Original Clinical Science—General

Evaluation of Immunocompetence and Biomarkers of Tolerance in Chimeric and Immunosuppression-Free Kidney Allograft Recipients

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Background. Thirty-seven patients have received a living-donor kidney transplant in a phase 2 study designed to induce tolerance with facilitated allogeneic hematopoietic stem cell transplant. The study protocol is based on tolerogenic CD8$^+$/T- cell receptor$^-$ facilitating cells (FCR001; also including hematopoietic stem cells and $\alpha\beta$-T-cell receptor$^+$ T cells) and low-dose, nonmyeloablative conditioning. Persistent chimerism allowing full immunosuppression (IS) withdrawal was achieved in 26 patients (time off IS 36–123 mo). **Methods.** We evaluated biomarkers of tolerance through urinary cell mRNA profilingand immunocompetence to respond to vaccination in these patients. We also assessed kidney function and metabolic parameters compared with standard-of-care patients on IS. **Results.** Persistently chimeric patients retained chimerism after removal of IS and remained rejection free without donor HLA–specific antibody development. The presence of donor chimerism at >50% correlated with a signature of tolerance in urinary cell mRNA profiles, with a uniquely elevated increase in the ratio of cytotoxic T lymphocyte–associated protein 4 to granzyme B mRNA. Tolerance was associated with protection from recurrence of immune-mediated causes of kidney disease. Tolerant participants were safely vaccinated, developed protective immune responses, and did not lose chimerism after vaccination. When compared with kidney transplant recipients treated with standard IS, tolerant participants showed stable kidney function and reduced medication use for hypertension and hyperlipidemia. **Conclusions.** These results suggest that elimination of IS has distinct advantages in living-donor kidney allograft recipients.

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INTRODUCTION

Kidney transplantation is the treatment of choice for most causes of end-stage kidney disease. However, prevention of kidney transplant rejection requires lifetime use of immunosuppression (IS; generally a combination of a calcineurin inhibitor, an antiproliferative agent, and corticosteroids).[1]

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Long-term outcomes following transplantation are suboptimal with prolonged drug-based IS. The 10-y graft survival rates for deceased-donor kidneys and HLA-mismatched living-donor kidneys remain low at 50% and 65%, respectively.[2] Death with a functioning allograft accounts for at least half of the recipient losses, and this is primarilya result of the toxic side effects associated with drug- based IS.[3] Therefore, development of alternate therapies to eliminate or minimize the need for lifelong IS through the induction of tolerance is a great unmet need. One strategy to eliminate the use of long-term IS is to induce donor-specific transplant tolerance by establishing hematopoietic chimerism.

We previously reported interim findings from the ongoing 2-center, single-arm, phase 2 study, FCR001A2201, evaluating the safety and efficacy of FCR001 in living-donor kidney transplant recipients receiving nonmyeloablative (NMA) conditioning and demonstrated that complete withdrawal of IS is pos- sible in kidney recipients when persistent, high levels of donor chimerism are established.[4,5] Thirty-seven patients received a transplant; all have had a follow-up period of >48 mo posttransplant. Twenty-six patients were successfully taken off IS (time off IS 36–123 mo) without rejection. All patients off IS continue to exhibit preserved kidney function, normal kidney allo- graft biopsies, and no donor-specific antibodies. The safety profile of FCR001 is consistent with standard- of-care (SOC) kidney transplant and hematopoietic stem cell transplant undergoing NMA conditioning, with patient and graft survivals similar to concurrent SOC kidney transplant recipients.

This report provides further analysis of the phase 2 trial. The molecular features associated with tolerance, including chimerism and urinary cell mRNA profiles as potential biomarkers of tolerance and immunocompetence,are evaluated. We also assessed metabolic parameters in FCR001 recipients compared with patients who received SOC. Notably, in FCR001 recipients, >50% donor T-cell chimerism at 6 mo predicted successful weaning and discontinuation of IS at 12 mo. Finally, patient immunocompetence was assessed through their ability to respond to vaccination.

MATERIALS AND METHODS

Chimerism Testing

Chimerism was determined by genotyping of simple sequence-length polymorphisms encoding short tandem repeats either at Northwestern University or at an independent laboratory (LabCorp, Burlington, NC). For lin- eage chimerism testing, $CD19^+$ (B cells), $CD3^+$ (T cells), and/or $CD66B^+$ (myeloid) cells were sorted from whole blood, then analyzed by molecular short tandem repeat typing. The sensitivity of the assay is approximately 1% to 2%. Internal controls to define the sensitivity were per- formed for each assay. Tolerance was defined by the ability to wean patients off IS and maintain stable kidney func- tion without biopsy-proven acute rejection (BPAR). The protocol was approved by the institutional institutional review boards and as an investigational device exemption by the s.

Vaccination of Phase 2 Trial Participants

Eligible participants who provided written consent received (or were vaccinated with) tetanus, diphtheria, and pertussis (TDaP); Pneumovax; hepatitis B virus (HBV); polio; meningococcal; *Haemophilus influenzae* type b;and influenza vaccines consistent with current American Society for Blood and Bone Marrow Transplant guidelines.[6] Revaccination data were compared between recipients with persistent chimerism off IS and patients whodid not achieve persistent donor chimerism. The serologic response for the HBV vaccine was a binary outcome of either seroconversion or no seroconversion. The outcomes were quantitative for TDaP vaccine and Pneumovax; a ratio of postvaccination to prevaccination titers of 1 to 1.99 was defined as "low response" and a ratio of >2 as "protective response." A "decreased response" was defined as titers that dropped after vaccination.

Urinary Cell mRNA Profiling

Urine specimens, approximately 50 mL, were collected and urinary cell pellets were prepared by centrifugationat the clinical sites, stored at −80 °C, and shipped to the Gene Expression Monitoring (GEM) Core at Weill Cornell Medicine, New York, for mRNA profiling. We measured absolute levels of urinary cell mRNAs using preamplification-enhanced, customized, real-time quantitative polymerase chain reaction (customized qPCR) assays developed in the Cornell GEM Core.[7]

At the GEM Core, total RNA was isolated from the urine cell pellets using a Rneasy kit (QIAGEN), and the quantity (absorbance at 260 nm) and purity (ratio of the absorbance at 260 and 280 nm) of the total RNA were measured using the NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific). Total RNA was reverse transcribed to complementary DNA using the TaqMan RT kit (cat. N808-0234; Applied Biosystems). Gene-specific oligonucleotide primers and TaqMan fluorogenic probes, designed by the GEM Core, were used forthe measurement of absolute copy numbers of mRNA for cytotoxic T lymphocyte–associated protein 4 (CTLA-4), T cell–surface glycoprotein CD3 epsilon chain (CD3ε), granzyme B, perforin (PRF1), interferon gamma-induced protein 10 (IP10), forkhead box protein 3 (FoxP3), transforming growth factor beta 1 (TGF-β1), and 18S ribosomal RNA (rRNA). These values were quantified using the Bak amplicon–based standard curve method developed and validated in the GEM Core.[7] The location and sequences of the oligonucleotide primer pairs and the gene-specific TaqMan probes used to quantify mRNA for CD3ε, GZMB, PRF1, IP10, FoxP3, TGF-β1, and 18S rRNA in the customized qPCR assays have been reported.[7] The sequence and location of the oligonucleotide primer pair and the TaqMan Probe for the measurement of CTLA-4 mRNA were sense primer, 5′-CGCCATACTACCTGGGCATAG-3′ (441–461), antisense primer, 5′-GATCCAGAGGAGGAA GTCAGAATC-3′ (529–506), and TaqMan Probe, 5′-FAM TATGTAATTGATCCAGAACCG MGB-3′ (479–499), accession no. BC074893. Abundance of mRNA was reported as copies per microgram total RNA isolated from the urinary cell pellet.

Urine specimens passed quality thresholds if the 18S rRNA copy number was $\geq 5 \times 10^7$ copies/μg total RNA

and if the TGF-β1 mRNA copy number was ≥100 copies/ µg total RNA, as previously specified.[7] If either threshold was not met, the urine specimen was classified as fail- ing quality control thresholds and excluded from down- stream data analysis.

