EXPANDED METHODS

Tem-EC Co-cultures

$CD4^+$ T cells were isolated by positive selection with antibody-conjugated magnetic beads (Invitrogen, Carlsbad, CA) and $CD45RO^+$ Tem were enriched (>95%) by negative selection using immunomagnetic beads to deplete $CD45RA^+$ naïve T cells, $CCR7^+$ central memory T cells, and HLA-DR^+ activated T cells and contaminating monocytes, B cells, and dendritic cells. EC were pretreated with IFN-γ (Invitrogen) at 50 ng/mL for 72 hr, washed, then plated together with $CD4^+$ Tem at $1x10^5$ and $3x10^6$ cells/2 cm^2, respectively in flat-bottomed, 24-well plates (BD Biosciences, San Jose, CA) in RPMI 1640 medium (Invitrogen) and treated with neutralizing monoclonal antibodies to TGF-β1/-β2/-β3 (clone 1D11, R&D Systems) and LAP-β1 (clone 27235, R&D Systems) at 10 μg/mL each, or irrelevant, isotype-matched antibody at 20 μg/mL. In experiments using the transwell system, $2x10^5$ untreated EC were plated on gelatin-coated 0.4 μm pore size membrane inserts (BD Biosciences) above co-cultures of $CD4^+$ Tem with either untreated or IFN-γ-pretreated EC.

Animals

C.B-17 SCID/beige mice (Taconic) were housed in microisolator cages and fed sterilized food and water. Serum IgG levels of 6-8 wk old animals were determined by ELISA using reagents from Fisher Scientific (Pittsburgh, PA). SCID/beige mice were considered “leaky” at IgG levels greater than 1 μg/ml and excluded from experimental use.

PBMC Reconstitution

Human PBMC, stored in 20% DMSO at -196 °C for up to 12 months, were thawed and washed before use. Viability after thawing was >95% as assessed by trypan blue exclusion.

SCID/beige mice were reconstituted with 0.5-3×10^8 human PBMC by i.p. inoculation at 1 wk postoperatively. The number of circulating human T cells was evaluated by flow cytometry. In brief, heparinized retro-orbital venous blood samples were obtained at 2 and 4 wk after adoptive transfer of human PBMC and the erythrocytes were lysed in ammonium chloride buffer. The leukocytes were incubated with FITC-conjugated mouse anti-human CD3 (Beckman Coulter, Miami, FL) and PE-conjugated rat anti-mouse CD45 (BD Biosciences) monoclonal antibodies and then analyzed using a FACS Calibur. Our definition of successful reconstitution was the detection of a discrete population of circulating human $CD3^+$ T cells with a frequency >0.1% of mouse leukocytes and was achieved in all mice in this study (no animals were excluded from analysis for failure to reconstitute).

Histology and Morphometry

The animals were exsanguinated at 3 or 5 wk post-op (2 or 4 wk after PBMC inoculation) by saline perfusion via the left ventricle at a pressure of 100 mm Hg. Artery grafts were excised, embedded in OCT compound, and 5 μm-thick transverse sections were stained with H&E or EVG using standard techniques to identify the vascular layers. Morphometric evaluations of microscopic images were performed using ImageJ software (http://rsbweb.nih.gov/ij/). In brief, the perimeters of the lumen, internal elastic lamina, and external elastic lamina were outlined and the intima area (between the lumen and internal elastic lamina perimeters) and vessel area (within the external elastic lamina perimeter) of 3-5 cross-sections, 150 μm apart, were assessed and averaged for each graft.

I**mmunofluorescence Microscopy**

Immunolabeling of 5 μm-thick, OCT-embedded artery sections was performed with the following primary antibodies: mouse anti-human CD31 (clone JC70A, DAKO, Carpinteria, CA), FITC-conjugated anti-α-smooth muscle actin (clone 1A4, Sigma-Aldrich, St. Louis, MO), mouse anti-human CD45RO (clone UCHL1, DAKO), and mouse anti-human LAP-β1 (clone 27235, R&D Systems). Isotype-matched, irrelevant IgG was used as negative controls. Detection of non-conjugated primary antibodies was visualized with Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen). The specimens were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Images were acquired using an Axiovert 200M microscopy system and processed using AxioVision 4.6 software (Carl Zeiss MicroImaging, Thornwood, NY). After merging the cell lineage marker and nuclei images, the number of luminal $CD31^+$ cells, medial SMA^+ cells, and intimal or medial $CD45RO^+$ cells were counted and averaged from 3-5 cross-sections, 150 μm apart, per graft.

