Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation.

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Fig. S1

A

A A' B C

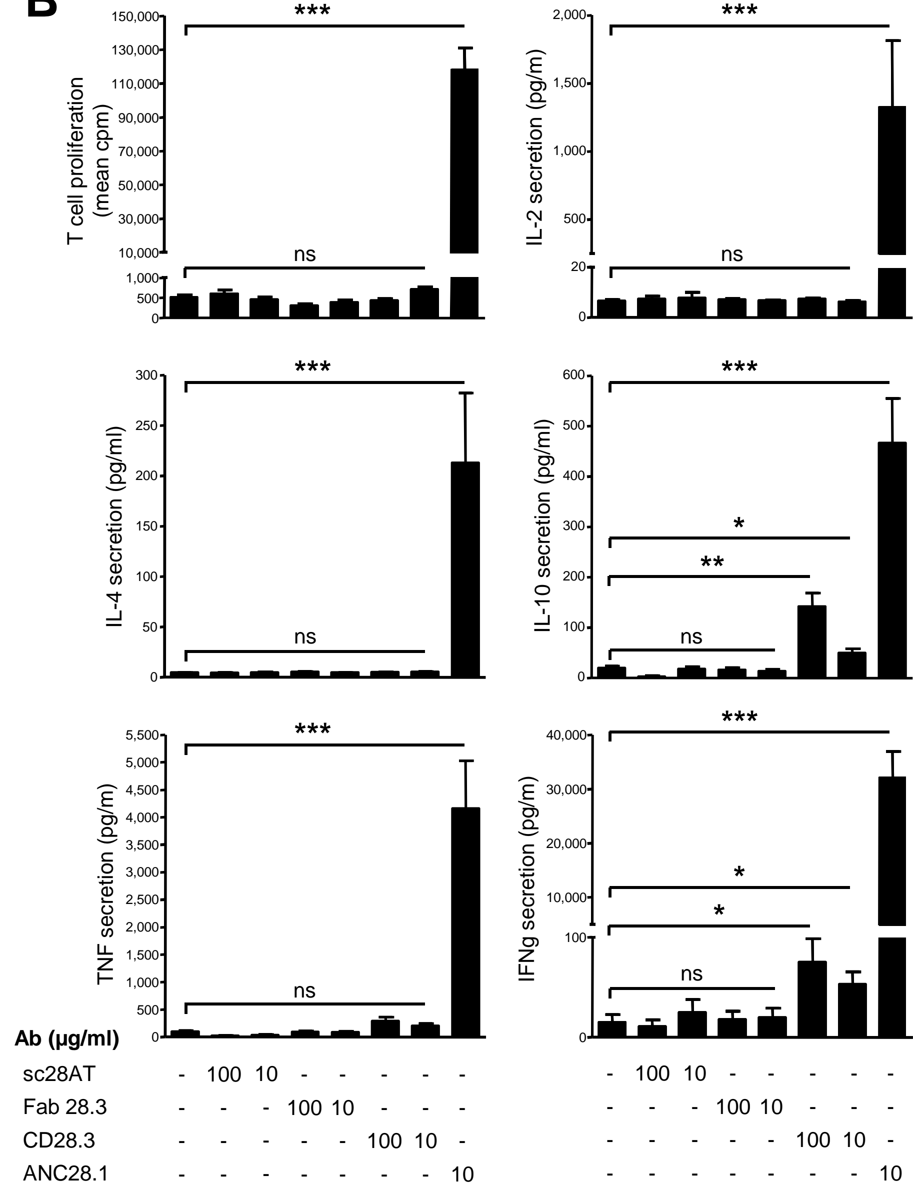
CD28 NKILVKQSPMLVAYDNAVNLSCKYSYNLFSREFRASLHKGLD

C' C'' D E

SAVEVCVVYGNYSQQLQVYSKTGFNCDGKLGNESVTFYLQNLYVNQ

F G G'

TDIYFCKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP



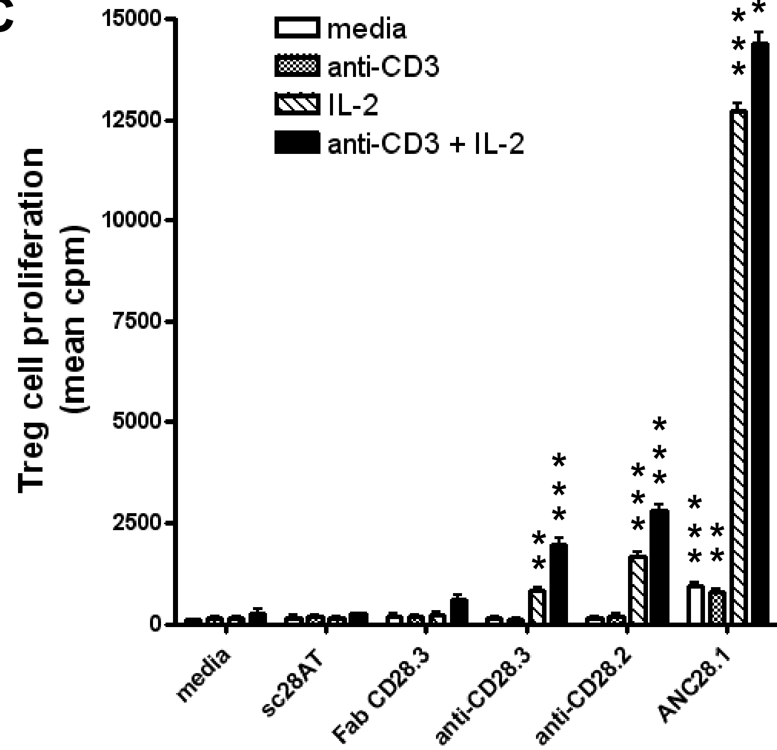


Fig. S1: Non agonistic properties of monovalent fragments from the CD28.3 antibody.

