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T Cell-Activated Macrophages Are Capable of Both Recognition and Rejection of Pancreatic Islet Xenografts

Shounan Yi,* Wayne J. Hawthorne,* Anne M. Lehnert,* Hong Ha,* Jeferey Kwok Wah Wong,* Nico van Rooijen,† Kelly Davey,* Anita T. Patel,* Stacey N. Walters,* Abhilash Chandra,* and Philip J. O’Connell²*

Macrophages have been proposed as the major effector cell in T cell-mediated xenograft rejection. To determine their role in this response, NOD-SCID mice were transplanted with fetal pig pancreas (FPP) before reconstitution with CD4⁺ T cells from BALB/c mice. Twelve days after CD4⁺ T cell reconstitution, purified macrophages (depleted of T cells) were isolated from CD4⁺ T cell-reconstituted FPP recipient mice and adoptively transferred to their nonreconstituted counterparts. After adoptive macrophage transfer, FPP recipient mice transferred with macrophages from CD4⁺ T cell-reconstituted mice demonstrated xenograft destruction along with massive macrophage infiltration at day 4 and complete graft destruction at day 8 postmacrophage transfer. By contrast, FPP recipients that received macrophages from nonreconstituted mice showed intact FPP xenografts with few infiltrating macrophages at both days 4 and 8 after macrophage transfer. The graft-infiltrating macrophages showed increased expression of their activation markers. Depletion of endogenous macrophages or any remaining CD4⁺ T cells did not delay graft rejection in the macrophage-transferred FPP recipients, whereas depletion of transferred macrophages with clodronate liposomally prevented graft rejection. Our results show that macrophages primed by FPP and activated by CD4⁺ T cells were attracted from the peripheral circulation and were capable of specific targeting and destruction of FPP xenografts. This suggests that in xenograft rejection, there are macrophage-specific recognition and targeting signals that are independent of those received by T cells. The Journal of Immunology, 2003, 170: 2750–2758.

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3 Abbreviations used in this paper: FPP, fetal pig pancreas; BSL, blood sugar level; iNOS, inducible NO synthase; Lip-C12 MDP, liposome-encapsulated dichloromethylene diphosphonate; MCP-1, monocyte chemoattractant protein-1; NOD, nonobese diabetic; TCRc, TCR β-chain C region.
Materials and Methods

Animals
Adult BALB/c mice obtained from the Animal Care Department, Westmead Hospital (Westmead, New South Wales, Australia), were used as CD4+ T cell donors or allogeneic islet donors. Adult NOD-SCID mice were purchased from Walter and Eliza Hall Institute (Melbourne, Australia) and served as FPP xenograft recipients. NOD-SCID mice were kept under specific pathogen-free conditions by housing them in microisolator cages with autoclaved food, water, and bedding. Pregnant sows purchased from a local inbred herd were used as FPP donors. In some experiments, adult Wistar rats from the Animal Care Department, Westmead Hospital, were used as islet xenograft donors.

Antibodies
Rat anti-mouse macrophage and CD4+ T cell mAbs were produced from the hybridomas Mac-1 and GK1.5, respectively, which were purchased from American Type Culture Collection (Manassas, VA). Rat anti-mouse CD3 (T lymphocytes) and B220 (B lymphocytes) mAbs were purchased from Cedarlane Laboratories (Ontario, Canada). Rabbit anti-mouse polyclonal Abs to inducible NO synthase (iNOS) (NOS2) and IL-15 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Unconjugated rat anti-mouse mAbs to IL-12, TNF-α, and DX5 (NK cells), and FITC-conjugated rat anti-mouse mAbs to MHC class II, ICAM-1, B7.1 (CD80), B7.2 (CD86), and TNF-α were purchased from BD PharMingen (San Diego, CA). PE-conjugated rat anti-mouse mAb to macrophages (F4/CD80) and FITC-conjugated sheep anti-rat and anti-rabbit IgG Abs were purchased from Serotech (Oxford, U.K.).