FCR001 Cohort

A total of 76 urine samples, collected posttransplantation from 24 FCR001 patients with persistent or tran- sient chimerism, were centrifuged at the clinical site to prepare urinary cell pellets, stored at −80 °C, and shipped to GEM Core for urinary cell mRNA profiling (Figure S1, SDC, http://links.lww.com/TP/C779). The Core was blinded to clinical information and biopsy diagnosis until the customized qPCR assay results were reportedback to the clinical center.

Among the 76 urine specimens, 62 urine samples from 22 patients passed quality control thresholds. Among these 62 urine samples, 51 samples were from 19 patients with persistent chimerism (≥50% donor T-cell chimerism for ≥6 mo) and 11 samples were from 3 patients withtransient chimerism. Among those with persistent chi- merism, 28 urine samples were from 14 patients off IS (as per protocol) and 23 urine samples were from 12 patients with persistent chimerism and on IS (as per protocol and within 12 mo of transplantation). Among the persistent chimerism group, 7 patients contributed urine samples both while on and off IS.

Clinical Trials of Transplantation-04 Cohort

In total, 485 kidney allograft recipients were prospectively enrolled in the Clinical Trials of Transplantation-04 (CTOT-04) study for urinary cell mRNA profiling. Among these patients, 220 patients underwent kid- ney allograft biopsies, and 244 biopsy-matched urine samples passed prespecified quality control thresholds. Among these urine samples, 163 were matched to 163 "no rejection" biopsies (from 126 patients), 43 were matched to 43 T cell–mediated rejection (TCMR) biop- sies (from 34 patients), 19 urine samples were matched to 19 borderline biopsies (from 17 patients), 10 urine samples were matched to 10 antibody-mediated rejec- tion biopsies (from 9 patients), and 9 urine samples were matched to 9 other biopsies (from 8 patients), as reported. In this study, the absolute levels of urinary cell mRNA in the 43 TCMR biopsy-matched urine samples matched from 34 patients (TCMR cohort) and in 161 no rejection biopsy-matched urine samples matched from 124 patients were compared with the absolute levels of urinary cell mRNAs measured in urine from the FCR001 cohort.

Statistical Analysis

Median absolute copy numbers (lower, upper quartiles) of each mRNA measure and of 18S rRNA were calculated. Before the calculation, any mRNA copy number <50 copies/µg total RNA (the lowest copy number in the PCR Bak standard curve), with cycle threshold (Ct) >35 in the PCR assay, was assigned a value of 12.5 (rounded to 13 in presented tables and figures); when no mRNA copy was detected after 40 Cts in the PCR assays, a value of 1 copy was assigned.

The Kruskal-Wallis test was used to compare mRNA copy numbers in >2 groups with Benjamini-Hochberg adjustment for multiple comparisons. The Wilcoxon rank-sum test was used for comparison of 2 groups with Benjamini-Hochberg adjustment for multiple comparisons. All analyses were performed using R data analysis software, version 3.3.3.

RESULTS

Protocol and Follow-Up

As of October 1, 2021, all 37 participants who received a kidney and FCR001 transplant have ≥4 y of follow-up, with a median follow-up of >6 y and longest follow-up of >12 y. Details of the conditioning and clinical outcomes have been previously reported.[4,5,8,9] A summary of the trial design is shown in Figure **S**2 (**SDC**, http://links.lww.com/TP/C779).

Chimerism Outcomes

Participants were categorized on the basis of observed patterns of peripheral blood donor stem cell chimerism. Persistent donor chimerism was observed in 27 patients, including 24 participants who developed high levels of mixed (>98%) donor whole-blood and T-cell chimerism beginning at 1-mo posttransplant. Three participants developed lower levels of mixed donor chimerism. The kinetics of whole-blood chimerism for participants with persistent and transient chimerism are shown in Figure 1. The pres- F1 ence of >50% donor chimerism at 3 mo was highly predictive of the ability to wean and remain off all IS.[4] None of the 26 participants who met this criterion developed donor-specific antibodies, experienced BPAR, lost chimerism, or had to resume IS for the duration of follow-up (1 patient developed graft-versus-host disease, returned to IS, and eventually expired[10]). Five participants were eventually withdrawn from IS whose 1-mo whole-blood donor chimerism levels ranged from 75% to 90%, noticeably below the 97% average for the other 21 individuals. Three of these participants experienced transient dips in chimerism levels that then approached nearly 100% by month 9 and remained at this level thereafter. The other 2 participants had progressively declining whole-blood chimerism levels over the first year posttransplant, stabilizing at 85% and 45%, respectively.

Transient donor chimerism (not maintaining ≥50% donor T-cell chimerism for 6 mo) was observed in 8 participants. Two of these participants received FCR001 with a reduced cell dose, as previously reported.[5] A third transiently chimeric participant was notable for being highly sensitized, with a historic maximum HLA class I panel-reactive antibody of 64%, and an actual panel-reactive antibody of 33% at the time of transplant. In 3 participants, transient chimerism was associated with the development of early posttransplant infections (*Clostridioides difficile*, gram-negative bacteremia, and cytomegalovirus reactivation). A total of 3 of the 8 participants lost their kidney allograft; 1 was because of a renal artery thrombosis during an episode of viral sepsis with hypotension, and the other 2 were because of rejection when both underwent substantial IS weaning during treatment for antibiotic-resistant infections. The remaining participants with

transient chimerism had normal kidney function and were weaned successfully to monotherapy with tacrolimus.

T-cell chimerism levels in FCR001-treated patients generally followed a similar trend to whole-blood levels (Figure 2). The observed lower average levels of T-cell chimerism compared with whole-blood donor chimerism in the first 3 mo could be a function of missing data, as 5 of 26 participants who were withdrawn from IS did not

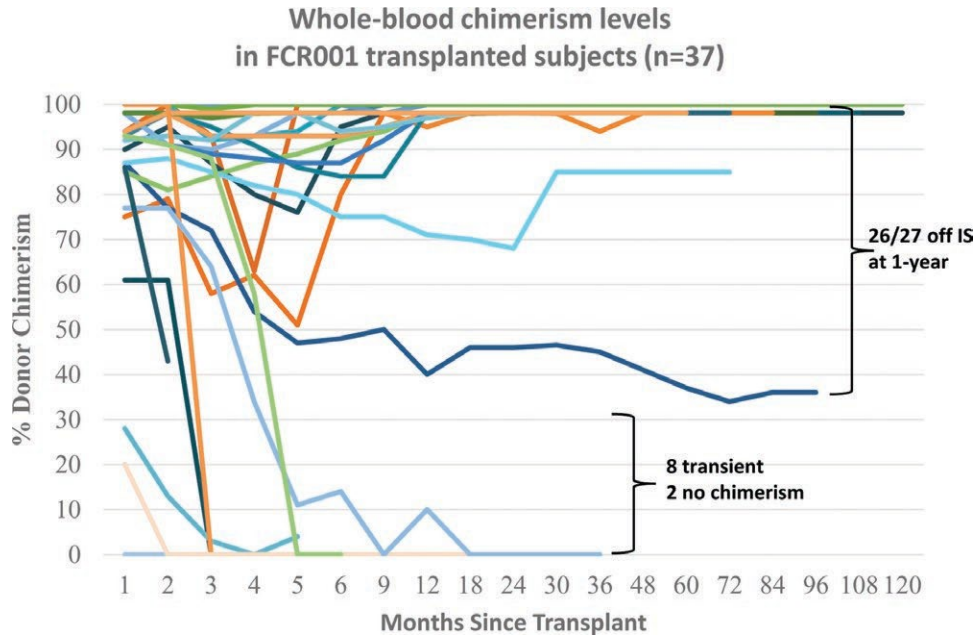

FIGURE 1. Kinetics of whole-blood chimerism in participants with persistent and transient chimerism (n = 37). Kinetics of whole-blood chimerism for participants with persistent and transient chimerism and their changes over time. Chimerism was measured using STR assays. Each line represents an individual patient. IS, immunosuppress.ion; STR, short tandem repeat.

