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Linked Suppression across an MHC-Mismatched Barrier in a Miniature Swine Kidney Transplantation Model

Adam D. Griesemer, John C. LaMattina, Masayoshi Okumi, Justin D. Etter, Akira Shimizu, David H. Sachs, and Kazuhiko Yamada

We have demonstrated previously that a 12-day course of FK506 permits the induction of tolerance to fully MHC-mismatched renal transplants in miniature swine. In the present study, we examined the mechanism of this tolerance by assessing the possibility that the survival of one-haplotype mismatched third-party kidneys might be prolonged via linked suppression. Ten SLA<sup>dd</sup> miniature swine received fully MHC-mismatched renal allografts from SLA<sup>cc</sup> donors with 12 days of FK506. Six animals received second SLA<sup>cc</sup> kidneys without immunosuppression to confirm tolerance. Regulatory mechanisms were assessed by mixed lymphocyte reaction (MLR) and cell-mediated lympholysis coculture assays and ELISA for regulatory cytokines. Linked suppression was investigated by transplanting SLA<sup>cc</sup> or SLA<sup>dd</sup> allografts into long-term tolerant recipients without immunosuppression. All recipients showed donor-specific unresponsiveness in standard cell-mediated lympholysis and MLR assays. Tolerant cells prestimulated with donor Ag and then cocultured with naive recipient MHC-matched cells inhibited antidonor responses, confirming the presence of regulatory cells. ELISA and MLR assays showed that TGF-β2 was involved in mediating the suppression in vitro. SLA<sup>dd</sup> renal allografts transplanted into tolerant recipients were rejected by postoperative day 8 (median, 7 days; range, 6–8). In contrast, SLA<sup>cc</sup> allografts showed markedly prolonged survival (median, 52 days; range, 28–78; p = 0.0246), suggesting linked suppression. Animals not challenged with a second donor-matched graft did not manifest linked suppression consistent with in vitro data showing that re-exposure to tolerated Ags is important for generation of regulatory cells. To our knowledge, these data represent the first evidence of linked suppression across fully MHC-mismatched barriers in a large animal model. The Journal of Immunology, 2008, 181: 4027–4036.

Tolerance induction across MHC-mismatched barriers would eliminate the need for continuous administration of immunosuppressive medications in the clinic. Recent reports have described tolerance across single haplotype HLA barriers in humans, although further studies are required to clarify the mechanism of tolerance (1). Preclinical models have shown that tolerance can be induced by deletion of alloreactive cells, the induction of anergy, and the generation of Ag-specific regulatory T cells (Tregs) (2, 3). Recently, much attention has been focused on the clinical potential of Tregs (4–6). Studies in rodents have demonstrated the importance of Tregs in the induction and maintenance of tolerance and that these Tregs are able to suppress a response to third-party Ags coexpressed with the tolerant Ag via linked suppression (7, 8). Such linked suppression could have implications for tolerance induction in patients. For example, if tolerance to common Ags could be induced by transplantation of immature dendritic cells (9–11) or by pretransplant administration of a single donor class I MHC molecule (12) in humans on transplant waiting lists, linked suppression might minimize the immune response to other Ags coexpressed on a transplanted organ, resulting in a decreased requirement for immune suppression.

Studies in large animal models are essential to bridge the gap between rodent studies and clinical application. However, confirmation of complex rodent models in large animals is difficult because multiple different MHC-defined haplotypes are required. Our herd of Massachusetts General Hospital (MGH) miniature swine, with defined MHC and intra-MHC recombinant strains that yield reproducible allograft transplantation results (13, 14), provide us with the unique opportunity to study the role of Tregs in prolonging survival of renal allografts via linked suppression.

We have previously demonstrated the induction of tolerance to fully MHC-mismatched renal allografts in MGH-inbred miniature swine using a short course of high-dose FK506 (15). Recipient animals uniformly accepted renal allografts with 12 days of FK506, demonstrated in vitro donor-specific unresponsiveness, and subsequently accepted a second donor-matched renal allograft without immunosuppression. Previous investigations suggested that regulatory mechanisms mediated tolerance in this model (15). In this study, we have assessed the possibility that one-haplotype donor-matched, one-haplotype third-party kidneys might be prolonged via linked suppression. We have also attempted to identify the cell populations responsible for mediating linked suppression and to characterize the mechanism of suppression in vitro.

