CTLA4Ig-Induced Linked Regulation of Allogeneic T Cell Responses

Richard S. Lee, James R. Rusche, Michaella E. Maloney, David H. Sachs, Mohamed H. Sayegh and Joren C. Madsen

*J Immunol* 2001; 166:1572-1582; doi: 10.4049/jimmunol.166.3.1572
http://www.jimmunol.org/content/166/3/1572

---

**References**
This article cites 42 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/166/3/1572.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
CTLA4Ig-Induced Linked Regulation of Allogeneic T Cell Responses

Richard S. Lee,* James R. Rusche,‡ Michaella E. Maloney,* David H. Sachs,§ Mohamed H. Sayegh,§ and Joren C. Madsen*†

The mechanisms by which CTLA4Ig exerts its powerful immunomodulatory effects are not clear. We show here that CTLA4Ig can induce linked regulation of allogeneic porcine T cell responses in vitro. Naive miniature swine SLA<sub>dd</sub> T cells were rendered hyporesponsive to specific allogeneic Ag after coculturing with MHC-mismatched SLA<sup>cc</sup> stimulators in the presence of CTLA4Ig. These Ag-specific hyporesponsive T cells were subsequently able to actively inhibit the allogeneic responses of naive syngeneic T cells in an MHC-linked fashion, as the responses of naive SLA<sub>dd</sub> responders against specific SLA<sup>cc</sup> and (SLA<sup>ac</sup>)<sub>F</sub><sub>1</sub> stimulators were inhibited, but allogeneic responses against a 1:1 mixture of SLA<sup>ww</sup> (I<sup>w</sup>, II<sup>w</sup>) and SLA<sup>cc</sup> (I<sup>c</sup>, II<sup>c</sup>) were maintained. This inhibition could be generated against either class I or class II Ags, was radiosensitive, and required cell-to-cell contact. Furthermore, the mechanism of inhibition was not dependent upon a deletional, apoptotic pathway, but it was reversed by anti-IL-10 mAb. These data suggest that CTLA4Ig-induced inhibition of naive allogeneic T cell responses can be mediated through the generation of regulatory T cells via an IL-10-dependent mechanism.


CTLA4Ig is a fusion protein that can induce allospecific hyporesponsiveness in vitro (1) and in vivo (2) by preventing CD28 activation by B7.1 and B7.2. The powerful immunosuppressive effects exerted by CTLA4Ig and the availability of a humanized CTLA4Ig variant (3) combine to make this an important agent in therapeutic strategies aimed at inducing tolerance in human allo- and xenograft recipients (4). Although a variety of mechanisms have been put forth to explain the immunosuppressive effects of CTLA4Ig (2, 5–7), including T cell anergy (8–10), the exact cellular mechanisms underlying the action of CTLA4Ig are unclear. It is known that the suppressive effects of CTLA4Ig can be observed in euthymic recipients in the absence of circulating levels of the agent (11). This observation suggests that the mechanism of action of CTLA4Ig is not simply T cell anergy, as maturing T cells would be expected to retain responsiveness to donor Ag in the absence of CTLA4Ig.

T cell anergy has traditionally been defined as the absence of a T cell response, when the TCR is engaged with an MHC receptor but a second costimulatory signal is not delivered (12). However, the conventional understanding of T cell anergy has been challenged by the recent observations by Lechler and others that anergic T cells can suppress the responses of other T cells (13–16). Thus, instead of simply not responding, certain anergic T cells can actively inhibit the alloresponses of other naive T cells. For instance, T cell clones rendered anergic either by soluble peptide cultures or immobilized anti-CD3 mAb have been shown to actively suppress the response of other T cell clones to Ags in an MHC-linked fashion (13–15). The ability of some anergic T cells to actively inhibit the response of other naive T cells represents a novel form of immune regulation and represents a potentially important strategy for achieving immune tolerance. However, the precise nature of this immune regulation is unknown.

The effect of CTLA4Ig in large animals is not well studied (2). We have recently investigated the in vitro effects of CTLA4Ig in functional porcine T cell assays and demonstrated that CTLA4Ig was able to induce Ag-specific T cell hyporesponsiveness in secondary allogeneic pig mixed lymphocyte cultures (3). We refer to these cells as hyporesponsive T cells as opposed to anergic T cells, as there is controversy concerning whether anergy requires signaling through CTLA4 (17). In this report, we extend previous findings by showing that Ag-specific hyporesponsive T (ASHT)<sup>3,4</sup> (3) cells induced by CTLA4Ig in mixed lymphocyte cultures were able to actively inhibit the allogeneic responses of naive, but not primed, porcine T cells. This inhibition occurred in an MHC-linked manner, required cell-to-cell contact, and appeared to be IL-10 dependent. These results suggest that the profound immunosuppressive effects of CTLA4Ig on T cell alloresponses is mediated in part through the generation of regulatory T cells using IL-10-dependent mechanisms.

