical elimination of alloreactive T cells early in transplantation may not be sufficient for long-term allograft survival and may require induced peripheral immunoregulatory mechanisms. We focused on CD4+CD25+FoxP3+ Treg cells because of their capacity to induce and maintain tolerance in various transplantation settings (35). Lymphocytes were isolated from the spleen of long-term graft recipients (>90 days) and phenotyped for CD4 and CD25 expression using flow cytometry (Fig. 5A). There was a significant increase in the percentage of CD4+CD25+ T cells (8.2 ± 1.3% of total lymphocytes) in long-term SA-FasL-treated graft recipients compared with results in naive rats (5.1 ± 0.4%). In contrast, rats with acute graft rejection had similar percentages of CD4+CD25+ T cells as naive animals whether they were treated with SA (5.7 ± 1.5%) or SA-FasL-engineered (5.1 ± 1.2%) splenocytes (Fig. 5B). The increased percentage of CD4+CD25+ Treg cells in long-term graft survivors was further confirmed with an Ab to the signature transcriptional factor FoxP3 (Fig. 5C; gated on CD4+ T cells).

Importantly, sorted CD4+CD25+ T cells from long-term graft survivors inhabited the proliferative responses of naive Teff cells to donor alloantigens when used at various ratios in a CFSE-based in vitro proliferation assay (Fig. 5D). This finding indicates that CD4+CD25+ T cells from long-term graft survivors are Treg cells, rather than newly activated Teff cells expressing CD25. Taken together, these data demonstrate that systemic immunomodulation with SA-FasL-engineered donor splenocytes induces long-term graft survival that is associated with increased percentages of CD4+CD25+FoxP3+ Treg cells in the periphery.

**Sorted CD4+CD25+FoxP3+ Treg cells from tolerant animals prevent the rejection of donor hearts in secondary naive recipients**

To provide direct evidence for the role of peripheral immunoregulatory mechanisms in the observed tolerance, 80–95 × 10^6 spleenocytes harvested from primary graft recipients were adoptively transferred into a second cohort of naive rats that had been subjected to 450 cGy total body irradiation 1 day earlier. All rats (n = 6) that received splenocytes from SA-FasL-engineered cells treated long-term primary graft survivors indefinitely accepted WF donor heart allograft (Fig. 6A). In marked contrast, animals (n = 4) receiving splenocytes from SA-engineered cell-treated primary
FIGURE 5. Long-term SA-FasL treated animals have high percentages of peripheral CD4^{-}CD25^{+} Treg cells. Splenocytes from long-term (>90 days) graft survivors treated with SA-FasL-engineered donor splenocytes (SA-FasL LT), acutely rejecting animals treated with SA-engineered splenocytes (SA Rej), or naive ACI animals (naive) were stained with fluorochrome-labeled Abs against CD4, CD25, and FoxP3 and analyzed using multiparameter flow cytometry. A, Dot plot pattern of splenocytes showing CD4^{-}CD25^{+} T cells in total lymphocyte gate. B, Tabulation of CD4^{-}CD25^{+} T cells for a minimum of three animals per group in the lymphocyte gate. *, p < 0.05 as compared with all the other groups. C, Long-term SA-FasL animals have high percentages of CD4^{-} T cells expressing intracellular FoxP3 (gated on CD4^{-} T cells). D, CD4^{-}CD25^{+} T cells sorted from long-term SA-FasL-splenocyte-treated graft recipients are suppressive in vitro. CFSE-labeled ACI naive splenocytes (1 × 10^5) were used as responder to irradiated WF splenocytes (1 × 10^5) in a 5-day proliferation assay in the presence of varying ratios of CD4^{-}CD25^{+} T cells sorted from long-term graft survivors. Naïve CFSE-labeled responder to CD4^{-}CD25^{+} T cell ratios are indicated in the right bottom corner of each graph. Percentages of proliferating allosreactive T cells (upper left) and added (ovals) CD4^{-}CD25^{+} T cells (lower left) are shown for each panel. All histograms show mean ± SD and significance was assessed using Mann-Whitney U test. APC, Allophycocyanin.

