Peripheral Tolerance to Class I Mismatched Renal Allografts in Miniature Swine: Donor Antigen-Activated Peripheral Blood Lymphocytes from Tolerant Swine Inhibit Antidonor CTL Reactivity

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Peripheral Tolerance to Class I Mismatched Renal Allografts in Miniature Swine: Donor Antigen-Activated Peripheral Blood Lymphocytes from Tolerant Swine Inhibit Antidonor CTL Reactivity

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Studies utilizing partially inbred miniature swine have demonstrated that a short course of cyclosporin A leads to indefinite survival of two haplotype class I mismatched renal allografts. In the present study, we have examined peripheral regulatory mechanisms that may be involved in maintenance of tolerance by coculturing PBL from long-term tolerant animals with naive recipient-matched PBL in cell-mediated lympholysis assays. We show that PBL from tolerant animals, primed in vitro with donor Ag, suppress antidonor CTL reactivity by naive recipient-matched PBL. Suppression was not observed when PBL from naive animals, primed with donor-matched PBL, were cocultured with PBL from a second naive animal, nor did PBL from either tolerant or naive recipient-matched control animals, primed with third-party Ag, suppress the generation of anti-third-party CTL by a second naive animal. The suppression was cell dose-dependent, radiation-sensitive, required cell-to-cell contact not reversed by the provision of exogenous IL-2, and associated with lower levels of IL-2R expression on the suppressive effector group (particularly the CD8 single positive cells) when compared with the control effector group. These data indicate an association between the presence of peripheral regulatory cells demonstrable in vitro and the maintenance of tolerance to renal allografts. The Journal of Immunology, 1999, 162: 550–559.
Materials and Methods

Animals

The surgical procedures, clinical course, and immunogenetic characteristics of the miniature swine used in this study have been described in detail elsewhere (Refs. 20, 23, and 26; Yamada et al., manuscript in preparation). Animals received two haplotype class I mismatched renal allografts (SLA<sup>dd</sup> (class I<sup>d</sup>, class II<sup>d</sup>) donor→SLA<sup>gg</sup> (class I<sup>g</sup>, class II<sup>g</sup>) recipient) and were treated with a 12-day course of CyA. In addition, the animals were either nonthymectomized (nos. 11468, 12019, and 11609) or thymectomized (nos. 11446, 11384, and 11207). The swine were thymectomized on day −42 (nos. 11446 and 11384) or day 0 (nos. 11207), in relation to the day of renal transplantation. Both thymectomized and nonthymectomized swine (hereafter referred to as thymectomized swine and non-thymectomized swine, respectively) accepted their SLA<sup>gg</sup> renal allografts long-term (>90 days). Two naive SLA<sup>dd</sup> swine (nos. 10785 and 10466) were used as control animals.

Medium

Tissue culture medium used for cell-mediated lympholysis (CML) assays consisted of RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 5% FCS (Sigma, St. Louis, MO), 100 U/ml of penicillin and 135 μg/ml of streptomycin (Life Technologies), 50 μg/ml of gentamicin (Life Technologies), 10 mM of HEPES (Fisher Scientific, Pittsburgh, PA), 2 mM of l-glutamine (Life Technologies), 1 mM of sodium pyruvate (BioWhittaker, Walkersville, MD), 0.1 mM of nonessential amino acids (BioWhittaker), and 5 × 10<sup>−5</sup> M of 2-ME (Sigma). The effector phase of the CML assay was performed using Eagle’s basal medium (Life Technologies) supplemented with 6% controlled processed serum replacement-3 (Sigma) and 10 mM of HEPES (Fisher Scientific).

Isolation of PBL

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, Durham, NC). The mononuclear cells were washed once with HBSS, and contaminating red cells were lysed with ammonium chloride potassium buffer (B&K Research Laboratory, Fiskeville, RI). Cells were then washed with HBSS resuspended in tissue culture medium and kept at 4°C until used in cellular assays or flow cytometry studies.

CML assays

Four types of CML assay systems were used in these studies.

Primary CML assays. Lymphocyte cultures containing 2 × 10<sup>6</sup> responder cells (PBL from naive SLA<sup>bb</sup>, nonthymectomized or thymectomized animals) and 4 × 10<sup>6</sup> stimulator PBL (SLA<sup>gg</sup> or third-party PBL, irradiated with 25 Gy) per well in a final volume of 2 ml of medium were incubated for 6 days at 37°C in 5% CO<sub>2</sub> using 24-well flat-bottom plates (Costar, Cambridge, MA). Bulk cultures were harvested, and effectors were tested for cytotoxic activity on target lymphocytes stimulated by phytohemagglutinin (M-Form, Life Technologies) previously titrated to give optimal proliferation. Target cells were labeled with <sup>51</sup>Cr (Amersham, Arlington Heights, IL) and incubated with the effector groups for 5.5 h using a negative control target (SLA-matched to the responders, i.e., SLA<sup>dd</sup>) and targets SLA-matched to the stimulators, which included donor-matched PBL (SLA<sup>gg</sup>; class I<sup>g</sup> and class II<sup>g</sup>) and third-party stimulators (SLA<sup>bb</sup>; class I<sup>b</sup> and class II<sup>b</sup>), E:T ratios of 100:1, 50:1, 25:1, and 12.5:1 were tested. Supernatants were harvested using the Skatron collection system (Skatron, Sterling, VA), and <sup>51</sup>Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as percent specific lysis (PSL) and calculated as:

\[
\text{PSL} = \frac{\text{experimental release (cpm) - spontaneous release (cpm)}}{\text{maximum release (cpm) - spontaneous release (cpm)}} \times 100\%.
\]

