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Specific Suppression of Human CD4+ Th Cell Responses to Pig MHC Antigens by CD8+CD28− Regulatory T Cells

Rodica Ciubotariu,* Adriana I. Colovai,* Giuseppina Pennesi,*‡ Zhouru Liu,* Douglas Smith,§ Pasquale Berlocco,¶ Raffaello Cortesini,¶ and Nicole Suciu-Foca2*

Evidence that T cells can down-regulate the immune response by producing or consuming certain cytokines or by lysing APCs or Th cells has been provided in various systems. However, the generation and characterization of suppressor T cell lines have met with limited success. Here we show that xenospecific suppressor T cells can be generated by in vitro stimulation of human T cells with pig APCs. Similar to allostimulatory suppressors, these xenospecific suppressor T cells carry the CD8+CD28− phenotype and react to MHC class I Ags expressed by the APCs used for priming. TCR spectratyping of T suppressor cells showed oligoclonal usage of TCR-Vβ families, indicating that xenostimulation of CD8+CD28− T cells results in Ag-driven selection of a limited Vβ repertoire. Xenospecific T suppressor cells prevent the up-regulation of CD254 molecules on the membrane of Th cells, inhibiting their ability to react against the immunizing MHC class II xenantigens. The mechanism of this suppression, therefore, appears to be blockade of CD154/CD40 interaction required for efficient costimulation of activated T cells. *The Journal of Immunology, 1998, 161: S193–S202.

The shortage of organ donors is an ever increasing problem in clinical transplantation. Although the use of pig organs may offer a solution, there are still several immunologic barriers that should be overcome before xenotransplantation can be envisioned. The first is the hyperacute rejection caused by the binding of naturally occurring Abs and complement, present in primates, to pig endothelial cells. Recent progress in the generation of transgenic pigs expressing human complement-regulatory molecules on vascular endothelium may solve this critical problem (1–4). However, Th cell recognition of xenogeneic MHC Ags via the direct and indirect pathways is likely to result in strong cellular immune responses that may be difficult to suppress using currently available strategies (4, 5). It is therefore apparent that the development of methods for specific suppression of xenograft rejection is an important objective for achieving successful xenotransplantation.

Although immunologic tolerance to allogeneic and xenogeneic tissues has been induced in a variety of experimental models (2, 6), attempts to ablate specifically the immune response to HLA-incompatible transplants in human patients have failed thus far. However, two recent reports have described strategies for in vitro education of regulatory T cells that suppress in a specific manner the direct recognition by CD4+ T cells of MHC class II Ags expressed on allogeneic APCs (7, 8). In one of these studies, regulatory T cells with suppressor activity were generated by stimulation of CD4+ T cells with allogeneic monocytes in the presence of IL-10. These regulatory T cells inhibited specifically the reactivity of CD4+ Th cells through the secretion of IL-10 and TGF-β (7).

In the other study, suppressor T cells were generated by multiple stimulations of human PBL with allogeneic APCs and shown to display the CD8+CD28− phenotype (8). These CD8+CD28− T cells recognized specifically HLA class I Ags expressed by the stimulatory APCs and suppressed the proliferative response of alloreactive CD4+ T cells against APCs used for priming. The suppressive effect was not mediated by lymphokines but instead required cell-to-cell interaction between CD4+ Th cells, CD8+CD28− T suppressor (Ts)3 cells, and allogeneic APCs expressing Ags against which the T cells were primed. In this system, Ts cells appeared to act by inhibiting costimulatory signals delivered by the allogeneic APCs, such as those provided by CD80/CD86 molecules (8).

This report demonstrates that xenospecific suppressor T cells can also be generated by multiple in vitro stimulations of human T cells with pig PBMCs. The CD8+CD28− population from these T cell lines (TCL) recognizes specifically xenogeneic MHC class I Ags and suppresses the proliferative response of Th cells to MHC class II Ags expressed by the xenogeneic APCs. Xenospecific Ts cells interfere with the expression of CD154, the CD40 ligand, on xenoreactive Th cells, further supporting the concept that the suppressor effect results from inhibition of costimulatory interactions between Th cells and APCs.

Materials and Methods

Pig specimens

Blood was obtained from outbred pigs and from Yucatan miniature swine (Sinclair Research Center, Columbia, MO). MHC haplotypes were defined by RFLP using swine histocompatibility leukocyte Ag (SLA) class I- and class II-specific probes (9–11). For experiments aimed at the identification of MHC Ags recognized by xenospecific Ts cells, blood was obtained from three SLA homozygous lines named W, Z, and Q. Line Q is homozygous for a crossover haplotype that carries the SLA class I genes of strain W and the SLA class II genes of Z (9–11).

3 Abbreviations used in this paper: Ts cell, T suppressor cell; PI, propidium iodide; TCL, T cell lines; SLA, swine histocompatibility leukocyte antigen; CD40L, CD40 ligand.
Human specimens
Blood was obtained from healthy blood donors typed for HLA class I and class II Ags by conventional serology and by genomic typing of in vitro amplified DNA with sequence-specific oligonucleotide probes.

