SDC, Material and Methods

Study design and population: immunosuppression

By design, the immunosuppression regimen used was left to the discretion of the treating physician. The standard immunosuppressive regimen used during the study period comprised basiliximab induction and a maintenance regimen by triple immunosuppression based on prednisone, tacrolimus and MMF.

Prospective monitoring, sample collection and treatment of viremia

Detailed clinical data, including demographics, kidney function, complete blood counts, and mediation (doses and blood levels) were recorded longitudinally. New medical diagnoses (in particular, infections, rejection, cardiovascular events and cancer) were recorded prospectively at each visit. In addition, serial monitoring for BKV was conducted routinely every 2 weeks during the first 12 months posttransplant and according to clinical suspicion after 12 months. Patients with confirmed BK viremia were switched from mycophenolate to leflunomide and kept under this regimen for several months after the viremia has cleared. Therefore, all the patients who were diagnosed with BK viremia received this treatment and were left on leflunomide for the remaining of the study period. CMV viremia was monitored weekly for 100 days following 100 days of prophylactic valganciclovir in CMV D+/R- recipients; CMV was tested in other patients according to clinical suspicion. Patients with asymptomatic CMV viremia were treated with valganciclovir until both of the following criteria were met: 2 weeks of treatment and negative PCR. Symptomatic CMV viremia received valganciclovir or IV ganciclovir until all of the following criteria were met: 4 weeks of treatment, symptom resolution and

negative PCR. In EBV R- patients, PCR and serology were obtained at every visit or at least every 2 weeks in the first year. One patient (ID32) was D-/R- and converted 22 months post transplant. Since then, this patient had a viremia at the log 3 level. Urinary tract infections were diagnosed based on urinalysis and culture and treated according to the antibiogram.

Cellular assay

For each experimental procedure, PBMCs for all time points from a given patient were thawed and cultured for 3 hours in RPMI 1640 containing 1% fetal bovine serum and IL-2 (25 U/ml, Peprotech, Rocky Hill, NJ). Adherent cells were detached by treatment with PBS containing 5 mM EDTA for 15 minutes at 4°C and were then resuspended in serum-free medium (Lonza, Walkersville, MD). PBMCs were distributed at 2×10^5 cells per well in round-bottomed 96-well plates (Sarstedt, Nümbrecht, Germany). Two separate plates were generated to study monocytes and T cells. The PBMCs were then incubated under the following conditions: under resting conditions, lipopolysaccharide (LPS) 1 µg /ml (Sigma-Aldrich, St-Louis, MO) for monocytes, anti-CD3/CD28 beads for T cells (according to the manufacturer's recommendations; the T cell activation kit, Miltenyi Biotec Inc., Auburn, CA) and EBV-derived peptides (according to manufacturer's recommendations; EBV Peptivator consensus, Miltenyi). The PBMCs were cultured overnight at 37°C under 5% CO_2 and then incubated for 5 hours with an exocytosis inhibitor (GolgiStop, BD Biosciences, San Diego, CA). MACS beads were used for leucocyte subset depletion, as per manufacturer's protocol (Miltenyi).

For monocytes, a viability marker was used prior to extracellular staining (efluor450, eBioscience, San Diego, CA). Samples in which the mortality rate was ≥30% were discarded. Cells were resuspended and blocked (FcR blocking reagent, Miltenyi), labeled with antibodies, and then fixed for 15 minutes in 4% paraformaldehyde (PFA) at 4°C. Intracellular labeling was carried out according to the manufacturer's protocol (Cytoperm, BD Biosciences). Background noise was determined using an isotype control. The following labeling antibodies were used: anti-CD14-PE-Vio770, anti-CD4-VioBlue, anti-CD8-VioGreen, anti-CD62L-PE-Vio770, anti-CD45RO-PerCP-Vio700, anti-IFN-γ-APC-Vio770 (Miltenyi), anti-CD16-BV510, and anti-TNF-α-FITC, (BioLegend, San Diego, CA). All antibodies were titrated against their respective isotype. PBMCs were analyzed by flow cytometry on a BD LSRFortessa instrument (BD Biosciences). Flow cytometry data analyses were performed with the FlowJo vX software (FlowJo LLC). As shown in this figure, we used the viability marker to exclude dead cells from the analysis.

