In vivo Prevention of Transplant Arteriosclerosis by *ex vivo* Expanded Human Regulatory T Cells

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Supplementary Figure 1

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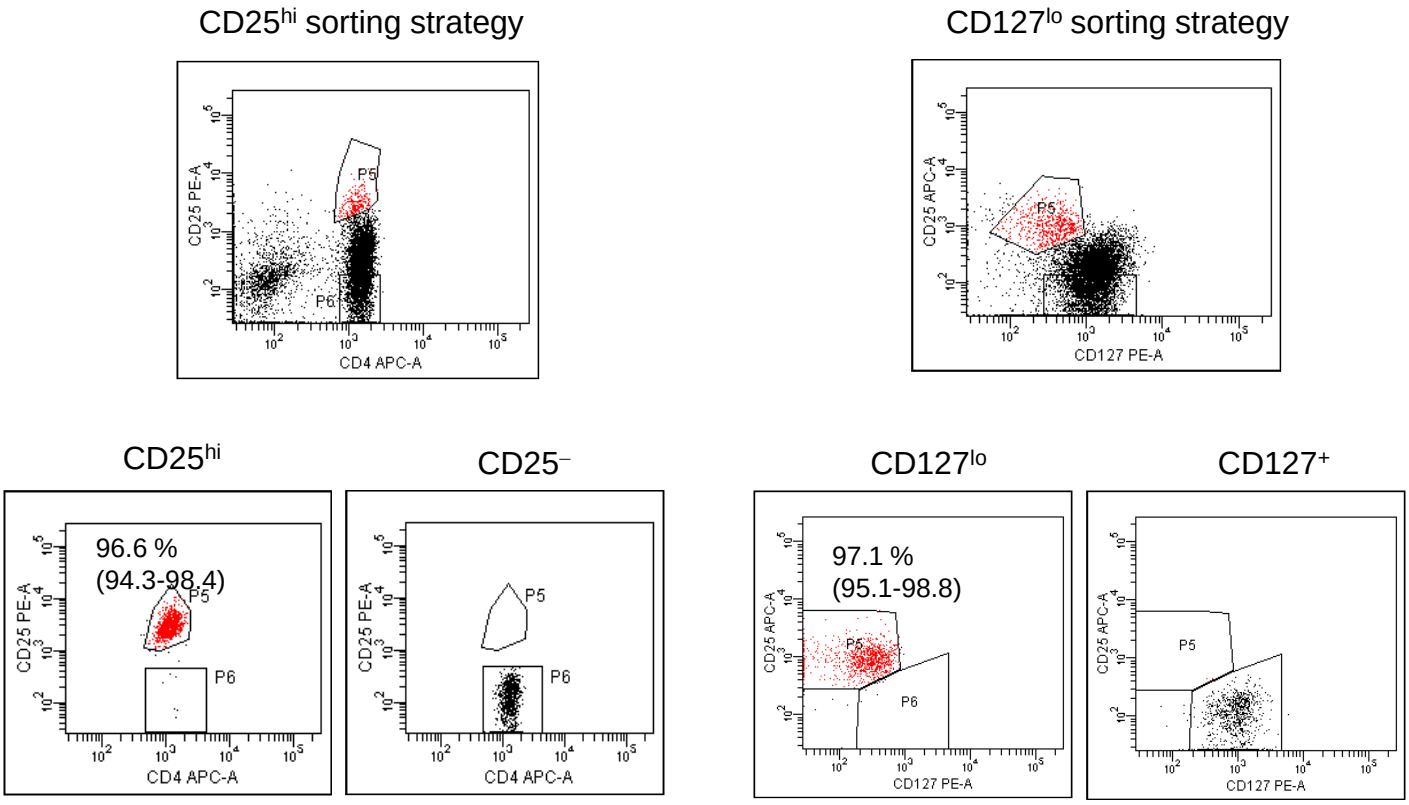

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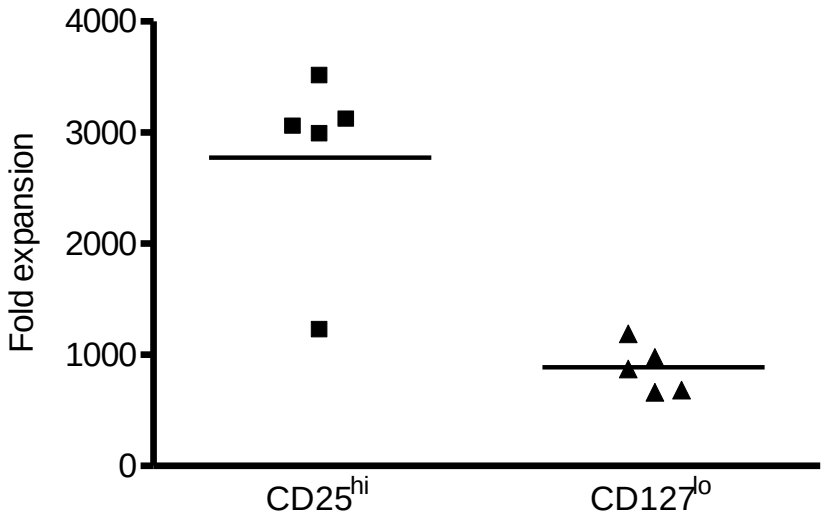

