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Article type : - Original Article

Interleukin-6 Blockade with Tocilizumab Increases Tregs and Reduces T Effector Cytokines in Renal Graft Inflammation: A Randomized Controlled Trial

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Abbreviations

AE	Adverse event
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CBC	Complete blood count
CHR	Committee on human research
CNI	Calcineurin inhibitor
CMV	Cytomegalovirus
CNS	Central nervous system
EBV	Epstein Barr virus
FDA	Food and Drug Administration
GFR	Glomerular filtration rate
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
IL	Interleukin
IL-6Rα	Interleukin-6 receptor α subunit
IND	Investigational new drug
IQR	Interquartile range
KO	Knockout
MDRD	Modification of Diet in Renal disease
MMF	Mycophenolate mofetil
nTreg	Natural regulatory T cells
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
RA	Rheumatoid arthritis
SCr	Serum creatinine
TB	Tuberculosis

TCZ	Tocilizumab
TGF-β	Transforming growth factor- beta
Teff	T effector cell
Treg	Regulatory T cell
UCSF	University of California, San Francisco
ULN	Upper limit of normal

Abstract

Interleukin-6 (IL-6) is a proinflammatory cytokine and key regulator of Treg: T effector cell (Teff) balance. We hypothesized that IL-6 blockade with tocilizumab, a monoclonal antibody to IL-6R, would increase Tregs, dampen Teff function, and control graft inflammation. We conducted a randomized controlled clinical trial (2014-2018) of clinically stable kidney transplant recipients on calcineurin inhibitor, mycophenolate mofetil and prednisone, with subclinical graft inflammation noted on surveillance biopsies during the first year post-transplant. Subjects received tocilizumab (8 mg/kg IV every 4 weeks; 6 doses; n=16) or no treatment (controls; n=14) on top of usual maintenance immunosuppression. Kidney biopsies pre- and post-treatment were analyzed using Banff criteria. Blood was analyzed for serum cytokines, Treg frequencies, and T cell effector molecule expression (IFN-γ, IL-17, granzyme B) post-stimulation *ex vivo*. Tocilizumab-treated subjects were more likely to show improved Banff ti-score (62.5% vs. 21.4%, p=0.03), increased Treg frequency (3.6±1.7% vs. 7.1±5.5%, p=0.0168), and a blunted Teff cytokine response compared to controls. Changes in Banff i- and t-scores were not significantly different. The treatment was relatively well-tolerated with no patient deaths or graft loss. Blockade of IL-6 is a novel and promising treatment option to regulate the T cell alloimmune response in kidney transplant recipients. NCT02108600

1. Introduction

Long-term renal graft loss rates remain largely static and are attributed to chronic immune-mediated injury [1]. Inflammation manifested by infiltration of mononuclear cells is noted in 11–44% of surveillance biopsies within the first year in patients with stable graft function [2-4], and is linked to progressive graft fibrosis and dysfunction [5,6]. Although there is emerging consensus that persistent graft inflammation is the result of an incompletely suppressed immune response to the allograft [5,7], the appropriate clinical management remains controversial and an area of active research.

Interleukin-6 (IL-6) has pleiotropic effects including granulopoiesis, B cell growth and maturation, and T cell proliferation and differentiation [8,9]. Notably, IL-6 occupies a unique position in determining the fate of naïve T cells. In the presence of IL-6 and TGF-β, naive T cells differentiate into pro-inflammatory Th17 cells, whereas in the absence of IL-6 and in the presence of IL-2, the same cells are induced to become FOXP3+ induced Tregs (iTregs) [10-13]. IL-6 interferes with the suppressive function of mouse natural Tregs (nTregs) by rendering effector T cells resistant to regulation [14,15], ablating FOXP3 expression by epigenetic silencing of the FOXP3 locus [16], and abrogating the FOXP3-centered transcriptional program down-regulating the essential FOXP3 partner Eos [17]. In addition, IL-6 has also been shown to increase the effector memory T-cell population due to its strong anti-apoptotic properties [18]. Therefore, IL-6 is a key regulator of T cell biology and blockade of IL-6 may favor immunological quiescence.

In mouse models of transplantation, IL-6 and interferon gamma (IFNγ) are the two major cytokines upregulated during allograft rejection [19,20]. IL-6 promotes T cell allo-immune responses and impairs Treg function [21]. Neutralization of IL-6 using a monoclonal antibody, transplanting in IL-6 knockout (KO) recipients, or using IL-6KO grafts was associated with decreased IFN-γ and IL-17 mRNA, reduced alloantigen-stimulated T cell proliferation, a higher proportion of Tregs, and prolonged allograft survival [22,23]. In humanized mouse models, IL-6 production plays an important role in chronic allograft vasculopathy and anti-IL-6 therapy results in reduction of intimal proliferation [24]. In renal transplant recipients, IL-6 levels in serum, urine and biopsy tissue are elevated during renal allograft rejection (45-49) and correlate with later graft loss (47) (50). Further, genetic polymorphisms of the IL-gene have been correlated with graft survival (51). These data collectively indicate that IL-6 plays a prominent role in immune mediated graft injury.

Tocilizumab (TCZ), a recombinant humanized monoclonal IgG1 antibody to IL-6 receptor alpha subunit (IL-6R-α), prevents IL-6 from binding to both membrane bound and soluble IL-6R-α, thereby blocking the pro-inflammatory effects of IL-6 [25]. TCZ is currently approved by the FDA for the treatment of rheumatoid arthritis (RA) and has a good record of overall safety [26,27]. Patients with RA have been observed to have a significant and sustained increase in circulating Tregs following treatment with TCZ [28,29]. Interestingly, TCZ has also shown efficacy in single-arm, pilot trials to reduce HLA antibody in highly sensitized patients awaiting kidney transplant (52) and those with chronic antibody-mediated rejection (AMR) (53, 54). We hypothesized that treatment with TCZ would increase circulating Tregs and lead to a reduction in renal allograft inflammation. We, therefore, conducted the first randomized controlled clinical trial of TCZ in kidney transplant recipients to assess rigorously the immune and clinical responses to inhibition of the IL-6 pathway.

2. Material and Methods

In this prospective, single-center, randomized, controlled, pilot study conducted at the University of California, San Francisco, kidney transplant recipients with borderline change on their 6- or 12- month post-transplant surveillance biopsy were randomized 1:1 using to receive either standard of care (control group) or tocilizumab (TCZ group) for 6 months. Subjects were enrolled by the study investigator. Randomization schedule used permuted block randomization (block size 6). Treatment assignments within blocks were assigned on a 1:1 basis to TCZ or control groups based on numbers generated using a commercial spreadsheet (Excel) random number generator. Subjects were assigned to the next available position in the randomization schedule by pharmacy personnel upon notification of enrollment by the study coordinator. Block size was not divulged to the investigators or their staff and randomization table was secured within the limited-access pharmacy.