Statistical Analyses

Test positivity was based on the presence of 2 consecutive values of TNF-α-positive cells that were below the established threshold. This threshold was used to describe the sensitivity, specificity, PPV and NPV. A similar analysis was conducted at the validation stage. The adjusted analyses were performed on the entire cohort using linear mixed models. Statistical analyses were performed using SPSS Statistics version 23 (IBM, Armonk, NY, USA). All tests were two-tailed, and a p value < 0.05 was considered statistically significant.

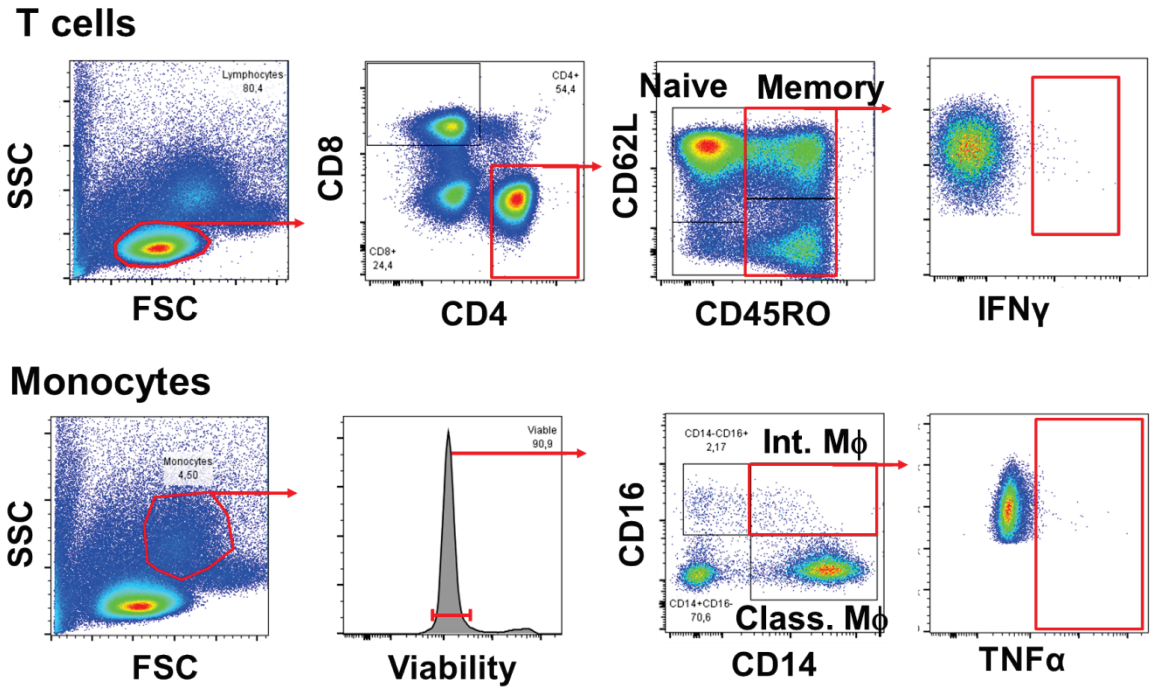

Figure S1. Gating strategy. The lymphocyte population was selected on the FSC/SSC gate, then targeted to CD4+CD8- or CD8+CD4- (top panel). The production profile of IFN-γ was analyzed for naive (CD45RO-CD62+), memory (CD45RO+), central memory (CD45RO+CD62+) effector memory (CD45RO+CD62-) subsets. The monocytes were selected on the FSC/SSC gate, then targeted on the viable cells (bottom panel). TNF-α production profile was analyzed for the CD14+CD16- and CD14+CD16+ monocytes Class. Mϕ: Classical monocyte (CD14+CD16-), Int. Mϕ: Intermediate monocyte (CD14+CD16+).

