Innate and Adaptive Immune Responses to Nonvascular Xenografts: Evidence That Macrophages Are Direct Effectors of Xenograft Rejection

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Innate and Adaptive Immune Responses to Nonvascular Xenografts: Evidence That Macrophages Are Direct Effectors of Xenograft Rejection

Annette Fox, Joanne Mountford, Andrea Braakhuis, and Leonard C. Harrison

Nonvascularized xenograft rejection is T cell mediated, but is dependent on initial macrophage (Mφ) infiltration. We developed an i.p. transplant model to define the roles of Mφ and T cells in xenograft rejection. Nonobese diabetic or BALB/c mice were injected i.p. with xenogeneic, allogeneic, or syngeneic cells, and the responding cells in subseuent lavages were assessed by flow cytometry and adoptive transfer. Neutrophils and monocytes/elicited Mφ were rapidly recruited in response to xenogeneic pig (PK15 or spleen) cells and, to a significantly lesser extent, allogeneic cells. These innate responses preceded T cell infiltration and occurred in their absence in SCID mice. Syngeneic cells induced negligible neutrophil or Mφ responses. Neutrophils and Mφ induced by xenogeneic cells in SCID mice stimulated T cell recruitment after transfer to immunocompetent mice. T cells in turn were required for Mφ activation and xenogeneic cell rejection. Thus, Mφ harvested from immunocompetent but not SCID mice injected with xenogeneic cells expressed activation markers and rejected xenogeneic cells when transferred into SCID mice. These findings demonstrate the interdependent roles of Mφ and T cells in xenograft rejection. The requirement for Mφ reflects their ability to mount a rapid, local innate response that stimulates T cell recruitment and, having received T cell help, to act as direct effectors of rejection. The Journal of Immunology, 2001, 166: 2133–2140.

The inadequate supply of human tissue for allografting has prompted interest in xenografts. Nonvascularized xenografts, such as tissue cultured fetal pig pancreas (FPP) or islets, are not prone to hyperacute rejection by preformed Abs, but are rejected within 10 days by an aggressive cell-mediated response that is difficult to prevent compared with allograft rejection (1–3). The mechanisms of cell-mediated xenograft rejection are poorly understood. Although nonvascularized xenograft rejection requires T cells, particularly CD4 T cells (4, 5), the exact role of T cells in graft rejection is not defined. Depletion and/or genetic manipulation studies have shown that B cells and Ab (6–8), IL-4, IL-5 and eosinophils (9), IFN-γ (10), Fc receptors (6), NK cells (11) and perforin (12) are each alone not essential for xenograft rejection.

It is increasingly recognized that innate immune responses mediated by monocytes/macrophages (Mφ), neutrophils, mast cells, and NK cells drive and shape adaptive immunity (13, 14). Cells of the innate immune system evolved to express receptors that recognize pathogen-associated molecular patterns (PAMPs), in particular distinctive oligosaccharide structures exquisitely different from self (14, 15). Generic expression of these germline-encoded, invariant receptors allows a rapid response to pathogens. In contrast, T cells of the adaptive immune system use randomly rearranged receptors that do not discriminate whether an Ag is or is not pathogen associated. However, innate immunity provides signals for T cell priming (15) and thereby focuses adaptive immunity on pathogen-associated Ags.

Mφ are the dominant infiltrating cell in pancreatic islet or FPP xenografts undergoing rejection, and some express an unusual CD4+ phenotype (11). Previously, we showed that Mφ are required for T cell infiltration and rejection of xenografts (16). In the present study, our aims were as follow: first, to investigate whether xenografts elicit an innate immune response; second, to define the interactions between Mφ and T cells in xenograft rejection; and finally, to determine whether Mφ are direct effectors of xenograft rejection. An i.p. transplant (IPT) model was developed in which xenogeneic cells are injected i.p., and responding cells are harvested for phenotypic and functional analysis by flow cytometry and adoptive transfer.

Materials and Methods

Animals

Nonobese diabetic (NOD), NOD-SCID, CB17-SCID, and BALB/c mice, aged 6–10 wk, were bred at the Walter and Eliza Hall Institute (Parkville, Australia). Outbred pregnant Landrace sows at 60–90 days of gestation were bred at the Victorian Institute of Animal Science (Werribee, Australia).

