Intragraft Th17 Infiltrate Promotes Lymphoid Neogenesis and Hastens Clinical Chronic Rejection

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*J Immunol* 2010; 184:5344-5351; Prepublished online 31 March 2010; doi: 10.4049/jimmunol.0902999

http://www.jimmunol.org/content/184/9/5344
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To evaluate the influence of intragraft inflammatory infiltrate on the course of chronic rejection, 11 human renal grafts, detransplanted for terminal failure, were analyzed. Samples were divided into two groups according to their graft survival (> or ≤8 y). In both groups, the main cell population infiltrating the graft interstitia was T lymphocytes. The extent of the lymphocytic infiltration and the distribution of naive and memory, CD4+ and CD8+ T cells, were similar in both groups. Although all types of Th polarization profiles can lead to terminal chronic rejection, a correlation between shorter graft survival and the presence of Th17 cells that produce IL-17 and IL-21 was observed. In contrast, grafts infiltrated by regulatory T cells survived significantly longer. The correlation between the expressions of activation-induced cytidine deaminase (the key enzyme of the germinal center reaction) and IL-21 suggests that Th17 could exert their deleterious effect by promoting lymphoid neogenesis, namely, the organization of inflammatory effectors into ectopic germinal centers in which a local humoral immune response is elicited. Further studies will determine whether Th17 infiltration can be used as a prognosis tool and whether the Th17 subset constitutes a therapeutic target for slowing down chronic rejection. The Journal of Immunology, 2010, 184: 5344–5351.

Despite progress achieved over the last two decades, the long-term outcome of transplanted kidneys has remained limited by chronic rejection (1). Immunosuppressive drugs that are currently available only partially control the recipient immune response and the persistent smoldering response is responsible for irreversible tissue destruction that inexorably leads to the decline of graft function. A better understanding of the immune mechanism is therefore a mandatory step in designing innovative therapeutic strategies that can efficiently control chronic rejection and improve long-term graft outcome.

Chronic rejection is characterized by typical histological changes that include interstitial fibrosis, vascular wall thickening, and mononuclear cell infiltration. Previous studies have demonstrated that among the immune effectors that infiltrate a chronically rejected graft, T lymphocytes are the main cell population (2, 3). Recent advances in the field of basic immunology have unraveled the heterogeneity of the CD4+ T cell population. It can now be split into a variety of subsets; classical Th1 cells and Th2 cells, the more recently defined Th17 cells and follicular helper T cells (Tfhs), and finally, regulatory T cells (Tregs) (4). The commitment of a T cell clone to one of these subsets is dependent on the expression of specific “master regulator” transcription factors; respectively, T-bet for Th1, GATA3 for Th2, RORC2 for Th17, Bcl6 for Tfhs, and Foxp3 for Tregs. Because each of these CD4+ T lymphocyte subsets displays distinct effector functions, a number of studies have been conducted to determine the impact of CD4+ T cell polarization on the rejection process. These works have led to the conclusion that the grafts may be destroyed indifferently by Th1, Th2 (5, 6), or Th17 (7, 8) cells, although with variable kinetics and through different pathogenic mechanisms. In contrast, Tregs appeared to exert a protective effect against rejection (9–11). However, because most of these studies have been conducted in transgenic murine experimental models, the validity of the findings in a clinical chronic rejection setting remains largely elusive.

In the current work, we have analyzed the influence of the polarization of intragraft CD4+ T cell infiltrate on the course of clinical chronic rejection.

Materials and Methods

Human samples

Eleven successive renal allografts, removed due to terminal chronic active rejection, were collected. The individual characteristics of these 11 patients are presented in Table I.

Fresh normal renal tissue was obtained from the intact portion of five kidneys removed for renal cancer. All the patients gave informed consent for the use of the samples for research purposes.

Histopathology

Fresh explanted renal allografts or control kidneys were dissected and embedded in paraffin or snap frozen immediately in OCT medium (Tissue-Tek, Agar Scientific) in liquid nitrogen.

Ten-micrometer-thick cryosections and 4 μm-thick paraffin sections were used. Routine examination was performed after Masson’s trichrome, H&E-Safran, periodic acid Schiff, and silver staining.
The percentages of lymphocytes, plasmacells, monocytes, eosinophils, and neutrophils infiltrating the renal grafts were defined by morphological analysis as follows: for each sample, 100 cells were counted on 10 randomly selected fields (original magnification ×200). The respective proportions of T CD4+, T CD8+, and B cells among the lymphocytes were then defined by flow cytometry analysis.

