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Role for Thymic and Splenic Regulatory CD4⁺ T Cells Induced by Donor Dendritic Cells in Allograft Tolerance by LF15-0195 Treatment

Elise Chiffoleau,* Gaëlle Bériou,* Patrick Dutartre,† Claire Usal,* Jean-Paul Soullilou,* and Maria Cristina Cuturi2*

A 20-day treatment with LF15-0195, a deoxyspergualine analogue, induced allograft tolerance in a fully MHC-mismatched heart allograft model in the rat. Long-term allografts displayed minimal cell infiltration with no signs of chronic rejection. CD4⁺ spleen T cells from tolerant LF15-0195-treated recipients were able to suppress in vitro proliferation of allogeneic CD4⁺ T cells and to transfer tolerance to second syngeneic recipients, demonstrating dominant suppression by regulatory cells. A significant increase in the percentage of CD4⁺CD25⁺ T cells was observed in the thymus and spleen from tolerant LF15-0195-treated recipient. In vitro direct stimulation with donor APCs demonstrated that CD4⁺ regulatory T cells proliferated weakly and expressed low levels of IFN-γ, IL-10, and IL-2. CD4⁺CD25⁺ cell depletion increased IL-2 production by CD4⁺CD25⁻ thymic cells, but not splenic cells. Moreover, tolerance was transferable with splenic and thymic CD4⁺CD25⁺ cells, but also in 50% of cases with splenic CD4⁺CD25⁻ cells, demonstrating that CD25 can be a marker for regulatory cells in the thymus, but not in the periphery. In addition, we presented evidences that donor APCs were required to induce tolerance and to expand regulatory CD4⁺ T cells. This study demonstrates that LF15-0195 treatment induces donor APCs to expand powerful regulatory CD4⁺CD25⁺ T cells present in both the central and peripheral compartments. The Journal of Immunology, 2002, 168: 5058–5069.

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Materials and Methods

Animals and transplantations

Eight-week-old male LEW.1W or Lewis rats served as heart donors and LEW.1A rats as allograft recipients (Center d’Elevage Janvier, Le Genest-Saint-Isle, France). Rats were congeneric and differed in haplotype. LEW.1W rats were RT1.u (A/B, u, C/D,u), Lewis rats were RT1.1 (A/B, I, C/D,1), and LEW.1A rats were RT1.a (A/B-a, C/D,a). Heterotopic heart grafts were performed using the Ono and Lindsey technique (33). The grafts were evaluated daily for function by palpation, and rejection was defined as the day of cessation of heartbeat.

Immunosuppression

LF15-0195 (Laboratoires Fournier, Daix, France) was prepared in PBS and delivered to allograft recipients by i.p. injection at 3 mg/kg for 20 days starting the day of cardiac transplantation.

In vivo transfer experiments

Irradiation. LEW.1A secondary recipients were treated with 4.5 Gy whole body irradiation (Center René Gauduchau, Nantes, France) 1 day before transplantation.

Cell transfer. Total spleen (2 × 10^6) or CD4^+ CD25^+ or CD4^+ CD25^- thymus and spleen T cells (5 × 10^5) from naive rats or from LF15-0195-treated recipients (>100 days) were injected i.v. into a secondary syngeneic recipient the day of cardiac transplantation.

Depletion of passenger leukocytes in allografts

Donor LEW.1W (RT1.u) rats received a single i.p. dose (300 mg/kg) of cyclophosphamide (Sigma-Aldrich, St. Louis, MO) 5 days before graft harvesting to deplete heart graft leukocytes, as previously described (34, 35). Grafts were extensively washed before transplantation.

Thymectomy of recipients

Thymectomy of LEW.1A recipients was performed 2 wk before cardiac transplantation.

Antibodies

The following hybridomas (mouse IgG) were obtained from the European Collection of Animal Cell Culture (Salisbury, U.K.) and were used to phenotype rat leukocytes: OX6 (anti-class II MHC), ED3 (recognizing sia-loadhesin on macrophages), Ox33 (anti-CD45 present on B cells), Ox1 and Ox30 (anti-CD45), R7-3 (anti-TCRβ), ED1 (recognizing CD68 on monocytes, macrophages, granulocytes, and dendritic cells (DCs)), W3/25 (anti-CD4), Ox8 (anti-CD8α), Ox 39 (anti-CD25), and Ox3 (anti-RT1.u). These mAbs were purified from hybridoma culture supernatants in our laboratory. T lymphocytes were purified from splenocytes and thymocytes by negative selection. Briefly, total spleen cells or thymocytes were incubated for 30 min on ice with a mixture of mouse anti-rat Abs: Ox6, ED3, Ox33, and Ox8. After two washes, cells were then incubated for 20 min under agitation with superparamagnetic beads with affinity-purified goat anti-mouse IgG covalently bound to the surface (Dynal, Oslo, Norway). These stained contaminating cells were then eliminated with a magnet. The purity of the collected CD4^+ T cells was controlled by FACS analysis (FACSScan; BD Biosciences, Mountain View, CA) with an anti-TCRβ mAb (R7-3) and an anti-CD4 (W3/25) (purity >95%).

CD25^+ T cell purification. CD25-positive cells were enriched using the MACS system (Magnetic Cell Sorting; Miltenyi Biotec, Paris, France). Briefly, spleen- or thymus-purified CD4^+ T cells were incubated with biotinylated Ox39 mAb (20 μg/1 × 10^6 cells, 30 min at 4°C). After two washes, cells were incubated with streptavidin Microbeads (200 μg/1 × 10^6 cells) for 30 min at 4°C. After two washes, bound cells were separated using a separation column placed in a strong magnetic field. The purity of the unbound or bound collected T cells was controlled by FACS analysis (FACSScan; BD Biosciences) with a FITC anti-CD25 mAb (Ox39). Purity was >90%.

Mixed leukocyte reaction (MLR)

Recovered low-density cells corresponding to APC-enriched cell populations from donor-type LEW.1W (RT1.u) or third-party Lewis (RT1.1) rats were irradiated and served as stimulator cells.

As a source of donor Ags for studies of indirect presentation, LEW.1W spleen cells were suspended at concentration of 1 × 10^8/ml in supplemented RPMI 1640 and lysed with three pulses of frozen step at −80°C, and then thawed at room temperature, as previously described (37). Any residual intact cells or cell membranes were removed by centrifugation at 1800 rpm for 10 min at 4°C. Then, LEW.1A syngeneic APC-enriched cell population was cultured for 4 h with LEW.1W splenocyte lysate before being irradiated.

Responder (T cells) (2 × 10^5) and stimulatory cells (5 × 10^5) were plated in 96-well round-bottom plates in triplicate in a volume of 200 μl of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 5 × 10^{-3} M 2-ME, 1 mM sodium pyruvate (Life Technologies), 1% nonessential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated (56°C, 30 min) FCS (Life Technologies).

