Tofacitinib Halts Progression of Graft Dysfunction in a Rat Model of Mixed Cellular and Humoral Rejection

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Background. The progression from acute to chronic antibody-mediated rejection in kidney transplant recipients is usually not prevented by current therapeutic options. Here, we investigated whether the use of tofacitinib (TOFA), a Janus kinase 3 inhibitor, was capable of preventing the progression of allograft dysfunction in a Fisher-to-Lewis rat model of kidney transplantation.

Methods. Rats were treated from the third week after transplantation to allow the development of rejection. Treatment was based on cyclosporin A, rapamycin or TOFA. Renal function was assessed at 1, 4, 8, and 12 weeks after transplantation, whereas rat survival, histological lesions, and infiltrating lymphocytes were analyzed at 12 weeks. **Results.** Tofacitinib prolonged graft survival, preserved tubular and glomerular structures and reduced humoral damage characterized by C4d deposition. Tofacitinib was able to reduce donor-specific antibodies. In addition, T and natural killer cell graft infiltration was reduced in TOFA-treated rats. Although rapamycin-treated rats also showed prolonged graft survival, glomerular structures were more affected. Moreover, only TOFA treatment reduced the presence of T, B and natural killer cells in splenic parenchyma. **Conclusions.** Tofacitinib is able to reduce the immune response generated in a rat model of kidney graft rejection, providing prolonged graft and recipient survival, better graft function, and less histological lesions.

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Despite new immunosuppressive strategies, long-term graft survival in kidney transplantation has not improved. Chronic allograft rejection represents indeed the main cause of graft failure after death with a functioning graft.^{1–4} Nowadays, immunosuppressive strategies mainly

inhibit the T-cell response, and only recently, the focus has switched on how to halt the antibody-mediated immune response. The humoral theory is based on the development of donor-specific antibodies (DSA) that are responsible for graft rejection.^{5–7} A complicated process mediates the development

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of antibodies upon exposure to antigens. Antigens are presented by either donor or recipient antigen-presenting cells to CD4⁺ T cells, which then activate B cells via cytokines and costimulatory factors. Immature B cells are differentiated into either memory B cells or antibody-forming plasma cells.⁸ Plasma cells subsequently produce antibodies for longer than a year without further implication of T cells.⁹

Recently, the innate immune system, in particular natural killer (NK) cells, has been related with antibody-mediated rejection due to antibody-dependent cellular cytotoxicity, direct lysis, IFN γ release and B cell activation.¹⁰ Natural killer cell transcripts have been detected in renal biopsies from patients under antibody-mediated rejection.¹¹

Allograft rejection is characterized by the production of a variety of cytokines including members of the IL-2, IL-12, IL-27 families, interferon, and growth factors. Upon engagement with their respective receptors, these cytokines activate downstream effector molecules, such as mammalian target of rapamycin (RAPA), protein kinase C, and Janus kinase (JAK)/signal transducer and activator of transcription (STAT).¹² Concretely, the JAK/STAT is a critical signaling pathway activated by many cytokines receptors. After cytokine engagement, JAK phosphorylates the cytoplasmic tail of its receptor, which becomes activated, leading to the recruitment of STATs. Afterwards, STATs translocate into the nucleus, where they regulate the expression of numerous genes.¹³ The pathological consequences of deregulated JAK/STAT signaling have been widely documented in many animal models of cancer, chronic inflammatory and autoimmune diseases.¹⁴ This association with severe immune dysfunction reveals the crucial role of the JAK/STAT pathway in the immune response. The JAK3 inhibitor, tofacitinib (TOFA), specifically binds to the ATP-binding pocket of JAK3 and is also capable to bind to both JAK1 and JAK2.^{15,16} Therefore, TOFA is categorized as a pan-JAK inhibitor preferentially inhibiting JAK3 and JAK1, and to a lesser extent JAK2. JAK3 is selectively expressed in hematopoietic cells, NK cells, thymocytes, T and B cells, and myeloid cells, and mutation in this kinase results in a loss of immunological function and in severe combined immunodeficiency.¹³ Tofacitinib has been approved for rheumatoid arthritis and is also being evaluated for the treatment of other autoimmune diseases. Furthermore, treatment with TOFA was beneficial in experimental lupus nephritis¹⁷ and has also been investigated for the prevention of rejection in solid organ transplantation.¹⁸⁻²¹ Nevertheless, TOFA development in transplant medicine was stopped after the results of a phase IIb trial.²²