CD3ε, Foxp3, IL10 or IL-17, and CD5b9 were sequentially communounstained on paraformaldehyde-fixed cryosections with various combinations of the following primary mAbs specific for human molecules: FOXP3 (clone 236A/4E7, biotinylated mouse IgG1, 10 μg/ml; Abcam), IL10 (polyclonal goat antiserum, 5 μg/ml; R&D Systems, Minneapolis, MN), IL17 (polyclonal goat antiserum, 5 μg/ml; R&D Systems), and CD5b9 (clone aE11, mouse IgG2a, 10 μg/ml; Dako, Carpinteria, CA). After incubation with each primary Ab, the appropriate secondary reagent (all from Invitrogen, San Diego, CA) was applied prior to immunostaining (AlexaFluor 546- streptavidin 10 μg/ml, and AlexaFluor 647 cross-absorbed and affinity purified rabbit-anti-goat IgG, 2 μg/ml). Finally, CD3ε cells were detected by using an Alexa Fluor 488 directly labeled mouse anti-human CD3 Ab (clone UCHT1, IgG1,k, from BD Biosciences, San Jose, CA). The nuclei were counterstained by DAPI. Slides were then permanently mounted with ProLong Gold antifade reagent (Invitrogen). Digital images of 0.3 μm intracellular sections were acquired using AxioVision software and a Zeiss 63 immersion objective equipped with the AxoTome and a cooled monochromatic digital camera (Zeiss, Oberkochen, Germany) and merged using Adobe Photoshop software.

**Cell suspension preparation**

Fresh explanted grafts were cut with a sterile razor blade into ~0.125 mm³ fragments that were incubated in a solution of 1 mg/ml collagenase A and 0.1 mg/ml DNAsase I (Roche, Manheim, Germany) for 1 h at 37°C. Cell suspensions were passed through a 70 μm cell strainer, and mononuclear cells were separated over Ficoll-Paque Plus (GE Healthcare, Velizy, France).

**Flow cytometry**

Ten million cells were incubated with a mixture of fluorescent mAbs specific for the following human cell surface markers: CD3 (PE-Texas Red, clone 7D6), CD4 (Alexa Fluor 700, clone RPA-T4), CD8 (Pacific Blue, clone RPA-T8), CD45 RA (FITC, clone HI100), CD45 RO (APC, clone 7D6), CD4 (Alexa Fluor 700, clone RPA-T4), CD8 (Pacific Blue, clone RPA-T8), and CD3 for the purified CD4+ T cells and GAPDH for the renal tissue. For flow cytometry analysis, 10 million cells were incubated with a mixture of fluorescent mAbs specific for the following human cell surface markers: CD3 (PE-Texas Red, clone 7D6) and CD4 (Alexa Fluor 700, clone RPA-T4). All these Abs were from BD Biosciences (San Jose, CA) except for the anti-CD3 from Caltag Laboratories (Burlingame, CA).

More than 1.10⁶ events in the lymphoid FSC/SSC gate were acquired on an LSRII flow cytometer and analyzed with DIVA software (BD Biosciences).

**Magnetic Ab cell sorting**

CD4+ T lymphocytes were purified from vials of frozen infiltrating cell suspension kept in liquid nitrogen. Frozen cells were thawed, counted, and plated in 80 μl cold separation buffer (composed of PBS supplemented with 0.5% BSA, and 2 mM EDTA). Magnetic microbeads (20 μl) coated with an anti-human CD4 Ab (Miltenyi Biotec, Auburn, CA) were added to the cell suspension that was then incubated for 15 min at 4°C. The cells were washed in 2 ml cold separation buffer centrifuged 10 min at 300 × g and resuspended in 500 μl CD4+ cells were sorted by positive immunomagnetic selection using the autoMACS system and the Possel separation program (Miltenyi Biotec).

**Molecular biology**

**mRNA extraction.** Purified CD4+ cells. The CD4 enriched fraction was centrifuged 5 min at 300 × g and the pellet was resuspended in 350 μl RLT lysis buffer. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA).

**Reverse transcription.** cDNAs were generated from 1 μg total RNA using SuperScript II reverse transcriptase (Invitrogen, Cergy Pontoise, France) and random decamers (Roche).