(A) Amino acid sequence of human CD28. Solid lines and letters: domains of the protein, as defined by Evans *et al*. (Nat Immunol, 6, 271-9, 2005). Boxed: CD28.3 Fab-contacts. Dashed underlining: target sequences of superagonistic antibodies, as defined by Lühder *et al*. (J Exp Med, 197, 955-66, 2003) (B) Proliferation and cytokine synthesis by human PBMC after 5 days in the presence of monovalent fragments of the conventionalCD28.3 anti-CD28 antibody (sc28AT or Fab fragments), intact divalent CD28.3 IgG1 antibodies or divalent superagonist ANC28.1 antibodies at the indicated concentrations. Results are means ±SD of results from 7unrelated healthy donors. *, ** and *** indicate a significant difference at $p<0.05$, 0.01 and 0.001, respectively.(C) Proliferation of human naturally regulatory T cell (CD4+ CD25+ CD127low, purity > 97%) after 3 days in the presence of monovalent fragments of the conventional CD28.3 anti-CD28 antibody (sc28AT or Fab fragments),intact divalent CD28.3 or CD28.2 or superagonist ANC28.1 antibodies at 10µg/ml. Treg cells were also cultured with or without IL-2 (50 UI/ml) and with or without anti-CD3 coating (1µg/ml). Results are mean cpm ± SD of a representative assay out of 4. ** and *** indicate a significant difference at $p<0.01$ and0.001, respectively, in comparison to control conditions.

Fig. S2

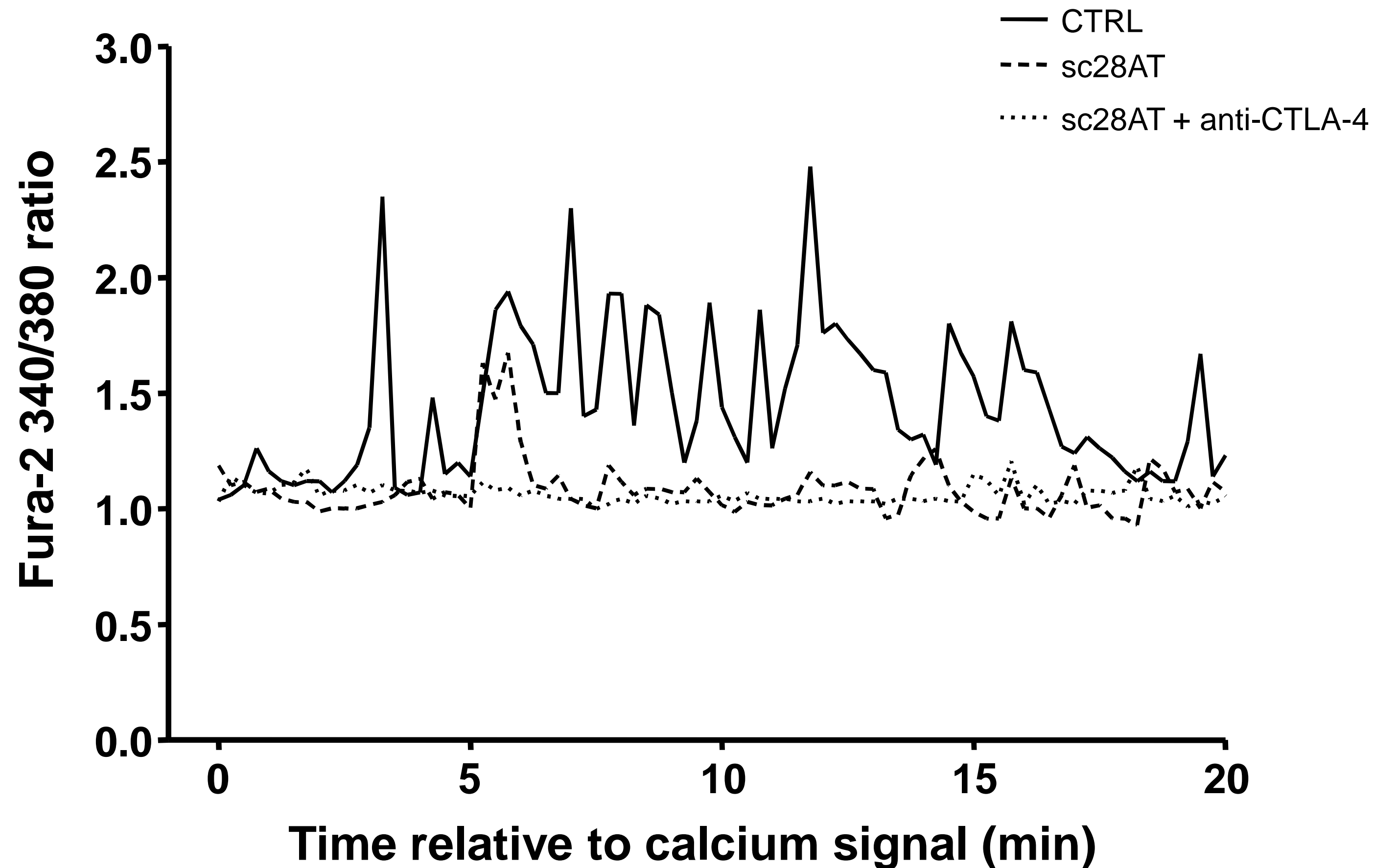


Fig. S2: Calcium flux profiles *in vitro*. T cells that had established a contact with an APC were tracked by time-lapse microscopy and the fluorescence of the FURA-2 probe was recorded over 20 min. One representative T cell is shown for for each condition. Cells were treated with 10µg/ml mouse Ig (CTRL), sc28AT or sc28AT + anti-CTLA-4 Fab fragments.