The cultures were incubated at 37°C, in 5% CO_2 and pulsed for the last 8 h with 0.5 μCi of [3H]TdR (Amersham, Les Ulis, France). The cells were then harvested on glass fiber filters, and [3H]TdR incorporation was measured using standard scintillation procedures (Packard Institute, Meriden, CT).

Determination of antigen alloantibodies

LEW.1W splenocytes from untreated or LF15-0195-treated recipients were incubated with decomplexed sera and diluted 1/4 in PBS containing 0.5% BSA (Sigma-Aldrich) and 0.02% sodium azide. To stain for IgG, cells were reacted with FITC affinity pure F(ab')2, mouse anti-rat IgG, Fcy fragment-specific Ab; mouse anti-rat IgG1, IgG2a, and IgG2b, Fcy fragment specific; and FITC goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cytokine assays

Supernatants from triplicate cultures of MLR were harvested and combined 72 h after stimulation. IFN-γ, IL-10, and IL-2 were measured using an ELISA from BD Pharmingen OptEIA (San Diego, CA) according to the manufacturer’s instructions.

Cell purification

Donor- and third-party APC. APC were enriched from spleen fragments digested with collagenase D (2 mg/ml; Boehringer Mannheim, Mannheim, Germany) for 30 min at 4°C. A total of 10 × 10^6 EDTA was added for 5 min, and cells were washed and resuspended in 5 × 10^5 EDTA-PBS containing 2% heat-inactivated FCS. Four milliliters of this suspension was layered onto a 14.5% Nicodenz gradient (Nycomed Pharma, Roskilde, Denmark) and centrifuged for 13 min at 2800 rpm at 4°C.

Total spleen and thymus cells. Cell suspensions from spleens and thymus were prepared, as described previously (35), from naive rats, from untreated recipients, or from LF15-0195-treated recipients sacrificed 100 days after transplantation.

Spleen and thymus CD4^+ T cell purification. T lymphocytes were purified from splenocytes and thymocytes by negative selection. Briefly, total spleen cells or thymocytes were incubated for 30 min on ice with a mixture of mouse anti-rat Abs: Ox6, Ed3, Ox33, and Ox8. After two washes, cells were then incubated for 20 min under agitation with superparamagnetic beads with affinity-purified goat anti-mouse IgG covalently bound to the surface (Dynal, Oslo, Norway). These stained contaminating cells were then eliminated with a magnet. The purity of the collected CD4^+ T cells was controlled by FACS analysis (FACSScan; BD Biosciences, Mountain View, CA) with an anti-TCRβ mAb (R7-3) and an anti-CD4 (W3/25) (purity >95%).

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Coupled to PE (Bioatlantic, Nantes, France). Secondary Abs included bi-
RNA extraction

Heart samples at 5 or 100 days after transplantation were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNAs from whole allografts were extracted according to the technique of Chirgwin (38). Total RNAs from purified cells were extracted using the technique of Chomczynski and Sacchi (39). The RNAs were quantified by UV absorbance at 260 nm.

Quantitative RT-PCR

Quantitative RT-PCRs were performed on the Applied Biosystems Prism 7700 (PE-Biosystems, Foster City, CA) using the TaqMan chemistry (PE Biosystems under license of Roche Molecular Systems, Pleasanton, CA). This TaqMan system performed real-time kinetic PCR and true quantitative gene analysis. The sequences of the gene-specific primers are given in Table I. Standards were prepared by PCR amplification of each target sequence using these primers. PCR products were extracted, and the A260 allowed the quantification of the template in the standards. The standards were diluted to load 10^2–10^3 copies/well. Total RNAs from grafts or from cells were reverse transcribed using oligo(dT), as previously described (40). A constant amount of cDNA corresponding to the reverse transcription of each cDNA sample was amplified using the SYBR Green PCR Core kit (PE-Biosystems) containing the primers of 100 ng of total RNA, or each dilution of the standard, was amplified (40). A constant amount of cDNA corresponding to the reverse transcription of 100 ng of total RNA, or each dilution of the standard, was amplified using the SYBR Green PCR Core kit (PE-Biosystems) containing the primers for hypoxanthine phosphoribosyltransferase (HPRT), IL-10, TGF-β, or CTLA-4 (cf Table I). The PCR efficiencies of all of the standards were >99%, and the fluorescence was always >0.95. Data were expressed as ratios of the number of copies of the specific gene to the number of copies of the HPRT gene.

Statistical analysis

Statistical evaluation was performed using the Student’s t test for unpaired data, and results were considered significant if p values were <0.05. Data were expressed as mean ± SD.

Results

Allografts from tolerant LF15-0195-treated recipients (>100 days) had no signs of chronic rejection

We previously described that a treatment for 20 days with LF15-0195 (3 mg/kg, i.p. daily) prolonged allograft survival to >100 days in a rat MHC-mismatched (LEW.1W to LEW.1A) heart allograft model (28) (see Table V). Acceptance of donor LEW.1W (RT1.u), but not of third-party Lewis (RT1), second heart allografts in the neck by tolerant long-term LF15-0195-treated recipients demonstrated donor-specific allograft tolerance (28).

Immunohistological analysis of allografts from tolerant LF15-0195-treated recipients (100 days) demonstrated a weak infiltration (6% of area infiltrate) by leukocytes (CD45-positive cells) (Fig. 1). For comparison, 29 and 11% of area infiltrate was observed, respectively, in rejected grafts from untreated recipients and in allografts from LF15-0195-treated recipients at day 5 after grafting. Four percent of area infiltrate in allografts from tolerant LF15-0195-treated recipients were CD68-positive cells and 1% were T cells. At >250 days after grafting, vessels of allografts from LF15-0195-treated recipients were not obstructed, and the intima was not thick and contained no lymphocytes. No signs of fibrosis were observed (Fig. 2). These data demonstrated that tolerant allografts displayed no signs of chronic rejection.

Quantitative analysis of cytokine mRNA expression in allografts

Cytokine mRNA expression analysis was performed in allografts from untreated or LF15-0195-treated recipients harvested 5 or 100 days after transplantation (n = 4). We demonstrated that IFN-γ and IL-10 mRNA expression was decreased in allografts from LF15-0195-treated recipients compared with allografts from untreated recipients at day 5 after grafting, whereas the mRNA expression of IL-13 and TGF-β was not different between the two groups (28) (Table II).

Low mRNA expression (100-fold less) of IL-2, IFN-γ, and IL-13 was observed in allografts from tolerant LF15-0195-treated recipients at day 100 after grafting compared with those at day 5 after grafting (data not shown). Allografts from tolerant LF15-0195-treated recipients expressed a weak level of TGF-β mRNA that was 20-fold less than at day 5 after grafting (Table II). We observed a significant increase (2-fold, p < 0.05) in IL-10 mRNA expression.