**Primer design.** The gene-specific primer sets are provided in Table I. All the primers were designed using the Universal ProbeLibrary from the Roche Applied Science Web site (www.roche-applied-science.com), except for the primers for human RORC2 (sequences kindly provided by Tom Cupedo). All the primers were obtained from Invitrogen.

**Quantitative real-time-PCR.** Quantitative real-time PCR (QRT-PCR) was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 20 μl, containing 5 μl cDNA sample, 5 μl diluted primer set, and 10 μl SYBR Green Master Mix (Qiagen). The thermal cycling was carried out by starting at 95°C for a 10 min hold, followed by 40 amplification cycles at 95°C for 15 s, and at 60°C for 60 s. Dissociation curve analysis was performed at the end of 40 cycles to check the identity of the PCR product. No signals were detected in no-template controls.

The results are expressed as threshold cycle (Ct) values that correspond to the cycle at which PCR enters the exponential phase. They were normalized to the Ct value obtained with housekeeping gene amplification; respectively, CD3 for the purified CD4+ T cells and GAPDH for the renal tissue. For each gene and each cDNA preparation, the PCR reactions were run three times and the results were averaged.

**Statistical analysis**

The data were analyzed using JMP 6.0 software (SAS Institute, Cary, NC); p values of <0.05 were considered statistically significant. Differences between the two groups of patients were tested by ANOVA with one-way ANOVA followed by Fischer’s PLSD tests. Correlations between continuous variables were tested using the linear regression model.

**Results**

**Chronically rejected kidneys display heterogeneous graft survival time**

For the current study, 11 human renal allografts, removed for terminal failure due to chronic active rejection, were used. The individual characteristics of the patients are presented in Table I.

<table>
<thead>
<tr>
<th>Characteristics of the Donor</th>
<th>Characteristics of the Recipient</th>
<th>Time on Dialysis (d)</th>
<th>Rank of Tr</th>
<th>IS Regimen</th>
<th>No. of AR</th>
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<td>CS, MMF, Siro</td>
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<td>1311</td>
<td>2</td>
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<td>1</td>
<td>CS, MMF, CsA</td>
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*No. of biopsy-proven episodes of acute rejection.

AF, African; APKD, autosomal dominant polycystic kidney disease; ATG, antithymocyte; Aza, azathioprine; Cs, corticosteroid; CsA, cyclosporine A; D, deceased; ESRF, end-stage renal failure; Etero, everolimus; F, female; FK506, tacrolimus; HUS, hemolytic and urticemic syndrome; IS, immunosuppressive; M, male; MMF, mycophenolate mofetil; NAS, nephroangiosclerosis; Siro, sirolimus; Tr, transplantation.
The graft survival time, that is, the time between the transplantation and the return to hemodialysis, ranged between 157 and 5467 d (0.43 and 14.97 y) and did not display a normal distribution. A recent study has established that the Kaplan-Meier half-life of renal grafts is ≈8 y (12). We therefore divided our cohort into two groups: patients whose graft survived less than 8 y (n = 8) were considered fast progressors for chronic rejection, and patients whose graft survived >8 y (n = 3) formed the slow progressor group (Fig. 1A).

To further establish the difference in chronic rejection kinetics between these two groups, all the biopsies of the 11 grafts were reviewed and a failure curve was plotted (Fig. 1B). As expected, grafts from the fast progressor group reached the Banff grade II chronic allograft nephropathy (13) more rapidly than grafts from the slow progressor group (p = 0.044).

**Histopathological features of chronically rejected kidneys**

Careful staging of the histopathological lesions was performed for each detransplanted graft using the Banff classification (13). Samples from the slow and the fast progressor groups displayed a similar level of acute (Fig. 1C) and chronic (Fig. 1D) rejection lesions, ruling out the possibility that the differences in graft survival time between these two groups were related to a difference of the moment of explantation during the course of chronic rejection.}

