Cardiovascular, Pulmonary and Renal Pathology

Intragraft Expression of the IL-10 Gene Is Up-Regulated in Renal Protocol Biopsies with Early Interstitial Fibrosis, Tubular Atrophy, and Subclinical Rejection

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Grafts with subclinical rejection associated with interstitial fibrosis and tubular atrophy (SCR+IF/TA) show poorer survival than grafts with subclinical rejection without IF/TA (SCR). Aiming to detect differences among SCR+IF/TA and SCR, we immunophenotyped the inflammatory infiltrate (CD45, CD3, CD20, CD68) and used a low-density array to determine levels of T_H1 (interleukin IL-2, IL-3, γ -interferon, tumor necrosis factor- α , lymphotoxin- α , lymphotoxin- β , granulocyte-macrophage colony-stimulating factor) and T_H2 (IL-4, IL-5, IL-6, IL-10, and IL-13) transcripts as well as of IL-2R (as marker for T-cell activation) in 31 protocol biopsies of renal allografts. Here we show that grafts with early IF/TA and SCR can be distinguished from grafts with SCR on the basis of the activation of IL-10 gene expression and of an increased infiltration by B-lymphocytes in a cellular context in which the degree of T-cell activation is similar in both groups of biopsies, as demonstrated by equivalent levels of IL-2R mRNA. These results suggest that the up-regulation of the IL-10 gene expression, as well as an increased proportion of B-lymphocytes in the inflammatory infiltrates, might be useful as markers of early chronic lesions in grafts with SCR. (*Am J Pathol* 2010; 176:1696–1704; DOI: 10.2353/ajpath.2010.090411)

Subclinical rejection (SCR), ie, the presence of histological lesions typical of acute allograft rejection (according to Banff criteria) in patients with stable renal function, has been

associated with silent progression of interstitial fibrosis and tubular atrophy (IF/TA).^{1–2} Furthermore, there is evidence that the simultaneous presence of subclinical rejection, interstitial fibrosis and tubular atrophy (SCR+IF/TA) could be associated with a poorer graft survival when compared with grafts with subclinical rejection without IF/TA (SCR), or with grafts with IF/TA but without subclinical rejection (IF/TA), suggesting that different mechanisms of immune response could be involved in the process of graft degeneration.³

CD4⁺ T helper (T_H) lymphocytes are essential regulators of the immune response. After activation by antigen-presenting cells, T_H lymphocytes differentiate into effector cells, specialized in cytokine secretion, that can be further classified as type 1 (T_H1) or type 2 (T_H2) based on their profiles of cytokine gene expression and immune regulatory function. The process of acute rejection has been related to the activation of the T_H1 response, with the subsequent production of interferon (IFN)- γ , interleukin IL-2, and other cytokines and chemokines, whereas the T_H2 response, which mediates humoral immunity, has been associated with chronic allograft rejection.^{4–6} However, cytokines of both T_H cell subtypes have been detected in patients during episodes of acute rejection, in which the levels of IL-7, IL-8, IL-10, IL-15, γ -IFN, perforin, granzyme-B, and Fas ligand transcripts, but not of IL-2, were found to be up-regulated.⁷

Studies of renal biopsies from clinically-stable transplanted patients, performed two to three years after transplantation, evidenced a low degree of immune

Supported by grants from Fundació Marató de TV3 (005310; to E.N.), Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, (PI020766 and PI040086; to E.N. and D.S., respectively), Red Temática de Investigación Cooperativa en Trasplantes (Red-064), and a Juan de la Cierva program fellowship (to M.H.).

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Accepted for publication December 14, 2009.

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activation in the allografts.⁵ Furthermore, in these biopsies, the severity of the inflammatory lesions associated with interstitial fibrosis and tubular atrophy could be correlated with an increase in IL-6 gene expression, a T_H2 cytokine.⁵ On the other hand, expression of TNF- α and IL-8 genes was only correlated with tubular atrophy, and no correlation was detected among changes in the expression of the IFN- γ , IL-1 β , IL-2, or IL-4 genes with any histological injury.⁵ Finally, in this complex context of pathophysiological alterations and changes in the expression of immune-related genes, no morphological trait has been found that could be used as a marker of the degree of immune activation to specifically detect those infiltrates associated with chronic lesions.

Low-density arrays (LDA), based on the use of real-time RT-PCR, have been introduced as a novel method for gene expression profiling and offer higher throughput than usual single gene analysis.⁸ We underwent the analysis of the expression of T_H1 (IL-2, IL-3, γ -IFN, tumor necrosis factor [TNF]- α , lymphotoxin- α , lymphotoxin- β , granulocyte-macrophage colony-stimulating factor [GM-CSF]) and T_H2 (IL-4, IL-5, IL-6, IL-10, and IL-13) cytokine genes in protocol biopsies of kidney allograft using LDA, with the aim to characterize the population of cells that infiltrated renal allografts, as well as to identify differences in gene expression that could facilitate the early detection of chronic lesions.

