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Bcl-2 Transduction Protects Human Endothelial Cell Synthetic Microvessel Grafts from Allogeneic T Cells In Vivo

Lian Zheng,* Thomas F. Gibson,* Jeffrey S. Schechner,†‡ Jordan S. Pober,*†‡ and Alfred L. M. Bothwell2*†

T cell interactions with vascular endothelial cells (EC) are of central importance for immune surveillance of microbes and for pathological processes such as atherosclerosis, allograft rejection, and vasculitis. Animal (especially rodent) models incompletely predict human immune responses, in particular with regard to the immunological functions of EC, and in vitro models may not accurately reflect in vivo findings. In this study, we describe the development of an immunodeficient SCID/bg murine model combining a transplanted human synthetic microvascular bed with adoptive transfer of human T lymphocytes allogeneic to the cells of the graft that more fully recapitulates T cell responses in natural tissues. Using this model, we demonstrate that transduced Bcl-2 protein in the engrafted EC effectively prevents injury even as it enhances T cell graft infiltration and replication. The Journal of Immunology, 2004, 173: 3020–3026.

The vascular endothelium of an allograft is a primary target for immune-mediated rejection (1, 2). Either CD4 or CD8 T lymphocytes can be mediators of this response. The CD4 T cell response depends on IFN-γ, because this cytokine is necessary for induction of MHC class II on endothelial cells (EC)3 (3–5), which are required for the recognition by CD4 T cells (6). MHC class I molecules, which are recognized by CD8 T cells, are constitutively expressed by EC at sufficient levels to trigger T cell recognition even without further induction by cytokine (7). In contrast to most non-bone marrow-derived cells, EC express functional costimulatory molecules that can result in activation of resting memory T cells (7). Although activation of resting CD8 T cells to effector CTL can occur after recognition of MHC class I-expressing cells and appropriate costimulatory molecules, clonal expansion of effector cell populations requires an adequate source of the growth factor IL-2, which often must be provided by CD4 T cells (8).

Calcineurin inhibitor-based immunosuppressive therapies, using cyclosporine or tacrolimus, are designed to prevent IL-2 synthesis and thus prevent CTL expansion, because, once generated, CTL may be resistant to these agents (9). Although calcineurin inhibitor-based immunosuppression has achieved remarkable success, it is often limited by toxicity, and, even when optimal, CTL-mediated rejection episodes can still occur. A complementary strategy, not yet applied in the clinic, would be to increase the resistance of graft cells, especially graft EC, to the effects of CTL. We have investigated the potential cytoprotective effects of overexpression of human Bcl-2 on the functional cytolytic activity of alloreactive CD8 T cells in vitro (10). HUVECs retrovirally transduced with Bcl-2 showed nearly complete resistance to a variety of inducers of apoptosis, including alloreactive CTL. It is difficult to predict whether Bcl-2 transduction will protect human EC from T cell injury in vivo. We have found that implantation of human skin or artery into immunodeficient SCID/bg mice followed by adoptive transfer of allogeneic human PBMC provides a useful model for analyzing in vivo T cell reactions. We use SCID/bg mice as hosts, because these animals have compromised NK cell as well as T and B cell functions and thus accept human lymphoid cells as well as solid tissue grafts. Following inoculation i.p. with 1–3 × 10^6 human PBMC, human CD4 and CD8 T cells appear in the circulation ~1 wk after injection and persist for 2 mo. B cells also engraft and produce human Ab, but human NK cells, mononuclear phagocytes, and dendritic cells do not engraft. In previous studies with this model, a human alloresponse to well-healed orthotopic skin or coronary artery interposition grafts occurs spontaneously by 2 wk and involves significant infiltration of both CD4 and CD8 T cells (11). Removal of B cells from the inoculum does not alter the response to human skin, indicating that this is a wholly T cell-mediated reaction (12). By 3 wk, there is extensive immune-mediated injury with nearly complete destruction of human vessels. Histopathologic analyses suggest that CTL are the major effector populations, but other mechanisms could also contribute.