Pancreatic islet preparation
For fetal pig pancreatic islet preparation, pancreas was removed from the fetuses obtained from a litter of donor pigs of ~70–90 days gestation. Then the pancreas was minced into 1- to 2-mm3-size fragments in cold PBS containing 5% FCS (Bioscience, New South Wales, Australia). Finally, the fragments of fetal pancreas were cultured on Millipore filters (Bedford, MA) and gelatin foam immersed in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS at 37°C, 10% CO2/90% O2 for 3 days and in 10% CO2/90% air for an additional day, as described elsewhere (19). Mouse pancreatic islets were isolated from adult BALB/c mice, as described previously (19). Briefly, the proximal common bile duct was cannulated and the pancreas was distended with 2 mg/ml collagenase (type IA, Sigma-Aldrich) density gradient in HBSS. Islets were collected, and the islet allograft recipients were transplanted with FPP under their renal capsule for RNA and DNA isolation. Cellular RNA was isolated using RNAzol B between the tip of the scapula. The skin graft site consisted of full-thickness incision, 2 mm in size. Hemostasis was achieved, and precut donor fetal pig split-thickness skin grafts were placed onto the site. The 10/0 polypropylene (Prolene, Ethicon, Johnson & Johnson Medical, Sydney, Australia) single interrupted sutures were used to secure the skin xenograft to the transplant site, which was covered with petroleum-jelly gauze. An adhesive bandage was placed over the graft site and around the torso to protect the grafts for 3 days. The grafts were then monitored and photographed daily for survival. Complete skin graft rejection was defined when less than 10% of the graft remained viable.

CD4+ T cell isolation and reconstitution
CD4+ T cells were prepared from spleens and lymph nodes isolated from female donor BALB/c mice. Briefly, mononuclear cells from spleens and lymph nodes were separated by Lymphprep (Pharmacia, Uppsala, Sweden). Purification of CD4+ T cells was performed by positive selection using Dynabeads mouse CD4 (Dyna, Oslo, Norway). After incubation with the Dynabeads, the bead-rosetted CD4+ T cells were separated and collected by DETACHaBEAD mouse CD4 (Dyna), according to the manufacturer’s instructions. The purity of the resulting CD4+ T cell population was 99% determined by FACS. A total of 1 or 2 × 106 CD4+ T cells was used to reconstitution of male FPP recipient NOD-SCID mice by tail vein injection 4 days after FPP transplantation. Male FPP recipient NOD-SCID mice injected with cold PBS were used as nonreconstituted controls.

Macrophage isolation and adoptive transfer
It has been shown in our previous study that CD4+ T cell-reconstituted, but not nonreconstituted FPP recipient SCID mice destroyed their grafts completely 12 days after reconstitution (17). Activated and nonactivated macrophages were obtained from the spleens, and FPP grafts were removed from male FPP recipient NOD-SCID mice 12 days after CD4+ T cell reconstitution or 12 days after PBS injection (nonreconstituted counterparts). For macrophage isolation, splenocytes and graft-infiltrating cells were treated with hemolytic buffer (8.3 g/L NH4Cl, 1 g/L KHCO3, 1 mL/L 0.1 M EDTA), followed by negative selection of macrophages using a mAb cocktail of rat anti-mouse CD3 (T lymphocytes), B220 (B lymphocytes), and DX5 (NK cells) mAbs, and sheep anti-rat IgG Dynabeads (Dyna) to deplete any residual T, B, and NK cells. After the selection, greater than 99% purity of the resulting macrophage population was obtained. Adoptive macrophage transfer was performed by i.v. injection of nonreconstituted female FPP recipient NOD-SCID mice with 1 or 2 × 106 purified macrophages. The recipients of both FPP and mouse islets with a BSL <10 nmol/L also received 2 × 106 purified macrophages from their FPP-alone counterparts that had been reconstituted with CD4+ T cells. In separate experiments, 2 × 106 purified macrophages obtained from CD4+ T cell-reconstituted FPP recipient NOD-SCID mice were incubated on ice with100 μL Lip-Cl2 MDP for 30 min before transfer to female recipient NOD-SCID mice by i.v. injection. FPP xenografts were taken at certain time points after adoptive macrophage transfer, and rejection was defined as no visible intact graft observed by histological examination.

Histology and immunohistochemistry
Grafts were removed from pancreatic islet xenograft recipients at selected time points after transplantation. The grafts were frozen in OCT compound (Tissue TCK, Miles, Elkhart, IN) and stored at −70°C. Cryostat sections (6–8 nm) were used for both histology examination by a routine H&E staining and immunohistochemical detection of CD4+ T cells (GK1.5) and macrophages (Mac-1), as described previously (17), using a peroxidase/anti-peroxidase rabbit kit (ScyTek, Logan, UT). Grafts isolated from macrophage-transferred recipients at the rejecting time point were also used for intracellular staining of the macrophage-related molecules IL-12, IL-15, TNF-α, and iNOS. For intracellular staining, the sections were treated with peroxidase suppressor and super block (ScyTek), according to the manufacturer’s instruction, followed by incubation for 15 min in permeabilization buffer (0.3% Triton X-100), then with primary anti-IL-12, anti-IL15, anti-TNF-α, or anti-iNOS Abs in permeabilizing buffer at 4°C before secondary Ab staining. After staining with the primary and secondary Abs, the sections were developed in diamobenzidine (ScyTek). Finally, all sections were counterstained with hematoxylin.