Supplementary Figure 1: see next page for figure legend

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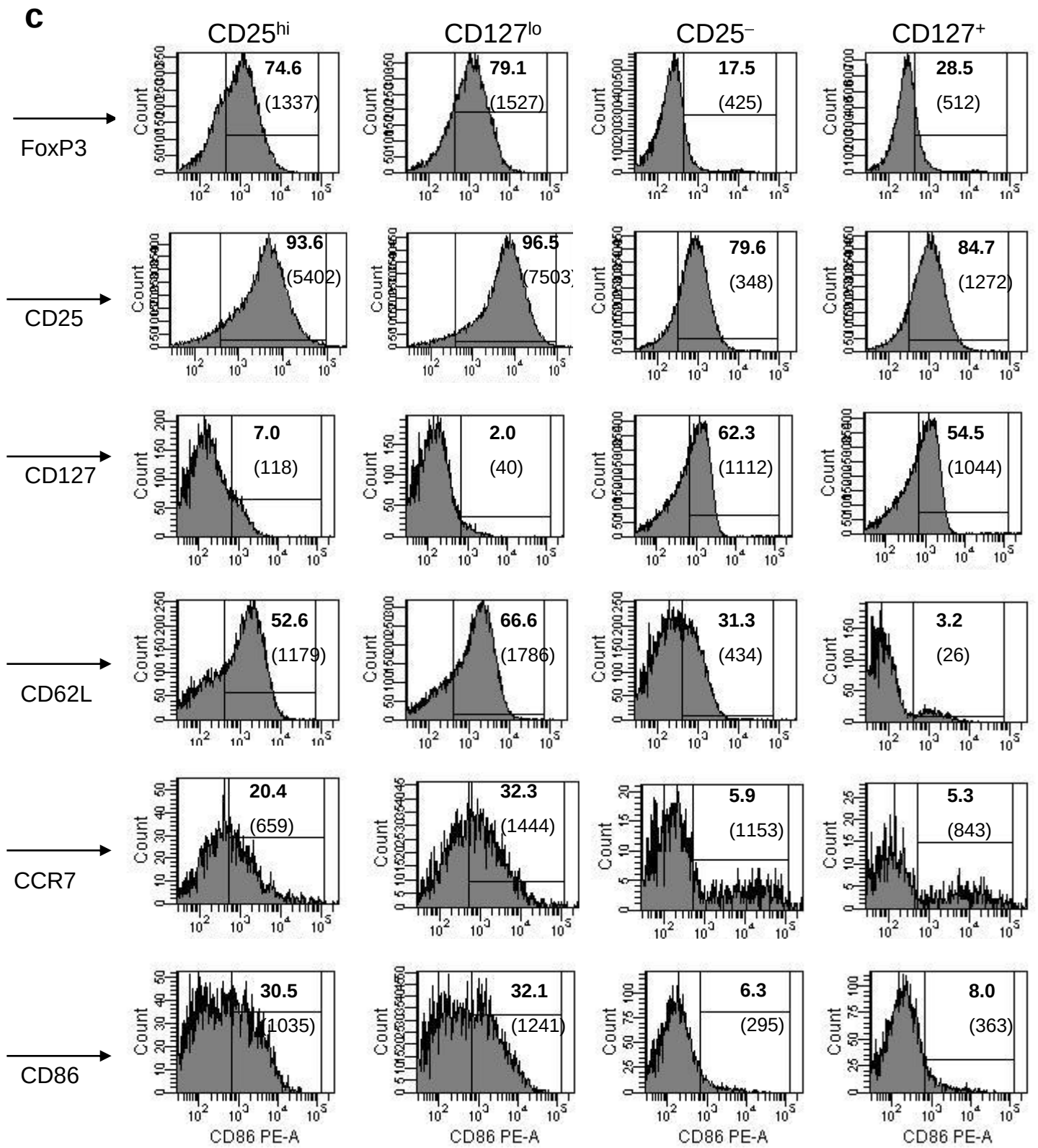