Table I. Oligonucleotides sequences (5’ to 3’) used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Forward HPRT</td>
<td>GCCAAGTTGGAAAAGCCAAGT</td>
</tr>
<tr>
<td>Reverse HPRT</td>
<td>GCCACATCAACAGGAGTCTGTAG</td>
</tr>
<tr>
<td>Forward IL-10</td>
<td>TCAAGCACTGCTATAGTGCCC</td>
</tr>
<tr>
<td>Reverse IL-10</td>
<td>CCTGTGTATATTCTAGAGG</td>
</tr>
<tr>
<td>Forward TGF-β</td>
<td>TGCTGGATCTACATTAAAGGCC</td>
</tr>
<tr>
<td>Reverse TGF-β</td>
<td>CTGCTGGATCTACATTAAAGGCC</td>
</tr>
<tr>
<td>Forward CTLA-4</td>
<td>GGCGACAATGAAGGCAAGT</td>
</tr>
<tr>
<td>Reverse CTLA-4</td>
<td>TCTGAATCTGGGCATGGTCTT</td>
</tr>
</tbody>
</table>

FIGURE 1. Cellular infiltrate phenotype in allografts from untreated or LF15-0195-treated recipients (days 5 and 100 after transplantation). Phenotypic analysis of cellular infiltrates in heart allografts from untreated recipients at day 5 (A), or LF15–0195-treated recipients at day 5 (B) or at day 100 (C) after transplantation. Sections were stained with Oxl/Ox30 (anti-CD45 present on all leukocytes), ED1 (recognizing CD68 present on monocytes, macrophages, granulocytes, and DCs), and R73 (anti-TCRαβ), and quantified as described in Materials and Methods. Results are expressed as the mean ± SD of percentage area infiltrate of four recipients in each group.

p < 0.05 (Student’s t test).

FIGURE 2. Histological analysis of long-term heart allografts from LF15-0195-treated recipients. Sections of long-term heart allografts from LF15-0195-treated recipients >250 days after transplantation were stained with hematoxylin-eosin-saffron. Micrograph is one allograft representative of five analyzed with the same procedure (objective ×40). Arrows showed clean vessels not obstructed. One contains RBCs (arrow in middle west).
expression in allografts from tolerant LF15-0195-treated recipients at day 100 after grafting compared with those at day 5 after grafting. IL-10 mRNA expression in allografts could be due to the restoration of IL-10 expression by CD68-positive cells (essentially macrophages) after treatment cessation, but also, IL-10 and TGF-β mRNA expression could be due to their expression by Th2 or regulatory T cells that could progressively infiltrate allografts (41).

**LF15-0195-treated recipients had decreased antidonor alloantibodies of the Th1-related isotype**

We have previously demonstrated that LF15-0195 treatment totally inhibited antidonor alloantibody production during treatment (28). To determine whether the production of alloantibodies was restored after treatment cessation in tolerant recipients at days 30 and 100 after grafting, we assessed antidonor IgG and isotype subclasses in sera, as described in Materials and Methods. Results are expressed as mean fluorescence channel for total IgG or for isotypes. Fig. 3 shows that 10 days after treatment cessation (day 30), the antidonor alloantibody response was totally inhibited in tolerant LF15-0195-treated recipients compared with rejecting untreated recipients (n = 4, p < 0.001). On day 100 after transplantation, total antidonor IgG levels were only partially restored in LF15-0195-treated recipients in contrast to untreated recipients (n = 4, p < 0.01). Analysis of the isotypes of alloantibodies produced showed that Th1-related IgG2b production was partially restored (n = 4, p < 0.005), whereas IgG2a and Th2-related isotype IgG1 were fully restored (n = 4). These results suggest that following LF15-0195 treatment cessation, tolerant animals recovered a partial antidonor alloantibody response with a preferential development of IgG1 alloantibodies, a Th2-related isotype, at the expense of IgG2b alloantibodies, a Th1-related isotype. Thus, LF15-0195 treatment could have modulated helper CD4+ T cells to inhibit production of Th1-related isotype Abs by B cells.

**Decreased donor-specific response of spleen T cells from tolerant LF15-0195-treated recipients (>100 days) in MLR**

Purified spleen T cells or total lymph node cells from LEW.1A (RT1.a)-untreated or LF15-0195-treated recipients (>100 days) were stimulated for 72 h with a donor LEW.1W (RT1.u) or third-party Lewis (RT1.l) APC-enriched population. We observed that spleen T cells from LF15-0195-treated recipients (>100 days) proliferated less (65% decrease) than those from untreated recipients when stimulated by donor LEW.1W APC (Fig. 4A). Proliferation was similar in the two groups when T cells were stimulated by third-party Lewis APC (Fig. 4B). In contrast, proliferation was similar when lymph node cells from LF15-0195-treated recipients or from untreated recipients were stimulated by donor (Fig. 4C) or third-party APC (Fig. 4D). These results demonstrated a compartmentalization of the donor-specific inhibition of the proliferative response of T cells from LF15-0195-treated recipients. This inhibition could be related to a clonal deletion of donor-specific T cells and/or the presence of regulatory cells.

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**Table II. Cytokines mRNA expression in allografts from LF15-0195-treated recipients**

<table>
<thead>
<tr>
<th>Cytokine/HPRT Transcripts Ratio (×10^-3)</th>
<th>TGF-β</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (5 days after grafting)</td>
<td>199.2 ± 65.99</td>
<td>162.7 ± 1.81</td>
</tr>
<tr>
<td>LF15-0195 treated (5 days after grafting)</td>
<td>112.3 ± 68.35</td>
<td>18.71 ± 11.19</td>
</tr>
<tr>
<td>LF15-0195 treated (&gt;100 days after grafting)</td>
<td>5.606 ± 5.486*</td>
<td>38.03 ± 3.429*</td>
</tr>
</tbody>
</table>

* Allografts from untreated or LF15-0195-treated recipients were harvested 5 or 100 days after transplantation and cytokine mRNA expression was analyzed by quantitative RT-PCR as described in Materials and Methods. Results are expressed as mean ± SD of the number of cytokine/number of HPRT transcript ratio for four animals in each group.

* p < 0.05 (Student’s t test).
Inhibition of in vitro proliferation of alloreactive CD4⁺ T cells by CD4⁺ spleen T cells from tolerant LF15-0195-treated recipients only with stimulation by donor APC (direct presentation of donor alloantigens)

To test the presence of regulatory cells able in vitro to inhibit alloreactive T cell proliferation, we performed a coculture system in which the same number of spleen CD4⁺ T cells from tolerant LF15-0195-treated recipients was added to spleen CD4⁺ T cells from untreated recipients (100 days after grafting). These cells were stimulated by either donor LEW.1W APC (direct presentation) or syngeneic LEW.1A APC pulsed with LEW.1W Ags (indirect presentation), as described in Materials and Methods (Fig. 5). We observed that CD4⁺ spleen T cells from tolerant LF15-0195-treated recipients proliferated less (80% decrease) compared with CD4⁺ spleen T cells from untreated recipients stimulated by direct presentation of donor Ags. Moreover, CD4⁺ spleen T cells from tolerant LF15-0195-treated recipients mixed with the same number of CD4⁺ spleen T cells from untreated recipients and stimulated by direct presentation of donor Ags reduced the proliferation by 50% compared with that of CD4⁺ spleen T cells from untreated recipients alone. In contrast, although the proliferation of CD4⁺ spleen T cells from tolerant LF15-0195-treated recipients stimulated by the indirect pathway was reduced compared with those from untreated recipients (70% decrease), suppression of proliferation was not observed in coculture. These results demonstrated, in this model, an in vitro dominant suppression by CD4⁺ spleen T cells from tolerant LF15-0195-treated recipients only with stimulation by direct presentation of donor Ags.