Generation of xenoreactive and alloreactive T cell lines
Human and pig PBMCs were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding human PBMCs (1 × 10^6/ml) were stimulated in 24-well plates with irradiated (1600 r) pig or human PBMCs (1 × 10^6/ml). Cells were cocultured for 7 days in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and 50 μg/ml gentamycin) (Gibco, Baltimore, MD). Responding cells were restimulated at 7-day intervals in medium containing 10 μM α-2,3 (Boehringer Mannheim, Indianapolis, IN).

Cell separation
NK cells were depleted from the alloreactive or xenoreactive TCLs before testing using goat anti-mouse magnetic beads (Dynal, Lake Success, NY) coupled with mAb anti-CD16 and CD56 (Becton Dickinson, San Jose, CA). Suspensions used in blastogenesis assays contained <2% CD16/CD56-positive cells, as indicated by flow cytometry. CD4^+ and CD8^+ T cells were separated from alloreactive and xenoreactive TNL by negative selection using Dynal CD4 and CD8 magnetic beads. T cell suspensions used as responders in blastogenesis assays were >98% positive for the CD4 and CD45RO markers. CD8^+ T cell suspensions were prepared by depletion of CD8^+ T cells from purified CD8^- T cell suspensions. For this procedure, goat anti-mouse Dynal beads were coupled with mAb anti-CD8 (Becton Dickinson, San Jose, CA), according to the manufacturer's instructions. The CD28-coupled beads were washed and incubated at 20 min at 4°C, with gentle end-over-end mixing. Rosetted CD8^-CD8^+ T cells were detatched from the beads by overnight incubation at 37°C and used in cell-mediated lysis experiments. Nonrosetted cells were collected, washed three times, and resuspended at 2.5 × 10^5 cells/ml in complete RPMI 1640 culture medium. The purity of the suspension was monitored by cytofluorographic analysis. The suspension was resorbed with CD28 beads when necessary, to obtain a population contaminated by <2% CD28^+ bright cells.

Proliferation assays
Blastogenesis assays were performed on day 14 or 21, after two or three stimulations, respectively, of human T cells with allogeneic or xenogeneic PBMCs (5 × 10^5 cells/well). CD4^+ and CD8^+ T cells were separated from alloreactive and xenoreactive TNL by negative selection using Dynal CD4 and CD8 magnetic beads. T cell suspensions used as responders in blastogenesis assays were >98% positive for the CD4 and CD45RO markers. CD8^+ T cell suspensions were prepared by depletion of CD8^+ T cells from purified CD8^- T cell suspensions. For this procedure, goat anti-mouse Dynal beads were coupled with mAb anti-CD8 (Becton Dickinson, San Jose, CA), according to the manufacturer's instructions. The CD28-coupled beads were washed and incubated at 20 min at 4°C, with gentle end-over-end mixing. Rosetted CD8^-CD8^+ T cells were detached from the beads by overnight incubation at 37°C and used in cell-mediated lysis experiments. Nonrosetted cells were collected, washed three times, and resuspended at 2.5 × 10^5 cells/ml in complete RPMI 1640 culture medium. The purity of the suspension was monitored by cytofluorographic analysis. The suspension was resorbed with CD28 beads when necessary, to obtain a population contaminated by <2% CD28^+ bright cells.

Diffusion chamber experiments
Xenoreactive CD4^+ T cells (2.5 × 10^5 cells/well) and irradiated xenogeneic APCs (5 × 10^5 cells/well) were cocultured in the bottom compartment of a transwell system (Nalge Nunc International, Roskilde, Denmark). Xenoreactive specific CD8^+ CD82^- T cells (1.25 × 10^5 cells/well) were added either to the bottom compartment or cocultured with specific pig APCs in the top compartment of the transwell system. After 48 h, the semipermeable membranes were removed, and the proliferative response of Th cells was measured by [3H]Tdr incorporation during the last 18 h of culture.

Flow cytometry
Human T cell subsets were defined using mAb CD4, CD8, CD3, CD45RO, and CD16/56 (Becton Dickinson). Cell suspensions were phenotyped before testing with a FACScan flow cytometer instrument (Becton Dickinson) equipped with a 15-mm argon laser. CaliBRITE flow cytometer beads and the FACScComp program (Becton Dickinson) were used for calibration of the cytomter.

