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Human TNF Can Induce Nonspecific Inflammatory and Human Immune-Mediated Microvascular Injury of Pig Skin Xenografts in Immunodeficient Mouse Hosts

Nancy C. Kirkiles-Smith,*‡ Dennis A. Tereb,*‡ Richard W. Kim,*‡ Jennifer M. McNiff,‡§ Jeffrey S. Schechner,*§ Marc I. Lorber,‡ Jordan S. Pober,*‡§ and George Tellides2‡†

TNF activates endothelial cells to express cell surface molecules that are necessary to recruit a local infiltrate of leukocytes. Because the actions of this proinflammatory cytokine are not species restricted, we investigated whether human TNF can up-regulate porcine endothelial adhesion molecules to elicit human T cell infiltration and damage of pig skin xenografts in a chimeric immunodeficient mouse model. We have previously demonstrated the vigorous rejection of human skin allografts and the absence of injury to porcine skin xenografts in human PBMC-SCID/beige mice. Intradermal administration of human TNF at high doses (600 or 2000 ng) caused nonspecific inflammatory damage of pig skin grafts, whereas low concentrations of TNF (60 or 200 ng) resulted in human PBMC-dependent injury of porcine endothelial cells. There was a strong correlation among pig skin xenograft damage, human T cell infiltration, and the TNF-induced up-regulation of swine MHC class I and class II molecules, VCAM-1, and, in particular, the de novo expression of porcine E-selectin. The microvascular damage and leukocytic infiltration elicited by TNF were enhanced by porcine IFN-γ, suggesting that xenografts may be less prone to cytokine-mediated injury due to the species-restricted effects of recipient IFN-γ. Our results indicate that maintenance of a quiescent endothelium, which does not express E-selectin or other activation-dependent adhesion molecules, is important in preventing human anti-porcine T cell xenoresponses in vivo and that TNF signaling molecules and TNF-responsive gene products are appropriate therapeutic targets to protect against human T cell-mediated rejection of pig xenografts. The Journal of Immunology, 2000, 164: 6601–6609.

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albeit in a delayed fashion (17). Because the sole source of porcine IFN-γ in a pig-to-human xenograft would be limited to pig passenger leukocytes, the quantity of donor cytokine production might predictably be minimal, particularly as donor leukocytes rapidly emigrate from the graft. We therefore investigated whether other human proinflammatory cytokines that are not species restricted, such as TNF, can substitute to initiate cellular xenogeneic immune responses in vivo. Furthermore, because TNF acts synergistically with IFN-γ on endothelial cells to recruit a local infiltrate of activated leukocytes (2), we also examined the interactions between human TNF and porcine IFN-γ on pig skin grafts in SCID/beige mice to determine whether cell-mediated xenograft rejection is modulated as a result of incompatible donor-host IFN-γ responses.

**Materials and Methods**

**Animals**

C.B-17 SCID/beige mice (Taconic Farms, Germantown, NY) were used at 5–8 wk of age. The animals were housed in microisolator cages and were fed sterilized food and water. Serum IgG levels were determined by sandwich ELISA using reagents from Cappel (Durham, NC) as previously described (22). SCID/beige mice were considered “leaky” at IgG levels >1 µg/ml and excluded from experimental use. Ventral abdominal skin was procured from 30- to 60-kg outbred Yorkshire pigs at a local abattoir.

**Skin grafting**

Pig skin was transplanted to SCID/beige mice as previously described (22) under a protocol approved by the Yale animal care and use committee. In brief, 0.5-mm-thick, partial thickness skin sheets were divided into 1-cm² pieces and fixed onto similar size defects on the dorsum of recipients using staples. The skin reproducibly engrafted with a >95% success rate and was allowed to heal for 5 wk before host reconstitution or graft treatment. The few animals with skin grafts that failed to completely heal were excluded from experimental use.

**PBMC isolation and reconstitution**

Human leukocytes were collected by leukapheresis of adult volunteer donors under a protocol sanctioned by the Yale human investigations committee. The PBMC were isolated using lymphocyte separation medium (Cappel) according to the manufacturer’s instructions. The cells were stored in 10% DMSO at −196°C and were thawed and washed before use. SCID/beige mice were reconstituted with 3 × 10⁶ human PBMC by i.p. inoculation 5 wk after skin engraftment. The number of circulating human T cells was evaluated by flow cytometry as previously described (22). In brief, heparinized retro-orbital venous samples were obtained 7 days after engraftment and the erythrocytes were lysed. The leukocytes were incubated with FITC-conjugated mouse anti-human CD3 (Immunotech, Westbrook, ME) and Quantum Red-conjugated rat anti-human CD45 (Sigma, St. Louis, MO). The number of circulating human T cells, with a frequency >0.5% of mouse leukocytes, was routinely detected in >95% of recipients. The few animals that failed to reconstitute were excluded from analysis.