Here, we performed a kidney transplant rat model with late introduction of immunosuppressive drugs, that is, immunological damage already developed at time of their introduction (3 weeks). We hypothesized that blocking JAK3 could effectively halt the progression of allograft dysfunction and graft loss. The results were compared with transplanted rats treated with conventional immunosuppressants that are used in clinical practice, namely, RAPA and the calcineurin inhibitor cyclosporin A (CsA).

MATERIALS AND METHODS

Animals and Surgical Technique

Male Lewis (LEW, RT1¹) rats were recipients of either male LEW or Fischer-344 (F344, RT1^{1v1}) grafts for syngeneic

and allogeneic renal transplants, respectively. F344 and LEW strains differ partially at major histocompatibility complexes and various non-MHC loci, conferring a weaker histocompatible combination. The animals were kept at constant temperature, humidity, and at a 12-hour light/dark cycle with free access to water and rat chow. The study was approved by and conducted according to the guidelines of the local animal ethics committee (Decret 214/97).

Donor organ procurement and kidney transplantation were performed under anesthesia with isoflurane. Briefly, donor kidneys were flushed with sterile physiologic saline at 4°C after systemic application of sodium heparin (1000 UI). Renal transplants were performed with an end-to-side anastomosis of the aortic stump of the donor kidney and recipient's aorta, and between the recipient inferior vena cava and donor renal vein, respectively, using 9-0 running nylon sutures.²³ The mean time for anastomosis was 24.8 $\pm$ 3.3 minutes. Uretero-ureterostomy was performed with an end-to-end interrupted sutures technique using 11-0 nylon sutures. Both native kidneys were removed at the time of engraftment.

Experimental Design and Follow-up

Three weeks after allogeneic transplantation, rats that were still alive were randomized into 5 groups. F-L + $\emptyset$; standard rat chow (n = 30); F-L + CsA: modified rat chow with 150 mg cyclosporin/kg of food (n = 11); F-L + RAPA: modified rat chow with 80 mg RAPA/kg of food (n = 13); F-L + TOFA50: modified rat chow with 50 mg TOFA/kg of food (n = 8); and F-L + TOFA100: modified rat chow with 100 mg TOFA/kg of food (n = 9). In addition, 11 syngeneic grafts were transplanted (L-L + $\emptyset$). One week after transplantation and monthly until the end of the study at 12 weeks, rats were weighed and placed in metabolic cages for 24-hour urine and tail-vein-blood collection. Blood urea nitrogen (BUN), urine and serum creatinine, and proteinuria (PU) were determined. At the end of the study, the animals were killed and kidney graft and spleen were harvested. Then, the kidney graft was cut transversally in 3 pieces: the middle piece was fixed in formalin, 1 cortex piece was used for infiltrating immune cell detection by flow cytometry, and an additional piece was kept for control analysis. Histological analysis and flow cytometry were performed only in surviving rats.

Drug Concentrations Analysis

Sirolimus whole blood concentrations were analyzed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method previously validated in our laboratory.²⁴ Cyclosporine was analyzed by enzyme multiplied immunoassay.