Despite their utility as models for studying T cell-mediated injury, skin or artery grafts have not lent themselves to studies of gene therapy-mediated cytoprotection. This is because human EC in these tissues have been difficult to transplant or transduce with high efficiency. In the present report, we extend the use of human-mouse chimeric models to study interactions between human lymphocytes and genetically modified EC in vivo by using a system we developed for forming a synthetic microvascular bed in SCID/bg mice (13). Normal HUVEC spontaneously form tubes when cast in collagen/fibronectin gels, which, when implanted into the abdominal wall of SCID/bg mice, spontaneously Anastomose with the mouse microvessels at the gel edge and become perfused with mouse blood after a period of 2–3 wk. The overexpression of Bcl-2 protein results in recruitment of smooth muscle-like cells (SMC) of mouse origin. Bcl-2-transduced HUVEC also form a more extensive network of tubes than do control-transduced or

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3 Abbreviations used in this paper: EC, endothelial cell; SMC, smooth muscle-like cell; DAB, diaminobenzidine.
untransduced HUVEC. In the present report, we have used this model to analyze the effect of Bcl-2 transduction on T cell-mediated injury in vivo. We find that Bcl-2 expression completely protects EC from T cell-mediated injury, although the persistence of human EC may actually enhance allogeneic T cell graft infiltration and expansion.

Materials and Methods

Animals

C.B-17 SCID/bg mice (Tacombit, Germantown, NY) were used at 5–6 wk of age. The animals were housed in microisolator cages and fed sterilized food and water. All animal experimental manipulations were performed under a protocol approved by the Yale Animal Care and Use Committee. Serum IgG levels were determined by ELISA using reagents from Cappel (Durham, NC). SCID/bg mice were considered leaky at IgG levels of >1 µg/ml and excluded from experimental use.

Cells and retroviral transduction

HUVEC were isolated by collagenase treatment of human umbilical veins under a protocol approved by the Yale Human Investigation Committee as previously described and cultured on 0.2% gelatin-coated plastic in Medium 199 with 20% FCS, 50 µg/ml EC growth supplement (Collaborative Research/BD Biosciences, Bedford, MA), 100 µg/ml porcine intestinal heparin (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. All of the HUVEC used in these experiments were at passage 1 through 6. Such cultures are homogeneous for EC markers (vWF, CD31, inducible E-selectin) and are free of contaminating CD45+ leukocytes.

Retroviral vectors expressing the caspase-resistant form of Bcl-2 have been previously described (14). HUVEC were infected with retroviral vectors in the presence of polybrene (hexadimethrine bromide; 8 µg/ml; Sigma-Aldrich) daily for up to 3 days. One day after the last infection, 95% of the cells expressed transgene.

Formation of HUVEC vascular construct in collagen/fibronectin gels

HUVEC were isolated and cultured as described. Microvessel grafts in collagen/fibronectin gels were prepared using a modified protocol from that previously described (13). EC (3 × 10^6 cells) were suspended in a 1.2 ml of a solution of rat tail type 1 collagen (1.5 mg/ml), human plasma fibronectin (100 µg/ml) (both from BD Biosciences), 25 mM HEPES, 1.5 mM NaOH, 10% FCS, and 10× Medium 199 (Sigma-Aldrich) for HUVEC at 4°C. The pH was adjusted to 7.5 by 0.1 M NaOH. The HUVEC collagen-fibronectin suspension was pipetted into rat tail type 1 collagen-coated C-6 Transwells (BD Biosciences) and warmed to 37°C for 15 min to allow polymerization of the collagen. M199 or DMEM medium with 10% FCS was added to the Transwells to cover the solidified gels. For implantation into SCID/bg mice, gels were harvested and trisected 24 h after gel formation. Each resulting 3 × 0.2-cm gel segment was implanted into a bluntly dissected s.c. pouch in the anterior abdominal wall of a 5- to 6-wk-old mouse. The wound was closed with a skin staple.