Secondary coculture assays. Control MLC were prepared using 24-well flat-bottom plates (Costar), each well containing 2 × 10<sup>6</sup> naive SLA<sup>dd</sup> responder PBL (naive1) and 4 × 10<sup>6</sup> stimulator PBL (irradiated with 25 Gy) in a final volume of 2 ml of medium. The same source of naive SLA<sup>dd</sup>-respondor PBL were used as indicator cells to detect suppression by PBL from tolerant animals in coculture assays. Similar MLC were prepared using PBL from tolerant SLA<sup>dd</sup> animals (nonthymectomized or thymectomized). Stimulator cells were donor-matched PBL (SLA<sup>gg</sup>) or third-party PBL (SLA<sup>bb</sup> or SLA<sup>aa</sup>), Cocultures contained 2 × 10<sup>6</sup> naive SLA<sup>dd</sup>-responder PBL, 2 × 10<sup>6</sup> PBL from tolerant animals, and 4 × 10<sup>6</sup> irradiated stimulator PBL per well in a final volume of 2 ml of medium. Additional cultures containing 4 × 10<sup>6</sup> responder PBL and 4 × 10<sup>6</sup> irradiated stimulator PBL were performed for naive and tolerant responder PBL to control for crowding effects due to increased numbers in the cocultures. Cultures were incubated for 6 days at 37°C in 7.5% CO<sub>2</sub> and harvested, and effectors were tested for cytotoxic activity as described above.

FIGURE 1. Schematic diagram of phase 1 and phase 2 of the secondary coculture assays for PBL from tolerant (nonthymectomized and thymectomized) animals (A) and naive (control) animals (B).
tolerant to SLA<sup>GG</sup>-renal allografts (non-thy1/tolerant-to-G<sup>G</sup>) or from thymecto-
mized swine tolerant to SLA<sup>GG</sup> (thy1/tolerant-to-G<sup>G</sup>) primed with SLA<sup>GG</sup> PBL (non-thy1/tolerant-to-G<sup>G</sup>-G<sup>G</sup> and thy1/tolerant-to-G<sup>G</sup>-G<sup>G</sup>), respectively) or with SLA<sup>hh</sup>
PBL (non-thy1/tolerant-to-G<sup>H</sup>-H<sup>H</sup> and thy1/tolerant-to-G<sup>H</sup>-H<sup>H</sup>), respectively (Fig. 1A, left), and 2) PBL from a naive swine (naïve2) primed with SLA<sup>GG</sup> PBL (naïve2-G<sup>G</sup>) or with SLA<sup>GG</sup> PBL (naïve2-H<sup>H</sup>) (Fig. 1B, left). These primed effectors were used in phase 2, as indicator cells.

Phase 2 (coculture phase, day 7–13) was as follows. On day 7, fresh control MLC were prepared in 24-well plates (Costar) containing 2 × 10<sup>5</sup> naïve PBL and 4 × 10<sup>5</sup> irradiated stimulator PBL (donor-matched, SLA<sup>GG</sup>); or third-party, SLA<sup>GG</sup> per well in a final volume of 2 ml of medium (Fig. 1A, right, lines 1 and 4; Fig. 1B, right, lines 1 and 4). The naïve1 PBL were from a different source than the naïve SLA<sup>GG</sup>-PBL used in the priming phase (naive2). The naïve PBL were used as indicator cells for the detection of suppression by the primed cells in coculture assays described below. The primed effectors from phase 1 were also harvested on day 7 and washed once in complete medium before being set up in the second phase. Control secondary MLC were prepared using both primed effector groups prepared in the first phase stimulated in a secondary MLC with the same irradiated stimulator cells used in the priming phase (Fig. 1A, right, lines 2 and 5; Fig. 1B, right, lines 2 and 5). Cocultures contained 2 × 10<sup>5</sup> naïve responder SLA<sup>GG</sup>-PBL, 2 × 10<sup>5</sup> primed effector cells from phase 1, and 4 × 10<sup>5</sup> irradiated stimulator PBL per well in a final volume of 2 ml of medium (Fig. 1A, right, lines 3 and 6; Fig. 1B, right, lines 3 and 6). Control cultures for crowding effects were set up containing 10<sup>6</sup> naïve1 PBL per well and 4 × 10<sup>5</sup> irradiated stimulator PBL, or 4 × 10<sup>5</sup> primed effectors from phase 1 and 4 × 10<sup>5</sup> irradiated stimulator PBL (not shown). Cultures in the second phase were incubated for 6 days at 37°C in 7.5% CO2 (day 7–13) and tested for cy-
totoxicity as described above. Target cells included PBL matched to the effectors (negative control) and PBL SLA-matched to the stimulators used in the first and second phase of the assay.