Materials and Methods

Patients

In this pilot study we used protocol biopsies from renal allografts performed during the first year of follow-up, obtained between 1995 and 1998, from which total RNA was available for analysis. Protocol biopsies were performed as previously described⁹ in patients who gave their informed consent and had a serum creatinine level below 250 μ mol/L, proteinuria below 1 g/day, and stable renal function defined as a variability of serum creatinine of less than 15% during the two weeks before and after of the biopsy.

For all biopsies, two cores of tissue were obtained, one of them was processed for conventional histology and the second was immediately snap frozen in liquid nitrogen and stored at -80°C for RNA extraction. Renal lesions were blindly evaluated according to the 2005 Banff working classification criteria.¹⁰ We identified nine patients with the simultaneous presence of SCR and IF/TA and 6 patients with SCR without IF/TA, and selected 16 additional normal protocol biopsies matched by the time of biopsy, to conduct a case-control study. Jurkat T-leukemia cells, activated with 20 nmol/L phorbol 12-myristate 13-acetate (PMA) and 1 μ mol/L ionomycin, were used as positive control for T-lymphocyte activation in the cytokine mRNA detection by real-time PCR. The Hospital Ethics Committee approved this study, and a written informed consent was obtained from each patient.

Immunohistochemical Analysis

For immunohistochemical studies, paraffin-embedded specimens were sliced at 4 μ m, incubated with monoclonal antibodies to CD45 (pan-leukocytes), CD3 (T-lymphocytes), CD68 (monocytes/macrophages), CD20 (B-lymphocytes; all from Master Diagnostica SL, Granada, Spain), or IL-10 (R&D Systems, Minneapolis, MN) and subsequently stained with a goat-anti-mouse secondary antibody labeled with horseradish peroxidase (Master Diagnostica SL) in an automated immunohistochemical stainer (Lab Vision, Fremont, CA).¹¹ In all cases, sections were counterstained with hematoxylin to make nuclei evident. Tonsil sections and biopsies from human kidney with acute rejection were used as positive controls in the identification of IL-10-expressing cells. A positive result was defined, by the pathologist, as the detection of IL-10-stained cells in the infiltrates. Positively-stained cells in the whole renal cortex were counted at $\times 20$ to $\times 40$ magnification and acquired with a digital camera. For the leukocyte markers, results were expressed as the number of positive cells/mm², or as the percentage of each population regarding the CD45 cells. With the aim to identify antibody-related rejection, C4d deposits were evaluated using a polyclonal antibody (Biomedica Medizinprodukte GmbH & co KG, Vienna, Austria). The negative control replaced the primary antibody by rabbit normal serum.¹¹ Positive C4d staining was defined as the presence of C4d deposition in more than 50% of peritubular capillary sections.

RNA Isolation and cDNA Synthesis

Total RNA isolation and purification, reverse transcription, and quality analysis of the synthesized cDNA were done as previously described.¹²

Low-Density Array

A sample of approximately 500 ng of cDNA was run (by duplicate) on the ABI PRISM7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA) using the LDA technology (microfluidic cards) with commercial probes and primers, and according to the manufacturer's protocol. The LDA contained predesigned primer probe sets for the following human cytokine and cytokine receptor genes (Applied Biosystems):

IL-2 (ID number: Hs00174114_m1); IL-2 receptor (ID number: Hs00166229_m1); IL-3 (ID number: Hs00174117_m1); IL-4 (ID number: Hs00174122_m1); IL-5 (ID number: Hs00174200_m1); IL-6 (ID number: Hs00174131_m1); IL-10 (ID number: Hs00174086_m1); IL-13 (ID number: Hs00174379_m1); lymphotoxin α (ID number: Hs00236874_m1); lymphotoxin β (ID number: Hs00242739_m1); γ -IFN (ID number: Hs00174143_m1); TNF- α (ID number: Hs00174128_m1); and GM-CSF (ID number: Hs00171266_m1); as well as the following endogenous controls glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) (ID number: s99999905_m1); and 18S rRNA, (ID number: 4342379-18S).

Table 1. Clinical Characteristics of Patients at Baseline, and at the Time of Protocol Biopsy, According to Histological Categories

	Normal <i>n</i> = 16	SCR <i>n</i> = 6	SCR+IF/TA <i>n</i> = 9	<i>P</i>
Donor age, years	41 ± 15	43 ± 23	47 ± 19	ns
Recipient age, years	51 ± 15	50 ± 04	45 ± 15	ns
Donor sex, male/female	8/8	4/2	2/7	ns
Recipient sex, male/female	10/6	5/1	7/2	ns
Number of transplant, 1st, 2 days	12/4	5/1	9/0	ns
PRA >20%	4 (25%)	1 (17%)	2 (22%)	ns
HLA mismatches	2.69 ± 1.25	2.50 ± 1.04	3.11 ± 1.45	ns
CIT, hours	21 ± 4	22 ± 3	23 ± 6	ns
ATN, no/yes	14/2	6/0	8/1	ns
Acute rejection, no/yes	14/2	4/2	4/5	0.07
At protocol biopsy				
Time after transplantation, days	189 ± 106	138 ± 39	242 ± 140	ns
Serum Creatinine level, μmol/L	129 ± 28	141 ± 41	184 ± 49*	0.006
Creatinine clearance, ml/min	58 ± 11	55 ± 13	50 ± 20	ns
Proteinuria, g/day	0.22 ± 0.10	0.18 ± 0.06	0.50 ± 0.29*†	0.001

PRA indicates panel reactive antibodies; HLA, human leukocyte antigen; CIT, cold ischemia time; ATN, acute tubular necrosis.