PBMC isolation and reconstitution

Normal PBMC were obtained from healthy donors by leukapheresis under a protocol approved by the Yale Human Investigation Committee. PBMC were isolated by density gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC) as previously described. The cells were stored in 10% DMSO in liquid nitrogen for up to 12 mo, thawed, and washed before use. Microvessel-grafted SCID/bg mice were reconstituted with 3 × 10^6 human PBMC by i.p. inoculation. The number of circulating T cells was evaluated by indirect immunofluorescence flow cytometry. In brief, heparinized retro-orbital venous blood samples were obtained at different time points after reconstitution. The erythrocytes were lysed using ammonium chloride, and leukocytes were incubated with PE-conjugated rat anti-mouse CD45 (BD Pharmingen, San Diego, CA) mAb and FITC-conjugated mouse anti-human CD3 mAb (Immunotech/Beckman Coulter, Marseilles, France), FITC-conjugated mouse anti-human CD4 mAb (Immunotech/Beckman Coulter), or FITC-conjugated mouse anti-human CD5 mAb (Immunotech/Beckman Coulter), and analyzed using a FACScan flow cytometer and CellQuest software (BD Biosciences).

Immunohistochemistry and quantitative analysis of sections

HUVEC gels were harvested from SCID mice at various times after PBMC cell injection, fixed in 10% buffered formalin, and embedded in paraffin. Sections (5-µm thick) were cut for immunostaining. In some experiments, specimens were instead snap frozen in Tissue-Tek OCT (Sakura Finetek, Zoeterwoude, The Netherlands) and 6-µm cryosections were prepared. Ab staining was conducted using the Vectastain ABC (avidin/biotin complex) kit (Vector, Burlingame, CA). Specifically, single Ab staining was performed with primary Ab, such as anti-human collagen IV (Sigma-Aldrich), anti-MHC class I (BD Pharmingen), anti-MHC class II (BD Pharmingen), anti-human ICAM-1 (BD Pharmingen), anti-human VCAM-1 (BD Pharmingen), anti-human CD45RO mAb (DakoCytomation, Carpinteria, CA), anti-human CD4 (Serotec, Raleigh, NC), anti-human CD8 mAb (Serotec, Raleigh, NC), or anti-human perforin (BD Pharmingen), for 1 h and washing with PBS, followed by incubation with biotinylated secondary Ab (Vector) for 1 h and washing with PBS. Slides were then incubated for 1 h with the ABC reagent and incubated with diaminobenzidine (DAB) peroxide substrate until the desired color developed, followed by a light hematoxylin stain (Sigma-Aldrich). For double staining with CD45RO and collagen IV Ab, the sections were first treated with anti-human CD45RO mAb, followed by incubation with biotinylated secondary Ab and visualized by DAB-NiCl2 (dark blue color). Then, the sections were incubated with anti-human collagen IV Ab, followed by incubation with biotinylated secondary Ab and visualized by DAB (brown color).

For quantitation of PBMC-mediated injury, the numbers of human type IV collagen-outlined human microvessels or human CD45RO T cells within the cross-section of the entire gel were either counted manually under the microscope, or a picture of the cross-section of the entire gel was taken, and the entire gel area was measured using Scion Image β 4.02 for Windows 95 software (downloaded from www.scioncorp.com/frames?download_now.htm). In that case, the density of human microvessels

| Table I. Preservation of human microvessels by Bcl-2 after injection of PBMCa |
|--------------------------------------|--------|---------|----------|
| Cells | No. Mice | Time after PBMC or saline | % Microvessel Destruction |
| HUVEC | 6 | 10 days | 0 |
| HUVEC-Bcl2 | 6 | 10 days | 0 |
| HUVEC+PBMC | 6 | 10 days | 0 |
| HUVEC-Bcl2+PBMC | 6 | 10 days | 0 |
| HUVEC | 3 | 2 wk | 0 |
| HUVEC-Bcl2 | 3 | 2 wk | 0 |
| HUVEC+PBMC | 3 | 2 wk | 0 |
| HUVEC-Bcl2+PBMC | 3 | 2 wk | 0 |
| HUVEC-Bcl2 | 10 | 3 wk | 0 |
| HUVEC+PBMC | 10 | 3 wk | 0 |
| HUVEC-Bcl2+PBMC | 12 | 3 wk | 0 |

* HUVEC or HUVEC transduced with Bcl-2 were used to form synthetic microvessels and implanted into SCID/bg mice. A total of 3 × 10^6 HUVEC was injected into grafted mice 2 wk after implantation. Grafts were recovered 10 days to 3 wk after PBMC injection and prepared as frozen sections. Anti-human collagen IV Ab staining was used to detect human microvessels. Experiments performed 3 wk after PBMC injection were done as four separate experiments with two or three mice in each group. Quantitation of microvessel destruction was done by visual enumeration as described in Materials and Methods.