Both groups remained on the same CNI-based immunosuppression regimen as at enrollment for the duration of the study. The TCZ group received an infusion (8 mg/kg per dose) of TCZ every 4 weeks (total 6 doses) in addition to their usual calcineurin inhibitor (CNI)-based immunosuppressive regimen. Subjects in each group underwent their standard of care follow-up surveillance kidney biopsy 6 months after the index biopsy and additional 6 months of follow-up for safety. See Figure 1 for study schema. This study was approved by the institutional review board at UCSF (CHR# 13-12062) and conducted under IND 120658. It is registered with ClinicalTrials.gov (NCT02108600).

2.1 Subjects:

This study enrolled kidney transplant recipients with stable kidney function (estimated glomerular filtration rate (GFR) ≥30 ml/min/1.73 m^2 using the Modification of Diet in Renal Disease or MDRD equation [30]) who were incidentally found to have graft inflammation on a surveillance biopsy within the first year post-transplant. Subjects had to be on maintenance immunosuppression consisting of a CNI (tacrolimus or cyclosporine) and mycophenolate mofetil (MMF)/ mycophenolic acid, with or without prednisone. Graft inflammation was defined as either Banff borderline change or 10-50% interstitial mononuclear inflammation (Banff i or ti1-2) without tubulitis (t0) [31].

Major exclusion criteria were: infection requiring treatment with oral antibiotics (within the preceding 2 weeks) or IV antibiotics (within 4 weeks); history of hepatitis B, hepatitis C, tuberculosis (TB); BK viremia >1000 copies/ml, any history of malignancy except adequately treated basal or squamous cell skin carcinoma or cervical carcinoma in situ; primary or secondary immunodeficiency; and inadequate peripheral venous access. In addition, those with the following conditions were excluded due to known risks of TCZ therapy: recent or planned major surgery (including joint surgery); history of lower GI conditions that might predispose to perforation such as diverticulitis, Crohn's disease, etc.; history of pre-existing central nervous system demyelination; and liver disease. Laboratory exclusion criteria were hemoglobin <8.5 g/dL, leukocytes <3 $X10^9$/L; neutrophils <2 $X10^9$/L, platelets <100 $X10^9$/L, total bilirubin, aspartate aminotransferase (AST) or alanine aminotransferase (ALT) >1.5 times the upper limit of normal (ULN).

2.2 Study Endpoints and Sample Size Estimates

The primary endpoint was a decrease in inflammation between kidney biopsies at baseline and 6 months later as measured by a 1-point reduction in the Banff "ti" score. Secondary endpoints included: (1) Incidence of *de novo* donor specific anti-HLA antibody (DSA) between baseline and 6 months follow-up (2) Incidence of clinical acute T cell-mediated rejections (TCMR), defined as Banff type 1A or greater on for-cause biopsies performed between enrollment and 6 months follow up. (3) Incidence of opportunistic infections, including bacterial urinary tract infections, pneumonia, sepsis and TB; viral infections with CMV, EBV, or BK virus; and any systemic fungal infections between enrollment and 6 months follow up. (4) Estimated GFR by MDRD at 6 months and 12 months following enrollment (5) Death or graft loss at 6 months and 12 months following

enrollment (6) Changes in circulating immune profile at 6 months and 12 months post enrollment compared to baseline.

The target enrollment for the study was 48, based on statistical estimates of the sample size required to show an absolute difference of 40% in the proportion of subjects with reduced graft inflammation post-treatment. Unfortunately enrollment was halted in late 2017 due to depletion of grant resources.

2.3 Study Therapy

Subjects continued their usual immunosuppression regimen in both groups. MMF was maintained at the same dose as at enrollment (500-3000 mg daily) and only adjusted if needed for cytopenias or other AEs. Tacrolimus dosing was maintained or adjusted to target trough levels of 4-10 mcg/L. Subjects in the TCZ group received, in addition, TCZ 8 mg/kg (maximum 800 mg per dose) intravenously at four-week intervals for a total of 6 doses. On the day of the infusion, a focused history and physical exam were performed and laboratory data reviewed prior to the infusion, which was administered over 60 minutes in the clinical research unit. Subjects were monitored for 30 minutes after the first infusion. If they had no infusion reactions to the first infusion, they were not monitored after subsequent infusions. Criteria for premature discontinuation of the study therapy included anaphylaxis or hypersensitivity reaction to TCZ, ALT or AST value >5X ULN or persistent elevation >3X ULN, platelet count <50 $X10^9$/L, absolute neutrophil count (ANC) <0.5 $X10^9$/L, biopsy proven acute rejection during the study, or serious infection including but not limited to sepsis, BK viremia (>10,000 copies/m/L), CMV or EBV infection, tuberculosis, or systemic fungal infections.

2.4 Safety

The duration of the study was 1 year for all participants. Follow-up assessments were conducted monthly for 6 months, then every 3 months. These included adverse event (AE) and medication review, physical exam, and laboratory assessments (CBC, metabolic panel, liver function tests, tacrolimus level, quantitative BK DNA PCR in plasma, lipid panel, HLA antibody, and urine pregnancy test when applicable). Mitigation strategies for neutropenia, thrombocytopenia and liver dysfunction were specified in the study protocol. An independent data and safety monitoring board reviewed cumulative safety data at 6-month intervals.

2.5 Graft Pathology

A standard of care surveillance kidney transplant biopsy was performed in all subjects at approximately 6 months following the baseline biopsy (obtained pre-enrollment). For-cause biopsies were performed for elevated SCr, proteinuria or rising DSA. Two cores of renal allograft tissue are usually obtained during the biopsy, both used for histopathology. All biopsies were scored by using the Banff classification of kidney allograft pathology. The pathologist was blinded to the study group assignment.

2.6 Mechanistic Studies

Blood was collected at enrollment (baseline) and 3, 6, 9 and 12 months later from subjects in both groups. Samples were processed into sera and PBMC and cryopreserved for later batched analysis. Concentrations of 14 cytokines including GM-CSF, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-17, IL-23, IFNγ, and TNFα in serum samples were determined using multiplex high-sensitivity Luminex (Eve Technologies, Vancouver, BC, Canada). Frequencies of Treg and T cell phenotypes were analyzed using flow cytometry. T cell expression of effector molecules IFN-γ, IL-17, and granzyme B were measured using flow cytometry after *ex vivo* PMA/ionomycin stimulation for 4 hours. Samples from subjects in the TCZ group were tested for immunogenicity (anti-drug antibodies).

2.7 Statistical Analysis

Continuous variables are presented as median (interquartile range); comparisons between groups were made using the Wilcoxon rank sum test. Categorical variables are presented as proportions; comparison between groups were made using Fisher's exact test. The difference between before and after observations was analyzed with the paired Wilcoxon signed rank test. Significance level was set at 0.05 Findings for secondary and other outcomes were considered exploratory. Hierarchical cluster analysis of serum cytokines was performed with the aid of JMP software v15 (SAS, Cary, NC) using the Ward method. Other statistical analyses of mechanistic data were performed with the aid of GraphPad Prism v9.0.0 for Mac OS X (GraphPad Software, La Jolla, CA). Statistical method used for a specific set of data is provided in the corresponding figure legend.