Transfer of tolerance with splenocytes

To test the possibility of the involvement of regulatory cells in tolerance maintenance, we performed spleen cell transfers from LF15-0195-treated recipients (>100 days) into secondary syngeneic graft recipients. When no cells were injected, irradiated LEW.1A secondary recipients rejected LEW.1W heart allografts in 18.2 ± 4.4 days (n = 5), demonstrating the immunocompetence of recipients (Table III). When splenocytes (2 × 10⁸) from naive rats were injected, LEW.1W heart allografts were rejected in 12.5 ± 2.9 days (n = 4). In contrast, when splenocytes (2 × 10⁹) from LF15-0195-treated recipients were injected, LEW.1W heart allografts were definitively accepted (>100 days) (n = 4; p < 0.01), whereas Lewis third-party allografts were rejected in 9 days (n = 3; p < 0.01). Infectious tolerance by in vivo adoptive transfer demonstrated the presence of potent donor-specific regulatory cells in splenocytes from LF15-0195-treated recipients.

Decreased percentage of CD4⁺ Thy-1⁻ T cells in the spleen and increased percentage of CD4⁺CD25⁺ T cells in the thymus and the spleen from long-term LF15-0195-treated tolerant recipients

To analyze whether a subpopulation of cells, which could be regulatory cells, was increased in LF15-0195-treated recipients, we performed a phenotypic analysis of cells from the thymus, spleen, and lymph nodes for different known markers of regulatory cells (41).

We observed no change in the absolute numbers of T cells, CD4⁺ T cells, or CD8⁺ T cells in spleen and lymph nodes from LF15-0195-treated recipients as compared with those from untreated recipients (data not shown). Interestingly, in the spleen T cells from LF15-0195-treated recipients, we observed a significant decrease in the percentage of Thy-1⁻ CD4⁺ T cells (recent thymic emigrants, RTE) (2.21% ± 1.39, n = 4) compared with spleens from either untreated recipients (>100 days) (6.9% ± 0.79, n = 4) (p < 0.001) or from naive rats (7.78% ± 1.74, n = 4) (p < 0.002) (Table IV).

In addition, we observed a higher percentage of CD25⁺ cells in CD4⁺ spleens T cells from LF15-0195-treated recipients (24.6% ± 6.21, n = 6) compared with those from naive rats (7.12% ± 0.63, n = 6) (p < 0.0003) or from untreated recipients (10.95% ± 2.36, n = 7) (p < 0.0003) (Table IV). A typical CD4⁺CD25 FACS analysis is shown in Fig. 6 that illustrates the increase in percentage of these cells in spleen T cells from LF15-0195-treated recipients (25%) compared with those from naive rats (6%). Moreover, the absolute number of CD4⁺CD25⁺ T cells in spleens from LF15-0195-treated recipients was increased (37 × 10⁶ ± 13 × 10⁶ cells (n = 5)) compared with one from untreated recipients (13.2 × 10⁶ ± 1.4 × 10⁶, n = 3; p < 0.03) or naive rats (12.7 ± 10⁶ ± 0.7 × 10⁶, n = 3; p < 0.03).

The same increase in percentage of CD4⁺CD25⁺ TCR⁺ cells was observed in thymocytes from LF15-0195-treated recipients (19.30% ± 8.44, n = 3) compared with thymocytes from untreated recipients (2.28% ± 0.30, n = 3; p < 0.03) or naive rats (4.21% ± 2.14, n = 3; p < 0.04) (Table IV). The increase in CD4⁺CD25⁺ was not observed in lymph nodes from LF15-0195-treated recipients (data not shown). Moreover, a 20-day treatment

Table III. Survival of allografts in secondary LEW.1A recipients after transfer of splenocytes from tolerant LF15-0195-treated recipients

<table>
<thead>
<tr>
<th>Cell Donor</th>
<th>Graft</th>
<th>Graft Survival (days)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive LEW.1A (2 × 10⁶)</td>
<td>LEW.1W</td>
<td>18.2 ± 4.4 (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Tolerant LF15-0195-treated LEW.1A (2 × 10⁶)</td>
<td>LEW.1W</td>
<td>&gt;100 (n = 4)</td>
<td>*</td>
</tr>
<tr>
<td>Tolerant LF15-0195-treated LEW.1A (2 × 10⁶)</td>
<td>LEW.1L</td>
<td>9 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>Thymectomized tolerant LF15-0195-treated LEW.1A (2 × 10⁶)</td>
<td>LEW.1W</td>
<td>&gt;100 (n = 3)</td>
<td></td>
</tr>
</tbody>
</table>

* Secondary LEW.1A recipients were irradiated with 4.5 Gy 1 day before the transfer of 2 × 10⁶ syngeneic spleen cells.

+, p < 0.05 (Student’s t test).

FIGURE 5. Spleen CD4⁺ T cells from tolerant LF15-0195-treated recipients suppress in vitro proliferation of alloreactive CD4⁺ T cells only with stimulation by donor APC. A total of 2 × 10⁸ spleen CD4⁺ T cells from untreated recipients or 2 × 10⁸ spleen CD4⁺ T cells from tolerant LF15-0195-treated recipients or both cocultured was stimulated by LEW.1W donor-irradiated APC-enriched cell population (direct presentation) or LEW.1A syngeneic irradiated APC-enriched cell population pulsed with donor Ags (indirect presentation). Values represent the cpm ± SD of all triplicates after 3 days of culture for thymidine incorporation. Results are representative of three independent experiments.
with LF15-0195 on naive, ungrafted LEW.1A rats did not increase the percentage of CD4+CD25+ T cells in spleen and thymus (data not shown), demonstrating that the increase of CD4+CD25+ T cell number is specific of tolerance model induced by LF15-0195 treatment.