**Cytokine treatment**

Recombinant human TNF (5 U/ng) was a gift from Dr. Dale Godson (VIDO, Saskatoon, Canada). The cytokines were diluted in saline at various concentrations, and 25-µl volumes were administered directly into the grafts by intradermal injection. The treatment was initiated 5 wk after skin grafting and 3 days after reconstitution in those animals that received human PBMC. The cytokines were administered every 48 h (days 1, 3, 5, and 7), and the skin was harvested 4 h after the final dose. Control animals received sterile, pyrogen-free saline alone.

**Histology and immunohistochemistry**

Pig skin grafts were harvested and bisected. Half the graft was fixed in 10% buffered formalin, and hematoyxlin and eosin (H&E) staining was performed on 3-µm-thick, paraffin-embedded sections using standard techniques. The other half was snap-frozen in OCT (Miles, Elkhart, IL), and 4-µm-thick sections were obtained. Immunostaining was performed as previously described (22). In brief, the sections were fixed in acetone for 10 min and incubated overnight at 4°C in the presence of isotype-matched, nonbinding control Abs or the following primary Abs: mouse anti-human CD3 (UCHT1, IgG1), mouse anti-human CD4 (MT310, IgG1), mouse anti-human CD8 (DK25, IgG1), mouse anti-human CD19 (HD37, IgG1), mouse anti-human CD45RA (4KB5, IgG1), mouse anti-human CD45RO (UCHL1, IgG2a), mouse anti-human CD56 (T199, IgG1), and mouse anti-human CD68 (EBM11, IgG1) from Dako (Carpenteria, CA); mouse anti-human perforin (69G, IgG2b) from Pharmingen (San Diego, CA); and mouse anti-swine MHC class I (74-11-10, IgG2b) and mouse anti-swine MHC class II (MSA3, IgG2a) from V around trans (Pullman, WA). Mouse anti-swine E-selectin (10H7, IgG1) and mouse anti-swine VCAM-1 (2A2, IgG1) were gifts from Drs. Scott Rollins and John Mueller (Alexandria Pharmaceuticals, New Haven, CT), mouse anti-swine CD54 (L525-IA6, IgG1) and mouse anti-swine platelet-endothelial cell adhesion molecule-1 (PECAM-1; LC1-4, IgG1) were obtained from Sorotec (Kidlington, U.K.), and rat anti-mouse CD45 (30-F11, IgG2b) and rat anti-mouse PECAM-1 (MEC 13.3, IgG2a) were purchased from PharMingen. The sections were then incubated with biotinylated goat anti-mouse or anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and binding of the secondary Abs was detected using avidin and biotinylated HRP (Elite ABC Vectorstain kit, Vector, Burlingame, CA). The peroxidase label was developed using 3-aminoethylcarbazole (Red AEC kit, Vector), and the sections were counterstained with hematoxylin.

**Scoring system**

The degree of graft microvascular injury was evaluated from H&E-stained sections by a dermatopathologist (J.M.M.), blinded to the treatment protocol. The scoring system was previously described (17). In brief, the percentage of dermal vessels showing endothelial cell loss or sloughing and intravascular thrombosis was assessed from an average of three high-power (∼200) microscopic fields using the following semiquantitative grading scale: grade 0, all vessels patent and uninvolved; grade 1, <25% of vessels show injury; grade 2, >25% of vessels show injury; and grade 3, >75% of vessels show injury. The staining intensity and distribution of porcine MHC Ags and adhesion molecules were evaluated by two independent observers (N.C.K.-S. and D.A.T.) in a blinded fashion as previously described (23). In brief, Ag immunostaining from an average of three high power (∼200) microscopic fields was assessed using the following semiquantitative grading scale: grade 0, absent staining or faint staining of an occasional vessel only; grade 1, faint staining of several vessels; grade 2, moderate intensity staining of most vessels; and grade 3, intense staining of most vessels. The staining intensity and distribution of human CD3 T cell infiltrates were similarly scored using the following semiquantitative grading scale: grade 0, none or occasional positive cells only; grade 1, sparse infiltration of positive cells; grade 2, moderate infiltration of positive cells; and grade 3, intense positively staining infiltrates.

