Expansion and tissue infiltration of an allospecific CD4+CD25+CD45RO+IL-7Rαhigh cell population in solid organ transplant recipients

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It has been recently shown (Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S.I. Alexander, R. Nanan, et al. 2006. J. Exp. Med. 203:1693–1700.) that the expression of interleukin (IL) 7 receptor (R) α discriminates between two distinct CD4 T cell populations, both characterized by the expression of CD25, i.e. CD4 regulatory T (T reg) cells and activated CD4 T cells. T reg cells express low levels of IL-7Rαlow, whereas activated CD4 T cells are characterized by the expression of IL-7Rαhigh. We have investigated the distribution of these two CD4 T cell populations in 36 subjects after liver and kidney transplantation and in 45 healthy subjects. According to a previous study (Demirkiran, A., A. Kok, J. Kwekkeboom, H.J. Metselaar, H.W. Tilanus, and L.J. van der Laan. 2005. Transplant. Proc. 37:1194–1196.), we observed that the T reg CD25+CD45RO+IL-7Rαlow cell population was reduced in transplant recipients (P < 0.00001). Interestingly, the CD4+CD25+CD45RO+IL-7Rαhigh cell population was significantly increased in stable transplant recipients compared with healthy subjects (P < 0.00001), and the expansion of this cell population was even greater in patients with documented humoral chronic rejection compared with stable transplant recipients (P < 0.0001). The expanded CD4+CD25+CD45RO+IL-7Rαhigh cell population contained allospecific CD4 T cells and secreted effector cytokines such as tumor necrosis factor α and interferon γ, thus potentially contributing to the mechanisms of chronic rejection. More importantly, CD4+IL-7Rα+ and CD25+IL-7Rα+ cells were part of the T cell population infiltrating the allograft of patients with a documented diagnosis of chronic humoral rejection. These results indicate that the CD4+CD25+IL-7Rα+ cell population may represent a valuable, sensitive, and specific marker to monitor allospecific CD4 T cell responses both in blood and in tissues after organ transplantation.

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The antillograft immune response remains an important cause of acute rejection and late graft dysfunction in solid organ transplantation. In addition to the clinical and pathological manifestations of graft dysfunction, various biological markers have been investigated to monitor graft rejection over the years. De novo donor-specific anti-HLA antibodies represent a valuable marker, as their development is generally associated with rejection (1). However, T cell immunity plays a pivotal role in graft rejection (2). In this regard, a series of biological markers related to the inflammatory response, such as cytokines, thromboxane B2, β2 microglobulin (3, 4), and other phenotypic and functional markers associated with T cell activation such as IL-2Rα (CD25) expression, CD69, HLA-DR (5–8), cell proliferation, cytokine secretion, and cytotoxicity (9–12), have been evaluated to monitor cell-mediated immunity. However, these markers are neither sensitive nor specific, and their
It has been recently reported that regulatory CD4 T cells (i.e., T reg cells) express low levels of IL-7Rα compared with other CD4 T cell subsets (15–17), and it has been proposed that IL-7Rα can be used to discriminate between T reg cells and activated CD4 T cells within the CD4+CD25+ cell population (15, 17). The CD4+CD25+IL-7Rαhigh cell population contains activated T cells and is poorly represented (~5% within CD4+CD25+ T cells) in healthy subjects. Conversely, after transplantation, both activated allospecific CD4 T cells and T reg cells may play a critical role by either participating in allograft rejection or promoting tolerance (18–25).

In this study, we have searched for sensitive and highly specific cellular markers that may define the population of allospecific CD4 T cells to quantify this cell population after transplantation, to monitor possible changes, and to determine the pattern of the allospecific population in chronic graft rejection. We demonstrate that a CD4 T cell population defined by the CD25+CD45RO+IL-7Rαhigh phenotype contains allospecific cells and is expanded in the blood of stable transplant recipients and even further expanded in patients with a documented diagnosis of chronic rejection. In these latter patients, CD4+CD25+IL-7Rα high cells were a major component of T cells infiltrating the allograft.