To study the expression of CD154 on responding human Th cells, cells were incubated for 6 or 18 h in MLC and then stained with saturating amounts of mAbs CD3-Per CP (peridinin chlorophyll protein-conjugated anti-CD3 mAb), CD154-PE, and CD4- FITC or CD8- FITC (Becton Dickinson, Alto, CA). The ability of xenoreactive CD8^+ CD82^- human T cells to induce apoptosis of pig PBMCs and of xenoreactive human CD4^+ Th cells after 4 h of coincubation at 37°C was tested by flow cytometry with the use of annexin V as a marker for apoptotic cells. As positive controls, cells treated with camptothecin (Sigma) were used. The ratio of pig PBMC, human Th cells, and human Ts cells was 1:0.5:0.25, as also used in blastogenesis assays. After incubation, cells were stained with mAb anti-human CD3-PE or CD4-PE, washed, and subsequently stained with annexin V-FITC and propidium iodide (PI) (R&D Systems). To analyze the population of pig PBMCs, log FL2 (CD3-PE) vs side scatter parameters were used to gate out human CD3^+ T cells. The percentage of apoptotic pig cells was determined from log FL1 (annexin-FITC) vs FL3 (PI) dot plots. To analyze the population of human CD4^+ Th cells undergoing apoptosis, log FL2 (CD4-PE) vs side scatter parameters were used to gate on CD4^+ positive cells. Log FL1 (annexin-V FITC) vs FL3 (PI) dot plots of the gated population provided the percentage of apoptotic CD4^+ Th cells.

Cytotoxicity assays
CD8^+ CD28^- and CD8^+ CD28^+ were isolated from activated CD8^+ cells and tested for cytotoxicity in a 51Cr release assay. Target cells were pig PBMCs stimulated with PHA (2 μg/ml) 3 days before the cytotoxicity assay. The cytotoxicity assay was performed with different E:T ratios. The percent cytotoxicity was calculated as % lysis = 100 × [(experimental release (cpm) – spontaneous release (cpm))] / [maximum release (cpm) – spontaneous release (cpm)].

TCR spectratyping
Total RNA was extracted using Qiagen columns (Qiagen, Valencia, CA) from xenoreactive human CD8^+ CD82^- Ts cells. RNA was reverse transcribed into cDNA in a reaction using Moloney murine leukemia virus reverse transcriptase primed with oligo(dT)18 (Clontech Laboratories, Palo Alto, CA), as recommended by the manufacturer. Aliquots of the cDNA synthesis reaction were amplified in 50-μl reactions with each of the 24 Vβ oligonucleotides (0.5 μM final concentration) and the β8 oligonucleotide (0.5 μM final concentration). Vβ and β8 primers were previously described (12, 13). As an internal control for the amount of cDNA used per reaction, a tube containing sense and antisense primers for the first exon of β8 region was included. Two microliters of the Vβ-β8 PCR products were subjected to elongation with a fluorophore-labeled Cα or β8-specific primer (0.5 μM final concentration) (12). The size and fluorescence intensity of labeled runoff products were determined on a 377 DNA sequencer (Perkin-Elmer Applied Biosystem Division, Foster City, CA) and analyzed by ABI PRISM 377 GENESCAN Analysis Program (Perkin-Elmer Applied Biosystem Division) (13).

The relative intensity of each Vβ family or β8-specific fragment was calculated as the peak area corresponding to each Vβ family or β8 fragment divided by the sum of all area peaks (12).

Statistical analysis
Statistical analysis of the results was performed using BMDP statistical software. Analysis of variance to assess significance of group differences (ANOVA) followed by Tukey's method for multiple comparison was applied. Correlation coefficients were obtained using Linear Regression Analysis. Student’s t test of significance was also used to access the differences between groups.
Results

Specificity of xenoreactive Ts cells

TCLs were generated by priming T cells from a healthy volunteer (SA) with PBMCs from an unrelated blood donor (BM) or with PBMCs from an outbred pig (pig A). The allospecific TCL (SA-anti-BM) as well as the xenospecific TCL (SA-anti-pig A) showed higher reactivity against APCs from the original stimulator after removal of CD8<sup>+</sup>CD28<sup>+</sup> Ts cells from the suspensions (Fig. 1).

Furthermore, when CD8<sup>+</sup>CD28<sup>+</sup> T cells were added to the cultures at the initiation of the blastogenesis assay, they inhibited significantly (<i>p</i> < 0.05) the reactivity of CD4<sup>+</sup> Th cells against APCs used for priming. The suppressive effect was species specific since CD8<sup>+</sup>CD28<sup>+</sup> Ts cells primed to pig APCs did not inhibit the response of CD4<sup>+</sup> Th cells primed to human APCs. Similarly, Ts cells primed to human APCs did not inhibit the response of CD4<sup>+</sup> Th cells primed to pig APCs, indicating that Ts cells recognize species-specific Ags (Fig. 1). Studies of an additional four xenospecific and allospecific TCLs yielded similar results.

To determine whether the suppressive effect correlates with the number of Ts cells present in the cultures, Ts cells from two xenoreactive TCLs, MN-anti-pig B and AP-anti-pig B, were tested at various concentrations for their ability to inhibit proliferation of Th cells from TCLs MN-anti-pig B and AP-anti-pig B, respectively. As illustrated in Table I, the strength of the suppressive effect increased with the number of Ts cells, indicating that suppression was dose dependent (<i>r</i> = 0.85, <i>p</i> < 0.008).