Materials and Methods

Animals

Transplant donors and recipients (5–7 mo of age) were selected from our herd of partially inbred miniature swine. The immunogenetic characteristics of this herd and the intra-MHC recombinant haplotypes have been described previously (Fig. 1) (14). Briefly, SLA<sup>dd</sup> (class I<sup>dd</sup>class II<sup>dd</sup>) animals served as recipients. SLA<sup>cc</sup> (class I<sup>cc</sup>class II<sup>cc</sup>) animals served as donors.
were cared for according to the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Histology and immunohistochemistry

When clinical rejection was suspected, allograft rejection was scored by the presence of anti-donor IgM and IgG deposition in renal allografts was examined by fluorescence microscopy using frozen sections stained with saturating concentrations of fluorescent isothiocyanate-labeled goat anti-swine IgM or IgG as previously described (17).

Preparation of PBL

Blood was drawn from the external jugular vein on PODs 30, 60, and >100. Freshly heparinized whole blood was diluted 1/2 with HBSS (Life Technologies) and the mononuclear cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (Organon Teknika). The mononuclear cells were washed once with HBSS and contaminating RBCs were lysed with ammonium chloride potassium buffer (B&K Research Laboratory). Cells were then washed with HBSS and resuspended in tissue culture medium. All cell suspensions were kept at 4°C until used in cellular assays.

Statistical analysis

Survival curves were plotted with GraphPad Prism software and p values were determined using the χ² test, and a p < 0.05 was considered significant. For in vitro assays, mean values of different assays were calculated using GraphPad Prism software and plotted with error bars expressing SE. Differences between groups were determined using the Student t test; p < 0.05 were considered significant.

Primary mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) assays

Both MLR and CML assays were performed as previously described (15, 16). [3H]Thymidine incorporation was counted for beta emission in MLR and 51Cr release was determined on a gamma counter (Micromedics) in CML. Cytotoxicity was expressed as percent specific lysis, calculated as:

\[
\text{percent specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100
\]

Coculture MLR

Peripheral regulatory mechanisms were investigated by in vitro coculture assays. The primary culture was set up as in primary MLR assays. After a 5-day priming with donor Ag, responder cells were then harvested and rested overnight at 4°C, after which they were coincubated with naive SLA-matched PBMCs and irradiated donor-type or third-party PBMCs for 5 additional days. Analysis of the MLR was performed as described for primary cultures above.

Coculture CML

Peripheral regulatory mechanisms and the role of CD25+ cells in mediating suppression were investigated by in vitro coculture assays. The primary culture was set up as in CML assays with either PMBCs, CD25-enriched, or CD25-depleted cells as responders. The primed responder cells were then harvested and rested overnight at 4°C, after which they were coincubated with naive SLA-matched PBMCs and irradiated donor-type or third-party PBMCs for 6 additional days. The cultures were harvested and results were analyzed as described above for primary cultures.

CD25 depletion/enrichment

CD25-positive cells were separated by magnetic cell separation (MACS; Miltenyi Biotec). PBL from experimental and naive control animals were incubated with FITC conjugated anti-CD25 (mAb 231-3B2, mouse IgG1) (18) and then separated with MACS anti-FITC beads via elution through two LS columns. CD25 depletion and enrichment was then confirmed by FACS analysis.

Cytokine-neutralizing MLR assays

Neutralizing MLR assays were performed as outlined above for primary MLR assays; however, 1 µg/ml mouse anti-human TGF-β1 (MAB240; R&D Systems; with no cross-reactivity with TGF-β2 (19)) and TGF-β2 IgG (MAB612; R&D Systems; with no cross-reactivity to other TGF-β isoforms) were added to the cell cultures before initiating the 5-day incubation period. Studies were repeated using 1 µg/ml Ab.