The number of porcine microvessels in the dermis of pig skin grafts was determined by counting the number of vessels whose endothelial cells stained positively for swine MHC class I Ag from an average of three high power (∼200) microscopic fields. In selected sections, the number of vessels staining positively for pig MHC class I molecules correlated with that for porcine PECAM-1.

**Data analysis**

Results are expressed as the mean ± SEM. The data were analyzed by Mann-Whitney U test. Differences between groups were described as significant when p < 0.05.

**Results**

Pig skin grafts were not rejected by SCID/beige mice

Initial experiments demonstrated that split-thickness pig skin grafts healed with a normal histological appearance 3–5 wk after engraftment to SCID/beige mouse recipients, as previously reported (17). Intradermal administration of saline did not elicit any pathologic changes (Fig. 1A). The epidermis displayed healthy keratinocyte maturation, the dermal microvasculature and collagen bundles appeared undamaged, and there was no evidence of inflammation. Immunohistochemical analysis with species-specific anti-PECAM-1 mAbs confirmed that porcine endothelial cells were retained by the pig skin microvasculature, which inosculated with invading mouse microvessels at the graft margins (Fig. 1A.
A moderate basal level of swine MHC class I and II molecules and VCAM-1 expression by dermal endothelial cells was observed 5 wk after engraftment (Fig. 2), which was mildly decreased compared with that in freshly procured pig skin that had not been transplanted (data not shown). The endothelial cells did not acquire an activated phenotype, as almost all the graft microvessels were E-selectin negative (Fig. 2). The expression of MHC Ags and adhesion molecules in saline-treated grafts (Fig. 3) was similar to that in untreated grafts (data not shown).

**Human T cells did not injure quiescent porcine endothelial cells**

Additional experiments investigated whether recipient reconstitution with human PBMC 5 wk after pig skin engraftment would result in porcine endothelial cell injury. Although the PBMC isolates from different donors contained variable numbers of lymphocytes, macrophages, and NK cells, only human T cells emerged in the mouse circulation after i.p. injection (D. A. Tereb, unpublished observations). Inoculation of $3 \times 10^8$ human PBMC into SCID/beige mice resulted in a significant leukocytic infiltrate and signs of endothelial cell injury.

**FIGURE 1.** Representative photomicrographs (at ×200 magnification) of H&E-stained pig skin grafts. The SCID/beige mouse recipients were unreconstituted (A and C) or inoculated with $3 \times 10^8$ human PBMC, i.p., 5 wk after engraftment (B and D). The grafts were then treated with saline (A and B) or human TNF at 60 ng (C and D), intradermally, every other day, for 7 days. Insets demonstrate porcine (stained crimson) and mouse (stained blue) PECAM-1 immunostaining (A) or higher magnification ($\times 1000$) of the dermal microvasculature (B–D). Only the TNF-treated graft from a recipient reconstituted with human PBMC contained a significant leukocytic infiltrate and signs of endothelial cell injury.

**FIGURE 2.** Representative photomicrographs (at ×200 magnification) demonstrating that human TNF up-regulated porcine endothelial expression of MHC and adhesion molecules. Pig skin grafts were engrafted onto SCID/beige mice and treated with saline (A, C, E, and G) or human TNF at 60 ng (B, D, F, and H) intradermally every other day for 7 days. Immunohistochemical analysis was performed with mAbs to swine MHC class I Ag (A and B), MHC class II Ag (C and D), VCAM-1 (E and F), and E-selectin (G and H).
beige mice resulted in the appearance of a discrete population of circulating human CD3+ T cells within 7 days, from 0.5–30% of the total circulating leukocytes. The pig skin grafts developed a sparse perivascular leukocytic infiltrate with no signs of endothelial cell injury 7 days after reconstitution (Fig. 1B). Porcine endothelial MHC Ag and VCAM-1 expression were slightly increased, and swine E-selectin expression was not induced (Fig. 3).

High concentrations of human TNF directly elicited microvascular damage of pig skin grafts

The effects of exogenous human TNF on pig skin grafts were assessed by histology. Intradermal injection of TNF at 2000 ng doses destroyed most of the pig microvessels, resulting in diffuse hemorrhagic necrosis of the grafts within 7 days (Table I). The phenotype of porcine endothelial cells at this dose of TNF therefore could not be evaluated. Administration of TNF in 600-ng doses for 7 days did not diminish the number of pig microvessels compared with that in saline-treated controls. Such pig skin grafts sustained significant endothelial cell injury, but minimal intravascular thrombosis (Table I). The dermis was edematous with focal areas of extravasated erythrocytes, and the epidermis had focal areas of necrosis. The microvascular damage at high doses of TNF was independent of recipient reconstitution with human PBMC (Table I).