Human CD3 T cell isolation and reconstitution

CD3 T cells were isolated from PBMC by negative selection. In brief, PBMC were first depleted of monocytes by adherence to culture flasks for 1 h at 37°C. Nonadherent cells were incubated with a mixture of mAb containing anti-MHC class II Ab (BD Pharmingen), anti-CD14 Ab (BD Pharmingen), anti-CD16 Ab (BD Pharmingen), anti-CD11b Ab (BD Pharmingen), and anti-CD56 Ab (BD Pharmingen) for 25 min at 4°C. The cells were then washed to remove excess Ab. Further enrichment was done by magnetic immunodepletion using BioMag goat anti-mouse IgG-bond bead (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The purity of the CD3 T cells was >90% as determined by direct immunofluorescence staining with anti-CD3 Ab (BD Pharmingen). Microvessel-grafted SCID/bg mice were reconstituted with 3 × 10^6 purified CD3 T cells by i.p. inoculation.
(numbers of vessels per square millimeter) or human CD45RO T cells was calculated by dividing the numbers of microvessels or human CD45RO T cells within the entire gel by the cross-sectional area of the gel.

For quantitation of CD3/H11001 T cell-mediated injury, the numbers of human type IV collagen-outlined human microvessels within the cross-section of the entire gel were counted manually under the microscope. The survival of the microvessels on CD3 T cell-injected samples was calculated by dividing the number of microvessels on CD3 T cell-injected samples by the number of microvessels on saline-injected samples. The p value was determined to be <0.001, indicating that there was significant difference of microvessel survival between the CD3 T cell- and saline-injected samples.

Results
Previously, we have described an in vivo model system for creating synthetic microvessels using HUVEC in SCID/bg mice (13). In the original description of these microvessels, there was a substantial recruitment of mouse SMC to the synthetic microvessels when the HUVEC were transduced with Bcl-2 compared with reporter gene (EGFP)-transduced HUVEC. The experiments described in this study use a minor modification, which is the inclusion of FBS when the gels are cast in the presence of collagen/fibronectin. This enhances the reproducibility and density of the microvessels that form from untransduced HUVEC. Moreover, it also allows HUVEC not transduced with Bcl-2 to recruit mouse SMC. Although Bcl-2-transduced HUVEC have even greater numbers of SMC surrounding the microvessels, the inclusion of serum enhances the utility of this model, because Bcl-2 transduction is not required to form good microvessels.

Because we had found a high degree of cytoprotection in vitro from human CTL by overexpression of Bcl-2 in HUVEC, we wished to assess whether this strategy would work in vivo. We first implanted groups of mice with gels containing either unmodified HUVEC or Bcl-2-transduced HUVEC as described above. The optimal conditions for reconstitution of these mice with PBMC had been established previously (15, 16). Intraperitoneal injection of 3 × 10⁸ PBMC per mouse results in effective reconstitution in

![FIGURE 1](http://www.jimmunol.org/)

Identification of synthetic human microvessels. HUVEC or HUVEC transduced with Bcl-2 were used to form synthetic microvessels and implanted into SCID mice. Either saline (control) or 3 × 10⁸ PBMC/mouse were injected i.p. into grafted mice 2 wk after implantation. Human microvessels were recovered 3 wk after PBMC injection and prepared as paraffin sections. Human collagen IV Ab staining was used to detect human microvessels. The experiment shown is representative of six similar experiments.

![FIGURE 2](http://www.jimmunol.org/)