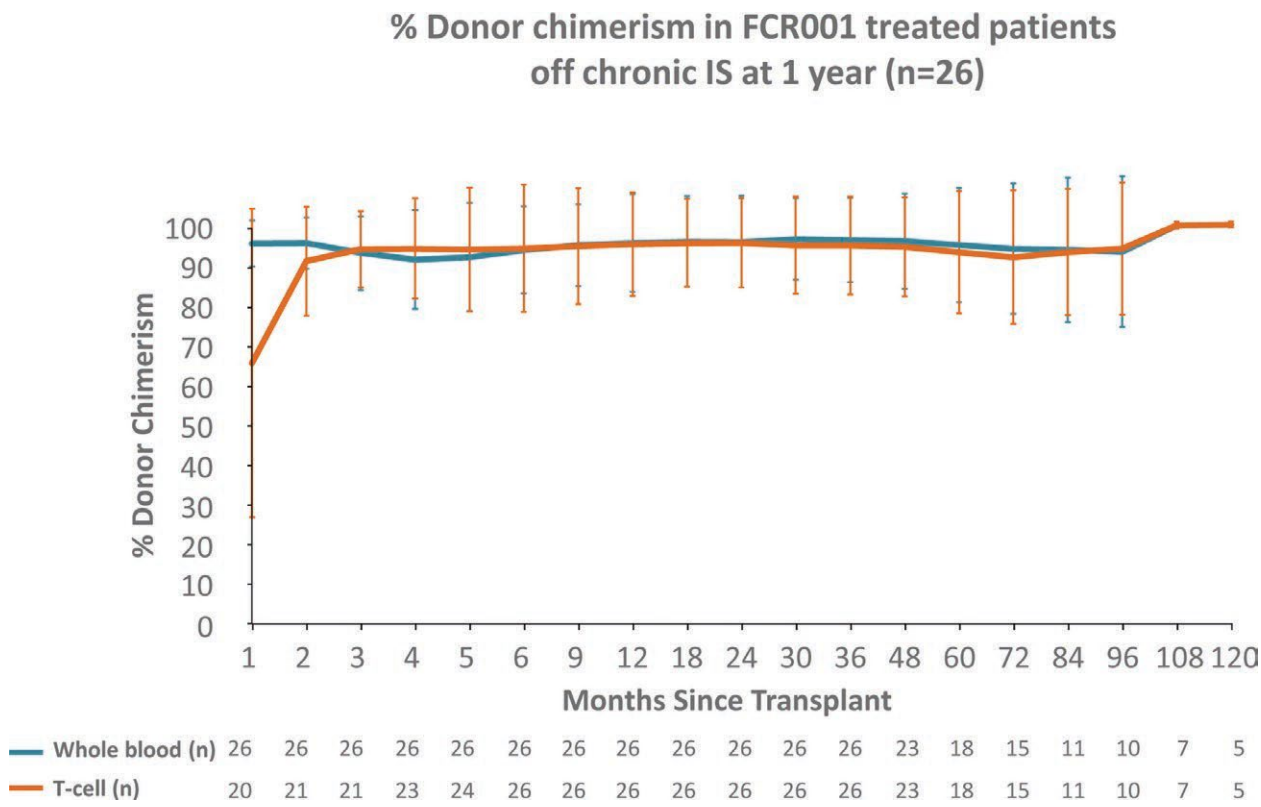

FIGURE 2. Percent donor whole-blood and T cell–chimerism levels in FCR001-treated patients off chronic IS over time (n = 26). Whole-blood and T cell–chimerism levels in FCR001-treated patients monitored up to 120 mo. Values are mean ± standard deviation. n indicates the number of FCR001-treated patients weaned off IS at approximately 1 y posttransplant for whom the percent whole-blood and T cell–donor chimerism were measured at that time point. IS, immunosuppression.

have routine T-cell chimerism levels measured in the first 6 mo posttransplant. It is also possible that T-cell chimerism levels could take longer to reach ≥95% compared with whole-blood levels.

The association of HLA match and donor–recipient relationship with persistent chimerism and weaning off IS is shown in Figure 3. Notably, there was no correlation between degree of HLA disparity and successful establishment of chimerism.

Persistent chimerism is associated with protection from recurrence of original kidney disease (Table 1). No patient with persistent chimerism experienced recurrence of their original cause of kidney disease. This included 2 patients with focal segmental glomerulosclerosis, a disease observed to recur in clinical trials that induced nonchimeric, operational kidney transplant tolerance.[5,11] Kidney disease recurrence, however, was observed in 2 of 3 patients with transient or no donor chimerism.

Comparison of Kidney Transplant Outcomes Following FCR001 or SOC

Among FCR001 recipients, data for rejection, kidney function, and medication use for hypertension and dyslipidemia were evaluated for participants who passed the 2-, 3-, and 5-y marks posttransplant. For comparison, 132 participants transplanted at Northwestern University between 2009 and 2012, determined to have been eligible for the FCR001 protocol, and maintained on SOC induc- tion and maintenance IS (alemtuzumab induction, tacroli- mus, and mycophenolate mofetil maintenance; the SOC cohort) were observed to have comparable patient and graft survival (Tables 2–4). The Northwestern SOC cohort was evaluated retrospectively as a third-party study and was not enrolled in the phase 2 study.

In the FCR001 cohort, a standardized measure of kid- ney function, estimated glomerular filtration rate (eGFR; measured by Modification of Diet in Renal Disease),among persistently chimeric participants improved over time compared with SOC controls at all time points fol- lowing 1 y posttransplant (Figure 4). BPAR occurred in 28.8% of SOC recipients at 2 y, 34.1% at 3 y, and 34.8%at 5 y posttransplant, whereas 0% of persistently chi- meric participants in the FCR001 cohort off IS exhibited BPAR at all time points. Treatment rates of hypertension and hyperlipidemia were also lower on FCR001. Among the SOC cohort, medication use for hypertension ranged from 79.3% to 82.8% compared with 18% to 27% in the persistently chimeric FCR001 cohort. Medication use for

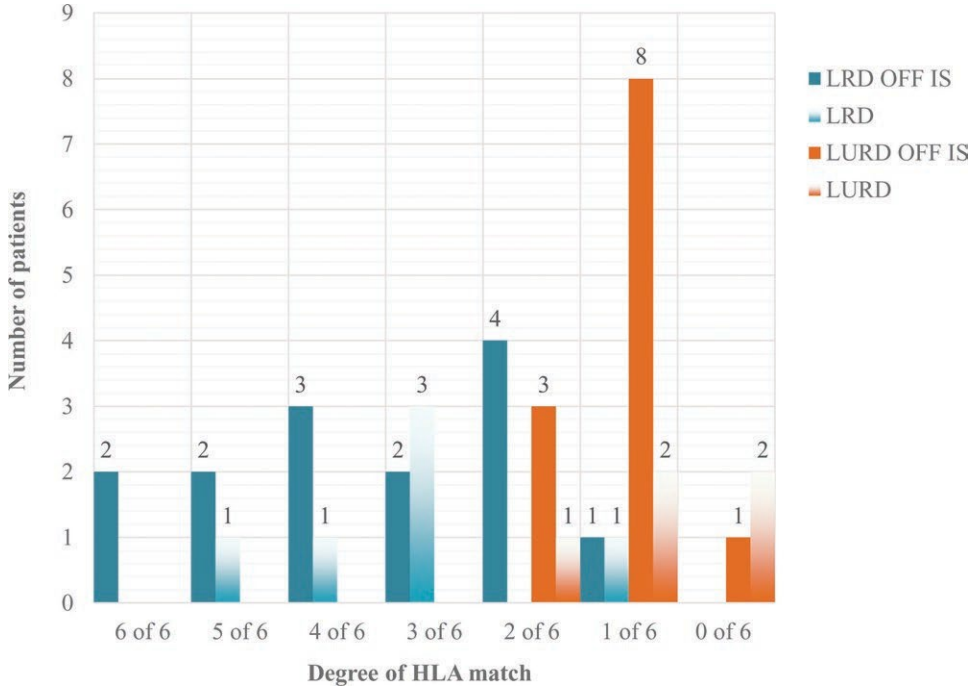

FIGURE 3. Association between persistent chimerism and weaning off IS in relation to HLA match and donor–recipient pairing. Association between donor–recipient HLA status, establishment of persistent chimerism, and the ability to wean off IS. Donor–recipient pairs listed by HLA status and relation. IS, immunosuppression; LRD, living related donor; LURD, living unrelated donor.

TABLE 1.