Low concentrations of human TNF initiated human T cell-dependent injury of pig endothelial cells

In contrast, human TNF in 60- or 200-ng doses for 7 days resulted in significant graft microvascular injury only among animals receiving human PBMC (Table I). Pig skin from reconstituted animals exhibited signs of leukocytic infiltration, edema, endothelial cell loss, and scattered fibrin thrombi (Fig. 1D). Grafts from unreconstituted SCID/beige mice had minimal signs of injury (Fig. 1B).

**Table I. Effects of human TNF on pig skin grafts in human PBMC-SCID/beige mice**

<table>
<thead>
<tr>
<th>Human TNF (ng)</th>
<th>Human PBMC</th>
<th>No. Skin Grafts</th>
<th>No. of Pig Vessels</th>
<th>Endothelial Injury</th>
<th>Vascular Thrombosis</th>
<th>Human CD3+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>−</td>
<td>23</td>
<td>48 ± 4</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>17</td>
<td>69 ± 11</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>1.6 ± 0.6*</td>
</tr>
<tr>
<td>60</td>
<td>−</td>
<td>4</td>
<td>82 ± 8*</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>2.1 ± 0.4*</td>
</tr>
<tr>
<td>60</td>
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<td>6</td>
<td>97 ± 14</td>
<td>1.7 ± 0.4*</td>
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<td>1.6 ± 0.6*</td>
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<tr>
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<td>8</td>
<td>54 ± 8</td>
<td>0.4 ± 0.3</td>
<td>0.3 ± 0.3</td>
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<tr>
<td>200</td>
<td>+</td>
<td>8</td>
<td>61 ± 20</td>
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<td>0.3 ± 0.2</td>
<td>2.1 ± 0.4*</td>
</tr>
<tr>
<td>600</td>
<td>−</td>
<td>10</td>
<td>58 ± 13</td>
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<td>0.2 ± 0.1</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>600</td>
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<td>8</td>
<td>66 ± 8</td>
<td>1.1 ± 0.2*</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>2000</td>
<td>−</td>
<td>13</td>
<td>11 ± 1*</td>
<td>1.3 ± 0.4*</td>
<td>0.3 ± 0.3</td>
<td>1.8 ± 0.3*</td>
</tr>
<tr>
<td>2000</td>
<td>+</td>
<td>16</td>
<td>12 ± 5*</td>
<td>1.5 ± 0.3*</td>
<td>0.8 ± 0.3*</td>
<td>1.8 ± 0.3*</td>
</tr>
</tbody>
</table>

* Pig skin was engrafted onto SCID/beige mice, which were either not reconstituted or inoculated with 3 × 10^8 human PBMC i.p. after 5 wk, and the grafts were then treated with various doses of human TNF, intradermally, every other day, for 7 days. Data represent mean values with SEM. Cytokine-treated groups were compared to untreated groups. Statistical significance was determined by the Mann-Whitney U test.

**, p < 0.05.
1C). TNF in 60-ng doses provided an angiogenic stimulus, with the number of porcine dermal microvessels increasing compared with that in saline-treated controls (Table I). TNF in 200-ng doses did not significantly alter the density of pig vessels, perhaps reflecting a balance of angiogenic and destructive effects.

Immunohistochemical analysis was performed using Abs to human leukocyte markers to determine whether the microvascular injury was associated with TNF-mediated recruitment and activation of human PBMC. A marked increase in graft-infiltrating CD3^+ T cells was observed with 60- and 200-ng doses of TNF compared with that in saline-treated skin (Table I and Fig. 4, A and B), consisting of approximately equal numbers of CD4^+ and CD8^+ cells (data not shown). The T cells were CD45RO^+ (Fig. 4C) and CD45RA^− (data not shown), and some human effector cells were perforin^+ (Fig. 4D). There were no infiltrating CD19^+ B cells (data not shown), CD56^− NK cells (Fig. 4E), or CD68^+ macrophages (Fig. 4F), consistent with the failure of these cell types to recirculate in the host after i.p. inoculation. Pig CD45^+ resident leukocytes or Langerhans cells persisted in the skin (Fig. 4G), and human TNF recruited a moderate number of mouse CD45^+ macrophages and neutrophils to the grafts (Fig. 4H).