Results

Naïve animals (positive control) showed strong anti-donor-
matched (SLA<sup>GG</sup>) and anti-third-party CTL reactivity in primary CML assays (Fig. 2A). Both nonthymectomized and thymecto-
mized tolerant animals demonstrated markedly lower antidonor CTL reactivity but maintained normal third-party responses (Fig. 2A). Unprimed PBL from tolerant swine were cocultured in a 1:1 ratio with naïve SLA<sup>GG</sup>-PBL to determine whether the hyporespon-
sive PBL from tolerant swine contained peripheral regulatory cells that could suppress the generation of CTL among naïve T cells. Antidonor and anti-third-party CTL responses of naïve animals were not suppressed in primary coculture assays with PBL from tolerant animals (compare Fig. 2B with the naïve control responses in Fig. 2A, left).

PBL from tolerant animals primed with donor Ag inhibited the generation of antidonor CTL reactivity by naïve PBL

We hypothesized that regulatory cells from tolerant animals may require activation with donor Ag before any suppressive properties could be observed. Therefore, PBL from tolerant swine, or naïve swine as controls, were prestimulated in culture for 6 days with donor Ag (SLA<sup>GG</sup>-PBL). After the priming phase, the effector groups were then rested overnight, harvested, washed, and set up in coculture assays as outlined in Fig. 1A (lines 1 to 3) and Fig. 1B (lines 1 to 3). Naïve1 was a second naïve animal that would be used in the cocultures as indicator cells whose response might be suppressed by putative regulatory cells from the tolerant animals. Naïve1 anti-SLA<sup>GG</sup> responses (Fig. 1A, line 1 and Fig. 1B, line 1) were the positive controls. The primed effector groups from the first phase were stimulated by SLA<sup>GG</sup> to determine the cytotoxicity of these effector groups alone (Fig. 1A, line 2 and Fig. 1B, line 2). Cocultures of the primed effectors with PBL from naïve1 were performed to determine whether the anti-SLA<sup>GG</sup> responses by naïve1 could be suppressed by the primed effectors (Fig. 1A, line 3 and Fig. 1B, line 3).

The pooled results for the anti-SLA<sup>GG</sup> responses in these assays are shown in Fig. 3. As expected, the naïve1 PBL generated strong anti-SLA<sup>GG</sup> CTL reactivity when cultured alone (Fig. 3A). These were similar to the cytotoxic responses of the naïve PBL controls in Fig. 2. When the primed effectors from phase 1 were stimulated a second time in phase 2 with SLA<sup>GG</sup>-PBL, the non-thy1/tolerant-to-G<sup>G</sup>-G<sup>G</sup> and thy1/tolerant-to-G<sup>G</sup>-G<sup>G</sup> effector groups produced minimal antidonor CTL reactivity, while the naïve2-G<sup>G</sup> effectors generated strong secondary anti-SLA<sup>GG</sup> cytotoxic responses (Fig. 34). mAb 36-7-5 (35) FITC-conjugated (anti-murine H2 K<sup>K</sup>, mouse IgG2a) and anti-Leu3a PE (Becton Dickinson, San Jose, CA) were used as nega-
tive control Abs. For three-color flow cytometry analysis, the staining pro-
cedure was performed as follows: 1) 10<sup>6</sup> cells per tube were incubated with purified swine IgG for 10 min to block nonspecific binding of conjugated Abs; 2) the first incubation was directly FITC-labeled Ab; 3) after a single wash, the second Ab, which was directly PE-labeled, was added; 4) after a further wash, the final biotinylated Ab was added; 5) the biotinylated Ab was developed using cy-chrome-streptavidin (PharMingen, San Diego, CA), which was incubated for 8 min with washes before and after the addition of the cy-chrome-streptavidin. mAb incubations were performed on ice for 30 min. The data acquisition was performed on a FACScan (Becton Dickinson) and analyzed using the Winlist program (Verity Software, Topsham, ME). Four PBL subpopulations were defined by staining for CD4 and CD8 using separate colors: CD4 single positive (SP), CD4/8 double positive (DP), CD4/8 double negative, and CD8 SP PBL. Three-
color flow cytometry analysis was performed by simultaneous staining of CD2, CD3, class I, class II, and CD25 with a third color.
from tolerant and naive animals were primed for 6 days in culture. Responses of naive cells was specific for the tolerant animals. (Fig. 3 compared with the cytotoxicity of each cocultured group alone (Fig. 1A, line 5 and Fig. 1B, line 5). Cocultures of the primed effectors with naive1 were performed to determine whether the anti-SLAbh responses by naive1 were suppressed by the primed effectors (Fig. 1A, line 6 and Fig. 1B, line 6). The results of the anti-SLAbh stimulated responses are shown in Fig. 4. There was no inhibition of anti-SLAbh CTL responses when naive1 was cocultured with non-thyx tolerant-to-G-H' or thyx tolerant-to-G-H' or naive2-H' primed effectors (Fig. 4, A and B, vs C).