Disease recurrence and donor chimerism

Condition	Persistent chimerism	Disease recurrence	Transient or no chimerism	Disease recurrence
FSGS	2	0	0	NA
IgAN	4	0	2	1
Membranous GN	1	0	1	1
Total	7	0	3	2

FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; IgAN, IgA nephropathy; NA, not available.

TABLE 2.

Outcomes in persistently or transiently chimeric FCR001 patients vs SOC patients (2-y follow-up posttransplant)

Total cohort	FCR001 (n = 37)		SOC (n = 132)
Cohort reaching 2-y follow-up	n = 31		n = 132
Survival	96.7%		97.7%
Graft survival[a]	93.3%		95.4%
Subcohort[b]	Persistently chimeric (n = 24)	Transiently chimeric (n = 6)	All (n = 128)
BPAR	0.0%	33.3%	28.8%
Hypertension	27.0%	66.6%	79.3%
Hyperlipidemia	18.0%	33.3%	45.2%

[a]Death-censored graft survival.
[b]Number of participants evaluable at 2 y.
BPAR, biopsy-proven acute rejection; SOC, standard of care.

TABLE 3.

Comparison of persistently chimeric and transiently chimeric patients with SOC patients (3-y follow-up posttransplant)

Total cohort	FCR001 (n = 37)		SOC (n = 132)
Cohort reaching 3-y follow-up	n = 28		n = 132
Survival	100.0%		96.2%
Graft survival	92.8%		92.4%
Subcohort[a]	**Persistently chimeric (n = 24)**	**Transiently chimeric (n = 6)**	**All (n = 104)**
BPAR	0.0%	33.3%	34.1%
Hypertension	18.0%	66.6%	76.6%
Hyperlipidemia	9.0%	33.3%	45.0%

[a]Number of participants with >3 y of follow-up.
BPAR, biopsy-proven acute rejection; SOC, standard of care.

TABLE 4.

Comparison of persistently chimeric and transiently chimeric FCR001 patients with SOC patients (5-y follow-up posttransplant)

Total cohort	FCR001 (n = 37)		SOC (n = 132)
Cohort reaching 5-y follow-up	n = 19		n = 132
Survival	94.7%		93.2%
Graft survival	88.8%		83.3%
Subcohort[a]	Persistently chimeric (n = 16)	Transiently chimeric (n = 4)	All
BPAR	0.0%	40.0%	34.8%
Hypertension	18.0%	60.0%	82.8%
Hyperlipidemia	9.0%	40.0%	43.0%

[a]Number of participants with ≥5 y of follow-up.
BPAR, biopsy-proven acute rejection; SOC, standard of care.

hyperlipidemia ranged from 43% to 45.2% in the SOC cohort compared with 9% to 18% in the persistently chimeric FCR001 cohort (Tables 2–4).

Vaccination Outcomes

To assess immunocompetence to respond to vaccination, recipients of FCR001 were vaccinated with TDaP, Pneumovax, HBV, polio, meningococcal, *Haemophilus influenzae* type b, and influenza vaccines consistent with current American Society for Blood and Bone Marrow Transplant guidelines.[6] Revaccination data were compared between recipients with persistent chimerism offIS and patients who did not achieve persistent donor chimerism. Persistently chimeric FCR001 patients showed higher rates of protective titers post–tetanus vaccination than the subset of patients with transient or no chimerism (Figure 5). Likewise, patients demonstrated higher protective response rates in 12 of 14 serotypes following Pneumovax (Figure 6). Rates of HBV seroconversion were also higher among tolerant patients: 3 of 4 patients studied did not have protective antibodies before vaccination, and all 3 had seroconversion/protection postvaccination. Only 1 of 3 patients without persistent chimerism seroconverted (data not shown). None of the chimeric patients lost chimerism after vaccination. F5 F6

Experience With Severe Acute Respiratory Syndrome Coronavirus 2 in FCR001 Patients

Twenty-eight participants from our phase 2 cohort with available data on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination and infection were identified (Table 5, Figure 7); 22 of 28 participants T5 F7

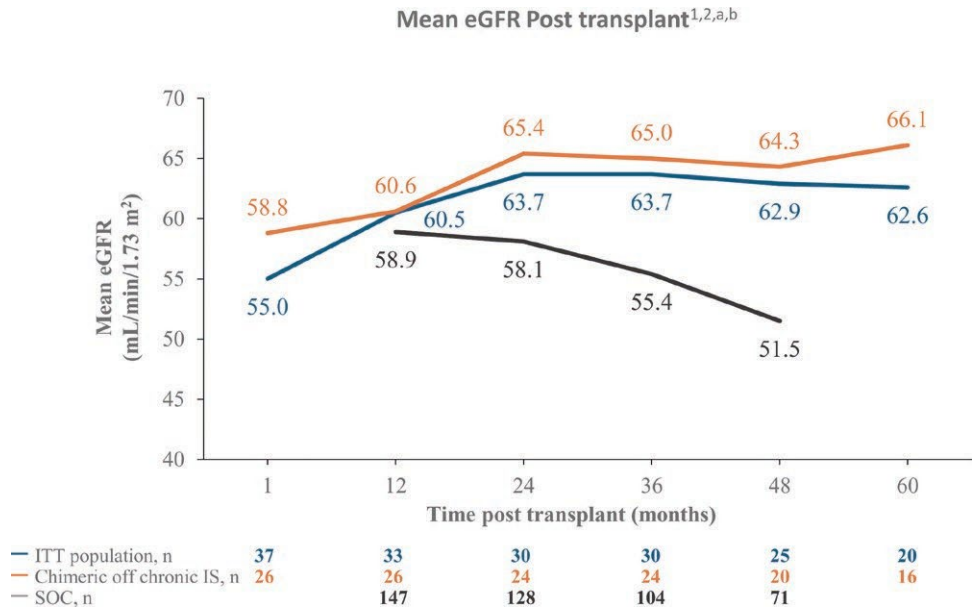

FIGURE 4. eGFR in FCR001 vs SOC cohort. Mean eGFR levels monitored in ITT, FCR001, and SOC patients during 5 y. eGFR, estimated glomerular filtration rate; IS, immunosuppression; ITT, intent-to-treat; SOC, standard of care.

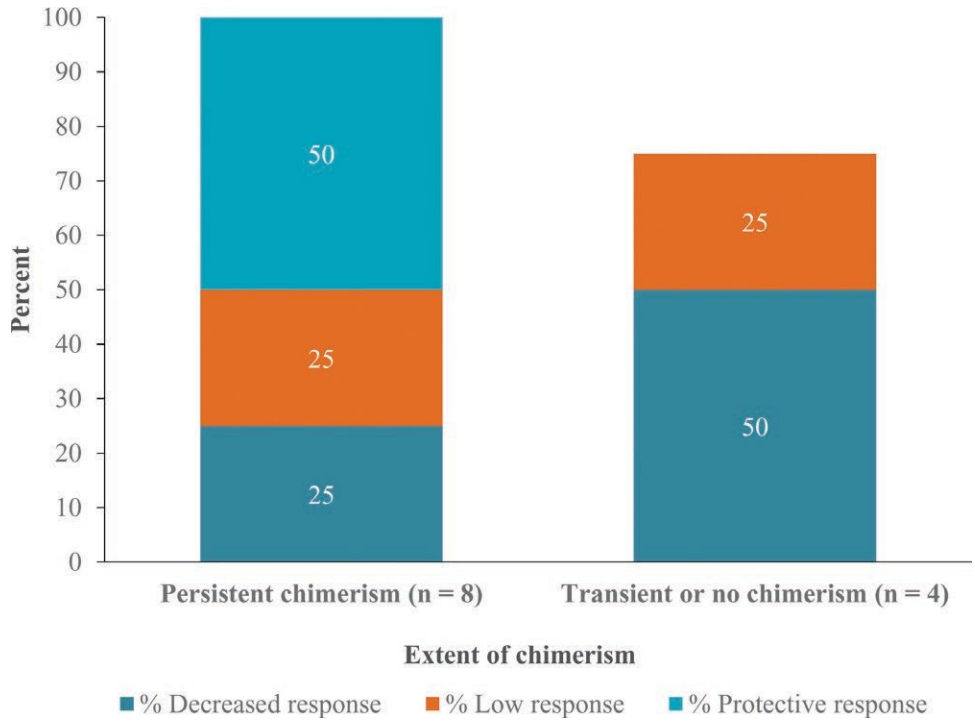

FIGURE 5. Response to tetanus vaccination in FCR001 cohort. Comparison of responses to tetanus vaccination between patients with persistent and transient/no chimerism.

were vaccinated. Eleven participants reported COVID-19 infection; symptoms (Table 6) were mild in all participants and did not require hospitalization. Neither vaccination against nor infection with SARS-CoV-2 was associated with a loss of peripheral blood chimerism in tolerant FCR001 participants. In addition, COVID-19 infection did not result in a significant change in kidney function (preinfection mean eGFR 68.1 mL/min/1.73 m^2 versus postinfection eGFR of 65.6 mL/min/1.73 m^2). Of the 4 vaccinated FCR001 participants who underwent antibody testing, all demonstrated strong humoral responses (Table 7).