A highly sensitive and specific LC/MS/MS method has been developed and validated for TOFA quantification in rat plasma. Briefly, to a 75- μ L aliquot of calibrators, quality controls and plasma from treated rats, 200 μ L of internal standard working solution was added, vortex-mixed, and added to the preconditioned HLB Oasis cartridge (Waters Corporation, Milford, MA). The analytes were eluted using 1-mL methanol, and the eluent was evaporated under gentle stream of nitrogen. The dried residue was reconstituted in 200- μ L acetonitrile: 0.2% formic acid in water (80:20, V:V) and 5- μ L clear aliquot was injected onto LC/MS/MS system. Analyte resolution was accomplished using an ACQUITY UPLC BEH C18, 1.7 μ m, 2.1 $\times$ 100 mm (Waters Corporation). A gradient mobile phase (MP) was used composed of 10 mM ammoniac

acetate (pH, 5) in water (MP-A) and acetonitrile (MP-B) at a flow rate of 0.4 mL/min. Runtime analysis was 7 minutes. Then, mass spectrometry analysis was performed by ACQUITY-Xevo TQ-S micro system in positive ionization mode (Waters Corporation). The lower limit of quantification for TOFA is 0.4 ng/mL. The specific transitions used were TOFA (313.2 and 149.0), and TOFA-13C3-15N (317.2 and 149.0).

DSA Detection

Donor-specific antibody levels were analyzed using flow cytometry. Serum samples from recipient animals were incubated with 5×10^5 splenocytes from Fisher rats, followed by FITC-anti-rat IgG. PE-anti-rat CD3, PE-Cy7-anti-rat CD8 and APC-anti-rat CD45RA surface markers were used to differentiate T cells, T cytotoxic cells, and B cells. The cells were fixed and analyzed using FACS Canto-II flow cytometer. The results were expressed as mean fluorescence intensity (MFI).

Conventional Histology and Immunohistochemistry

Formalin-fixed tissue was embedded in paraffin, and 3- μ m sections were cut. A renal pathologist (E.V.M.) evaluated hematoxylin-eosin, periodic acid Schiff and Sirius Red stains for each graft to evaluate tubular atrophy (TA), fibrosis (F), tubulitis, capillaritis peritubular and glomerular pathology. TA and F were scored from grade 0 to 3 (0, none [$<10\%$]; 1, mild [10 to 25%]; 2, moderate [26 to 50%]; 3, severe [$>50\%$]). Tubulitis was scored from grades 0 to 3 according to Banff criteria (1, 1-4 lymphocytes per tubular section, 2, 5-10 lymphocytes per tubular section and 3, >10 lymphocytes). peritubular capillary (PTC) was scored from grade 0 to 3: 1, at least one leukocyte cell in $\geq 10\%$ of cortical PTCs with three or four leukocytes in most severely involved PTC; 2, at least one leukocyte in $\geq 10\%$ of cortical PTCs with five to 10 leukocytes in most severely involved PTC; 3, at least one leukocyte in $\geq 10\%$ of cortical PTCs with >10 leukocytes in most severely involved PTC. Glomerular pathology was classified as minimal change (minimal mesangial enlargement or cellularity), glomerulitis (monocytes or lymphocytes are presents into the lumen of glomerular capillaries), focal segmental glomerulosclerosis (FSGS), and transplant glomerulopathy (TG). Glomerulitis was analyzed using a semiquantitative scoring system from 0 to 3 (0, no glomerulitis; 1, glomerulitis in $<25\%$ of glomeruli; 2, segmental or global glomerulitis in 25% to 75% of glomeruli; 3, glomerulitis in $>75\%$ of glomeruli). Focal segmental glomerulosclerosis was analyzed using a semiquantitative scoring system from 0 to 3 (0, no affection; 1, $<25\%$ of glomerular area affected; 2, 25% to 75% affected; 3, $>75\%$ affected). Transplant glomerulopathy was analyzed using a semiquantitative scoring system from 0 to 3 (0, no GBM double contours; 1, double contours of the GBM in 1% to 25% of capillary loops in the most affected nonsclerotic glomerulus by light microscopy; 2, double contours affecting 26% to 50% of peripheral capillary loops in the most affected glomerulus; and 3, double contours affecting $>50\%$ of peripheral capillary loops in the most affected glomerulus).