PCR and RT-PCR
FPP xenografts removed from FPP recipient NOD-SCID mice were used for RNA and DNA isolation. Cellular RNA was isolated using RNAzol B...
CD4⁺ T CELL-ACTIVATED MACROPHAGES REJECT XENOGRAFTS

(Tel-Test, Friendswood, TX). cDNA was synthesized by incubating 5 μg RNA in a reaction mixture consisting of 2 μg of oligo(dT)₁₂₋₁₈, 2.5 mM dNTP mix, 10 mM DTT, 1 μ/μl RNase inhibitor (Promega, Madison, WI), and 200 U of SuperScript II reverse transcriptase (Life Technologies) at 42°C for 1 h. DNA was obtained by incubating an aliquot of graft samples in 50 μl of extraction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 0.1 μg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20) and 10 μg proteinase K for 1 h at 55°C. A total of 5 μl of each cDNA product was used for amplification by PCR using Red Hot DNA polymerase (Advantec Biotechnologies, Epikon) and specific primers for mouse IL-12 p40 subunit (sense, 5'-ATCAAGGGAATCTCAAAACAGC-3', antisense, 5'-AACGCAACCAAGCAGAAGA-3'), TNF-α (sense, 5'-CCACGACGCTTACTACACTGAT-3', antisense, 5'-CCTGCCACCACACTCAGAT-3'), IL-15 (sense, 5'-CCACCTCGACTTTGTG-3', antisense, 5'-CTGTTTGCAGAAGTACCCG-3'), iNOS (sense, 5'-GAGACGACAAGAGTTGTCAG-3', antisense, 5'-TGCCGAGATTGGTTCTATT-3'), and TCR β-chain C region (TCRcβ) (sense, 5'-GACCTCTGAGTCCTATG-3', antisense, 5'-GATCTCATAGAGGATGGTTGGC-3'). A total of 1 μl of each DNA extraction was amplified by Red Hot DNA polymerase and specific primers for sex-determining region of mouse Y-chromosome (sense, 5'-CTTCGTGATGCAAACTTTCG-3', antisense, 5'-TGGTGTGCTGATGGTGGG-3') (21). The thermal cycling program used for PCR was one cycle for 3 min at 94°C and 35 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 5 min at 72°C as a final step. Mouse GAPDH was used as a housekeeping gene control (sense, 5'-TGAGTGCTGATGGGTGACGG-3', antisense, 5'-TGAGTGCTGATGGGTGACGG-3'). PCR products were analyzed by electrophoresis on a 2% agarose gel.

Flow cytometry

Grafts were removed from CD4⁺ T cell-reconstituted FPP NOD-SCID recipients either 12 days after they had been reconstituted with CD4⁺ T cells or 8 days after adoptive transfer with activated macrophages. The grafts were gently ground with frosted objective slides in RPMI 1640 medium (Life Technologies) containing 10% FCS and 0.06 mM EDTA, followed by single cell suspension preparation. Cells were washed twice in PBS and then incubated at 4°C for 20 min with Fc Block (BD PharMingen) to block Fe receptors and avoid nonspecific staining. For dual-color staining of macrophages coexpressing cell surface activation markers, graft-infiltrating cells were incubated with mAb F4/80-PE plus mAb ICAM-1, nonreconstituted NOD-SCID recipients destroyed their FPP grafts (Table I and Fig. 1). Moreover, CD4⁺ T cell-reconstituted recipients, the destroyed FPP grafts were infiltrated intensively by macrophages and CD4⁺ T cells, whereas very few infiltrating macrophages were observed in nonreconstituted NOD-SCID recipients (Fig. 1). Consequently, CD4⁺ T cell reconstitution of FPP recipient NOD-SCID mice induced up-regulated expression of activation markers, in particular MHC class II, ICAM-1, B7.1, IL-12, IL-15, and TNF-α in the graft-infiltrating cells for 30 min at 4°C in Perm/Wash solution with FITC-conjugated sheep anti-rat IgG (Serotec) for mAbs IL-12 and TNF-α, or FITC-conjugated sheep anti-rabbit IgG (Serotec) for Ab IL-15. Finally, the cells were incubated with mAb F4/80-PE in staining buffer for 30 min at 4°C. After Ab staining, cells were analyzed by flow cytometry on a FACScan (BD Biosciences, Mountain View, CA) gated to exclude nonviable cells. At least 10,000 viable cells were analyzed per sample, and the percentage of macrophages coexpressing ICAM-1, MHC class II, B7.1, B7.2, IL-12, IL-15, and TNF-α, respectively, was calculated as the proportion of double-positive staining cells/total F4/80-staining positive cells detected by FACS. Cells stained with isotype-matched Abs were used as isotype controls for FACS analysis, and the percentage of individual mAb-staining positive cells was determined by cumulative subtraction of the isotype controls.