Preparation and injection of cells

The adherent epithelial-like pig kidney cell line, PK15, was obtained from American Type Culture Collection (Manassas, VA) and grown in Eagle’s MEM with nonessential amino acids and 5% FCS. Similarly, the adherent fibroblast-like BALB/c-3T3 cell line was obtained from American Type Culture Collection and grown in DMEM containing 5% FCS. Cell lines were harvested by incubation with trypsin and washed three times with mouse tonicity PBS. Spleens, harvested from mice and adult or fetal pigs, were disrupted by gentle extrusion through a fine wire mesh. The resultant single-cell suspensions were treated with 0.16 M NH4Cl to lyse RBC, then washed three times with PBS. All cells were resuspended at 2 × 10^7/ml in PBS, and 1 × 10^7 cells were injected i.p. Syngeneic splenocytes were
buffer maintained at 37°C. Cells at 2–10^3 through a 0.45-
2% BSA, 0.1% sodium azide, and 0.1% EDTA that had been filtered
were then centrifuged, and the pellet was resuspended in PBS containing
4.8 to 5.5 ml. Cell counts were performed with a hemocytometer. Cells
were then centrifuged, and the pellet was resuspended in PBS containing
2% BSA, 0.1% sodium azide, and 0.1% EDTA that had been filtered
through a 0.45-μm pore size sterile filter (wash buffer).

Flow cytometry

The mAbs used for flow cytometry, their specificities, and their sources are
listed in Table I. mAbs were either directly fluorochrome conjugated or
biotinylated and detected with streptavidin-PE (Caltag, Burlingame, CA).
Cells were kept at 4°C throughout the staining procedure. Cells (at least
1 × 10^6/well) were incubated with FcγRII receptor block (Table I) for 10
min, then incubated with primary mAbs for 30 min. When using biotinyl-
ated primary mAbs, cells were washed twice with wash buffer, then in-
cubated with streptavidin-PE for 15 min. After staining, cells were washed
twice with wash buffer, then fixed with 3% (v/v) formaldehyde in PBS.
When staining for FcγRII receptors, blocking was omitted and the pri-
mary mAb was detected with FITC-conjugated anti-RIg (Vector Laborato-
ries, Burlingame, CA). The number of cells expressing a particular
marker was calculated by multiplying percentages obtained from flow cy-
tometry by the concentration of cells in lavage fluid. In this way, variability
due to lavage volumes recovered was avoided. To discriminate between
responding cells and injected splenocytes, two-color analysis was per-
formed with an mAb specific for H-2D^d (Table I) to detect allogeneic
BALB/c splenocytes and by carboxyfluorescein diacetate succinimidyl es-
ter labeling to detect syngeneic NOD splenocytes.

Intracellular cytokine analysis

Mice were given either a single i.p. injection of cells or two injections (1
mo apart) and i.p. cells were harvested 1–5 days later for intracellular
cytokine analysis. Lavages were performed as previously described, with
addition of 10 μg/ml brefeldin A (Sigma, St. Louis, MO) to the lavage
buffer maintained at 37°C. Cells at 2–10 × 10^6/ml were incubated in this
buffer for an additional hour at 37°C to facilitate intracellular accumula-
tion of cytokines. After staining for surface markers, as described above, cells
were fixed, permeabilized, and stained with cytokine-specific mAbs (Table
I) according to the protocols of PharMingen (San Diego, CA).