Materials and Methods

Antibodies

The CTLA4Ig used in these studies was human CTLA4IgG4 purified from plasmid-transformed NSO cells. CTLA4IgG4 is a fusion protein that combines the human T cell surface receptor CTLA4 with the constant region of human (h) IgG4. The extracellular domain of hCTLA4 was cloned as a

*Transplantation Biology Research Center and †Division of Cardiac Surgery, Department of Surgery, Massachusetts General Hospital, Boston, MA 02114; ‡Repligen Corporation, Needham, MA 02494; and §Laboratory of Immunogenetics and Transplantation, Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115

Received for publication June 12, 2000. Accepted for publication November 2, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported in part by the National Heart, Lung, and Blood Institute of the National Institutes of Health (RO1-HL54211). R.S.L. is a recipient of the American College of Surgeon’s Resident Research Fellowship and a Research Fellowship Award from the International Society of Heart and Lung Transplantation.

Address correspondence and reprint requests to Dr. Joren C. Madsen, EDR 105, Department of Surgery, Massachusetts General Hospital, Boston, MA 02114. E-mail address: madsen@helix.mgh.harvard.edu

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00

3 Abbreviations used in this paper: ASHT cell, Ag-specific hyporesponsive T cell; h, human; SLA, swine leukocyte Ag.

4 We are using the nomenclature of ASHT only to simplify the description of these cells in this paper and are not suggesting that the term necessarily be adopted for use beyond this purpose.
fusion protein to hinge, CH2, and CH3 domains of a mutant hlgG4 deficient in Fc receptor binding (data not shown). This hlgG4 sequence contains L235G and G237A missense mutations. CTLA4IgG4 cross-reacts with the porcine molecule as previously described (3). Isotype control Abs included h60.1, a humanized anti-CD11b IgG4 that binds human, but not porcine, CD11b.

Animals

The inbred miniature swine used in this study have been described in detail previously (18, 19). Presently, inbred swine of three homozygous MHC (swine leukocyte Ag (SLA) in swine) haplotypes are maintained: SLA^a (F19*), SLA^h (F19*), and SLA^k (F19*). In addition, four in-mHC recombinants of the SLA^a (F19*), SLA^h (F19*), and SLA^k (F19*) haplotypes have been derived by spontaneous recombination events during the breeding of heterozygotes as part of the breeding program. All animal care and procedures were in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animals Resources and published by the National Institutes of Health.

Preparation of PBMC

For preparation of pig PBMC from freshly collected, heparinized whole blood, the blood was diluted 2/3 with HBSS (Life Technologies, Grand Island, NY), and 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 RBC were lysed with ACK lysing buffer (BioWhittaker, Walkersville, MD). Cells were washed again with HBSS and resuspended in complete tissue culture medium.

Mixed leukocyte reaction

Responder PBMC (2 4 10^5) and irradiated (2500 cGy) PBMC (2 4 10^5) were added to 200 ml of complete tissue culture medium in U-bottom wells in triplicate. Complete tissue culture medium for MLR assays consisted of RPMI 1640 (Life Technologies) supplemented with 6% fetal porcine sera (Sigma, St. Louis, MO), 100 U/ml penicillin, 155 µg/ml streptomycin (Life Technologies), 50 µg/ml gentamicin (Life Technologies), 10 mM HEPES (Cellgro, Agawam, MA), 2 mM t-glutamine (Life Technologies), 1 mM sodium pyruvate (BioWhittaker), nonessential amino acids (BioWhittaker), and 5 10^-3 M 2-ME (Sigma). The cultures were incubated at 37°C in humidified air containing 7% CO2 for 5 days, then assayed for [3 H]thymidine incorporation as described above. Results are expressed as the mean counts per minute as described above.

In vitro peptide proliferation assay

Approximately 2 wk after immunization with the PC1 allogeneic peptide, PBMCs from the peptide-immunized pig were tested against the same PC1 allogeneic peptide (aa 3–27) in 96-well U-bottom plates at 37°C in 5% CO2. Naive syngeneic SLA^ad (F19*) or (SLA^ac) (F19* × F19*) nylon wool-adherent PBMCs, used as APCs (2 4 10^5), were preincubated with 50 µg of the class I allopeptide for 2 h at 37°C in 5% CO2. After incubation, peptide-loaded APCs were washed in fresh medium and added to the appropriate wells (2 4 10^5). After 5 days of incubation, [3 H]thymidine incorporation was measured as the mean counts per minute as described above.

