

# Interferon- $\gamma$ plays a nonredundant role in mediating T cell-dependent outward vascular remodeling of allogeneic human coronary arteries<sup>1</sup>

YINONG WANG,\* WILLIAM R. BURNS,\* PAUL C. Y. TANG,\* TAI YI,\*  
JEFFREY S. SCHECHNER,<sup>†</sup> HANS-GUENTER ZERWES,<sup>‡</sup> WILLIAM C. SESSA,<sup>§</sup>  
MARC I. LORBER,\* JORDAN S. POBER,<sup>†,¶,||</sup> AND GEORGE TELLIDES\*<sup>2</sup>

Interdepartmental Program in Vascular Biology and Transplantation, and the Departments of \*Surgery, <sup>†</sup>Dermatology, <sup>§</sup>Pharmacology, <sup>¶</sup>Immunobiology, and <sup>||</sup>Pathology, Yale University School of Medicine, New Haven, Connecticut, USA; and <sup>‡</sup>Transplantation & Immunology Research, Novartis Pharma AG, Novartis Pharma AG, CH-4002 Basel, Switzerland

## SPECIFIC AIMS

We investigated the cellular and molecular mechanisms of T cell-mediated outward vascular remodeling using a human/mouse chimeric model.

## PRINCIPAL FINDINGS

### 1. In vivo model of allogeneic human coronary artery rejection

Human epicardial coronary arteries (6–8 mm long and 0.5–1 mm in diameter) were bisected and microsurgically interposed into the aortae of paired severe combined immunodeficient (SCID)/beige mice. One animal received an adoptive transfer of  $3 \times 10^8$  allogeneic (to the artery) human peripheral blood mononuclear cells (PBMCs) i.p. 1 wk postoperatively and the other recipient was untreated. The adjacent human arterial segments were used as matched investigational and control grafts to account for size and morphological differences between individual donor vessels. T cells were detected only in the circulation of animals that received PBMCs ( $9.5 \pm 1.5\%$  human CD3<sup>+</sup> cells/mouse CD45<sup>+</sup> cells after 2 wk). Paired animals ( $n=64$ ) were killed at 2, 4, or 6 wk after immune reconstitution and grafts were removed for analysis.

### 2. PBMC-dependent injury and remodeling of coronary arteries

Human coronary artery grafts in control SCID/beige mice maintained a normal vascular architecture and histological appearance for the duration of the studies, though the thickness of the intima in vessels from different donors varied from minimal to equal to that of the media. Coronary arteries from allogeneic human PBMC-reconstituted hosts demonstrated marked inflammation and enlargement of the intima and adventitia. The adjacent mouse aorta was unaffected.

We quantified morphological changes between

paired human coronary artery grafts from reconstituted vs. untreated recipients. After 2 wk, a mild expansion of the graft vascular compartments in PBMC-treated animals did not reach statistical significance and the lumen area was relatively unchanged compared with controls (Fig. 1A). By 4 wk, the lumen area was not significantly diminished due to a corresponding increase of the total area circumscribed by the external elastic lamina compared with that of uninflamed grafts (Fig. 1B). At 6 wk, the lumen was strikingly compromised (Fig. 1C). Over time, there was a slight expansion of the media, a >10-fold increase of the intima area and a tripling in vessel size in response to the presence of allogeneic PBMCs.

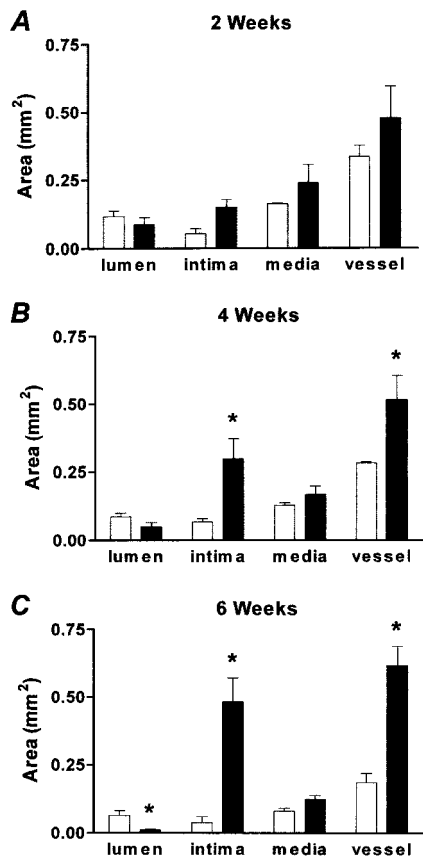
### 3. Graft infiltrating cells are human T lymphocytes