3. Results

3.1 Enrollment and Subject Characteristics

33 subjects were enrolled in the study between 2014 and 2017. Of these, 30 were evaluated for the primary endpoint; 29 of 30 completed the entire study follow-up. Three subjects were withdrawn from the study (all in the control arm) due to: noncompliance with study visit 0 (n=1), subject withdrew consent after randomization (n=1), inability to obtain follow-up biopsy and care at UCSF due to change in insurance coverage at 6 months post-enrollment (n=1). One subject was lost to study follow-up (in the TCZ arm) after 7 months but continued to receive routine clinical care. Study therapy was prematurely discontinued in 3 subjects in the TCZ group due to AEs (see Section 3.4). See Figure 2 for subject disposition in the study.

Baseline characteristics of enrolled subjects are listed in Table 1. Although patients on steroid-free immunosuppression were eligible for the study, all enrolled subjects happened to be on steroid maintenance (prednisone 5 mg daily) at the time of enrollment. The two groups were similar, except for a significantly lower eGFR in the control group (difference 10.8 ml/min/1.73 m^2, 95% CI 0.46 to 21.14). Although subjects were allocated randomly to each group, randomization was not stratified for eGFR, which permitted this difference to occur and created baseline heterogeneity between the two groups.

3.2 Graft Pathology

Table 2 lists the Banff i, t and ti-scores of the baseline and follow up biopsies in all participants. All subjects had a diagnosis of borderline change (Banff t1 or t2 with i0 or i1) on the qualifying biopsy. No subjects had Banff i-score >1, t-score >2, or ti-score >2 at baseline. The scores were similar at baseline in both groups. The biopsies of subjects in the TCZ group appear to be slightly more inflamed at baseline, but this difference was not statistically significant (Table 1). Median Banff t-scores improved in both groups (p=0.009 for TCZ, p=0.004 for control). Interestingly however, the median Banff i- and ti-scores stayed the same in the control group, while they decreased in the TCZ group, but these differences over time between groups were not statistically significant (p=0.45 and p=0.09 for i- and ti-scores, respectively) (Table 2). Notably, a higher proportion of subjects in the TCZ group than the control group had a decline in the ti-score (10/16 vs. 3/14, p=0.03) (Table 3).

3.3 Acute Rejection and Graft Function

No subjects developed ≥Banff IA TCMR or AMR during the study. No subjects developed *de novo* DSA during the study. Graft function was relatively unchanged in both groups at 6 and 12 months compared to baseline (see Table 4) and there was no significant difference between the groups.

3.4 Safety

There were no cases of death, graft loss, anaphylaxis or hypersensitivity reaction to TCZ, drug-induced liver injury, severe thrombocytopenia (platelet count <50 X109/L) or severe neutropenia (ANC <0.5 X109/L) in the study. No subjects on treatment developed anti-TCZ antibodies. Study-related AEs are summarized in Table 5. Bacterial infections, BK viremia, decrease in WBC and platelet count, and mild elevations in transaminases were numerically more common in the TCZ group, although these differences were not statistically significant. No EBV, CMV, fungal or mycobacterial infections occurred during the study. In all, 3 of 16 subjects discontinued TCZ prematurely (after 2, 4 and 5 doses for BK viremia, pneumonia/sepsis and transplant pyelonephritis respectively) and 4 of 16 subjects got reduced-dose TCZ after the 3rd dose due to treatment-emergent cytopenias. All subjects recovered without sequelae or adverse impact on graft function.

3.5 Immunological and Cytokine Parameters

To determine the immunological impact of TCZ treatment, we analyzed serum and PBMC focusing on Teff function and Treg frequencies. We first analyzed serum samples collected at baseline and month 6 for the concentration of 14 cytokines that included those associated with Th1 effectors, (IFNγ, IL-2, and IL-12p70), Th17 (IL-17A and IL-23), Th2 (IL-4, IL-5, IL-13), innate immune cytokines (TNFα, IL-1γ, IL-8, and GM-CSF), and IL-6. Cytokines were well above the limit of detection in most samples. Moreover, the concentrations of the cytokines detected were within similar ranges of those in a reference data set of 650 samples across 10 unrelated studies (Table S1, data provided by Eve Technology Technical Support Team). Hierarchal cluster analysis showed that these 14 cytokines formed two polarized clusters of Th1/17 consisted of IFNγ, IL-17A, IL-12p70 and IL-23 and Th2 cluster of IL-4, -5, -13 along with IL-6 and IL-8. IL-2 and IL-1b were also found to be coordinately expressed whereas IL-10, TNFα, and GM-CSF had their own expression patterns distinct from these clusters or each other (Figure S1A). Such pattern of expression suggests that this cytokine panel can detect immunological skewing i.e. Th1, Th17, Th2 and innate immune biases in patients. Review of the baseline data from subjects randomized to control and TCZ groups show that these immune types were

evenly distributed in the two groups (Figure S1B) and there were no significant differences in any of the cytokines between the groups (Figure 3A).

We then analyzed changes in serum cytokines in samples collected at month 6 in both groups. Samples in the TCZ group for serum cytokine analysis were available from 2 subjects who received shorter TCZ treatment, 3 of the 4 subjects who were switched to half dose TCZ (4 mg/kg) after the third dose due to AEs, and 9 subjects who received full dose of the 6 month course. We thus separated the TCZ group into TCZ partial- (in duration or total dose) and TCZ full-dose subgroups when analyzing post treatment samples. In subjects who stopped TCZ treatment early, samples collected within one month after the last dose of TCZ, instead of those collected at month 6, were analyzed. We noted a dramatic rise of serum IL-6 after TCZ treatment in partial-TCZ (fold increase mean=11.4; median=6.9) and full-TCZ (fold increase mean=18.1, median=3.8) subgroups (Figure 3B and C). In contrast, IL-6 levels in the control group were mostly unchanged in the 6-month period. TCZ-induced increase of serum IL-6 was likely due to the reduced bioavailability of IL-6R and consequently lower IL-6R mediated consumption of IL-6 as reported previously [32]. This demonstrated that the dose of TCZ used was sufficient to block IL-6 interaction with its receptor in our study population, even in those who exhibited elevated serum IL-6 at baseline (Figure 3C). We also noted a trend of decreases in serum IL-17 in the full-TCZ group although the decrease was not statistically significant (Figure 3B and 3D). Other cytokines including IFNγ remained relatively unchanged (Figure 3B and E).