**P* ≤ 0.005 vs Normal (Bonferroni test); †*P* = 0.006 vs SCR (Bonferroni test).

Transcript levels were calculated using the delta threshold cycle (Ct) method based in the relative quantification of cytokine expression normalized to the house-keeping gene after determining the first cycle of fluorescence detection (Ct), and calculating the difference of these threshold cycles. In a second step we normalized the results observed in grafts with SCR to the expression in normal protocol biopsies by applying the $2^{-\Delta\Delta Ct}$ method, as described by the manufacturer (Applied Biosystems; User Bulletin 2). The normalization to GAPDH or 18S rRNA as internal controls gave comparable results. In the case of the data presented in this manuscript, normalization was referred to the 18S rRNA internal control. When no detectable level of transcript was found, a value equal to half the minimum observed 18S-normalized level was assigned.¹³

Statistical Analysis

To analyze the normal distribution of variables, the Kolmogorov-Smirnov test was applied. Student *t* test and the analysis of variance were used in normal quantitative variables. Mann-Whitney *U* test and Kruskal-Wallis test were applied to those not following a normal distribution and to ordinal variables. Differences between categorical data were assessed using a 2 × 2 contingency table analysis and the Fisher exact tests. Correlation between transcript levels and lymphocyte infiltration was analyzed by the Spearman rank correlation coefficient. Results were expressed as mean ± SD. *P* values were corrected for the number of variables compared according to the Bonferroni method. The outlier values (values outside 1.5 times the interquartile range above the third quartile or below the first quartile) in box-plot graphs were not considered for the quantification of cytokine transcript levels. All *P* values were two-tailed, and a *P* value <0.05 was considered significant.

Results

Patients and Biopsies

We studied 31 patients (22 males and 9 females) with a mean age of 48 ± 12 years and with a protocol biopsy performed at 194 ± 112 days after transplantation (ranging from 95 to 485 days). Sequential protocol biopsies were available for 18 patients.

All patients received a cyclosporine (CsA)-based immunosuppression associated either with antilymphocytic antibodies (*n* = 14), azathioprine (*n* = 8), or mycophenolate mofetil (*n* = 9). Table 1 shows the baseline clinical characteristics and clinical evolution after transplantation in patients with normal protocol biopsies (*n* = 16), with protocol biopsies displaying subclinical rejection with interstitial fibrosis and tubular atrophy (SCR+IF/TA; *n* = 9), or showing subclinical rejection without interstitial fibrosis nor tubular atrophy (SCR; *n* = 6). Grafts with SCR+IF/TA showed the poorest creatinine levels (184 ± 49 μmol/L in SCR+IF/TA patients versus 141 ± 41 μmol/L in SCR patients or 129 ± 28 μmol/L in normal controls; *P* = 0.006) and proteinuria (0.50 ± 0.29 g/day in SCR+IF/TA patients versus 0.18 ± 0.06 g/day in SCR patients or 0.22 ± 0.10 g/day in normal controls; *P* = 0.001) of all groups studied.

All renal biopsies contained at least one arterial section, and the mean number of glomeruli in the biopsy core was 10 ± 6. Sample size distribution according to 1997 Banff criteria were as follows: 16 (51.6%) were adequate (≥10 glomeruli, more than 2 arteries), 10 (32.2%) represented minimum sample adequacy (between seven and nine glomeruli, and at least one arterial section), and 5 (16.1%) biopsies were inadequate (between one and six glomeruli and at least one arterial section). The severity of IF/TA, according to the Banff score, in the group of patients of SCR+IF/TA was mild in eight cases and moderate in one case. Table 2 scores acute and chronic lesions in all patients of the study according to Banff criteria. The

Table 2. Severity of Acute and Chronic Lesions According to Banff Criteria

	Normal	SCR	SCR+IF/TA	P
n	16	6	9	
g	0.00 ± 0.00	0.17 ± 0.40	0.33 ± 0.50	0.055
i	0.19 ± 0.40	1.00 ± 0.00	1.00 ± 0.00	<0.0001
t	0.00 ± 0.00	1.00 ± 0.00	0.78 ± 0.44	<0.0001
v	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
ah	0.06 ± 0.25	0.33 ± 0.51	0.13 ± 0.35	0.27
cg	0.00 ± 0.00	0.17 ± 0.40	0.44 ± 0.52	0.012
ci	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.50	<0.0001
ct	0.06 ± 0.25	0.00 ± 0.00	1.00 ± 0.50	<0.0001
cv	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.35	0.26

Shown are the mean ± SD of the Banff scores. The abbreviations used in the above Table correspond to the following pathological categories: g, glomerulitis; i, interstitial infiltrate; t, tubulitis; v, vasculitis; ah, arteriolar hyalinosis; cg, chronic glomerular lesions; ci, chronic interstitial lesions; ct, chronic tubular lesion; cv, chronic vascular lesions.

histological diagnosis of marginal biopsies was normal in 4 cases, and the fifth case displayed subclinical rejection with interstitial fibrosis and tubular atrophy (SCR+IF/TA).