Identification of human CD45RO T cells in human microvessels 3 wk after PBMC injection. Mice were injected with PBMC 2 wk after implantation. Human microvessels were recovered 3 wk after PBMC injection and embedded in paraffin. A (saline control) and B (+PBMC) contain HUVEC microvessels stained with anti-CD45RO; C (saline control) and D (+PBMC) contain HUVEC-Bcl-2 microvessels stained with anti-CD45RO; E (saline control) and F (+PBMC) contain HUVEC-Bcl-2 microvessels stained with anti-CD45RO (dark blue), anti-collagen IV (brown), and a light blue counterstain. Human CD45RO Ab staining was used to detect human CD3 T cells. The experiment shown is representative of six similar experiments.
all recipients. Based on a series of pilot experiments, we allowed the microvascular bed to heal for 2 wk in vivo and then injected i.p. either saline (control) or PBMC. Grafts were harvested and stained with anti-CD45RO to visualize T cells at various times after PBMC injection. At 10 days following introduction of the allogeneic PBMC, there is no alteration of the structure of HUVEC-derived microvessels compared with control animals, and no T cells are detected within the gel graft (Table I). Untransduced microvessel grafts analyzed at 2 wk show minimal evidence of microvessel destruction as well as the presence of a few T cells in samples that received PBMC (one mouse of three showed a 50% reduction in the number of microvessels). When mice were examined 3 wk after introduction of PBMC, we note consistent destruction of untransduced microvessels in the grafts (60–100%) and infiltration by a significant number of T cells within grafts containing either untransduced or HUVEC-Bcl-2-transduced microvessels in animals receiving PBMC but not in no-PBMC control animals (Figs. 1 and 2; Table I). In other words, despite the fact that microvascular bed grafts formed from Bcl-2-transduced HUVEC showed infiltration of T cells in all samples that received PBMC, there was little evidence of microvessel loss.

To quantify these responses, the microvessel grafts harvested at 3 wk from four independent experiments were characterized for the presence of infiltrating T cells by immunostaining with anti-CD45RO Ab and for human microvessels by staining for human type IV collagen (see Fig. 2). The range in vessel density is 200–400 vessels/mm² and the no-PBMC controls in each experiment are assigned as 100% (see Table II). By this detailed quantitation, the microvesSEL destruction is similar to that shown in Table I. Quantitation of T cell infiltration reveals some variation in the density of T cells in each experiment but ∼10-fold greater numbers of T cells are observed in the HUVEC-Bcl-2 microvesSEL grafts than in control HUVEC grafts. When samples are simultaneously stained for CD45RO (dark blue), collagen IV (brown), and a hematoxylin counterstain to reveal all cells in the slide, infiltrating T cells are observed primarily in the proximity of the microvessels. This observation implies that the T cells are responding to the EC in the microvessels. We also observed expansion of human T cells in the peripheral blood (see Fig. 3), and there are greater numbers of T cells at all time points in the animals with HUVEC-Bcl-2 grafts than with HUVEC microvesSEL grafts. There are about twice as many circulating CD8 T cells as CD4 T cells. This observation suggests that, despite this resistance to injury, Bcl-2-transduced HUVEC actually appear to stimulate expansion of T cell numbers as well as recruitment. The mechanism of this response is not known, but it could result from presentation of allogeneic MHC molecules by the EC.

To further characterize the T cell reaction to the grafts, we performed immunohistochemical analyses on frozen sections from untransduced and Bcl-2-transduced HUVEC injected with saline or PBMC. In general, HUVEC or HUVEC-Bcl-2, whether analyzed in cell culture before implantation or after implantation, do

![FIGURE 3. Enumeration of human T cells in peripheral blood of grafted SCID/bg mice. The number of circulating human T cells was evaluated by indirect immunofluorescence flow cytometry. The leukocytes were incubated with PE-conjugated rat anti-mouse CD45 mAb, FITC-conjugated mouse anti-human CD3 mAb, FITC-conjugated mouse anti-human CD4 mAb, and FITC-conjugated mouse anti-human CD8 mAb, and analyzed using a FACScan flow cytometer. The experiment shown is representative of three similar experiments.](http://www.jimmunol.org/)

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### Table II. Quantitation of microvesSEL and infiltrating T cell density