To determine if these changes in serum cytokines were due to changes in T cell composition or activation by TCZ treatment, we analyzed PBMC samples using flow cytometry. The frequencies of naïve ($CCR7^+CD45RO^-$), central memory ($CCR7^+CD45RO^+$), effector memory ($CCR7^-CD45RO^+$), and TEMRA ($CCR7^-CD45RO^-$) in $CD4^+$ and $CD8^+$ T cell compartments were similar between groups at baseline and remained stable throughout the treatment period (data not shown). The frequencies of $CD4^+CD25^+FOXP3^+$ Tregs were similar in the two groups at baseline (5.1±2.4% vs 4.7±3.4%, p=0.9554, Figure 4A and B). In the TCZ group, Treg frequency increased as early as 3 months after starting treatment (7.1±5.5%) and remained high until the end of the treatment (6.7±3.8%, Figure 4A and B). In contrast, frequencies of $CD4^+CD25^+FOXP3^+$ Tregs decreased in the control group (3.6±1.7% at month 3, 3.7±1.8% at month 6), resulting in significant different between the control and TCZ full-dose group. Baseline Treg frequencies varied between 1.8 to 10.8%. We thus normalized the data to patient's own baseline to better discern the changes in Tregs. This analysis showed a persistent

overall increase in Treg frequency at month 3 and 6 in the TCZ full dose group when compared to controls (Figure 4C) although there was some heterogeneity in the magnitude of increase within the treatment group. Additionally, we tested remaining available samples using $FOXP3^+HELIOS^+$ to define Tregs among $CD4^+$ T cells and found similar results of increased $FOXP3^+HELIOS^+$ Tregs in full TCZ group (Figure 4D to F). For this additional analysis, we included samples collected and month 12 that had not been collected during previous analyses of $CD4^+CD25^+FOXP3^+$ Tregs and observed a trend of decrease in Treg frequencies after the completion of TCZ treatment.

To determine if Teff function was altered by TCZ treatment, we further analyzed the PBMC samples for effector molecule expression after short-term *ex vivo* stimulation with PMA and ionomycin. At baseline, IFNγ, IL-17, and granzyme B expressing $CD4^+$ and $CD8^+$ T cells were readily detected in samples collected from subjects assigned to either groups (Figure 5). While frequencies of CD4 and CD8 T cells expressing these effector molecules remained stable or increased in the control group during study follow-up, their frequencies decreased in the TCZ-treated subjects, particularly those who received full dose treatment. Thus at 6 months study follow up in the TCZ group, there was a significant decrease in percentages of IFNγ-, IL-17-, and granzyme B producing $CD4^+$ T cells (Figure 5), significant decrease of IFNγ-producing $CD8^+$ T cells, and trend of decrease of IL-17-producing $CD8^+$ T cells and granzyme B+ $CD8^+$ T cells (Figure 5) when compared to control group. These results by flow cytometric analysis parallel the serum cytokine findings, demonstrating a decrease of Th1 and Th17 responses after TCZ treatment.

4. Discussion

In this prospective randomized controlled study in subjects with subclinical graft inflammation, we found that the majority of those treated with TCZ showed an improvement in the ti-score compared to a small proportion of controls (62.5% vs. 21.4%, p=0.03). This finding supports the hypothesis that IL-6 plays a key role in renal graft inflammation. The median ti-score also improved in the TCZ group while it remained the same in the control group, although this difference did not reach statistical significance (p=0.14), possibly due to the small number of subjects. TCZ therapy was relatively safe and well-tolerated in this pilot study with no infusion reactions or unexpected AEs. Cytopenias were the most frequent complication in the TCZ group (similar to the non-transplant population) and readily managed by dose modifications.

At our center, patients with early subclinical graft inflammation undergo a follow-up biopsy 6 months later as standard of care. We chose to conduct the pilot trial of TCZ in this population because it allowed us to direct observe the changes in graft inflammation and link them to TCZ therapy by comparison with an untreated control group. One disadvantage of this approach may have been the relatively short duration of therapy, since the consensus in the RA population is that clinical response to biological agents "may take ≥24 weeks" [33] and previous studies show that a substantial proportion of non-responders within the first 8 weeks of therapy achieve improvement after 24 weeks of TCZ [34].

The most notable finding of our study is the impact of TCZ on the circulating T cell profile and function in kidney transplant recipients. Treatment with TCZ was strongly associated with a significant increase in the number of circulating Tregs and decrease in Teff function during the 6 months that the subjects were receiving drug, although individual subjects' responses can be variable. These findings mirror the increase in circulating Tregs and decrease in Th17 cells observed at 4-6 months following the initiation of TCZ in patients with RA [28,29]. Treg frequencies gradually returned to baseline over the course of the following 6 months, although changes in Teff cytokine production persisted. In the TCZ group, increase in circulating Tregs appeared to peak and stabilize at 3 months. However, the maximal impact on decreasing Teff cytokine production was not seen till 6 months, suggesting that at least 6 months of therapy is needed to elicit a biologic response. It is possible that a longer duration of therapy would have demonstrated an even more profound impact on effector cytokines.

An important limitation is that the study could not meet its recruitment target of 48 subjects, and consequently, is underpowered statistically. However, we observed a strong mechanistic signal in the T cell compartment associated with TCZ treatment, which leads us to believe that the difference seen in the evolution of graft inflammation in the TCZ-treated group is not merely statistically significant but also clinically meaningful. Our pilot study is the first prospective randomized controlled trial of TCZ therapy in kidney transplant recipients, and we have established that short-term treatment with TCZ (superimposed on CNI-based triple immunosuppression) is safe and feasible in the early post-transplant population. This is also the first time that a pharmacologic treatment has been shown to have such a robust impact on the number of peripheral Tregs in subjects while they remain on CNI-based immunosuppression. We conclude that therapy with TCZ induced a significant shift in the immune balance in favor of immune regulation of kidney transplant

recipients and was associated with improvement in graft inflammation. Inhibition of the IL-6 pathway with TCZ or other agents is an important therapeutic innovation and a useful addition to the pharmacologic armamentarium in solid organ transplant.

Author Contributions

S.C. and F.G.V. designed the study; C.H. and J.L. carried out experiments; S.C., J.L, Z.G.L, Q.T. and F.G.V analyzed the data; S.C., J.L., and Q.T. made the figures; S.C., Q.T., and F.G.V. drafted and revised the paper; all authors approved the final version of the manuscript

Acknowledgments

This investigator-initiated clinical trial was supported by a grant from Genentech-Roche group, who also supplied the study drug, tocilizumab. The mechanistic studies were supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number U01- AI113362-01 (F.G.V.). Statistical support was expertly provided by the Biostatistics Core which is funded by the UCSF Department of Surgery.

Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. S.C. has received consulting fees from Everest Clinical Research. F.G.V. has received research grants from Genentech-Roche group, Bristol-Myers Squibb Company, Pfizer Inc., Novartis International AG, Angion Biomedica Corp., Vitaeris Inc., Sanofi S.A., Merck & Co. Inc., Astellas Pharma Inc., Hookipa Pharma, Alnylam Pharmaceuticals Inc., and Viela Bio Inc. Q.T. is a co-founder of and scientific advisor to Sonoma Biotherapeutics.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Figure Legends

Figure 1. Study Schema

Figure 2. Subject disposition in the study: CONSORT flow diagram

Figure 3. Alterations of serum cytokines in control of TCZ-treated subjects. A. Comparison of baseline concentrations of 14 cytokines in the serum collected at trial enrollment in control (n=11) and TCZ groups (n=15). **B.** Median fold change of cytokines concentrations at Month 6 ($Concentration^{M6}/Concentration^{baseline}$) is shown as heat map (Control n=11, TCZ partial n=5, TCZ full n=9). **C to E.** Concentrations of IL-6, IL-17, and IFNγ at baseline (M0) and Month 6 (M6). Each circle represents one individual subject and bars are mean of the group for each time point. Significance of the differences between baseline and M6 for each group was determined using 2-way ANOVA with Sidak multiple comparison posttest. All significant findings were marked with p values on the graphs.