RESULTS AND DISCUSSION
Distribution of CD4+CD25+ T cell populations defined by the expression of IL-7Rα (i.e., IL-7Rαlow vs. IL-7Rαhigh) in stable organ transplant recipients

We have studied the distribution of T reg cells and activated CD4 T cells in blood mononuclear cells of 32 stable transplant recipients, 21 liver and 11 kidney transplant recipients, and 4 patients with documented chronic kidney rejection, as well as 45 healthy subjects.

In accordance with recent studies (15, 17), the majority (88.2%) of CD4+CD25+ T cells were CD45RO+FOXP3+ and IL-7Rαlow in a representative healthy subject (1 out of 45), as shown in Fig. 1 A. This is the typical phenotype of T reg cells (26–28). Only a small percentage (3.1% in the example shown in Fig. 1 A) of CD4+CD25+ T cells expressed IL-7Rα high, and these cells were FOXP3 negative. We then performed the same analysis in stable transplant recipients. In one representative stable liver transplant recipient (1 out of 32), the CD4+CD25+CD45RO+FOXP3+IL-7Rαlow cell population was reduced (66.1%; Fig. 1 A) compared with the representative healthy subject. Interestingly, we observed a major increase (29.1%) in the CD4+CD25+CD45RO+FOXP3+IL-7Rαhigh cell population (Fig. 1 A). The differences in the distribution of IL-7Rαlow and IL-7Rαhigh within the CD4+CD25+CD45RO+ cell population between healthy subjects and stable transplant recipients were confirmed by the analysis of a larger number of subjects.

In agreement with a previous report (25), the percentage of total CD4+CD25+ T cells was significantly reduced (P < 0.00001) in stable transplant recipients receiving immunosuppressive therapy compared with healthy subjects. The reduction was in the range of 40%; the mean percentage of CD4+CD25+ T cells was 2.23 ± 0.12% in healthy subjects (n = 45) compared with 1.31 ± 0.14% in stable transplant recipients (n = 32; Fig. 1 B).

The analysis of IL-7Rα and FOXP3 expression allowed the discrimination between T reg cells (26–28) and activated CD4 T cells. This analysis showed that the percentage of T reg cells (the IL-7Rαlow-FOXP3+ cell population) was significantly lower (P < 0.00001) in stable transplant recipients (mean = 62 ± 1.54%) compared with healthy subjects (mean = 29.37 ± 2.33%; Fig. 1 B). Therefore, these results indicate that the reduction in T reg cells in stable transplant recipients is even greater than that estimated on the basis of the analysis of total CD4+CD25+ T cells. Furthermore, the population of activated CD4 T cells (i.e., the CD4+CD25+CD45RO+IL-7Rαhigh cell population) was greatly expanded (mean = 18.38 ± 1.05%) in stable transplant recipients compared with healthy donors (mean = 6.16 ± 0.76%; Fig. 1 B). These differences were highly significant (P < 0.00001). On the basis of the present results and of recent studies (15, 17), we then analyzed the correlation between IL-7Rα and FOXP3 expression in CD4+CD25+ T cells in stable transplant recipients. This analysis confirmed a negative correlation between IL-7Rα and FOXP3 expression in CD4+CD25+ T cells (Fig. 1 C). The IL-7Rαlow cells were contained within the FOXP3-positive cell population, whereas IL-7Rαhigh cells were within the FOXP3-negative cell population (Fig. 1 C). Collectively, these results indicated that IL-7Rα is a valuable marker to discriminate between T reg cells and activated CD4 T cells within the CD4+CD25+ T cell population in stable recipients of organ transplants.