**Human TNF up-regulated porcine endothelial MHC Ags and adhesion molecules**

To further analyze the basis for human TNF-induced activation and recruitment of human T cells to pig skin grafts, immunohistochemical analysis was undertaken to evaluate the expression of endothelial MHC Ags and adhesion molecules. Swine-specific mAbs to MHC class I molecules (24), MHC class II molecules (25), VCAM-1 (13), and E-selectin (10) were used, which did not cross-react with either human or murine Ags (data not shown). The phenotype of endothelial cells from dermal capillaries or from larger vessels in the superficial vascular plexus were similar and were thus evaluated as a single entity. Administration of human TNF was associated with a dose-dependent up-regulation of porcine MHC class I Ag and VCAM-1 expression on endothelial cells (Fig. 3). Similarly, the expression of these molecules was induced on basal keratinocytes and vascular smooth muscle cells (Fig. 2).

A modest increase in endothelial MHC class II molecule expression reached statistical significance at the 200-ng dose (Fig. 3). TNF markedly induced endothelial E-selectin expression to a similar degree at all doses tested (Figs. 2 and 3). Recipient reconstitution with human PBMC did not significantly modulate the TNF-induced up-regulation of porcine MHC Ags or adhesion molecules (Fig. 3).

**Human TNF-mediated injury of pig skin grafts was potentiated by porcine IFN-γ**

Finally, we examined whether porcine IFN-γ modulated human TNF-induced activation and injury of pig endothelial cells. Concurrent administration of IFN-γ in 200-ng doses and TNF in 60-ng doses for 7 days resulted in significant microvascular damage compared with the minimal endothelial cell injury resulting from treatment when either cytokine was used alone (Table II). Strikingly, the combination of cytokines elicited thrombosis of many graft vessels (Fig. 5), which was not seen with TNF alone at up to 30-fold higher doses (Table I). Host reconstitution with human PBMC did not augment the injury or thrombosis of porcine microvessels, although a marked CD3^+ T cell infiltrate was observed with the combined cytokine treatment (Table II).

Porcine IFN-γ in 200-ng doses for 7 days up-regulated swine MHC class I and II Ag expression, minimally increased porcine VCAM-1 expression, and did not induce pig E-selectin expression (Fig. 6). Concomitant intradermal administration of human TNF in 60-ng doses augmented the up-regulation of pig MHC class II and VCAM-1 by porcine IFN-γ, but had no additional effect on swine MHC class I or E-selectin expression compared with either cytokine alone (Fig. 6). Recipient reconstitution with human PBMC had little effect on the regulation of endothelial Ag expression by porcine IFN-γ or by the combined cytokine treatment (Fig. 6).

**Discussion**

This report describes the in vivo effects of human TNF on pig skin xenografts in the presence or the absence of human PBMC using chimeric immunodeficient murine hosts. Although the human PBMC-SCID/beige mouse model does not recreate a complete human immune system, it nevertheless provides a useful strategy to study the interaction of human T cells with allogeneic or xenogeneic endothelial cells. The microvascular damage of human skin in
this model is mediated by CTL, is attenuated by immunosuppressive agents, and does not require Ab-dependent mechanisms (20). The lack of preformed Abs and the failure of human NK cells to reconstitute the host enable the study of cell-mediated xenoresponses in the absence of hyperacute or acute vascular rejection. It is generally predicted that human anti-porcine T cell xenoresponses will be greater than alloresponses (3, 4). The finding that pig skin xenografts are not rejected by human PBMC in SCID/beige mice (17), in contrast to the marked infiltration and destruction of allografts in the same model (18–21), is unexpected. We confirm that pig skin grafts, in which the microvascular endothelial cells are of a basal quiescent phenotype, are minimally infiltrated and injured by adoptively transferred human T cells in immunodeficient mouse hosts. The brisk rejection of allografts in parallel experiments excludes T cell anergy as a reason for the absence of pig xenograft injury in human PBMC-SCID/beige mice.