Flow cytometry

The presence of anti-donor class I (SLA class Iα and Iγ) and class II (SLA class Iβ and Iγ) IgM and IgG Abs in the serum of experimental swine was detected by indirect flow cytometry. FACS was performed using a BD FACSAria III cell sorter (Becton-Dickinson) and analyzed with BD FACSDiva software (Becton-Dickinson).
Biosciences FACScan microfluorimeter and recombinant SLA PBL to determine the SLA-binding specificity of the Ab. FITC-labeled goat anti-swine IgM or IgG polyclonal Abs were used as secondary reagents (Kirkegaard & Perry Laboratories). Staining was performed with 1/100,000 of donor-type PBL (SLAa/a and SLAc/c) resuspended in 100 μl of HBSS (Life Technologies) containing 0.1% BSA and 0.05% NaN3. Cells were incubated for 30 min at 4°C with 10 μl of decomplemented test sera, and after two washes a saturating concentration of FITC-labeled goat anti-swine IgM or IgG was added and incubated for 30 min at 4°C. After a final wash, cells were analyzed by flow cytometry using propidium iodide gating to exclude dead cells. Both normal pig serum and pretransplant sera from each experimental animal were used as controls for specific binding.

**ELISA**

ELISA was used to determine quantitative changes of PBL cytokine secretion patterns in response to self, donor, and third-party Ag. The secretion of IL-10, IFN-γ, TGF-β1, and TGF-β2 was evaluated. IL-10 and IFN-γ ELISA were performed using commercially available CytoScreen ELISA kits (BioSource International) as previously reported (21).

**Results**

**Induction of tolerance to fully mismatched kidneys with 12 days of FK506**

All animals were treated with 12 days of FK506 with a target level of 35 ng/ml following fully MHC-mismatched renal transplantation. The animals maintained stable renal function for 120 days and demonstrated donor-specific unresponsiveness on both primary CML and MLR assays (Fig. 2, A–C). To assure that the animals were truly tolerant to their fully MHC-mismatched kidneys, the primary graft was removed at POD 120 in 6 of 10 animals. A second kidney that was MHC matched to the primary graft (but mismatched at minor Ags) was then transplanted without immunosuppression. All animals had stable renal function (D) and maintained hyporesponsiveness to SLA+c PBL by CML (E; n = 6) and hyporesponsiveness to SLA+c and SLA+e stimulation by MLR (F; n = 6) assays, while a strong response was seen to outbred (Out) PBL in both CML and MLR assays. Re-Txp, Retransplantation.

**In vitro studies of regulatory mechanisms**

**Primary CML using bulk PBMCS and CD25-negative populations demonstrated that CD25+ cells mediate suppression.** To assess regulatory mechanisms in vitro, we used MACS to deplete CD25+ cells and show in vitro unresponsiveness to donor PBMCs while maintaining a response to third-party stimulators (Fig. 2, E and F).

**% Specific Lysis (E:T ratio 100:1)**

![Graph showing % Specific Lysis](http://www.jimmunol.org/)

**CD25 depletion CML assays.** To determine whether CD25+ cells are involved in suppressing antidonor responses, we used MACS to deplete CD25+ cells from tolerant PBL, which were then added to naive PBL and tested for cytotoxicity. CD25+ depletion almost completely restored the CTL of tolerant PBL, whereas naive PBL was unaffected. Data are from two separate animals before the second SLA+c kidney transplantation.

