

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008;358:353-61.

Supplementary Appendix

Supplementary Table 1. Recipient/Donor histocompatibility

	A	B	Cw	DR	DR other	DQB1
Subject 1	2, 30	8, 18	05, 07	3	DR52	0201
Donor	2, 30	50, 18	05, 06	3, 7	DR52, 53	0201, 0202

	A	B	Cw	DRB1	DR other	DQB1
Subject 2	1, 11	08	07	0301	DRB3	0201
Donor	3, 11	08, 18	07	0301, 0901	DRB3, DRB4	0201, 0303

	A	B	Cw	DRB1	DR other	DQ
Subject 3	02, 26	07, 57	06, 07	07, 15	DRB4, DRB5	0303, 0602
Donor	02	07, 44	06, 07	04, 07	DRB4	0301, 0303

	A	B	Cw	DR	DR other	DQ
Subject 4	03, 26	07, 13	06, 07	2, 7	51, 53	1, 2
donor	01, 03	07, 08	07	2, 17	51, 52	2, 6

	A	B	Bw	DR	DR other	DQ
Subject 5	2	44	4	1, 2	51	1
Donor	2, 11	35, 44	4, 6	1, 7	53	1, 2

Supplementary Table 2. Sequence and Location of Oligonucleotide Primers and Probes

Gene-specific oligonucleotide primers and probes were designed using Primer Express software (PE Applied Biosystems, Foster City, CA). The probes were labeled with 6-carboxy-fluorescein (FAM) at the 5' end and 6-carboxy-tetramethylrodamine (TAMRA) at the 3' end. FAM functioned as the reporter dye and TAMRA as the quencher dye.

Accession		
Gene	Number	Sequences (Location)
FoxP3	NM_014009	Sense: 5'-GAGAAGCTGAGTGCCATGCA-3' (939-958) Antisense: 5'GGAGCCCTTGTCGGATGAT3' (1025-1007) Probe: 5'-FAM-TGCCATTTTCCCAGCCAGGTGG-TAMRA-3' (962-983)
Granzyme B	J04071	Sense: 5'-GCGAATCTGACTTACGCCATTATT-3' (534-557) Antisense: 5'-CAAGAGGGCCTCCAGAGTCC-3' (638-619) Probe: 5'-FAM-CCCACGCACA ACTCAATGGTACTGTGCG-TAMRA-3' (559-585)
18S rRNA	K03432	Sense: 5'-GCCCGAAGCGTTTACTTT GA-3' (929-948) Antisense: 5'-TCCATTATTCCTAGCTGCGGTATC-3' (1009-986) Probe: 5'-FAM-AAAGCAGGCCCGAGCCGCC-TAMRA-3' (965-983)

Protocol for Pre-amplification Enhanced Real-Time Quantitative PCR Assay

Pre-amplification enhanced real-time quantitative PCR assay was performed as reported (Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB,

Hartono C, Li B, Sharma VK, Seshan SV, Kapur S, Hancock WW, Schwartz JE, Suthanthiran M. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med.* 2005 Dec 1; 353 (22):2342-51). In the first step, a pre-amplification reaction was set up in a GeneAmp 9600 thermal cycler with 3 μ l cDNA and 7 μ l of dNTP, 10X PCR buffer, Taq DNA polymerase, and gene specific oligonucleotide primer pairs.

In the second step, PCR reaction for each sample was set up in duplicate as a 20 μ l reaction volume using 10 μ l TaqMan Fast Universal PCR Master Mix, 2.5 μ l pre-amplified template cDNA, gene specific primers (300 nM primers for Foxp3 and granzyme B and 200 nM for 18S) and 200 nM gene specific TaqMan probes. PCR amplification protocol included 40 cycles of denaturation at 95°C for 3 sec and primer annealing and extension at 60°C for 30 sec. Transcript levels were calculated using an absolute standard curve method (Ding R, Medeiros M, Dadhania D, Muthukumar T, Kracker D, Kong JM, Epstein SR, Sharma VK, Seshan SV, Li B, Suthanthiran M. Noninvasive diagnosis of BK virus nephritis by measurement of messenger RNA for BK virus VP1 in urine. *Transplantation.* 2002 Oct 15; 74(7):987-94).