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**FIGURE 1.** Chronically rejected grafts are infiltrated by T lymphocytes. *A,* The graft survival time of each chronically rejected kidney was plotted. A vertical line indicates the Kaplan-Meier half-life for renal grafts as reported by Meier-Kriesche et al. (12). Samples with shorter survival time formed the fast progressor group (n = 8; black dots). The grafts that lasted >8 y (n = 3; white dots) constituted the slow progressor group. *B,* All the biopsies performed on the 11 renal grafts of the cohort (n = 30, median = 3 per graft, range 0–6) were blindly reviewed by a pathologist and the chronic lesions were scored according to the Banff scale (13). A failure curve was obtained for the two groups (fast progressor group: red curve; slow progressor: blue curve) by plotting, for each sample, the time between the transplantation and the first biopsy showing grade II chronic allograft nephropathy. Samples for which the last biopsy was below this threshold were right censored. *C and D,* The rejection lesions of detransplanted renal grafts were blindly scored (from 0 to 3) by a pathologist using the Banff semiquantitative scale (13), in which four histological compartments are evaluated: the glomeruli (g), the interstitium (i), the tubules (t), and the blood vessels (v). The mean score, for each histological compartment, is provided for the fast and slow progressor groups for acute (*C*) and chronic (*D*) rejection lesions. *E,* The composition of intragraft inflammatory infiltrates was defined by combining histological and flow cytometry analysis. The percentages of eosinophils, neutrophils, monocytes, plasma cells, and lymphocytes were evaluated by morphological analysis. The proportions of T CD4⁺, T CD8⁺, and B cells among the lymphocytes were then defined by flow cytometry. No statistically significant difference was observed between the two groups. *F,* The distribution of naive (CD45RA⁺) and memory (CD45RO⁺), CD4⁺, and CD8⁺ T cell subsets, among the CD3⁺ T cell infiltrating chronically rejected grafts was determined by flow cytometry for the two groups. *ns,* p > 0.05.
The size of the interstitial inflammatory infiltrates (i Banff score) was similar for the samples of the two groups (slow versus fast: 1.67 ± 1.15 versus 1.78 ± 0.99; p = 0.87).

Histological and flow cytometry analysis were combined to more precisely define the composition of the intragraft infiltrates. No significant difference was observed between the two groups (Fig. 1E). In line with previous studies (2, 3, 14), CD3⁺ T lymphocytes were the main cell population infiltrating the interstitium of the chronically rejected grafts. The distribution of naïve and memory, CD4⁺ and CD8⁺ subsets among the CD3⁺ cells was equally similar (Fig. 1F).

Fast and slow progressors display opposite intragraft Th polarization profiles

The polarization profile of the intragraft CD4⁺ T cell population was determined for each sample as follows: total RNA was extracted from cell suspensions of purified intragraft CD4⁺ T lymphocytes (sorting purity 90.64 ± 9.49%). The relative gene expression for the five specific “master regulator” transcription factors, namely, Tbet for Th1, Gata3 for Th2, Rorc2 for Th17, Bcl6 for Tfh, and Foxp3 for Treg, was quantified by QRT-PCR (list of primers in Table II). The data were subsequently processed by hierarchical clustering in an attempt to group samples according to their polarization profile.

Ward’s clustering method efficiently discriminated the three samples of the slow progressor group from the eight samples of the fast progressor group (Fig. 2A). Slow progressors were characterized by the absence of detectable expression of Rorc2 and a high level of expression of Foxp3, whereas fast progressors displayed the opposite polarization profile (slow versus fast progressor: Rorc2 = 0.00 ± 0.00 versus 0.68 ± 0.04; p < 0.0001 and Foxp3 = 0.93 ± 0.01 versus 0.84 ± 0.04; p = 0.009). Of note, the two samples from the fast progressor group that did express Foxp3 concomitantly with Rorc2 (CR1 and CR6) were characterized by a significantly longer graft survival time than the six remaining samples of the fast progressor group (2428 ± 672 versus 1109 ± 1025 d; p = 0.04). The level of expression of Bcl6 (0.95 ± 0.05 versus 0.85 ± 0.04; p = 0.012) and Gata3 (0.94 ± 0.01 versus 0.86 ± 0.04; p = 0.02) was significantly higher in the slow progressor group and the level of expression of Foxp3 was similar in the two groups (0.84 ± 0.04 versus 0.81 ± 0.02; p = 0.12).

Table II

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<th>Amplicon Length (bp)</th>
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<td>5'-TCA GAC TGA GGT TGG GGT TC-3'</td>
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<td>BCL6</td>
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<td>5'-TGT GAC GGA AAA GCC GCA GGT TA-3'</td>
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<td>158</td>
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The size of intragraft Th17 infiltrate is correlative to the kinetics of chronic rejection

Pairwise correlation analysis was performed to determine how the expressions of the 5 “master regulator” transcription factors are related to each other as well as their relation to the graft survival time (Fig. 2B).