Next, it was of interest to determine the suppressive activity of CD4+CD25+CD45RO+IL-7Rαlow and IL-7Rαhigh cells. For these purposes, sorted purified IL-7Rαlow and IL-7Rαhigh cell populations isolated from stable transplant recipients were assessed for their suppressive activity in a mixed lymphocyte reaction (MLR). The IL-7Rαlow cell population (T reg cells) strongly suppressed the MLR (83% inhibition of proliferation; P = 0.02), whereas the IL-7Rαhigh cell population did not show any significant suppressive activity (P = 0.2; Fig. 1 D). These results obtained in stable transplant recipients are in agreement with those previously shown in healthy individuals (15, 17). Furthermore, the CD4+CD25+CD45RO+IL-7Rαhigh cell population was also evaluated for the ability to secrete cytokines such as IFN-γ and TNF-α after polyclonal stimulation with anti-CD3 plus anti-CD28 antibodies. This analysis performed in blood mononuclear cells of 29 stable transplant recipients showed that a substantial percentage of CD4+CD25+CD45RO+IL-7Rαhigh cells secreted inflammatory cytokines, such as TNF-α (~15%) and IFN-γ (~4%), that may be involved in the mechanisms of chronic rejection (Fig. S1, http://www.jem.org/cgi/content/full/jem.20062120/DC1). Collectively, these results indicated that the CD4+CD25+CD45RO+IL-7Rαhigh cell population...
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Figure 1. Expression of CD25, CD45RO, IL-7Rα, and FOXP3 on blood CD4 T cells of healthy subjects and stable transplant recipients.

Blood mononuclear cells from 45 healthy subjects and 32 stable organ transplant recipients (11 kidney and 21 liver transplant recipients) were analyzed for the surface expression of CD4, CD25, CD45RO, and IL-7Rα and for the intracellular expression of FOXP3. (A) Surface expression of CD45RO and IL-7Rα in CD4+CD25+ T cells. Representative flow cytometry profiles of one healthy donor (1 out of 45; top) and one stable liver transplant recipient (1 out of 32; bottom). The vast majority of CD4+CD25+ T cells are CD45RO+IL-7Rα+ and FOXP3+ in the healthy donor, while a substantial percentage of CD4+CD25+ T cells are CD45RO+IL-7Rαhigh and FOXP3− (green) in the stable transplant recipient. (B) Cumulative data on the proportion of CD4+CD25+ T cells (top left), CD4+CD25+FOXP3+ IL-7Rαlow T cells (top right), and CD4+CD25+CD45RO+IL-7Rαhigh T cells (bottom) in stable transplant recipients and healthy donors. (C) Correlation between the expression of FOXP3 and IL-7Rα within CD4+CD25+ T cells. The percentage of FOXP3+ T cells correlated with that of IL-7Rαlow cells within CD4+CD25+ T cells, and the percentage of FOXP3− cells correlated with that of IL-7Rαhigh cells within CD4+CD25+ T cells. The analyses were performed in 26 stable transplant recipients. In both cases, these correlations were statistically significant (P < 0.05). (D) Suppressive activity of IL-7Rαlow and IL-7Rαhigh CD4+CD25+CD45RO+ T cell populations in stable transplant recipients. CD4+CD25+CD45RO−IL-7Rαhigh and CD4+CD25+CD45RO+IL-7Rαlow cell populations were sorted from blood mononuclear cells of five stable liver transplant recipients, and their suppressive activity was evaluated in a MLR. The extent of cell proliferation in a MLR was assessed in the absence (positive control) or in presence of the sorted IL-7Rαlow and IL-7Rαhigh cell populations. Cell proliferation was measured by [3H]thymidine incorporation. The data shown are expressed as the mean ± SE and were obtained from five independent experiments.

does not contain T reg cells and suggested that the expansion of this cell population in transplant recipients is associated with the presence of the allograft.

Larger expansion of the CD4+CD25+CD45RO+IL-7Rαhigh cell population in transplant recipients with chronic rejection

To better support this hypothesis, we examined the presence of the CD4+CD25+CD45RO+IL-7Rαhigh cell population in kidney transplant recipients with documented chronic humoral rejection. The total CD4+CD25+ T cell population was not significantly different in kidney recipients with documented chronic rejection compared with stable transplant recipients (mean = 33.66 ± 3.43% vs. 18.38 ± 1.05%; P = 0.01; Fig. 2 B). Interestingly, an almost doubled percentage of CD4+CD25+CD45RO+IL-7Rαhigh cells within the total CD4+CD25+ cell population was observed in patients with documented chronic rejection (51.07 ± 5.8%) compared with stable transplant recipients (62.02 ± 1.54%; Fig. 2 B). These differences were highly significant (P < 0.0001).

