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Interleukin-6 in islet xenograft rejection

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Abstract Earlier work on primate cardiac xenotransplantation has demonstrated a correlation between interleukin (IL)-6 levels and severity of vascular rejection. IL-6 was originally identified as a lymphokine inducing final maturation of B lymphocytes into antibody-secreting cells. The present study aimed to evaluate the role of IL-6 in fetal porcine islet-like cell cluster (ICC) xenograft rejection. Moreover, other authors have reported that eosinophils dominate the cellular response following discordant islet xenograft transplantation. Here, a technique for specific detection of eosinophils was applied. IL-6-deficient mice and wild-type controls were implanted with fetal porcine ICCs under the kidney capsule and killed 4-, 7-, and 10 days after transplantation. Xenografts were histologically evaluated, and serum samples were analyzed for IgM and IgG antibodies against ICC membrane antigens. IL-6-deficient mice and wild-type controls readily rejected the xenograft. On day 7 after transplantation, abundant numbers of F4/80$^+$ and Mac-1$^+$ cells were found distributed throughout the collapsing graft accompanied by small amounts of eosinophils and peripherally accumulated CD3$^+$ T cells (predominantly CD4$^+$). Significantly lower serum levels of IgM and IgG antibodies against ICC membrane antigens were observed in IL-6-deficient mice on day 4 or 7 after transplantation when compared to wild-type controls. No significant differences were seen on day 10 after transplantation. In both experimental groups, specific IgM and IgG antibody levels remained stable over time. In the pig-to-mouse model, IL-6 seems to be of minor importance to fetal porcine ICC xenograft rejection. Macrophages, and not eosinophils, dominate the cellular response associated with this process.

Keywords Xenotransplantation · Islet · Interleukin-6 · eosinophils · In vivo animal model · Immunochemistry

Abbrevations *BSA* Bovine serum albumin · *DTH* Delayed-type hypersensivity · *ICC* Islet-like cell cluster · *IL* Interleukin · *PBS* Phosphate-buffered saline · *RT* Room temperature · T_H T-helper

Introduction

Interleukin (IL)-6 is a multifunctional cytokine exhibiting a wide array of biological effects. It regulates immune responses, hematopoiesis, and acute phase reactions, indicating that it plays a central role in host defense mechanisms (reviewed in [1, 11]).

Earlier work on primate cardiac xenograft transplantation [23] has demonstrated that discordant pig-to-baboon xenografts induce more intense IL-6 responses

than concordant rhesus monkey-to-baboon xenografts. In this study, the IL-6 level appeared to correlate with the intensity of histologically detected rejection, especially the severity of acute vascular rejection, indicating that IL-6 may be particularly significant to discordant xenograft rejection. Interestingly, IL-6 production in T cells has been shown to be induced by T cell mitogens or antigenic stimulation in the presence of direct contact with macrophages [9]. Further, a recent study on the activity of IL-6 in the differentiation of monocytes to macrophages and dendritic cells has shown that IL-6 inhibits the differentiation of monocytes to dendritic cells by promoting their differentiation toward macrophages [25]. In addition, IL-6 has been used as a marker of mononuclear cell activation in a recent study investigating the protective effect of human decay accelerating factor in a porcine hearts perfused by human blood [40].

Findings in our earlier work [2, 3, 10, 17, 37, 38, 50] suggest that the process of islet-like cell cluster (ICC) rejection in both rats and mice is a T helper (T_H)1-dependent delayed-type hypersensitivity (DTH)-like reaction. Similarly, experimental autoimmune encephalomyelitis, an inflammatory demyelinating disease that serves as a model for multiple sclerosis, is a DTH-like reaction believed to be mediated by T_H1-cytokines [48]. Okuda and co-workers investigated the role of IL-6 in this autoimmune disease model and demonstrated that IL-6-deficient mice are resistant to the induction of autoimmune encephalomyelitis [34], suggesting that IL-6 might be of importance in the pig-to-mouse ICC xenograft rejection model.

Pancreatic islet xenograft rejection is dependent on $CD4^+$ T cells bearing T cell receptor $\alpha\beta$ chains [3, 6, 41, 42, 51]. Still, macrophages and eosinophils have been designated the main cellular subtypes infiltrating a discordant islet xenograft after transplantation in rodents. As mentioned above, results obtained in our previous studies [2, 3, 10, 17, 37, 38, 50] demonstrate that ICC xenograft rejection in rodents resembles the immune response associated with a T_H1-dependent DTH-reaction with infiltration of macrophages and peripherally accumulated T cells. In contrast, other investigators have reported that eosinophils dominate the cellular response following discordant islet xenotransplantation [20, 24, 28, 42, 43]. In these studies, eosinophils were either identified with azo eosin, an acid dye that stains the acidophilic granules of eosinophils, or with detection of endogenous peroxidase. Identification of eosinophils by detection of cells expressing endogenous peroxidase may result in an overestimate of the number of eosinophils present within the xenograft. Allergic airway inflammation-models, in which eosinophils are extensively studied, depend on reliable techniques for specific detection of eosinophils. One generally accepted technique, which has been used in mouse [16, 53] and human [5] models, is based on the histochemical visualization of cyanide-resistant endogenous peroxidase activity [45]. Here, eosinophils are distinguished from other cell types expressing endogenous peroxidase.