In accordance with the data presented above, we observed a negative correlation between the expressions of Rorc2 and Foxp3 (r = -0.73; p = 0.01). Moreover, there was a significant negative correlation between graft survival time and Rorc2 expression (r = -0.82; p = 0.001) and a positive correlation between graft survival time and Foxp3 expression (r = 0.73; p = 0.01).

Of note, we also observed an unexpected positive correlation between Tbet and Gata3 expressions (r = 0.69; p = 0.02), two transcription factors that respectively control the commitment in the Th1 and Th2 pathways, which are known to be antagonistic (15).

Influence of Th17 infiltrate on the intragraft microenvironment

The cytokine microenvironment of explanted kidney allografts was analyzed by QRT-PCR. Pieces of renal tissue, obtained from the intact portion of five kidneys removed for renal cancer, were used as control tissue (“normal kidneys”).

The expression of IL-10, a key regulatory cytokine, was increased in all chronically rejected grafts, but this increase was less pronounced in the fast progressor group (Fig. 2C, left panel). In contrast, IL-17 expression was only increased in the fast progressor group (Fig. 2C, middle panel). This dichotomy in the cytokine microenvironment of the two groups of chronically rejected grafts, observed at the gene expression level, was confirmed at the molecular level by immunofluorescence analyses (Fig. 3A). In the interstitium of kidneys from the fast progressor group, T lymphocytes were principally IL-17-producing Th17 cells. IL-10–producing Tregs were barely detectable. The opposite was true for the graft with the longest survival time (Fig. 3B).

In addition to producing IL-17, Th17 cells have been shown to produce a large amount of IL-21 (16). In line with these data a significant increase of the expression of IL-21 was observed in the samples from the fast progressor group (Fig. 2C, right panel).
Intragraft IL21 promotes lymphoid neogenesis

In accordance with previous studies (17, 18), B cell nodular aggregates were found in the interstitium of chronically rejected kidneys, but in none of the five normal kidneys. At the center of certain of these aggregates, a core of B cells was expressing activation-induced cytidine deaminase (AID), suggesting that these nodules were functional intragraft germinal centers (Fig. 3C). Indeed, the B lymphocyte maturation in germinal centers is characterized by the expression of AID, the key enzyme that controls class switch recombination and somatic hypermutations (19).

In an attempt to quantify lymphoid neogenesis, namely, the process by which de novo organized lymphoid structures emerge from chronic inflammatory infiltrates, the relative expression of AID was measured by QR-PCR. The level of expression of AID was correlative to that of RORC2 (r = 0.59; p = 0.05) and the linear regression model showed the closest correlation when AID was plotted against IL-21 (Fig. 3D). (r = 0.87; p < 0.0001).

In contrast, no correlation was found between the levels of expression of AID and BCL6, the “master regulator” transcription factor for Tfh (20), or between IL-21 and BCL6 (data not shown). In line with these results, the percentages of Tfh infiltrating the chronically rejected grafts were not different in the slow and fast progressor groups (Fig. 3E).

The correlation between Th17 infiltration and AID expression suggests that Th17 cells could hasten graft destruction by promoting lymphoid neogenesis. Several studies have indeed reported that intragraft ectopic germinal centers are the site of elicitation of a local humoral immune response (18, 21–23).