To determine the nature of the SLA Ags recognized by CD8<sup>+</sup>CD28<sup>+</sup> Ts cells on pig stimulating cells, xenoreactive TCLs were generated by stimulating PBMCs from a human blood donor (ES) with irradiated APCs from three different strains of inbred swine, Q, W, and Z. Strain Q shares class I Ags with W and class II Ags with Z, being homozygous for a recombinant haplotype which carries the SLA class I Ags of W and the class II Ags of Z.

Table II shows the results of independent experiments in which TCL generated on three different occasions, by priming PBMC from individual ES with APCs from strain Q, W, and Z, were used. In these experiments, CD4<sup>+</sup> Th cells from ES-anti-swine Q were tested for reactivity in cultures without Ts cells or with Ts cells from ES-anti-Q, ES-anti-W, and ES-anti-Z.

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**Table I.** Dose-dependent suppression of CD4<sup>+</sup> Th reactivity to irradiated pig APCs in the presence of CD8<sup>+</sup>CD28<sup>+</sup> Ts cells

<table>
<thead>
<tr>
<th>No. of Cells/ Well (&lt;i&gt;x&lt;/i&gt;10&lt;sup&gt;4&lt;/sup&gt;)</th>
<th>Reactivity (mean cpm) of Th Cells from TCL&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APCs</td>
</tr>
<tr>
<td></td>
<td>MN-anti-B</td>
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<tr>
<td></td>
<td>AP-anti-B</td>
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<tr>
<td></td>
<td>Z</td>
</tr>
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<td></td>
<td>W</td>
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<td></td>
<td>Z</td>
</tr>
</tbody>
</table>

<sup>a</sup> All reactions were performed in triplicate. The SD to the mean was <10%.

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**Table II.** Reactivity of CD4<sup>+</sup> Th cells from TCL ES-anti-swine Q in the presence of CD8<sup>+</sup>CD28<sup>+</sup> Ts cells

<table>
<thead>
<tr>
<th>Ts from</th>
<th>Source of APCs Used for Priming of Ts</th>
<th>Genotype of APCs</th>
<th>Reactivity (mean cpm) of CD4&lt;sup&gt;+&lt;/sup&gt; Th from TCL ES-anti-Q&lt;sup&gt;W&lt;/sup&gt; at Following Source of APCs Used in Blastogenesis Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Class I</td>
<td>Class II</td>
</tr>
<tr>
<td>Expt. 1</td>
<td>No Ts added</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ES-anti-Q</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>ES-anti-W</td>
<td>W</td>
<td>W</td>
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<tr>
<td></td>
<td>ES-anti-Z</td>
<td>Z</td>
<td>Z</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>No Ts added</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ES-anti-Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ES-anti-W</td>
<td>W</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>ES-anti-Z</td>
<td>Z</td>
<td>Z</td>
</tr>
<tr>
<td>Expt. 3</td>
<td>No Ts added</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ES-anti-Q</td>
<td></td>
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<td></td>
<td>ES-anti-W</td>
<td>W</td>
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<td></td>
<td>ES-anti-Z</td>
<td>Z</td>
<td>Z</td>
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</tbody>
</table>

<sup>a</sup> For each experiment, reactions were set up in triplicate cultures. The SD to the mean of triplicate reactions was <10%.
The reactivity of Th cells primed to APCs of strain Q to the specific stimulator Q was inhibited efficiently by autologous Ts cells primed to Q or to W (which shares MHC class I Ags with Q), but not by Ts cells primed to Z (which is MHC class II identical, yet class I different from the specific stimulator Q) \((p < 0.05)\). CD4\(^+\) T cell reactivity to strain Z was inhibited only by Ts cells primed to Z, but not by Ts cells primed to strain Q or W which are class I different from Z \((p < 0.05)\) (Table II). This indicates that CD8\(^+\) CD28\(^-\) Ts cells are activated by SLA class I Ags on xenogeneic APCs and inhibit the response of CD4\(^+\) Th cells against class II Ags expressed by the same stimulating target cells. The MHC class II specificity of Th cell reactivity was confirmed by the fact that human CD4\(^+\) T cells primed to APCs from a strain Q swine reacted to APCs from strain Z (class II identical with Q) but not from strain W (class II different from Q).

To establish whether the suppressive activity of CD8\(^+\) CD28\(^-\) T cells requires the direct interaction of these cells with the APCs that trigger Th cell reactivity, cell-mixing experiments were performed. In these experiments, mixtures of APCs from strain Z and W were used to stimulate the reactivity of Th cells from TCLs ES-anti-Q. The reactivity of Th cell anti-Q was tested in cultures with or without Ts cells primed to Q, W, or Z. In cultures without Ts cells, Th cells primed to Q proliferated vigorously, consistent with the specific recognition of MHC class II Ags shared by strains Q and Z. This response, however, was not inhibited by Ts cells primed to Q or W, indicating that Ts cells do not inhibit Th cell reactivity to SLA class II Ags unless the SLA class I Ags which they recognize are coexpressed by the same APCs. Indeed inhibition of the response to mixtures of APCs from W and Z was observed only in the presence of Ts cells primed to Z \((p < 0.05)\), further demonstrating that the interaction of Ts cells and Th cells with the same APCs is required for suppression. This finding is consistent with the hypothesis that Ts cells interfere with the delivery of costimulatory signals by APCs to CD4\(^+\) Th cells (8).