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Mice</th>
<th>Vessels/mm²</th>
<th>Vessel Density as % of Control</th>
<th>T Cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>3</td>
<td>205 ± 147</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC-Bcl-2</td>
<td>2</td>
<td>252 ± 8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC+PBMC</td>
<td>3</td>
<td>33 ± 8</td>
<td>16</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>HUVEC-Bcl-2+PBMC</td>
<td>3</td>
<td>315 ± 120</td>
<td>125</td>
<td>20 ± 25</td>
</tr>
<tr>
<td>HUVEC</td>
<td>3</td>
<td>309 ± 110</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC-Bcl-2</td>
<td>3</td>
<td>338 ± 123</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC+PBMC</td>
<td>2</td>
<td>90 ± 51</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC-Bcl-2+PBMC</td>
<td>3</td>
<td>374 ± 222</td>
<td>111</td>
<td>106 ± 105</td>
</tr>
<tr>
<td>HUVEC</td>
<td>3</td>
<td>298 ± 72</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC-Bcl-2</td>
<td>2</td>
<td>510 ± 91</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC+PBMC</td>
<td>3</td>
<td>150 ± 62</td>
<td>50</td>
<td>28 ± 44</td>
</tr>
<tr>
<td>HUVEC-Bcl-2+PBMC</td>
<td>3</td>
<td>435 ± 114</td>
<td>85.3</td>
<td>203 ± 179</td>
</tr>
<tr>
<td>HUVEC</td>
<td>3</td>
<td>124 ± 57</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC-Bcl-2</td>
<td>2</td>
<td>188 ± 81</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC+PBMC</td>
<td>3</td>
<td>89 ± 12</td>
<td>72</td>
<td>7.9 ± 8</td>
</tr>
<tr>
<td>HUVEC-Bcl-2+PBMC</td>
<td>3</td>
<td>215 ± 12</td>
<td>114</td>
<td>55 ± 53</td>
</tr>
</tbody>
</table>

*The density of microvessels and T cells was measured 3 wk after introduction of PBMC, as described in Materials and Methods.*
not show any differences in cell surface expression of any marker tested (data not shown). Within implanted gels, both untransduced and Bcl-2-transduced HUVEC express comparable expression of HLA class I, and these levels are not affected by injection of PBMC (see Fig. 4), although there are many fewer stained cells in Fig. 4B, presumably because the untransduced HUVEC were destroyed by the injected PBMC. Analysis of HLA class II expression in the gels shows absence of this molecule on both untransduced and Bcl-2-transduced HUVEC after saline injections (Fig. 5, A and C), similar to cultured HUVEC. There is substantial induction of HLA class II after introduction of PBMC in the HUVEC-Bcl-2 (Fig. 5D) (again, there are few positive cells in Fig. 5B due to destruction of HUVEC by the PBMC). We deem this induction of MHC class II to be a consequence of cytokines released by the expanding T cells in the SCID/bg mice, most likely human IFN-γ.

The HUVEC-Bcl-2 implants were also analyzed for expression of adhesion molecules (Fig. 6). Expression of human VCAM-1 and ICAM-1 is significant only after introduction of PBMC. These molecules may well contribute to the recruitment of T cells into the grafts. Again, the cytokines released by the expanding human T cells in vivo are likely to be responsible for adhesion molecule induction, and the most important of these may be IFN-γ and TNF. To characterize the mechanism of injury, we have stained the infiltrating T cells with various Abs (see Fig. 7). The vast majority of the infiltrating cells (at least 90%) express CD8 and perforin, although no expression of Fas is detected (data not shown). No significant staining of cells with anti-CD4 mAb is observed (data not shown).

To assess the cellular requirements for the induction of the T cell response, we have tested whether purified T cells can substitute for unfractionated PBMC. If not, then one might suspect the participation of an APC in the PBMC (e.g., monocytes or DC). T cells were purified from fresh PBMC and injected into mice bearing untransduced HUVEC implants. As shown in Fig. 8, there is ~90% destruction of microvessels when purified CD3⁺ T cells are injected. This experiment would support the data shown in Fig. 3, suggesting that there is direct recognition of the graft EC resulting in activation of alloreactive cytolytic CD8⁺ T cells. It also suggests that neither human alloantibody, macrophages, nor NK cells, participate in the effector phase of the reaction, because these potential effectors are not transferred with purified T cell populations.