We analyzed coronary artery grafts by immunohistochemistry and enumerated various cell types to characterize the inflammation and injury of individual arterial compartments that are associated with outward vascular remodeling. The number of luminal endothelial cells remained stable at 2 and 4 wk ( $64 \pm 13$  vs.  $68 \pm 68$  and  $58 \pm 7$  vs.  $64 \pm 18$  cells per cross section in untreated vs. PBMC-treated grafts, respectively). Loss of endothelial cells was noted in rejecting arteries at 6 wk ( $68 \pm 11$  vs.  $39 \pm 9$  cells per cross section in untreated vs. PBMC-treated grafts) and may reflect immunologic injury or simply loss of luminal perimeter as their density did not diminish.

Grafts from PBMC-reconstituted animals had progressively fewer medial  $\alpha$ -smooth muscle (sm) actin<sup>+</sup> cells than those from paired untreated animals at 2, 4, and 6 wk ( $536 \pm 10$  vs.  $457 \pm 85$ ,  $465 \pm 88$  vs.  $349 \pm 85$ , and  $275 \pm 73$  vs.  $132 \pm 38$  cells per cross section in untreated vs. PBMC-treated grafts, respectively). Since there was a

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<sup>2</sup> Correspondence: Boyer Center for Molecular Medicine, Rm. 454, 295 Congress Ave., New Haven, CT 06510, USA. E-mail: [george.tellides@yale.edu](mailto:george.tellides@yale.edu)



**Figure 1.** Intimal expansion and inadequate outward vascular remodeling resulted in luminal compromise. Lumen, intima, media, and vessel areas of human coronary artery grafts were determined 2 (A,  $n=3$ ), 4 (B,  $n=6$ ), and 6 (C,  $n=3$ ) wk after PBMC reconstitution (filled bars) or no treatment (open bars) of SCID/beige mouse recipients. Data are means  $\pm$  SE. \* $P < 0.05$  vs. paired untreated control grafts (Student's  $t$  test).

small increase in the media area after adoptive transfer of allogeneic PBMCs, the density of vascular smooth muscle cells in inflamed arteries decreased with time compared with paired control grafts.

Large numbers of human CD45<sup>+</sup> leukocytes progressively infiltrated the grafts of reconstituted hosts at 2, 4, and 6 wk ( $2 \pm 1$  vs.  $2242 \pm 403$ ,  $27 \pm 14$  vs.  $4671 \pm 1115$ , and  $71 \pm 39$  vs.  $7577 \pm 2511$  cells per cross section in untreated vs. PBMC-treated grafts, respectively). The adventitia contained more leukocytes than the intima and the medial infiltrate was sparse. The number of infiltrating cells correlated with the degree of expansion of the vascular compartment resulting in a relatively constant density of CD45<sup>+</sup> cells. The graft infiltrating cells were almost all CD3<sup>+</sup> T lymphocytes expressing the CD45RO marker, with one- to twofold as many CD4<sup>+</sup> as CD8<sup>+</sup> T cells. There were very few CD68<sup>+</sup> macrophages and no detectable mouse CD45<sup>+</sup> leukocytes.