Supplemental Figures

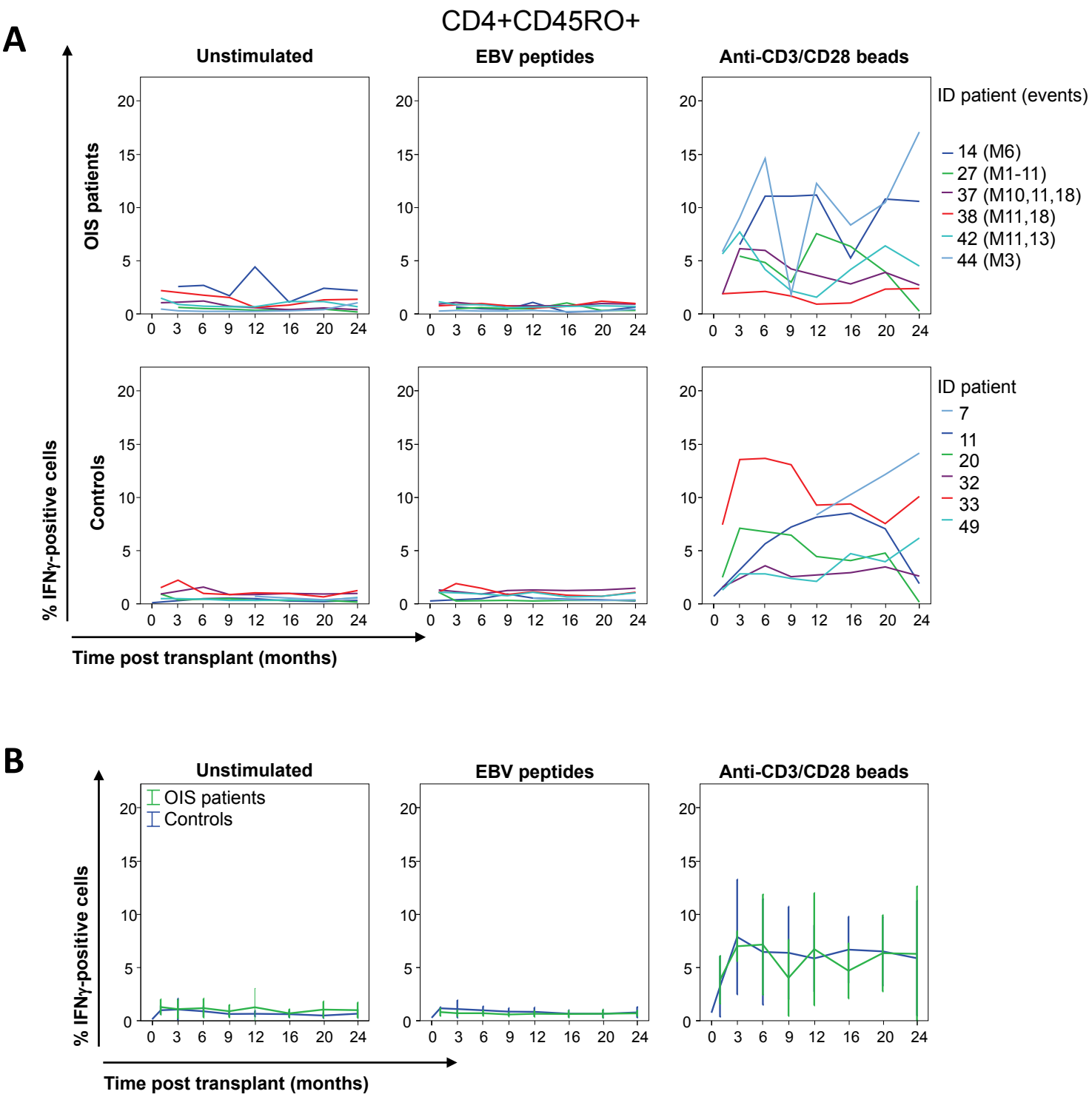

Figure S2. Memory CD4+ T cell response. (A) Within-patient analysis of CD4+CD45RO+ T cells producing IFN-γ, showing each patient over time as a separate line. The cells were studied following no stimulation (left panels), stimulation with EBV peptides (middle panels) and stimulation with anti-CD3/CD28 beads (right panel). (B) Within-group percentages of the same data. Data are presented as mean ± SD by time point for each group.