**Weak secretion of IFN-γ, IL-2, and IL-10 by CD4+ spleen T cells from tolerant LF15-0195-treated recipients on stimulation with donor APC (direct presentation)**

Spleen CD4+, CD4+CD25−, or CD4+CD25+ cells were stimulated 3 days by LEW.1W donor APC-enriched populations. As previously demonstrated in Fig. 5, CD4+ spleen T cells from tolerant LF15-0195-treated recipients proliferated less (67 × 10^3 cpm) than CD4+ spleen T cells from either naive rats (172 × 10^3 cpm) or untreated recipients (>100 days) (289 × 10^3 cpm) (Fig. 7A). Total CD4+ T cells from tolerant LF15-0195-treated recipients secreted less IFN-γ (10 × 10^3 pg), IL-2 (1.6 × 10^3 pg), and IL-10 (5 × 10^3 pg) than CD4+ T cells from untreated recipients (55 × 10^3, 4 × 10^3, and 18 × 10^3 pg, respectively) (Fig. 7, B–D). Surprisingly, in contrast to CD4+CD25− from naive rats that did not proliferate in MLR (data not shown), CD4+CD25+ spleen T cells from tolerant LF15-0195-treated recipients stimulated by donor APC were able to proliferate (10^5 × 10^3 cpm) as total CD4+ T cells (67 × 10^3 cpm), and depletion of this subpopulation (CD4−CD25− subpopulation only) did not increase proliferation (84 × 10^3 cpm) (Fig. 7A). CD4+CD25+ and CD4+CD25− subpopulation of T cells from tolerant LF15-0195-treated recipients secreted the same level of IFN-γ (9 × 10^3 and 7 × 10^3 pg, respectively) and IL-10 (6 × 10^3 and 5 × 10^3 pg, respectively). No IL-2 was detected in supernatants of CD4+CD25+ spleen T cells from tolerant LF15-0195-treated recipients, and depletion of this subpopulation did not restore production of IL-2 by CD4+CD25− subpopulation (1.5 × 10^3 pg) (Fig. 7, B–D). These results demonstrated that splenic CD4+CD25+ T cells from tolerant LF15-0195-treated recipients stimulated by donor APC (direct presentation) were able to proliferate, but did not produce IL-2. Moreover, the CD4+CD25− subpopulation produced low level of IL-2, suggesting that donor-specific regulatory cells maintaining the inhibition of IL-2 could also be present in the CD25− subpopulation.

**CD4+CD25+ thymocytes inhibited IL-2 production by CD4+CD25− thymocytes**

CD4+ thymocytes from LF15-0195-treated recipients stimulated by donor APC proliferated as well as CD4+ thymocytes from naive rats (30 × 10^3 cpm vs 24 × 10^3 cpm, respectively) (Fig. 8A). However, we denoted that CD4+ thymocytes from naive rats proliferated less in MLR than spleen CD4+ T cells (30 × 10^3 cpm vs 172 × 10^3 cpm, respectively). CD4+ thymocytes from LF15-0195-treated recipients expressed the same level of IFN-γ (1.9 × 10^3 pg) and IL-10 (1.6 × 10^3 pg), but less IL-2 (88 pg) than CD4+ thymocytes from naive rats (1.5 × 10^3, 1.2 × 10^3, 375 pg, respectively) (Fig. 8, B–D). As for spleen cells, no difference in proliferation was observed between CD4+CD25+ (21 × 10^3 cpm) and CD4+CD25− thymocyte subpopulation from LF15-0195-treated recipients (25 × 10^3 cpm) (Fig. 8A). However, CD4−CD25+ thymocytes produced 2-fold less IFN-γ (420 pg) than CD4+CD25− thymocytes (950 pg) (Fig. 8B). No difference in production of IL-10 was observed between CD4+CD25+ and CD4+CD25− thymocytes (Fig. 8C). Interestingly, CD4−CD25− thymocytes produced no IL-2, and depletion of this population (CD4−CD25− cells only) restored the high production of IL-2 (540 pg) to the same level as the production in naive rats (375 pg) (Fig. 8D). These results demonstrate that CD4+CD25+ thymocytes from tolerant LF15-0195-treated recipients proliferated in vitro by stimulation with donor APC. However, CD4+CD25− thymocytes expressed no IL-2, and depletion of the CD4+CD25+ population restored secretion of IL-2 by CD4+CD25− cells. These results demonstrated that CD4+CD25− thymocytes from LF15-

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**Table IV. Percentage of CD4+Thy1+ cells and CD4+CD25+ cells in thymocytes and CD4+ spleen T cells**

<table>
<thead>
<tr>
<th></th>
<th>% CD4+Thy1+</th>
<th>% CD4+CD25+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive rats</td>
<td>7.78</td>
<td>4.21</td>
</tr>
<tr>
<td>Untreated recipients (&gt;100 days)</td>
<td>6.90</td>
<td>2.28</td>
</tr>
<tr>
<td>Tolerant LF15-0195-treated recipients (&gt;100 days)</td>
<td>2.11</td>
<td>19.30</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>1.74</td>
<td>0.30</td>
</tr>
</tbody>
</table>

---

**Figure 6.** Representative FACS staining of CD4+CD25− cells in spleen T cells from naive rats or LF15-0195-treated recipients. Spleen T cells from naive rats or from tolerant LF15-0195-treated recipients were purified and double stained for FACS analysis, as described in Materials and Methods, with W3/25-PE (anti-CD4) and Ox39-FITC (anti-CD25). Results are expressed as the percentage of CD4+CD25− in spleen T lymphocytes from naive rats or from LF15-0195-treated recipients.
0195-treated recipients stimulated by donor APC were able to inhibit the IL-2 production by CD4\(^+\)CD25\(^+\) thymocytes.

Transfer of tolerance with CD4\(^+\)CD25\(^+\) thymocytes and spleen T cells

To determine in which CD25\(^+\) or CD25\(^-\) subpopulation of CD4\(^+\) T cells were regulatory cells, we performed transfers of these cells into LEW.1A-irradiated recipients. When naive syngeneic irradiated recipients did not receive cell transfers, allografts were rejected in 18.2 ± 4.4 days (n = 5; Fig. 9). When 5 × 10\(^6\) CD4\(^+\)CD25\(^+\) thymocytes from tolerant LF15-0195-treated recipients were transferred, allografts were rejected in 19.8 ± 11.9 days (n = 5). When 5 × 10\(^6\) CD4\(^+\)CD25\(^+\) thymocytes from tolerant LF15-0195-treated recipients were transferred, grafts survived indefinitely in three of four recipients (n = 4, p < 0.02). One allograft was rejected at day 41. CD4\(^+\)CD25\(^-\) spleen T cells from tolerant LF15-0195-treated recipients (5 × 10\(^6\)) were also able to transfer tolerance (>100 days, n = 3) as were CD4\(^+\)CD25\(^-\) spleen T cells (5 × 10\(^6\)), which were able to transfer tolerance (>100 days) in four of eight recipients (n = 8). These results demonstrate that spleen and thymus CD4\(^+\)CD25\(^+\) cells from tolerant LF15-0195-treated recipients contained regulatory cells capable of transferring tolerance, demonstrating a dominant immune regulation. In the periphery, regulatory cells were also present in the CD4\(^+\)CD25\(^-\) T cell subpopulation, but these cells were less numerous or and/or less efficient in transferring tolerance since 50% of recipients were tolerant. Moreover, regulatory cells from LF15-0195-treated recipients were donor specific since transfer of