**Figure 2.** SLA+id recipients accepted SLA+c/c kidneys after 12 days of FK506. All animals accepted full MHC-mismatched kidneys with stable renal function (A). In vitro assays showed hyporesponsiveness to SLA+c and SLA+e stimulation, while maintaining strong responses to SLA+a and outbred PBL. Shown are CML responses at day 30 (n = 6 animals; B) and MLR responses at day 120 (n = 6 animals; C). Animals accepted second SLA+c kidneys without immunosuppression: in 6 of 10 animals, the first kidney was removed 120 days after primary kidney transplantation and a second SLA+c kidney was transplanted without immunosuppression. All animals had stable renal function (D) and maintained hyporesponsiveness to SLA+c PBL by CML (E; n = 6) and hyporesponsiveness to SLA+c and SLA+e stimulation by MLR (F; n = 6) assays, while a strong response was seen to outbred (Out) PBL in both CML and MLR assays. Re-Txp, Retransplantation.
FIGURE 4. Coculture assays to confirm regulatory cell function in vitro. A, Bulk MLR and CML coculture assays to demonstrate the function of regulatory cells from tolerant animals in vitro: MLR coculture assays showed that antidonor responses were inhibited ~70%, while anti-third-party responses were not inhibited. Bar 1 (blue) is the naive response to donor (top) and third-party (bottom) PBL. Bar 2 (red) is the response of PBL from the tolerant animal. Bar 3 represents the hypothetical summation of bars 1 and 2, while Bar 4 (yellow) shows the actual response of cocultured PBL from the tolerant and naive animal. B, CML coculture assays: primed PBL from the tolerant animal inhibited a naive CTL response by 86% (red bar shows CTL for the coculture; naive CTL response, black bar), while priming PBL from a naive animal resulted in a sensitized response (blue bar). Data represent the results from three different animals before the second SLAc/c kidney transplant, two of which received SLAa/c kidneys and one of which received an SLAa/d kidney. C, Isolation of CD25-negative and CD25-enriched cell fractions to determine the role of CD25 cells in the suppression of the antidonor response in vitro: Nearly complete depletion of CD25 cells (middle panel) was achieved, while CD25 enrichment increased the percentage of CD4/CD25 double-positive cells from 2.4% (first panel) to nearly 35% (third panel). D, Coculture CML assays set up using different ratios of primed CD25 cells to naive recipient-matched PBL: unseparated PBL from the long-term tolerant animal showed suppression (red bar), but the unseparated PBL from the naive animal showed a sensitized response (light blue bar). To determine whether CD25 cells mediate this suppression, we primed CD25 cells from both
cells from the PBMCs of tolerant and naive SLA-matched animals in CML assays. Although there was no difference in CTL between bulk PBMCs and CD25-depleted PBMCs from naive animals, bulk PBMCs from tolerant animals, which contains CD25-positive cells, showed a marked decrease in anti-donor CTL responses (Fig. 3). This CTL response was partly restored in tolerant animals by CD25 depletion, but it was still slightly decreased when compared with the naive control, suggesting either a second regulatory cell population or a slight loss of donor-specific alloreactive T cells following separation.

MLR and CML coculture assays showed suppression of antidonor responses. To determine whether the Tregs could demonstrate suppression of a naive antidonor response, we set up both MLR and CML coculture assays. PBMCs from tolerant animals and naive controls were primed with donor PMBCs in primary cultures and then cocultured with SLA-matched PMBCs to determine whether the primed could inhibit a naive response. Primed PBMCs from tolerant animals inhibited the naive antidonor response by 70%, whereas naive anti-third-party responses were not inhibited (Fig. 4A). Cocultured CML assays demonstrated that primed PBMCs from tolerant animals inhibited a primary antidonor response by 86%, while primed PMBC from a naive animal augmented the primary response (Fig. 4B). These assays thus demonstrated that PBMCs from tolerant animals contained a cell population capable of suppressing the antidonor response in vitro and were likely responsible for mediating tolerance in vivo.

We attempted to determine whether the CD4/CD25 double-positive cells were responsible for the suppression we observed in bulk CML coculture assays by depleting CD25+ cells from the PMBCs by MACS from both tolerant animals and naive SLA-matched controls. We also used the CD25-enriched population eluted from the LS columns after MACS depletion in the coculture assays. FACS analysis of the cell populations showed that depletion of CD25-positive cells was almost complete (Fig. 4C) while the CD25-enriched fraction contained ~35% of CD4/CD25 double-positive cells (compared with ~3% pre-MACS). The two cell populations were primed with donor PMBCs in primary cultures and then added to naive anti-donor CML cultures in ratios from 1:10 to 1:1000 (primed:naive cells). Although the CD25-positive cell population from both tolerant and naive animals suppressed a naive antidonor response at a 1:1 ratio, only the CD25-negative cell population from the tolerant animals was able to suppress a naive antidonor response (Fig. 4, D and E). This result suggested that nonspecific regulatory cells were present in both naive and tolerant animals, and also that a regulatory cell population is circulating in the periphery of the tolerant animals that, at its resting state, was not CD4/CD25 double positive.