Cultures to determine whether primed non-thyx tolerant-to-G-G' or thyx tolerant-to-G-G' effectors could suppress naive1 anti-third-party reactivity (e.g., (thyx tolerant-to-G-G' + naive1) anti-third-party response) were also performed. Controls for these cultures included determining whether naive2-G' effectors would suppress a second naive1 anti-third-party response (i.e., (naive1 + naive2-G') anti-third-party response). These studies showed that non-thyx tolerant-to-G-G' or thyx tolerant-to-G-G' primed effectors were able to suppress naive1 anti-third-party reactivity partially and to variable degrees (data not shown). However, unlike the specific inhibition observed for antidonor reactivity above, this inhibition was also seen for naive2-G' primed effectors. Similarly, non-thyx tolerant-to-G-H', thyx tolerant-to-G-H', and naive2-H' primed effectors were able to variably suppress naive1 anti-SLAbh responses. These assays indicated that this suppression was nonspecific, and most importantly, was unrelated to the tolerant state induced in vivo. Previous reports of mice have demonstrated such nonspecific suppression of generation of CTL responses by alloactivated lymphocytes from naive mice (36). Since this kind of nonspecific suppression could be distinguished from the specific suppression in our assays, it was not further characterized.

In contrast, the suppression observed with PBL from tolerant animals primed with donor cells was specific for tolerant animals, and also specific for donor SLA Ag, provided the same stimulator was used in the priming phase and coculture phase of the assay (Figs. 3 and 4). Therefore, subsequent experiments were focused on determining the mechanism of the regulatory response in these assays. For this purpose, the tolerant recipient that demonstrated the greatest degree of suppression (thymectomized animal 11446) was chosen for further study.

Suppression of naive PBL in coculture assays was cell-dose dependent

All coculture assays described above were performed with a naive SLAdid-thyx tolerant-to-G-G' PBL ratio of 1:1. In this analysis, the concentration of naive SLAdid PBL (2 × 10^6 cells/ml) was kept constant, and cocultured with primed thyx tolerant-to-G-G' PBL added at decremental concentrations (2 × 10^5 cells/ml to 0.001 × 10^5 cells/ml). Coculture of naive SLAdid PBL with primed thyx tolerant-to-G-G' effector PBL led to a titratable inhibition of the naive anti-SLAdid cytotoxic response (Fig. 5). Significant inhibition (>50% inhibition of the naive SLAdid antidonor cell response alone) was still observed at a thyx tolerant-to-G-G'-naive SLAdid cell ratio of 1:4. At lower concentrations of the regulator cells, the inhibitory effect was not detectable.

Suppression of CML reactivity is mediated by nonadherent, radiation-sensitive cells

Since previous studies suggested that miniature swine contain an adherent cell population with suppressive properties (37, 38), naive SLAdid PBL (2 × 10^6 cells/ml) were cocultured with unseparated thyx tolerant-to-G-G' PBL (2 × 10^6 cells/ml) or an adherent cell-depleted thyx tolerant-to-G-G' effector group (2 × 10^6 cells/ml) (Fig. 6). The nonadherent cell population completely inhibited the naive anti-SLAdid response. Ten percent of the primed...
thyx tolerant-to-G effector group were adherent cells, and when this number of adherent cells (0.2 x 10^6 cells/ml) was cocultured with naive SLA\(^{dd}\) responders, the naive anti-SLA\(^{gg}\) response alone (PIL, 38.5%; E:T ratio, 100:1) was inhibited to a PIL of 25% (E:T ratio, 100:1). Therefore the suppressive effect was predominantly in the nonadherent cell population.

If the suppressive response required DNA synthesis during the cell cycle, one might predict that the regulatory cells would be inactivated by radiation. Therefore, the suppressive thyxtolerant-to-G cells were either untreated or irradiated with 25 Gy. The results showed that the nonirradiated thyxtolerant-to-G, cocultured with naive SLA\(^{dd}\) PBL, inhibited the naive anti-SLA\(^{gg}\) response as expected. However, the suppression was completely eliminated when the thyxtolerant-to-G cells were irradiated before the coculture. In contrast, the anti-SLA\(^{hh}\) cytotoxic response of the naive PBL alone and the thyxtolerant-to-G primed effector group alone were augmented when the naive PBL were cocultured with thyxtolerant-to-G-H effector groups. Irradiated before the coculture (naive + thyxtolerant-to-G-H 25 Gy), the cytotoxicity was similar to the cytotoxicity of the naive PBL alone.