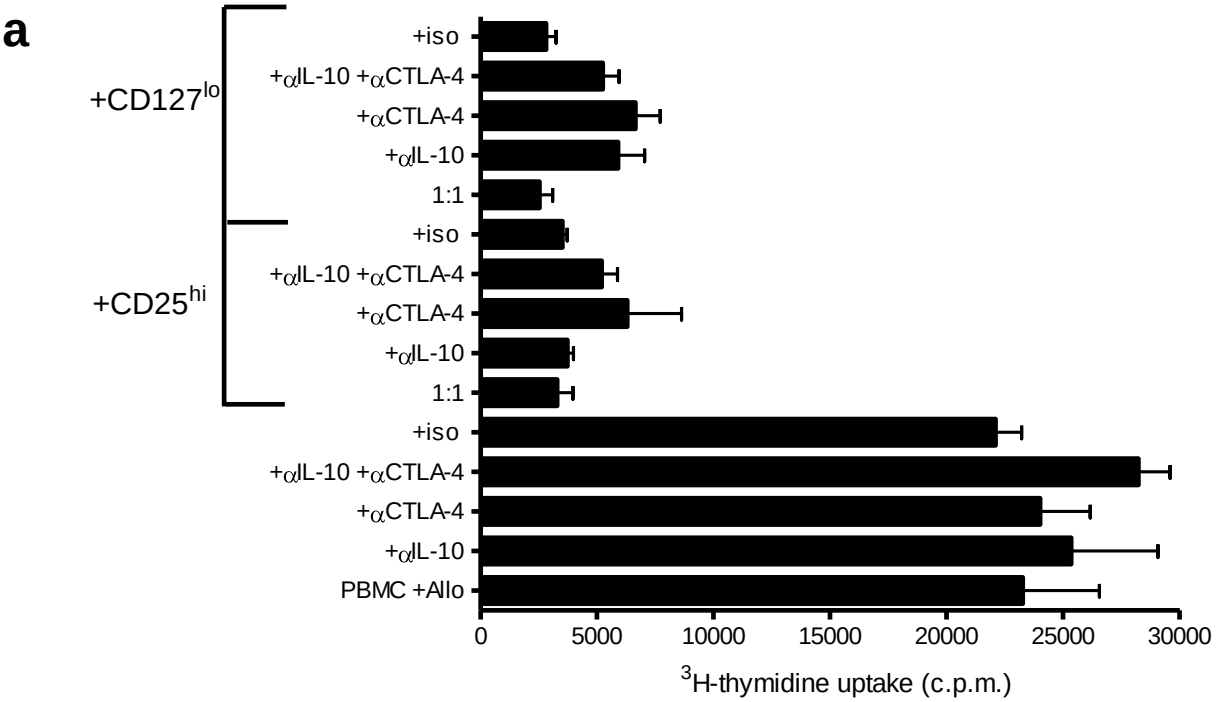
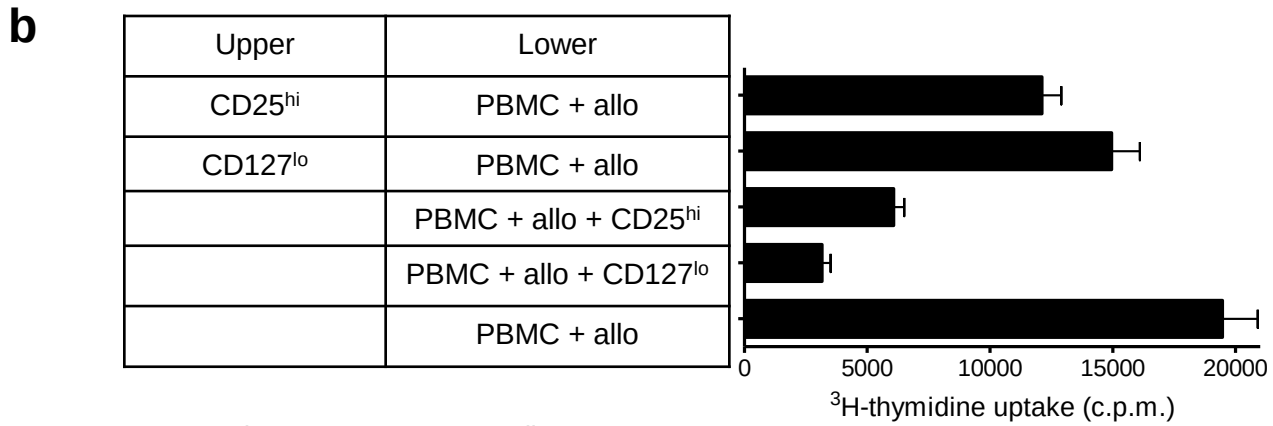
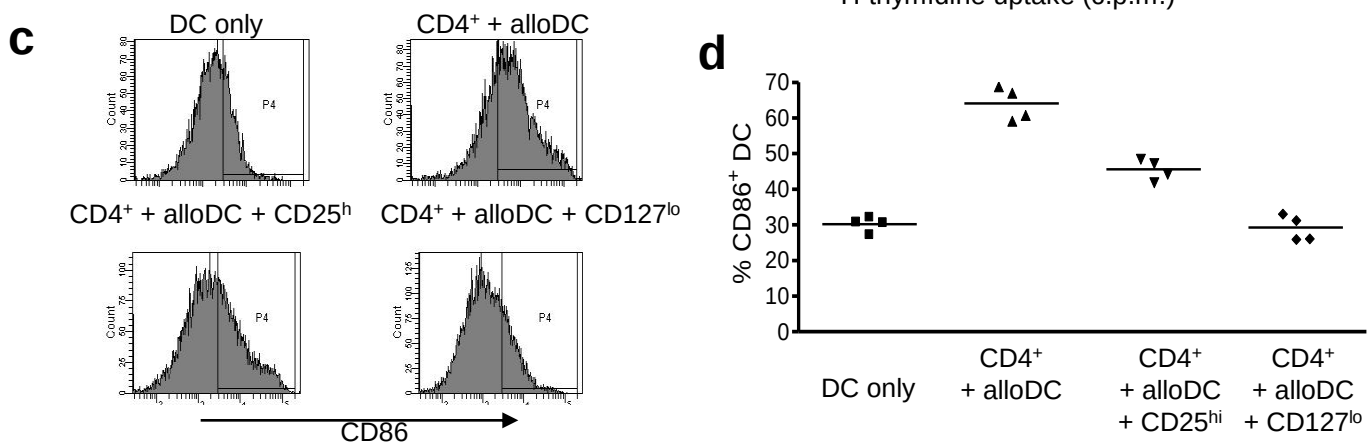