Fig. S3

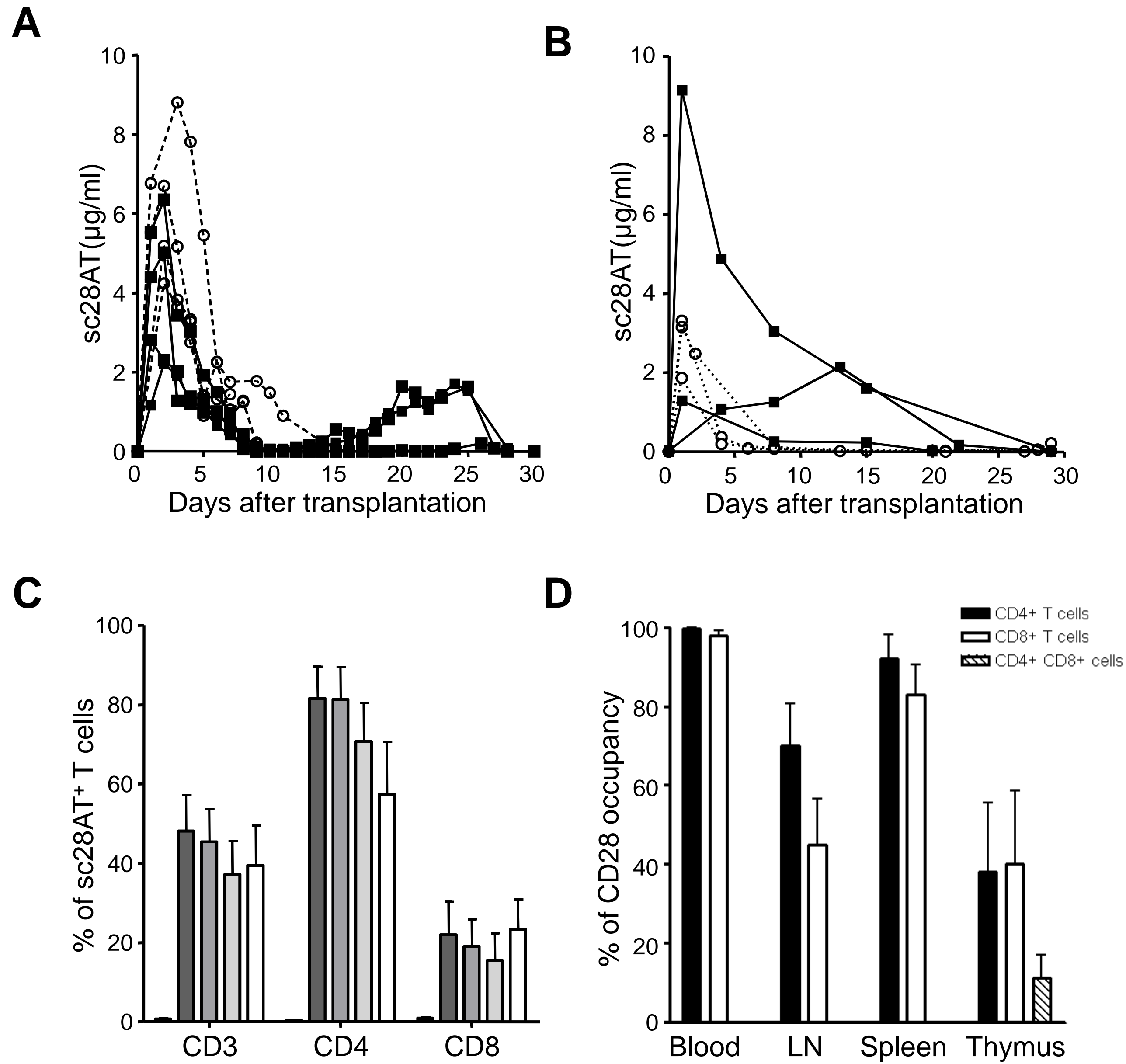


Fig. S3: Parmacokinetic and pharmacodynamic aspects of sc28AT. (A) Blood samples were drawn at the indicated time points, just before the new daily injection, from baboon recipients of kidney allograft under sc28AT monotherapy (○) or sc28AT + calcineurin inhibitor bitherapy (■). Trough levels were measured by ELISA in the serum. Different lines represent individual animals. (B) Same as in (A), in the cardiac transplant model in macaques. (C) In the baboon kidney graft model, the percentage of CD3+, CD4+ and CD8+ blood T cells loaded with sc28AT was recorded by flow cytometry (mean ± SD; n=4). For each phenotype, from left (dark) to right (white), analyses on day 0 and 1, 2, 4 and 6 post transplantation. (D) Tissue penetration of sc28AT, 16h after injection (2mg/Kg), in naïve monkeys. Data are mean ± SD and normalized to the % of CD28+ cells of the indicated phenotype (n=6 for blood and n=4 for lymph node (LN), spleen and thymus).