Cell-mediated xenograft rejection requires the specific recognition of porcine Ags by human T cells. In vitro studies have documented vigorous direct activation of human CD4+ and CD8+ T cells by pig endothelial cells (5–7) and robust indirect presentation of porcine peptides to human CD4+ T cells by human APC (7–9). We demonstrate that MHC class I and II molecules are expressed by porcine dermal endothelial cells up to 6 wk after skin engraftment to SCID/beige mice. The basal expression of MHC class I and II Ags in pig skin grafts may be an intrinsic property of microvascular endothelial cells or may be induced by the large number of resident pig leukocytes. In contrast, the expression of MHC class I and II molecules by arterial endothelial cells is lost within 7–14 days of transplanting pig coronary arteries into immunodeficient mice, in which there is complete emigration of graft passenger leukocytes (26). Administration of human TNF increases the basal expression of swine MHC class I Ags on endothelial cells of pig skin grafts, extending similar in vitro findings (14). The up-regulation of porcine MHC class II molecules by human TNF is surprising, although this may be an indirect effect mediated through activation of resident pig leukocytes. Human TNF has been reported to induce the expression of MHC class II Ags by cultured porcine endothelial cells (14, 15); however, these experiments did not rigorously exclude contaminating leukocytes or an IFN-γ-dependent response. In human endothelial cells, it is well documented that TNF increases the level of expression of MHC class I, but not class II, Ags (27), although TNF can increase IFN-γ-induced MHC class II expression on other human cell types, such as pancreatic endocrine cells (28) and various tumor cells (29). Predictably, both basal and TNF-induced expression of endothelial MHC Ags are up-regulated by porcine IFN-γ treatment of pig skin grafts. In addition to stimulation of human T cells by swine MHC xenoinigenes, there is potent costimulation provided by porcine endothelial B7-2 (5, 30). We have documented a constitutive expression of B7-2 by microvascular endothelial cells of pig skin grafts that is not modulated by cytokine treatment (unpublished observations). In contrast, human endothelial cells do not express B7-2 or costimulate T cells through a CD28 pathway (5). Thus, the unresponsiveness of human T cells to saline-treated, pig skin grafts in SCID/beige mice cannot be explained by diminished graft immunogenicity.

An increase in the surface expression of adhesion molecules is a fundamental characteristic of endothelial cell activation by proinflammatory cytokines (2). Cultured porcine endothelial cells are similar to human cells in that they do not express E-selectin or

**Table II. Effects of porcine IFN-γ and human TNF on pig skin grafts in human PBMC-SCID/beige mice**

<table>
<thead>
<tr>
<th>Porcine IFN-γ (ng)</th>
<th>Human TNF (ng)</th>
<th>Human PBMC</th>
<th>No. of Skin Grafts</th>
<th>No. of Pig Vessels</th>
<th>Endothelial Injury</th>
<th>Vascular Thrombosis</th>
<th>Human CD3+ Cells</th>
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<tr>
<td>0</td>
<td>0</td>
<td>−</td>
<td>23</td>
<td>48 ± 4</td>
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<td>0.0 ± 0.0</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>0</td>
<td>0</td>
<td>+</td>
<td>17</td>
<td>69 ± 11</td>
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<td>0.1 ± 0.1</td>
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<td>−</td>
<td>8</td>
<td>74 ± 11</td>
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<td>13</td>
<td>60 ± 9</td>
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<td>0.2 ± 0.2</td>
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<td>0</td>
<td>60</td>
<td>−</td>
<td>4</td>
<td>82 ± 8*</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>1.6 ± 0.6*</td>
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<tr>
<td>0</td>
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<td>6</td>
<td>97 ± 14</td>
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<td>0.2 ± 0.2</td>
<td>2.6 ± 0.2*</td>
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<tr>
<td>200</td>
<td>60</td>
<td>−</td>
<td>5</td>
<td>78 ± 7*</td>
<td>1.8 ± 0.6*</td>
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<tr>
<td>200</td>
<td>60</td>
<td>+</td>
<td>6</td>
<td>94 ± 17</td>
<td>2.0 ± 0.5*</td>
<td>0.6 ± 0.2*</td>
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</table>

* Pig skin was engrafted onto SCID/beige mice, which were either not reconstituted or inoculated with 3 × 10^8 human PBMC i.p. after 5 wk, and the grafts were then treated with porcine IFN-γ and/or human TNF, intradermally, every other day, for 7 days. Data represent mean values with SEM. Cytokine-treated groups were compared to untreated groups. Statistical significance was determined by the Mann-Whitney U test.

* p < 0.05.