Supplementary Figure 1: T_{reg} cells sorting strategy and *ex vivo* expansion.
(**a**) Cells stained with CD4 APC and CD25 PE were isolated as $CD25^{hi}CD4^{+}$ along with $CD25^{-}CD4^{+}$ by high speed cell sorting. Additionally, cells stained with CD127 PE, CD25 APC and CD4 PerCP were sorted as $CD127^{lo}CD25^{+}CD4^{+}$ and $CD127^{+}CD25^{-}CD4^{+}$. Mean purity and range of each sorted population and range of purity achieved using PBMC from five individual blood donors are shown. (**b**) Fold expansion data at the end of the expansion protocol (d16) are shown for $CD25^{hi}$ and $CD127^{lo}$ T_{reg} cells. (**c**) Sorted and *ex vivo* expanded $CD25^{hi}$ and $CD127^{lo}$ T_{reg} cells, as well as $CD25^{-}$ and $CD127^{+}$ effector T cells, were stained for surface and intracellular antigens. A representative example of expanded cells from the same PBMC donor is shown. Percentage of positive cells is shown in bold whilst mean fluorescence intensity (MFI) is shown in brackets**.**

Supplementary Figure 2





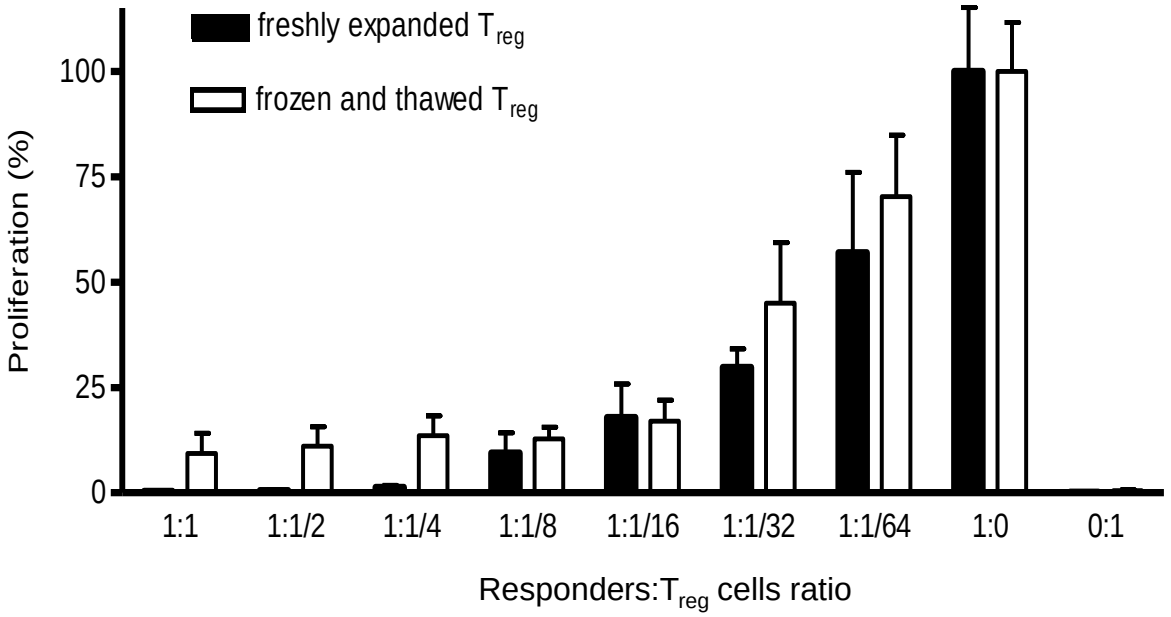


Supplementary Figure 2: Suppressive effect of expanded T regulatory cells *in vitro*.

(**a**) PBMC were co-cultured with irradiated allogeneic PBMC and expanded T_{reg} cells for 7 days at a 1:1 ratio in the presence or absence of either antibody to IL-10 (1μg ml^{-1}), antibody to CTLA-4 (1μg ml^{-1}), the combination or isotype control antibody. ^{3}H-thymidine incorporation was measured to assess proliferation. Each test consisted of 3–6 replicates. (**b**) Expanded T_{reg} cells were cultured in 1:1:1 ratio with autologous PBMC (PBMC) and irradiated allogeneic PBMC (allo) in either upper or lower Transwell chamber as indicated. Proliferation was assessed by ^{3}H-thymidine incorporation after 7 days of culture. Each test consisted of 4 replicates. (**c**,**d**) CD14 microbead-enriched monocytes were cultured *in vitro* in the presence of recombinant human IL-4 and GM-SCF. The resultant monocyte-derived DC were cultured in quadruplicates with allogeneic $CD25^{-}CD4^{+}$ cells and corresponding expanded T_{reg} cells in 1:1:1 ratio for further 3 days. CD86 expression on DC was analysed by flow cytometry. Histograms of CD86 expression (**c**) and percentage of $CD86^{+}$ DC (**d**) are presented.