Identification of a Tolerance Signature Through Urinary Cell mRNA Profiling

Urinary cell mRNA profiling has emerged as a powerful tool for the noninvasive monitoring of kidney allografts, including patients with immune kidney transplant tolerance.[7,12] We hypothesized that tolerant FCR001 patients would be associated with a unique urinary cell mRNA

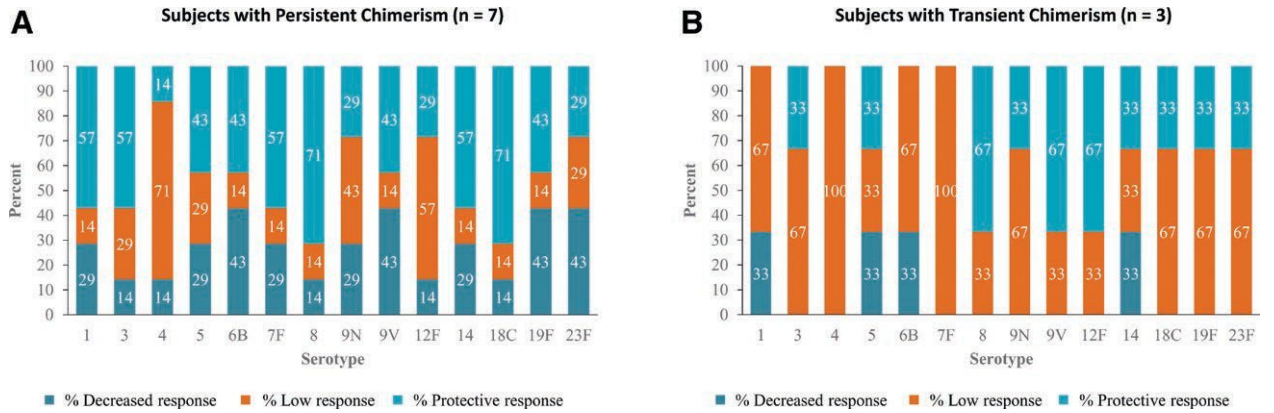

FIGURE 6. Response to pneumococcal vaccination in patients who received FCR001. A, Participants with persistent chimerism (n = 7). B, Participants with transient chimerism (n = 3). Patient response to pneumococcal vaccination with persistent (A) and transient (B) chimerism, separated by serotype.

signature distinct from that of SOC patients. To identify the discriminating signature, we focused on FCR001 patients with persistent chimerism. Urine specimens from 3 distinct groups of patients were compared for urinary cell mRNA abundance: 28 urine specimens from 14 FCR001 patients with persistent chimerism and off IS per protocol and normal allograft biopsies (fludarabine, cyclophosphamide, and rituximab FCR persistent group), 161 no rejection biopsy-matched urine specimens from 124 patients from the CTOT-04 study (no rejection group), and 43 Banff grade 1A or higher TCMR biopsy-matched urine specimens from 34 patients from the CTOT-04 study (TCMR group; Figure 8). F8

TABLE 5.

Demographics and available SARS-CoV-2 data

Demographics	FCR001/LDKT patients (n = 28)
Age range	28-73 y
Male	25
Race, White	25
Fully vaccinated[a]	22
Pfizer-BioNTech	16
Moderna	3
Johnson & Johnson	3
Positive PCR test	11
Serology performed	4

[a]At least 2 doses of Pfizer-BioNTech or Moderna vaccines or 1 dose of the Johnson & Johnson vaccine.
LDKT, living-donor kidney transplant; PCR, polymerase chain reaction.

Kruskal-Wallis testing of the urinary cell copies of mRNA for CTLA-4 (Figure 8A; Kruskal-Wallis adjusted [Adj]$P = 6 \times 10^{-20}$), CD3ε (Figure 8B; Adj $P = 3 \times 10^{-10}$), granzyme B (Figure 8C; Adj $P = 6 \times 10^{-11}$), perforin (Figure 8D; Adj $P = 1 \times 10^{-10}$), IP10 (Figure 8E; Adj $P = 5 \times 10^{-10}$), FoxP3 (Figure 8F; Adj $P = 6 \times 10^{-5}$), TGF-β1 (Figure 8G; Adj $P = 5 \times 10^{-7}$), and 18S rRNA (Figure 8H; Adj $P = 2 \times 10^{-5}$) as the dependent variable showed that the copy numbers are significantly different among the FCR persistent group, no rejection biopsy group, and the TCMR biopsy group.

In pairwise comparisons, urinary cell copies of mRNA for CTLA-4 were significantly higher in the FCR persis-tent group than in the no rejection group (median 439 copies/µg total RNA in the FCR persistent group versus 13 copies/µg total RNA in the no rejection group, Adj $P = 5 \times 10^{-14}$; Table 8). CD3ε mRNA copies were also numerically higher in the FCR persistent group than inthe no rejection group (median 1239 versus 360 copies/µgtotal RNA, respectively, Adj $P = 0.12$; Table 8). Urinary cell copies of mRNA for granzyme B were significantly lower in the FCR persistent group than in the no rejection group (median 106 versus 337 copies/µg total RNA, respectively; Adj $P = 0.03$). Urinary cell copies of mRNA for TGF-β1 were also significantly lower in the FCR persistent group T8

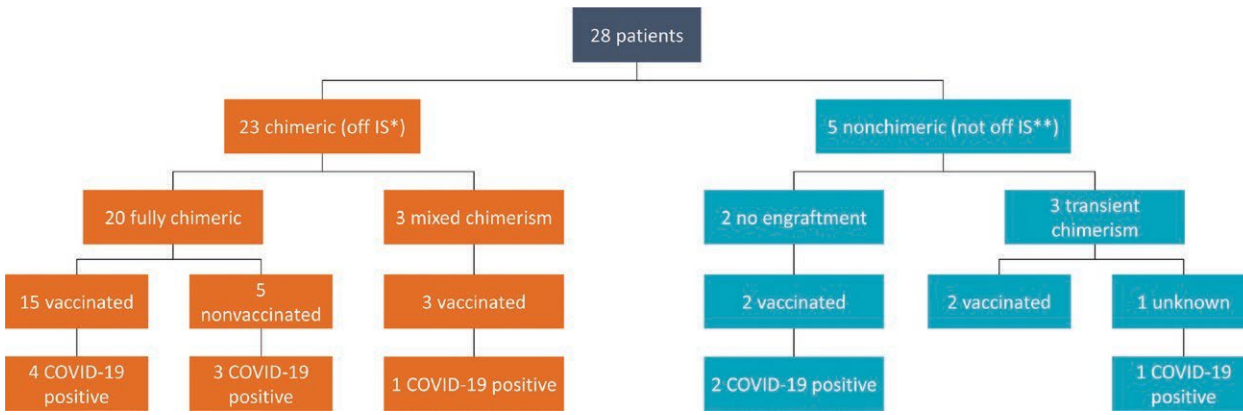

FIGURE 7. Outcomes with SARS-CoV-2 in FCR001 cohort. * Range 51.5–139.4 mo. ** One patient was on low-dose mTOR-based IS. IS, immunosuppression; mTOR, mammalian target of rapamycin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

than in the no rejection group (median versus 4589 copies/μg total RNA, respectively, Adj $P = 0.04$). The copies of mRNA for perforin, IP10, FoxP3, and 18S rRNA were not different between the FCR persistent group and the no rejection group (Adj $P > 0.05$; Table 8).