**Discussion**

The results presented in this study establish that Bcl-2 can provide an in vivo cytoprotective effect for EC using a synthetic graft in a model of human immune-mediated injury. The model is a synthesis of our in vitro studies characterizing human T cells and the development of in vivo models using reconstituted immunodeficient SCID/bg mice. Previous studies with human skin or artery grafts showed that the introduction of human PBMC resulted in a significant spontaneous alloresponse. We see a similar response using synthetic microvessel beds in which the only human cell
component are EC. Furthermore, purified CD3+ T cells were able
to develop cytolytic function in vivo. This indicates that Ab is
unnecessary for the response. The mice are screened for lack of
murine Ab before use in our experiments and there is no evidence
indicating the presence of alloreactive human Abs. The expansion
of the number of circulating T cells in animals bearing grafts is
also consistent with prior observations that EC are capable of ac-
tivating a CD8 T cell response in the absence of professional APC
(17). This would also be consistent with direct T cell recognition
of the HUVEC lining the microvessels. Interestingly, the degree of
cytoprotection we observe in vivo is essentially complete, whereas
it was manifested as a major shift in the E:T cell ratios required for
lysis using in vitro assays (10). Our observations also appear to
uncouple T cell infiltration from T cell-mediated injury, a phe-
nomenon long appreciated by transplant pathologists attempting to
evaluate allograft rejection.

The mechanism of the development of T cell cytolytic function
appears consistent with certain expectations. The T cells expand in
vivo, and activation of human T cells, especially CD8 cells, results
in secretion of cytokines. Expression of HLA class II is virtually
undetectable in the absence of PBMC but dramatically increased
after injection, probably as a consequence of IFN-γ. Adhesion
molecules ICAM-1 and VCAM-1 are induced on the HUVEC-
Bcl-2 microvessels that are resistant to cytolysis as a consequence
of Bcl-2 expression.

In murine systems, CD4 T cells play an important role in facilitat-
ing secondary expansion and development of a CD8 memory
response (18). The role of CD4 T cells in a human CD8 response
is less clear. The provision of growth factors, especially IL-2, may
be the only requirement. In vitro-purified human CD8 T cells do
generate an effector T cell response to HUVEC (19, 20). This in
vivo model should be very valuable in these types of studies of
basic requirements for development of an immune response. Re-
cently, there has been considerable evidence that regulatory T cells
can have a potent effect in promoting graft survival. This system
is also well suited to examine at a mechanistic level the function of
human regulatory cells in an in vivo model.

Mechanistic studies and preclinical evaluation of therapeutics of
human lymphocyte interactions with blood vessels and tissues in
vivo are severely limited for ethical reasons, and proof of principle
of gene therapy has almost exclusively depended upon animal
models or in vitro systems. The results presented clearly show that
our model is well suited to assess the functional properties of a
broad range of gene products and it is possible that other protective
genes that show limited effects in in vitro assays may show sig-
nificant functional phenotypes in the in vivo model. The genetic
modifications that could be made to the EC are essentially unlim-
ited. Factors that enhance recognition can be overexpressed or in-
hibited in HUVEC. For example, assessment of genes that affect
homing or activation of lymphocytes (e.g., adhesion molecules,
cytokines, chemokines, or costimulators) could also be effectively
studied.

In conclusion, we have developed a novel model system involv-
ing transplantation of cultured human EC into immunodeficient
mice, resulting in formation of a synthetic microvascular bed. We
have shown in this study that this system can be combined with
adoptive transfer of human T cells to evaluate the ef
cacy of gene
therapy as a strategy for reducing allograft injury. Strikingly, the
protective effect of Bcl-2 is even more pronounced in vivo, where
the possibilities of injury are more complex, than it was in vitro.

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![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Expression of ICAM-1 and VCAM-1 on synthetic human
microvessels. Experiments were performed as described in Fig. 1, but only
HUVEC-Bcl-2 implants were analyzed for expression using frozen sec-
tions. The experiment shown is representative of three similar experiments.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Expression of CD8 and perforin on synthetic human mi-
crovessels. Experiments were performed as described in Fig. 1, but only
HUVEC-Bcl-2 implants were analyzed for expression using frozen sec-
tions. The experiment shown for CD8 expression is representative of six
similar experiments and three for the analysis of perforin expression.

![FIGURE 8](http://www.jimmunol.org/)

**FIGURE 8.** Purified CD3+ T cells develop cytotoxic function in vivo. T
cells were purified from fresh PBMC, and 3 × 10⁷ CD3+ T cells were
injected i.p. 2 wk following gel implantation and harvested 6 wk later.
Boyer Center for Molecular Medicine Tissue Culture facility for providing HUVEC. Retroviral vectors and packaging cell lines were obtained from Dr. Garry Nolan at Stanford University (Stanford, CA).

References