Grafts with SCR+IFTA were clinically stable, and graft function was followed for 118 ± 27 months. Figure 1 demonstrates that grafts diagnosed of SCR did not dis-

Figure 1. Clinical evolution of serum creatinine levels in the three groups of patients studied in this work. Serum creatinine levels were determined at the time of the protocol biopsy (time = 0) and at the stated times. Values show that the renal function was stable in the different groups and demonstrate that grafts actually suffered a process of subclinical rejection, rather than a non-overt clinical rejection. Bx followed by a number is our identification code for the protocol biopsies.

play a non-overt clinical rejection, because creatinine levels were mostly stable in the different groups. In this period of time, one patient from the group of normal protocol biopsies lost their graft due to chronic allograft dysfunction associated with nephrotic proteinuria 93 months after transplantation (no diagnostic biopsy was performed), and another three died with a functioning graft (one due to a sepsis, and other two due to coronary syndrome). In the group with SCR only, one patient lost their graft due to chronic allograft dysfunction 43 months after transplantation (no diagnostic biopsy was available for further study), but this patient died later of myeloma. Finally, three patients in the group of SCR+IF/TA lost their grafts, two due to biopsy-proven IF/TA at 98 and 120 months after transplantation, and one due to a “*de novo*” glomerulonephritis. In addition, one patient died with a functioning graft due to acute myocardial infarction in the group of SCR+IF/TA.

SCR+IF/TA Allografts Showed More Infiltrating B-Lymphocytes than SCR Allografts

We studied the cellularity of interstitial infiltrates by immunohistochemistry (IHC) using antibodies specific for B-lymphocytes (anti-CD20), T-lymphocytes (anti-CD3), macrophages (anti-CD68), and total leukocytes (anti-CD45). Table 3 shows that grafts with interstitial fibrosis and tubular atrophy (SCR+IF/TA) displayed a more severe infiltration of total (T and B) lymphocytes, but not of macrophages, expressed as the absolute number of infiltrating cells per mm², when compared with renal tissue from normal protocol biopsies (598 ± 268 CD3⁺ cells/mm² in SCR+IFTA grafts versus 253 ± 149 CD3⁺ cells/mm² in normal protocol biopsies, $P = 0.005$; 353 ± 264 CD20⁺ cells/mm² in SCR+IFTA grafts versus 79 ± 108 CD20⁺ cells/mm² in normal protocol biopsies, $P = 0.015$; 261 ± 99 CD68⁺ cells/mm² in SCR+IFTA grafts versus 193 ± 133 CD68⁺ cells/mm² in normal protocol biopsies, $P = 0.112$). When grafts with SCR+IFTA and grafts with SCR were compared, no significant differences in the absolute number of infiltrating cells were found.

We also determined the relative weight of each cell population in the composition of the inflammatory infiltrate

Table 3. Density of Infiltrating Cells in Protocol Biopsies According to Their Immunophenotype (in Number of Cells per mm²)

mAb	Normal n = 11	SCR n = 6	SCR+IF/TA n = 7	P
CD45	460 ± 334	696 ± 293	1034 ± 500*	0.019
CD3	253 ± 149	435 ± 176	598 ± 268†	0.005
CD20	79 ± 108	175 ± 154	353 ± 264*	0.015
CD68	193 ± 133	326 ± 116	261 ± 99	0.112

Cellularity of interstitial infiltrates was studied by immunohistochemistry using antibodies specific for B-lymphocytes (anti-CD20), T-lymphocytes (anti-CD3), macrophages (anti-CD68), and total leukocytes (anti-CD45). Positively stained cells in the whole renal cortex were counted at ×40 magnification, and results were expressed as the mean ± SD of the number of positive cells per mm². The number of patients studied is stated below each clinical condition.

* $P = 0.01$ vs Normal (Bonferroni test); † $P = 0.005$ vs Normal (Bonferroni test).

Table 4. Relative Proportions of CD3⁺, CD20⁺, and CD68⁺ Cells Regarding CD45⁺ Cells in Protocol Biopsies

mAb	Normal n = 11	SCR n = 6	SCR+IF/TA n = 7	P
CD3	61 ± 18%	64 ± 10%	62 ± 21%	ns
CD20	12 ± 10%	23 ± 18%	35 ± 23%*	0.04
CD68	44 ± 19%	51 ± 16%	28 ± 9% [†]	0.04

For each case, we calculated the number of CD3⁺, CD20⁺, and CD68⁺ infiltrating cells as percentage of total CD45⁺ cells, and expressed the result as the mean ± SD.