![FIGURE 7. Proliferation and production of IFN-γ, IL-10, and IL-2 by spleen CD4\(^+\), CD4\(^+\)CD25\(^+\), and CD4\(^+\)CD25\(^-\) T cells from LF15-0195-treated recipients. Purified total CD4\(^+\) or CD4\(^+\)CD25\(^+\) or CD4\(^+\)CD25\(^-\) thymocytes from naive or from untreated or tolerant LF15-0195-treated recipients (100 days after transplantation) were stimulated by a LEW.1W donor-irradiated APC-enriched cell population for 72 h. Values represent the cpm ± SD of all triplicates after 3 days of culture for thymidine incorporation (A). IFN-γ (B), IL-10 (C), and IL-2 (D) were measured in the supernatants of triplicates by ELISA, as described in Materials and Methods, and the results are expressed in picograms per milliliter. Results are representative of three independent experiments.](http://www.jimmunol.org/)

![FIGURE 8. Proliferation and production of IFN-γ, IL-10, and IL-2 by thymus CD4\(^+\), CD4\(^+\)CD25\(^+\), and CD4\(^+\)CD25\(^-\) cells from LF15-0195-treated recipients. Purified total CD4\(^+\) or CD4\(^+\)CD25\(^+\) or CD4\(^+\)CD25\(^-\) thymocytes from naive or from tolerant LF15-0195-treated recipients (100 days after transplantation) were stimulated by a LEW.1W donor-irradiated APC-enriched cell population for 72 h. Values represent the cpm ± SD of all triplicates after 3 days of culture for thymidine incorporation (A). IFN-γ (B), IL-10 (C), and IL-2 (D) were measured in the supernatants of triplicates by ELISA, as described in Materials and Methods, and the results are expressed in picograms per milliliter. Results are representative of three independent experiments.](http://www.jimmunol.org/)
thymic and splenic cells from LF15-0195-treated recipients into secondary recipients induced Lewis third-party allograft rejection (18/11006, 2.8 days, n/11005, 2, and 9 days, n/11005, 3 (Table III), respectively).

Transfer of the same number of thymic and splenic CD4⁺CD25⁺ T cells (5/11003, 10⁶) from naive rats into syngeneic recipients did not protect from allograft rejection (18/11006, 2.8 days, n/11005, 2, and 21.5/11006, 2.1 days, n/11005, 2, respectively), demonstrating the specificity of CD4⁺CD25⁺ regulatory T cells from LF15-0195-treated recipients in this model of tolerance.

Donor APC were required to mediate allograft tolerance

On day 5 after grafting, we observed, by immunohistology, numerous donor MHC class II-positive cells in allografts from LF15-0195-treated recipients in contrast to allografts from untreated recipients (data not shown). Moreover, the number of donor MHC class II-positive cells was dramatically increased at day 30 after grafting in allografts from LF15-0195-treated recipients, suggesting that donor APC had expanded (Fig. 10A). Subsequently, on day 100, donor MHC class II-positive cells were found in heart allograft (Fig. 10B) and the thymus (Fig. 10D). No donor MHC class II-positive cells were observed in the thymus from untreated recipients on day 100 after grafting (Fig. 10C).

These results demonstrated the presence of a microchimerism in tolerant LF15-0195-treated recipients at 100 days after transplantation and suggested that donor APC could play a role in tolerance.

Therefore, to investigate the involvement of donor APC in allograft tolerance, LEW.1W heart allografts were depleted of passenger leukocytes by donor treatment with cyclophosphamide, as previously described (35). We observed that depletion of passenger leukocytes did not prolong allograft survival in untreated recipients (7.7/11006, 0.8 days, n/11005, 6, vs 7/11006, 0.1 days, n/11005, 12) (Table V). When donor grafts were depleted of APC, LF15-0195-treated recipients rejected their grafts in /11011, 23.2/11006, 12.2 days (n/11005, 6) in contrast to >100 days for untreated donor grafts (p < 0.001). These results demonstrated that donor APC were required for allograft tolerance. Moreover, LF15-0195-treated, but APC-depleted graft recipients had dramatically decreased percentage of CD4⁺CD25⁺ cells in the spleen (8.6% ± 0.5) and thymus (9.8% ± 0.7) compared with the spleen (19.5% ± 4.7) (p < 0.02)

FIGURE 9. Survival of allografts in secondary LEW.1A recipients after transfer of CD4⁺CD25⁺ or CD4⁺CD25⁻ splenic or thymus T cells from tolerant LF15-0195-treated recipients. Secondary LEW.1A recipients were irradiated with 4.5 Gy 1 day before transfer of 5 × 10⁶ syngeneic CD4⁺CD25⁺ or CD4⁺CD25⁻ spleen or thymus T cells from tolerant LF15-0195-treated recipients and were transplanted with LEW.1W heart allografts. *, p < 0.05 (Student’s t test).

FIGURE 10. Presence of donor APC in allograft and thymus from tolerant LF15-0195-treated recipients. Sections of allograft at days 30 (A, ×400) and 100 (B, ×400) after transplantation from tolerant LF15-0195-treated recipients, and thymus at day 100 after transplantation from untreated (C, ×200) or tolerant LF15-0195-treated recipients (D, ×200) were stained with Ox3 (anti-RT1.u), as described in Materials and Methods. Representative micrographs demonstrated the positive staining of donor MHC class II in the allograft (A and B) and thymus (D) of tolerant LF15-0195-treated recipients.
and thymus (19.3% ± 8.4) from LF15-0195-treated recipients (n = 3). These results suggest that donor APC were able to expand and colonize lymphoid compartments, and that direct presentation of donor Ags was required to expand powerful regulatory cells in central and/or peripheral compartments.

Presence of the thymus was not required to induce allograft tolerance and to induce regulatory cells in the periphery

To determine whether the thymus was required for tolerance, we performed thymectomy of adult recipients 2 wk before transplantation.

Untreated thymectomized LEW.1A recipients rejected LEW.1W heart allografts in 7 days (n = 3; Table V). A 20-day treatment with LF15-0195 induced allograft tolerance, despite the absence of the thymus in two of three recipients, suggesting that the adult thymus was not essential for allograft tolerance. Moreover, the transfer of splenocytes from thymectomized tolerant animals to second syngeneic recipients led to transfer of tolerance, demonstrating that splenocytes contained regulatory cells and that the thymus was not required to induce regulatory cells (Table III). Moreover, we observed a higher percentage of CD4+CD25+ cells in purified CD4+ spleen T cells from thymectomized tolerant LF15-0195-treated recipients (27% ± 1.41, n = 2) compared with those from untreated recipients (10.95 ± 2.36, n = 7). This percentage (27%) was similar to the percentage in nontreated tolerant LF15-0195-treated recipients (24.60% ± 6.21, n = 6) (Table VI).