When the CD4+CD25+CD45RO+IL-7Rαhigh population from patients with chronic rejection was compared with the one of stable kidney transplant recipients, the difference was also significant (P = 0.0003; unpublished data). Therefore,
although the expansion of the CD4+CD25+CD45RO+IL-7Rαhigh cell population in patients with chronic rejection was associated with a decrease of T reg cells, the reduction of the T reg cell population was not sufficient to explain the increase of the CD4+CD25+CD45RO+IL-7Rαhigh cell population, thus indicating an absolute increase in the number of this latter population. Indeed, the absolute number of CD4+CD25+CD45RO+IL-7Rαhigh cells was also significantly increased (1.5 ± 0.4 × 10^3 cells per 100 μl of blood) in patients with chronic rejection compared with stable transplant recipients (0.8 ± 0.1 × 10^3 cells per 100 μl of blood; P = 0.04). However, the absolute number of T reg cells did not significantly change between the stable transplant recipients (3.8 ± 0.5 × 10^3 cells per 100 μl of blood) and the patients with chronic rejection (3 ± 0.5 × 10^3 cells per 100 μl of blood; P = 0.3).

Collectively, these results indicate (a) an association between the presence of an expanded CD4+CD25+CD45RO+IL-7Rαhigh cell population and the occurrence of chronic allograft rejection, and (b) the expansion of the activated CD4+CD25+CD45RO+IL-7Rαhigh cell population defined by the expression of IL-7Rα cannot be explained only by the result of the decrease of the T reg cell population defined by the expression of FOXP3. Therefore, monitoring IL-7Rα may be a more valuable tool than monitoring FOXP3 to assess the patient’s immune status and/or the occurrence of allograft chronic rejection.

**The CD4+CD25+CD45RO+IL-7Rαhigh cell population contains allospecific CD4 T cells**

To have insights on the possible involvement of the CD4+CD25+CD45RO+IL-7Rαhigh cell population in the allospecific response to the graft, we examined the allospecific response of the transplant recipient to the donor in a MLR. Indeed, we had the opportunity to address this issue in a case of documented kidney chronic humoral rejection in which blood mononuclear cells of the (living) donor were available. The CD4+CD25+CD45RO+IL-7Rαhigh cell population was largely expanded (~40% of the CD4+CD25+ T cells) at the time of the diagnosis of chronic humoral rejection (time point 1; Fig. 3). The MLR was performed using the transplant recipient–sorted CD4+CD25+CD45RO+IL-7Rαhigh cells mixed with irradiated transplant donor or MHC unrelated blood mononuclear cells. Interestingly, we found that the proliferation of the CD4+CD25+CD45RO+IL-7Rαhigh cell population was substantially higher (stimulation index [SI] = 66) in the presence of irradiated transplant donor blood mononuclear cells compared with the proliferation in the presence of MHC unrelated blood mononuclear cells (SI = 13; Fig. 3). Because of the diagnosis of chronic humoral rejection, this

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**Figure 2.** Expression of CD25, CD45RO, IL-7Rα, and FOXP3 on blood CD4 T cells of stable transplant recipients and kidney transplant recipients with chronic rejection. Blood mononuclear cells from 32 stable transplant recipients (11 kidney and 21 liver transplant recipients) and 4 kidney transplant recipients with biopsy-proven chronic rejection were analyzed for the surface expression of CD4, CD25, CD45RO, and IL-7Rα and for the intracellular expression of FOXP3. (A) Means ± SE of cumulative data on the proportion of CD4+CD25+ T cells in stable transplant recipients and transplant recipients with chronic rejection. The percentage of CD4+CD25+ T cells in total CD4+ T cells was slightly increased in transplant recipients with chronic rejection compared with stable transplant recipients, but these differences were not significant (P = 0.21). (B) Means ± SE of cumulative data on the proportion of CD4+CD25+FOXP3+IL-7Rαhigh T cells in stable transplant recipients and transplant recipients with chronic rejection. The proportion of FOXP3+IL-7Rαhigh T cells within the CD4+CD25+ T cell population was slightly reduced in the patients with chronic rejection compared with stable transplant recipients, and these differences were significant (P = 0.01). (C) Means ± SE of cumulative data on the proportion of CD4+CD25+CD45RO+IL-7Rαhigh T cells in stable transplant recipients and transplant recipients with chronic rejection. The proportion of CD45RO+IL-7Rαhigh T cells within the CD4+CD25+ T cell population was significantly increased in the chronic rejection group compared with the stable group (P < 0.0001).
were co-cultured with 10^5 irradiated blood mononuclear cells (40 Gy) either points of a kidney transplant recipient with biopsy-proven chronic rejection (time point 2), the IL-7Rα population 15 mo after the first determination (time point 1) in the IL-7Rα high–activated cell population 15 mo after the first determination (time point 1) in the IL-7Rα high–activated cell population.