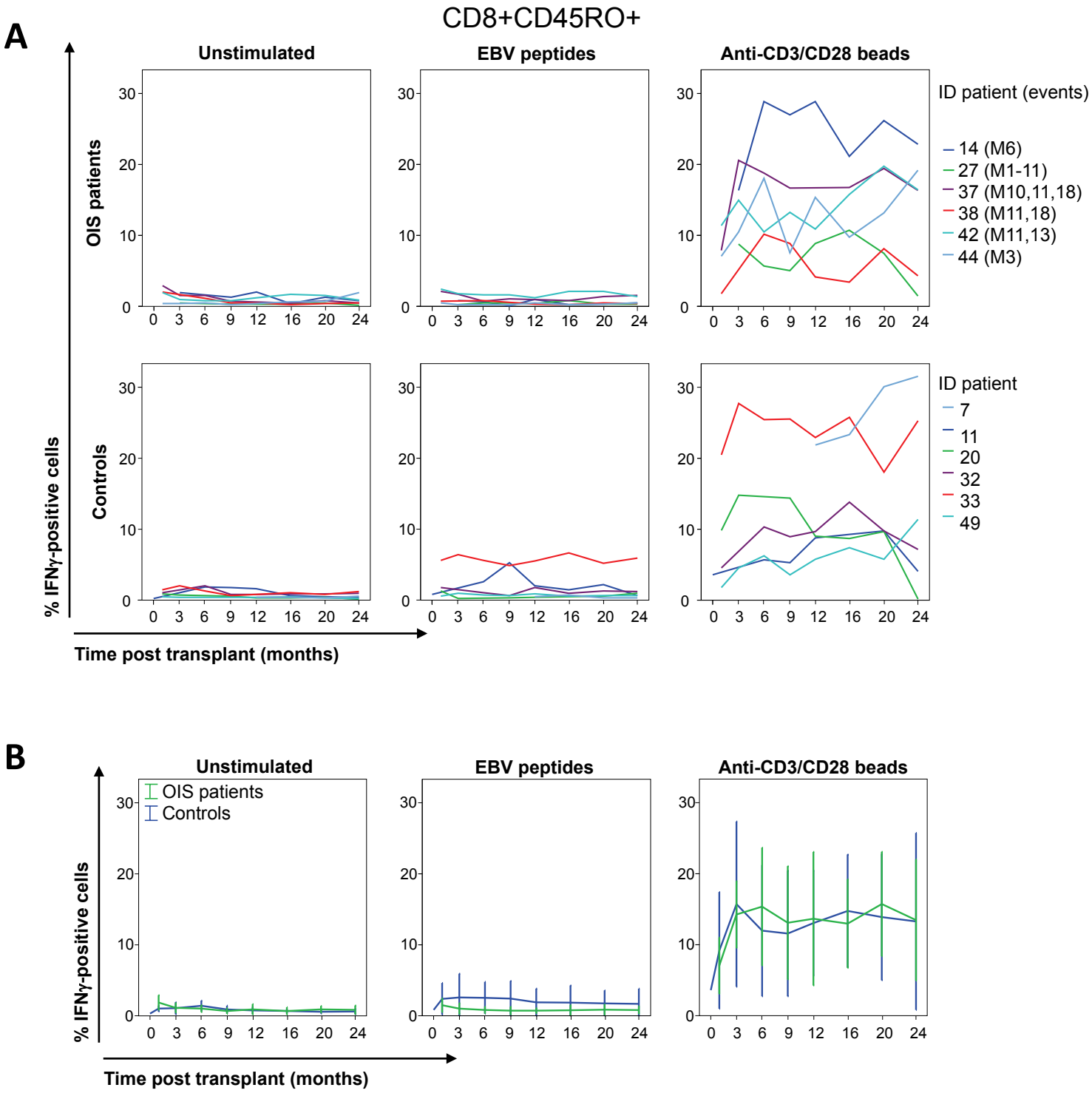

Figure S3. Memory CD8+ T cell response. (A) Within-patient analysis of CD8+CD45RO+ T cells producing IFN-γ, showing each patient over time as a separate line. The cells were studied following no stimulation (left panels), stimulation with EBV peptides (middle panels) and stimulation with anti-CD3/CD28 beads (right panel). (B) Within-group percentages