To investigate the role of IL-6 in fetal porcine ICC xenograft rejection, mice with a targeted mutation of the gene encoding IL-6 [13, 15] and wild-type controls were implanted with fetal porcine ICCs under the kidney capsule and killed at 4, 7, or 10 days after transplantation. Xenograft rejection was evaluated with regard to the different phenotypes of the cells infiltrating the xenograft and to antibody and complement deposition within the ICC xenograft using immunohistochemical and immunofluorescence techniques respectively. Eosinophils were detected by histochemical visualization of cyanide-resistant endogenous peroxidase activity. Serum samples were collected at the time of killing and analyzed for IgM and IgG antibodies against ICC membrane antigens.

Materials and methods

Ethics

All animal experiments were approved by the Research Ethics Committee of Uppsala University and performed in accordance with local institutional and Swedish national rules and regulations. "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) were followed.

Preparation and culture of fetal porcine pancreas

Fetal pigs served as donors for discordant xenogeneic implantation. Pregnant sows from a local stock were killed by means of a slaughtering mask at 70 ± 5 days of gestation (full-term is 115 days]. The fetuses (6–14 per litter) were then immediately collected from the uterus and placed on ice during transport to the laboratory. After aseptic removal, the pancreatic glands were minced into 1- to 2-mm^3 fragments in cold Hank's solution, and then treated with collagenase (≈ 10 mg/ml; Boehringer Mannheim, Mannheim, Germany) during vigorous shaking as previously described [18, 19]. The digested tissue was washed and explanted into culture dishes, allowing cellular attachment (Nunclon 90 mm Ø; Nunc, Kamstrup, Denmark). The culture medium was RPMI 1640 (11.1 mM glucose; Sigma Chemicals, St. Louis, MO) supplemented with 10 mM nicotinamide (Sigma Chemicals). Human serum (10% [v/v] heat inactivated; The Blood Center, Huddinge Hospital, Huddinge, Sweden) was added after 24 h. The culture dishes were kept at 37 °C in 5 % CO_2 in humidified air, and the culture medium was changed every second day. On day 4 of culture, most of the ICCs were free-floating, or could easily be detached by gently flushing the culture medium. All free-floating fragments (diameter < 0.7 mm) were considered to be ICCs and were harvested without any further purification step.

Animals (Table 1)

Male, inbred B6,129 mice, aged between 20 and 22 weeks, with a targeted mutation of the gene encoding IL-6 (IL-6 -/-; The Jackson

Table 1 Morphological evaluation of fetal porcine ICC xenografts after transplantation under the kidney capsule of mice with a targeted mutation of the gene encoding IL-6 (IL-6 -/-) and wild-type controls (WT)[a]

Recipient animal	Observation time (days)	*n*	ICC	F4/80	Mac-1	MHC class II	CD3>	CD4	CD8	Eosinophils
IL-6 -/-	4	7 (5b)	+ +	+	+	+	+	+	+	0
WT	4	6	+ +	+	+	+	+	+	+	0
IL-6 -/-	7	6 (5b)	+ (+)	+ + +	+ + +	+ +	+ (+)	+	+	+
WT	7	5	+ (+)	+ + +	+ + +	+	+	+	+	+
IL-6 -/-	10	6 (5b)	0	+ + +	+ + +	+ + +	+ +	+	+	+ +
WT	10	4	0	+ + +	+ + +	+ + +	+ +	+	+	+ +

[a] The number of remaining ICCs was assessed semiquantitatively and graded in three different categories: 0, no surviving cells; + , a few intact ICCs; and + + , intact xenograft with ICCs arranged in chords and duct-like structures. Consecutive tissue sections from each transplant were stained with the different antisera and the number of the different cell phenotypes infiltrating the xenograft was assessed semi-quantitatively and graded in four degrees of severity: 0, no infiltrating cells; + , few infiltrating cells; + + , moderate number of infiltrating cells; and + + + , abundant number of infiltrating cells

[b] Indicates the number of recipient animals surviving the period of observation and analyzed by immunohistochemistry

Laboratory, Bar Harbor, ME) were used as recipients. Controls, aged between 18 and 20 weeks, were provided from the B6129F2/J colony (The Jackson Laboratory). All rodents had free access to tap water and pelleted food (Type R34; AB AnalyCen, Lidköping, Sweden) throughout the experimental period.