Primed tolerant PBL require cell-to-cell contact to suppress antidonor CTL reactivity by naive SLA\(^{dd}\) PBL

Separation of the naive SLA\(^{dd}\) PBL and the primed thyxtolerant-to-G cells by a semipermeable membrane that allowed soluble factors, but not cells, to pass through (6-well transwell chambers in culture plates) (Costar) was performed to distinguish the hypothesis that thyxtolerant-to-G cells required cell-to-cell contact with the naive PBL to inhibit the generation of CTL as opposed to the possibility that soluble factors were involved in the mechanism of suppression. Thymectomized PBL were prestimulated for 6 days in phase 1 (thyxtolerant-to-G), and subsequently, the following cultures were established in phase 2, as described in Materials and Methods: 1) naive PBL and SLA\(^{gg}\) stimulators in the bottom chamber of the transwell and medium only in the top chamber, 2) thyxtolerant-to-G-G and SLA\(^{gg}\) stimulators in the top chamber of the transwell and medium only in the bottom chamber, 3) naive PBL cocultured with thyxtolerant-to-G-G and SLA\(^{gg}\) stimulators in the same well, and 4) naive PBL with SLA\(^{gg}\) stimulators cultured in the bottom chamber of the transwell.

FIGURE 3. Anti-SLA\(^{gg}\) cytotoxicity (PIL at E:T ratio of 100:1) in phase 2 of CTL assays. Nonthymectomized, thymectomized, and naive2 PBL were prestimulated for 6 days with SLA\(^{gg}\) PBL in phase 1 (non-thyx tolerant-to-G-G, thyxtolerant-to-G-G, and naive-2G, respectively). In the second phase of the assay, anti-SLA\(^{gg}\) CTL responses were determined for a second naive animal, naive1 (n = 14(A); the primed effector groups from phase 1: non-thyx tolerant-to-G-G (n = 2), thyxtolerant-to-G-G (n = 9), and naive2G (n = 3(B); and cocultures of naive1 PBL with the primed effector groups: naive1 + non-thyx tolerant-to-G-G (n = 2), naive1 + thyxtolerant-to-G-G (n = 9), naive1 + naive2-G (n = 3(C)). Results represent the mean ± SD.

FIGURE 4. Control anti-SLA\(^{hh}\) cytotoxic responses (PIL at E:T ratio of 100:1) in phase 2 of CTL assays. Nonthymectomized, thymectomized, and naive2 PBL are prestimulated for 6 days with SLA\(^{hh}\) PBL in the phase 1 (non-thyx tolerant-to-G-H, thyxtolerant-to-G-H, and naive-2H, respectively). In the second phase of the assay, anti-SLA\(^{hh}\) CTL responses are determined for a second naive animal, naive1 (n = 10(A); the primed effector groups from phase 1: non- thyxt tolerant-to-G-H (n = 2), thyxt tolerant-to-G-H (n = 5), and naive-2H (n = 3(B); and cocultures of naive1 PBL with the primed effector groups: naive1 + non-thyx tolerant-to-G-H (n = 2), naive1 + thyxt tolerant-to-G-H (n = 5), naive1 + naive2-H (n = 3(C)). SLA\(^{gg}\) stimulators were used in four assays for the thymectomized group and showed identical results (data not shown). Results represent the mean ± SD.
The nonadherent cell population contained most of the suppressive properties of the primed thyx tolerant-to-G’ effector group. Naive SLA<sup>dd</sup> PBL (2 × 10<sup>6</sup> cells/ml) were cultured alone, cocultured with unseparated thyx tolerant-to-G’ PBL (2 × 10<sup>6</sup> cells/ml), or cocultured with an adherent cell-depleted thyx tolerant-to-G’ effector group (2 × 10<sup>6</sup> cells/ml). Complete inhibition of the naive anti-SLA<sup>gg</sup> CTL response was observed with the nonadherent cell population.

anti-SLA<sup>gg</sup> CTL response of the naive PBL to the level of the positive control (i.e., CTL response of the naive PBL cultured alone). These results indicated that cell-to-cell contact was required for the suppression of the naive PBL and that soluble factors were not able to mediate suppression between the transwell chambers. Control assays that examined anti-SLA<sup>hh</sup> responses of naive PBL alone, thyx tolerant-to-G’ alone, naive PBL cocultured with nonirradiated thyx tolerant-to-G’ effectors, and naive PBL cocultured with irradiated (25 Gy) thyx tolerant-to-G’ effectors. B, Anti-SLA<sup>hh</sup> CTL activity of naive effectors alone, thyx tolerant-to-H’ effectors alone, naive PBL cocultured with nonirradiated thyx tolerant-to-H’, and naive PBL cocultured with irradiated (25 Gy) thyx tolerant-to-G’, H’ effectors.