Flow cytometry

Flow cytometry was analyzed using a Becton Dickinson FACScan microfluorometer (San Jose, CA). Swine IgG was used to block FcR binding for porcine cells. Apoptotic cells were detected using TdT- and FITC-labeled nucleotides (ApopTag; Intergen, Purchase, NY) according to the manufacturer’s instructions. Biotinylated mouse anti-class I IgG,G,M (2,12,3) (21) was used to stain for SLA class I expression. PE-avidin (Becton Dickinson) was used as the secondary staining reagent. 74-12-4-P-E or CyChrome Ab was used to stain swine CD4 cells, and 76-2-11-P-E or FITC Ab was used to stain swine CD8 cells (22). Naive SLA^ad PBMCs, irradiated (600 cGy) SLA^ad PBMCs (6 h after irradiation), MLCs containing naive SLA^ad PBMC (4 10^5) and irradiated (2500 cGy) stimulator SLA^ac PBMCs (4 10^5), and cocultures containing CTLA4Ig-induced hyporesponsive SLA^ad PBMCs (4 10^5) and irradiated (2500 cGy) stimulator SLA^ac PBMCs (4 10^5) were analyzed for apoptosis. Cells taken from culture were washed with 25-cm2 flasks at 37°C in 7% CO2. For staining, 5 10^5 cells/tube of porcine or human cells were resuspended in 100 µl of HBSS (Life Technologies) containing 0.1% BSA and 0.1% NaN3 (FACS medium). Ten microliters of primary or isotype control Ab at ~1 µg/10^6 cells was added to the appropriate tubes for 30 min at 4°C. After two washes, a saturating concentration of secondary Ab was added and incubated for 30 min at 4°C. Cells were washed with FACS medium twice and then analyzed by double-color flow cytometry.

Results

We have previously shown that miniature swine T cells cultured with complete SLA-mismatched stimulators in the presence of CTLA4Ig were hyporesponsive to specific stimulators upon re-stimulation in the absence of CTLA4Ig, but maintained primary
responses to third-party allogeneic stimulators (3). For clarity, we have labeled these CTLA4Ig-modulated T cells ASHT cells (3).

To further characterize ASHT cells that were generated after exposure to CTLA4Ig, secondary MLRs were performed (Fig. 1A). SLA<sub>dd</sub> (I<sub>b</sub>II<sub>b</sub>) T cells were incubated with fully allogeneic SLA<sup>cc</sup> (I<sub>c</sub>II<sub>c</sub>) stimulators in the presence of CTLA4Ig or isotype control IgG4 during the primary MLC culture. After 7 days of incubation, responder cells were collected, washed, and rested in fresh medium. Subsequently, the responders were replated in secondary MLR cultures in the absence of any Ab. SLA<sub>dd</sub> T cells incubated with allogeneic SLA<sup>cc</sup> stimulators in the presence of CTLA4Ig were hyporesponsive to specific SLA<sup>cc</sup> stimulators upon restimulation in the absence of CTLA4Ig compared with responders incubated originally with control IgG4 (Fig. 1B). This inhibition was significant compared with the control IgG4 group; however, a residual proliferative response could be detected on days 2 and 3 of the secondary MLC. This residual response could not be eliminated in multiple assays, even with higher titrations of CTLA4Ig, suggesting that either CD28-negative populations of T cells were responding or alternative pathways of costimulation were being used.

When the ASHT cells were restimulated with (SLA<sup>aa</sup>)<sub>F1</sub>, (I<sub>c</sub>II<sub>c</sub>), no primary proliferative response was observed on days 4 and 5 despite a vigorous primary response to SLA<sup>aa</sup> (I<sub>c</sub>II<sub>c</sub>) and (SLA<sup>aa</sup>)<sub>F1</sub>, (I<sub>c</sub>II<sub>c</sub>), and a 1/1 mixture of SLA<sup>aa</sup> and SLA<sup>cc</sup> stimulators on days 4 and 5 (Fig. 1A). In fact, the kinetics and magnitude of proliferation to (SLA<sup>aa</sup>)<sub>F1</sub>, stimulators mirrored that observed against SLA<sup>cc</sup> stimulators, suggesting that the presence of the SLA<sup>c</sup> Ag on the APC prevented a primary response from T cells that would otherwise react to the SLA<sup>c</sup> Ag (linked Ag). This inhibition was not observed when a 1/1 mixture of SLA<sup>aa</sup> and SLA<sup>cc</sup> stimulators (unlinked Ag) was used (Fig. 1A). The proliferative curve against the 1/1 mixture occurred earlier than that observed with SLA<sup>aa</sup> stimulators. Although the reason for this change in kinetics was unclear, the result was observed consistently in four independent assays. Perhaps, the residual proliferative response of the ASHT cells to SLA<sup>cc</sup> stimulators generated early production of IL-2 and facilitated an accelerated response of naive cells to SLA<sup>aa</sup> stimulators through the action of secreted cytokines.

In contrast, SLA<sub>dd</sub> T cells incubated with specific SLA<sup>cc</sup> stimulators in the presence of control IgG4 during the primary MLC showed brisk and vigorous secondary responses on days 2 and 3 to SLA<sup>cc</sup>, (SLA<sup>aa</sup>)<sub>F1</sub>, and a 1/1 mixture of SLA<sup>aa</sup> and SLA<sup>cc</sup> stimulators (Fig. 1B). The primary response of the control group to third-party SLA<sup>aa</sup> stimulators peaked on day 4 (Fig. 1B). Of note, the lack of a primary response to SLA<sup>cc</sup> cells was not due to the immunodominance of SLA<sup>c</sup> Ags on the (SLA<sup>ac</sup>)<sub>F1</sub> cells, as SLA<sub>dd</sub> responders primed with (SLA<sup>aa</sup>)<sub>F1</sub> cells in vitro responded to both SLA<sup>aa</sup> cells and SLA<sup>cc</sup> cells upon restimulation (data not shown).