Results

Reconstitution of NOD-SCID recipients with CD4⁺ T cells resulted in FPP graft destruction and full macrophage activation

Our previous studies have shown that reconstitution of FPP recipient SCID mice (deficient of both T and B lymphocytes) with CD4⁺ T cells causes complete graft destruction along with a massive accumulation of activated CD4⁺ T cells, NK cells, and macrophages at day 12 after CD4⁺ T cell reconstitution (17). To confirm that CD4⁺ T cells were able to initiate xenograft rejection and mediate macrophage activation in NOD-SCID mice (deficient of T and B lymphocytes and NK cells), NOD-SCID mice were transplanted with FPP xenografts, and 4 days after transplantation the FPP recipients were reconstituted with 2 × 10⁶ purified CD4⁺ T cells obtained from BALB/c mice. FPP graft survival was evaluated by histology, demonstrating that CD4⁺ T cell-reconstituted, but not nonreconstituted NOD-SCID recipients destroyed their xenografts completely at day 12 after reconstitution (Table I and Fig. 1). In CD4⁺ T cell-reconstituted recipients, the destroyed FPP grafts were infiltrated intensively by macrophages and CD4⁺ T cells, whereas very few infiltrating macrophages were observed in nonreconstituted NOD-SCID recipients (Fig. 1). Moreover, CD4⁺ T cell reconstitution of FPP recipient NOD-SCID mice induced up-regulated expression of activation markers, in particular MHC class II, ICAM-1, B7.1, IL-12, IL-15, and TNF-α in the graft-infiltrating cells.

Table I. Morphological evaluation of FPP xenograft survival in recipient NOD-SCID mice

<table>
<thead>
<tr>
<th>FPP Recipients</th>
<th>n</th>
<th>Observation Time (Days)</th>
<th>Remaining Endocrine Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reconstitution</td>
<td>3</td>
<td>100 (postreconstitution)</td>
<td>+ + +</td>
</tr>
<tr>
<td>CD4⁺ T cell reconstitution</td>
<td>12</td>
<td>12 (postreconstitution)</td>
<td>0 to +</td>
</tr>
<tr>
<td>Nonactivated MØ transferred</td>
<td>5</td>
<td>8 (posttransfer)</td>
<td>+ + +</td>
</tr>
<tr>
<td>Activated MØ transferred</td>
<td>1 x 10⁶</td>
<td>3</td>
<td>8 (posttransfer)</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁶</td>
<td>6</td>
<td>4 (posttransfer)</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁶/GK1.5</td>
<td>8</td>
<td>0 to +</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁶/Lip-C12 MDP</td>
<td>4</td>
<td>8 (posttransfer)</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁶/Lip-C12 MDP treated</td>
<td>5</td>
<td>12 (posttransfer)</td>
</tr>
</tbody>
</table>

* Rejection time was determined by histological examination of FPP grafts, as described in Materials and Methods.

† The number of remaining endocrine cells was assessed semiquantitatively, as described by Sandberg et al. (24), and graded in four different categories: 0, no surviving cells; +, occasional surviving cells; + +, a few partially intact FPP grafts; and + + +, completely intact FPP grafts.

‡ Female FPP recipients transferred with 2 x 10⁶ macrophages (MØ) from nonreconstituted male FPP recipients.

§ Female FPP recipients transferred with macrophages (MØ) from CD4⁺ T cell-reconstituted male FPP recipients.

‖ Female FPP recipients transferred with 2 x 10⁶ activated macrophages and treated with CD4⁺ T cell-depleting mAb GK1.5.

¶ Female FPP recipients depleted of endogenous macrophages with Lip-C12 MDP prior to transfer with 2 x 10⁶ activated macrophages.

‖ Female FPP recipients transferred with 2 x 10⁶ activated macrophages pretreated with Lip-C12 MDP.
tution (16 days after FPP transplantation). Macrophages were pu-
recipient NOD-SCID mice who had not undergone prior reconsti-
istent NOD-SCID mice were adoptively transferred to female FPP 
other effectors, especially CD4

cells (A and B) as well as in the splenic 
intravasating macrophages (Table II and Fig. 2) as well as in the splenic 
macrophages from these reconstituted recipients (data not shown).