A comparison of urinary cell copies of mRNA in FCR persistent group with those in the TCMR group showed that copies of CTLA-4 mRNA, as compared with all other mRNAs measured, were uniquely higher in the FCR persistent group (439 copies/μg total RNA in the FCR persistent group versus 135 copies/μg total RNA in the TCMR group; Adj $P = 0.006$; Table 8). In contrast, urinary cell copies of mRNA for CD3ε (Adj $P = 7 \times 10^{-5}$), granzyme B (Adj $P = 2 \times 10^{-8}$), perforin (Adj $P = 1 \times 10^{-7}$), IP10 (Adj $P = 2 \times 10^{-5}$), FoxP3 (Adj $P = 0.007$), TGF-β1 (Adj $P = 4 \times 10^{-7}$), and the level of 18S rRNA (Adj $P = 2 \times 10^{-5}$) were all significantly lower in the FCR persistent group compared with the TCMR group (Table 8).

A comparison of copies between the TCMR group and the no rejection group showed that urinary cell copies of CTLA-4 mRNA were higher in the TCMR group versus no rejection group (Adj $P = 2 \times 10^{-11}$). In accordance with earlier studies, copies of mRNA for CD3ε, granzyme B, perforin, IP10, FoxP3, TGF-β1, and 18S rRNA were all significantly higher in the TCMR group than in the no rejection group (Adj $P < 0.05$; Table 8).

TABLE 6.

COVID-19 symptoms in FCR001 cohort

Signs and symptoms	COVID-19–positive patients n=11 (%)
Cough	5 (45%)
Fatigue	4 (36%)
Body aches	3 (27%)
Fever	2 (18%)
Loss of smell	2 (18%)
Sore throat	2 (18%)
Runny nose	1 (9%)
Headache	1 (9%)
Diarrhea	1 (9%)
Shortness of breath	1 (9%)

A summary of COVID-19 symptoms for FCR001 patients. Data collected from 11 patients.
COVID-19, coronavirus disease 2019; .

Among the urine specimens profiled for urinary cell mRNA copies (Figure **S**1, **SDC**, http://links.lww.com/TP/C779), 23 urine specimens were from 12 FCR patients with persistent chimerism and on IS and 28 urine specimens were from 14 FCR patients with persistent chimerism and off IS. To assess the impact of IS on urinary cell mRNA copies, we compared the levels between these 2 groups. Table 9 shows that IS did not affect the number of copies of any of the mRNAs (Adj $P > 0.05$ for all mRNAs compared). The median (interquartile range [IQR]) CTLA-4 mRNA copy number was 575 (323–1004) copies/μg total RNA isolated from the 23 urine specimens from the 12 patients on IS compared with 439 (146–947) copies/μg total RNA isolated from the 28 urine specimens from the 14 patients off IS (Wilcoxon rank-sum test, Adj $P = 0.65$). T9

Figures S3 and S4 (**SDC,** http://links.lww.com/TP/C779) provide additional information regarding urinary cell CTLA-4 mRNA copies in the FCR patients with persistent chimerism. Figure S3 (**SDC,** http://links.lww.com/TP/C779) shows the CTLA-4 mRNA copy number in relation to posttransplant day and whether the patient was on or off IS. Figure S4 (SDC, http://links.lww.com/TP/ C779) shows urinary cell CTLA-4 mRNA copies from the 7 patients while on IS and subsequently off IS. A comparison of urinary cell CTLA-4 mRNA copies restricted to the same patients providing urine samples while on or off IS showed no significant difference in the levels in urine samples collected while on IS or not (Adj $P = 0.94$; Wilcoxon signed-rank test, paired analysis comparing levels in urine collected at the closest time before stopping IS and the closest time after stopping IS).

We also calculated the ratio of the mRNA encoding negative immunoregulatory protein CTLA-4 to the mRNA encoding the cytotoxic protein granzyme B and compared the ratios among the FCR persistent group, TCMR biopsy group, and no rejection biopsy group. The ratio was highest in the FCR persistent group and differed significantly among the 3 groups ($P = 1 \times 10^{-14}$, Kruskal-Wallis test; Figure 9). A pairwise comparison showed that the median ratio of CTLA-4 mRNA to granzyme B mRNA in the FCR persistent group was significantly higher compared with the no rejection group (5.62 versus 0.04, $P = 7 \times 10^{-15}$) and the TCMR group (5.62 versus 0.03, $P = 6 \times 10^{-16}$). F9

TABLE 7.

COVID-19 serologic results in FCR001/LDKT patients

Participant	Antibody test	Result	Time point	Participant details
NU17	Siemens ADVIA Centaur SARS-CoV-2 total assay	>10.00 index (ref ≤1.00)	28 d postvaccination with Pfizer-BioNTech	Off all IS, mixed chimerism
NU27	Siemens ADVIA Centaur SARS-CoV-2 total assay	>10.00 index (ref ≤1.00)	176 d postvaccination with Johnson & Johnson	Off all IS, with h/o GvHD
	Siemens ADVIA Centaur SARS-CoV-2 total assay	>10.00 index (ref ≤1.00)	15 d postvaccination with Pfizer-BioNTech	Off all IS, mixed chimerism
NU22	Beckman Access SARS-CoV-2 IgG	7.02 index (ref ≤1.00)	177 d postvaccination with Pfizer-BioNTech	Off all IS, mixed chimerism
NU11	Quest Diagnostics SARS-CoV-2 total antibody, spike, semi quantitative	35.5 U/mL (ref <0.8 U/mL)	222 d postvaccination with Moderna	On CNI-based IS, transient chimerism

A summary of antibody tests used and the results in 4 FCR001/LDKT participants.
CNI, calcineurin inhibitor; GvHD, graft-versus-host disease; h/o, history of; IgG, immunoglobulin G; IS, immunosuppression; LDKT, living-donor kidney transplant; ref, reference; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