**FIGURE 5.** Representative photomicrographs (at ×200 magnification, insets at ×1000 magnification) of H&E-stained pig skin grafts. The SCID/beige mouse hosts were not reconstituted. The grafts were treated with porcine IFN-γ alone at 200 ng (A) or a combination of porcine IFN-γ at 200 ng and human TNF at 60 ng (B) intradermally every other day, for 7 days. Administration of both cytokines resulted in marked endothelial cell injury and microvascular thrombosis.
VCAM-1 under quiescent conditions, but do so after TNF exposure (10–13). In accord with studies of human skin engrafted to SCID mice (31), administration of human TNF induces the de novo expression of E-selectin on microvascular endothelial cells of pig skin grafts. Porcine IFN-γ does not enhance TNF-mediated effects on E-selectin expression. Human TNF also up-regulates VCAM-1 expression by porcine dermal endothelial cells. However, pig skin grafts express basal levels of VCAM-1, in the absence of cytokine treatment, for up to 6 wk after engraftment, whereas microvascular endothelial cells of human skin transplanted to SCID mice are VCAM-1 negative (18). We have consistently detected a basal expression of endothelial VCAM-1, but not E-selectin, in porcine skin, cardiac, and renal vessels (unpublished observations), and the constitutive expression of VCAM-1, but not E-selectin, by certain pig organs has been previously reported (32). Porcine IFN-γ modestly augments TNF-induced up-regulation of VCAM-1 expression. In contrast to exogenous administration of cytokines, circulating human T cells do not significantly activate pig endothelium in SCID/beige mice. Despite the specificity of IFN-γ, the readily inducible expression of E-selectin and the significant up-regulation of VCAM-1 expression on porcine endothelial cells by human TNF suggest that activation of graft endothelial cells by proinflammatory cytokines will not be a limiting factor in human anti-porcine cell-mediated xenoresponses in vivo.

Lymphocyte homing requires adhesion to endothelial cells by multiple steps, including initial tethering to selectins and subsequent firm attachment to Ig superfamily members, such as VCAM-1 and ICAM-1 (33). In vitro studies with blocking mAbs have established that pig E-selectin and VCAM-1 bind to ligands on human T cells (10–13) and that these adhesion receptors are important for attachment of human leukocytes to activated porcine endothelial cells under simulated flow conditions (34). In addition, animal experiments have documented the role of porcine E-selectin and VCAM-1 in recruiting pig lymphocytes to sites of immune-mediated dermal inflammation (32, 35). This report correlates the infiltration of pig skin grafts by human CD45RO+/CD3+ memory T cells with the TNF-induced, de novo expression of endothelial E-selectin and augmented levels of endothelial VCAM-1. The results imply that porcine E-selectin or another surface marker of endothelial cell activation is more critical than porcine VCAM-1 in the emigration of human lymphocytes across pig vessels, since saline- or IFN-γ-treated grafts, with moderate basal levels of VCAM-1 and little expression of E-selectin, have minimal human T cell infiltrates. Our observations are consistent with previous findings that E-selectin recruits a local infiltrate of memory T cells (36, 37). ICAM-1 expression was not studied due to a lack of suitable reagents. The difficulty in identifying swine ICAM-1 suggests a significant difference from its human homologue, although studies of the corresponding human ligand, LFA-1, have inferred that pig ICAM-1 or an alternative LFA-1 receptor is functional across the species differences (6, 38). Our data indicate that adhesion molecules expressed by activated, but not quiescent, porcine endothelial cells are sufficient to recruit an infiltrate of human T cells to pig xenografts under physiologic flow conditions.

Cytokines are effectors of tissue injury in addition to mediators of inflammatory responses (2). High concentrations of TNF result in pig skin graft damage in the absence of human PBMC. This may represent a direct cytotoxic effect on porcine endothelial cells, and TNF is described to cause apoptosis of cultured endothelial cells under certain conditions (39). However, TNF-induced activation of resident pig leukocytes and infiltrating mouse leukocytes undoubtedly occurs in the grafts, and indirect cytolytic mechanisms cannot be excluded in this model. Moreover, TNF-treated endothelial cells are more susceptible to lysis by neutrophils (40). In contrast, pig skin graft injury at low concentrations of TNF is dependent on the presence of human PBMC. A strong correlation exists between infiltration of pig skin grafts by human T cells in response to low dose TNF treatment and signs of microvascular damage. The presence of perforin-positive effector cells supports a mechanism of cell-mediated cytotoxicity of xenogeneic cells. Surprisingly, TNF, which promotes endothelial thrombogenicity (41),

![FIGURE 6](http://www.jimmunol.org/)
did not alone result in significant intravascular thrombosis unless administered together with porcine IFN-γ or the hosts reconstituted with human PBMC. IFN-γ may augment the procoagulant actions of TNF or may activate resident leukocytes to secrete factors such as porcine IL-1. IL-1 acts additively with TNF to increase the expression of endothelial procoagulant molecules (41) and interacts synergistically with TNF in priming the local Shwartzman reaction (42). The species specificity of IL-1 is controversial, as some investigators have found that human IL-1 activates porcine endothelial cells (11, 12), whereas others have not (10, 13, 15). Furthermore, mouse IL-1 does not activate human or porcine cells (43, 44). The complexity of interactions among the three species comprising the chimeric model underscores the molecular incompatibilities inherent in xenogeneic cellular responses.