Expression of IL-2R on the inhibitory effector group and effect of exogenous IL-2 on the suppressive response

One model for T cell-mediated suppression suggests that Ag-specific T cells that have been rendered anergic suppress other T cells with the same specificity by competing for IL-2 and for the Ag-presenting cell (8). If competition or absorption of IL-2 was a major mechanism of suppression, the α-chain of the IL-2R might be expected to be expressed at increased levels on the thyx tolerant-to-G’ effectors stimulated by SLA<sup>gg</sup> at the end of phase 2, when compared with the control thyx tolerant-to-H’ effectors stimulated by SLA<sup>hh</sup>. Flow cytometry demonstrated that thyx tolerant-to-G’ anti-SLA<sup>gg</sup> effectors, which suppressed the generation of CTL by naive PBL, expressed lower levels of IL-2R than thyx tolerant-to-H’ effectors (Fig. 9A) when compared with thyx tolerant-to-H’ anti-SLA<sup>hh</sup> effectors, which did not demonstrate a suppressed response (Fig. 9B). The greatest difference in expression of IL-2R on the thyx tolerant-to-G’ anti-SLA<sup>gg</sup> effectors and the thyx tolerant-to-G’ anti-SLA<sup>hh</sup> effectors was observed in the CD8 SP cell population (Fig. 9, C vs D).

To further assess competition for IL-2 as a mechanism of suppression, exogenous IL-2 was added in titrating concentrations (0–50 U/ml) to anti-SLA<sup>gg</sup> responses of naive PBL alone, thyx tolerant-to-G’ alone, and cocultures of naive PBL with thyx tolerant-to-G’ with SLA<sup>gg</sup> stimulators cultured in the top chamber of the transwell. These were all cultured for 6 days, harvested, and tested for cytotoxicity. The results of one representative experiment are presented in Fig. 8. As expected, the naive PBL alone stimulated by SLA<sup>gg</sup> PBL generated strong anti-SLA<sup>gg</sup> cytotoxic responses, while thyx tolerant-to-G’ alone stimulated by SLA<sup>gg</sup> PBL did not generate anti-SLA<sup>gg</sup> cytotoxic responses. Cocultures of naive PBL with thyx tolerant-to-G’ and SLA<sup>gg</sup> stimulators in the same well led to complete suppression of the naive anti-SLA<sup>gg</sup> response as described above. However, separation of the naive and thyx tolerant-to-G’ groups by a transwell restored the suppression of the naive PBL and that soluble factors were not able to mediate suppression between the transwell chambers. Control assays that examined anti-SLA<sup>hh</sup> responses of naive PBL alone, thyx tolerant-to-G’ alone, naive PBL cocultured with nonirradiated thyx tolerant-to-G’ effectors, and naive PBL cocultured with irradiated (25 Gy) thyx tolerant-to-G’ effectors.

Expression of IL-2R on the inhibitory effector group and effect of exogenous IL-2 on the suppressive response

One model for T cell-mediated suppression suggests that Ag-specific T cells that have been rendered anergic suppress other T cells with the same specificity by competing for IL-2 and for the Ag-presenting cell (8). If competition or absorption of IL-2 was a major mechanism of suppression, the α-chain of the IL-2R might be expected to be expressed at increased levels on the thyx tolerant-to-G’ effectors stimulated by SLA<sup>gg</sup> at the end of phase 2, when compared with the control thyx tolerant-to-H’ effectors stimulated by SLA<sup>hh</sup>. Flow cytometry demonstrated that thyx tolerant-to-G’ anti-SLA<sup>gg</sup> effectors, which suppressed the generation of CTL by naive PBL, expressed lower levels of IL-2R than thyx tolerant-to-H’ effectors (Fig. 9A) when compared with thyx tolerant-to-H’ anti-SLA<sup>hh</sup> effectors, which did not demonstrate a suppressed response (Fig. 9B). The greatest difference in expression of IL-2R on the thyx tolerant-to-G’ anti-SLA<sup>gg</sup> effectors and the thyx tolerant-to-G’ anti-SLA<sup>hh</sup> effectors was observed in the CD8 SP cell population (Fig. 9, C vs D).

To further assess competition for IL-2 as a mechanism of suppression, exogenous IL-2 was added in titrating concentrations (0–50 U/ml) to anti-SLA<sup>gg</sup> responses of naive PBL alone, thyx tolerant-to-G’ alone, and cocultures of naive PBL with thyx tolerant-to-G’ with SLA<sup>gg</sup> stimulators cultured in the top chamber of the transwell. These were all cultured for 6 days, harvested, and tested for cytotoxicity. The results of one representative experiment are presented in Fig. 8. As expected, the naive PBL alone stimulated by SLA<sup>gg</sup> PBL generated strong anti-SLA<sup>gg</sup> cytotoxic responses, while thyx tolerant-to-G’ alone stimulated by SLA<sup>gg</sup> PBL did not generate anti-SLA<sup>gg</sup> cytotoxic responses. Cocultures of naive PBL with thyx tolerant-to-G’ and SLA<sup>gg</sup> stimulators in the same well led to complete suppression of the naive anti-SLA<sup>gg</sup> response as described above. However, separation of the naive and thyx tolerant-to-G’ groups by a transwell restored the suppression of the naive PBL and that soluble factors were not able to mediate suppression between the transwell chambers. Control assays that examined anti-SLA<sup>hh</sup> responses of naive PBL alone, thyx tolerant-to-G’ alone, naive PBL cocultured with nonirradiated thyx tolerant-to-G’ effectors, and naive PBL cocultured with irradiated (25 Gy) thyx tolerant-to-G’ effectors.
thyx tolerant-to-G' effector groups in which the naive PBL responses were inhibited. The addition of IL-2 at doses $>5$ U/ml produced nonspecific cytotoxicity (lymphokine-activated killer (LAK) cell) at the lower range of concentrations of IL-2 and induced complete unresponsiveness in naive anti-SLA$^{gg}$ CTL responses at the higher concentrations of IL-2. The unresponsiveness of the naive anti-SLA$^{gg}$ CTL responses with the addition of high doses of IL-2 was consistent with previous studies demonstrating that similar concentrations of IL-2 induced apoptosis and cell death of activated CD4 and CD8 T cells (39). Therefore, the optimal concentration of IL-2 that would allow interpretation of the results was determined to be $<5$ U/ml. However, the addition of $<5$ U/ml of IL-2 was not able to overcome the suppression of naive PBL when cocultured with the primed thyx tolerant-to-G' effector group (Fig. 10).