It is possible, however, that in addition to interacting with APCs, Ts cells and Th cells also “communicate” with each other, recognizing TCR determinants or other structures in an MHC-restricted manner (14, 15). To explore this possibility, TCLs were generated by stimulating PBMCs from two HLA-disparate individuals, AP (HLA-A30, B35, DR\(^{B1\ast}0701\), 1301) and MN (HLA-A1, A32, B8, B44, DR\(^{B1\ast}0101\), 0301) with APCs from the same outbred pig (pig B). The blastogenic response of both TCLs (MN-anti-pig B and AP-anti-pig B) to pig APCs was significantly stronger \((p < 0.01)\) when CD8\(^+\) CD28\(^-\) Ts cells were depleted from the cell suspensions, indicating that CD4\(^+\) Th cell responses were suppressed by autologous Ts cells (Fig. 2). The reactivity of CD4\(^+\) Th cells from both lines to stimulating APCs was inhibited by CD8\(^+\) CD28\(^-\) Ts cells from either of these lines \((p < 0.01)\). The difference between the suppressor activity of Ts cells from MN-anti-pig B and AP-anti-pig B was not statistically significant. These results were confirmed in two additional experiments for which other TCLs were used. Hence, no MHC-restricted interaction between Th cells and Ts cells is required for suppression to occur.

To determine whether Ts cells secrete inhibitory factors, coculture experiments using semipermeable membranes, for separating Ts cells from Th cells, were performed. Th cells from TCL ES-anti-W were stimulated with irradiated xenogeneic APC from strain W in the bottom compartment, whereas Ts cells were stimulated with the same APCs in the top compartment. Xenoantigen-specific stimulation of Th cells was inhibited significantly only when Ts cells, Th cells, and APCs were in close contact, but not when Ts cells and Th cells were separated by a membrane \((p = 0.0001)\), indicating that cell-to-cell interaction is required for the suppressive effect induced by CD8\(^+\) CD28\(^-\) Ts cells to occur (Fig. 3A).

To further explore the possibility that suppression is mediated by inhibitory cytokines, such as IL-10 or TGF-β, experiments were performed in which mAbs to IL-10 and to TGF-β were added to cultures containing only Th cells or both Th cells, Ts cells, and stimulating APCs. These mAbs had no significant effect on Th cell proliferation in the absence of Ts cells (data not shown) and failed to abrogate or decrease the inhibitory effect induced by Ts cells on Th cell reactivity (Fig. 3B).

Cyttofluorographic analysis of Ts cells from three different TCLs (ES-anti-Q, ES-anti-W and ES-anti-Z) showed that they produced high levels of IFN-γ and moderate amounts of IL-2, yet no detectable levels of IL-4 and IL-10. Th cells from the same cultures produced high levels of IL-2 and IFN-γ, moderate amounts of IL-4, and no IL-10 (Fig. 4).

Study of Ts cell-induced apoptosis

We explored the possibility that the suppressive activity of xenogeneic Ts cells may be due to killing of pig APCs. Ts cells from a human TCL (GC-anti-swine Z), which inhibited by 88% the response of autologous Th cells to the specific stimulator, were tested for their ability to induce apoptosis or lysis of pig APC. Flow cytometry studies of apoptosis were performed by incubating Ts cells for 4 h with pig APCs in the presence or absence of xenogeneic CD4\(^+\) Th cells and then staining the cultures with annexin V. The percentage of annexin V-positive APCs was not significantly different in cultures with or without Ts cells, indicating that no apoptosis of pig APCs was induced (Fig. 5A). Also, the percentage of necrotic pig cells stained by PI was not significantly different in cultures with or without human Ts cells. Furthermore, cell-mediated lysis experiments in which PHA-activated pig lymphocytes were used as targets showed lysis when CD8\(^+\) CD28\(^-\) T cells
were used as effectors, but not when CD8^+ CD28^+ T cells from the same line were tested. This demonstrates that Ts cells do not kill xenogeneic APCs used for priming (Fig. 5B).

We next investigated the hypothesis that Ts cells may cause apoptotic death of xenoreactive Th cells. In these experiments, CD4^+ Th cells from TCL GC-anti-swine Z were incubated for 4 h with APCs from swine Z, in the presence or absence of autologous Ts cells. The percentage of annexin V-positive CD4^+ Th cells in cultures with Ts cells was not significantly different from the percentage found in cultures without Ts cells, indicating that suppression is not mediated by killing of xenoreactive Th cells (Fig. 5C). Hence, Ts cell suppressive activity is not due to killing of either Th cells or stimulatory APCs.