Supplementary Figure 3

a



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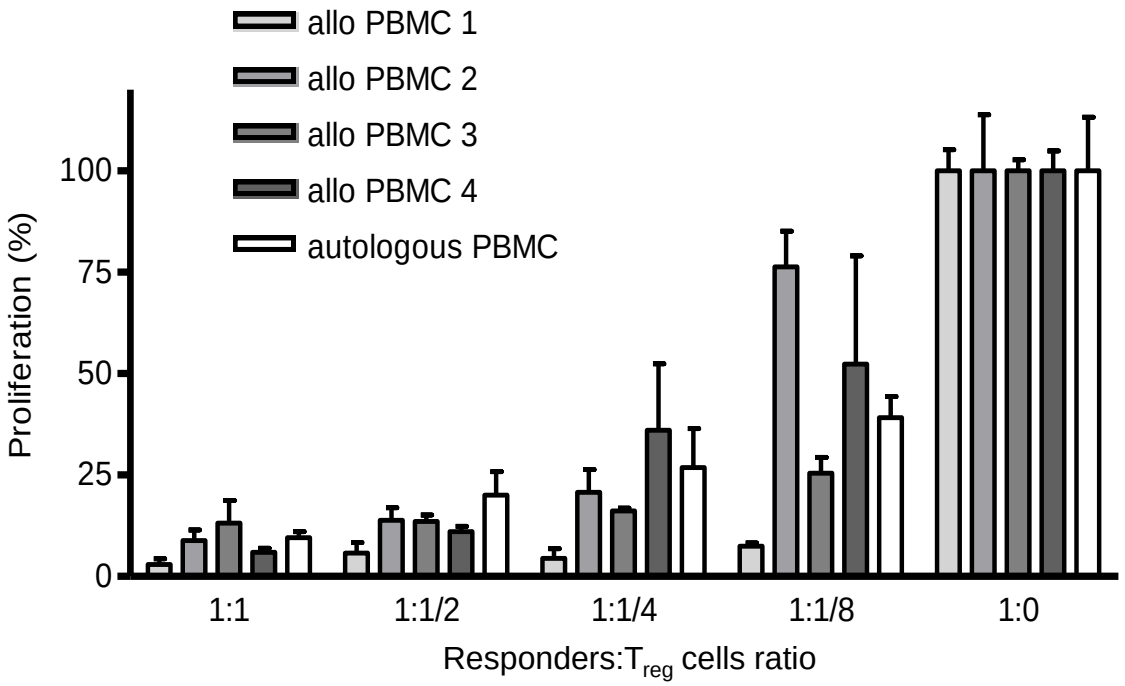
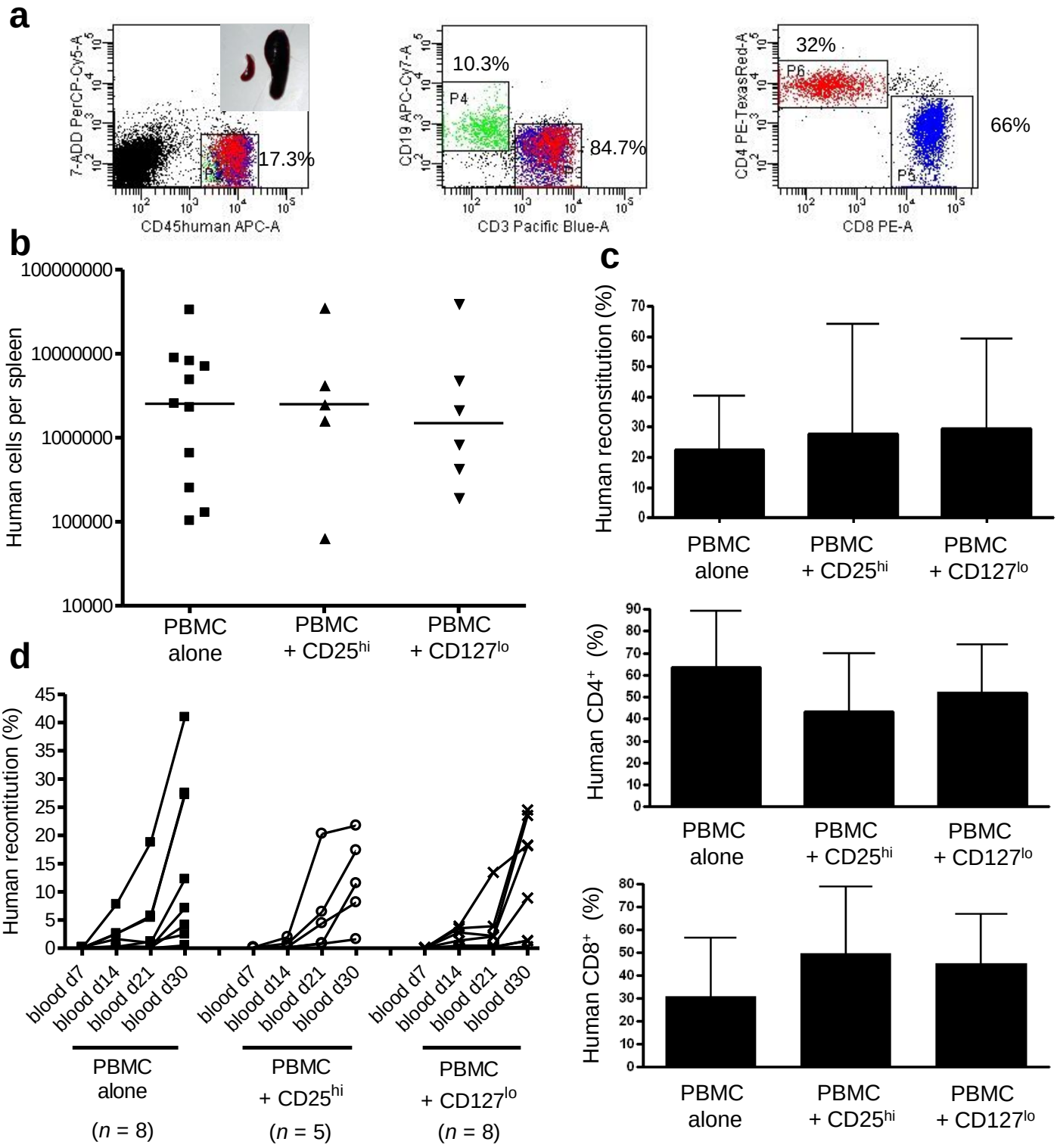


Supplementary Figure 3: Analysis of specificity and the effect of freezing and thawing on expanded T_{reg} cells function *in vitro*.