*P = 0.038 vs Normal (Bonferroni test); [†]P = 0.056 vs Normal (Bonferroni test).

by normalizing the number of infiltrating cells to the total number of CD45 cells, expressing the result as percentage. Table 4 shows that the value for B lymphocytes was significantly higher in SCR+IF/TA grafts than in SCR grafts or in normal tissue (35 ± 23% in SCR+IF/TA biopsies versus 12 ± 10% in normal protocol biopsies, and 23 ± 18% in SCR biopsies, *P* = 0.04), whereas no significant differences were found for T lymphocytes (62 ± 21% in SCR+IF/TA biopsies versus 61 ± 18% in normal protocol biopsies and 64 ± 10% in SCR biopsies *P* = ns). Regarding macrophages, a higher proportion was detected in normal biopsies and SCR grafts than in SCR+IF/TA grafts (44 ± 19% in normal protocol biopsies, 51 ± 16% in SCR biopsies versus 28 ± 9% in SCR+IF/TA grafts, *P* = 0.04).

With the aim to study a potential pathogenic role of the humoral immunity in the development of SCR and SCR+IF/TA, we analyzed the diffuse deposits of C4d along the peritubular capillaries from 26 biopsies, but no differences were evident among the groups (with 2 of 12 normal protocol biopsies, 2 of 6 SCR biopsies, and 3 of 8 SCR+IF/TA grafts presenting C4d deposits, *P* = ns). In addition, the number of infiltrating B lymphocytes detected did not correlate with the diffuse C4d deposition found in peritubular capillaries (161 ± 195 B cells/mm² in grafts without C4d staining versus 196 ± 159 in grafts with diffuse C4d staining, *P* = ns).

SCR+IF/TA Allograft Biopsies Showed Significantly Higher Levels of IL-10 mRNA than Protocol Biopsies with SCR

We studied the expression of 13 cytokine genes (T_H1 and T_H2 responses) in our group of protocol biopsies by using the LDA technology, and the results obtained are shown in Table 5. The most significant finding was the frequent detection of IL-10 transcripts in SCR+IF/TA grafts (detected in 77.8% of SCR+IF/TA grafts versus 33.3% of SCR grafts and 37% of normal protocol biopsies; *P* = 0.06). On the contrary, we could not detect IL-4 or IL-13 transcripts in any of our samples, and IL-2 (6.5%), IL-3 (9.7%), IL-6 (19.4%), and GM-CSF (3.2%) mRNAs were detected only in a small number of the entire group of protocol biopsies.

Expression data were also quantitatively analyzed. Regarding the expression of T_H1-associated cytokines that were prevalently detected in protocol biopsies (γ-IFN,

Table 5. Number of Protocol Biopsies in which the Stated Cytokine Transcripts Were Detected

Cytokines	Normal n = 16	SCR n = 6	SCR+IF/TA n = 9	P
IL-2	2 (12%)	0	1 (11.1%)	ns
IL-2 receptor	9 (56%)	5 (83.3%)	6 (66.7%)	ns
IL-3	3 (19%)	0	0	ns
IL-4	0	0	0	ns
IL-5	0	0	1 (11.1%)	ns
IL-6	3 (19%)	2 (33.3%)	1 (11.1%)	ns
IL-10	6 (37%)	2 (33.3%)	7 (77.8%)	0.06
IL-13	0	0	0	ns
γ-IFN	7 (43%)	3 (50%)	6 (66.7%)	ns
TNF-α	12 (75%)	5 (83.3%)	6 (66.7%)	ns
Lymphotoxin α (LTA)	3 (19%)	2 (33.3%)	3 (33.3%)	ns
Lymphotoxin β (LTB)	10 (62%)	5 (83.3%)	8 (88.9%)	ns
GM-CSF	0	0	1 (11.1%)	ns

Total RNA was extracted from the protocol biopsies, reverse transcribed to cDNA, and used for cytokine transcript quantification by real-time RT-PCR using low-density arrays (Applied Biosystems). Shown are the number of protocol biopsies in which expression of the stated cytokine genes was detected in the groups studied.

IL indicates interleukin; γ-IFN, gamma interferon; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte-macrophage colony-stimulating factor.

TNF-α, lymphotoxin-α, lymphotoxin-β), no differences were found among normal, SCR, and SCR+IF/TA grafts (data not shown). On the other hand, expression analysis of T_H2-associated cytokines showed a significant increase in the expression of IL-10 transcripts in biopsies with SCR+IF/TA when compared with normal or SCR biopsies (−19 ± 3 cycles in biopsies with SCR+IF/TA, −24 ± 3 cycles in biopsies with SCR, and −23 ± 3 cycles in normal biopsies, *P* = 0.017) as shown in Figure 2A. To determine the relative increase of IL-10 gene expression in SCR+IF/TA over SCR levels, we normalized values of IL-10 mRNA expression in both groups, relative to normal biopsies, by applying the 2^{−ΔΔCt} method (Figure 2B). One outlier at the SCR+IF/TA group and one extreme value at the SCR group were detected and excluded from the analysis. As shown in Figure 2B, levels of the IL-10 mRNA were shown to be increased in biopsies with SCR+IF/TA when compared with levels present in protocol biopsies with SCR (131.9 ± 112-fold increase in IL-10 transcript levels in SCR+IF/TA biopsies regarding normal protocol biopsies, versus 3.5 ± 3-fold increase in IL-10 levels in SCR biopsies regarding normal protocol biopsies, *P* = 0.011).