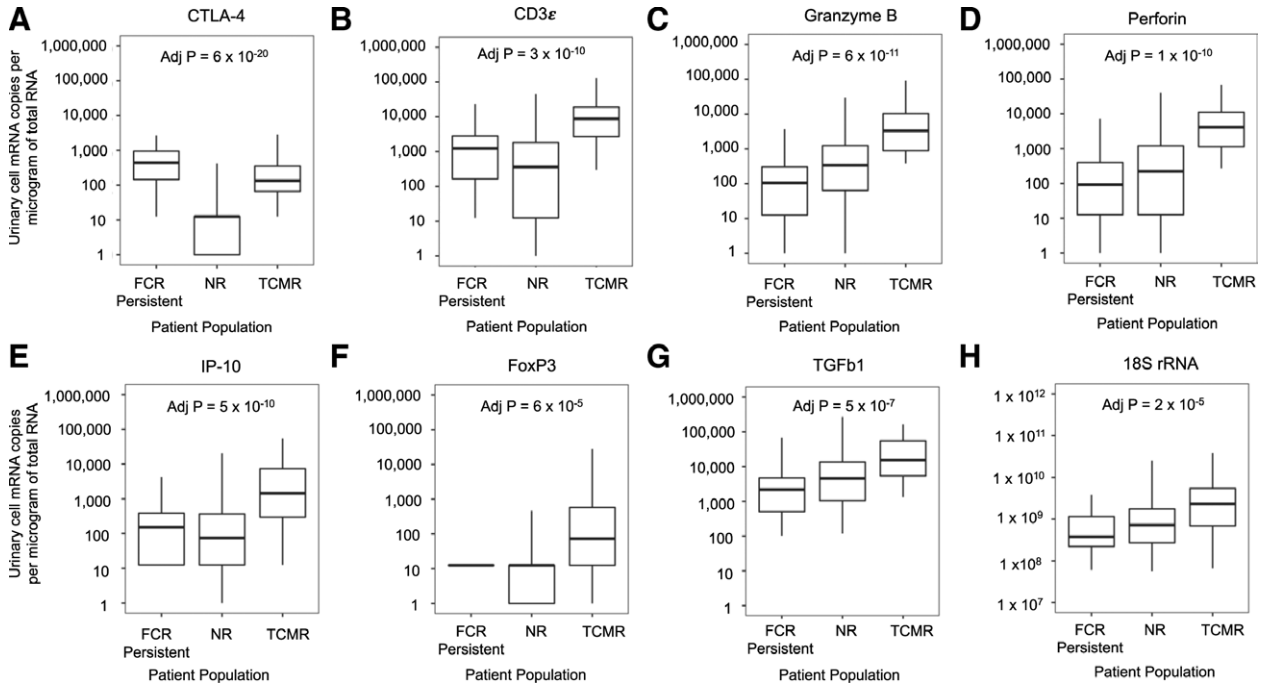

FIGURE 8. Absolute copy numbers of mRNAs in urinary cells. Box and whisker plots show the median (horizontal line within each box), the 25th and 75th percentile values (bottom and top of each box, respectively), and the largest value no >1.5 times the interquartile range from the hinge (whiskers) of mRNA copy number per microgram of total RNA isolated from urinary cells and 18S rRNA copy number per microgram of total RNA isolated from urinary cells. Urinary cell mRNA copy numbers in 28 urine specimens from 14 FCR001 patients with persistent chimerism and off IS therapy (FCR Persistent), 161 urine specimens from 124 patients with biopsies without acute or chronic rejection features (NR), and 43 urine specimens from 34 patients with biopsies classified as Banff acute TCMR were measured using preamplification-enhanced real-time quantitative PCR assays. Any mRNA copy number <50 copies, the lowest copy number in the PCR standard curve, was assigned a value of 12.5 in PCR assays; when no mRNA copies were detected after 40 Cts in the PCR assays, a value of 1 copy was assigned. Urinary cell levels of mRNA for CTLA-4 (A), CD3ε (B), granzyme B (C), perforin (D), IP10 (E), FoxP3 (F), TGFβ1 (G), and 18S rRNA (H) are shown. Adjusted *P* values within each box are based on the Kruskal-Wallis test comparing the 3 groups with Benjamini-Hochberg adjustment for multiple comparisons, with mRNA copy numbers used as the dependent variable. Adj, adjusted; CD3ε, T cell–surface glycoprotein CD3 epsilon chain; Ct, cycle threshold; CTLA-4, cytotoxic T lymphocyte–associated protein 4; FCR persistent, FCR001 patients with persistent chimerism and off IS; FoxP3, forkhead box P3; IP10, interferon gamma-induced protein 10; IS, immunosuppression; NR, no rejection; PCR, polymerase chain reaction; rRNA, ribosomal RNA; TCMR, T cell–mediated rejection biopsy; TGFβ1, transforming growth factor beta 1.

TABLE 8.

Absolute copy numbers of urinary cell mRNAs

Gene	FCR persistent group (n = 28 urine specimens, 14 patients)	No rejection group (n = 161 urine specimens, 124 patients)	TCMR group (n = 43 urine specimens, 34 patients)	Kruskal-Wallis Adj *P*[a]	FCR persistent vs no rejection Adj *P*+[b]	FCR persistent vs TCMR Adj *P*+[b]	TCMR vs no rejection Adj *P*+[b]
CTLA-4	439 (146-947)	13 (1-13)	135 (66-350)	6×10^{-20}	5×10^{-14}	0.006	2×10^{-11}
CD3ε	1239 (165-2852)	360 (13-1812)	8826 (2767-19140)	3×10^{-10}	0.12	7×10^{-5}	2×10^{-10}
Granzyme B	106 (13-304)	337 (64-1246)	3304 (897-10 438)	6×10^{-11}	0.03	2×10^{-8}	1×10^{-9}
Perforin	92 (13, 404)	223 (13, 1208)	4119 (1144-11 110)	1×10^{-10}	0.28	1×10^{-7}	2×10^{-10}
IP10	153 (13-391)	75 (13-370)	1445 (305-7356)	5×10^{-10}	0.23	2×10^{-5}	3×10^{-10}
FoxP3	13 (13-13)	13 (1-13)	73 (13-577)	6×10^{-5}	0.33	0.007	2×10^{-5}
TGF-β1	2182 (510-4787)	4589 (1047-13 267)	15142 (5400-55 288)	5×10^{-7}	0.04	4×10^{-7}	1×10^{-5}
18S rRNA	4×10^{8} (2×10^{8}-1×10^{9})	7×10^{8} (3×10^{8}-2×10^{9})	2×10^{9} (7×10^{8}-5×10^{9})	2×10^{-5}	0.12	2×10^{-5}	6×10^{-5}

Absolute copy numbers of transcripts were measured using the preamplification-enhanced real-time quantitative PCR assays. Median absolute copy number (lower, upper quartiles) of each mRNA measure and median absolute copy number (lower, upper quartiles) of 18S rRNA are shown. Any mRNA copy number <50 copies, the lowest copy number in the PCR standard curve, was assigned a value of 12.5 (rounded to 13 in the table); when no mRNA copies were detected after 40 Cts in the PCR assays, a value of 1 copy was assigned for data analysis.

[a]Adjusted *P* values were calculated using the Kruskal-Wallis test of no differences among the FCR persistent group, NR group, and TCMR group with Benjamini-Hochberg adjustment for multiple comparisons.

[b]Adjusted *P* values were calculated using the Wilcoxon rank-sum test for pairwise comparisons with Benjamini-Hochberg adjustment for multiple comparisons.

Adj, adjusted; CD3ε, T cell-surface glycoprotein CD3 epsilon chain; Ct, cycle threshold; CTLA-4, cytotoxic T lymphocyte-associated protein 4; FCR persistent, FCR001 patients with persistent chi-merism and off IS; FoxP3, forkhead box P3; IP10, interferon gamma-induced protein 10; IS, immunosuppression; NR, no rejection; PCR, polymerase chain reaction; rRNA, ribosomal RNA; TCMR, T cell-mediated rejection biopsy; TGF-β1, transforming growth factor beta 1.

TABLE 9.

Absolute copy numbers of mRNAs in urine from FCR001 participants with persistent chimerism and either on or off IS

Gene	FCR001 persistent group on IS (n = 23 urine specimens, 12 patients)	FCR001 persistent group off IS (n = 28 urine specimens, 14 patients)	Adj *P*[a]
CTLA-4	575 (323-1004)	439 (146-947)	0.65
CD3ε	1782 (594-4100)	1239 (165-2852)	0.37
Granzyme B	207 (63-728)	106 (13-304)	0.29
Perforin	277 (77-882)	92 (13-404)	0.29
IP10	266 (56-921)	153 (13-391)	0.51
FoxP3	13 (13-41)	13 (13-13)	0.65
TGF-β1	3045 (1426-10 013)	2182 (510-4787)	0.29
18S rRNA	6×10^8 (2×10^8-1×10^9)	4×10^8 (2×10^8-1×10^9)	0.65

Absolute copy numbers of transcripts were measured using the preamplification-enhanced real-time quantitative PCR assays. Median absolute copy number (lower, upper quartiles) of each mRNA measure and median absolute copy number (lower, upper quartiles) of 18S rRNA are shown.

[a]Adjusted *P* values were calculated using the Wilcoxon rank-sum test for pairwise comparisons with Benjamini-Hochberg adjustment for multiple mRNA comparisons.

Adj, adjusted; CD3ε, T cell-surface glycoprotein CD3 epsilon chain; CTLA-4, cytotoxic T lymphocyte-associated protein 4; FCR001 Persistent, FCR001 patients with persistent chimerism; FoxP3, forkhead box P3; IP10, interferon gamma-induced protein 10; IS, immunosuppression; PCR, polymerase chain reaction; rRNA, ribosomal RNA; TGFβ1, transforming growth factor beta 1.