Table I. mAbs used for flow cytometry

<table>
<thead>
<tr>
<th>mAb</th>
<th>Specificity</th>
<th>Source</th>
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<tr>
<td>F4/80</td>
<td>F4/80 (Mφ/monocytes)</td>
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<td>2A42</td>
<td>FcγRII/II receptors</td>
<td>PharMingen</td>
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<tr>
<td>R6-6C5</td>
<td>GR1 (neutrophils/mcocytes)</td>
<td>PharMingen</td>
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<td>DX5</td>
<td>Pan-NK cells</td>
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<td>M142</td>
<td>MHC class I</td>
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<td>16/10A1</td>
<td>CD80</td>
<td>PharMingen</td>
</tr>
<tr>
<td>3/23(b)</td>
<td>CD40</td>
<td>PharMingen</td>
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<td>34-2-12</td>
<td>H-2D^d</td>
<td>PharMingen</td>
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<tr>
<td>RA3.6B2</td>
<td>B220 (B cells)</td>
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<td>29B</td>
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<td>H129.19</td>
<td>CD4</td>
<td>Sigma</td>
</tr>
<tr>
<td>53-6.7</td>
<td>CD8α</td>
<td>Sigma</td>
</tr>
<tr>
<td>53-5.8</td>
<td>CD8β</td>
<td>Sigma</td>
</tr>
<tr>
<td>OX-6</td>
<td>Rat RT1-B Ag; cross-reacts with mouse H2-A molecules including I-A^b, but not H-2A^d</td>
<td>Serotec (Kidlington, U.K.)</td>
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<td>2G9</td>
<td>I-A^d II</td>
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<td>TNF-α</td>
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<td>R3-34</td>
<td>Rat IgG1 isotype control</td>
<td>PharMingen</td>
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<tr>
<td>R35-38</td>
<td>Rat IgG2b isotype control</td>
<td>PharMingen</td>
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</tbody>
</table>

Induction and recovery of elicited Mφ and neutrophils for adoptive transfer

CB17-SCID mice were injected i.p. with xenogeneic PK15 cells. Mice were
killed 1 day later, and peritoneal lavages were performed. Lavaged
cells were pelleted by centrifugation and resuspended in 30% Percoll (Am-
ersham Pharmacia Biotech, Uppsala, Sweden), then loaded onto a discon-
tinuous 45 and 70% Percoll gradient. The gradient was centrifuged at
400 × g_max for 20 min at room temperature in a swing-out rotor. PK15 cells
were mainly retained in the 30% layer that was discarded. Cells at the
45–70% Percoll interface, enriched for elicited Mφ and neutrophils, were
collected, washed twice with PBS, and resuspended in PBS for i.p. injec-
tion. The compositions of lavaged cells and subsequent Percoll fractions
were determined by flow cytometry. In several experiments lavage cells
were stained with M142, the mAb specific for mouse MHC class I (Table
I), to enable neutrophils and elicited Mφ to be differentiated from other
cells, including PK15 cells (see Fig. 4A), and then sorted to >99% purity
using the MoFlo (Cytomation, Fort Collins, CO).

Induction and recovery of Mφ for adoptive cotransfer

BALB/c mice were injected i.p. with xenogeneic, allogeneic, or syngeneic
cells and peritoneal lavages were performed 5 days later. For Mφ enrich-
ment, CD4-, CD8-, and B220-positive cells were depleted using MACS
magnetic microbeads conjugated to mAbs specific for these markers
(Milenyi Biotec, Auburn, CA). The cellular composition of lavages, be-
fore and after MACS separations, was determined by flow cytometry.
Equal numbers of postdepletion, xenogeneic-, allogeneic-, or syngeneic-stim-
ulated lavage cells were injected i.p. into CB17-SCID recipients along
with 1 × 10^7 PK15 cells. Recipient mice were also given 0.1 mg i.p. of the
CD4 T cell-depleting mAb, GK1.5, to deplete any remaining CD4 T cells
that may have been transferred.

Statistics

Results were analyzed by Student’s two-tailed t test, using Microsoft Excel
software. A p < 0.05 was considered to be significant.