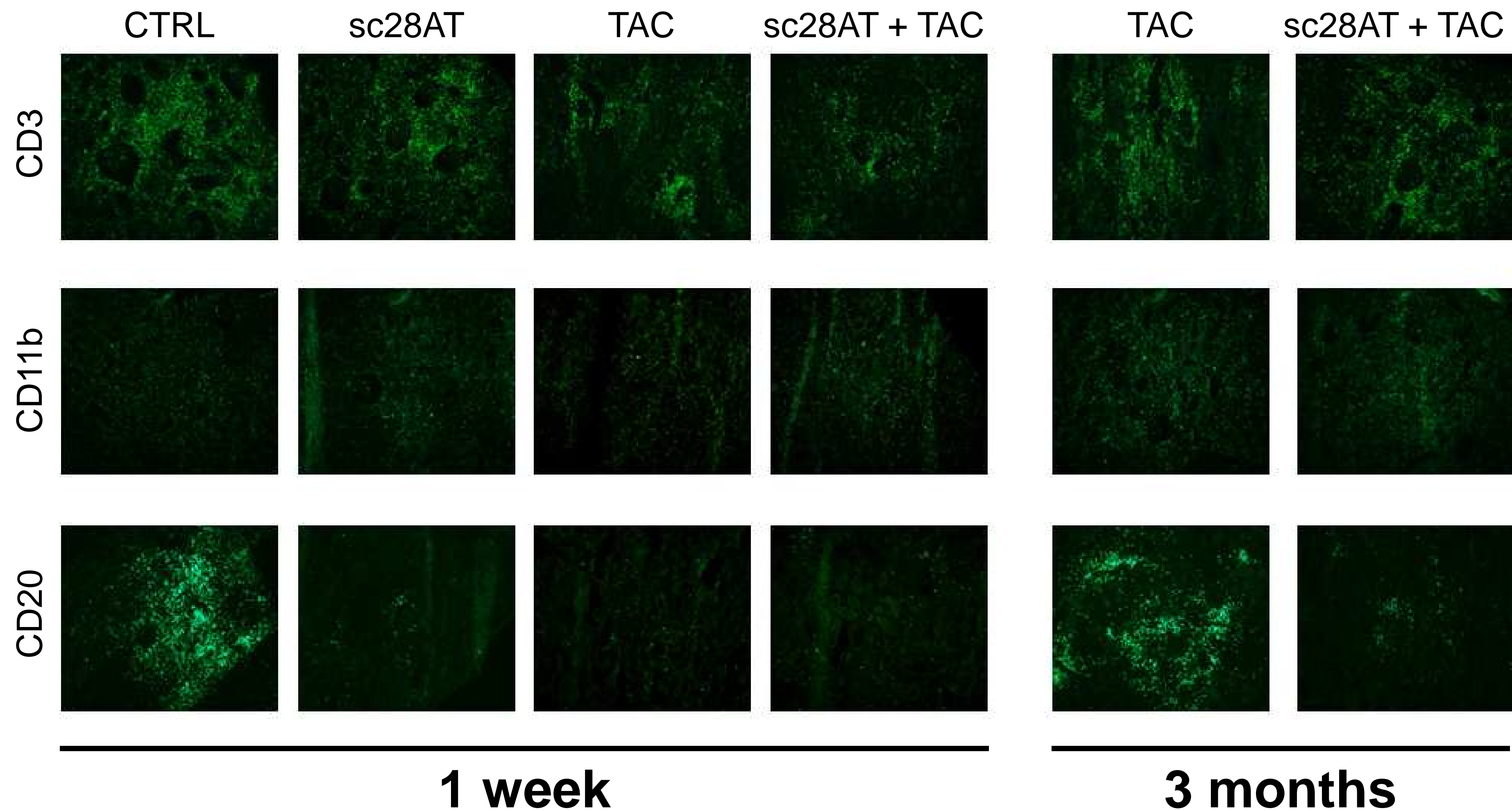


Fig. S4: Immunohistological analyses of kidney graft biopsies. Representative biopsies from kidney transplants in the baboon model, at 1 week post transplantation for controls (CTRL) and animals treated with sc28AT monotherapy (sc28AT), and 1 week or 3 months post transplantation for animals treated with Tacrolimus (TAC) and sc28AT + Tacrolimus. FITC-labeled antibodies against CD3 (T cells), CD11b (monocytes/macrophages) and CD20 (B cells) were used.