Sections (3- μ m thick) mounted on xylene glass slides (Dako, Carpinteria, CA) were used for immunohistochemistry. After antigen retrieval had been carried out, endogenous peroxidase blocking for 10 minutes in 3% hydrogen peroxide (Merck, Darmstadt, Germany) was performed before primary

antibody incubation. The primary antibody, rat anti-C4d (Hycult Biotech, PA), was incubated overnight at 4°C. Envision system-specific anti-rabbit secondary antibody labeled with horseradish peroxidase polymer (Dako, Glostrup, Denmark) was applied for 1 hour. All sections were counterstained with Mayer hematoxylin. Immunohistochemical procedure was performed at the same time to avoid possible day-to-day variations in staining performance. All images were acquired using an Olympus BX51 clinical microscope and DP70 digital camera and software (Olympus, Tokyo, Japan).

Flow Cytometry

Spleens were dissected manually into single-cell suspension. A single-cell suspension from kidney grafts was obtained after enzyme digestion (collagenase type IV; Thermo Fisher Scientific, Waltham, MA) and mechanical dissociation by GentleMacs (Miltenyi Biotec GmbH, Germany). Flow cytometry analysis was performed on a FACS Canto II (BD Biosciences, Heidelberg, Germany). For surface marker staining, cell preparations were stained with mAbs indicated in Table S1, SDC (<http://links.lww.com/TP/B556>), according to the specific experiment. Cells were stained intracellularly with Foxp3-specific mAbs using the intracellular Foxp3 staining kit from eBiosciences. In all experiments, dead cells were unambiguously excluded using the Aqua Live/Dead fixable dead cell kit (Thermo Fisher Scientific). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical Analysis

Statistical analysis was performed using the SPSS 14.0 statistics package. Univariate analysis using the log-rank test (Kaplan-Meier curves) was conducted to assess rat survival (time from kidney transplantation to death). Values are given as mean $\pm$ standard deviation. Drug levels are given as median. The Kruskal-Wallis or Mann-Whitney U tests were used where applicable.

RESULTS

We performed kidney transplantation from F344 to LEW to analyze allogenic response. LEW recipients developed a progressive renal allograft dysfunction in the absence of treatment. A group of 5 rats were killed after 3 weeks to analyze the histological lesions. Twenty-one days after transplantation, BUN levels increased from 10.5 ± 1.08 to 41.6 ± 10.01 mg/dL ($P = 0.0018$). Three weeks after transplantation, 75 LEW recipients of F344 kidney graft were randomized in 5 groups according to the treatment incorporated into the food ($\emptyset$, without treatment; CsA RAPA, TOFA50/TOFA100) that received until the end of the study, 12 weeks after transplantation.

Renal Function and Survival

The allogenic group without immunosuppressive treatment (F-L + $\emptyset$) showed a progressive impairment of renal function, with an increase of BUN, serum creatinine and PU levels along the experiment and a reduction of urine creatinine (Figure 1). Cyclosporin A treatment showed the same results or even worse than the F-L + $\emptyset$ group. Tofacitinib treatments stabilized BUN and serum creatinine levels from the randomization time until the end of the study. Meanwhile, rapamycin was only able to control BUN and serum creatinine levels until week 8 (Figures 1A and B). Considering PU, no treatment completely blocked the progression, only TOFA treatments showed lower levels of PU at each time analyzed after the randomization (Figure 1C). Tofacitinib

FIGURE 1. Effect of JAK-3 inhibition on renal function. A, BUN levels. B, Serum creatinine. C, Proteinuria. D, Urine creatinine. L-L, LEW-to-LEW; F-L, F344-to-LEW; Ø, without treatment; RAPA, rapamycin; TOFA, tofacitinib. *Significantly different when compared to F-L + Ø group at the same time of analysis (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

treatment, at higher dose, led to higher urine creatinine levels compared with all the other treatments (Figure 1D).