**Discussion**

The present study was undertaken to determine the mechanism of tolerance to porcine class I mismatched renal allografts, and in particular, to test for the presence of peripheral regulatory cells. Since both thymectomized and nonthymectomized animals were shown to accept two haplotype class I mismatched renal allografts with a 12-day course of CyA (23, 26), both groups of animals were examined. While only nonthymectomized animals should be capable of utilizing central mechanisms of tolerance to maintain their allografts. The presence of such regulatory cells activated by donor Ag expressed by the allograft could confer local protection in vivo. The present study demonstrates that the regulatory cells require cell-to-cell contact to exert their suppressive effect, and this property would ensure that the immunoregulatory responses would be limited to sites where donor Ag and recipient donor-reactive T cells were present. We speculate that such cells may play an important role in the maintenance of tolerance in this preclinical large animal model of allograft transplantation and would have implications in the application of peripheral regulatory mechanisms of tolerance to clinical transplantation.

Results from previous studies indicated that local regulation or suppression may play a role in the maintenance of tolerance to class I mismatched renal allografts in miniature swine (24, 40). One study demonstrated that the administration of exogenous IL-2 ("help") during the induction of tolerance on postoperative day 8, 9, and 10, led to acute rejection of allografts, suggesting that a
limitation of help was an important mechanism leading to the induction of tolerance (24). In contrast, the provision of exogenous IL-2 did not abrogate tolerance in long-term acceptors. Mechanisms of tolerance that may be operating in long-term tolerant swine include deletion, anergy, or suppression of helper cells and/or CTL. Deletion or anergy of the antidonor helper cells in the maintenance period would lead to failure to activate antidonor CTL. However, since anti-donor precursor CTL are not deleted in long-term tolerant animals (40), the provision of exogenous IL-2 during the maintenance phase would be expected to abrogate tolerance. The findings in the present study support the alternative hypothesis that regulatory cells could inhibit the generation of CTL in vivo. Further studies are required to determine whether the regulation acts at the level of the helper cell or directly on the CTL. However, the concordance of the in vivo data demonstrating that exogenous IL-2 did not abrogate tolerance in long-term acceptors (24) and the in vitro findings that IL-2 was not able to overcome the suppression in vitro in the present study, argues against a deficiency of help as the major mechanism of tolerance in the maintenance phase of tolerance.

The presence of local regulatory cells is supported by studies of cocultures of graft-infiltrating cells from miniature swine that became spontaneously tolerant to single haplotype class I mismatched renal allografts. These cells were able to suppress antidonor CTL responses of naive T cells in vitro (41, 42). However, in contrast to the regulatory cells in the present study, the graft-infiltrating cells did not require preactivation in vitro to demonstrate their inhibitory effect in CML assays, probably because these cells were purified from a site where donor Ag was at high concentration (i.e., directly from the allograft).

Studies in miniature swine have indicated that PBL from tolerant and naive animals contain an adherent, non-T cell population that is able to nonspecifically suppress the generation of CTL (37, 38). The data in the present study demonstrate that the putative regulatory cells in primed PBL of tolerant swine were distinct from the suppressive adherent cell population described in the previous reports. Although a minimal degree of inhibition was evident in the adherent cell population, profound suppression of the naive antidonor response was observed in the nonadherent cell population (Fig. 6). A further differentiating property of the regulatory cells in the current study, compared with the adherent suppressive cells, was the complete inactivation of the inhibition by irradiating the thyG tolerant-to-G effector group with 25 Gy (Fig. 7). The previous adherent suppressive cells were shown to be resistant to irradiation with 25 Gy but were sensitive to 50 Gy (37). The suppression observed with the adherent cell population was nonspecific and observed in naive (and tolerant) animals (43, 44), in contrast to the suppression observed in the present study in which the inhibition was demonstrated only in tolerant animals by priming PBL specifically with donor Ag. Since the tolerance in this large animal model is specific for donor Ag, it is likely that the nonadherent cells are more important than the adherent cells in the maintenance of tolerance.