Fig. S5

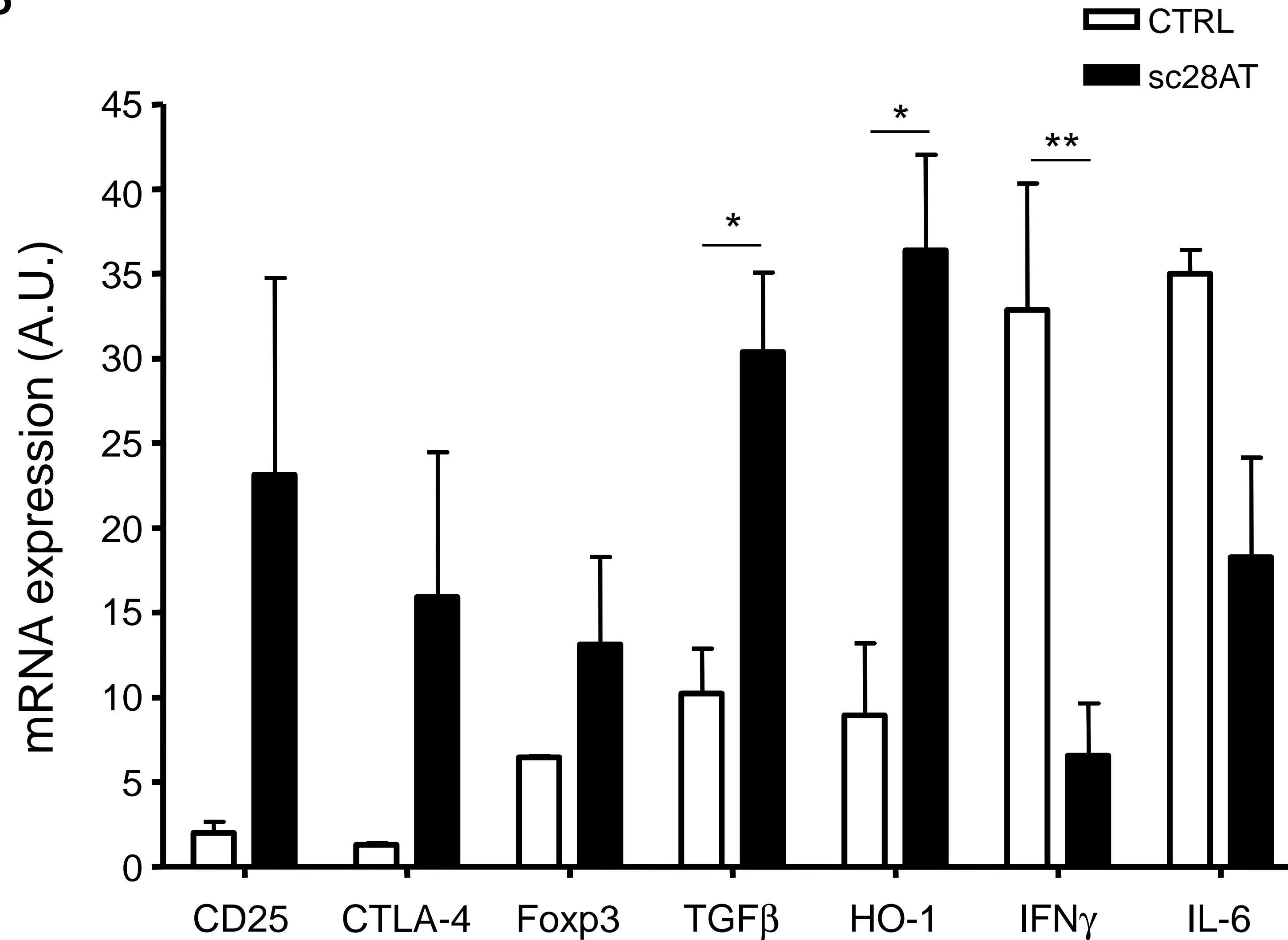


Fig. S5: qPCR measurement of mRNA transcripts in kidney graft biopsies. Kidney graft biopsies were harvested 1 week after transplantation from baboon recipients either untreated (CTRL;n=2) or treated with sc28AT (n=4) and processed for mRNA analysis by qPCR as indicated in the Methods section. Results are means ± SD of the expression level normalized to HPRT expression. *, $p<0.05$ and **, $p<0.01$.

Fig. S6

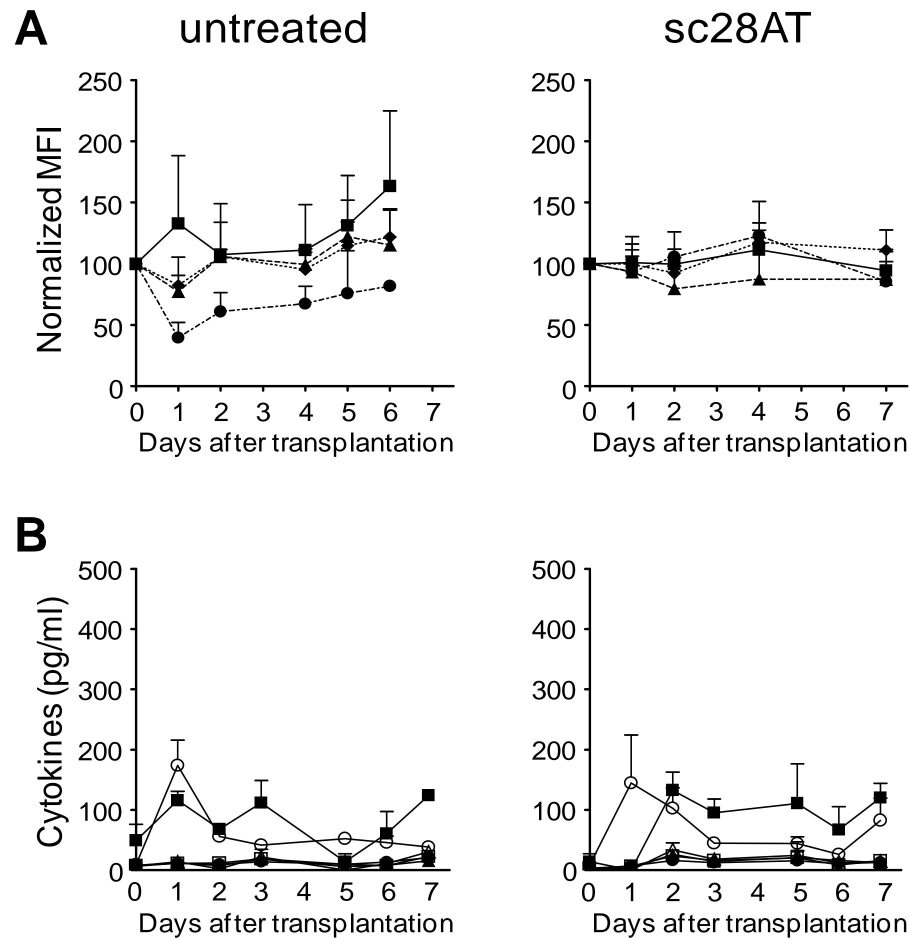


Fig. S6: Non agonistic properties of sc28AT *in vivo*.

(A) Phenotypic analyses of peripheral blood T cells during the first week post transplantation in controls (n=3) and sc28ATmonotherapy recipients (n=4). Curves indicate the median fluorescence intensity (MFI) of the following markers on T cells: CD25 (■, solid black lines), MHC-DR (▲, dashed black lines), CD62L (♦, dotted gray lines), CD45RO (●, dashed gray lines). Data are means ± SD and normalized to the initial MFI on day 0 that was arbitrarily attributed a value of 100. (B) Cytokines measured in the blood during the first week following kidney transplantation in untreated recipients (n=3) and recipients treated with sc28AT monotherapy (n=4). ■, IL-2; ▲, IL-4; ●, IL-5; □, TNFa; Δ, IFNg; ○, IL-6. Results are expressed as mean ± SD.