During the first 20 days after randomization, 40% of LEW recipients rejected the F344 kidney grafts (F-L + Ø group) and died. Both TOFA, at 50 and 100 mg/kg of food, and rapamycin treatments clearly prolonged graft survival ($P = 0.0422$, $P = 0.0313$, and $P = 0.0328$, respectively), whereas CsA treatment only led to a tendency towards better graft survival without reaching statistical significance (Figure 2).

Donor-specific Antibodies

DSA detected on F-L + Ø rats over the time was presented in Figure S1, SDC (<http://links.lww.com/TP/B556>). Only DSA that bind to CD4 and CD8 T cells increased in time dependent manner until week 4, then MFI declined at 8 and 12 weeks. The MFI decline observed could be related with rat loses over the time. The effect of the immunosuppression on DSA was presented in Figure 3. One week after

FIGURE 2. Effect of immunosuppression on recipient survival. *Significantly different when compared to F-L + Ø group at the same time of analysis (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). F-L, F344-to-LEW; Ø, without treatment.

randomization (week 4), CsA and rapamycin-treated rats presented higher titres of DSA, whereas there were no differences between the F-L + Ø group and TOFA-treated rats. Tofacitinib treatments could reduce statistically DSA presence from week 8 (Figures 3D-F) to week 12 (Figures 3G-I).

Histology and Immunohistochemistry

Natural Course Without Immunosuppression

Allogeneic grafts (F-L + Ø) showed tubular atrophy at 3 weeks after transplantation, which increased significantly at 12 weeks (Figure 4A). After 12 weeks of transplantation, kidney grafts from the surviving animals in the allogeneic group showed tubular atrophy, tubulitis, capillaritis in PTCs, and fibrosis (Figure 5). Tubulitis, capillaritis and fibrosis could be significantly reduced with either TOFA or rapamycin treatment (Figures 5B-D). In addition, rapamycin could also reduce tubular atrophy (Figure 5A). Cyclosporin A treatment could not reduce any of these parameters. Considering the glomerular compartment, kidney grafts presented heterogeneous lesions, including minimal changes, glomerulitis, FSGS, and TG (Figures 5E-G). At 3 weeks after transplantation (before randomization), all allogeneic renal grafts showed glomerular minimal changes and glomerulitis. In addition, 9 weeks later, FSGS and TG were observed in 53% and 69%, respectively (Figure 4E). Allogeneic grafts showed an increase of C4d deposition in PTCs and glomeruli after 3 weeks and an increase up to 12 weeks after transplantation (Figures 4F and G).

Evolution With Immunosuppressive Treatments

Cyclosporin A treatment did not lead to improvement in FSGS and TG compared with placebo. Rapamycin treatment led to reduction of TG score, whereas the incidence of FSGS

FIGURE 3. Detection of DSAs at 4 weeks after transplantation (A-C), 8 weeks after transplantation (D-F) and twelve weeks after transplantation (G-I). We can distinguish DSA that recognize antigens expressed by B cells, CD4 or CD8 T cells. *Significantly different when compared to F-L+ ∅ group at time (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). #Statistical difference between groups.

lesions did not change. In contrast, TOFA treatments at both doses showed a slight reduction of FSGS score without reaching statistical differences and reduced two-thirds the TG score (Figures 5F and G). Both TOFA and rapamycin

treatments reduced C4d deposition on PTCs and glomeruli (Figure 6). Cyclosporin A treatment could not reduce C4d deposition on PTC, but higher C4d staining was observed on glomeruli compartment (Figure 6).