Adoptive transfer of CD4

capable of mediating FPP xenograft rejection in the absence of 
phages isolated from CD4

To determine whether CD4

cells and macrophages (C and D). Positive cells were stained 
bron. Arrows indicate remaining islet cells.

trating macrophages (Table II and Fig. 2) as well as in the splenic 
macrophages from these reconstituted recipients (data not shown).

Adoptive transfer of CD4

cel-activated macrophages caused rapid and specific FPP xenograft rejection

To determine whether CD4

cells, highly purified macrophages isolated from CD4

infiltration (data not shown) in these CD4

cells that might have been transferred along with 
macrophages. In a separate experiment, FPP NOD-SCID rece-

cipients transferred with activated macrophages were also given 
0.3 mg i.p. of the CD4

cell-depleting mAb, GK1.5, on days 0, 1, and 3 of adoptive macrophage transfer. Histological and immu-
nological analyses demonstrated a similar FPP xenograft rejecting 
time point (Table I) and macrophage infiltration (data not shown) in 
these CD4

cell-depleting mAb-treated recipients when com-
pared with their counterparts without the mAb treatment. Rejecting 
exonografts were also examined by immunohistochemistry for the 
presence of leukocyte subsets other than macrophages. No other 
effector cells, including CD4

A

B

C

D

FIGURE 1. Histology and immunohistochemistry of FPP xenograft re-
jection after CD4

cell reconstituted. FPP grafts from NOD-SCID re-
ipients reconstituted with (FPP + CD4) or without (FPP alone) CD4

T cell reconstitution at day 12 after reconstitution were stained for CD4

T cells (A and B) and macrophages (C and D). Positive cells were stained 
brown. Arrows indicate remaining islet cells.

rified from the grafts and spleens of CD4

T cell-reconstituted FPP recipient mice, as described. T cells were not detectable in the 
purified macrophage populations by FACS (data not shown). When FPP recipients were transferred with 1 \times 10^6 macrophages from their CD4

T cell-reconstituted counterparts, extensive xenograft destruction was apparent by day 8 after macrophage transfer (Table I), whereas in FPP recipients that received nonactivated macrophages from nonreconstituted FPP recipients, their xenografts remained intact with few infiltrating macrophages detected at both days 4 and 8 after adoptive macrophage transfer (Fig. 3A, a and b). Adoptive transfer of NOD-SCID recipients of FPP with 2 \times 10^6 purified macrophages from their CD4

T cell-reconstituted counterparts caused severe graft destruction along with a large accumulation of infiltrating macrophages at day 4 and complete graft destruction with massive macrophage infiltration at day 8 after macrophage transfer (Table I and Fig. 3A, c and d), indicating that the rapidity and severity of FPP xenograft rejection were dependent on the number of exogenous macrophages trans-

Several experiments were performed to show that rejection was 
dependent on transferred activated macrophages rather than the 
activation of endogenous macrophages within the host animal. 
First, Y chromosome was detected in rejecting xenografts of 
female NOD-SCID mice, indicating that at least some of the in-
filarating macrophages were of donor origin (Fig. 4). Next, we 
examined whether the FPP xenograft rejection in the macrophage-

tration of FPP graft-infiltrating 
cells isolated from the grafts and spleens of CD4

T cel-reconstituted recipients when com-
pared with their nonreconstituted (FPP/nonactivated Mφ) male recipients, their xeno-
graft destruction was apparent by day 8 after macrophage transfer (Table I and Fig. 3A, 1 and 3) of adoptive macrophage transfer. Histological and immu-
nological analyses demonstrated a similar FPP xenograft rejecting 
time point (Table I) and macrophage infiltration (data not shown) in 
these CD4

T cell-depleting mAb-treated recipients when com-
pared with their counterparts without the mAb treatment. Rejecting 
exonografts were also examined by immunohistochemistry for the 
presence of leukocyte subsets other than macrophages. No other 
effector cells, including CD4

T (Fig. 3A), CD8

T, B, and NK 
cells (data not shown), were found infiltrating the rejecting grafts 
of any macrophage-transferred FPP recipient. Furthermore, no 
TCRβ expression was detected by RT-PCR in FPP grafts in 
which activated macrophages were transferred to NOD-SCID re-
cipients. This further excluded the potential for xenograft rejection

<table>
<thead>
<tr>
<th>Source of Graft-Infiltrating Cells</th>
<th>% of Mφ in Graft-Infiltrating Cells</th>
<th>% of Mφ-Coexpressing Cell Surface Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPP alone</td>
<td>UD</td>
<td>UD</td>
</tr>
</tbody>
</table>
| FPP/CD4