These results demonstrate that the presence of the thymus was not required to induce tolerance and expansion of CD4+CD25+ regulatory T cells in the periphery.

Thymic and splenic CD4+CD25+ T cells from tolerant LF15-0195-treated recipients or from naive rats expressed the same level of CTLA-4, TGF-β, and IL-10 mRNA

We performed quantitative analysis of mRNA expression of CTLA-4, TGF-β, or IL-10 in unstimulated CD4+CD25+ or CD25− thymus and spleen T cells from tolerant LF15-0195-treated recipients or from naive rats. We observed in Fig. 11 that CD4+CD25+ thymus cells from naive rats or from LF15-0195-treated recipients expressed more CTLA-4, TGF-β, and IL-10 mRNA than thymus CD25− cells from naive rats or from LF15-0195-treated recipients, respectively. However, no difference in expression of CTLA-4 or TGF-β was observed between thymic CD4+CD25+ from naive rats and CD4+CD25+ from tolerant LF15-0195-treated recipients. In the spleen, CD4+CD25+ or CD4+CD25− from naive rats or from LF15-0195-treated recipients expressed the same level of CTLA-4 and TGF-β mRNA. In contrast, spleen CD4+CD25+ cells from naive rats or from LF15-0195-treated recipients expressed more IL-10 mRNA than CD4+CD25− from naive rats or from LF15-0195-treated recipients, respectively. However CD4+CD25− from naive rats expressed the same level of IL-10 mRNA as CD4+CD25− cells from

Table V. Effects of passenger leukocyte depletion of donor LEW.1W heart allografts or thymectomy of LEW.1A recipients on allograft survival

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Treatment</th>
<th>Donor</th>
<th>Graft</th>
<th>Graft Survival (days)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEW.1A</td>
<td>LF15-0195a</td>
<td>LEW.1W</td>
<td>Heart</td>
<td>6, 7 (×10), 9</td>
<td>7 ± 0.1</td>
</tr>
<tr>
<td>LEW.1A</td>
<td>LF15-0195a</td>
<td>LEW.1W</td>
<td>Heart (CPA treated)</td>
<td>7 (×3), 8 (×2), 9</td>
<td>7.7 ± 0.8</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>LF15-0195a</td>
<td>LEW.1W</td>
<td>Heart (CPA treated)</td>
<td>7, 15 (×2), 34 (×3)</td>
<td>23.2 ± 12.2*</td>
</tr>
<tr>
<td>LEW.1Aa</td>
<td>LF15-0195a</td>
<td>LEW.1W</td>
<td>Heart</td>
<td>7 (×3)</td>
<td>7</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>LF15-0195a</td>
<td>LEW.1W</td>
<td>Heart</td>
<td>60, &gt;100 (×2)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* Twenty-day treatment with LF15-0195 at 3 mg/kg i.p.

a LEW.1A recipients were thymectomyzed 2 wk before LEW.1W grafting.

*, p < 0.05 (Student’s t test).

Table VI. Percentage of CD25+ cells in CD4+ spleen T cells from thymectomized LF15-0195-treated recipients

<table>
<thead>
<tr>
<th>CD4-purified T cells</th>
<th>Average</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated recipients (&gt;100 days)</td>
<td>10.95</td>
<td>2.36 (n = 7)</td>
</tr>
<tr>
<td>Tolerant LF15-0195-treated recipients (&gt;100 days)</td>
<td>24.60</td>
<td>6.21 (n = 6)</td>
</tr>
<tr>
<td>Thymectomized tolerant LF15-0195-treated recipients (&gt;100 days)</td>
<td>27.00</td>
<td>1.41 (n = 2)*</td>
</tr>
</tbody>
</table>

*, p < 0.05 (Student’s t test).
LF15-0195-treated recipients. These results demonstrated that thymus and spleen CD4⁺CD25⁺ cells from LF15-0195-treated recipients expressed the same quantity of mRNA for CTLA-4, TGF-β, and IL-10 as thymus and spleen CD4⁺CD25⁺ cells from naive rats, suggesting that they could be the same cell population as that described in other models as naturally suppressive cells (24). However, the lack of specific Abs for CTLA-4 in the rat did not allow us to perform analysis of cell surface expression of CTLA-4.

Discussion
In this study, we demonstrated that a short-term treatment with LF15-0195 induced donor-specific allograft tolerance. Allografts were poorly infiltrated by macrophages and T cells and developed no signs of chronic rejection. Allografts expressed no IL-2, no IFN-γ, and a low quantity of IL-10 and TGF-β mRNAs. IL-10 mRNA expression in allografts could be due to restoration of IL-10 expression by macrophages after treatment cessation, but also IL-10 and TGF-β mRNA expression could be due to expression by Th2 or regulatory T cells that could progressively infiltrate allografts (28, 41). We demonstrated an inhibition in specific antitumor response and the presence of donor-specific regulatory cells in spleen from tolerant LF15-0195-treated recipients. Spleen regulatory CD4⁺ T cells were able in vitro to suppress the proliferation of allogeneic CD4⁺ T cells in a coculture system and were able in vivo to suppress allograft rejection after adoptive transfer, demonstrating their infectious tolerance properties. Regulatory cells were not present or not in a sufficient number in the lymph nodes from tolerant LF15-0195-treated recipients, suggesting compartmentalization of regulatory T cells in transplant recipients, as previously described in transplantation tolerance induced by CD4-targeted mAb therapy (42). Moreover, CD4⁺CD25⁺ cell subpopulation of thymus or spleen, but not of lymph nodes from tolerant LF15-0195-treated recipients was increased. Several reports have demonstrated splenic and thymic CD4⁺CD25⁺ naturally suppressive T cells as not secreting IL-2, as inhibiting IL-2 expression by CD4⁺CD25⁻ cells, and as expressing CTLA-4, TGF-β, and IL-10 (19, 21–24). We demonstrated that thymic and splenic CD4⁺CD25⁺ cells from naive rats or from LF15-0195-treated recipients expressed the same level of mRNA for CTLA-4, TGF-β, and IL-10, suggesting that these cells could derive from the same population. Moreover, CD4⁺CD25⁺ from tolerant LF15-0195-treated recipients proliferated poorly, but did not produce IL-2. Depletion of CD4⁺CD25⁺ subpopulation restored the IL-2 production by CD4⁺CD25⁻ thymocytes, but not spleen cells. In addition, allograft tolerance was transferable to a second recipient by splenic and thymic CD4⁺CD25⁻ T cells, but also in 50% of recipients with splenic CD4⁺CD25⁻ T cells, demonstrating that in our model, CD25 is a marker of regulatory cells in the thymus, but not in the periphery. It has been shown that naturally suppressive CD4⁺CD25⁺ T cell transfer can protect mice from the development of autoimmune disease in half the cases (43). Moreover, Mason et al. (15) have shown that in rat, CD4⁺CD25⁺ spleen and thymus T cells were able to protect from the development of autoimmune diabetes, but also spleen CD4⁺CD25⁺ T cells when RTE were deleted. They suggested that the CD25 RTE⁺ cells contained diabetogenic cells that were insufficiently regulated by the CD25 regulatory cells; thus, the CD4⁺CD25⁻ subpopulation of regulatory cells could have been therefore not in a sufficient number to protect from disease when RTE⁺ cells were present. They speculated that these cells could have been CD25⁻ cells that were generated in the thymus, which had lost the marker in the periphery. In our model, we observed a decrease in the percentage of RTE⁺ cells (Thy-1⁺) in spleens from tolerant recipients, and these results could explain why we succeeded in transferring tolerance in 50% of the recipients with peripheral CD4⁺CD25⁻ T cells. However, we cannot exclude the possibility that peripheral CD4⁺CD25⁻ regulatory T cells from LF15-0195-treated recipients come from a distinct lineage than CD4⁺CD25⁺ regulatory T cells.