FIGURE 4. Quantification of different damage entities observed in renal allograft during rejection process. Tubular atrophy (A), tubulitis (B), capillaritis (C) and fibrosis (D). E, Quantification of glomerular lesions including MC, glomerulitis, FSGS, and TG. F, Quantification of C4d deposition on PTCs. G, Percentage of glomerulus with C4d deposition. 3w and 12w, 3 and 12 weeks after transplantation. *Significantly different when compared with F-L (3w) group (* $P < 0.05$; ** $P < 0.01$). MC, minimal changes.

FIGURE 5. Effect of JAK-3 inhibition on renal damage induced by rejection process. Representative photomicrographs from different entities observed in allogeneic renal graft after 12 weeks of transplantation: tubular atrophy (A); tubulitis (B); capillaritis (C); and fibrosis (D). Its quantification is presented to its right side. A representative image from different glomerular lesions was presented: glomerulitis (E), FSGS (F) and transplant glomerulopathy (G). H, Quantification of glomerular lesions in allogeneic renal graft after 12 weeks of transplantation. Periodic acid Schiff stain was used in A-C and E-G whereas Sirius Red stains was used in D. Scale bar is 200 μ m (D), 100 μ m (A-B) or 50 μ m (C, E, F, G). #Significantly different when compared to L-L group. *Significantly different when compared to F-L + Ø group (* P < 0.05; ** P < 0.01; *** P < 0.001).

Flow Cytometry: Lymphocyte Graft Infiltration

The analysis of immune infiltrating cells into the renal graft is shown in Figure 7. Allogeneic grafts were infiltrated with T cells; T_{helpers} , regulatory T (Treg) cells, and $T_{\text{cytotoxic}}$ (Figures 7A-D); and NK cells (Figure 7E). Tofacitinib significantly reduced T-cell infiltration of the graft. Considering NK cells, rapamycin and TOFA blocked to the same extent NK cell infiltration (Figure 7E). Cyclosporin A treatment significantly reduced Treg cells and NK cell infiltration.

Flow Cytometry: Spleen Immune Cells

The analysis of immune cells in the spleen is shown in Figure 8. Allogeneic grafts showed an increased proportion of CD8 cells, follicular (FO) and marginal zone (MZ) B cells and NK cells in the spleen compared to syngeneic grafts. Tofacitinib significantly reduced T (helpers, regulatory, and cytotoxic), B (FO and MZ) and NK cells. Rapamycin and CsA only reduced NK cells.

Safety

No animal in the TOFA group died. At 12 weeks, the TOFA-treated animals showed the same body weight as the

syngeneic group (data not shown). No clinical manifestations of infections were observed in any of the groups.

Drug Levels

Regarding food intake, there were no differences between the different study groups. The drug levels remained stable throughout the day (Figure S2, SDC, <http://links.lww.com/TP/B556>). Before killing, immunosuppressant blood levels were determined. Food-treated rats (rapamycin 80 mg/kg) showed rapamycin blood concentrations of 4.5 ng/mL, whereas CsA blood level was 459.9 ng/mL in CsA 150 mg/kg treated rats. Tofacitinib 50 mg or 100 mg/kg of food treatments were translated in TOFA blood levels of 17.15 and 42.3 ng/mL, respectively.

DISCUSSION

The F344-to-LEW renal transplantation is a model of chronic rejection, both T cell and antibody mediated, where F344 allografts develop clinical and histopathological features of immune-mediated chronic allograft dysfunction.^{25,26} Donor-specific antibodies against both glomerular and tubular basement membranes have been detected in the sera of LEW who received F344 grafts.²⁷ Previous reports have

FIGURE 6. Effect of JAK-3 inhibition on C4d deposition. A-C, Representative images of C4d deposition on peritubular capillaries and glomeruli. D, Quantification of C4d deposition on PTCs. E, Percentage of glomerulus with C4d deposition. #Significantly different when compared to L-L group. *Significantly different when compared to F-L + Ø group (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

demonstrated that early introduction of CsA could completely block acute rejection; however, these grafts subsequently develop signs of chronic rejection after 6 to 7 months.²⁸ In our approach, all immunosuppressive regimens assessed have been started 3 weeks after transplantation to study the utility of each immunosuppressant when the rejection process is already activated. At that time, BUN levels were twice as high in the F-L group compared with the L-L group, and kidney allografts showed signs of tubular atrophy, tubulitis, capillaritis, glomerulitis, and interstitial infiltration.