During humoral rejection, the binding of alloantibodies to the graft leads to the activation of the classical pathway of the complement system that in turn promotes tissue destruction.
FIGURE 3. Th17 cells produce IL-21 that promotes lymphoid neogenesis. A and B, Representative findings of immunofluorescence analyses performed on the two groups of detransplanted renal grafts are shown. A, Cytokinetic microenvironment was analyzed at the cellular level as follows: cryosections were stained with Abs specific for human IL-10 (left panel) or IL-17 (right panel; red). DAPI (blue) was used to counterstain the nuclei. Upper row: fast progressor; lower row: slow progressor. Original magnification ×630. B, To detect Tregs and Th17 cells, cryosections were stained with Abs specific for human CD3 (green), IL-10 or IL-17 (red), and FOXP3 (pink). DAPI (blue) was used to counterstain the nuclei. The lowest thumbnails of each column show merged stainings. Original magnification ×630. C, Histological analysis was carried out on successive serial sections of kidney grafts explanted for terminal failure. Representative findings characterizing chronically rejected grafts are shown (original magnification ×50). H&E staining showed the nodular organization of the inflammatory infiltrate. Nodules were composed of a core of CD20 positive B cells. In certain nodules a subset of B cells, located at the center of the nodule, was expressing AID, namely, the key enzyme controlling class switch recombination and somatic hypermutations. D, The relative gene expression levels of IL-21 and AID were measured as described in Fig. 2C. Individual values of the 11 detransplanted grafts (eight fast progressors, black dots; three slow progressors, white dots) and the five normal kidneys (gray dots) were plotted. The linear regression model shows a close positive correlation between IL-21 and AID expressions. The formula of the linear regression is provided, as well as \( r \), the coefficient of determination, and the \( p \) value of the regression. E, The percentage of TfhS, defined as CD3+ CD4+CXCR5+ CD28+ (44), among the CD3+ CD4+ T cells infiltrating chronically rejected grafts was determined by flow cytometry. Representative findings of the flow cytometry analyses are shown. Box plots represent the distribution of the percentages of TfhS in the slow progressor (Slow prog), and fast progressor (Fast prog) groups. ns, \( p > 0.05 \). F, Representative findings of immunofluorescence analyses performed on the two groups of detransplanted renal grafts are shown. Complement activation was detected by staining the cryosections with anti-human C5b9 (pink). DAPI (blue) was used to counterstain the nuclei. Original magnification ×630.
Th17 INFILTRATE IN CHRONIC REJECTION

product of the inactivation of C4b by factor I, is currently used in clinic as an indirect marker for the activation of the classical pathway (24). Although reliable for the diagnosis of acute humoral rejection, several reports have pointed out the fluctuations of C4d staining in time, which reduces its sensitivity for the diagnosis of chronic humoral rejection (25). In addition, C4d staining is very difficult to interpret on explanted renal grafts because of false-negative results of the staining in areas of necrosis [(25), O. Thaunat and N. Patey, unpublished data]. Therefore, in an attempt to reliably analyze the participation of the humoral arm of the immune system, we performed immunofluorescence staining for the membrane attack complex (C5b9), the terminal complement complex, in the 11 detransplanted kidneys (Fig. 3F). In agreement with our hypothesis, C5b9 staining was principally detected in the grafts with a high level of Th17 infiltration, namely, the fast progressors.

Discussion

Although animal experimental models have provided important insights into the pathophysiology of chronic rejection (3, 26), the validity of these findings in the clinical setting is sometimes difficult to assess. Complementary studies, using human samples, are therefore mandatory for collecting valid information on pathophysiology of clinical diseases.

Chronically rejected human kidneys are sometimes removed, providing a large amount of diseased tissue. We took advantage of this opportunity to evaluate the influence of intragraft inflammatory infiltrate on the course of clinical chronic rejection.

We are perfectly aware that working with detransplanted human kidneys has several drawbacks: 1), the fact that only chronically rejected renal grafts with terminal failure were analyzed precluded a kinetic analysis of the process, and 2), the correlative nature of our observations was inherent to study in humans. In contrast, the advantages of using detransplanted grafts largely outweigh the drawbacks: they enable us to analyze the whole organ, both at the cellular and the molecular levels, thus avoiding two important limitations of studies using graft biopsies, 1), the problem of the representativeness of the sample, and 2), the risk for misinterpretations due to the limited number of techniques that can be used when studying biopsies (27).