FIGURE 6. Tolerance is maintained by CD4^{-}CD25^{+} Treg cells. A, Adoptive transfer experiments into irradiated secondary graft recipients. Splenocytes (80–95 × 10^5) harvested from long-term (>90 days; SA-FasL) or acutely rejecting (SA or SA-FasL) recipients were transferred into 450-cGy-irradiated naïve ACI animals 1 day before WF heart transplantation. B, Adoptive transfer experiments into nonirradiated secondary graft recipients. Experimental conditions are as in A. C, Sorted CD4^{-}CD25^{+} T cells from long-term but not from acutely rejecting animals prevent rejection of WF hearts in naïve ACI recipients. Unmanipulated naïve ACI rats were adoptively transferred 1 day before WF heart transplants with 5–9 × 10^6 CD4^{-}CD25^{+}FoxP3^{+} T (Treg) cells sorted from SA-FasL long-term graft survivors, 50–90 × 10^6 Treg-depleted splenocytes from these animals, or 5–9 × 10^6 CD4^{-}CD25^{+}FoxP3^{+} Treg cells sorted from acutely rejecting (SA-engineered splenocytes treated and normal controls).
To provide direct evidence for the contribution of Treg cells in the observed tolerance, CD4+/CD25+ Treg cells were positively sorted from tolerant splenocytes using Miltenyi beads. Sorted Treg cells (5–9 × 10^6 cells/recipient) as well as Treg-depleted splenocytes (50–90 × 10^6 cells/recipient) were adaptively transferred into naive ACI recipients of WF hearts 1 day before transplantation. All recipients (n = 4) adoptively transferred with Treg cells accepted their grafts (Fig. 6C). In marked contrast, all grafts in the group receiving Treg cell-depleted splenocytes underwent rejection with a MST of 23.4 ± 3.4 days. However, this rejection time was significantly delayed as compared with normal controls (MST 9 ± 0.4 days), suggesting either the presence of other immune regulatory cells or contamination with T cells. Importantly, Treg cells sorted from acutely rejecting animals (SA-splenocyte treated or normal controls) did not prevent the rejection of donor grafts following adoptive transfer into naive secondary recipients under similar conditions as Treg cells sorted from FasL-treated long-term graft survivors. Taken together, these data demonstrate that Treg cells are critical to the observed donor-specific peripheral tolerance achieved by systemic immunomodulation using SA-FasL-engineered splenocytes.

**Discussion**

Immunomodulation with genetically engineered cells expressing FasL has been extensively tested for the induction of tolerance to auto- and alloantigen with reported efficacy in various preclinical settings (14–18, 20, 36–40). However, the translation of this approach to the clinic remains to be realized primarily due to difficulties associated with efficient and rapid manipulation of donor primary cells under clinically applicable conditions to reproducibly express FasL and safety concerns of the gene therapy. Furthermore, FasL has pleiotropic effects on immune and nonimmune cells, and as such long-term stable expression of this molecule using gene therapy might have detrimental consequences. In the present study, we overcame these difficulties by transient display of a recombinant form of FasL protein with potent apoptotic activity (25) on donor splenocytes in a rapid (~2 h) and efficient manner (~100% of the targeted cells). Systemic immunomodulation with FasL-engineered donor cells resulted in peripheral tolerance to cardiac allografts without the use of any additional immunosuppression.

Although a series of studies using various APC genetically engineered to express FasL for immunomodulation reported alloantigen-specific immune nonresponsiveness, none of these studies demonstrated that such nonresponsiveness eventually leads to long-term allograft tolerance (11, 16, 37, 40). To our knowledge, this is the first study to demonstrate that systemic immunomodulation with donor cells engineered to express FasL on their surface induces long-term peripheral tolerance to cardiac allografts in a totally allogeneic rat strain combination. The mechanistic basis of the induced peripheral tolerance involves physical elimination of alloreactive cells by activation-induced cell death early after transplantation and maintenance of tolerance by CD4+CD25+FoxP3+ Treg cells. The enhanced immunomodulatory effect of repeated splenocyte infusion reported in the present communication can be attributed to effective elimination of alloreactive immune effector cells at remote sites from the graft, robust induction/expansion of Treg cells, or both. T cells require Ag-specific activation and several rounds of sensitization to acquire sensitivity to Fas/FasL-mediated apoptosis (10). Also, it is well-established that memory immune cells reside within nonlymphoid target tissues, plausibly to generate a rapid and effective secondary immune response to recurrent infection (41). Therefore, systemic and repeated administration of engineered donor splenocytes may have the advantage of tolerizing compartmentalized memory responses defined as heterologous immunity that serves a major barrier for tolerance induction in the clinic (4).