### FIGURE 1

**A**. CTLA4Ig-induced ASHT cells inhibited naive allogeneic T cells in a linked fashion. SLA<sub>dd</sub> (DD) responder cells (4 x 10<sup>5</sup>) were incubated with allogeneic SLA<sup>cc</sup> (CC) irradiated stimulator cells (4 x 10<sup>5</sup>) in the presence of CTLA4Ig (50 μg/ml) during the primary MLC for 7 days. On day 7, responder cells were collected, washed with fresh medium, and rested for 3 days at 37°C. After 3 days of resting, the bulk SLA<sub>dd</sub> (DD) lymphocyte culture (2 x 10<sup>5</sup>) was restimulated in a secondary MLC without any Ab using self (DD), specific SLA<sup>cc</sup> (CC; 2 x 10<sup>5</sup>), third-party SLA<sup>aa</sup> (AA; 2 x 10<sup>5</sup>), (SLA<sup>aa</sup>)<sub>F1</sub> (AC; 4 x 10<sup>5</sup>), a 1/1 mixture of SLA<sup>cc</sup> and SLA<sup>aa</sup> (AA + CC; 2 x 10<sup>5</sup> and 2 x 10<sup>5</sup>), or (SLA<sup>aa</sup>)<sub>F1</sub> (AD; 4 x 10<sup>5</sup>) irradiated stimulator cells. Trinitiated thymidine incorporation was measured on the indicated days postrestimulation. Data are representative of four similar experiments performed in triplicate. **B**. Secondary MLR of SLA<sub>dd</sub> (DD) responders originally incubated with allostimulator (SLA<sup>cc</sup>; CC) in the presence of control IgG4 (50 μg/ml) and restimulated in a secondary MLC without any Ab using self (DD), specific SLA<sup>cc</sup> (CC; 2 x 10<sup>5</sup>), third-party SLA<sup>aa</sup> (AA; 2 x 10<sup>5</sup>), (SLA<sup>aa</sup>)<sub>F1</sub> (AC; 4 x 10<sup>5</sup>), a 1/1 mixture of SLA<sup>cc</sup> and SLA<sup>aa</sup> (AA + CC; 2 x 10<sup>5</sup> and 2 x 10<sup>5</sup>), or (SLA<sup>aa</sup>)<sub>F1</sub> (AD; 4 x 10<sup>5</sup>) irradiated stimulator cells. Trinitiated thymidine incorporation was measured on the indicated days postrestimulation. Data are representative of four similar experiments performed in triplicate.
cultured with or without ASHT cells did not respond to third-party SLA^cc stimulators alone (Fig. 2B). The early proliferation in response to SLA^cc stimulators in cocultures of ASHT cells with naive T cells (Fig. 2A) observed on days 2 and 3 was probably due to the residual response of the ASHT cells, consistent with the data in Fig. 1A. Thus, ASHT cells were able to regulate the response of naive T cells to both specific and third-party MHC Ags provided that the third-party Ags were expressed on the same cell as the original Ag, i.e., linked regulation.

**Flow cytometric analysis of ASHT cells**

We analyzed the phenotype of the ASHT cells after 7 days of incubation with fully allogeneic stimulators and CTLA4Ig. Double staining for CD4 and CD8 (22) revealed that 44.1% of the population were CD8^+ T cells, 3.3% were CD4^+ CD8^low T cells, and 49.7% were double-positive T cells (Fig. 3A). The simultaneous expression of CD4 and CD8 Ags by a large population of resting peripheral T lymphocytes has been well documented in swine (6–60% in swine vs 0.5–8% in humans) (23, 24). The porcine CD8^+ CD4^+ T cell subset contains both mature resting T cells (25) and Ag-activated memory Th cells (26). Furthermore, after Ag exposure, single-positive CD4^+ T cells in swine may acquire a double-positive CD4^+ CD8^+ phenotype (27). Consistent with the literature, we found a 6-fold increase (6.8–38.4%) in the double-positive population after Ag exposure (Fig. 3, C and B). The presence of a significant population of CD8^+ CD4^+ T cells in swine and the dynamic nature of the expression of CD4 and CD8 after Ag exposure made definitive T cell subset phenotyping of ASHT cells difficult to interpret.