TGF-β2, but not TGF-β1, assisted in the maintenance of in vitro tolerance in long-term tolerant animals. We have previously demonstrated that partial inhibition of the MLR was seen in Transwell MLR assays, suggesting that inhibitory cytokines were involved in suppression (15). To identify the soluble factors that were involved in suppressing naive responses, we investigated the level of cytokines in culture medium. ELISA analysis demonstrated that the secretion of TGF-β2 by long-term tolerant PBMCs was enhanced after incubation in the presence of donor Ag relative to TGF-β2 secretion by tolerant PBMCs after incubation with third-party Ag (Fig. 5A). To confirm that TGF-β2 was involved in mediating the suppression in this model, we performed MLR assays using PBL from long-term tolerant and naive animals in the presence of anti-human TGF-β2. The provision of 1 μg/ml anti-TGF-β2 demonstrated an 11-fold increase in reactivity (p = 0.0005) when applied to the tolerant cells (Fig. 5, B1 and B2), eliminating the donor-specific hyporesponsiveness witnessed in standard MLR studies. Slight stimulatory effects were seen in anti-third party MLR assays with the provision of 1 μg/ml anti-TGF-β2, but the same trend was seen in control assays with naive cells (Fig. 5, C1 and C2), but this increase did not achieve statistical significance. Reactivity of long-term tolerant PBL was not affected by anti-human TGF-β1.

Evidence for in vitro-linked suppression in long-term tolerant animals. To determine whether cells responsible for suppressing an antidonor response in vitro could suppress a response to third-party Ags on PMBCs that coexpressed the donor Ag, we set up primary MLR and CML assays using SLAa/c (class Ia/c, class IIa/c) PMBCs. These assays showed that PMBCs from long-term tolerant animals had a decreased response to SLAa/d PMBCs compared with the response to SLAa/c PMBCs, but the response was greater than that to SLAa/c PMBCs (Fig. 2, B, C, E, and F). To confirm that the decrease in reactivity was due to regulatory mechanisms, we performed coculture assays by priming cells from long-term tolerant and naive animals with donor SLAa/c PMBCs and then cocultured the primed cells with naive responders and SLAa/c stimulators to see whether the primed cells would suppress the SLAa/c response in vitro. The cells from the tolerant animal completely inhibited the naive anti-SLAa/c response, whereas cells from the naive animal enhanced it, further suggesting a role for regulatory cells in this model (Fig. 6A).

In vivo-linked suppression

Retransplantation to assess linked suppression. Approximately 90 days following SLAa/c retransplantation (6 of 10 animals, group A) or 120 days following primary SLAa/c transplantation (4 of 10 animals, group B), SLAa/d recipients that were tolerant to SLAa/c renal allografts underwent retransplantation in a similar manner to that described above. Half of the animals in groups A and B received recombinant haplotype SLAa/c (class Ia/c/class IIa/c) kidneys to test for linked suppression and half received SLAa/d (class Ia/d/class IIa/d) kidneys to prove specificity of the suppression. The recombinant SLAa/c haplotype presented Ag on both SLAa/c (third-party type) and SLAa (original donor type) class I and class II molecules, while the recombinant SLAa/d haplotype presented Ag on both SLAa (third-party-type) and SLAa (recipient-type) class I and class II molecules. No immunosuppression was administered following transplantation.

Prolonged survival of one-haplotype third-party grafts by linked suppression. To test whether the linked suppression that was seen in vitro would also occur in vivo, we transplanted SLAa/c kidneys without immunosuppression to three long-term tolerant SLAa/d animals 90 days after they had accepted the second SLAa/c kidney.