Results

Kinetics of i.p. responses to xenogeneic, allogeneic, or syngeneic cells

Xenogeneic pig (PK15 or spleen) cells stimulated rapid recruitment of neutrophils, identified by their GR-1^hi (17), intermediate
side scatter (SSC) profile when injected i.p. into NOD (Fig. 1A) or
BALB/c mice (data not shown). Within 1 day, peritoneal neutro-
phils were markedly and significantly increased (~10-fold; p < 0.05) in response to xenogeneic cells compared with syngeneic
spleen cells or PBS (Fig. 1B). Neutrophil numbers increased slightly 1 day after injecting allogeneic spleen cells, but not significantly compared with controls (Fig. 1B). Neutrophil responses to xenogeneic cells were significantly greater than those to allogeneic cells (xenogeneic vs allogeneic spleen cells, 5.8-fold difference (p < 0.003; Fig. 1B); PK15 cells vs 3T3 cells, 5.1-fold difference (p = 0.008)). Xenogeneic cell stimulated-monocytes/elicited Mφ were identifiable by their F4/80<sup>int</sup>, GR1<sup>int</sup>, intermediate SSC profile (17). This profile was clearly distinct from the F4/80<sup>very high</sup>, GR1<sup>low</sup>, high SSC profile of resident Mφ (17) recovered from PBS-injected mice (Fig. 1A). Subsequent differentiation of elicited Mφ was evidenced by the gradual increase in F4/80 and GR1 intensity, intermediate SSC profile of resident Mφ (17) recovered from PBS-injected mice (Fig. 1A). Within 2 days of injecting xenogeneic cells, monocyte/elicited Mφ numbers were significantly higher than in PBS or syngeneic cell recipients (Fig. 1B). However, monocyte/elicited Mφ responses to xenogeneic cells were significantly greater than to allogeneic cells (xenogeneic vs allogeneic spleen cells, 2.4-fold difference (p < 0.005); PK15 cells vs 3T3 cells, 2.1-fold difference (p = 0.007)).

T cell responses to allogeneic and xenogeneic cells were not detected until day 2 and peaked around day 5 (Fig. 1B). All CD4 cells coexpressed CD3, and an mAb specific for the CD8 β-chain was used to demonstrate that CD8 T cells expressed the CD8αβ heterodimer (data not shown). Initially, CD4 T cells exceeded CD8 T cells in all responses (Fig. 1B), but by day 5 of xenogeneic responses there were as many CD8 T cells (CD4:CD8 T cell ratio, 1.09 ± 0.18). In contrast, allogeneic 3T3 cells always elicited more CD4 than CD8 T cells, with a significantly higher CD4:CD8 T cell ratio (2.14 ± 0.48; p = 0.002) by day 5. B cell numbers increased 1.6- to 2.5-fold in response to either xenogeneic cells or allogeneic cells compared with PBS-injected controls (data not shown). DX5<sup>+</sup> NK cells increased in response to allogeneic (days 1 and 2) and especially xenogeneic splenocytes (days 2–5) in BALB/c mice, but NK cell numbers were very low (Fig. 1B) and were not increased at all in some experiments. NK cell responses were not assessed in NOD mice, which are NK cell deficient. There was little or no response of any kind to syngeneic cells (Fig. 1B), demonstrating that the changes detected were to foreign cells rather than to trauma or cells per se. Similar results were obtained in C57BL6 and BALB/c mice (data not shown).

**Intraperitoneal responses in SCID mice**

Xenogeneic or allogeneic cells were injected into SCID mice to investigate the T cell dependence of responses. As in immunocompetent mice, neutrophils and elicited Mφ dominated in lavages from xenogeneic PK15 cell- and PBS-injected NOD mice 1 day after injecting xenogeneic PK15 cells (Fig. 2A). In contrast, few or no neutrophils or elicited Mφ were detected in response to syngeneic, allogeneic, or xenogeneic spleen cells or PBS (Fig. 1B). Neutrophil numbers increased slightly 1 day after injecting allogeneic spleen cells, but not significantly compared with controls (Fig. 1B). Neutrophil responses to xenogeneic cells were significantly greater than those to allogeneic cells (xenogeneic vs allogeneic spleen cells, 5.8-fold difference (p = 0.003; Fig. 1B); PK15 cells vs 3T3 cells, 5.1-fold difference (p = 0.008)). Xenogeneic cell stimulated-monocytes/elicited Mφ were identifiable by their F4/80<sup>int</sup>, GR1<sup>int</sup>, intermediate SSC profile (17). This profile was clearly distinct from the F4/80<sup>very high</sup>, GR1<sup>low</sup>, high SSC profile of resident Mφ (17) recovered from PBS-injected mice (Fig. 1A). Subsequent differentiation of elicited Mφ was evidenced by the gradual increase in F4/80 and GR1 intensity, intermediate SSC profile of resident Mφ (17) recovered from PBS-injected mice (Fig. 1A). Within 2 days of injecting xenogeneic cells, monocyte/elicited Mφ numbers were significantly higher than in PBS or syngeneic cell recipients (Fig. 1B). However, monocyte/elicited Mφ responses to xenogeneic cells were significantly greater than to allogeneic cells (xenogeneic vs allogeneic spleen cells, 2.4-fold difference (p < 0.005); PK15 cells vs 3T3 cells, 2.1-fold difference (p = 0.007)).