Flow Cytometry

For expression of surface molecules, the cells were labeled with PE-conjugated, mouse anti-human LAP-β1 (clone 27232, R&D Systems), PE-conjugated, mouse anti-human TGF-β RII (clone 25508, R&D Systems), FITC-conjugated, mouse anti-human HLA-DR (clone B8.12.2 Beckman Coulter), or fluorescence-conjugated, isotype-matched, irrelevant IgG, and analyzed using a FACS Calibur (BD Biosciences). For intracellular cytokine analysis, the cells were collected after 24 hr of co-culture, stimulated with PMA at 10 ng/mL and ionomycin at 1 μM for 30 min in the presence of brefeldin A at 10 μg/mL, labeled with APC-conjugated, mouse anti-human CD4 (clone OKT4, Beckman Coulter), fixed with 4% paraformaldehyde and permeabilized with 0.1% w/v saponin, then labeled with PE-conjugated, mouse anti-human IL-17A (clone eBio64DEC17, eBioscience, San Diego, CA) and FITC-conjugated, mouse anti-

human IFN-γ (clone 4S.B3, eBioscience) or fluorescence-conjugated, isotype-matched, irrelevant IgG, and analyzed using a LSR II (BD Biosciences).

ELISA

Supernatant and plasma levels of cytokines were determined using sandwich ELISA Duoset kits (R&D Systems) for TGF-β1, IL-5, IL-10, IL-17, and IFN-γ according to the manufacturer's instructions.

Real-time Quantitative RT-PCR

Serial sections of OCT-embedded arteries or centrifuged cell pellets were immersed in RLT lysis buffer (QIAGEN, Valencia, CA), vigorously vortexed, and total RNA was isolated using RNeasy mini kits (QIAGEN) according to the manufacturer's protocol. RT with random hexamer and oligo-dT primers was performed according to the Multiscribe RT system protocol (Applied Biosystems, Foster City, CA). RT-PCR reactions were prepared with TaqMan PCR Master Mix and predeveloped assay reagents from Applied Biosystems. Samples were analyzed on an iCycler (Bio-Rad Laboratories). RNA samples processed without the RT enzyme were used as negative controls. The expression level of each transcript was normalized to that of GAPDH.

Table S1. Expression of cytokine transcripts in artery grafts pooled from independent experiments[a].

Cytokine	IgG	TGF-β Ab	*P* value
IFN-γ/GAPDH ($x10^{-2}$)	3.138±1.039	5.918±1.210	0.0002
IL-4/GAPDH ($x10^{-5}$)	1.154±0.597	3.959±2.444	0.7646
IL-10/GAPDH ($x10^{-3}$)	2.383±0.882	1.702±0.686	0.7744
IL-17/GAPDH ($x10^{-4}$)	2.313±1.466	0.542±0.410	0.1055
IFN-γ/CD3ε ($x10^{-1}$)	2.182±0.517	5.094±1.972	0.0335
IL-4/CD3ε ($x10^{-4}$)	0.832±0.598	1.209±0.691	0.5703
IL-10/CD3ε ($x10^{-2}$)	6.685±3.425	1.533±0.723	0.3755
IL-17/CD3ε ($x10^{-4}$)	9.322±5.530	1.670±1.088	0.1309

[a]Data were pooled from the results shown in Fig. 5, 6, and S1. Human coronary arteries were grafted to SCID/beige mice, reconstituted with 0.5-3$x10^{8}$ allogeneic human PBMC at 1 wk post-op, and the recipients were treated with non-immune IgG or TGF-β antibody 3x per wk. The artery grafts were removed at 2 or 4 wk after PBMC inoculation. T cell cytokine transcripts were measured by quantitative RT-PCR and normalized to GAPDH or CD3ε. Data are means±SE (*n*=21-23), are non-Gaussian in distribution, and comparisons are by Wilcoxon signed rank test.