(**a**) Thawed *ex-vivo* expanded T_{reg} cells show similar *in vitro* suppression capacity as expanded cells analyzed before freezing. PBMC were co-cultured with irradiated allogeneic PBMC and serial dilutions of expanded Treg cells for 7 days. ^{3}H-thymidine incorporation was measured to assess cellular proliferation. Data shown represent percentage of proliferation compared to stimulated PBMC control. Each test consisted of 4 replicates. (**b**) Autologous PBMC or non-matched allogeneic PBMC were co-cultured with irradiated allogeneic PBMC and serial dilutions of expanded T_{reg} cells for 7 days. ^{3}H-thymidine incorporation was measured to assess cellular proliferation. Data shown represent percentage proliferation compared to that of the stimulated PBMC control. Each test consisted of 4 replicates. The data shown are representative of two independent experiments.

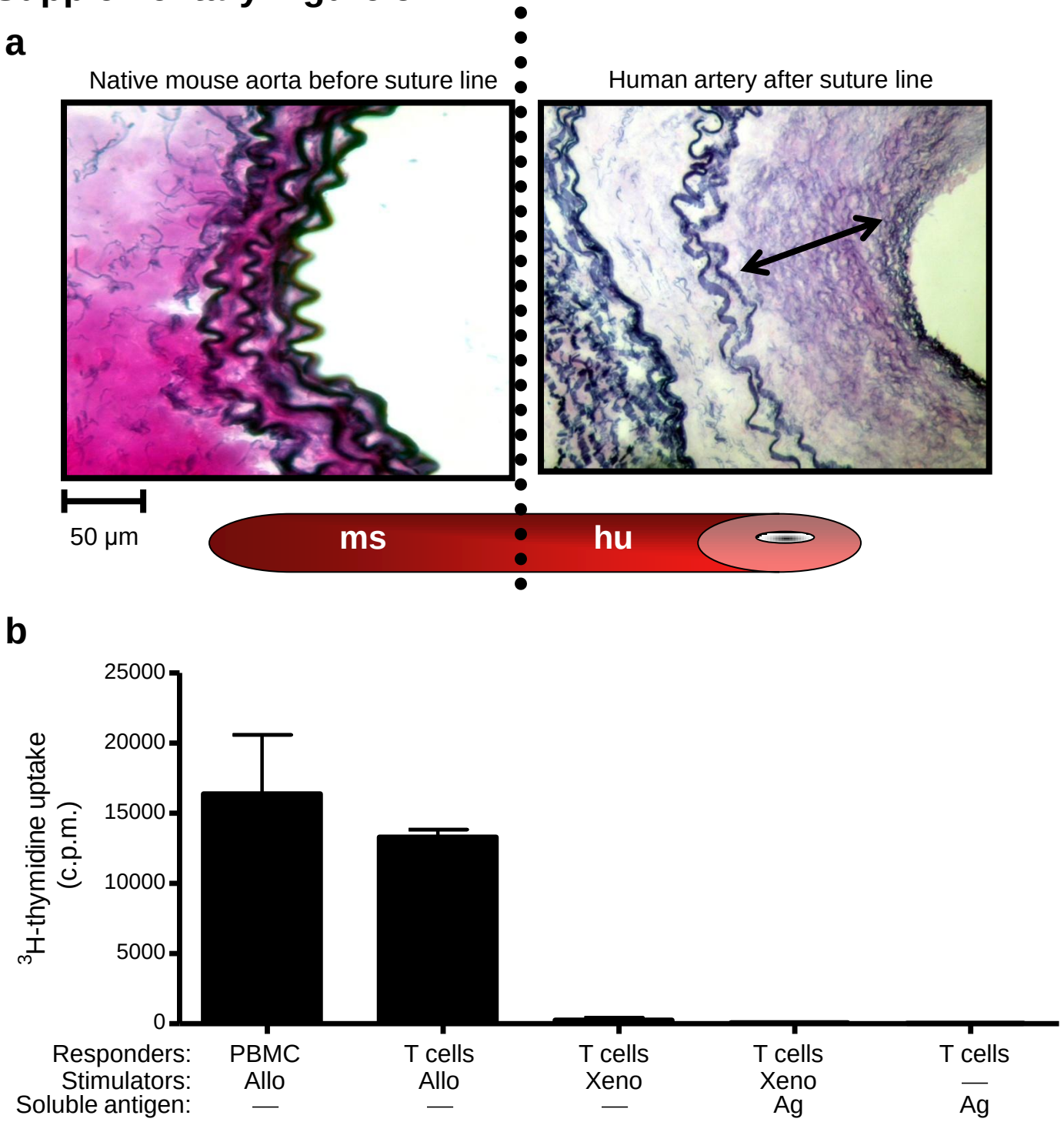
Supplementary Figure 4



Supplementary Figure 4: Human cell reconstitution of immunodeficient mouse.

(**a**) Representative plots of human PBMC-engrafted mouse splenocytes are shown. In mice reconstituted with 10^7 human PBMC, human cell subsets were gated on live human CD45$^+$ cells isolated from spleens of inoculated mice 30 days after human cell transfer. Engrafted spleens showed splenomegaly and marked hepatization (**a** insert). (**b**) Number of human cells (CD45$^+$) in a spleen of reconstituted mice on day 30. (**c**) The percentage of human CD45$^+$, CD4$^+$, or CD8$^+$ reconstitution in the spleen as detected by FACS analysis. (**d**) Kinetics of repopulation with human leukocytes was measured in the blood on day 7, 14, 21 and 30 after adoptive transfer of human PBMC. Each line represents one mice, the percentage of human CD45$^+$ cells within live leukocyte gate is shown. (**a**–**c**) Samples from mice transplanted with human artery and inoculated with human PBMC, (**d**) samples from untransplanted mice inoculated with human PBMC.