In support of the association between the magnitude of the expansion of the IL-7Rα high–activated cell population in two additional cases of stable kidney transplant recipients in which blood mononuclear cells of (living) donors were available. The percentage of the IL-7Rα high cell population during the first year after transplantation in 11 transplant recipients. We recently had the opportunity to prospectively monitor the CD4+CD25+IL-7Rαhigh cell population during the first year after transplantation in 11 kidney transplant recipients receiving calcineurin-based immunosuppression with tacrolimus and mycophenolate mofetil (Fig. 5).

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**Figure 3. Analysis of allospecific CD4 T cell responses.** Sorted purified CD4+CD25+CD45RO−IL-7Rαhigh T cells (7 × 10^5 cells) from two time points of a kidney transplant recipient with biopsy-proven chronic rejection were co-cultured with 10^5 irradiated blood mononuclear cells (40 Gy) either from the organ donor or from an MHC unrelated subject. After 7 d of culture, cell proliferation was measured by [H]thymidine incorporation. Cell cultures containing only irradiated cells were used as negative controls.

Furthermore, to better characterize the influence of immunosuppressive therapy on the IL-7Rα high–activated cell population, we studied five additional transplant recipients and seven subjects with various autoimmune diseases receiving immunosuppressive therapy. An augmentation of immunosuppressive therapy in the transplant recipients was consistently associated with a significant subsequent reduction in the percentage of the IL-7Rα high cell population from 31.17 ± 6.33% to 17.33 ± 4.98% (P = 0.002). The percentage of this population in patients with autoimmune diseases (n = 7) receiving various types of immunosuppressive therapy was not significantly different from the one of healthy subjects (P = 0.39; unpublished data).

**CD4+CD25+IL-7Rα+ cells infiltrate the allograft**

We investigated the phenotype of the cells infiltrating the kidney tissue allograft in five patients with a documented diagnosis of chronic humoral rejection. For this purpose, frozen kidney biopsies were simultaneously stained with the following antibody combinations: CD4 plus CD25, CD4 plus IL-7Rα, CD25 plus IL-7Rα, CD4 plus FOXP3, IL-7Rα plus FOXP3, and CD25 plus FOXP3. Variable numbers of cells infiltrating the allograft were consistently found in all of the cases studied. Of interest, with regard to CD4 T cells, ~20% were CD25+ and ~50% were IL-7Rα+; all CD25+ cells coexpressed IL-7Rα (Fig. 4 A). The estimates of the percentage of infiltrating CD4+CD25+ and CD4+ IL-7Rα+ cells have been generated from counting ~80 infiltrating CD4 T cells in 18 different microscopic fields of the five kidney biopsies. We also investigated the expression of FOXP3 in the CD4 T cells infiltrating the allograft, but CD4+FOXP3+ cells were not found in the five kidney biopsies examined (Fig. 4 B). However, CD4+FOXP3+ cells were consistently found in the lymph node biopsies used as control tissue (Fig. 4 C). It is also worth mentioning that cellular infiltrates were very scarce in the biopsy of patients with a kidney disease such as interstitial nephritis, and in this case, infiltrating CD4 T cells were CD25− and IL-7Rα− (unpublished data). Therefore, these results indicate that the predominant CD4 T cell population infiltrating the allograft in patients with chronic rejection is composed of CD4+CD25+ and CD4+IL-7Rα− cells.