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splenocytes or PBS were also compared in CB17-SCID mice (Fig. 2B). As with immunocompetent mice, xenogeneic splenocytes elicited stronger neutrophil and elicited Mφ responses than allogeneic splenocytes, and the response to syngeneic splenocytes was minimal (Fig. 2B).

**Fate of xenogeneic cells injected i.p.**

To monitor rejection, xenogeneic PK15 cells were injected into immunocompetent or SCID mice, and recoveries were compared over time. Within the first 2 days, there was considerable variability and no clear difference between immunocompetent and SCID mice (Fig. 3D). However, by day 3 PK15 cell recovery from immunocompetent mice was markedly reduced, and thereafter absent, in contrast to the continued recovery of PK15 cells from SCID mice (Fig. 3, B–D). The rapid loss of PK15 cells from immunocompetent compared with SCID mice along with the strong T cell response in the former indicates that xenogeneic cells are subject to T cell-dependent rejection. Additionally, in three experiments we found that PK15 cells were rejected within 1 day of injection into immunocompetent mice (n = 4) that had been primed with PK15 cells 28 days previously, consistent with a memory T cell response.

**Effect of xenogeneic-elicited Mφ and neutrophils on T cell recruitment**

Mφ- and neutrophil-enriched PK15 cell-depleted lavage cells from PK15 cell-injected SCID mice were transferred i.p. to BALB/c mice to examine their effect on T cell recruitment. PK15 cell-elicited, Percoll-enriched lavage cells from SCID mice, comprising 78% neutrophils and elicited Mφ and 3% PK15 cells (Fig. 4A), stimulated significantly more CD4 and CD8 T cell recruitment within 1 day of transfer to BALB/c mice than did control cells from PBS-injected SCID mice, comprising 15% (1.1 × 10^6) neutrophils and Mφ (Fig. 4B), or PK15 cells transferred alone in excess (5 × 10^6/mouse; Fig. 4B). By day 3, about 4.5 times more CD4 T cells had been recruited after transfer of PK15 cell-elicited neutrophils and Mφ compared with cells from PBS-injected SCID mice (Fig. 4C). However, by this time PK15 cells alone had induced more T cell recruitment, presumably because of the greater number transferred (Fig. 4C). When elicited Mφ and neutrophils from SCID mice injected with PK15 cells were sorted to >99% purity and transferred i.p. to BALB/c mice, significantly higher numbers of CD4 and CD8 T cells were recruited within 1 day than after transfer of control lavage cells from PBS-injected SCID mice (Fig. 4D).
Requirement for T cells in MΦ responses to xenogeneic cells

The requirement for T cells in MΦ responses to xenogeneic cells was examined by comparing MΦ from PK15 cell-injected immunocompetent and SCID mice. Within 5 days of PK15 cell injection into immunocompetent NOD mice, there was a 3.5-fold increase in MΦ that expressed MHC class II (p = 0.005) compared with mice injected with syngeneic splenocytes (Fig. 5). This was reflected by an increase in the total number of MHC class II MΦ from \(3.5 \times 10^5 \) to \(4.9 \times 10^5\) /ml. MΦ harvested from immunocompetent NOD mice 5 days after xenogeneic vs syngeneic stimulation also expressed significantly higher levels of CD80 (1.6-fold increase; p = 0.0001; Fig. 5), CD40 (1.4-fold increase; p = 0.04; data not shown), and FcR\(\gamma\)III/II (1.9-fold increase; p = 0.003; data not shown). In contrast, MΦ harvested from NOD-SCID mice 5 days after PK15 cell injection did not express MHC class II or increased levels of CD80 (Fig. 5) or CD40 (data not shown) compared with MΦ from syngeneic cell-injected controls. FcR\(\gamma\)III/II expression was not examined in SCID mice. Similar results were obtained when responses to xenogeneic and allogeneic cells were compared in BALB/c and CB17-SCID mice (data not shown).