This is the first study of TOFA in a rat model of renal transplantation. In our model, TOFA monotherapy was able to

prolong renal graft survival, preserving tubular and glomerular structures and reducing humoral damage characterized by C4d depositions. In addition, T and NK cell graft infiltration was reduced in TOFA-treated rats. Although rapamycin-treated rats also have prolonged renal graft survival, glomerular structures were less protected against the progression of rejection, and T-cell infiltration was not reduced. Moreover, TOFA, but neither rapamycin nor CsA, treatment reduced the amount of T (CD4 and CD8), B (FO and MZ) and NK cells in the spleen.

The worse results obtained in CsA-treated rats may also be explained by the high blood trough levels finally encountered

FIGURE 7. Effect of JAK-3 inhibition on immune cell infiltrating renal graft. A, T cells, CD3⁺. B, T_{helpers} + Treg cells, CD3⁺CD4⁺. C, Treg cells, CD3⁺CD4⁺CD25⁺FOXP3⁺. D, T_{cytotoxic} cells, CD3⁺CD8⁺. E, NK cells, CD3⁺CD314⁺CD161⁺. #Significantly different when compared to L-L group (# $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$). *Significantly different when compared to F-L + Ø group (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

FIGURE 8. Effect of JAK-3 inhibition on immune cells in spleen. A, T cells, CD3⁺. B, T_H + Treg cells, CD3⁺CD4⁺. C, T_C cells, CD3⁺CD8⁺. D, FO B cells, CD3⁺CD161⁺His24⁺His57⁺. E, MZ B cells, CD3⁺CD161⁺His24⁺His57⁺. F, NK cells, CD3⁺CD314⁺CD161⁺. #Significantly different when compared to L-L group (*P < 0.05). *Significantly different when compared with F-L + $\emptyset$ group (*P < 0.05; **P < 0.01; ***P < 0.001).

in this group. High CsA levels could induce nephrotoxicity that has been related with an increase of several components of complement system (C3, C4d, and MAC) in mice kidneys.²⁹ In our study, drug delivery is completely different from the human setting. The introduction of drugs in food improves the welfare of rats, but completely changes the pharmacokinetics of the drugs (Figure S2, SDC, <http://links.lww.com/TP/B556>). A high exposure of CsA was achieved, and despite this, the control of the immune response was not sufficient, and the animals showed signs of toxicity and worse kidney function. However, both TOFA doses achieved better results and less toxicity.

Our results are in line with previous studies in nonrenal murine models, which have evaluated the utility of TOFA treatment in the prevention of allograft rejection. Allograft vasculopathy was reduced in rat aorta transplantation by inhibiting both innate and adaptive immune systems and reducing the production of alloantibodies.³⁰ Tofacitinib treatment reduced splenic NK and CD8 T-cell proliferation in a murine allogeneic mixed lymphocyte reaction.³¹ Tofacitinib monotherapy was able to prolong cardiac allograft survival in a mouse model. In addition, coadministration of low doses of TOFA and suboptimal CsA showed a marked improvement in efficacy.³¹ Deuse et al³² demonstrated in orthotopic trachea transplantation that JAK3 inhibition more potently diminished CD3 and macrophage infiltration than everolimus without adverse effects.