**Ag specificity of the regulatory properties of ASHT cells**

To define the specificity of ASHT cells, SLA^dd responders were cultured with either class I-disparate SLA^ag (I^c II^d) or class II-disparate SLA^ak (I^d II^c) stimulators in the presence of CTLA4Ig during the primary MLR culture. After 3 days of rest, the responder cells were cocultured with naive SLA^dd cells and restimulated in a secondary MLR culture without Ab using allogeneic stimulators that were linked with the original Ag or a third-party Ag. Fig. 4A demonstrates that ASHT cells were generated after incubation of SLA^dd cells with class I-disparate SLA^ag stimulators in the presence of CTLA4Ig during the primary culture. The proliferative response of cocultured naive SLA^dd cells against (SLA^ag)F_1, (I^d II^c), stimulators, which linked the original class I^d Ag to third-party class II^c Ags, was markedly inhibited. However, the response to (SLA^ak)F_1, (I^c II^d) stimulators, which presented a new class II^c Ag, was comparable to that observed with third-party SLA^ak and SLA^kk cells.

Similar results were observed when SLA^dd cells were incubated with class II-disparate SLA^ak (I^d II^c) stimulators in the presence of CTLA4Ig during the primary MLR culture (Fig. 4B). The primary response of cocultured naive SLA^dd cells against (SLA^ak)F_1 and SLA^ak stimulators, but not (SLA^ag)F_1, stimulators, was inhibited by the SLA^dd ASHT cells. The primary response to third-party SLA^aa cells remained intact. As the specificity of CD8^+ and CD4^+
Inhibition mediated by ASHT cells was dependent on cell-cell contact

To determine whether this regulatory phenomenon was contingent on cell-cell contact, coculture assays with Transwells were performed. SLA<sup>dd</sup> ASHT cells and SLA<sup>cc</sup> stimulators were cultured in the upper wells, while naive SLA<sup>dd</sup> T cells were cultured with SLA<sup>cc</sup> stimulators in the lower wells. Fig. 5 demonstrates that naive SLA<sup>dd</sup> T cells were able to respond to SLA<sup>cc</sup> stimulators when the SLA<sup>dd</sup> ASHT cells were separated from responders by a semipermeable membrane. In contrast, when the ASHT cells were cocultured with naive SLA<sup>dd</sup> T cells in the absence of a membrane, full inhibition of the primary allogeneic MLR was observed (Fig. 5). In addition, no inhibition was observed when naive SLA<sup>dd</sup> T cells were tested against third-party SLA<sup>cc</sup> stimulators in the lower wells. For a positive control, exogenous IL-2 (100 IU/ml) was added to the upper well. This led to maximal proliferation to SLA<sup>cc</sup> stimulators on day 5 (Fig. 5). Thus, the regulation of naive T cells by ASHT cells required cell-cell contact.

ASHT cells cannot regulate allogeneic responses of primed T cells

Next, we investigated whether T cells rendered hyporesponsive by CTLA4Ig could inhibit the response of in vitro and in vivo primed T cells. When SLA<sup>dd</sup> T cells that were primed in vitro against SLA<sup>cc</sup> stimulators were cocultured with ASHT cells in a secondary MLR, the response against specific SLA<sup>cc</sup> stimulators at the same or double the responder concentration (CC-2 or CC-4) was not inhibited (Fig. 6A). Instead, the Ag-activated T cells displayed primed MLR responses against specific allogeneic stimulators, with brisk and robust responses on days 2 and 3 after restimulation.
These results suggested that, unlike naive T cells, directly primed T cells were resistant to the regulation mediated by CTLA4Ig-induced ASHT cells.

To examine whether ASHT cells could regulate the alloreponses of T cells primed in vivo through the indirect pathway of allorecognition, PBMCs from a SLA<sup>dd</sup> pig immunized to an SLA class I<sup>c</sup> peptide (PC1 aa 3–27) in CFA were cultured with irradiated self (DD), SLA-matched stimulators loaded with allospecific class I<sup>c</sup> peptide (DD + PC1 peptide 1), or SLA<sup>cc</sup> (CC) stimulators. For coculture assays, PBMCs from the immunized pig (SLA<sup>dd</sup>) were cultured with ASHT cells (SLA<sup>dd</sup>; A-DD) and stimulated with irradiated (SLA<sup>cc</sup>)F<sub>1</sub> (CD), SLA<sup>cc</sup> (CC), or (SLA<sup>dd</sup>)F<sub>1</sub> stimulators loaded with PC1 peptide 1 (CD + PC1 peptide 1). The proliferative responses of an unimmunized naive SLA<sup>dd</sup> pig are shown against self (DD), SLA-matched stimulators loaded with allospecific class I<sup>c</sup> peptide (DD + PC1 peptide 1), and SLA<sup>cc</sup> (CC) stimulators as a control. Data are representative of two similar experiments performed in triplicate.