**Expression of CD40 ligand (CD40L) (CD154) on xenoreactive Th cells**

We have explored the possibility that Ts cells interfere with the costimulatory interaction between CD154 on Th cells (CD40L, T-BAM, p39, or TRAP) and CD40 on xenogeneic APCs. For this, we studied the expression of CD154 on xenoreactive CD4^+ Th cells which were stimulated with pig APCs in the presence or in the absence of Ts cells. After 6 h of incubation, cells were stained with mAbs anti-CD3, CD154, and either CD4 or CD8. Analysis of the results obtained in independent experiments, using six different TCLs, showed that the level of CD154 expression on CD4^+ Th cells was significantly higher (p < 0.01) in cultures containing pig APCs than in cultures without stimulating cells (Fig. 6A and 6B). However, expression of CD154 on CD4^+ Th cells was drastically reduced in the presence of Ts cells (Fig. 6C), indicating that Ts cells prevent Ag-induced up-regulation of CD154 on CD4^+ Th cells. There was a statistically significant difference between the level of CD154 expression on Th cell cultures with and without Ts cells (p < 0.01) in all six experiments. The up-regulation of CD154 was Ag specifc, requiring TCR activation, since it did not occur on CD4^+ Th cells challenged with APCs from an SLA class II-different pig (Fig. 6D). The expression of CD154 on xenoreactive CD4^+ Th cells was maximal after 6 h and decreased significantly after 18 h of incubation with stimulating APCs (data not shown). No expression of CD154 was observed on Ts cells at any time point studied. Hence, Ts cell-induced events that result in Th cell inhibition occur within the first 6 h of stimulation.

**FIGURE 3.** A, Diffusion chamber experiments. Th cells from TCL CG anti-W were tested for reactivity to APC from the specific stimulator (strain W) on close contact with Ts cells (from the same TCL) or separated from Ts cells by a semipermeable membrane. Percent suppression of Th cell reactivity by Ts cells is indicated. B, Th cells from TCL ES-anti-W were tested for reactivity to APC from the specific stimulator (strain W) in the presence of autologous Ts cells and the indicated mAbs.

**FIGURE 4.** Cytokine profile of xenoreactive Th cells and Ts cells. Th cells and Ts cells from TCL CG-anti-pig Z were activated with PMA and ionomycin. Cells were treated with brefeldin A and then fixed and stained with mAbs specific for IL-2, IFN-γ, IL-4, and IL-10. Histograms obtained for the activated samples (solid line) and resting control samples (dotted lines) are presented. The results are representative of three independent experiments.

**T helper cells**

![IL-2](image1)

![IFN-γ](image2)

![IL-4](image3)

![IL-10](image4)

**T suppressor cells**

![IL-2](image5)

![IFN-γ](image6)

![IL-4](image7)

![IL-10](image8)
Spectratyping of TCLs expressed by Ts cells

The V\textbeta\ gene usage of Ts cells from four human anti-pig TCLs (MN-anti-pig B, ES-anti-Q, ES-anti-W, and ES-anti-Z) was determined by spectratyping (Figs. 7 and 8). Ts cells from each of these xenoreactive TCL showed a restricted TCR V\textbeta\ gene usage. The side-by-side comparison of the V\textbeta\ repertoire expressed in un-

stimulated and stimulated CD8\textsuperscript{+}CD28\textsuperscript{−} T cells indicates that after two stimulations with xenogeneic APCs, there was oligoclonal ex-

FIGURE 5. Failure of Ts cells to induce killing of pig APCs or human xenoreactive Th cells. A. Pig APCs were incubated for 4 h with Th cells, Ts cells, or both Th and Ts cells. The percent of early apoptotic (annexin V positive, PI negative) and late apoptotic/necrotic (annexin V positive, PI positive) pig APCs in cultures with and without xenoreactive human T cells was determined by flow cytometry. Camptothecin-treated APCs were used as positive controls for apoptosis. B. CD8\textsuperscript{+}CD28\textsuperscript{−} and CD8\textsuperscript{+}CD28\textsuperscript{+} T cells from TCL ES-anti-Q were tested for their ability to kill PHA-

stimulated target cells from strain Q in a \textsuperscript{51}Cr release assay. Results are expressed as percent lysis. C. Human Th cells were incubated with pig APCs in the presence or absence of Ts cells. The percent of CD4\textsuperscript{+} human T cells undergoing apoptosis was determined by staining with annexin V and PI. The percent of early apoptotic (annexin V positive, PI negative) and late apoptotic/necrotic (annexin V positive, PI positive) Th cells is shown. CD4\textsuperscript{+} Th cells treated with camptothecin served as positive controls.