**Importance of the analysis of the CD4+CD25+CD45RO+ IL-7Rαhigh cell population in the clinical monitoring of transplant recipients**

In this study, the results shown in Figs. 1 and 2 demonstrating the expansion in blood of the CD4+CD25+CD45RO−IL-7Rαhigh cell population were obtained from a cross-sectional analysis performed in 32 (21 liver and 11 kidney) organ transplant recipients. We recently had the opportunity to prospectively monitor the CD4+CD25+CD45RO−IL-7Rαhigh cell population during the first year after transplantation in 11 kidney recipients receiving calcineurin-based immunosuppression with tacrolimus and mycophenolate mofetil (Fig. 5).
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A
Double positive T-cells
IL-7R⁺ T-cells
CD4⁺ T-cells

Double positive T-cells
IL-7R⁺ T-cells
CD25⁺ T-cells

Double positive T-cells
CD25⁺ T-cells
CD4⁺ T-cells

B
Kidney biopsy
Double positive T-cells
CD4⁺ T-cells
FOXP3⁺ T-cells

Double positive T-cells
CD25⁺ T-cells
FOXP3⁺ T-cells

Double positive T-cells
IL-7R⁺ T-cells
FOXP3⁺ T-cells

C
Lymph node
Double positive T-cells
CD4⁺ T-cells
FOXP3⁺ T-cells
At study entry (before transplantation), there were no differences in the percentage of CD4⁺CD25⁺CD45RO⁺IL-7Rα<sup>high</sup> cells between the transplant recipients and living donors. The mean percentage in transplant recipients (5.04 ± 0.75%) and in living donors (4.65 ± 0.7%) was consistent with that observed in the large cohort of healthy subjects (n = 45; 6.16 ± 0.76%; Fig. 5). Of interest, the CD4⁺CD25⁺CD45RO⁺IL-7Rα<sup>high</sup> cell population was significantly increased (up to ~14% of the CD4⁺CD25⁺ T cells; P < 0.005) 1 mo after transplantation as compared with study entry, and the activated cell population remained expanded during the year of observation (Fig. 5). Interestingly, kidney allograft function in these recipients was stable during the study period, and there was no evidence of allograft rejection. These data further support the relationship between the expansion of the IL-7Rα<sup>high</sup> CD4 T cell population and the presence of the allograft and the usefulness of implementing the determination of this cellular marker in the immunologic monitoring of the transplant recipients.

CONCLUSIONS
We have identified the presence of a CD4 T cell population that is selectively expanded in patients after organ transplantation and is phenotypically defined by the expression of CD25, CD45RO, and high levels of IL-7Rα. Of interest, this cell population is expanded in stable transplant recipients in the presence of the allograft and the usefulness of implementing the determination of this cellular marker in the immunologic monitoring of the transplant recipients.

MATERIALS AND METHODS
Patients. 21 liver and 15 kidney transplant recipients were studied at the Centre Hospitalier Universitaire Vaudois. All patients gave informed written consent before participating in the study, which was approved by the local institutional review board. 45 healthy volunteers were included as controls.

Peripheral blood was obtained from 21 clinically stable liver transplant recipients, all of whom had received a first cadaveric graft and were studied for >12 mo after transplantation. This group was composed of 15 males and 6 females, with a median age of 51.2 yr (range = 20–67 yr) at the time of the study and a median time from transplantation to the study of 7.6 yr (range = 1–19 yr). Their immunosuppressive therapy was based on cyclosporine or tacrolimus with prednisone (n = 7) in combination with mycophenolate mofetil or azathioprine (n = 2).