of the same data. Data are presented as mean ± SD by time point for each group.

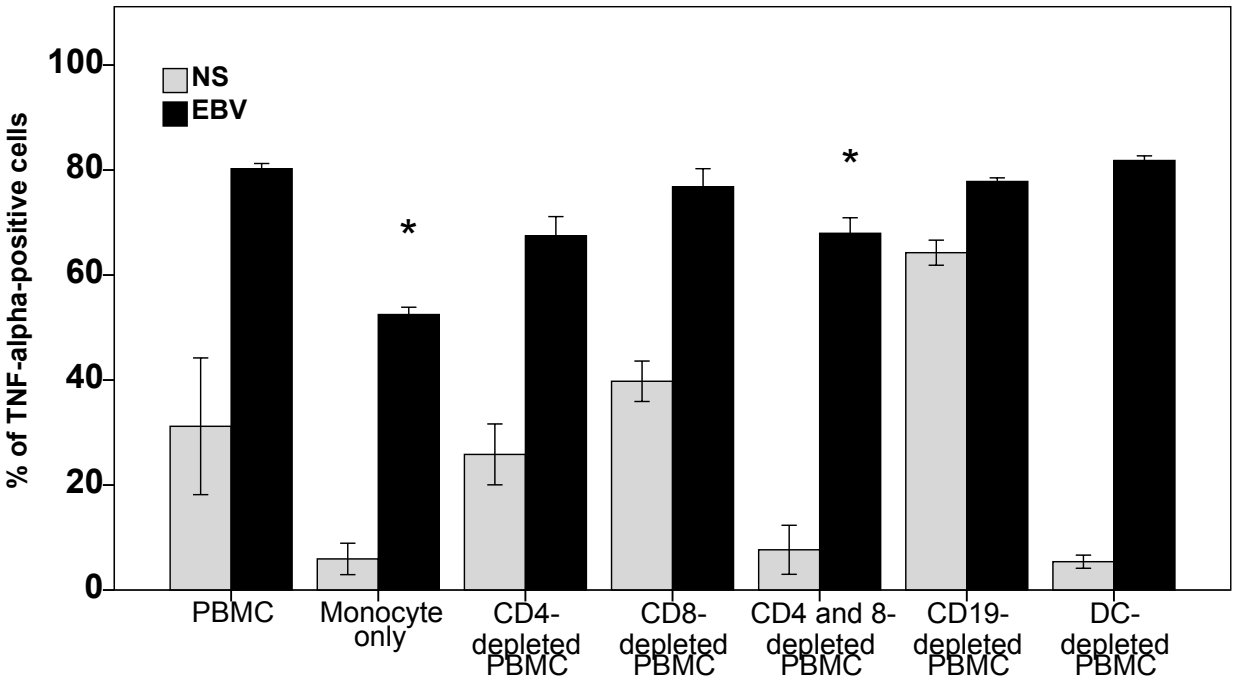

Figure S4. Monocyte response following depletion of leucocyte subsets. We cultured monocytes alone following negative selection. For the other cell subset depletion, positive selection of the depleted cells was used. Data are presented as mean ± SE; n=3; *, <0.05.