The standard curve was established using PCR generated 73bp mouse Bak amplicon as the standard. The Bak amplicon was generated in a PCR reaction using GeneAmp 9600 thermal cycler and with 3 μ l cDNA and 22 μ l of dNTP, 10X PCR buffer, Taq DNA polymerase, and Bak specific oligonucleotide primer pair

(Sense primer: 5' CCCACATCTGGAGCAGAGTCA 3' [192-212]; Antisense primer: 5' CAGATGCCATTTTTTCAGGTCTTG 3' [264-242], Accession Number Y13231). The PCR product was separated by electrophoresis with a 2% agarose gel and the amplicon size (73bp) was confirmed using a DNA size standard of pUC mix marker 8 (Crystalgen, Plainview, NY). The Bak amplicon was isolated and purified from the gel with QIAquick gel extraction kit (Qiagen, Valencia, CA). The absolute quantity of the purified amplicon was measured by A260 and converted to the number of copies using the molecular weight of DNA. The Bak amplicon was diluted to the concentration of 10^7 copier/ μ l (stock solution).

When a standard curve was to be established for the real-time quantitative PCR assay, the stock solution was diluted over 6 orders of magnitude (1000000, 100000, 10000, 1000, 100, 10 copies per 1 μ l) (work solution). 2.5 μ l of the work solution was added to duplicate wells and amplified with Bak specific primer pair and Bak specific fluorogenic TaqMan probe (5' FAM CAGGTGACAAGTGACGGTGGTCTCCA-TAMRA 3' [215-240]). The threshold cycles (Ct) were then plotted versus the log of the initial amount of the BAK amplicon to develop the standard curve. The thresholds were in the exponential phase of the PCR and the higher the initial copy number of the BAK amplicon, the lower was the CT value.

We measured the levels of mRNA using an Applied Biosystems 7500 Fast Real-Time PCR System. 2.5 μ l of pre-amplified cDNA were amplified in duplicate with gene specific primer pair and gene specific TaqMan probe and the Ct values for

Foxp3, granzyme B and 18s rRNA in the target sample were determined. The copy numbers for Foxp3, granzyme B and 18S rRNA was calculated by comparing their respective Ct to the standard curve.

Statistical analysis was performed after normalization of Foxp3 mRNA copy number and granzyme B copy number using 18S rRNA copy numbers (mRNA copies in one μg RNA/18S rRNA copies in one fg RNA) and log transformation. The log – transformed mean ($\pm\text{SE}$) ratio of foxp3 mRNA copies to 18S rRNA copies was 5.48 ± 0.31 in the Stable IS Free group, 3.90 ± 0.27 in the Stable on IS group, 2.85 ± 0.34 in the Normal Kidney group, and 7.09 ± 0.38 in the Acute Rejection group. The log – transformed mean ($\pm\text{SE}$) ratio of granzyme B to 18S rRNA was 5.12 ± 0.69 in the Stable IS Free group, 4.53 ± 0.59 in the Stable on IS group, 3.41 ± 0.75 in the Normal Kidney group, and 9.76 ± 0.84 in the Acute Rejection group.

Differences in mRNA levels among groups were examined using Nested Analysis of Variance (Nested ANOVA) with repeated observations nested within patients. When group differences were found to be statistically significant, Tukey's honestly significant differences (HSD) method was used to test for differences between all possible pairwise comparisons. By this analysis, the intragraft level of foxp3 mRNA in the Stable IS Free group was significantly higher than in the Stable on IS group (Tukey-Kramer Adjusted $P=0.0073$) or the Normal Kidney group ($P=0.0002$), but lower than in the Acute Rejection group

($P < 0.0001$). Intragraft level of granzyme B mRNA in the Stable IS Free group was not significantly different from that in the Stable on IS group ($P = 0.91$) or the Normal Kidney group ($P = 0.36$) and significantly lower than that in the Acute Rejection Group ($P = 0.0002$).

Figure Legend

Supplementary Fig. 1

Representative glomeruli from protocol biopsies in figure 1B of article. A) Patient 1 (3 year post transplantation), B) Patient 2 (3 year post transplantation), C) Patient 4 (day 731 post transplantation), and D) Patient 5 (day 360 post transplantation). Each is normal, except for C, which shows segmental duplication of the glomerular basement membrane (arrows) and a few mononuclear cells in capillary loops. Original magnifications 40x; PAS stains.