Peripheral blood was also obtained from 15 kidney transplant recipients divided into two groups: (a) 11 patients with a stable graft function defined by a stable serum creatinine with values inferior to 150 μmol/liter and by a 24-h proteinuria inferior to 0.5 g/day, and (b) 4 patients with biopsy-proven chronic rejection. The stable kidney group was composed of seven males and four females, with a median age of 48.8 yr (range = 24–70 yr) at the time of the study and a median time from transplantation to the study of 8.6 yr (range = 1–19 yr). All patients had received a first graft: eight from a deceased donor, two from a result of the merging of the two single-color images. (bottom) CD25⁺ T cells (green) and CD4⁺ T cells (red). Double-positive CD4⁺CD25⁺ T cells (yellow arrowheads) are colored in orange as a result of the merging of the two single-color images. (B, top) CD4⁺ T cells (green) infiltrating the kidney of a patient with documented chronic rejection are FOXP3 negative (red). (middle and bottom) CD25⁺ T cells (green) and IL-7Rα⁺ T cells (green) are also negative for FOXP3 (red). (C) CD4⁺ T cells (yellow) in the lymph node are FOXP3 positive (green). Bars, 10 μm.
living/unrelated donor, and one from a living/unrelated donor. No patient in this group had circulating anti-HLA antibodies. The chronic rejection group was composed of two males and two females, with a median age of 42.8 yr (range = 26–56 yr) at the time of the study and a median time from transplantation to the study of 9 yr (range = 6–16 yr). Also in this group, patients had received a first graft: three from a deceased donor and one from a living/unrelated donor (father to daughter). In the four patients with biopsy-proven chronic rejection (29, 30), circulating donor-specific anti-HLA antibodies (DSA) were detected in serum by ELISA (One Lambda, Inc.), and capillary C4d deposits were demonstrated in kidney graft biopsies. In three out of four biopsies, typical lamination with multilayering of the basement membrane of peritubular capillaries was also demonstrated by electron microscopy. In the living/donor recipient with chronic rejection, a repeat B cell cross match was positive (T and B cell cross matches were negative at the time of transplantation). In all four patients, circulating DSA were directed against defined class II antigens (anti–class II DSA). The median serum creatinine at the time of diagnosis of chronic rejection was 247 μmol/liter (range = 184–350 μmol/liter), whereas the median 24-h proteinuria was 2.1 g/day (range = 1–3.5 g/day).

A second group of 11 kidney transplant recipients was monitored prospectively at the Hôpitaux Universitaires de Genève and was composed of 7 males and 4 females, with a median age of 42.7 yr (range = 23–81 yr) at the time of the transplantation. The group of kidney donors (living donors) was composed of two males and nine females, with a median age of 52.8 yr (range = 32–73 yr) at the time of the donation. All of these 11 kidney transplant recipients received a 3–4-d thymoglobulin (1.5 mg/kg) course as induction therapy, with a calcineurin-based immunosuppressive regimen. The group of patients with various autoimmune diseases studied at the Centre Hospitalier Universitaire Vaudois was composed of one male and six females, with a median age of 37.9 yr (range = 21–55 yr) at the time of the study. The patients received various regimens of immunosuppressive drugs.

FACS analysis and sorting. Peripheral blood mononuclear cells were isolated using standard Ficoll-Hypaque (GE Healthcare) gradient centrifugation. The antibodies used for flow cytometric analyses included PerCP, PerCP-Cy5.5, or PE-conjugated mouse anti–human CD4 (Becton Dickenson); allophycocyanin (APC)-conjugated mouse anti–human CD25 (BD Biosciences), FITC (BD Biosciences), or ECD (Beckman Coulter)-conjugated mouse anti–human CD45RO; and PE (Beckman Coulter)- or APC (R&D System)-conjugated mouse anti–human IL-7Rα. For intracellular FoxP3 analysis, cell preparations were fixed and permeabilized with fixation/permeabilization buffers (eBioscience) after staining of cell surface markers and stained with FITC-conjugated rat anti–human FOXP3 (eBioscience). For cell sorting experiments, CD4+CD25+CD45RO+IL-7Rαhigh, CD4+CD25−CD45RO+IL-7Rαhigh, and CD4+CD25− cells were isolated from the peripheral blood of transplant recipients. The grade of purity in all of the sorting experiments ranged between 92 and 98%. All flow cytometric analyses were performed on a FACS Calibur and LSR II, while cell sorting was performed on a FACSVantage SE and FACSAria (Becton Dickenson).

Suppressive function assay. The suppressive function of freshly sorted CD4+CD25+CD45RO+IL-7Rαhigh and CD4+CD25−CD45RO+IL-7Rαhigh cells isolated from the peripheral blood of liver transplant recipients was assessed in a MLR.