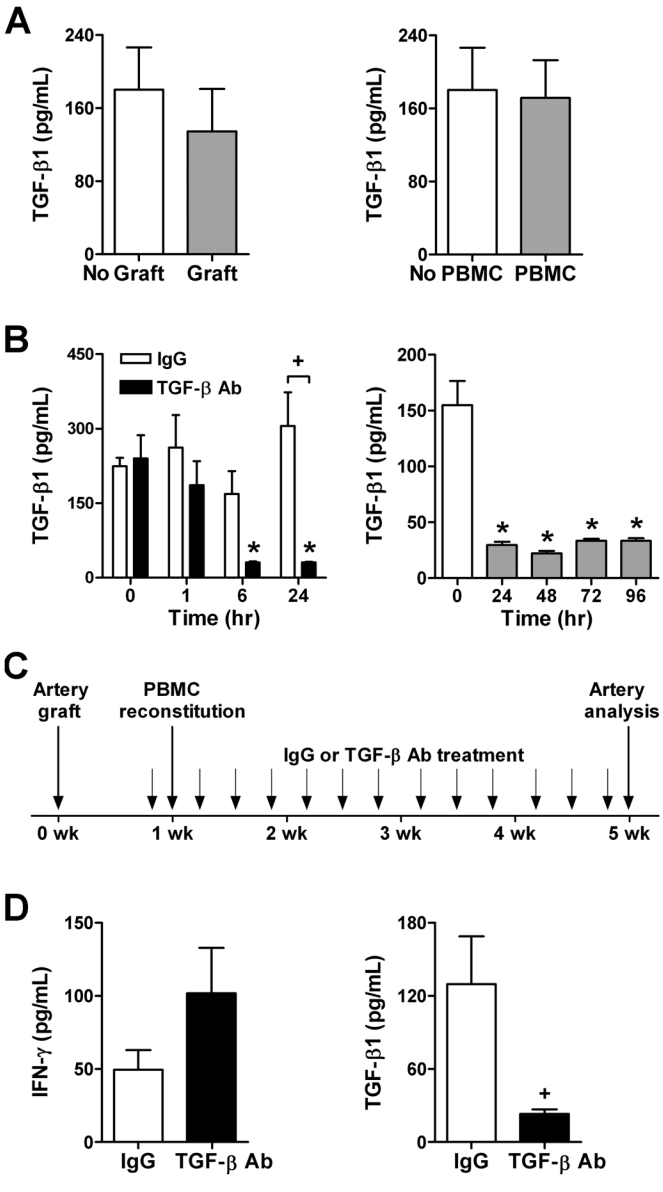
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Effects of TGF-β antibody therapy in humanized mice (*A*) TGF-β1 was measured by ELISA in the plasma of SCID/beige mice either untreated, grafted with human coronary arteries, or reconstituted with 1.5-3x10^8 human PBMC (*n*=6-10). (*B*) Plasma levels of TGF-β1 in SCID/beige mice at 1-24 hr or 24-96 hr after a single dose of antibody at 250 μg s.c. (*n*=5-6). **P*<0.05, Post-treatment vs. Pre-treatment, repeated measures ANOVA. $^+$*P*<0.05, TGF-β Ab vs. IgG, one-way ANOVA. (*C*) Timeline of multiple antibody dosing to artery grafting, PBMC reconstitution, and artery procurement. (*D*) Frequency of human $CD3^+$ vs. mouse $CD45^+$ circulating cells at 3 and 5 wk post-op (or 2 and 4 wk after adoptive transfer of 1.5-3x10^8 human PBMC) in SCID/beige mice treated with IgG or TGF-β antibody at 250 μg s.c., 3x per wk, from 1-5 wk post-op (*n*=13). (*E*) Plasma levels of human IFN-γ or active TGF-β1 in these animals (*n*=10). $^+$*P*<0.05, TGF-β Ab vs. IgG, paired t test.

Figure S2. TGF-β antibody therapy does not modulate the morphology of non-rejecting artery grafts. Human coronary arteries were interposed into the aorta of SCID-beige mice that did not receive PBMC. The animals received no treatment or were treated with IgG or TGF-β antibody at 250 μg s.c., 3x per wk, from 1-5 wk post-op (*n*=3 from 3 artery donors). Graft sections were stained with (*A*) H&E or (*B*) EVG at 5 wk post-op and representative photomicrographs are shown (bar, 300 μm). Intima and total vessel areas were calculated from EVG-stained graft sections. The artery grafts were also analyzed by immunofluorescence microscopy using (*C*) PE-labeled anti-CD31 (red color), (*D*) FITC-labeled anti-smooth muscle α-actin (SMA) (green color), and (*E*) PE-labeled anti-CD45RO (red color). The internal elastic lamina is visible due to auto-fluorescence (green color). Representative photomicrographs are shown at high magnification (bar, 50 μm). The number of $CD31^+$ luminal cells and SMA^+

medial cells were counted per cross (x)-section of the grafts. There were no infiltrating $CD45RO^{+}$ cells detected in the absence of PBMC reconstitution and counting of memory T cells was not done (N.D.).