Spectratyping of TCLs expressed by Ts cells

The V\textbeta\ gene usage of Ts cells from four human anti-pig TCLs (MN-anti-pig B, ES-anti-Q, ES-anti-W, and ES-anti-Z) was determined by spectratyping (Figs. 7 and 8). Ts cells from each of these xenoreactive TCL showed a restricted TCR V\textbeta\ gene usage. The side-by-side comparison of the V\textbeta\ repertoire expressed in un-

FIGURE 6. Xenoreactive CD8\textsuperscript{+}CD28\textsuperscript{−} Ts cells prevent up-regulation of CD40L expression on xenoreactive CD4\textsuperscript{+} T cells. Human CD4\textsuperscript{+} T cells were incubated for 6 h without APCs (A), with APCs from the specific xenostimulator (pig W) (B), with APCs and Ts cells (C) or with control APCs from a pig (pig Z) that has different SLA class II Ags (D). CD154 expression on CD3\textsuperscript{+}CD4\textsuperscript{+} human T cells was analyzed by flow cytometry. The percent CD154-positive T cells and the mean fluorescence intensity (MFI) are indicated. The results obtained with this TCL (CO-anti-pig W) are representative of data obtained from six TCLs.

FIGURE 7. V\textbeta\ repertoire of unstimulated CD8\textsuperscript{+}CD28\textsuperscript{−} T cells from individual MN and of xenoreactive Ts cells from TCL MN-anti-pig B expressed as relative intensity. To analyze spectratypes, relative intensity was calculated as the peak area corresponding to each V\textbeta\ family divided by the sum of all peak areas.
and the relative intensities of V\(\beta\)23 were 0.48, 0.38, 0.30, and 0.23, respectively. V\(\beta\)16 was highly represented in all TCL generated from individual ES yet was absent in TCL obtained from another human subject (MN). V\(\beta\)5 was also expressed by Ts cells from all TCL derived from responder ES, but with lower intensity than V\(\beta\)16. In two of the TCLs from individual ES (ES-anti-pig W and ES-anti-pig Z), V\(\beta\)14 was represented with high intensity (Fig. 8, B and C). Other V\(\beta\) families such as V\(\beta\)15 were uniquely represented in one suppressor cell line (ES-anti-pig Z) (Fig. 8C), while absent from the other lines (ES-anti-Q, ES-anti-W, and MN-anti-pig B). Analysis of the CDR3 size distribution revealed a unimodal or bimodal distribution for each V\(\beta\) family, except V\(\beta\)23 that showed a multipeak gaussian-like distribution (Fig. 9). The oligoclonality of the V\(\beta\) repertoire expressed by xenoreactive Ts cells was also confirmed by analyzing the J\(\beta\)-V\(\beta\) fragments of V\(\beta\) families (Fig. 10).

**Discussion**

The phenotypic characteristics of suppressor cells as well as the mechanisms that underlie their function have been the object of numerous studies (16–25). Both CD4\(^+\) and CD8\(^+\) T cells with suppressive activity have been described, although Ag-specific suppressor cell lines have been difficult to generate.

CD4\(^+\) T cells producing TGF-\(\beta\), IL-4, and IL-10 were shown to play an important role in protecting animals from experimental autoimmune encephalomyelitis after oral feeding with Ag (21). IL-10 was recently shown to induce in vitro differentiation of regulatory CD4\(^+\) T cells with suppressor activity and inhibit alloantigen-specific reactivity of CD8\(^+\) T cells (7, 23). MHC class II-restricted CD8\(^+\) Ts cells that release IL-4 and suppress Th1 cell proliferation were described in human leprosy (24, 25). In the mouse model, CD8\(^+\) Ts cells were also described, yet these cells were restricted by nonclassical MHC class I Ags (Qa-1) expressed
by B cells and inhibited Th2 responses by production of IFN-γ (18). In other studies, suppression was mediated by Qa-1-restricted CD8+ T cells, which recognize TCR determinants on the membrane of CD4+ Th cells (14, 15, 25). The mechanism of antidiotypic suppression involved Th cell lysis or induction of Th cell apoptosis via ligation of Fas (15, 19).

An alternative mechanism of suppression seems to reside in inhibition of TCR-mediated cytotoxicity by CD8+CD28+ and CD4+CD28+ T cells, which express NK inhibitory receptors (26–28). The inhibitory effect of these killing-inhibitory receptors results from mobilization of protein tyrosine phosphatases on the cytoplasmic tail of killing-inhibitory receptor molecules (28).

In a previous study, we have shown that human CD8+CD28+ Ts cells, which inhibit alloreactive CD4+ Th cells, recognize HLA class I Ags on the surface of allogeneic APCs used for priming (8). The suppression was mediated by down-regulation of CD80 and CD86 expression on the allogeneic APCs and, thus, by impairment of their ability to deliver the costimulatory signals required for the activation of CD4+ Th cells in response to HLA class II alloantigens.

The present study demonstrates for the first time that the xenospecific response of human CD4+ Th cells to pig MHC class II Ags can be also suppressed by CD8+CD28− T cells immunized in vitro against xenogeneic MHC class I Ags. The suppressive effect was not mediated by idiotypic interactions between xenoreactive Ts cells and Th cells, since Th cells primed to APCs from an individual pig were efficiently suppressed not only by autologous but also by allogeneic human Ts cells immunized against the same SLA class I Ags.