tolerant and naive animals with donor PMBCs. Primed CD25+ enriched cells from both long-term tolerant animals at a ratio of 1 CD25+ cell to 10 naive cells (yellow bar) and naive PBL at a ratio of 1 CD25+ cell to 10 naive cell (brown bar) suppressed a naive SLAa/d antidonor response. Data shown are mean values for two animals, one of which received an SLAa/c kidney and one of which received an SLAa/d kidney. E. Coculture CML assay using CD25-negative cells from both long-term tolerant animals and naive animals: CD25-negative cells from the long-term tolerant animal suppressed a naive antidonor response at a 1:1 ratio (yellow bar), while CD25-negative cells from a naive animal showed a response similar to bulk PBMCs (brown bar), suggesting that a regulatory population that is CD25 negative in the tolerant animal’s PBL can suppress an antidonor response after activation in culture. Data shown are mean values for two animals, one of which received an SLAa/c kidney and one of which received an SLAa/d kidney.
FIGURE 5. A, ELISA showed increased levels of TGF-β after stimulation with donor PBL. We performed ELISA to evaluate the cytokine secretion of tolerant and naïve PBL after stimulation with either donor (blue bars) or third-party (red bars) PBL. The OD at 450 nm for tolerant animals is shown on the left and that for naïve controls on the right. The levels of IFN-γ, TGF-β1, and TGF-β2 were elevated by stimulation with donor PBL, while IL-10 secretion was inhibited. B, MLR with cytokine-neutralizing Ab: the standard MLR is shown in purple, the response with control Ig shown in blue, the response with anti-human TGF-β1 shown in red, and the response with anti-human TGF-β2-neutralizing Ab shown in green. There was a substantial increase in the antidonor MLR of tolerant animals after the addition of TGF-β2-neutralizing Ab (B1; p = 0.0005). Although the tolerant anti-third party had a nonsignificant increase in the proliferation (B2), the same trend was seen for naïve PMBCs after both donor (C1) and third-party (C2) stimulation. Data represent results from two separate assays.
As controls, three long-term tolerant animals received SLA\textsuperscript{al/d} (one-haplotype third-party, one-haplotype recipient-matched) kidneys without immunosuppression. The ureter of the second SLA\textsuperscript{a/c} kidney was ligated and the graft left in so that we could determine whether tolerance to the SLA\textsuperscript{a/c} graft was broken if the SLA\textsuperscript{a/c} or SLA\textsuperscript{a/d} graft was rejected. The median graft survival of SLA\textsuperscript{a/c} kidneys was 52 days, which was significantly longer than the controls (median = 7 days, p = 0.0246; Fig. 6B). Two of three experimental animals maintained their grafts for >52 days postoperatively, while the third rejected the graft on POD 28 after maintaining Cre below 3 for the first postoperative week (Fig. 6C). Biopsy results obtained on the day of sacrifice demonstrated a diffuse mononuclear cell infiltrate that was consistent with rejection. The degree of rejection was scored as less severe than that seen in the recipient that rejected its SLA\textsuperscript{a/d} allograft on POD 6.

**FIGURE 6.** Linked suppression prolonged SLA\textsuperscript{a/c} kidney graft survival. A, Linked suppression in vitro: coculture CML demonstrated linked suppression in vitro. We primed PBL from naive and long-term tolerant animals with donor SLA\textsuperscript{c/a} PBL and then cocultured them with naive recipient-matched SLA\textsuperscript{d/a} cells with SLA\textsuperscript{a/c} stimulators to determine whether the Tregs could show linked suppression in vitro. The naive PBL did not mediate suppression (black bar), while PBL from the tolerant animal completely eliminated the anti-SLA\textsuperscript{a/c} response (red bar; p = 0.04). Data from two animals after the second SLA\textsuperscript{a/c} kidney, one received SLA\textsuperscript{a/c} and the other the SLA\textsuperscript{a/d} kidneys. B, Survival: Kaplan-Meier survival analysis demonstrated that the graft prolongation of the SLA\textsuperscript{a/c} grafts was statistically significant with p = 0.0246. C, Linked suppression: recipients of SLA\textsuperscript{a/c} kidneys maintained their grafts for 28, 54, and 80 days until termination of this experiment. D, Control animals: recipients of SLA\textsuperscript{a/d} kidneys all had a sharp rise in creatinine at POD 5 and went on to reject their grafts completely by POD 8. E, In vitro responses before and after SLA\textsuperscript{a/c} transplantation: Recipients of SLA\textsuperscript{a/c} kidneys were hyporesponsive to SLA\textsuperscript{a/c} stimulators (red bar, left side) at the time of the SLA\textsuperscript{a/c} transplant, but had a good response to SLA\textsuperscript{a/d} stimulators (dark blue bar, left side). However, following SLA\textsuperscript{a/c} transplant, two animals developed hyperresponsiveness to SLA\textsuperscript{a/d} stimulators (dark blue bar, right side; p = 0.007), suggesting spreading of tolerance to the SLA\textsuperscript{a}Ag (p = 0.007). Data are from CML assays in two different animals. F, In vitro response after rejection of SLA\textsuperscript{a/d} kidney: rejectors of SLA\textsuperscript{a/d} kidneys had a sensitized response to SLA\textsuperscript{a/d} PBL (red bar), while maintaining hyperresponsiveness to SLA\textsuperscript{a/c} PBL (blue bar) at the time of SLA\textsuperscript{a/d} kidney rejection, indicating that T cell tolerance to SLA\textsuperscript{a} persisted. Naive anti-SLA\textsuperscript{a/d} response is shown in green. Data represent CML assays from two different animals.
SLA<sup>ac/c</sup> kidneys (Fig. 7A). In recipients of SLA<sup>a/d</sup> kidneys, FACS analysis confirmed the development of anti-SLA<sup>a</sup> IgM in the serum, but no IgM or IgG developed to the SLA<sup>ac</sup> Ag (Fig. 7C). Additionally, the SLA<sup>ac/c</sup> grafts in the control animals did not have cellular infiltration or Ab deposition at the time of SLA<sup>a/d</sup> graft rejection, indicating that tolerance to the SLA<sup>ac</sup> Ag was not broken (Fig. 7A).