Figure 4. Alteration of Treg frequencies in control of TCZ-treated subjects. A. Examples of flow cytometic analyses of CD4+CD25+FOXP3+ Tregs in peripheral blood collected at baseline, month 3 and 6. Numbers in the plot are percentages of Tregs. **B.** Summary of CD4+CD25+FOXP3+ Tregs in peripheral blood collected at baseline, month 3 and 6 (at baseline and month 3: control n=14, TCZ partial n=5, TCZ full n=9; at month 6: control n=13, TCZ partial n=4, TCZ full n=9). **C.** Data in panel B are normalized to subjects' own baseline and graphed. **D.** Examples of flow cytometic analyses of CD4+HELIOS+FOXP3+ Tregs in peripheral blood collected at baseline, month 3, 6, and 12. **E.** Summary of CD4+HELIOS+FOXP3+ Tregs in peripheral blood collected at baseline, month 3, 6, and 12. Each circle in panels B and D represents one individual subject and bars are mean of the group for each time point (at baseline and month 3: control n=7, TCZ partial n=3, TCZ full n=7; at month 6: control n=6, TCZ partial n=3, TCZ full n=7; at month 12: control n=6, TCZ partial n=3, TCZ full n=5). F. Data in panel E are normalized to subjects' baseline and graphed. Significance of the differences was determined using 2-way ANOVA with Tukey multiple comparison posttest. All significant findings were marked with p values on the graphs.

Figure 5. Alteration of T cell effector molecule expression in control of TCZ-treated subjects. A. Examples of flow cytometic analyses of IFNg, IL-17, and granzyme B expression by T cells in the peripheral blood collected at baseline and month 6. **B.** Summary of flow cytometic analyses of IFNg, IL-17, and granzyme B expression by T cells in the peripheral blood collected at baseline, month 3 and 6. Each circle in panels represents one

individual patient and bars are mean of the group for each time point (at baseline and month 3: control n=14, TCZ partial n=5, TCZ full n=9; at month 6: control n=13, TCZ partial n=4, TCZ full n=9). Significance of the differences was determined using 2-way ANOVA with Tukey multiple comparison posttest. All significant findings were marked with p values on the graphs.

References

1. Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? *Am J Transplant.* 2004;4(8):1289-1295.
2. Heilman RL, Devarapalli Y, Chakkera HA, et al. Impact of subclinical inflammation on the development of interstitial fibrosis and tubular atrophy in kidney transplant recipients. *Am J Transplant.* 2010;10(3):563-570.
3. Park WD, Griffin MD, Cornell LD, Cosio FG, Stegall MD. Fibrosis with inflammation at one year predicts transplant functional decline. *J Am Soc Nephrol.* 2010;21(11):1987-1997.
4. Thierry A, Thervet E, Vuiblet V, et al. Long-term impact of subclinical inflammation diagnosed by protocol biopsy one year after renal transplantation. *Am J Transplant.* 2011;11(10):2153-2161.
5. Lefaucheur C, Gosset C, Rabant M, et al. T cell-mediated rejection is a major determinant of inflammation in scarred areas in kidney allografts. *Am J Transplant.* 2018;18(2):377-390.
6. Nankivell BJ, Shingde M, Keung KL, et al. The causes, significance and consequences of inflammatory fibrosis in kidney transplantation: The Banff i-IFTA lesion. *Am J Transplant.* 2018;18(2):364-376.
7. Nankivell BJ, Agrawal N, Sharma A, et al. The clinical and pathological significance of borderline T cell-mediated rejection. *Am J Transplant.* 2019;19(5):1452-1463.
8. Holsti MA, Raulet DH. IL-6 and IL-1 synergize to stimulate IL-2 production and proliferation of peripheral T cells. *J Immunol.* 1989;143(8):2514-2519.
9. Lotz M, Jirik F, Kabouridis P, et al. B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *J Exp Med.* 1988;167(3):1253-1258.
10. Fujimoto M, Nakano M, Terabe F, et al. The influence of excessive IL-6 production in vivo on the development and function of Foxp3+ regulatory T cells. *J Immunol.* 2011;186(1):32-40.
11. Haruta H, Ohguro N, Fujimoto M, et al. Blockade of interleukin-6 signaling suppresses not only th17 but also interphotoreceptor retinoid binding protein-specific Th1 by promoting regulatory T cells in experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci.* 2011;52(6):3264-3271.
12. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol.* 2010;40(7):1830-1835.
13. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 2006;24(2):179-189.

14. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science.* 2003;299(5609):1033-1036.
15. Wan S, Xia C, Morel L. IL-6 produced by dendritic cells from lupus-prone mice inhibits CD4+CD25+ T cell regulatory functions. *J Immunol.* 2007;178(1):271-279.
16. Lal G, Yin N, Xu J, et al. Distinct inflammatory signals have physiologically divergent effects on epigenetic regulation of Foxp3 expression and Treg function. *Am J Transplant.* 2011;11(2):203-214.
17. Sharma MD, Huang L, Choi JH, et al. An inherently bifunctional subset of Foxp3+ T helper cells is controlled by the transcription factor eos. *Immunity.* 2013;38(5):998-1012.
18. Rochman I, Paul WE, Ben-Sasson SZ. IL-6 increases primed cell expansion and survival. *J Immunol.* 2005;174(8):4761-4767.
19. Riella LV, Yang J, Chock S, et al. Jagged2-signaling promotes IL-6-dependent transplant rejection. *Eur J Immunol.* 2013;43(6):1449-1458.
20. Zhao X, Boenisch O, Yeung M, et al. Critical role of proinflammatory cytokine IL-6 in allograft rejection and tolerance. *Am J Transplant.* 2012;12(1):90-101.
21. Shen H, Goldstein DR. IL-6 and TNF-alpha synergistically inhibit allograft acceptance. *J Am Soc Nephrol.* 2009;20(5):1032-1040.
22. Lei J, He F, Wu M, Zheng X, Chen X, Chen Z. Administration of anti-interleukin-6 monoclonal antibody prolongs cardiac allograft survival. *Transpl Int.* 2010;23(12):1271-1281.
23. Wang H, Guan Q, Lan Z, et al. Prolonged renal allograft survival by donor interleukin-6 deficiency: association with decreased alloantibodies and increased intragraft T regulatory cells. *Am J Physiol Renal Physiol.* 2012;302(2):F276-283.
24. Fogal B, Yi T, Wang C, et al. Neutralizing IL-6 reduces human arterial allograft rejection by allowing emergence of CD161+ CD4+ regulatory T cells. *J Immunol.* 2011;187(12):6268-6280.
25. Mihara M, Kasutani K, Okazaki M, et al. Tocilizumab inhibits signal transduction mediated by both mIL-6R and sIL-6R, but not by the receptors of other members of IL-6 cytokine family. *Int Immunopharmacol.* 2005;5(12):1731-1740.
26. Genovese MC, Rubbert-Roth A, Smolen JS, et al. Longterm safety and efficacy of tocilizumab in patients with rheumatoid arthritis: a cumulative analysis of up to 4.6 years of exposure. *J Rheumatol.* 2013;40(6):768-780.