Figure S1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80
0
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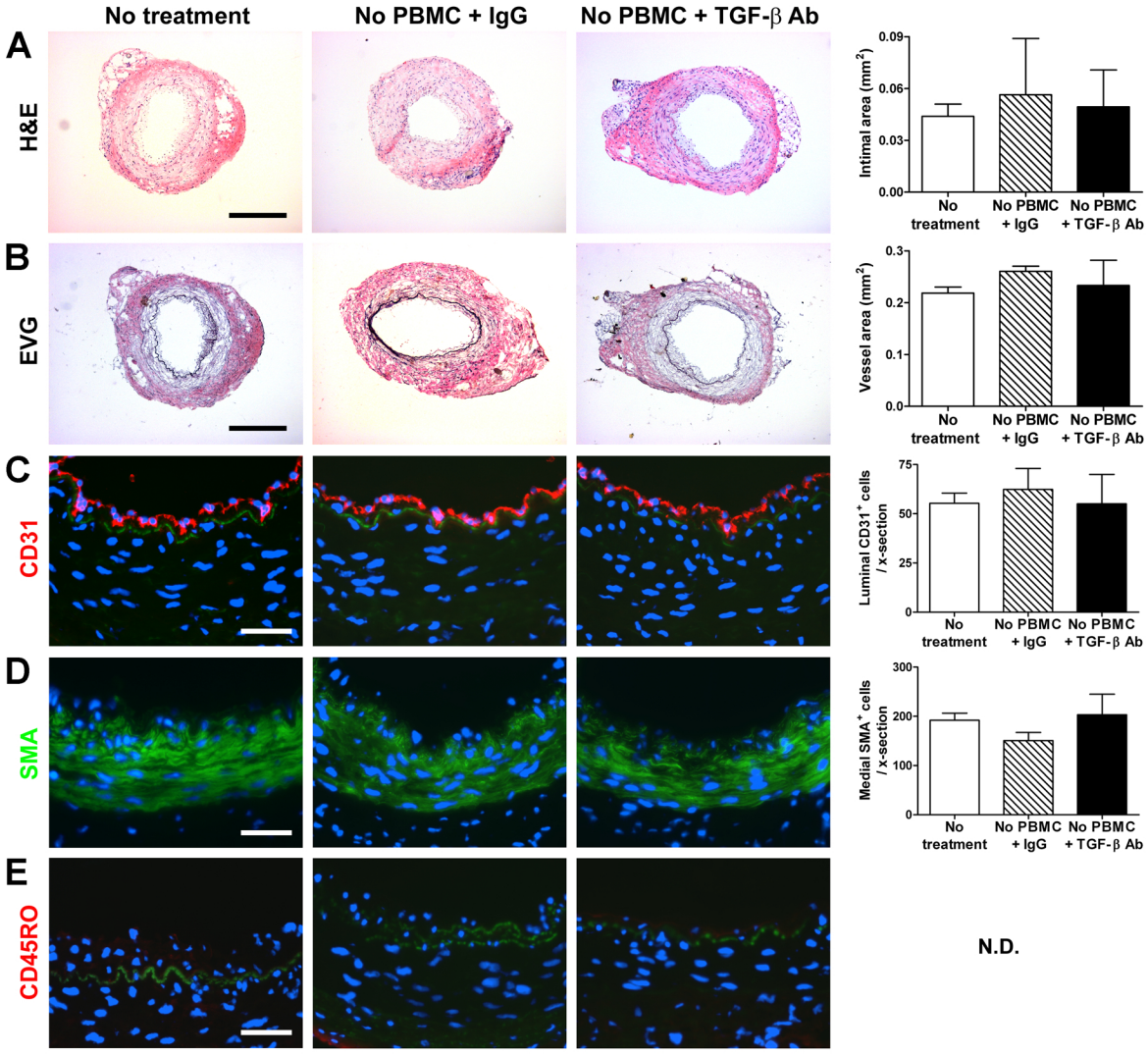
Graft
No PBMC
PBMC
B
IgG
TGF-β Ab
450
300
150
0
+
*
0
1
6
24
Time (hr)
200
150
100
50
0
24
48
72
96
C
Artery graft
PBMC reconstitution
IgG or TGF-β Ab treatment
Artery analysis
0 wk
1 wk
2 wk
3 wk
4 wk
5 wk
D
IFN-γ (pg/mL)
150
100
50
180
120
60

Figure S2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No PBMC + TGF-β Ab
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H&E
B
EVG
C
CD31
D
SMA
E
CD45RO
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0.09
0.06
0.03
0.00
No treatment
No PBMC + IgG
No PBMC + TGF-β Ab
Vessel area (mm2)
0.3
0.2
0.1
0.0
Luminal CD31+ cells / x-section
75
50
25
0
Medial SMA+ cells / x-section
300
200
100
0
N.D.