For these purposes, 10^6 irradiated (40 Gy) allogeneic blood mononuclear cells (stimulator cells) and 5 × 10^4 CD4+CD25−T cells isolated from liver transplant recipients (responder cells) were co-cultured either alone (positive control) or in the presence of 5 × 10^3 freshly sorted CD4+CD25+CD45RO+IL-7Rαhigh or CD4+CD25−CD45RO+IL-7Rαhigh cells. Proliferation was measured at day 7 by [3H]thymidine incorporation.

Allospecific T cell response. Allospecific T cell proliferation of the CD4+CD25+CD45RO+IL-7Rαhigh cells isolated from one kidney recipient with documented chronic rejection was assessed by co-culturing 10^6 irradiated (40 Gy) donor blood mononuclear cells with 7 × 10^4 sorted purified recipient CD4+CD25−CD45RO+IL-7Rαhigh cells. Cultures were made in triplicates, and proliferation was measured at day 7 by [3H]thymidine incorporation.

At a control, the same sorted purified population was co-cultured with irradiated MHC-unrelated blood mononuclear cells. The SI was calculated on the basis of the fold increase in the proliferation observed in the culture containing CD4+CD25+CD45RO+IL-7Rαhigh cells and irradiated donor or MHC-unrelated cells compared with the negative control, i.e., cultures containing irradiated cells alone.

Cytokine production. 2 × 10^6 cryopreserved blood mononuclear cells from liver and kidney transplant recipients were prestained with anti–IL-7Rα PE and mouse anti–human CD25 PE-Cy7 (BD Biosciences) antibodies and stimulated overnight with soluble 1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 antibodies (BD Biosciences) in 1 ml of complete medium (RPMI plus 10% FBS) containing 1 μl/μl each of GolgiPlug and GolgiStop (BD Biosciences). At the end of the stimulation period, the cells were washed and stained with CD4 PerCP-Cy5.5 and CD45RO ECD antibodies. The cells were permeabilized with FACS Permeabilizing Solution 2 (BD Biosciences) and stained with mouse anti–human IFN-γ or TNF-α FITC and rat anti–human IL-2 APC (BD Biosciences). Data were acquired on an LSRII and analyzed using DiVa software (Becton Dickenson). Unstimulated cells were used as negative control for the production of cytokines. The background never exceeded 0.02%.

Immunohistochemistry. Frozen tissues from kidney biopsies were incubated at room temperature for 20 min with a blocking solution (5% normal goat serum) and immediately stained overnight at 4°C with a mix of primary antibodies: IgG1 mouse anti–human IL-7Rα with IgG2a mouse anti–human CD4 (Beckman Coulter) or with IgG2b rat anti–human IL-25 (Serotec) and mouse anti–human CD4 with rat anti–human CD25 (Serotec) and mouse anti–human CD4 with rat anti–human IL-7Rα. Secondary antibodies (goat IgG specific) were added after 16 h (after washing the unbound primary antibodies) and incubated for 1 h at room temperature in the dark. Goat anti–mouse IgG1 Alexa Fluor 488, goat anti–mouse IgG2a Alexa Fluor 594, goat anti–mouse IgG (H+L) Alexa Fluor 532, goat anti–rat IgG (H+L)+ FITC, biotinylated goat anti–rat IgG (H+L), streptavidin Texas red, and streptavidin FITC were all obtained from Invitrogen. The tissues were mounted with antifade reagent (ProLong Gold; Invitrogen) and observed on an imaging microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with epifluorescence using single-band excitation filters: Alexa Fluor 488, ex. 480/40 LP; Alexa Fluor 594, ex. 564/12; and Alexa Fluor 532, ex.535. Photos were taken using an AxioCam and AxioVision software (version 4.6; Carl Zeiss MicroImaging, Inc.). Each fluorochrome was imaged separately, and the resulting images were automatically pseudocolored and merged by the AxioVision software.

Statistical analysis. Statistical significance was calculated by the two-tailed t test and linear regression analysis. P < 0.05 was considered significant.

Online supplemental material. Fig. S1 shows that CD4+CD25+CD45RO+IL-7Rαhigh cells secrete inflammatory cytokines. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062120/DC1.

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