Conklyn et al³³ have demonstrated that TOFA selectively reduced NK and CD8 T cells in cynomolgus monkey blood. The TOFA effect on NK and CD8 cells was dose- and time-dependent, and importantly after cessation of dosing the concentration of CD8 T lymphocytes increases to levels that were higher than the original ones. Another group demonstrated that TOFA was able to prevent kidney allograft rejection and to prolong kidney graft survival in nonhuman primates.^{19,34} Furthermore, they postulated that TOFA in combination with mycophenolate mofetil (MMF) can be used

to prevent kidney allograft rejection in nonhuman primates. However, monkeys under TOFA and MMF treatment showed dose-dependent anemia and gastrointestinal side effects.³⁵

Only 3 clinical studies have been published about TOFA treatment in kidney transplant recipients. First, van Gurp et al²⁰ developed a phase I study to demonstrate safety and tolerability of TOFA treatment at different doses up to 30 mg BID in combination with MMF in renal transplant patients with stable renal function. Their results showed an acceptable safety and tolerability, however, a high rate of infections was observed with a dose-dependent correlation to severity. In addition, a partial depletion of CD4⁺CD25^{bright} (Treg cells) in 30-mg BID doses were detected. Sewgobind et al³⁶ have been demonstrated in vitro that low dose of TOFA preferentially inhibit T effector cells (CD4⁺CD25^{dim}) whereas high dose reduce the activity of T conventional and Treg cells.

In the second and third studies, phase IIa and IIb, TOFA was used to replace calcineurin inhibitor therapy.^{21,22} Both studies demonstrated efficacy and noninferiority against CsA; however, high doses of TOFA were related with severe infections. In particular, in the phase IIa study, TOFA was associated with higher rates of opportunistic viral infections (CMV and BK). The concentration of circulating NK cells was lower indeed in TOFA-treated patients.²¹ In the phase IIb study, TOFA regimens had increased rates of infections as well and posttransplant lymphoproliferative diseases. CMV disease incidence was increased while BK virus nephropathy showed a slightly higher incidence. Histologically, TOFA-treated patients presented less interstitial fibrosis and tubular atrophy compared with CNI-treated patients. Anemia and neutropenia were more frequent in TOFA groups. Tofacitinib could inhibit JAK2, which is indeed a mediator of hematopoiesis.³⁷ Previous studies have demonstrated that high mycophenolic acid exposure could induce hematological abnormalities, including anemia and neutropenia.³⁸ Fewer TOFA-treated patients developed new onset diabetes after transplantation compared to CsA-treated patients.

After these clinical studies, no additional ones have been performed and the pharmaceutical company decided not to proceed to phase III in kidney transplantation. However, Vincenti et al³⁹ reevaluated the phase IIb trial to determine the implication of TOFA exposure. The post hoc analysis showed that patients with below-median exposure of TOFA had better clinical outcome than CsA-treated patients without increasing infection rate. Several recent reviews postulated again TOFA as an immunosuppressive tool, as long as the infectious problems were solved.^{40–42} Thus, our results could serve as preliminary experimental data in the reevaluation of TOFA treatment in kidney transplantation. More precisely, we explored the possibility to use TOFA in a particular kind of patients, namely, those patients with antibody-mediated and mixed rejection who are likely to develop chronic allograft injury. This point is worthy of attention, especially if we take into account that the available therapies for the treatment of humoral rejection are little effective to date. Our promising results about the efficacy of TOFA in this model could pave the way for future clinical studies in this very delicate subset of transplanted patients. Importantly, immunomonitoring of Tofacitinib has to be strict, in that way, the analysis of STAT5 phosphorylation by phosphoflow may be used as functional assay for cellular pharmacodynamics monitoring of TOFA activity.⁴³

In conclusion, our results showed the utility of TOFA treatment to prevent the progression of renal allograft rejection in a rat model. Tofacitinib treatment could be an addition to the armamentarium in solid organ transplant medicine. Nevertheless, additional experience and phase IIc-controlled studies are needed to demonstrate non inferiority against tacrolimus. To minimize infections and bone marrow toxicities associated with the TOFA and MMF combination, TOFA exposure should be monitored and other concomitant medicines need to be identified. In this sense, mTOR inhibitors might have a more favorable safety profile in terms of the risk of viral infections and hematological malignancies.

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