T cells                         | 58.5 ± 6.5                        | 45.6 ± 5.0 SD 76.0 ± 7.1                  |
| FPP/nonactivated Mφ              | UD                                | UD                                      |
| FPP/activated Mφ                 | 98.3 ± 1.8*                       | 70.4 ± 7.2* SD 93.3 ± 7.9*               |

* FPP graft-infiltrating cells isolated from male NOD-SCID recipients of FPP without reconstitution (FPP alone) or with 
CD4

T cell reconstitution (FPP/CD4

T cells) at day 12 after reconstitution, or female NOD-SCID recipients of FPP trans-
ferred with macrophages (Mφ) from their nonreconstituted (FPP/nonactivated Mφ) or CD4

T cell-reconstituted (FPP/activated Mφ) male counterparts, respectively, at day 8 after transfer, were used for dual-color FACS analysis of proportion of macrophages coexpressing cell surface activation markers. Proportion of macrophages coexpressing cell surface activation markers was calculated as % double-staining positive cells divided by % total individual cell surface marker-positive cells. The data are the mean ± SD of at least three individual samples.

UD, Undetectable.

*, Value of p < 0.05 compared with CD4

T cell-reconstituted counterparts.
to be initiated by any remaining CD4+ T cells in the transferred recipient mice (Fig. 5). Thus, the xenograft rejection response shown in this study was initiated predominantly by the transfer of activated macrophages and was not dependent upon the activation of either endogenous or exogenous macrophages mediated by transferred CD4+ T cells.

To further examine whether activated macrophages were specific for xenografts, streptozotocin-induced diabetic NOD-SCID mice were transplanted with a BALB/c mouse islet allograft under the left kidney. After the BSL was normalized to <10 mmol/L, these recipients were then transplanted with a FPP xenograft under their right renal capsule before macrophage transfer. The immature FPP xenograft did not secrete insulin in response to glucose, while the adult allograft was functional and normalized the BSL. Eight days after macrophage transfer from CD4+ T cell-reconstituted (GF/FPP + CD4) or activated macrophage-transferred (GF/FPP + activated Mφ) FPP recipient NOD-SCID mice, respectively, and splenocytes from nonreconstituted (SP/FPP alone) or nonactivated macrophage-transferred (SP/FPP + nonactivated Mφ) recipients, respectively. The numbers indicated in upper left and right quadrants represent the percentage of single-color positive macrophages and dual-color positive macrophages coexpressing intracellular cytokines, respectively. Data shown are from one of three independent experiments.

To determine whether macrophages sensitized to pig islets were capable of destroying other pig tissue, fetal pig skin grafts were transplanted on NOD-SCID recipients of FPP before receiving macrophages from CD4+ T cell-reconstituted recipients of FPP alone. The results showed that while both fetal pig skin and FPP grafts remained intact in the recipients without macrophage transfer, the recipients that received CD4+ T cell-activated macrophages rejected their FPP and fetal pig skin grafts at the same time point (8–10 days after macrophage transfer), indicating that there was no organ specificity of the response (data not shown).

Endogenous macrophages were not necessary for FPP xenograft destruction initiated by adoptive transfer of exogenous activated macrophages

To evaluate further the relative importance of endogenous macrophages compared with exogenous transferred macrophages in the rejection response, an efficient macrophage-depleting reagent, Lip-C12 MDP, was given i.p. to FPP recipient NOD-SCID mice (100 μl/10 g body weight) 2 days before adoptive macrophage transfer. A single injection of Lip-C12 MDP into mice has been reported to induce depletion of macrophages within 2 days (12, 22). In our Lip-C12 MDP-treated animals, ≥95% of splenic macrophages were depleted 2 days after administration and at the time of donor macrophage infusion. Similar to the FPP recipients that received macrophages from CD4+ T cell-reconstituted FPP recipients, but without Lip-C12 MDP treatment, the FPP recipients that received Lip-C12 MDP rejected their xenografts completely at day 8 after adoptive transfer with activated macrophages (Table I). Similar to the original histology seen in macrophage-transferred recipients, those animals that received Lip-C12 MDP treatment showed a large accumulation of graft-infiltrating macrophages (data not shown), indicating that depletion of endogenous macrophages did not affect xenograft rejection.
not delay FPP xenograft rejection. The FPP xenograft destruction initiated by adoptive transfer of exogenous activated macrophages was further confirmed by reconstituting recipient NOD-SCID mice with activated macrophages that were treated with Lip-C12 MDP before the transfer. In these recipients, FPP xenograft survival was prolonged at least beyond 12 days after the macrophage transfer. Graft histology showed intact FPP grafts with substantially reduced macrophages (Fig. 3A, e and f).