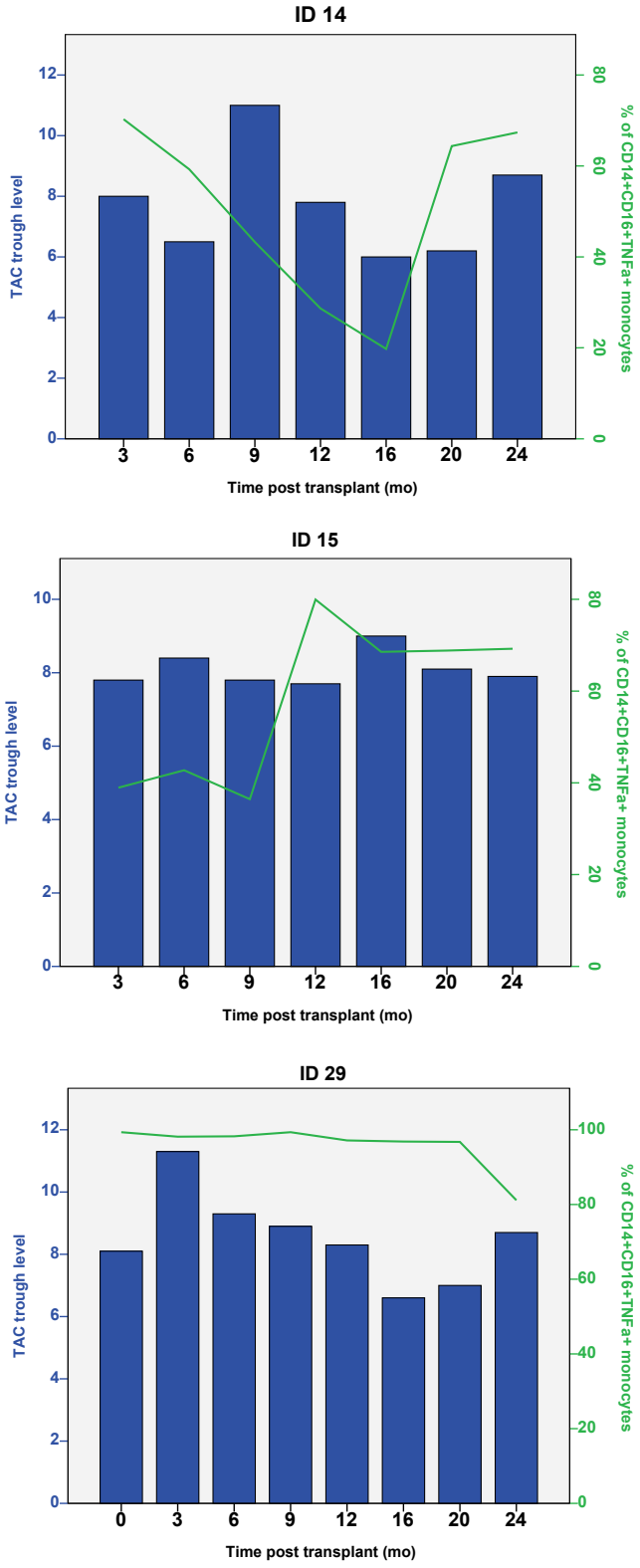

Figure S5. Monocyte response and TAC through levels over time. The three illustrative examples show the lack of correlation between the proportion of TNF-α-positive monocytes and the TAC through levels for each of these patients. Each figure illustrates the course of a single patient. The multivariable linear mixed model presented in the main text is consistent with these examples. IDxx, ID study patient number.