MLR and CML assays performed the day of SLA<sup>a/c</sup> retransplantation demonstrated donor-specific unresponsiveness to SLA<sup>ac/c</sup> Ag, with intact responses to SLA<sup>a/c</sup> and SLA<sup>a/a</sup> Ag. However, by POD 30 after SLA<sup>a/c</sup> kidney transplantation, both MLR and CML revealed the continued unresponsiveness to SLA<sup>ac/c</sup>, but also the loss of anti-SLA<sup>a/c</sup> and SLA<sup>a/a</sup> response, with maintenance of a robust response to outbreds third-party PMBCs, suggesting spreading of tolerance to the SLA<sup>a</sup> Ag in vitro (Fig. 6E, MLR not shown). Control animals displayed an increased response to SLA<sup>a/d</sup> PMBCs after rejection of their SLA<sup>a/d</sup> kidneys, consistent with a sensitized response (Fig. 6E).

**FIGURE 7.** Histology and Ab production. A, SLA<sup>a/d</sup> kidneys were rejected by day 8. Histology of SLA<sup>a/d</sup> kidneys showed severe hemorrhage and mononuclear cell and neutrophil infiltration, while SLA<sup>a/c</sup> kidneys only showed mild fibrosis resulting from the ligation of the ureter. B, Immunohistochemical analysis of Ab deposition. SLA<sup>a/d</sup> kidneys showed dense deposition of IgM (left panels) and IgG (right panels) on the glomeruli and peritubular capillaries. C, FACS analysis of Ab in the sera of animals that rejected SLA<sup>a/d</sup> kidneys. Ab FACS showed that IgM and IgG Ab developed against SLA<sup>a/a</sup> cells, but not to the SLA<sup>a/c</sup> cells, demonstrating that although the third-party graft was rejected, tolerance to SLA<sup>a</sup> was not broken. Day of SLA<sup>a/d</sup> transplant shown in black histogram and day of SLA<sup>a/d</sup> rejection shown in red line. Histology of SLA<sup>a/c</sup> kidneys at POD 21. D and E, POD 54, H&E; original magnification, ×100). SLA<sup>a/c</sup> kidneys showed a moderate cellular infiltrate at POD 21, but the infiltrate had almost completely resolved by POD 54.

Requirement for restimulation of Tregs to SLA<sup>a/c</sup> kidneys for in vivo-linked suppression. Since six of six animals became tolerant to the first SLA<sup>a/c</sup> kidney with a short course of immunosuppression and then accepted a second donor-matched kidney without additional immunosuppression, demonstrating that all animals had true tolerance, we also asked whether in vivo-linked suppression would occur without restimulating the Tregs with the donor-matched kidney. Two animals received SLA<sup>a/c</sup> kidneys 120 days after the primary kidney transplant without immunosuppression. The surgical procedure was identical to the animals that displayed linked suppression. These two animals both rejected their grafts via cellular and humoral mechanisms (average graft survival = 6.5 days), despite showing in vitro unresponsiveness to donor and hyperresponsiveness to SLA<sup>a/c</sup> in MLR and CML assays (data not shown). Two additional control animals received SLA<sup>a/d</sup> kidneys 120 days after the primary SLA<sup>a/c</sup> transplant. There was no significant difference in the time of rejection of SLA<sup>a/d</sup> and SLA<sup>a/c</sup> kidneys in these animals (n = 2, average graft survival = 7 days).
This result suggested that restimulation of the Tregs was required to enhance regulatory mechanisms to a level that would then be able to suppress responses to third-party Ags coexpressed with the tolerant Ag and show to linked suppression.