With the aim to identify the source of IL-10 we performed IHC analysis in 18 biopsies from which paraffin-embedded material was still available (eight biopsies with normal histology, five biopsies with SCR, and five biopsies with SCR+IF/TA). However, very few IL-10-positive cells were evident in the inflammatory infiltrates, even in the control biopsies of clinical acute rejection, thus making double staining worthless. Thus, biopsies were classified merely by the presence or absence of IL-10-producing cells in the cellular infiltration. In this way, we detected IL-10-positive cells in three of the eight biopsies with normal histology, three of the five SCR

Figure 2. Box-plot showing intragraft expression of IL-10 mRNA levels in protocol biopsies. Total RNA was extracted from the protocol biopsies, reverse-transcribed to cDNA, and used for IL-10 transcript quantification by RT-PCR using LDAs (Applied Biosystems). Levels of IL-10 mRNA were quantified by two different methods (see below). **A:** Levels of IL-10 mRNA were calculated by using the Ct method, as the Ct difference with 18S rRNA (18S_C-IL10_C). When no detectable levels of transcript were found, a value equal to half the minimum observed 18S-normalized level was assigned. Values represent the *n*-fold differences in the number of cytokine transcripts. **B:** Levels of IL-10 mRNA were normalized according to the expression in normal protocol biopsies by applying the $2^{-\Delta\Delta CT}$ method. Values represent the *n*-fold differences in the number of cytokine transcripts related to the expression in normal protocol biopsies. In both cases, each diagram represents the median, quartiles, outliers (○), and the extreme values (*). The colored box represents the interquartile range that contains 50% of the values. The whiskers are lines that extended from the box to the highest and lowest values, excluding outliers. A line across the box represents the median value.

biopsies, and three of the five biopsies with SCR+IF/TA ($P = ns$; see Figure 3).

Next, we analyzed the possible relationship among IL-10 expression and lymphocyte infiltration with progression to IF/TA, in sequential protocol biopsies from 18 patients. From these, nine patients displayed histology without IF/TA in both biopsies, in five patients there were a progression of lesion to IF/TA (three biopsies with a normal histology and two biopsies with SCR), and four patients displayed IFTA in both biopsies. In two patients, the progression of lesions was associated with clinical deterioration (one case with a previous normal biopsy and one case with a previous biopsy with SCR lesions). Furthermore, we detected that allografts with IF/TA in both biopsies displayed higher levels of IL-10 mRNA (-18 ± 1 cycles in grafts with IF/TA in both biopsies, $n = 4$; versus -21 ± 3 cycles in grafts without IF/TA in both biopsies $n = 9$; and versus -24 ± 2 cycles in grafts with a progression to IF/TA, $n = 5$; $P = 0.031$), but no differences in the proportion of infiltrating B-lymphocytes versus CD45⁺ cells were observed ($26 \pm 2\%$ in grafts with IF/TA in both biopsies, $n = 2$; versus $23 \pm 16\%$ in grafts without IF/TA in both biopsies, $n = 7$; and versus $13 \pm 12\%$ in grafts with a progression to IF/TA, $n = 5$; $p = ns$), suggesting that these markers had no predictive value.

We also analyzed IL-10 gene expression and cell infiltration in protocol biopsies from grafts that were lost due to IF/TA, with the aim to identify markers associated to the outcome. Grafts that were lost due to IF/TA displayed non-significantly higher levels of IL-10 mRNA (-20 ± 3 cycles in grafts lost due to IF/TA $n = 4$ versus -23 ± 3 cycles in long-lasting grafts, $n = 22$; $P = 0.17$), a non-significantly higher degree of T-lymphocyte infiltration regarding CD45⁺ cells ($82 \pm 18\%$ in grafts lost due to IF/TA, $n = 3$ versus $61 \pm 15\%$ in long-lasting grafts, $n = 18$; $P = 0.08$) and of B-lymphocytes versus CD45⁺ cells

Figure 3. Detection of IL-10-positive cells in protocol biopsies with SCR+IF/TA or SCR. Shown are the two protocol biopsies, together with a sample from human tonsils and another one from a renal allograft suffering an episode of acute allograft rejection as positive controls. Tissue samples were prepared for IHC, stained with an anti-IL-10-specific antibody, counterstained with hematoxylin to make nuclei evident, and scored at $\times 20$ magnification. Shown are representative individual sections of the ten studied for each sample in which positive cells can be observed in the biopsy with SCR+IF/TA and not in the SCR biopsy. In all of the panels, arrows indicate IL-10-positive cells. Shown are also several renal tubuli (t) and glomeruli (G). Bx followed by a number is our identification code for the protocol biopsies.