SLA<sup>dd</sup> cells to (SLA<sup>cc</sup>)F<sub>1</sub> stimulators without loaded peptide was higher than the response directed against SLA<sup>cc</sup> stimulators, but less than the response directed against peptide-loaded (SLA<sup>dd</sup>)F<sub>1</sub> cells. This suggests that (SLA<sup>dd</sup>)F<sub>1</sub> cells naturally present class I<sup>c</sup> peptides in association with class II<sup>d</sup> molecules, which is consistent with previous studies (28). Thus, ASHT cells were not able to inhibit the allogeneic responses of T cells primed in vivo through the indirect pathway of Ag presentation, although it is not known whether this failure of inhibition was due to the fact that the response was a primed one or an indirect one.

**Effects of IL-2 on ASHT cells**

The absence of a secondary proliferative response to SLA<sup>cc</sup> or SLA<sup>cc</sup> stimulators by SLA<sup>dd</sup> ASHT cells could be due to deletion of responding cells, which would not be expected to be sensitive to exogenous IL-2. To test this hypothesis, IL-2 was added to appropriate wells during the secondary MLR. The addition of exogenous IL-2 restored proliferative responses of SLA<sup>dd</sup> ASHT cells to specific SLA<sup>cc</sup> stimulators, with peak proliferation 4 days postrestimulation (Fig. 7). Exogenous IL-2 also restored the primary response of the bulk culture to (SLA<sup>cc</sup>)F<sub>1</sub> stimulators, which presented a linked Ag. In the absence of IL-2, specific hyporesponsiveness to SLA<sup>cc</sup> stimulators was observed (Fig. 7). The kinetics of the proliferative response appeared to be accelerated by 1 day compared with the normal naive response (cf Figs. 1A and 7).

(Fig. 6A). These results suggested that, unlike naive T cells, directly primed T cells were resistant to the regulation mediated by CTLA4Ig-induced ASHT cells.

**FIGURE 6.** A, ASHT cells cannot inhibit allogeneic responses of T cells primed in vitro. SLA<sup>dd</sup> (DD) responders were cultured with irradiated SLA<sup>cc</sup> (CC) stimulators in the presence of control IgG4 (50 μg/ml) for 7 days. On day 7 responder cells were collected, washed with fresh medium, and rested at 37°C. After 3 days of rest, bulk cultures were cocultured with SLA<sup>dd</sup> ASHT cells in a secondary MLR. Two concentrations of SLA<sup>cc</sup> stimulator were used, 2 × 10<sup>5</sup>/well (CC-2) and 4 × 10<sup>5</sup>/well (CC-4). Data are representative of two similar experiments performed in triplicate.

B, ASHT cells cannot inhibit allogeneic responses of T cells primed in vivo through the indirect pathway of allorecognition. PBMCs from a SLA<sup>dd</sup> pig immunized to an SLA class I<sup>c</sup> peptide (PC1 aa 3–27) in CFA were cultured with irradiated self (DD), SLA-matched stimulators loaded with allospecific class I<sup>c</sup> peptide (DD + PC1 peptide 1), or SLA<sup>cc</sup> (CC) stimulators. For coculture assays, PBMCs from the immunized pig (SLA<sup>dd</sup>) were cultured with ASHT cells (SLA<sup>dd</sup>; A-DD) and stimulated with irradiated (SLA<sup>cc</sup>)F<sub>1</sub> (CD), SLA<sup>cc</sup> (CC), or (SLA<sup>dd</sup>)F<sub>1</sub> stimulators loaded with PC1 peptide 1 (CD + PC1 peptide 1). The proliferative responses of an unimmunized naive SLA<sup>dd</sup> pig are shown against self (DD), SLA-matched stimulators loaded with allospecific class I<sup>c</sup> peptide (DD + PC1 peptide 1), and SLA<sup>cc</sup> (CC) stimulators as a control. Data are representative of two similar experiments performed in triplicate.
The reason for this acceleration was not apparent. Possibly, the ASHT cells were more responsive to exogenous IL-2 because of previous exposure to allogeneic stimulators. In any case, the ability of IL-2 to restore the proliferative response to specific and linked stimulator argues against a deletional mechanism of T cell regulation. Furthermore, regulation mediated by the ASHT cells was not simply due to consumption of nutrients and IL-2 within the medium during the early response of the ASHT cells (Fig. 1A), as supernatants from MLCs were harvested on day 4 and used to replate primary allogeneic MLRs. Vigorous primary allogeneic proliferative responses were observed after plating MLRs with the culture supernatants (data not shown).

ASHT cells secreted sustained levels of IL-10 but minimal levels of IFN-γ

The in vivo administration of CTLA4Ig has been shown to suppress Th1 responses but spare Th2 responses (7). To ascertain the phenotype of CTLA4Ig-induced, ASHT cells, we examined the cytokine profile of the ASHT T cells in the presence of specific SLA<sup>dd</sup> stimulators. Fig. 8A demonstrates that ASHT T cells secreted sustained low levels of IL-10 (15–25 pg/ml) throughout the culture period, with peak production 2 days after restimulation. The level of IL-10 production was approximately one-tenth of the amount secreted by primed T cells in response to specific SLA<sup>cc</sup> stimulator (Fig. 8B). In contrast, ASHT cells produced only minimal amounts of IFN-γ throughout the culture period (Fig. 8C), while control primed responders generated high levels of IFN-γ for the duration of the culture period (Fig. 8D). This cytokine profile was consistent with a Th2 or possibly a Th3 phenotype (29). Further analysis of the cytokine profile of the ASHT cells was precluded by the lack of available swine-specific reagents.