In line with what has previously been reported (2, 3, 14), we found that T lymphocytes represented the main subset (60%) of inflammatory cells infiltrating chronically rejected grafts. We therefore focused our attention on this cell population. Neither the size of the interstitial lymphocytic infiltration nor the distribution of naive and memory, CD4+ and CD8+ T cells, correlated with the kinetics of chronic rejection. Interestingly, we observed that the polarization profile of the Th infiltrate displayed opposite features in slow and fast progressors. Indeed, Tregs were the main subset found in the samples of the slow progressor group. In contrast, Th17 infiltration was correlated with a faster progression toward terminal chronic rejection. These findings were not unexpected given that 1), the destructive potential of Th17 cells has been documented in various chronic inflammatory diseases (28, 29), and 2), Treg and Th17 differentiation pathways of are known to be antagonistic (16, 30). However, the slow progressor group raises an intriguing question: why did these three grafts, all characterized by a high Treg/Th17 ratio, also develop terminal chronic rejection? The fact that the high number of Tregs failed to control the destruction of the grafts was probably not related to a defective regulatory function of these cells. Indeed, IL-10–producing Tregs were detected in the interstitium of rejected grafts and enrichment in IL-10 was observed in the cytokinic microenvironments of the slow progressor grafts. These data suggest that Tregs play an important role in slowing down the chronic rejection process, as previously proposed (9–11). On the other hand, it is important to note that renal grafts from the two groups displayed similar levels of infiltration by immune effectors. Only the T lymphocyte polarization profiles were different: in the slow progressor group Th17 were replaced by Th1 and Th2 cells, two potent effector subsets which can induce chronic rejection (6). It therefore appears that Th17 cells, although very effective in accelerating the process, are not necessary to induce chronic rejection.

The recruitment of neutrophils can be considered to be one of the plausible mechanisms by which Th17 cells could hasten chronic destruction of the graft. Indeed, IL-17 has been shown to mobilize neutrophils through granulopoiesis and CXC chemokine induction, as well as increased survival locally (31), and previous studies have reported the destructive potential of neutrophils on allografts (32). However, we did not observe an increased proportion of neutrophils in the samples of the fast progressor group. Instead, we found that an increased proportion of Th17 cells in the infiltrate was not only associated with an enrichment of IL-17 but also of IL-21. IL-21 is a type I cytokine that amplifies Th17 responses (16). Recent studies have demonstrated that IL-21 not only acts as a growth factor for Th17 cells, but also has critical functions for the development of humoral immune responses. In fact, IL-21 is both 1), necessary for the differentiation of T(H)s (33, 34), a subset of CD4+ T cells that has a crucial role in T cell help for B cells (35), and 2), able to directly promote the activation and the differentiation of B cells (36, 37).

The importance of the humoral arm of immune response in the pathophysiology of chronic rejection has long been demonstrated (38–40). Our group (18, 21, 23) and others (17, 41) have contributed to document the role of lymphoid neogenesis, namely, the progressive organization of inflammatory cells into structures that morphologically resemble secondary lymphoid organs (42) during chronic rejection. Accumulating evidence indeed suggests that intragraft ectopic germinal centers support the maturation of a local humoral immune response, which in turn contributes to tissue destruction (21–23). The close correlation observed between the levels of expression of AID (the key enzyme in the germinal center reaction) and IL-21 leads us to propose that Th17 cells hasten graft destruction by promoting lymphoid neogenesis through the production of IL-21. This hypothesis is further substantiated by evidence of complement activation detected in grafts with a high level of Th17 infiltration.

Preliminary data suggest that IL-21 produced by Th17 cells directly stimulates intragraft B cells rather than through the generation of T(H)s, which is in line with the conclusions of Nurieva et al. (33).

Finally, it is interesting to note that in using detransplanted kidneys, we reached conclusions compatible with those proposed by Burlingham et al. who recently published the first study reporting the involvement of Th17 cells in clinical lung chronic rejection (43).

In conclusion, although Th17 cells are not necessary for chronic rejection, their presence within the graft was correlative to a faster progression of the destruction process. In contrast, grafts infiltrated by regulatory T cells survived significantly longer.

The deleterious effect of Th17 could be mediated by their production of IL-21, a cytokine able to promote intragraft lymphoid neogenesis, which in turn support the development of a local humoral immune response.

Further studies are necessary to determine whether Th17 infiltration can be used as a prognosis tool and whether the Th17 subset constitutes a valuable therapeutic target for slowing down chronic rejection.
Acknowledgments
We thank the urologists, pathologists, and nephrologists from the Edouard Herriot, Foch, Henri Mondor, Necker, and Pasteur Hospitals for assistance in the collection of the samples. We are grateful to Dr. Laure-Hélène Noel and Dr. Dominique Desvaux for expertise in renal pathology. We also thank Anne Durandy for anti-AID Abs and Tom Cupedo for the primers specific for human RORC2. We are indebted to Antonino Nicoletti and Jean-Baptiste Michel for mentorship.

Disclosures
The authors have no financial conflicts of interest.

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