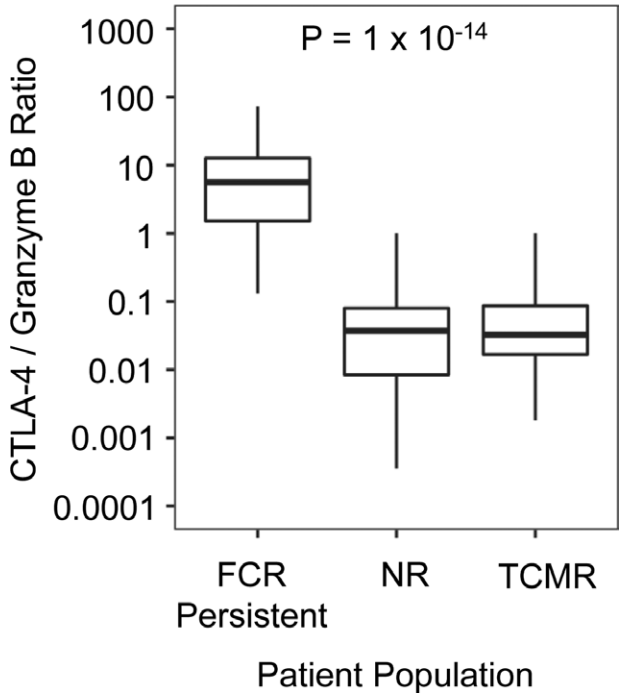

FIGURE 9. Ratio of CTLA-4 mRNA to granzyme B mRNA in urinary cells. Box and whisker plot shows the median (horizontal line within each box), the 25th and 75th percentile values (bottom and top of each box, respectively), and the largest value no >1.5 times the interquartile range from the hinge (whiskers) of the ratio of CTLA-4 mRNA copy number to granzyme B mRNA copy number. Urinary cell mRNA copy numbers in 28 urine specimens from 14 FCR001 patients with persistent chimerism and off IS (FCR Persistent), 161 urine specimens from 124 patients with biopsies without acute or chronic rejection features (NR), and 43 urine specimens from 34 patients with biopsies classified as Banff acute TCMR were measured using preamplification-enhanced real-time quantitative PCR assays. Any mRNA copy number below 50 copies, the lowest copy number in the PCR standard curve, was assigned a value of 12.5 in PCR assays; when no mRNA copies were detected after 40 Cts in the PCR assays, a value of 1 copy was assigned. *P* value is based on the Kruskal-Wallis test comparing the 3 groups, with mRNA copy numbers used as the dependent variable. Ct, cycle threshold; CTLA-4, cytotoxic T lymphocyte–associated protein 4; FCR persistent, FCR001 patients with persistent chimerism and off IS; IS, immunosuppression; NR, no rejection; PCR, polymerase chain reaction; TCMR, T cell–mediated rejection biopsy.

We also measured transcript levels in 11 additional urine samples from 3 FCR recipients with transient chi- merism (on IS as per protocol; FCR transient group). Data analysis showed that urinary cell copies of CTLA-4 mRNA were numerically higher in the FCR persistent group than in the FCR transient group (439 versus 257 copies/μg total RNA, respectively; Adj *P* = 0.90; Table 10). Furthermore, mRNAs encoding cytotoxic proteins gran- zyme B and perforin and the mRNA for chemokine IP10 were numerically lower in the FCR persistent group com- pared with the FCR transient group (Adj *P* > 0.05). All other comparisons yielded Adj *P* > 0.05 (Table 10). The ratio of CTLA-4 to granzyme B mRNA was higher in the FCR persistent group compared with the FCR transientgroup, but the difference was not statistically significant (median ratio 5.62 versus 1.99, respectively; *P* > 0.05). T10

DISCUSSION

FCR001 combined with living-donor kidney transplant plus reduced-intensity NMA conditioning has shown great promise for the establishment of chimerism and donor-specific tolerance.[8] The mechanism of tolerance induction through chimerism continues to be defined and is likely multifactorial, as evidenced by a higher ratio of regulatory T/effector T cells.[13,14] In the present studies, we identified potential biomarkers of tolerance through urinary cell mRNA profiling and the correlation of donor chimerism with immune tolerance.

Comparison of clinical outcomes in tolerant chimericand SOC participants showed comparable patient survival and graft survival at 2, 3, and 5 y. In addition, tolerant FCR001 participants exhibited stable kidney function compared with SOC patients whose kidney function declined over time. Treatment for hypertension and hyper- lipidemia was more common in SOC than in tolerant FCR001 participants. Finally, patients whose underlying end-stage kidney disease was caused by an autoimmune condition did not experience recurrence. We conclude that there are meaningful long-term medical benefits to estab- lishing tolerance in kidney transplant recipients, which become apparent within 2 y of transplantation.

Vaccination did not alter the stability of donor chimer-ism. Moreover, response to vaccination was superior in

TABLE 10.

Absolute copy numbers of mRNAs in urine from FCR001 participants with persistent chimerism and transient chimerism

Gene	FCR001 persistent group (n = 28 urine specimens, 14 patients)	FCR001 transient group (n = 11 urine specimens, 3 patients)	*Adj P* [a]
CTLA-4	439 (146-947)	257 (197-504)	0.90
CD3ε	1239 (165-2852)	1062 (473-3568)	0.90
Granzyme B	106 (13-304)	208 (47-745)	0.90
Perforin	92 (13-404)	106 (35-830)	0.90
IP10	153 (13-391)	449 (101-1077)	0.90
FoxP3	13 (13-13)	13 (13-13)	0.90
TGFβ1	2182 (510-4787)	1532 (150-11226)	0.90
18S rRNA	4×10^8 (2×10^8-1×10^9)	5×10^8 (2×10^8-1×10^9)	0.90

Absolute copy numbers of transcripts were measured using the preamplification-enhanced real-time quantitative PCR assays. Median absolute copy number (lower, upper quartiles) of each mRNA measure and median absolute copy number (lower-upper quartiles) of 18S rRNA are shown.

[a]Adjusted *P* values were calculated using the Wilcoxon rank-sum test between 2 groups with Benjamini-Hochberg adjustment for multiple mRNA comparisons.

Adj, adjusted; FCR Persistent, FCR001 patients with persistent chimerism; FCR Transient, FCR001 patients with transient chimerism; CD3ε, T cell-surface glycoprotein CD3 epsilon chain; CTLA-4, cytotoxic T lymphocyte-associated protein 4; FoxP3, forkhead box P3; IP10, interferon gamma-induced protein 10; IS, immunosuppression; PCR, polymerase chain reaction; rRNA, ribosomal RNA; TGFβ1, transforming growth factor beta 1.

persistently chimeric participants off IS compared with transiently chimeric participants on IS, supporting the hypothesis that these tolerant participants are immunocompetent to antigenic challenges. The immunocompetence of tolerant FCR001 participants is further supported by data showing mild forms of infection with SARS-CoV-2, a contrast to the well-described increased morbidity and mortality observed in immunosuppressed solid organ transplant recipients. FCR001 participants were safely and successfully vaccinated against SARS-CoV-2 without affecting the degree of chimerism.

Urinary cell mRNA profiling of a subgroup of FCR001 participants identified a potential signature of tolerance, characterized by increased copies of CTLA-4 mRNA, and a significantly higher ratio of CTLA-4 mRNA to granzyme B mRNA. CTLA-4 is a molecule involved in the suppres- sion of T-cell responses and is considered a "master regu- lator" of peripheral tolerance.[15] The immunoregulatory phenotype deciphered in this study—the high abundance of CTLA-4 mRNA and the low abundance of mRNAs encoding immunostimulatory molecules (eg, IP10) and cytotoxic attack molecules (eg, granzyme B)—suggests tipping of the immune balance toward negative regulation and away from cytopathic mechanisms and a proinflam- matory milieu. The unique urinary cell mRNA signatureidentified in this study needs to be validated in a larger cohort of patients. In this regard, we previously found that urinary cell CTLA-4 mRNA levels were significantly higher in the 5 urine specimens from 5 recipients of HLA- identical kidney allografts rendered tolerant by alemtu- zumab induction therapy and donor $CD34^+$ cell infusion[16] compared with the CTOT-04 no rejection biopsy group (median CTLA-4 copies/μg total RNA, 256 versus 13, respectively; $P = 0.002$) and numerically higher compared with the CTOT-04 TCMR biopsy group (median CTLA-4 copies/μg total RNA, 256 versus 135, respectively; $P = 0.62$). Our findings, in addition to suggesting a novel signature of tolerance, raise the intriguing hypothesis that IS drugs could be safely reduced in SOC kidney transplant participants exhibiting the urinary cell mRNA signatureidentified in this study. In this regard, IS did not appear to impact urinary cell CTLA-4 mRNA copy number in this study. Thus, the CTLA-4 signature identified here may be evident in patients treated with IS.

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