27. Schiff MH, Kremer JM, Jahreis A, Vernon E, Isaacs JD, van Vollenhoven RF. Integrated safety in tocilizumab clinical trials. *Arthritis Res Ther.* 2011;13(5):R141.
28. Pesce B, Soto L, Sabugo F, et al. Effect of interleukin-6 receptor blockade on the balance between regulatory T cells and T helper type 17 cells in rheumatoid arthritis patients. *Clin Exp Immunol.* 2013;171(3):237-242.
29. Samson M, Audia S, Janikashvili N, et al. Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. *Arthritis Rheum.* 2012;64(8):2499-2503.
30. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med.* 1999;130(6):461-470.
31. Solez K, Colvin RB, Racusen LC, et al. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant.* 2008;8(4):753-760.
32. Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. *Blood.* 2008;112(10):3959-3964.
33. Furst DE, Keystone EC, So AK, et al. Updated consensus statement on biological agents for the treatment of rheumatic diseases, 2012. *Ann Rheum Dis.* 2013;72 Suppl 2:ii2-34.
34. Keystone EC, Anisfeld A, Ogale S, Devenport JN, Curtis JR. Continued benefit of tocilizumab plus disease-modifying antirheumatic drug therapy in patients with rheumatoid arthritis and inadequate clinical responses by week 8 of treatment. *J Rheumatol.* 2014;41(2):216-226.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table 1. Study subjects: baseline demographics and transplant characteristics

	Control group (n=14)	TCZ group (n=16)	p-value
Age in years, median (IQR)	51.5 (40, 56)	53.5 (41.5, 59)	0.82
Sex, male, n (%)	8 (57.1)	9 (56.2)	0.99
Race, n (%)			
White	3 (21.4)	3 (18.8)	0.58
African-American	1 (7.1)	0	
Hispanic	7 (50.0)	11 (68.8)	
Other	3 (21.4)	2 (12.5)	
Cause of ESRD, n (%)			
Glomerulonephritis	3 (21.4)	8 (50.0)	0.43
Tubulo-interstitial disease	3 (21.4)	1 (6.3)	
Diabetes mellitus	5 (35.7)	3 (18.8)	
Hypertension	1 (7.1)	1 (6.3)	
Unknown etiology	2 (14.3)	3 (18.6)	
cPRA≥80%, n (%)	3 (21.4)	6 (37.5)	0.44
Repeat transplant, n (%)	1 (7.1)	4 (25.0)	0.34
Deceased donor, n (%)	9 (64.3)	12 (75.0)	0.69
Depleting antibody induction, n (%)	7 (50.0)	12 (75.0)	0.26
Maintenance IS at enrollment, n (%)			
Tacrolimus	13 (92.9)	16 (100)	0.47
Cyclosporine	1 (7.1)	0 (0.0)	
MMF/ mycophenolic acid	14 (100)	16 (100)	
Prednisone	14 (100)	16 (100)	
MDRD eGFR in ml/min/1.73 m^2, median (IQR)	52 (46, 59)	62.5 (56, 74)	0.046
Urine protein/creatinine in mg/g, median (IQR)	0.1 (0.1, 0.2)	0.1 (0.1, 0.2)	0.76

Banff scores on baseline biopsies, median (IQR)			
Interstitial inflammation (i)	0 (0, 1)	1 (0, 1)	0.49
Tubulitis (t)	1 (1, 1)	1 (1, 1.5)	0.44
Total inflammation (ti)	1 (0, 1)	1 (0.5, 1)	0.38

Table 2. Graft pathology of the baseline and follow up biopsies analyzed as change in median Banff i, t and ti-scores

	Timing of kidney biopsy	Banff i-score, median (IQR)	p-value*	Banff t-score, median (IQR)	p-value*	Banff ti-score, median (IQR)	p-value*
Control group (n=14)	Baseline	0 (0, 1)	0.38	1 (1, 1)	0.004	1 (0, 1)	0.73
	Follow up	0 (0, 0)		0 (0, 1)		1 (0, 1)	
TCZ group (n=16)	Baseline	1 (0, 1)	0.07	1 (1, 1.5)	0.009	1 (0.5, 1)	0.14
	Follow up	0 (0, 0)		0 (0, 1)		0 (0, 1)	

* Paired Wilcoxon signed rank test

Table 3. Graft pathology of the baseline and follow up biopsies analyzed as the proportion of subjects with improvement in Banff scores and diagnosis

	Control group (n=14)	TCZ group (n=16)	p-value**
Subjects with a decrease in Banff-i score			
1-point, n (%)*	4 (28.6)	7 (43.8)	0.47
Subjects with a decrease in in Banff t-score			
1-point, n (%)	8 (57.1)	7 (43.7)	
2-points, n (%)	0	3 (18.7)	
Total with any decrease	9 (64.3)	10 (62.5)	0.99
Subjects with a decrease in Banff ti-score			
1-point, n (%)	3 (21.4)	9	
2-point, n (%)	0	1	
Total with any decrease	3 (21.4)	10 (62.5)	0.03
Subjects with resolution of borderline change, n (%)	8 (57.1)	9 (56.2)	0.99

*No subject had >1-point reduction in Banff i-score as there were no baseline biopsies with i-scores >1 **All p-values are from Fisher's exact tests comparing any decrease (yes/no) between groups

Table 4. Change in renal function during the study

	Control group (n=14)	TCZ group (n=16)	p-value
Change in MDRD eGFR at 6 months follow up from Baseline in ml/min/1.73 m^2, median (IQR)	3 (-1, 11)	5 (-0.5, 13)	0.59
Change in MDRD eGFR at 12 months follow up from Baseline in ml/min/1.73 m^2, median (IQR)	4.5 (-1, 10)	1.5 (-1.5, 4)	0.36

Table 5. Summary of study-related adverse events

Adverse event	Control group (n=14)	TCZ group (n=16)	p-value
Bacterial infection	0	3	0.23
BK viremia >1000 copies/ml	0	2	0.48
Neutropenia			
1000-1500 X 10^9/L	1	3	
500-999 X10^9/L	1	2	
<500 X10^9/L	0	1	
Total	2	6	0.22
Thrombocytopenia			
50-100 X10^9/L	1	2	
<50 X10^9/L	0	0	
Total	1	2	1.00
Elevated AST or ALT			
>1.5 and <3 times ULN	2	3*	
>3 times ULN	0	0	
Total	2	3*	1.00

*One additional subject had mild elevation of AST and ALT seen 7 months after the last dose of TCZ. This abnormality resolved with discontinuation of statin and was not felt to be related to TCZ therapy.