Lysis of APCs in the stimulator cell population of the secondary cultures could induce apparent suppression by the primed tolerant-G effector cells in phase 2 of the CTL assays. Although this possibility was not formally excluded, this would be an unlikely explanation since the suppressive primed non-thyG tolerant-to-G and thyG tolerant-to-G-G effector groups prepared in phase 1 (Fig. 1A, left) had negligible cytotoxic reactivity against SLA-G stimulators (e.g., anti-SLA PSL of 6.0% and 0.14% for two separate animals). In contrast, suppression of the naive PBL was not observed when nontolerant animals that generated marked anti-SLA CTL responses were the source of coculture primed cells in the secondary cultures.

Inhibition of naive PBL responders was not observed when cells were separated by transwells (Fig. 8). The transwell studies suggest that either soluble factors (e.g., cytokines) are not involved in the suppressive effect observed in vitro, or that soluble factors are operative but only when delivered at high concentrations, requiring cell-to-cell contact or close proximity of the regulatory cells with the donor reactive T cell. Alternatively, soluble factors may influence antidonor T cell responses by exerting their effects on the Ag-presenting cell. The presence of suppression in some models may result from differential secretion of T cell-derived cytokines by Th1 and Th2 cells (45, 46). Graft acceptance has been associated with a preferential shift toward a Th2 response, since Th2 cytokines are able to inhibit the Th1 program of cytokines (IL-2 and IFN-γ) consistently observed in rejecting allografts (47, 48). Most attention has been focused on CD4+ Th1/Th2 cells, however similar patterns have been described for CD8+ cells (49). Studies from our laboratory support the speculation that regulatory cytokines are involved in the mechanisms leading to acceptance of class I mismatched renal allografts in miniature swine, since tolerant animals have high levels of IL-10 gene transcripts locally in the graft, while rejector animals express high levels of the IFN-γ transcript (28). This cytokine pattern would be consistent with the differential activation of Th1 and Th2 cells in the tolerated allograft. Future studies will address the cytokine production by these primed regulatory cells, which could provide important information about soluble mediators of suppression.

![FIGURE 10. The effect of adding titrating concentrations of exogenous IL-2 to anti-SLA responses of naive PBL alone and cocultures of naive PBL with thyG tolerant-to-G effector cells. The responses of the thyG tolerant-to-G-G' alone was the same as the responses of the cocultures of naive PBL with thyG tolerant-to-G-G' effectors. The anti-SLA PSL responses are shown for cultures in which 0 U/ml, 0.75 U/ml, 1.0 U/ml, and 3.0 U/ml of IL-2 was added.](http://www.jimmunol.org/)
Alternative mechanisms of tolerance may require cell-to-cell contact. Anergic T cells were shown to suppress Ag-specific T cells in vitro by competition for locally produced IL-2R and for the generation of CTL in the secondary cocultures. However, the hypothesis that inhibition could be mediated by anergic T cells expressing increased levels of IL-2R that absorbed IL-2 (8, 50) seems an unlikely explanation for the suppression observed in the present analysis. The inhibitory effector group, thymi\textsuperscript{t}-to-G\textsuperscript{G} anti-SLA\textsuperscript{hh} PBL, expressed relatively lower levels of IL-2R when compared with the nonsuppressive effector groups, thymi\textsuperscript{t}-to-G\textsuperscript{G} H\textsuperscript{anti-SLA}\textsuperscript{hh} PBL (Fig. 9). Furthermore, anergy and suppression by anergic cells can generally be reversed by the addition of exogenous IL-2 (8). The inhibition by primed tolerant PBL could not be overcome by addition of exogenous IL-2. The non-thymi\textsuperscript{t}-to-G\textsuperscript{G} and thymi\textsuperscript{t}-to-G\textsuperscript{G} effector cells could represent anergic cells that suppress naive PBL by competition for the Ag-presenting cell (8). The fact that the cell-dose dependency studies demonstrated that the inhibition was rapidly lost after the ratio of thymi\textsuperscript{t}-to-G\textsuperscript{G} naive PBL was reduced to <1:4, and that cell-to-cell contact was required for the inhibition, is consistent with competition for ligand as a mechanism for the inhibition. A similar result would be achieved if the regulatory cells described in the current study were anti-idiotypic T cells (11), or veto cells (17, 18) that would require cell-to-cell contact to mediate their inhibitory effects.

Activation or maintenance of suppressor cells in mixed lymphocyte reaction has been described in rodent models (15, 51–54). Regulatory cells generated from MLC were shown to prolong cardiac allograft survival when adoptively transferred in vivo using a rat model (53). Our miniature swine are inbred for the MHC but not for non-MHC loci. Inbreeding of a miniature swine line is currently underway, and adoptive transfer studies will be possible when the inbred line is established.

The data described in the present study is consistent with the hypothesis that regulatory cells are involved in the maintenance of tolerance to renal allografts in miniature swine, and concur with other studies from our laboratory suggesting that local suppressive factors are implicated in mechanism of tolerance (24, 40, 41, 42). Since the immunololigic characteristics of miniature swine are similar to those of humans (20), isolation of the regulatory cells identified in the current report could provide important information for establishing donor-specific tolerance in clinical transplantation.

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