The results of our experiments identify TNF as a key mediator of human T cell recruitment and activation by pig xenografts and that the absence of porcine endothelial cell activation may prevent cell-mediated xenoresponses. Thus, TNF signaling molecules and TNF-responsive gene products are appropriate therapeutic targets for xenotransplantation immunosuppressive strategies. Inhibitory anti-TNF Abs or soluble TNF receptor fusion proteins are successful in treating chronic inflammatory diseases (45, 46) and could be administered to xenograft recipients. A more selective approach would be through genetic engineering of pig donors. Because gene deletion by homologous recombination technology is not available at present for mammals other than mice, breeding of TNF receptor- or E-selectin-deficient pigs is not yet possible. However, transgenes can be introduced into the pig genome (47). In vitro experiments have demonstrated that porcine endothelial cells transfected with adenoviral vectors containing human genes for inhibitors (48) or dominant negative mutants (49) of TNF signaling molecules are resistant to cytokine-induced activation. Creation of such transgenic pigs has been proposed to protect against acute vascular rejection (50) and may prevent T cell xenoresponses as well.

We conclude that human T cells adoptively transferred to SCID/beige mice are not anergic, that pig skin grafts remain immunogenic after transplantation, that porcine endothelial cells are readily activated by exogenous human TNF, that activated porcine endothelial cells recruit human T cells, and that infiltrating human lymphocytes differentiate into effector cells and elicit xenograft injury. Therefore, the absence of spontaneous infiltration and injury of pig skin grafts in human PBMC-SCID/beige mice is probably due to insufficient activation of human xenoreactive T cells in this model. Indeed, a different strategy of adoptively transferring sensitized human PBMC to recombine-activating gene-1-deficient mice does result in pig skin graft damage (51, 52). Sensitization of human PBMC to porcine cells in vitro before adoptive transfer may enhance direct presentation of pig Ags due to increased intercellular contact, but may also allow indirect presentation of porcine peptides by human APC. Because human CD68+ macrophages and CD19+ B cells remain in the peritoneal cavity of the hosts and do not recirculate, indirect presentation of porcine xenogenitans by human APC is unlikely to occur in the SCID/beige mouse model. Thus, direct recognition of foreign Ags may be sufficient for allograft injury in this model, but insufficient for xenograft rejection due to the greater dependence of xenogeneic cellular responses on indirect presentation pathways (53, 54). An alternative explanation is that quiescent porcine endothelial cells do not express sufficient adhesion molecules to allow for the attachment and activation of human T cells. Regardless of the mechanism, the chimeric immunodeficient mouse model identifies a significant difference between human xenogeneic vs allogeneic cellular responses and establishes that pig xenografts are not injured by human T cells under the same conditions that result in vigorous rejection of allografts.

In summary, intradermal administration of human TNF to pig skin grafts in SCID/beige mice elicits nonspecific inflammatory and immune-mediated injury of porcine endothelial cells after 1 wk of treatment, depending on the concentration of cytokine and the presence of human PBMC. High concentrations of TNF elicit microvascular damage due to direct toxicity and/or participation of murine innate immune responses. Low concentrations of TNF induce human PBMC-dependent endothelial cell injury. The recruitment and activation of human T cells by pig skin grafts in response to human TNF correlate with an up-regulation of porcine endothelial MHC and adhesion molecules, in particular with induction of E-selectin expression. In contrast, saline- or porcine IFN-γ-treated pig skin grafts, which do not express E-selectin, are not infiltrated or injured by human T cells within 1 wk of adoptive transfer to immunodeficient mouse hosts. TNF-induced microvascular damage, especially as manifested by thrombosis, is enhanced by porcine IFN-γ, which suggests that xenografts may be relatively less prone to cytokine-mediated injury due to the species-restricted effects of recipient IFN-γ. We have not proven that porcine endothelial activation, human T cell accumulation, and pig skin graft damage are interdependent, although the correlation of these cytokine-induced events suggests a causal relationship. Our results indicate that TNF signaling molecules and TNF-responsive gene products are appropriate therapeutic targets to prevent human anti-porcine cell-mediated xenoresponses.

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References
cytokines on porcine endothelial cell MHC molecule expression: unique role for TNF-α in MHC class II induction. Transplant. Proc. 26:1152.

15. Batten, P., M. H. Yacoub, and M. L. Rose. 1996. Effect of human cytokines (IFN-γ, TNF-α, IL-1β, IL-4) on porcine endothelial cells: induction of MHC and adhesion molecules and functional significance of these changes. Immunology 87:127.