The possibility that suppression of CD4+ Th cells was mediated by lymphokines secreted by CD8+CD28− Ts cells is also unlikely, since the suppressive activity required the interaction between Th cells and Ts cells with the same APCs. Thus, Th cell inhibition occurred only when the immunizing SLA class I and II Ags were coexpressed on the membrane of stimulating APCs, but not when these Ags were expressed by two distinct populations of APCs. Furthermore, diffusion chamber experiments in which Ts and Th cells were separated by semipermeable membranes showed that Th cell reactivity to xenogeneic APCs was not inhibited, indicating that suppression is not mediated by soluble factors. Cytolymphographic analysis of CD8+CD28− Ts cells showed that these cells produce IL-2 and IFN-γ, but not IL-4 and IL-10. Moreover, experiments using mAbs against inhibitory cytokines, such as IL-10 and TGF-β, excluded their contribution to the suppressor effect. Hence, neither the production nor the consumption of lymphokines by Ts cells (22, 24, 29) can explain their inhibitory effect on Th cells in this system.

In the allogeneic system, we demonstrated that Ts cells interfere with Th cell-induced up-regulation of B7 (CD80, CD86) expression on APC (8). The interaction between CD40 on APC and CD40L (CD154), a transiently expressed CD4+ T cell molecule, is essential for the induction of accessory molecules on APCs, in

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**FIGURE 9.** Vβ spectratyping of Ts cells from TCL MN-anti-pig B. Only the families with positive signal are shown. x-axis, fragment lengths on base pairs; y-axis, fluorescence amplitude.
particular CD80, CD86, and 4–1BB ligand, and for the initiation of Ag-specific T cell reactivity (30–35). However, blockade of either CD28/B7 or CD40L/CD40 pathways does not inhibit completely T cell-mediated alloimmune responses, indicating that, although interrelated, the CD28 and CD40L pathways serve as independent regulators of T cell responses (36).

We have explored the possibility that Ts cells interfere with the expression of CD40L (CD154) on activated CD4+ Th cells. Cytofluorographic analysis showed that up-regulation of CD154 expression on xenoreactive CD4+ Th cells was induced by pig APCs, indicating that human CD154/pig CD40 interaction contributes to the strong proliferative response occurring on recognition by human TCRs of SLA class II Ags. Hence, in the human-pig system, xenogenotyp-specific CD4+ Th cell responses involve not only the CD28/B7 and CD2/LFA1 costimulatory pathways, as previously described (37), but also the CD154/CD40 pathway. However, the expression of CD154 on xenoreactive CD4+ Th cells was significantly reduced in the presence of Ts cells. The molecular mechanism of CD154 down-regulation on xenoreactive CD4+ Th cells by Ts cells is currently under investigation. The possibility that Ts cells prevent up-regulation of CD40L on CD4+ T cells by killing the xenogeneic stimulating cells or by inducing Th cell apoptosis was ruled out since no evidence of Ts cell-induced cell death was found by either flow cytometry of 51Cr release studies. Proliferation of CD4+ T cells was not restored in the presence of cells expressing constitutively CD40L, suggesting that costimulation of xenogeneic APCs through the CD40–CD40L pathway is not sufficient to circumvent the suppressive effect of Ts cells (A. I. Colovai, manuscript in preparation). Down-modulation of CD154 by Ts cells may lead, however, to disengagement of Th cells from the targets, preventing full activation and proliferation of these cells.

T cell reactivity to allogeneic and xenogeneic MHC Ags bears resemblance to TCR activation by nominal Ags and pathogens, as it involves recognition of targets expressing novel MHC/peptide complexes. Since the generation of allo- or xenospecific Ts cells in vitro requires multiple rounds of stimulation, it is possible that the chronic exposure to Ag is also required in vivo for the induction of Ts cells. The oligoclonal expansion of a few TCR Vβ families observed within the population of xenoreactive Ts cells is reminiscent of the skewed TCR repertoire displayed by T lymphocytes with HLA class I-specific NK-inhibitory receptors (27), a phenomenon suggested to result from chronic antigenic stimulation.

MHC class I-restricted Ts cells may play a physiologic role in regulating the immune response of Th cells against self or nonself peptide/MHC class II complexes. The finding that there is cross-talk between the MHC class I and class II pathways of peptide processing supports the notion that the same APCs present both helper- and suppressor-inducing peptides (38). It is possible that recognition by Ts cells of MHC class I-bound peptides helps control local inflammation caused by Ag-specific Th cells. Identification of suppressor-inducing peptides may be useful for induction of unresponsiveness to auto-, allo-, or xenogeneic T cells. Furthermore, understanding of the mechanism of Ts cell-mediated down-regulation of CD154 expression on activated Th cells may contribute to the development of new immunotherapeutic strategies.

This issue becomes particularly important in view of the recent finding that Th cells condition the APCs to directly stimulate T killer cells by CD154–CD40 signaling, rather than by delivering short range acting lymphokines (39–42). The emerging picture from our studies is that Ts cells down-regulate the immune response by interfering with CD154–CD40 signaling, thus preventing the up-regulation of costimulatory (B7) molecules on APCs.

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References