Table S1. Descriptive of the clinical events

Patient ID	EBV status	Main clinical event(s)
TIS patients		
10	D+/R+	BK viremia M4, TCMR M6, secondary CMV viremia M8, borderline changes suspicious for TCMR M12, M24.
13	D+/R+	Urinary infection tract M3, M6, M16, borderline changes suspicious for TCMR M6, cellulitis M19.
14	D+/R+	BK viremia M6.
15	D+/R+	BK viremia M4, pneumonia M6, cellulitis and influenza M12.
16	D+/R+	Secondary CMV viremia M7, TCMR M24.
21	D-/R+	Clostridium difficile colitis M3, borderline changes suspicious for TCMR M3, pyelonephritis M6, diverticulitis M13, bacteriuria with infection M13, repeated > 3 times thereafter
27	D+/R+	Borderline changes suspicious for TCMR M1, BK viremia M11
28	D-/R+	BK viremia M12.
34	D+/R+	BK viremia M12.
37	D+/R-	Lymphoma (PTLD) M10, BK viremia M11, BK viremia M18.
38	D+/R+	BK nephropathy M11, zona M18.
39	D+/R+	Persistent BK viremia M7, pyelonephritis with sepsis M10, BK nephropathy M20.
42	D+/R+	Secondary CMV viremia M11, BK viremia M13, TCMR.
44	D+/R+	BK viremia M3.
45	D+/R+	Borderline changes suspicious for TCMR M6, BK viremia M12, died M16.
46	D+/R+	BK viremia M4.
Controls		
7	D+/R+	No event.
9	D+/R+	Borderline changes suspicious for TCMR M10.
11	D+/R+	TCMR M2.
12	D+/R+	No event.
17	D+/R+	TCMR M1, borderline changes suspicious for TCMR M3.
18	D+/R+	TCMR M0, M5, lost of follow-up M16.
19	D+/R+	No event.
20	D+/R+	No event.
22	D+/R+	No event.
25	D+/R+	No event.
26	D+/R+	TCMR M6, M9, borderline changes suspicious for TCMR M20.
29	D+/R+	No event
30	D+/R+	No event, lost of follow-up M12.
31	D+/R+	No event
32	D-/R-	No event
33	D-/R+	No event
35	D+/R+	No event, lost of follow-up M12.
36	D+/R+	No event
40	D-/R+	No event
41	D+/R+	No event
43	D+/R+	No event
47	D+/R+	No event
48	D-/R+	No event
49	D+/R+	Signs of ABMR M6 (Isolated Banff score glomerulitis score = 1)

Mx, month x post transplant, TCMR, T cell mediated rejection; ABMR, antibody-mediated rejection; D, donor EBV status; R, recipient EBV status.

Table S2. Assessment of the coefficients of variability (CV)

A. Intra-assay (variation between duplicates)

Sample	Triplicate 1 (%)	Triplicate 2 (%)	Triplicate 3 (%)	Triplicate Mean (%)	Standard Deviation	% CV
A	70.3	68.5	73.8	70.9	2.7	3.8
B	78.2	88.5	79.7	82.1	5.5	6.8
C	76.0	76.7	73.1	75.2	1.9	2.6
D	74.5	77.9	77.9	76.8	1.0	2.6
	Intra-assay CV = 3.9%					

B. Inter-assay (variation between experiments)

Sample	Experiment 1 (%)	Experiment 2 (%)	Experiment 1 & 2 Mean (%)	Standard Deviation	% CV
A	70.9	74.3	72.6	2.4	3.4
B	82.1	86.8	84.5	3.3	4.0
C	75.2	73.5	74.4	1.3	1.7
D	76.8	78.1	77.4	0.9	1.2
	Inter-assay CV = 2.6%				

C. Inter-operator

Sample	Operator 1 (%)	Operator 2 (%)	Operator 1 & 2 Mean (%)	Standard Deviation	% CV
A	70.9	71.8	71.3	0.7	0.9
B	82.1	75.2	78.7	4.9	6.2
C	75.2	80.9	78.1	4.0	5.1
D	76.8	80.7	78.0	3.8	4.9
	Inter-assay CV = 4.3%				

D. Fresh vs. frozen cells

Sample	Frozen cells (%)	Fresh cells (%)	Fresh & Frozen Mean (%)	Standard Deviation	% CV
E	74.3	71.6	72.9	1.9	2.6
F	67.5	72.1	69.8	3.2	4.6
G	79.9	72.6	76.3	5.2	6.8
H	65.1	67.0	66.0	1.3	2.0
	Inter-assay CV = 4.0%				