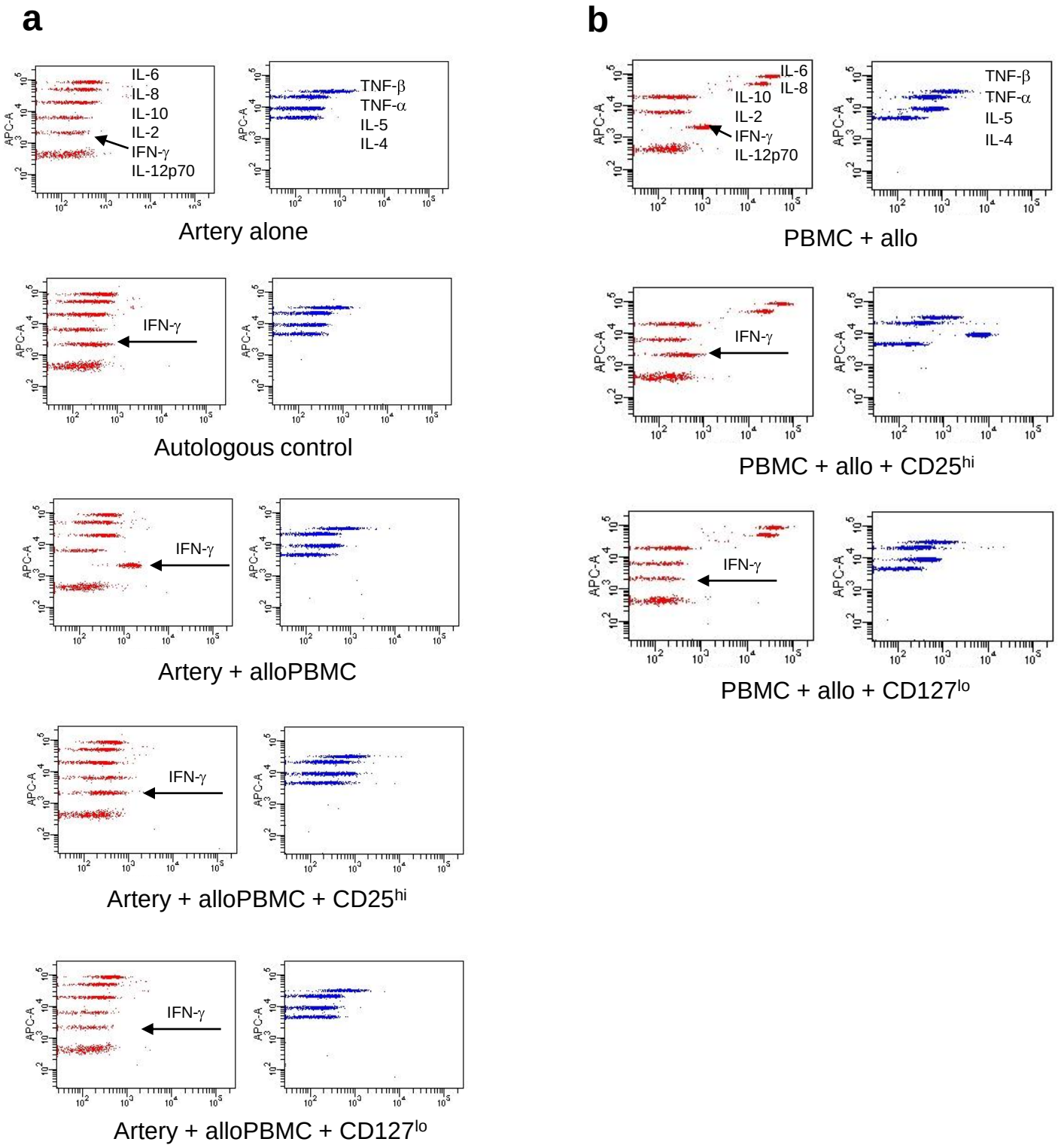
Supplementary Figure 5



Supplementary Figure 5: Human allo-responses predominate over xeno-responses both *in vitro* and *in vivo.*

(**a**) Representative EvG staining (*n* = 6) shows no TA in the native mouse aorta when compared to marked intimal proliferation (arrow) just after the suture line in the human arterial segment. (**b**) PBMC or purified human T cells were cultured with irradiated allogeneic PBMC (allo) or BALB/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ splenocytes (xeno; direct pathway) or BALB/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ splenocytes presenting sonicated human antigen (xeno + Ag; indirect pathway). ^{3}H-thymidine incorporation was measured. Error bars represented as standard deviations. *In vitro* results shown are representative of three independent experiments. ms = mouse; hu = human