#### 4. Infiltrating T lymphocytes are predominantly interferon $\gamma$ (IFN- $\gamma$ ) -producing cells

We characterized graft infiltrating cells by their expression of cytokine production to determine if a particular

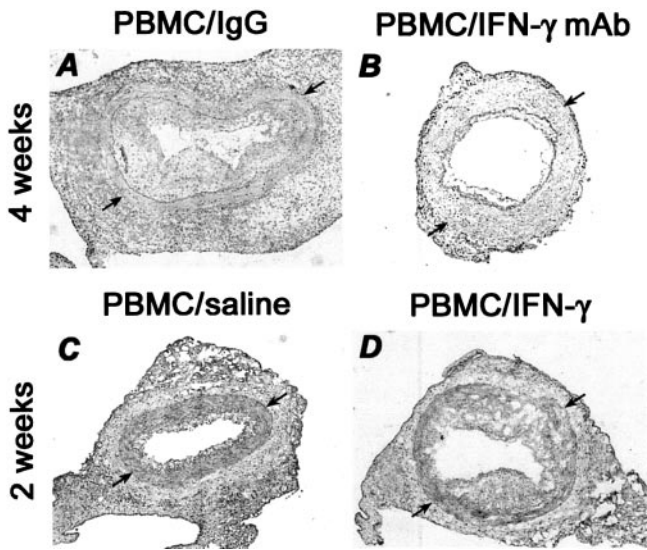
T lymphocyte subset was associated with outward vascular remodeling. Specific mRNA expression was quantified from the whole vessel wall or separately from the intima, media, and adventitia. Transcripts encoding CD3 $\epsilon$  (pan-T cell marker) and IFN- $\gamma$  were present in the PBMC-treated whereas interleukin (IL)-5 and IL-10 were largely undetectable. CD3 $\epsilon$  mRNA increased progressively from 2, 4, and 6 wk and most abundant within the adventitia ( $0.50 \pm 0.31$ ,  $0.58 \pm 0.12$ , and  $3.26 \pm 2.56$  transcripts/genome, respectively). A similar pattern of expression was seen for IFN- $\gamma$  ( $0.40 \pm 0.24$ ,  $1.55 \pm 0.99$ , and  $3.73 \pm 2.15$  transcripts/genome). Immunohistochemistry confirmed that CD3 antigen expression was greatest in the adventitia and least in the media of inflamed vessels. Together, the data demonstrate that cellular infiltrates in these rejecting human arteries are composed mainly of IFN- $\gamma$ -secreting T lymphocytes.

Circulating human lymphocytes produced type 1 cytokines. Intracellular flow cytometric analysis demonstrated that 16–26% of PMA/ionomycin-activated human CD3<sup>+</sup> T cells expressed IFN- $\gamma$  at 4 wk. Human IFN- $\gamma$  was detected only in the plasma of PBMC-reconstituted animals and increased to  $122 \pm 46$ ,  $574 \pm 121$ , and  $492 \pm 204$  pg/ml at 2, 4, and 6 wk after adoptive transfer, respectively.

#### 5. IFN- $\gamma$ is required for T cell-mediated vascular injury and remodeling

To determine if there is an essential role for IFN- $\gamma$  in human arterial rejection, we examined the effects of neutralizing antibody or exogenous cytokine. Interventions did not significantly modulate host PBMC reconstitution. Treatment with a monoclonal antibody to human IFN- $\gamma$  for 4 wk significantly inhibited intimal hyperplasia and outward vascular remodeling of coronary artery grafts in response to allogeneic PBMCs (Fig. 2). The intimal area decreased from  $0.36 \pm 0.05$  to  $0.14 \pm 0.03$  mm<sup>2</sup> ( $P < 0.05$ ), the vessel area decreased from  $0.64 \pm 0.07$  to  $0.58 \pm 0.08$  mm<sup>2</sup> ( $P < 0.05$ ), and the lumen area increased from  $0.03 \pm 0.01$  to  $0.21 \pm 0.05$  mm<sup>2</sup> ( $P < 0.05$ ) in anti-IFN- $\gamma$  vs. irrelevant antibody-treated recipients, respectively ( $n=5$  in each group). There was an associated trend of fewer infiltrating leukocytes, a greater preservation of endothelial and vascular smooth muscle cells, and a decreased expression of type I cytokine mRNA. Type II cytokine transcripts were not induced in response to diminished IFN- $\gamma$  production and activity.