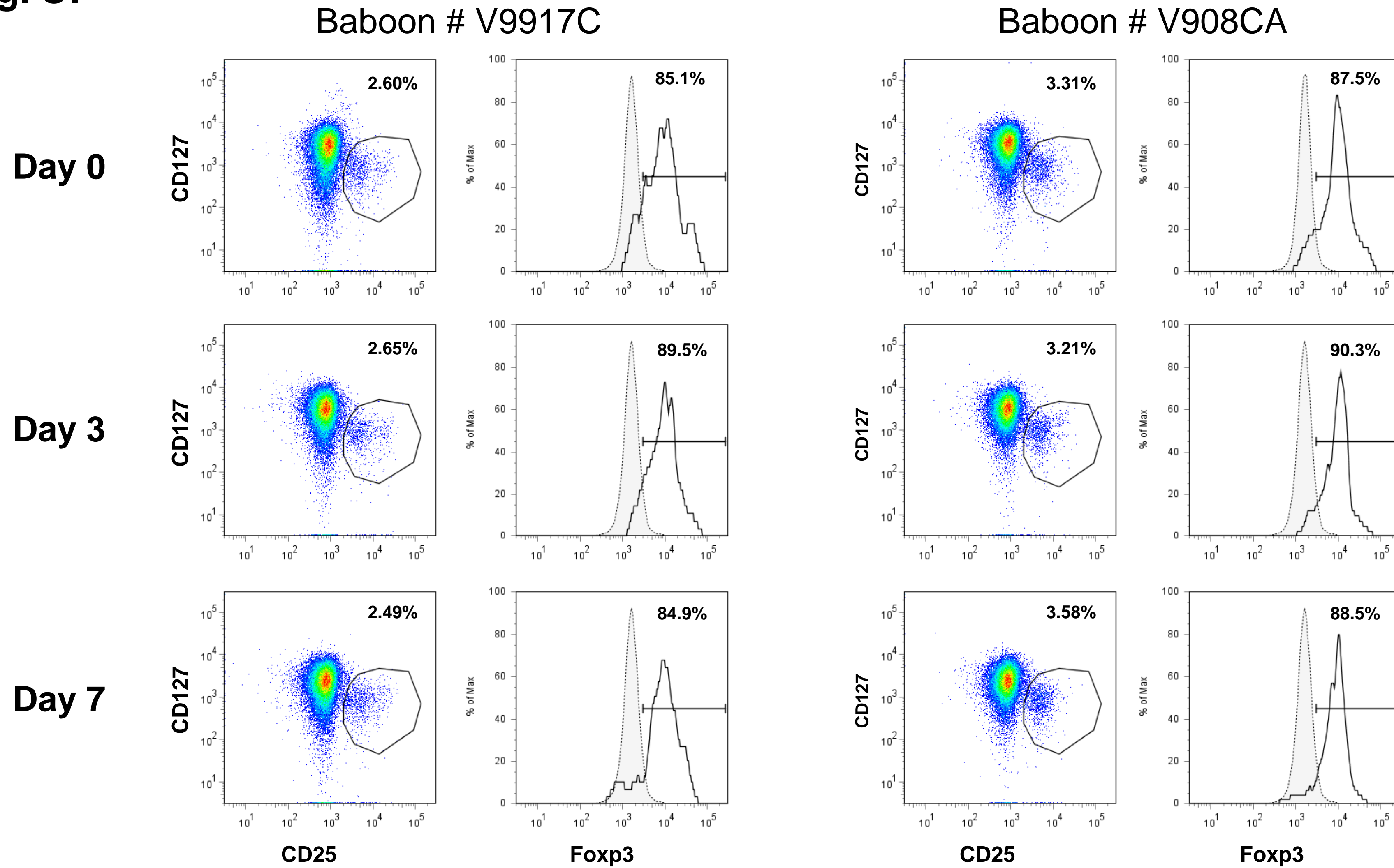
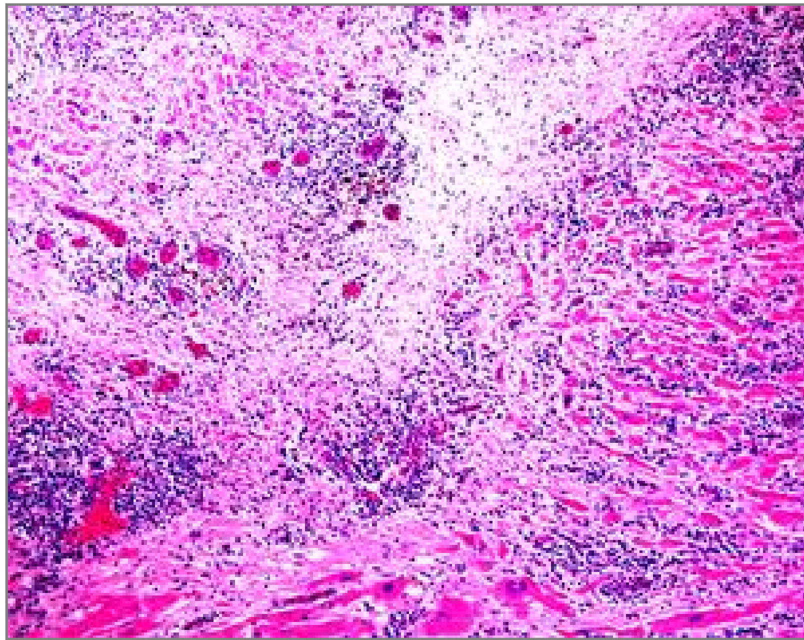


Fig. S7: Sc28AT daily injection in naïve baboons does not enhance peripheral regulatory T cells. Two baboons received daily i.v. injections of 4mg/Kg sc28AT for one week. Regulatory T cells were monitored at day 0, 3 and 7 after the first injection by flow cytometry. Data are % of CD25+ CD127 lo in CD4+ T cells and % of Foxp3+ in CD25+ CD127lo CD4+ T cells.