As shown in SCID mice (Fig. 3), MΦ were not able to kill xenogeneic cells efficiently, if at all, in the absence of T cells. To determine whether MΦ could kill xenogeneic cells if given T cell help, they were recovered from BALB/c mice 5 days after PK15 cell injection, purified, and cotransferred with PK15 cells to CB17-SCID mice. As before, we found that the number of MHC class II+ MΦ increased after xenogeneic PK15 cell, but not syngeneic 3T3 cell, injection. Approximately 70% of the transferred cells were MΦ, up to 7% were neutrophils, and <10% were T cells. The SCID recipients were also injected with a CD4 T cell-depleting mAb (GK1.5), and T cells were not detected in peritoneal lavages. Recovery of PK15 cells from recipients of PK15 cell-stimulated MΦ was approximately 2.6-fold lower than that from recipients of 3T3 cell-stimulated MΦ after only 1 day and was very low or negligible after 2 days, demonstrating that PK15 cell-stimulated MΦ transferred rapid rejection (Fig. 6). Subsequently, transfer of at least \(5 \times 10^6\) MΦ was shown to be required for efficient rejection of \(1 \times 10^7\) PK15 cells. PK15 cell rejection was faster after the transfer of MΦ than after the transfer of primed, unfractioned lavage cells (Fig. 6) or primed T cells alone (data not shown).

![FIGURE 4.](http://www.jimmunol.org/) Effect of xenogeneic-elicited MΦ and neutrophils on T cell recruitment. Cells lavaged from PK15 cell-injected SCID mice were enriched for neutrophils and MΦ and depleted of PK15 cells after Percoll density gradient separation (A). These cells (\(7 \times 10^6\)), lavage cells from PBS-injected SCID mice (\(7 \times 10^6\)), or \(5 \times 10^6\) PK15 cells only were injected i.p. into BALB/c mice (n = 4–7). CD4 and CD8 T cell numbers (mean and SD) in BALB/c lavages were determined 1 and 3 days later by flow cytometry and cell counts. CD4 and CD8 T cells in BALB/c lavages were also enumerated 1 day after transfer of \(1 \times 10^6\) PK15 cell-elicited, MoFlo sorted (>99% purity) neutrophils and MΦ from SCID mice (n = 6) or lavage cells from PBS-injected SCID mice (n = 10; D). *, p value compared with PK15-SCID cell transfer.

![FIGURE 5.](http://www.jimmunol.org/) MΦ phenotype in response to xenogeneic vs syngeneic cells in immunocompetent and SCID mice. Comparison of the MHC class II and CD80 profiles of F4/80-stained MΦ in lavages performed 5 days after injecting NOD (n = 4–6) or NOD-SCID (n = 3) mice with syngeneic splenocytes (dashed lines) or xenogeneic PK15 cells (solid lines).
Cytokine responses to i.p. xenogeneic, allogeneic, or syngeneic cells

To further examine the mechanisms of elicited Mφ- and neutrophil-mediated T cell recruitment and T cell-mediated activation of Mφ during xenoresponses, cytokine expression by NOD Mφ and CD4 T cells was examined by two-color flow cytometry. The proportion of cells expressing IFN-γ or TNF-α, but not IL-4, increased within 1 day of secondary PK15 cell injection (Fig. 7A), as did cell numbers (data not shown). In the xenogeneic response, 47 ± 7% of the TNF-α+ cells were F4/80+ Mφ (Fig. 7A), with a corresponding increase in F4/80+ TNF-α+ cells in xenogeneic-stimulated compared with control lavages (Fig. 7B). Approximately 15% of CD4+ cells expressed TNF-α, but GR1+ cells did not appear to be TNF-α+ (data not shown). In the xenogeneic response, 21 ± 6% of IFN-γ+ cells were CD4+ T cells, with an absolute increase in the numbers of CD4+ IFN-γ+ cells (Fig. 7B). Restimulation in vitro with PMA and ionomycin led to a further increase in the numbers of TNF-α+ and IFN-γ+ cells, but IL-4+ cells remained undetectable (data not shown). Similar results were obtained with cells lavaged 2 and 4 days after secondary or 1 and 5 days after primary PK15 cell injection (data not shown).