In models of allograft tolerance in mice, CD4⁺ regulatory T cells have been described as being generated by indirect presentation and as exerting their suppressive properties when stimulated by donor Ags presented in the context of recipient APC (44, 45). However, in our model, a high number of donor APCs was observed in allografts from LF15-0195-treated recipients and depletion of passenger leukocytes from grafts before transplantation abrogated tolerance, suggesting that direct presentation of donor Ags was required for tolerance. In addition, we observed a low percentage of CD4⁺CD25⁺ T cells in the thymus and spleen from APC-depleted allograft recipients, suggesting that donor APCs were required for regulatory CD4⁺CD25⁺ cell expansion and their presence in the thymus and in the periphery. In vitro, spleen regulatory CD4⁺ T cells proliferated poorly, expressed low levels of IFN-γ and IL-2, and were able to suppress the proliferation of allogeneic CD4⁺ T cells, but only with stimulation by donor APC. Moreover, thymic CD4⁺ T cells from LF15-0195-treated recipients stimulated by donor APC poorly secreted IL-2, and CD4⁺CD25⁺ cells were able to suppress IL-2 production by CD4⁺CD25⁻, demonstrating that regulatory cells expanded or exerted their suppressive properties when they were stimulated by direct presentation. Recent reports have shown that regulatory CD4⁺CD25⁺ T cells were able to expand ex vivo by direct stimulation with allogeneic APC (46, 47).

It has been described that donor interstitial DCs were able to proliferate in untreated rat cardiac allografts before migrating to the spleen (48). We suggested that donor APCs could proliferate under LF15-0195 treatment in allografts and could then, after treatment, be able to colonize other organs such as the thymus. Donor APCs could serve as a potent source of allogeneic Ags in the thymus and in the periphery, and under particular conditions could lead to the deletion of alloreactive cells and/or the development of powerful donor-specific regulatory cells (2, 49–52). Moreover, donor APCs could be involved in the homeostasis of regulatory CD4⁺ T cells to induce stable tolerance. Indeed, it has been described that regulatory CD4⁺ T cells required the continuous presence of tolerizing Ags to survive in allograft tolerance models and recently that CD4⁺CD25⁺ regulatory T cells required interactions with MHC class II for in vivo proliferation and homeostasis (4, 53, 54).

DCs have been described to play a role in transplantation tolerance and to induce regulatory T cells by their tolerogenic properties (55, 56). Thomas et al. (57, 58) demonstrated that treatment with DSG combined with anti-CD3 immunoxin induced transplantation tolerance in macaques and was associated with reduction in number of mature DC in graft. In addition, DSG has been described to inhibit APC Ag-processing and class I and class II expression (59–61). However, we observed no effect of LF15-0195 on in vitro splenic DC maturation (E. Chifolleau and M. C. Cuturi, unpublished results). Moreover, preliminary studies showed that graft-infiltrating recipient DC expressed high levels of class II MHC and B7-2 costimulatory molecules. In contrast, donor interstitial resident DC showed low level of B7-2 expression, suggesting different effect of LF15-0195 on recipient or donor interstitial DC maturation (Chifolleau et al., unpublished results). Further investigations will determine whether LF15-0195 could act directly on interstitial donor or recipient APC maturation or function to induce regulatory T cells.

Thymectomy was not essential for the induction of allograft tolerance and for the expansion of CD4⁺CD25⁺ T cells in the
periphery. Therefore, presence of donor-specific CD4+CD25+ regulatory T cells in the thymus related to the presence of donor APC was not the only mechanism involved in allograft tolerance, and expansion of CD4+CD25+ T cells occurs in the periphery.

Naturally suppressive CD4+CD25+ T cells have been reported to be generated in the thymus since the postnatal development (15, 16, 18, 20), suggesting that peripheral CD4+CD25+ regulatory T cells derive from thymic precursors, and their expansion and functional development could occur extrathymically dependent on the presence of Ags. Indeed, as previously reported in mice (26), thymic and splenic CD4+CD25+ T cells from naive rats were not able to transfer protection from allograft rejection, demonstrating that priming with alloantigen is required for their donor-specific expansion. It is difficult to imagine how CD4+CD25+ regulatory T cells in the periphery could be specific for alloantigens since they were generated in the thymus in absence. However, it has been shown that Ag-specific regulatory CD4+CD25+ T cells can be expanded in the periphery by i.v. or oral administration of foreign Ag as OVA (27). Moreover, expansion of donor-specific regulatory CD4+CD25+ T cells able to transfer tolerance to second recipients has also been described in models of allograft tolerance (26, 45). Alloantigen-specific CD4+CD25+ regulatory T cells were described as being able to expand ex vivo by direct stimulation via costimulatory blockade (46, 47). Indeed, a high frequency of T cells cross-reacting with foreign Ags whose alloantigens has been reported (62–64). Therefore, the natural repertoire of CD4+CD25+ cells could exhibit cross-reactions with limited sets of alloantigens, and in some circumstances and because of linked suppression, CD4+CD25+ would be able to tolerate a full organ with numerous alloantigens. Several studies have demonstrated that deletion or inactivation of alloreactive cells was necessary to induce allograft tolerance (9, 45, 53, 65–67). Deletion or inactivation of these alloreactive cells could occur during the induction phase and could enable regulatory cells to expand and establish a stable donor-specific tolerance. Indeed, we have previously demonstrated that under treatment, LF15-0195 modulates Th1-type alloreactive cell function (28). In our model, we speculated that the maintenance of the tolerance state could be linked to a beneficial balance in favor of expanded donor-specific CD4+ regulatory T cells compared with allogeneic reactive cells.

In conclusion, we have demonstrated in this work that tolerant LF15-0195-treated recipients displayed donor-specific CD4+CD25+ regulatory cells in the thymus and CD4+CD25+ in spleen. Moreover, tolerance and expansion of CD4+CD25+ regulatory cells were dependent on donor-passenger leukocytes, suggesting that direct presentation of alloantigens led in part to the expansion of powerful CD4+ regulatory cells able to establish a stable tolerance.

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