Fig. S8

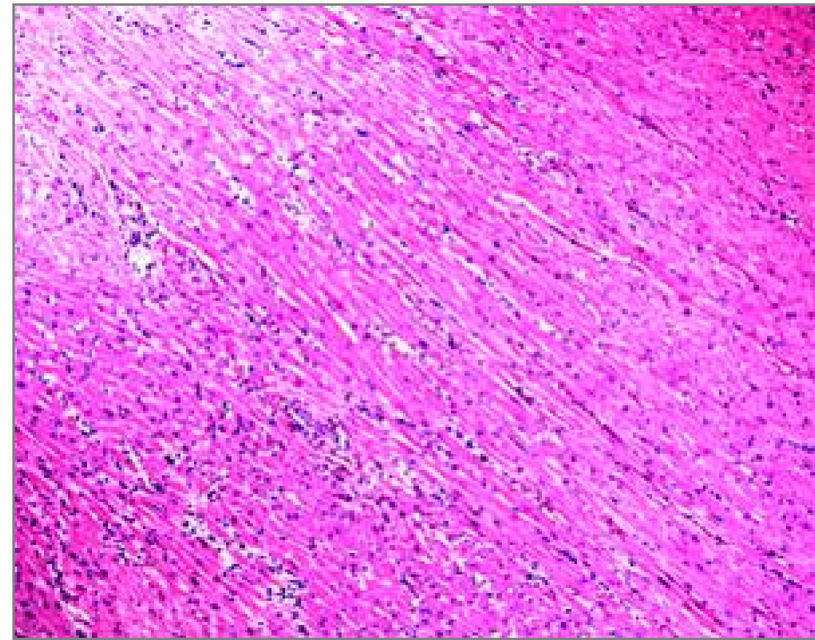
A



Explanted heart graft at day 72
CsA alone

ISHLT Grade 2

B



Explanted heart graft at day 80
CsA + sc28AT (2mg/kg)

ISHLT Grade 1A

Fig. S8: Representative histological analysis of cynomolgus monkey heart allografts at 3 months after transplantation (H&E staining).

A recipient treated with Cyclosporine A (A) shows intra-graft cellular infiltrate and edema (ISHLT Grade 2), whereas a recipient treated with Cyclosporine A + sc28AT (B) shows preserved myocardial structure (ISHLT Grade 1A).