Discussion

The IPT model demonstrates that xenografts and, to a lesser extent, allografts rapidly elicit innate neutrophil and Mφ responses that preclude the influx of T cells and occur in the absence of T cells in SCID mice. Innate immunity is attributed to receptors that evolved to recognize PAMPs clearly distinguishable from self (14, 15). Our findings imply that grafts express PAMP-like molecular patterns recognized by innate immune receptors. The PAMPs recognized by many of the innate receptors identified to date are oligosaccharides (14). Oligosaccharide differences between species are exemplified by the strong, preformed Ab response of primates to Galα(1,3)Gal expressed on nonprimate, xenogeneic endothelial cells (18). Primate NK cells directly recognize oligosaccharides, including Galα(1,3)Gal moieties, expressed by porcine endothelial cells in vitro (19, 20), and a range of oligosaccharides mediate interactions between monocytes and xenogeneic RBC (21, 22). The stronger stimulation of innate immunity by xenografts compared with allografts would be consistent with the idea that they express disparate nonpolymorphic molecules, whereas allograft disparities are largely restricted to the polymorphic MHC proteins.

Innate immune responses to xenografts may impact on the cell-mediated response and contribute to the strength and complexity of
xenograft compared with allograft rejection. The current study provides evidence for a role of innate immunity in the recruitment of T cells. MΦ and neutrophils elicited by xenogeneic cells in SCID mice induced T cell recruitment upon transfer to immunocompetent mice. Furthermore, we have previously demonstrated that MΦ are required, at least locally, for T cell infiltration of FPP xenografts (16). These results are consistent with the well-documented chemoattractant properties of neutrophils and monocytes (23, 24), mediated by the release of chemokines such as IFN-γ-inducible protein-10 and cytokines such as TNF-α. In turn, chemokines, including macrophage inflammatory protein-1α and -β, IL-8, and KC (the murine homologue of growth-related oncogene-α) have been detected in skin allografts during the innate response before the induction of alloreactive T cells (25, 26), and blockade of monocyte induced by IFN-γ prevents circulating alloantigen-specific T cells from entering grafts (27). As in responses to various pathogens (24), neutrophils appeared to be the first cells recruited into xenografts. Neutrophils release both monocyte and T cell chemoattractants, and it has been suggested that they respond directly to pathogens and initiate cell-mediated immunity (24, 28). Cell-specific expression of cytokine protein was quantified during the xenograft response for the first time in the present study. TNF-α+ cells, approximately 50% of which were monocytes/elicited MΦ, were induced by xenogeneic cells consistent with previous semi-quantitative PCR studies of cytokine RNA expression in FPP and pig proislet xenografts (29). IFN-γ+ CD4+ cells were also induced by xenogeneic cells, consistent with previous studies (30). The phenotype of the remaining IFN-γ− cells was not investigated in detail, although IFN-γ−, CD8+ cells have been detected (A. Fox, unpublished observation), and other cells, such as NK cells and MΦ, may express IFN-γ (31). IL-4+ cells were not detected, although others have reported that levels of IL-4 RNA are increased in xenografts (29, 30). Cell-associated IL-4 protein may be harder to detect than whole graft-associated IL-4 RNA, or IL-4 expression may differ in the different xenograft models.