Anti-IL-10 mAb reversed the inhibition mediated by ASHT cells but did not reverse hyporesponsiveness

Since ASHT cells secreted IL-10, but not IFN-γ, neutralization and supplementation MLRs were performed to further characterize the nature of the T cell regulation mediated by these cells. Fig. 9A demonstrates that the addition of anti-swine IL-10 mAb (5 µg/ml) to cocultures containing ASHT cells and naïve SLA<sup>dd</sup> T cells restored the primary allogeneic response to SLA<sup>dd</sup> stimulators. The primary response to third-party SLA<sup>aa</sup> stimulators was also maintained. In contrast, anti-swine IL-4 Ab (10 µg/ml), exogenous swine IFN-γ (1000 U/ml), and control IgG had no effect on reversing the inhibition (Fig. 9, B–D). Thus, the immunoregulatory effects mediated by the ASHT cells appeared to be IL-10 dependent.
Since IL-10 leads to inactivation of APCs with a reduction in the expression of class II molecules and costimulatory ligands (CD80, CD86) (30), we examined the expression of both B7 and class II on APCs after coculture with ASHT and naive T cells. No significant differences in the level of B7 or class II expression were detected at 12, 24, and 36 h of coculture (data not shown). Thus, the IL-10-dependent immune regulation mediated by CTLA4Ig-induced ASHT cells did not appear to be related to the down-regulation of class II on CD80/CD86 by APCs.

The effects of anti-IL-10 mAb might be contributing to the direct reversal of hyporesponsiveness, which would, in effect, lead to the reversal of suppression indirectly. To distinguish whether anti-IL-10 mAb was directly reversing the suppression mediated by the regulatory cells or actually reversing the hyporesponsiveness of the ASHT cells, neutralization MLRs were performed using ASHT cells for responders and specific SLA cc or linked SLA ac stimulators. If anti-IL-10 mAb were reversing hyporesponsiveness directly, then a primary MLR should be detected against both specific and linked stimulators. If, however, anti-IL-10 mAb were reversing the suppression, then a primary MLR should be observed for only the linked stimulator. Fig. 10 demonstrates the addition of anti-IL-10 mAb restored the proliferative response to linked SLA ac stimulators, but not to specific SLA cc stimulators, while the response to third-party SLA ac stimulators remained intact. Thus, anti-IL-10 was effecting the reversal of suppression and not hyporesponsiveness.

**ASHT cells did not induce apoptosis**

Another possible hypothesis to explain the mechanism of immune regulation mediated by ASHT cells is the deletion of naive cells by either the APC or the regulatory T cells themselves. This would be similar to a veto-like mechanism found in mice (31, 32). To address this hypothesis, we examined whether the level of apoptosis in responder class I+ cells after coculture with ASHT cells was increased relative to that in cultures with naive responders and stimulator cells alone. Two-color flow cytometric analysis was performed using a mouse mAb specific for class I (responder haplotype; 2.12.3) (21) and TdT- and FITC-labeled nucleotides, which indicate DNA fragmentation, the hallmark of apoptosis. Fig. 11 demonstrates that the percentage of responder class I+ cells that stained brightly for dNTP-FITC after coculture of naive SLA dd cells with SLA dd ASHT cells and irradiated SLA cc stimulators was ~3% at 60 h of culture (Fig. 10D) and ~10% at 84 h of culture (Fig. 11E). These levels were actually lower than those observed after culturing naive SLA dd cells with irradiated SLA cc stimulators alone (8.5% at 60 h (Fig. 11C) and 30.6% at 84 h (Fig. 11E)). Irradiated (600 cGy) SLA dd cells were analyzed after 6 h of incubation for a positive apoptotic control (Fig. 11B), while naive SLA dd cells served as a negative control (Fig. 11A). The failure to observe a significant population of apoptotic class I+ cells (i.e., SLA cc irradiated stimulators) in the cultures was probably due to complete cell death and fragmentation by 60 and 84 h of incubation. Flow cytometric analysis revealed very few class I+ cells (<7%) remaining as early as 12 h postculture using irradiated (2500 cGy) SLA cc cells (data not shown). Finally, SLA dd ASHT cells did not lyse specific SLA cc or syngeneic SLA dd targets in chromium release assays (data not shown), arguing against a direct cytotoxic mechanism of regulation.