**FIGURE 3.** Histology and immunohistochemistry of xenograft rejection after activated macrophage transfer. A, FPP grafts stained for CD4$^+$ T cells (a, c, and e) and macrophages (b, d, and f). a and b, FPP grafts from NOD-SCID recipients that received macrophages without Lip-C12 MDP treatment from nonreconstituted counterparts; c and d, FPP grafts from NOD-SCID recipients that received macrophages without Lip-C12 MDP treatment from CD4$^+$ T cell-reconstituted counterparts at day 8 after macrophage transfer; e and f, FPP grafts from NOD-SCID recipients that were transferred with Lip-C12 MDP-pretreated macrophages from CD4$^+$ T cell-reconstituted counterparts at day 12 after macrophage transfer. B, Mouse islet grafts stained by H&E (a) and anti-macrophage mAb (b). Mouse islets were isolated from FPP and mouse islet recipients that received macrophages from CD4$^+$ T cell-reconstituted NOD-SCID recipients of FPP alone at day 20 after macrophage transfer. Positive cells were stained brown. Arrows indicate remaining islet cells.
Following adoptive transfer, graft-infiltrating macrophages demonstrated an activated phenotype

The above data support the hypothesis that activated macrophages are capable of destroying FPP xenografts in the absence of other effector cells, confirming macrophages as a major mediator of FPP xenograft destruction. To identify potential effector pathways used by macrophages, we evaluated infiltrating macrophages for expression of specific macrophage-related cell surface and intracellular activation markers. Two-color FACS staining of graft-infiltrating macrophages showed that a large percentage of macrophages from adoptively transferred recipients coexpressed macrophage cell surface activation markers, such as MHC CII, ICAM-1, and B7.1, while there was only modest up-regulation of B7.2 (70% coexpressing MHC CII, 93% coexpressing ICAM-1, 48% coexpressing B7.1, and 6% coexpressing B7.2, respectively). The level of macrophage activation marker expression was substantially higher in activated macrophage-transferred recipients compared with their expression in CD4⁺ T cell-reconstituted recipient NOD-SCID mice (FPP + CD4⁺ T cells) (59% coexpressing MHC CII, 76% coexpressing ICAM-1, 20% coexpressing B7.1), or nonreconstituted (FPP alone) or nonactivated macrophage-transferred mice (FPP + non-activated Mø) (undetectable) (Table II). Intracellular expression of the cytokines IL-12, IL-15, and TNF-α was assessed by two-color FACS analysis. In nonactivated macrophage-transferred recipients, cytokine-coexpressing macrophages were undetectable, while in activated macrophage-transferred recipient NOD-SCID mice, the infiltrating macrophages demonstrated up-regulated intracellular expression of the cytokines IL-12, IL-15, and TNF-α (proportions of IL-12-, IL-15-, and TNF-α-coexpressing macrophages were approximately 29, 35, and 42%, respectively) (Fig. 2).

The up-regulated expression of IL-12, IL-15, TNF-α, and iNOS by graft-infiltrating macrophages in the activated macrophage-transferred recipient mice was further confirmed by both immunohistochemical and RT-PCR analyses of graft-infiltrating cells showing IL-12, IL-15, TNF-α, and iNOS mRNA expression by the graft-infiltrating cells (Fig. 6) and IL-12-, IL-15-, TNF-α-, and iNOS-positive staining cells in the grafts (Fig. 7). Taken together, these results demonstrated an activated macrophage phenotype by xenograft-infiltrating macrophages following adoptive macrophage transfer, further confirming the role of macrophages as the major effector cell in the initiation and/or mediation of FPP xenograft rejection response.

Discussion

Cellular xenograft rejection is CD4⁺ T cell dependent, but the actual effector mechanisms involved have been a matter of controversy. Macrophages have been proposed as one of the major effector cells responsible for CD4⁺ T cell-mediated xenograft destruction. Unlike allografts in which the predominant cells seen are T cells, in rejecting xenografts the predominant cell is the macrophage (18, 23–25). The preponderance of macrophages in rejecting xenografts has led us and others to propose that these cells are primarily responsible for graft destruction (11–13, 17). Recently, we have shown in a model of FPP xenograft rejection that CD4⁺ T cells are required for the initiation of the xenograft rejection response in mice. Once activated, CD4⁺ T cells recruit macrophages and NK cells to the graft via an IFN-γ-dependent pathway, which ultimately leads to graft destruction (17). However, in all these studies, the evidence supporting macrophages as the predominant effector cell in xenograft rejection was indirect. The difficulty has been the lack of an effective way of depleting host macrophages at the time of transplantation. In this study in which potential effector cells were adoptively transferred to NOD-SCID recipients of FPP, macrophages were found to be capable of both specific recognition and rejection of pancreatic islet xenografts. This response was relatively specific, as macrophages sensitized to pig islet grafts were not able to nonspecifically destroy mouse islet allografts or rat islet xenografts.