In our view, induction of CD4+CD25+FoxP3+ Treg cells using SA-FasL-engineered donor splenocytes is the most significant finding of the present study. The essential role of Treg cells in tolerance was demonstrated by adoptive transfer experiments where cells sorted from lymphoid organs of long-term graft acceptors transferred tolerance to naive, unmanipulated secondary recipients (Fig. 6C). Importantly, CD4+CD25+FoxP3+ T cells sorted from acutely rejecting animals did not prevent the rejection of donor grafts in naive secondary recipients following adoptive transfer. This may be because the sorted CD4+CD25+FoxP3+ Treg cells were not donor specific, contaminated with newly activated alloreactive Teff cells expressing CD25, or rat Teff cells transiently express FoxP3 following activation as reported for human Teff cells (42). The presence of Treg cells, other than CD4+CD25+FoxP3+ Treg cells, in our model is consistent with our observations that Treg cell-depleted total splenocytes from SA-FasL-treated long-term graft survivors did not prevent the rejection of donor grafts upon adoptive transfer into secondary naive graft recipients but caused significant prolongation as compared with controls (Fig. 6C). These regulatory T cells may include CD4+CD8+TCR− T cells (43), CD8+FoxP3+ T cells (44), and/or newly described CD8+CD45RChlow T cells expressing IFN-γ (45). These cell types are also implicated in allotransplantation achieved using donor-specific transfusion. For example, infusion of allogeneic donor splenocytes into graft recipients was shown to generate CD4+CD8+T− Treg cells that used FasL to physically eliminate alloreactive T cells for tolerance induction (43). Furthermore, tolerance to skin allografts disparate for H-Y Ag has recently been shown to require FasL on donor cells used for infusion and Fas in graft recipients (46). Therefore, the Fas/FasL system may serve as a common denominator of mechanisms responsible for tolerance achieved by immunomodulation of graft recipients using donor-specific transfusion in form of donor lymphocytes.

We envision three possible mechanisms for the induction/expansion of Treg cells by SA-FasL-engineered splenocytes. First, FasL interaction with Fas up-regulated on the surface of Treg cells in response to donor Ags may transduce a mitogenic signal, leading to their expansion. Although Fas signaling was shown to be involved in the physiological process of Teff cell activation under selected conditions (47, 48), it remains to be determined whether such a function also applies to Treg cells. Second, Treg cells may be less sensitive to Fas-mediated apoptosis as compared with Teff cell effects. Immunomodulation with SA-FasL-engineered splenocytes may preferentially eliminate Teff effector cells, thereby tipping the balance toward the expansion of Treg cells. However, the sensitivity of Treg cells to Fas/FasL-mediated apoptosis has been the subject of few studies with conflicting observations. Although some studies reported that Treg cells are more sensitive to FasL-mediated apoptosis than Teff effector cells (49, 50), we (51) and others indicated the exact opposite (52). These conflicting observations may be due to the study designs, such as lack of comparative analysis of Treg and Teff effector cell sensitivity to FasL-mediated apoptosis in the course of an immune response to Ags under inflammatory conditions. Third, Teff cells undergoing apoptosis may give rise to the generation of adaptive Treg cells or expansion of naturally occurring Treg cells. Consistent with this notion are the observations that apoptotic lymphocytes release two cytokines, IL-10 and TGF-β, that play important roles in the generation and function of Treg cells (53). Furthermore, apoptotic bodies from dying cells were shown to contribute to the generation
of Treg cells through mechanisms that involved TGF-β and macrophages (54). Further studies are needed to establish mechanisms that are responsible for the generation of Treg cells in our model and whether these cells are of adaptive or natural type.

The immunomodulatory approach presented here has several attractive features with direct relevance to the clinic. First, it allows for rapid, efficient, and transient display of exogenous immunomodulatory proteins on the cell membrane with immediate function without a time lag required for gene transfer-based expression. Second, the transient display of immunomodulatory proteins with pleiotropic effects may minimize the potential undesired effects arising from their stable and long-term expression achieved by gene therapy. Third, several proteins with synergistic functions may be simultaneously displayed on the cell surface to maximize their therapeutic efficacy (R. K. Sharma and H. Shirwan, unpublished data). In particular, our approach may be incorporated into cell-based immunomodulation approaches, such as donor-specific leukocyte transfusion or hemopoietic stem cell transplantation, for tolerance induction in the clinic. Infusion of unmodified donor leukocytes or bone marrow cells into graft recipients for the purpose of immunomodulation has been extensively tested with observed beneficial effects in various preclinical and clinical settings (55, 56). The presence of SA-FasL on donor leukocytes and bone marrow cells may further improve their therapeutic efficacy by eliminating alloreactive pathogenic lymphocytes and/or inducing peripheral immunoregulatory mechanisms as shown by the present study. In conclusion, this protein display approach possesses the simplicity, safety, and efficacy required to make it a clinically relevant and practical alternative to gene therapy for immunomodulation with broad application to cell-based therapies, regenerative medicine, autoimmunity, and transplantation.

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Disclosures

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