($40 \pm 42\%$ in grafts lost due to IF/TA, $n = 3$ versus $18 \pm 13\%$ in long-lasting grafts, $n = 18$; $P = ns$).

IL-10 Expression Correlated with B-Lymphocyte Infiltration in Protocol Renal Allograft Biopsies

We analyzed the correlation between transcript levels, for all of the above T_H1 and T_H2 cytokines, and infiltrating cells in protocol biopsies. We found that IL-10 mRNA levels were significantly correlated with the number of CD20⁺ cells/mm² ($r = 0.69$, $P = 0.009$) but not with CD45⁺ cells/mm², CD3⁺ cells/mm², or CD68⁺ cells/mm². Furthermore, IL-10 mRNA levels did not correlate with the time of the protocol biopsies. Finally, and for the rest of the cytokine genes studied, we could not detect any correlation among graft infiltration and level of expression (data not shown).

SCR+IF/TA Allograft Biopsies Did Not Show Higher T-Lymphocyte Activation than Biopsies with SCR

Finally, because the expression of functional IL-2 receptor (IL-2R) is induced on activation of naïve and effector

T-cells, and it is always expressed in regulatory T-cells, which might be permanently activated by self antigens, we analyzed the expression of the IL-2R mRNA in our group of protocol biopsies. No significant differences in IL-2R mRNA levels were detected among the different groups studied (-17 ± 1 cycles in biopsies with SCR+IF/TA, -17 ± 3 cycles in biopsies with SCR, and -18.1 ± 2 cycles in normal biopsies, $P = 0.18$), suggesting that the degree of T-cell activation was similar in the patients from these groups.

In conclusion, here we show that grafts with early IF/TA and SCR (subclinical rejection associated with interstitial fibrosis and tubular atrophy) can be distinguished from grafts with SCR on the basis of the higher IL-10 mRNA expression and the increased infiltration by B-lymphocytes shown by the former, in a cellular context in which the degree of T-cell activation is similar in both populations, as demonstrated by the equivalent levels of IL-2R mRNA. However, these markers did not seem to have predictive value, suggesting that the higher IL-10 mRNA levels and increased B-lymphocyte infiltration could be surrogate markers for chronic inflammation in SCR+IF/TA.

Discussion

Previous studies of protocol biopsies showed that subclinical rejection associated with interstitial fibrosis and tubular atrophy (SCR+IF/TA) implied a poorer graft outcome than SCR or IF/TA alone, suggesting that different mechanisms of immune response could be causing the graft degeneration in these clinical conditions.¹⁻³ Among these pathophysiological mechanisms, graft infiltration by mononuclear cells should be considered influential because of its potential to produce inflammatory lesions that would eventually lead to fibrosis.

In this study, we describe the characterization of the lymphocytic infiltrate in protocol biopsies from renal allografts. We show that infiltrates in SCR+IF/TA grafts displayed a higher proportion of B-lymphocytes, and a similar degree of T-cell activation, than grafts with SCR only. We also analyzed the expression of T_H1 and T_H2 cytokines and found that IL-10 was the only cytokine whose expression discriminated SCR+IF/TA from SCR, in contrast with another report in which more cytokine genes were found to be overexpressed.¹⁴ This difference was presumably attributable to the different techniques used, which probably will have differences in dynamic ranges, detection limits, or in their ability to cause false positive results, although because no comparative analysis has been published it is very difficult to ascertain the actual cause. In the work here presented, we also amplified the housekeeping 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as internal controls for each sample analyzed. In this way, 18S rRNA was readily detectable after 18 cycles (18 ± 3 cycles, considering all of the samples), and the GAPDH mRNA was usually detected at cycle 27 (27 ± 2 , considering all of the samples, data not shown) so that lack of detection of other cytokine transcripts was probably attrib-

utable to their very low expression in our group of protocol biopsies.

Our results suggest that SCR does not represent a single homogeneous clinical condition, and that changes in the composition of the infiltrating mononuclear cell population could result in increased risks to develop chronic lesions. Regarding the fact that SCR+IF/TA displayed a higher proportion of B-lymphocytes in the interstitial infiltrate than SCR grafts, this result complements those previously published by our group that demonstrated, in a large group of protocol biopsies, that the presence of B-lymphocytes was associated with histological damage and poor outcome.¹¹ Here, we have shown that this pathophysiological role is restricted to B-lymphocytes, because neither T-cells nor macrophages were differentially detected in the inflammatory infiltrates, and no differential T-cell activation was observed because the expression of IL-2 receptor was similar in SCR+IF/TA, SCR, and normal grafts. These results are in agreement with the observation, by others, that the degree of $CD20^+$ B-cell infiltration in renal biopsies was associated with the severity of the acute graft rejection and with graft loss,¹⁵ and highlight the controversial role of B-cells in solid organ transplantation, because they participate, as antigen-presenting cells, in the indirect pathway of alloantigen presentation, as well as in the cellular regulation of T-lymphocytes and dendritic cells.¹⁶ In addition, it has been reported that the time after transplantation and the degree of interstitial inflammation were independent predictors of B-cell-associated transcript and of immunoglobulin associated transcript scores.¹⁷ When we tested sequential biopsies from normal grafts or from grafts with SCR for B-cell infiltration, we did not observe a predictive value for the progression to IF/TA, although these results were hindered by the few cases available for study. Thus, we propose that detection of B-lymphocytes in interstitial infiltrates could be a surrogate marker for chronic inflammation, but not for progression to IF/TA.