Conventional wisdom has suggested that T cells are recruited to the graft via secondary lymphoid organs. Once they have reached
the graft, they provide the signals necessary for ongoing macrophage recruitment (26). However, in our model, an alternative mechanism for macrophage involvement has been proposed. Macrophages were activated in a different host before adoptive transfer. Once activated by CD4\(^+\) T cells, they were able to localize to the graft. To do this, the graft itself must provide further signals that are responsible for macrophage recruitment from the circulation to the site of inflammation. Furthermore, the response was relatively specific for pig tissue, suggesting that specific macrophage receptors, such as pattern-recognition receptors, are involved. These signaling mechanisms are important, but at this stage remain speculative. Given that this is a strong inflammatory reaction, it is likely that they are under the control of proinflammatory signaling molecules such as NF-kB. Chemokines are likely to be involved in attracting macrophages to the graft site, and macrophages were detected in both the mouse allograft as well as the FPP xenograft. In a different experimental model, monocyte chemoattractant protein-1 (MCP-1) was shown to be essential for attracting macrophages to the site of inflammation. In the work by Palframan et al. (27), inflamed epithelium was shown to secrete MCP-1 that was transported to the draining lymph nodes, where it was responsible for attracting circulating monocytes. A similar mechanism may be present in our system. In immune-competent mice, we have identified high levels of MCP-1 early in the rejection response (J. Wong and P. O’Connell, unpublished observations), and it is possible that MCP-1 or a similar chemokine could be responsible for attracting circulating monocytes to the graft via the draining lymph node. Experiments to test this hypothesis are ongoing.

Once attracted to the xenograft, macrophages are capable of graft destruction. This cytotoxic process has been shown to be relatively specific. Work by Korsgren and Jansson (28) has shown that when syngeneic mouse and pig islets were admixed and placed under the kidney capsule, only the pig islets were destroyed, suggesting that specific cytotoxicity occurred. In the study reported in this work, cytolytic molecules such as NO and TNF-α were identified in the macrophages of rejecting grafts. Although these products may have been involved in graft destruction, there was no direct evidence supporting this. Macrophage-mediated cytotoxicity can occur via Ab-dependent cellular cytotoxicity and through cell-cell contact (29). Although Ab-dependent cellular cytotoxicity may be an important mechanism of graft destruction in situations in which there are preformed Abs to the xenograft, this was not the case in this study, as NOD-SCID mice lack B cells and Ab. Therefore, cytotoxicity was most likely the result of cell-cell contact and the secretion of cytotoxic or cytostatic substances. Once activated macrophages secrete many products, including reactive oxygen species, TNF-α, and complement factors (29), which, depending on the circumstances, lead to cell necrosis or apoptosis (30, 31). A better understanding of the macrophage recognition and effector pathways is essential if rational therapeutic strategies to prevent this phenomenon are to be developed.

This study provides direct evidence that macrophages are the major effector cell in T cell-mediated xenograft rejection. Previous studies have shown this to be a strong immune response that is difficult to suppress (5, 32–34). It is different mechanistically from allograft rejection in that CD8\(^+\) T cells are not involved, and more macrophage-mediated destruction occurs at early time points (35, 36). Xenografts are phylogenetically more distant than allografts, and therefore there is the potential for greater involvement of the innate immune system. Our findings suggest that in xenograft rejection, macrophages receive additional signals other than via T cells. Macrophages have a large array of pattern-recognition receptors that recognize conserved residues from bacteria and viruses (37–39) and may recognize conserved structural or genetic differences from phylogenetically distant species such as pigs. Such receptors may be important for the specificity of the macrophage response and allow appropriately activated macrophages to differentiate between pig xenograft and mouse allograft islet tissue. If this hypothesis is proven correct, it could have important implications for the design of future antirejection strategies, and the prevention of non-T cell-mediated recruitment of macrophages to the islet xenograft may be a necessary component of any future xenograft immunosuppressive protocol.

In conclusion, this study confirms that macrophages are an important effector mechanism for the destruction of pancreas islet xenografts. The fact that macrophages were capable of rejecting xenografts in the absence of any other effector cells, and were able to migrate to the graft site and identify the graft, independent of other signals from T cells, was a surprising feature of this response. To explain this phenomenon, we propose that macrophages receive signals other than those received from T cells that allow them to identify and destroy xenografted tissue. In addition, this phenomenon could explain why immunosuppressive strategies that inhibit the alloimmune response are ineffective at suppressing T cell-mediated xenograft rejection.

References