Figure 1

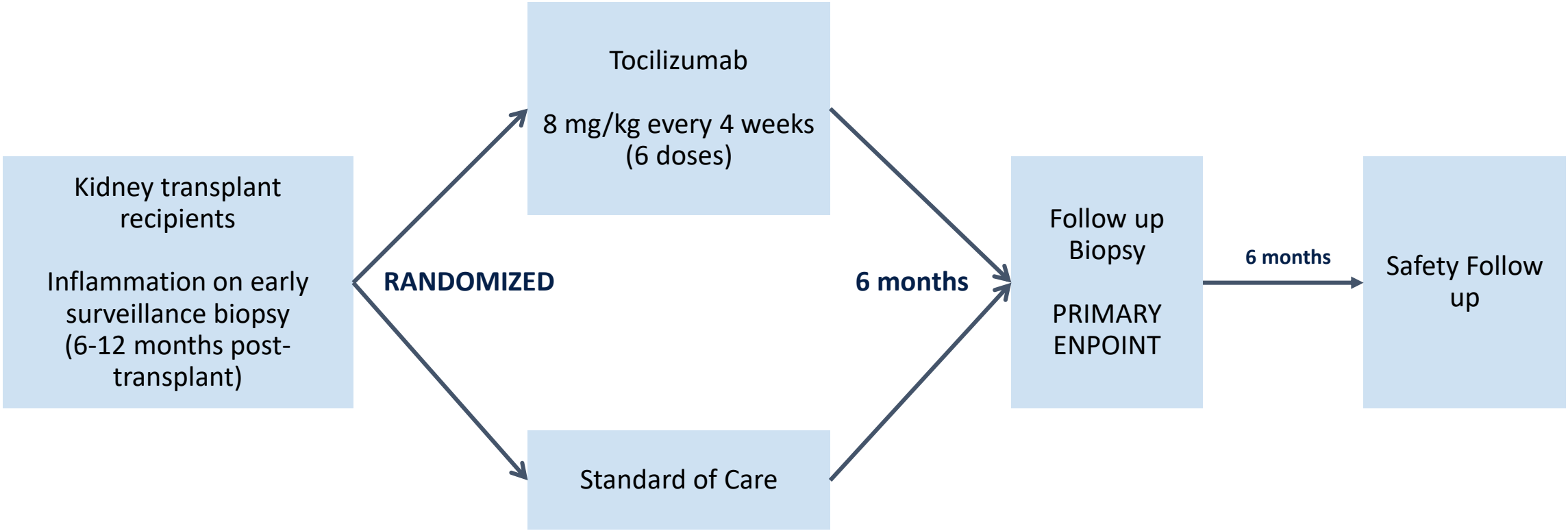


Figure 2

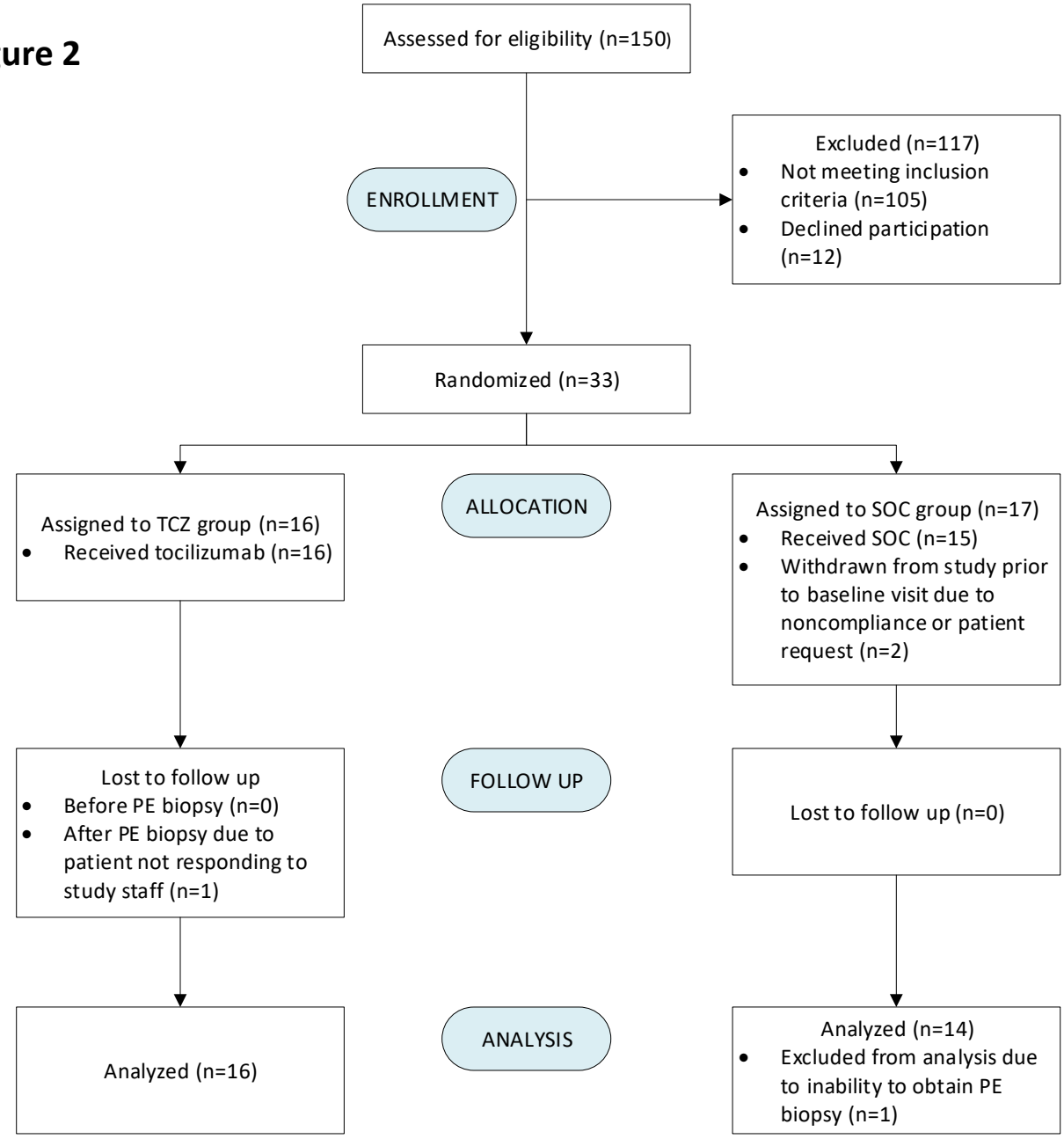