On the other hand, the observation that the IL-10 gene expression was specifically induced in SCR+IF/TA grafts raised the question of whether this resulted from T_H2 polarization, a fact that could partially explain the poor prognosis of stable grafts with SCR and IF/TA in early protocol biopsies, or if, alternatively, IL-10 could be produced by macrophages or regulatory T-cells. We aimed to determine the lineage of the IL-10-producing cells by performing double IHC on paraffin-embedded samples of protocol biopsies, but few IL-10-producing cells were detected, even in control samples of clinical acute rejection. Furthermore, we observed a lack of correlation of IL-10 detection among qPCR and IHC, probably because of the different sensitivities of the two techniques.

Our results agree with the previous observation that grafts with acute rejection showed an increased IL-10 expression,⁷ although the role of IL-10 in graft rejection is yet controversial because it has been reported as inducing both anti-inflammatory and pro-inflammatory effects. In this sense, IL-10 secreted by T-regulatory cells was recently shown to suppress antigen-specific effector cell responses via a cytokine-dependent mechanism that included the down-regulation of pro-inflammatory T_H1 cy-

tokines.¹⁸ Furthermore, in a recent work using a rat renal acute rejection model to study changes in gene expression subsequent to the grafting, Edemir et al detected the overexpression of genes related to the acute immune response, the activation of the T_H1 response, as well as the up-regulation of the IL-10 gene, and suggested that the activation of this last could have a counterregulatory role to limit the inflammatory response.¹⁹ This protective action of IL-10 has been experimentally confirmed by demonstrating that transduction of the IL-10 gene in a rat model of kidney allograft rejection improved renal function and prolonged allograft survival.²⁰ However, IL-10 is also a stimulator of the immune system, because it induces the differentiation and proliferation of B cells, leading the immune response toward the humoral pathway and enhancing antibody responses against the graft,²¹ a result that would be partially in agreement with our current observation that patients with SCR and IF/TA showed a more severe B cell interstitial infiltrate than patients displaying SCR without IF/TA. Furthermore, and accordingly with our previous results,¹¹ here we show that grafts with SCR+IF/TA did not display a higher proportion of peritubular C4d staining, and that there were no correlation between C4d and the degree of B-lymphocyte infiltration, suggesting that B-lymphocyte infiltration may not necessarily be a surrogate marker of antibody-mediated chronic allograft rejection.

On the other hand, the high IL-10-producer genotype has been associated with episodes of steroid-resistant renal allograft rejection and with SCR in six-month protocol biopsies,^{22–23} and high pretransplant IL-10 secretion, measured in mixed lymphocyte cultures, were also correlated with acute renal allograft rejection.^{24–25} Furthermore, Hribova et al^{26–27} showed, by real-time RT-PCR, that IL-10 gene expression was up-regulated during acute rejection when compared with nonrejecting controls, as well as in patients suffering chronic allograft dysfunction or delayed graft function, whereas pretransplant serum IL-10 levels were shown to be up-regulated in patients who would undergo rejection.²⁸ Interestingly, Everolimus (an immunosuppressant drug that prevents tubular atrophy and interstitial fibrosis) had been previously shown to reduce IL-10 synthesis in renal allograft recipients.^{29–30} Thus, the up-regulation of IL-10 mRNA levels in stable protocol biopsies displaying SCR, as well as the increase in the proportion of B-lymphocytes, would suggest the presence of a chronic deleterious infiltrate. Whether these B-lymphocytes were working as antigen-presenting cells, as indirect effector cells, or as antibody-producing cells is not yet known. Furthermore, and although we cannot discard that intragraft B-lymphocytes might play a direct role in the development of the low-grade inflammatory lesions that conduce to the scarring of the graft, our results indicate that they could be considered as a histological marker of a previous severe inflammation leading to a poorer prognosis, but would not be useful to predict progress to IF/TA in normal grafts or grafts with SCR without IF/TA.

In summary, this study shows that protocol biopsies with subclinical rejection associated with interstitial fibrosis and tubular atrophy (SCR+IF/TA) presented a more

severe infiltrate of B lymphocytes, a similar degree of T-cell activation, and higher IL-10 mRNA levels than protocol biopsies with SCR but without chronic lesions. Although not supporting a pathogenic role for infiltrating B-lymphocytes, our results suggest the utility of using B-cell infiltrates and IL-10 mRNA levels as markers of the low-grade inflammatory lesions that conduce to the scarring of the graft.

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