In contrast, administration of exogenous human IFN- $\gamma$  for 2 wk enhanced intimal and vessel expansion with a reciprocal modulation of inflammatory markers and indices of vascular injury compared with the blocking anti-serum strategy (Fig. 2). These results establish that IFN- $\gamma$  has a requisite and non-redundant role in the pathologic manifestations of human vascular rejection.



**Figure 2.** Neutralization of IFN- $\gamma$  inhibits intimal expansion and outward vascular remodeling, whereas supplementation of IFN- $\gamma$  enhances these effects. Microscopic appearance of two paired human coronary artery grafts at 2 (bottom panels) and 4 (top panels) wk after PBMC reconstitution of SCID/beige mouse recipients. One pair of animals was treated with irrelevant IgG (A) or anti-IFN- $\gamma$  monoclonal antibody (mAb, B), and a second pair of animals received saline (C) or IFN- $\gamma$  (D). Sections were lightly stained with anti- $\alpha$ -sm actin antibody to assist in defining the vascular compartments. The external elastic laminae are marked with arrows. Representative photomicrographs of graft cross sections were obtained at the same low magnification.

### CONCLUSIONS AND SIGNIFICANCE

IFN- $\gamma$  is present in clinical specimens of atherosclerosis and graft arteriosclerosis. In mouse models of cholesterol-induced vasculopathy or allogeneic cardiac transplantation, IFN- $\gamma$  is required to induce intimal thicken-

ing. In these clinical and experimental studies, arterial disease was defined exclusively by intimal expansion. We extend these previous observations by demonstrating that vascular remodeling, the most significant pathologic factor determining arterial lumen size, is also dependent on IFN- $\gamma$  during immunologic injury of human arteries. We did not find evidence of Th2-associated mechanisms for arterial enlargement as recently described in atherosclerotic aortic aneurysms.

The arterial wall contained exclusively type 1 cytokine-secreting T cells. The presence of IFN- $\gamma$  within the graft may inhibit the production of IL-5 as Th1 and Th2 responses are cross-regulated. Alternatively, IFN- $\gamma$ -producing, but not IL-5-secreting, T cells may be selectively recruited to the allogeneic arteries from the periphery. Our study does not assess the relative importance of locally produced vs. circulating IFN- $\gamma$ , although it is likely that both contribute. Our neutralizing Ab experiment validates the concept that the IFN- $\gamma$  axis is a therapeutic target in clinical graft rejection. Paradoxically, a potential risk of these agents is arterial lumen loss if the inhibition of outward remodeling exceeds that of intimal expansion. The enhanced vasculopathy in response to exogenous IFN- $\gamma$  provides an alternative interpretation why infections which elicit Th1 responses, such as those due to *Chlamydia pneumoniae* or cytomegalus virus, may exacerbate atherosclerosis or arteriosclerosis without necessarily being causative.

Our model of human allogeneic arterial injury is an important experimental advancement as it reproduces key remodeling manifestations of clinical vasculopathy. We previously reported that IFN- $\gamma$  is sufficient to elicit intimal expansion and enlargement of coronary arteries in the absence of leukocytes. We now provide evidence that IFN- $\gamma$  is necessary for T cell-mediated injury and outward vascular remodeling of human coronary arteries. EJ

**Figure 3.** Schematic illustration depicting the proposed role of IFN- $\gamma$  and morphological changes of T cell-mediated outward vascular remodeling of allogeneic human coronary arteries in our experimental model. T cells are recruited to the intima from the arterial lumen and to the adventitia from the microvasculature (dashed lines). Locally secreted and circulating IFN- $\gamma$  act on the media in a paracrine and endocrine fashion, respectively (dotted lines). The cytokine effects on medial vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM) result in outward vascular remodeling. Shaded areas represent the intima, the dashed lines demonstrate the extent of outward vascular remodeling; dotted lines indicate the amount of luminal loss. The outward vascular remodeling is not a linear process nor does it plateau for luminal loss to occur. If there were no outward vascular remodeling, the increasing intimal area would have equaled that of the original internal elastic lamina area, thus occluding the lumen, by 4 wk.