Figure 3

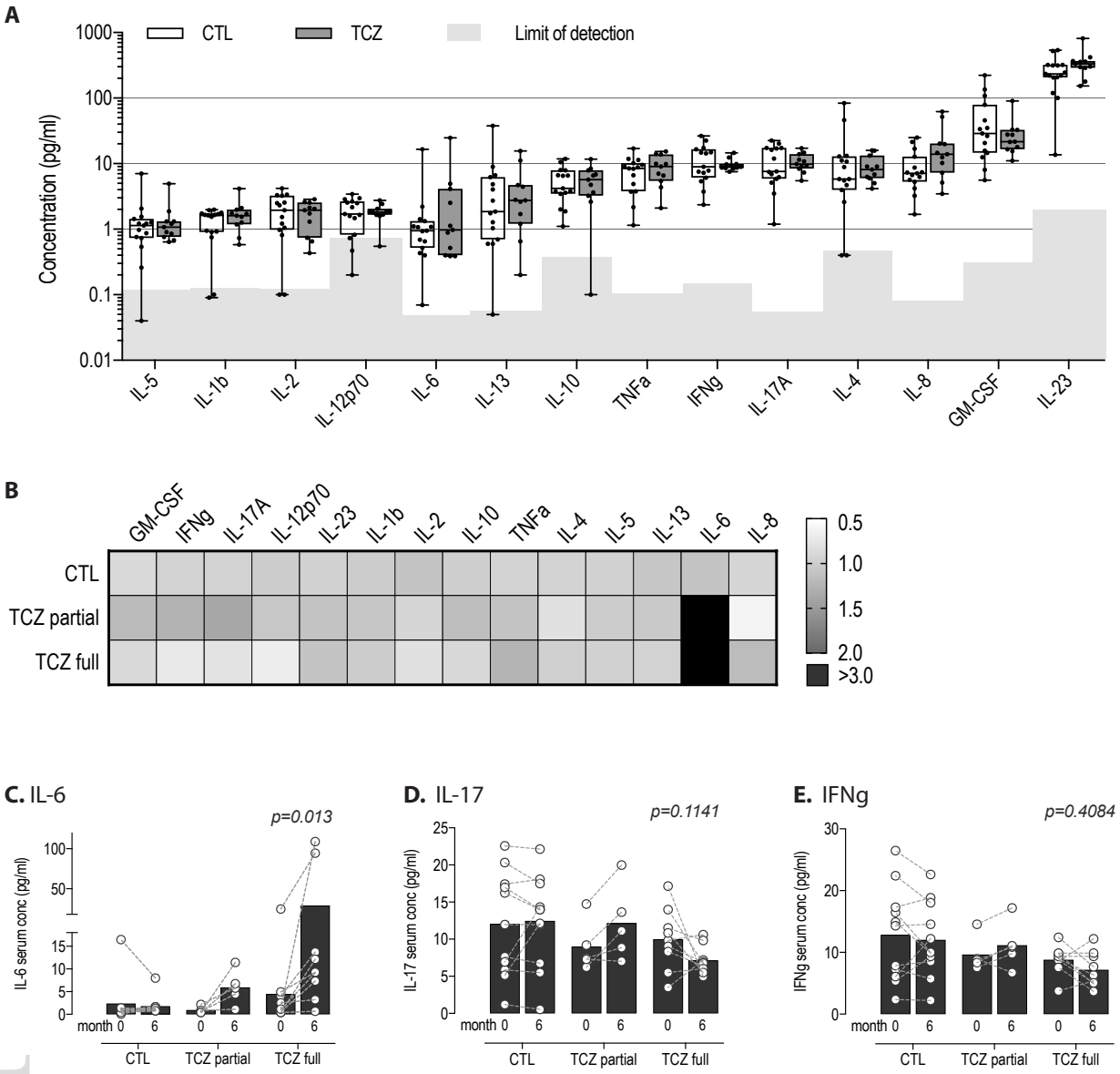
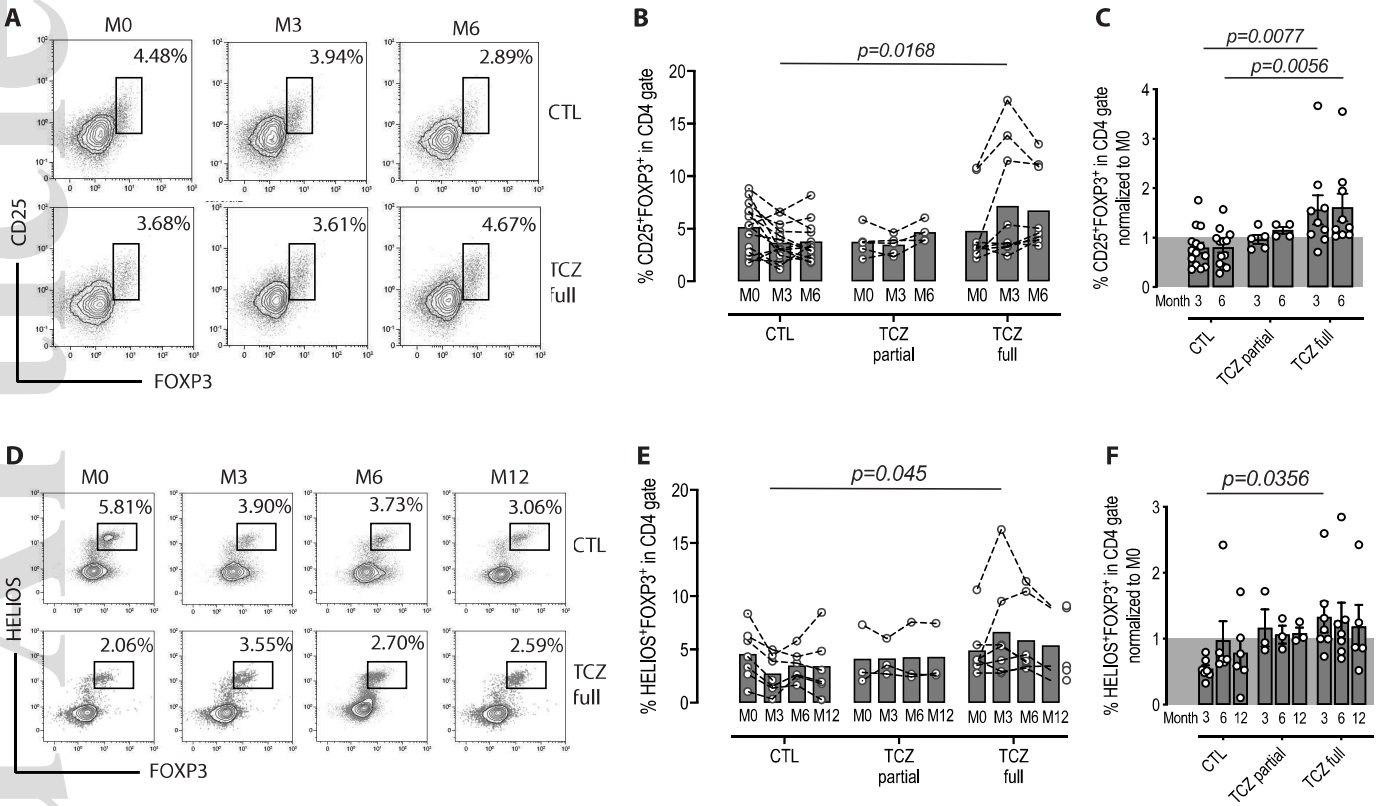


Figure 4



Figurer 5