**Discussion**

Our results demonstrate that blockade of the B7-CD28 pathway by CTLA4Ig gives rise to hyporesponsive T cells, which can actively inhibit the allogeneic responses of naive, but not primed, T cells to specific alloantigen or to third-party alloantigens expressed on the same APC as the specific Ag (i.e., linked regulation). We have referred to these CTLA4Ig-induced regulatory cells as hyporesponsive T cells as opposed to anergic T cells as there is controversy concerning whether anergy requires signaling through
The immunoregulatory effects of IL-10 are well documented (30). IL-10 directly inhibits T cell proliferation and cytokine production in response to Ag (34) and indirectly inhibits T cell function through its effects on the APC (30). Regulatory CD4+ T cells are characterized by the production of high levels of IL-10, but no IL-4 (29), and IL-10 not only leads to the generation of regulatory T cells, but also serves as a mechanism through which regulatory T cells exert their effect (29). Recently, a surface-bound form of IL-10 was described on human PBMCs (35). The existence of a surface form of IL-10 might explain our seemingly paradoxical finding that anti-IL-10 mAb could reverse the inhibition mediated by CTLA4Ig-induced regulatory cells, yet cell-cell contact was required for the regulatory cells to effect inhibition. Perhaps, the regulatory T cells interacted with APCs and delivered a negative signal through a surface form of IL-10, which led to inactivation of the APC and prevention of a primary response by a naive T cell interacting with that APC. One would expect the effects of this regulation to be very local, since the surface-bound IL-10 would be required to interact with a surface receptor. An alternative interpretation is that the regulatory T cells inactivated naive T cells after direct cell-cell contact with the naive T cell through a veto-like mechanism. While this is formally possible, we think that this is unlikely, as a 1/1 mixture of SLAaa and SLA cc stimulators did not lead to suppression. In bulk culture it seems statistically unlikely that a T cell interacting with SLA cc APC would not also be in contact with a T cell that is recognizing SLA cc APC. Furthermore, no evidence for a deletional or cytotoxic mechanism was found.

To our knowledge, this represents the first report describing the ability of blocking the B7-CD28 pathway alone with CTLA4Ig to mediate linked regulation of allogeneic T cell responses. Early models of anergic T cells with regulatory properties were based on the use of immobilized anti-CD3 Ab, soluble peptides, or T cell presentation of Ag and were not dependent upon inhibitory cytokines, such as IL-10 (13–16). Although there are some similarities between these models of immune regulation and our own, our model appears to be distinct because 1) primed cells were resistant to regulation, 2) bulk populations of cells were used to generate regulatory cells and not Ag-specific T cell clones, and 3) linked
regulation was dependent upon the actions of IL-10 (13–16). Blockade of the CD40-CD154 pathway alone has been shown to induce linked suppression to MHC-matched, minor Ag-disparate skin grafts in mice, but anti-CD8 mAb was also required for graft prolongation (36). Very recently, blockade of both CD40 and CD86 pathways was shown to induce regulatory human T cells; however, the degree of inhibition was only partial (up to 60% maximal) compared with the level of inhibition in our studies (37). In the human study regulation required the use of both anti-CD40 and anti-CD86 mAbs (CTLA4Ig was not addressed), while our study showed complete suppression with the use of CTLA4Ig alone. IL-10 also appeared to play a role in the mechanism of regulation in the human study; however, neutralization of IL-10 only partially restored the allogeneic response in that study. These differences suggest that the characteristics of CTLA4Ig-induced regulatory cells and the nature of their regulation are probably distinct from those exhibited by the anergic regulatory T cells previously described.

Evidence for the in vivo generation of a regulatory T cells by CTLA4Ig has been suggested in several rodent models of allotransplantation. The adoptive transfer of CTLA4Ig-treated CD4+ cells along with naive T cells led to donor-specific tolerance of mouse allogeneic islets (38). Rat cardiac allograft recipients treated with CTLA4Ig and donor-specific transfusion led to indefinite graft survival in 50% of recipients (39). Furthermore, the transfer of cells from the CTLA4Ig-treated rats led to infectious tolerance in naive hosts (39). The ability of costimulatory blockade with CTLA4Ig to mediate immune regulation in a linked fashion is reminiscent of previous reports demonstrating linked suppression through the use of nondepleting CD4 Abs in vivo (40, 41). Indeed, linked suppression mediated by regulatory T cells may be a general mechanism of immune regulation that contributes to various models of peripheral tolerance (42).

Our model could also explain the observation that immune modulation by CTLA4Ig often does not require continued administration or persistent circulating Ab, since Ag-specific hyposensitive T cells induced by CTLA4Ig in our system were able to regulate the responses of naive T cells even after cessation of therapy. In light of the recent phase I clinical trial of CTLA4Ig in psoriasis vulgaris patients (11) and the use of CTLA4Ig to prevent graft-vs-host disease in bone marrow transplant recipients (43), the ability of CTLA4Ig-induced regulatory cells to suppress the naive T cell response could have significant implications for clinical transplantation and therapy for autoimmunity (44).

Acknowledgments

We thank Drs. Hugh Auchincloss, Jr., Henry Winn, and Kathryn J. Wood for their suggestions and critical review of this manuscript and Laurie Niederer for preparation of this manuscript.

References