Supplementary Figure 6

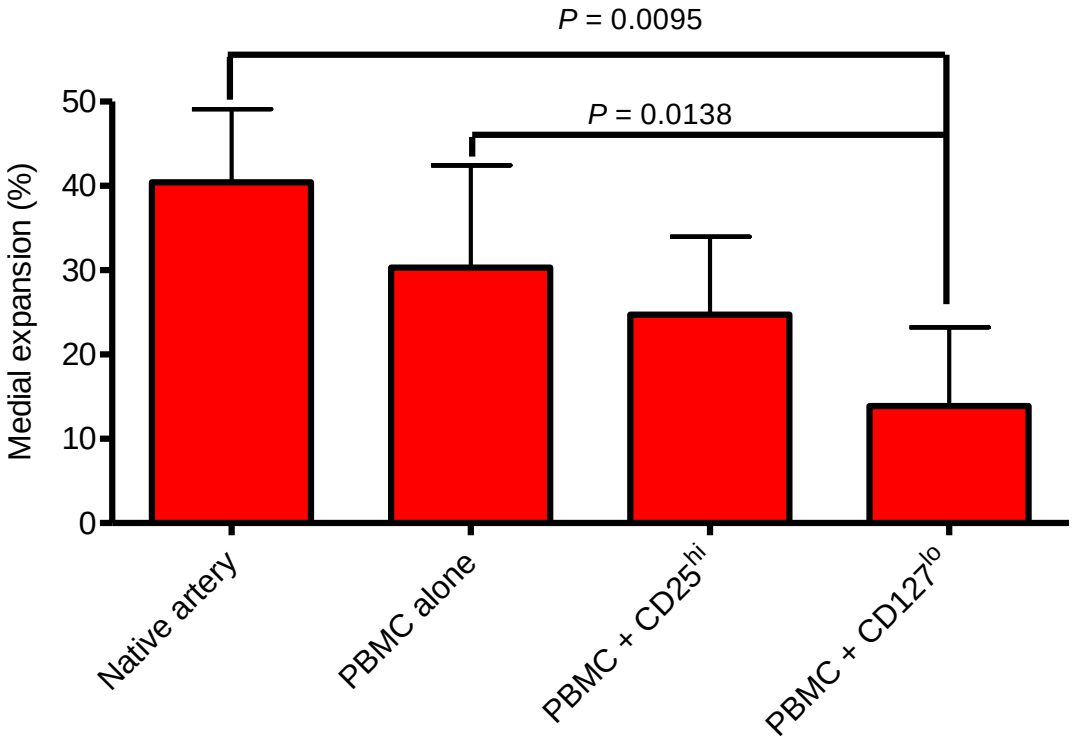


Supplementary Figure 6: Analysis of *in vivo* and *in vitro* cytokine release in the presence of T regulatory cells.

(**a**) Cytokine bead array was performed on serum samples harvested from mice transplanted with an arterial graft and reconstituted with allogeneic PBMC in the presence or absence of *ex vivo* expanded $CD25^{hi}$ or $CD127^{lo}$ T_{reg} cells. (**b**) Cytokine bead array performed on supernatants taken after 5 days of culture of PBMC stimulated with irradiated allogeneic PBMC in the presence or absence of T_{reg} cells. Representative dot plots from at least 3 independent experiments are shown.

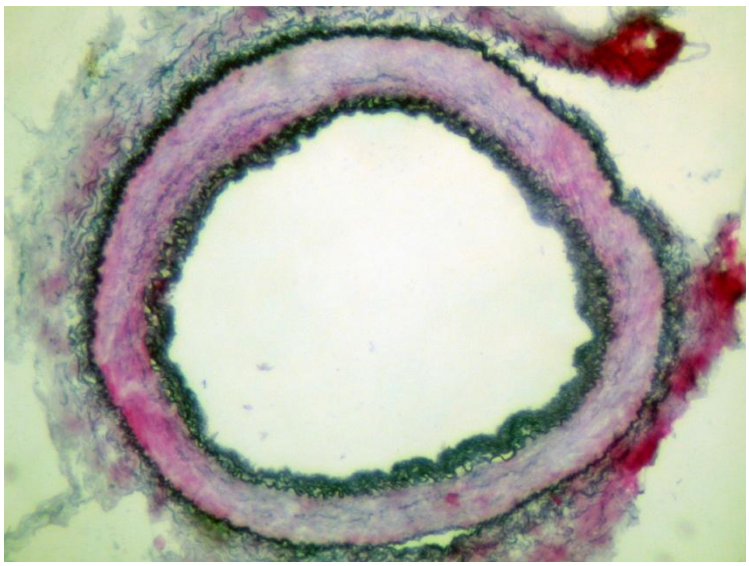
Supplementary Figure 7

a



b

Native artery



Supplementary Figure 7: Changes in medial area of transplanted human arteries.

(**a**) Medial area of untransplanted (native) human arteries and grafts procured 30 days after transplantation ($n \geq 5$ in all groups). (**b**) A representative photomicrograph of a native untransplanted human artery is shown.

Supplementary Table 1: HLA typing

Experimental Groups	PBMC Donor	IMA Sidebranch Donor
1	A : 3, 24 B: 7,38 DR*: 13,15	A: 1, 2 B: 8, 44 DR: 17,11
2	A:11, 2 B: **60**, 27 DR*: 8, 10	A: 32, 3 B: **60** DR: 4
3	A:29, 26 B:44, 55 DR*: 7, 11	A: 24, 25 B: 27, 18 DR*: 16, 15
4	A:1, **2** B:39, 60 DR*: 13, 8	A: **2**, 31 B: 27, 51 DR*: 4
5	A:32, 3 B: 18, 56 DR*: 1, 11	A: 11, 26 B: 44, 35 DR*: 103, 14

*HLA – DR molecular type analysed from locus DRB1
IMA= Internal Mammary Artery