Discussion

We have previously demonstrated the induction of tolerance to fully MHC-mismatched renal allografts in a miniature swine model using only a short course of FK506 (15). This present study sought to investigate the possibility that donor-specific tolerance in this model was secondary to the existence of Treg populations. In this study, we report that 1) Tregs from tolerant animals suppress naive antigen responses in vitro, 2) TGF-β is involved in the mechanism of in vitro suppression, 3) suppression of rejection of kidneys bearing third-party Ags coexpressed with Ags to which the recipient is tolerant is associated with Tregs, and 4), “boosting” of such Treg function by challenge with a second donor-matched graft appears to be required to detect linked suppression in vivo.

Our initial studies of Tregs in miniature swine demonstrated that priming is required in vitro to see suppression in coculture assays (22). In this study, we showed that priming via a donor-matched transplant was also required for linked suppression to be seen in vivo. This suggests that to observe clinically significant Treg activity in vivo, repeated stimulation of Tregs may be required. Once these Tregs were primed in vivo, they suppressed third-party allogeneic responses only if donor MHC existed on the same cell, while conferring no survival advantage to control grafts displaying self-MHC class I and class II with third-party class I and class II. This suggests that Tregs require stimulation by the “tolerated” MHC to exert their effects, as has been suggested in rodent studies (23), and Treg function in this model is not dependent on presentation of self-class II by the graft, as has also been reported (24, 25).

Previous studies investigating the mechanism of tolerance in this model showed that suppression could be seen in Transwell assays designed to detect a soluble mediator of regulatory function, but that inhibition in Transwell assays was not as robust as when cell-cell contact was allowed (15). Because few commercially available reagents cross-react with pig cytokines, we were limited to examining the involvement of IL-10, IFN-γ, TGF-β1, and TGF-β2 in the mechanism of the in vitro suppression. We showed that TGF-β2 is secreted by cells from tolerant animals and that anti-TGF-β2-neutralizing Ab decreased the inhibition by Tregs. This correlates with a report in monkeys that showed infiltration of tolerant kidneys with T cells positive for TGF-β (26). We also showed that CD4+CD25+, as well as CD4+CD25− cells from tolerant animals, suppress naive antigen responses. CD4+CD25+ cells have been characterized as natural Tregs and are thought to act via cell-cell contact (27). Several reports have described the induction of adaptive regulatory cells by natural Tregs (25, 28, 29). These adaptive Tregs, IL-10-secreting Tr1 (30), or TGF-β secreting Th3 (31) do not require cell-cell contact to exert their regulatory effects. In our model, we found evidence for suppression by both natural CD4+CD25+ Tregs and adaptive CD4+CD25+ Tregs that may act by secretion of TGF-β. Graca et al. (32) have reported that both CD4+CD25+ and CD4+CD25− cells were able to prolong skin graft survival in mice. When they compared the efficacy of the CD4+CD25+ cells to CD4+CD25− cells isolated from tolerant mice, they found that 105 CD4+CD25− cells had similar suppressive activity to 106 CD4+CD25− cells. Our in vitro studies (Fig. 4, D and E) found a similar relationship in the suppressive activity of these cells. Also consistent with our data, they reported that CD4+CD25− cells from both tolerant and naive animals possessed suppressive activity, whereas CD4+CD25− cells were only suppressive when isolated from tolerant animals. Evidence for the function of CD4+CD25low adaptive Tregs has recently been reported in humans (33), suggesting that the mechanism of tolerance in these models may be similar to that observed in humans.

Linked suppression may have clinical applications. If a protocol can be developed that can induce tolerance in patients to prevalent MHC Ags, possibly through the use of immature dendritic cells (9–11) or peptides (12), it may be possible to induce tolerance to MHC Ags prevalent in a recipient population before cadaveric transplantation. Since in cadaveric transplants, the donor is not known until just before transplant, the induction of tolerance to a panel of MHC Ags would allow for a high probability of a single MHC Ag match to a donor. The Tregs would then decrease allo-reactive responses to the other MHC on the graft, decreasing the required immunosuppression. It is also possible that these Tregs could induce spreading of tolerance, as was seen in CML and MLR assays in our animals, and that immunosuppression could eventually be weaned completely. The demonstration that linked suppression occurs in large animals thus supports further validation of this approach in the clinic.

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References


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