Xenogeneic cells elicited T cell responses that coincided with rejection. Furthermore, rejection was accelerated in mice that had previously been primed and did not occur in T cell-deficient SCID mice, demonstrating its T cell dependence. However, it has never been clear whether T cells directly reject grafts or are required to activate other effectors. MΦ are, in fact, the dominant infiltrating cell in xenografts undergoing rejection and are well-known effectors against autoimmune or infected target cells (32–34). We show here that T cells are required for MΦ activation and for the first time that activated MΦ can directly reject xenogeneic cells.

Whether rejection by xenoactivated MΦ is specific and, if so, by what mechanism is currently being investigated. Activation of MΦ that could kill cells, including self cells, nonspecifically would be deleterious. That self cells are normally not killed by activated MΦ indicates that mechanisms operate at the level of either MΦ or the T cells involved in their activation to direct effector potential toward foreign targets. Also, the range of targets that activated MΦ can kill in vitro is limited by the cytotoxic potential that MΦ acquire during activation and by the sensitivity of different targets to such toxicity (35). For example, L929 cells are sensitive to TNF-α-mediated killing and P815 cells to NO-mediated killing by LPS/IFN-γ-activated, casein-induced MΦ, whereas allogeneic cells are not sensitive to either (36). In preliminary experiments we have found that PK15 cell rejection in SCID mice is greater after transfer of xenogeneic- than allogeneic-stimulated MΦ. While this suggests a degree of MΦ specificity, it may be simply that xenogeneic cells are a stronger stimulus for induction of MΦ with greater cytotoxicity than allogeneic cells. At least two reports indicate MΦ specificity for targets. First, allogeneic fetal pancreas segments were rejected at a normal rate when cotransplanted with a xenograft, even though the xenograft was rejected more rapidly (4). Second, MΦ that dominated peritoneal infiltrates after IPT of allogeneic Meth A tumor cells killed allogeneic, but not syngeneic, cells in vitro and in vivo, and this killing could only be inhibited by cells of the same allogeneic haplotype and was cell-cell contact dependent (36, 37). Mechanisms for specific recognition of targets by effector MΦ have not been described. As we have demonstrated that xenogeneic cells are recognized by the innate immune system, it is tempting to speculate that receptors such as the PAMP recognition receptors may be involved in directing and limiting the cytotoxicity of effector MΦ toward foreign targets.

T cells were required for the generation of MΦ that could reject xenografts, a requirement classically attributed to the provision of IFN-γ (33, 38, 39). We demonstrated that xenogeneic cells induce IFN-γ+ CD4 T cells. However, as islet xenograft rejection is only slightly delayed in IFN-γ-deficient mice (10), other cytokines may be involved in MΦ activation. Signals from Ag-specific T cells may also influence the cytotoxic potential of MΦ and hence the targets they can kill. In preliminary experiments we could recover MHC class II+ MΦ from PK15 cell-injected SCID mice if they were also given PK15 cell-stimulated, but not allogeneic cell-stimulated, T cells. This coincided with PK15 cell rejection specifically in the former. The sites at which activated MΦ can act as effectors may also be governed by Ag-specific T cells. This is indicated by various studies demonstrating a requirement for T cell infiltration of grafts for MΦ to be present in large numbers (40, 41). Whether this results from increased MΦ infiltration or proliferation locally is not known. The requirement for local T cells is particularly apparent in studies showing that i.v. injected xenograft-activated MΦ do not infiltrate and reject renal subcapsular FPP grafts in T cell-deficient SCID mice (A. Fox, unpublished observation). This need for T cells is bypassed in the IPT model, by delivering activated MΦ to the graft site.

Concluding remarks

Xenografts in particular are recognized by the innate immune system, and xenograft rejection requires interactions between innate and adaptive immunity. Although attention has been focused on the role of innate immunity in Ag presentation and T cell priming, the present study highlights the important role of innate immunity in T cell recruitment. In turn, T cell help is required for MΦ activation and MΦ-mediated graft rejection. It is likely that general adjuvant effects of innate immunity mediated by cytokines and chemokines impact on many aspects of the cell-mediated response and contribute to the strength and complexity of xenograft compared with allograft rejection. Innate immunity is a fertile area for the development of novel therapies to prevent adaptive immunity and graft rejection.

References

Innate and Adaptive immune responses to nonvascular xenografts