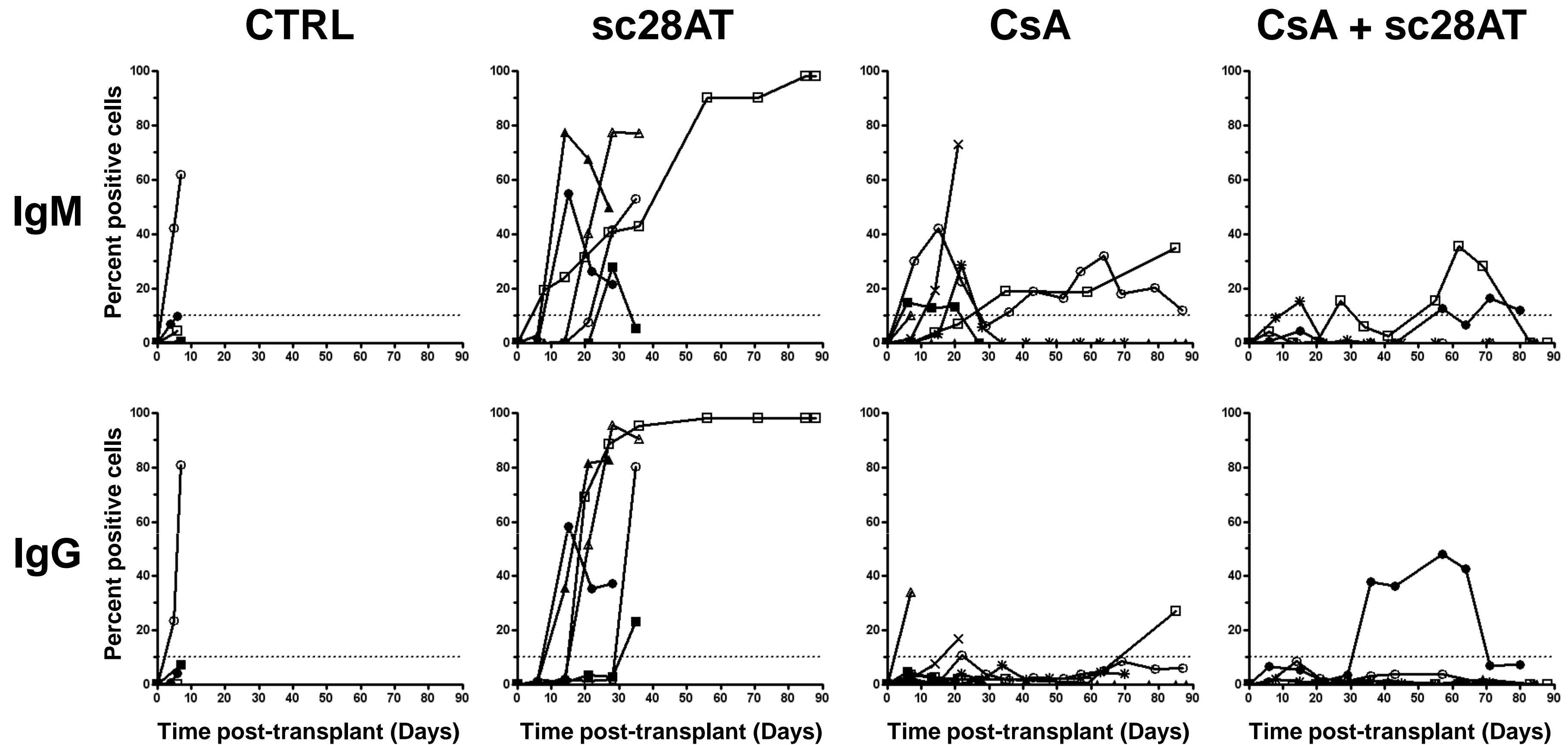


Figure S9. Long-term graft survival induced by CsA and CD28 blockade is associated with control of alloantibody production.

The presence of anti-donor IgM and IgG antibodies was measured by flow cytometry using recipient serum collected at regular intervals after transplant until graft rejection, and donor splenocytes as source of antigen. Results above 10% (dashed line) are considered positive. All animals treated with sc28AT alone (middle left panel) elaborated IgM and IgG (6/6) alloantibody. Most CsA-treated animals (middle right panel) consistently elaborated IgM (5/7) and some made IgG (3/7) alloantibody. With CsA+sc28AT (right-hand panels), consistent IgM and IgG was only detected in 3 and 1 of 5 animals treated respectively.

Fig. S10

A

Treatment	Graft survival (days)	p-value	
Subtherapeutic CsA	12, 15, 20, 38, 42, 43, 49		
sc28AT	8*, 13*, 21*, 22#, 34#, 36#		
Subtherapeutic CsA + sc28AT	21*, 33#, 55*, 62#, 72*, >88#	p=0,021 (*vs* sc28AT)	p=0,028 (*vs* sub-CsA)

B

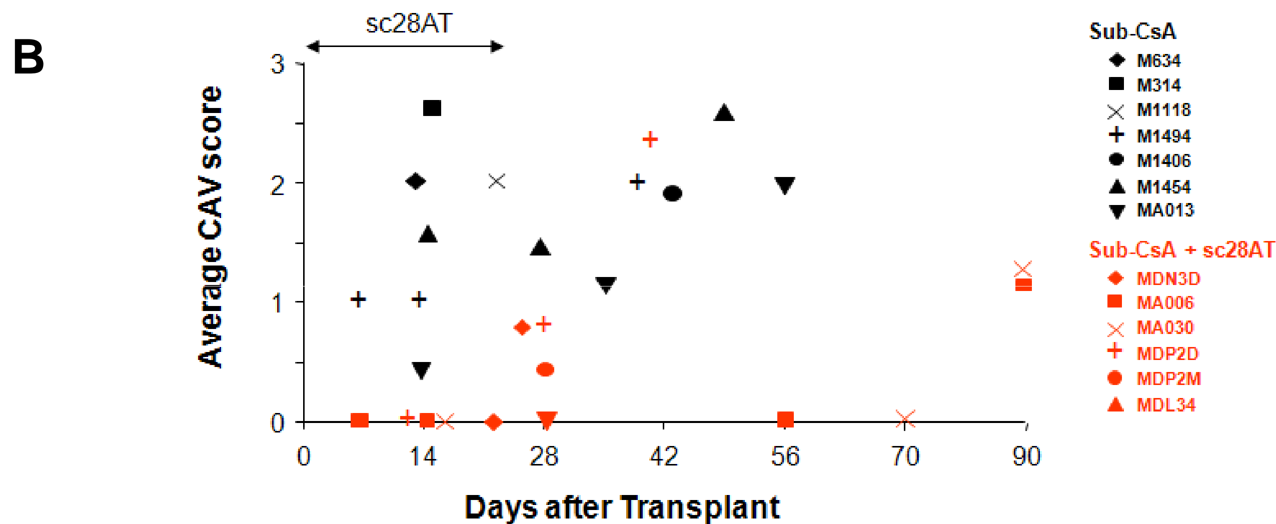


Fig. S10: Selective CD28 blockade synergized with low dose of Cyclosporine A to delay cardiac allograft rejection and cardiac allograft vasculopathy in macaques.

(A) Cardiac allograft survival for monkeys treated with low dose of Cyclosporine A (subtherapeutic CsA; n=7), treated with sc28AT monotherapy at 2 mg/Kg (*; n=3) or 10 mg/Kg (#; n=3) or low dose of Cyclosporine A + sc28AT at 2 mg/Kg (*; n=3) or 10 mg/Kg (#; n=3). (B) CAV appearance in sub-CsA or sub-CsA + sc28AT treated monkeys after transplantation. Data are individually represented at the indicated time of biopsy after transplantation and expressed as CAV severity score.

Video S1, S2, S3 online: Time lapse microscopy analyses of T cell motility *in vitro*.

A human anti-EBV T cell clone, labeled with a FURA-2 fluorescent probe, was mixed with allogeneic EBV-positive B cells and tracked over 20 min. One representative movie was recorded for each condition. Supplementary Video 1, control, addition of 10µg/ml mouse Ig. Supplementary Video 2, addition of 10µg/ml sc28AT. Supplementary Video 3, addition of 10µg/ml sc28AT + 10µg/ml anti-CTLA-4 Fab fragments. Please note the formation of stable T-APC conjugates in Videos 1 and 3 but not in video 2 (e.g. in the presence of sc28AT alone without CTLA-4 blockade). Calcium fluxes were indicated by changes in T cell fluorescence as visualized by green fluorescence in the absence of calcium flux versus yellow to red fluorescence in cells displaying calcium flux.