Transplantation procedures

At the time of transplantation, 3 µl of fetal porcine ICCs (i.e., ≈ 300 ICCs) were implanted through an incision in the left renal capsule of avertin-anesthetized mice [18].

Histology

Animals were killed at 4, 7, and 10 days after transplantation. The ICC grafts, clearly visible as a whitish spot under the renal capsule, were excised with a margin of approximately 3 mm and stored in a histological transport medium (Histocon, Histolab, Betlehem Trading, Gothenburg, Sweden) at 4 °C until snap-frozen in isopentane and subsequently stored at –70 °C. Serial sections (6 µm thick) were cut in a cryostat (–20 °C), air-dried, and then stored at –70 °C. After storage, the slides were fixed in cold acetone, diluted 1/2 in distilled water for 30 s, followed by final fixation in cold acetone (100%) for 5 min. Tissues were routinely processed for histology and stained with hematoxylin.

Immunohistochemistry

Slides were fixed as described in Histology. Subsequent incubations of the sections with antibodies were carried out sequentially for 30 min, followed by one wash for 5 min in phosphate-buffered saline (PBS) between each step. Unspecific antibody binding was blocked by incubation with PBS containing 4% (w/v) bovine serum albumin (BSA) and 10% (v/v) normal rabbit serum (code no. X0902; Dako, Glostrup, Denmark) for 5 min. Incubations with monoclonal antibodies (antibodies to F4/80, code no. MCA497, clone CI:A3-1; Mac-1/CD11b (CR3), code no. MCA711, clone 5C6; MHC class II/H-2I-A, code no. MCA09, clone P7/7; CD3, code no. MCA500G, clone KT3; and CD8, code no. MCA609G, clone KT15, were obtained from Serotec, Kidlington, UK; antibodies to CD4/L3T4, code no. 1440, clone GK1.5, were obtained from Becton Dickinson Labware, Bedford, MA) were followed by rabbit anti-rat IgG antibody (code no. Z494; Dako), diluted 1/50 in PBS containing 4% (w/v) BSA, and 10% (v/v) normal rabbit serum (Dako). After a final incubation with a monoclonal rat alkaline phosphatase-anti-alkaline phosphatase reagent (code no. D488; Dako), the alkaline phosphatase reaction was developed using BCIP/NBT/INT (code no. K599; Dako), diluted 1/2 in levamizole (code no. X3021; Dako), an endogenous alkaline phosphatase inhibitor solution, for 2 × 10 min. The slides were counter-stained with hematoxylin and mounted in glycerine gelatin. Control experiments were performed by omitting the primary antibody. The frequency of the different cell phenotypes infiltrating the xenograft was expressed semiquantitatively and graded in four degrees of severity, as specified in Table 1.

Histochemistry

Slides were fixed as described in Histology. Eosinophils were detected by histochemical visualization of cyanide-resistant endogenous peroxidase activity [45]. In brief, tissue sections were incubated for 8 min at room temperature (RT) in PBS buffer supplemented with 3.3-diaminobenzidine tetrahydrochloride (60 mg/100 ml; code no. D 5637; Sigma Chemicals), 30% H_2O_2 (0.3 ml/100 ml; code no. 12201; KEBO Lab AB, Stockholm, Sweden), and NaCN (120 mg/100 ml). After rinsing in water, slides were counterstained with hematoxylin and mounted in glycerine gelatin. Eosinophils were identified by their dark brown reaction product. The frequency of eosinophils infiltrating the xenograft was expressed semiquantitatively and graded in four degrees of severity, as specified in Table 1.

Immunofluorescence

Stainings were performed on unfixed tissue sections. Subsequent incubations of the sections were carried out sequentially for 30 min, followed by one wash for 5 min in PBS between each step. The following were used for direct immunofluorescence staining: fluorescein-labeled rat anti-mouse IgM heavy chain (code no. MCA199F; Serotec), diluted 1/100 in PBS containing 4% (w/v) BSA; fluorescein-labeled rat anti-mouse IgG heavy chain (code

Table 2 Serum levels of IgM (anti-ICC IgM) and IgG (anti-ICC IgG) antibodies against ICC membrane antigens in IL-6-deficient mice (IL-6 -/-) and wild-type controls (WT) after transplantation of fetal porcine ICC under the kidney capsule[a]

Recipient animal	Observation time (days)	*n*	Anti-ICC IgM	An ti-ICC IgG
IL-6 -/-	4	5	0.28 ± 0.05	0.23 ± 0.05
WT	4	6	1.22 ± 0.19	0.95 ± 0.13
IL-6 -/-	7	5	0.45 ± 0.07	0.28 ± 0.03
WT	7	5	1.19 ± 0.19	1.01 ± 0.10
IL-6 -/-	10	5	0.56 ± 0.11	0.38 ± 0.05
WT	10	4	1.15 ± 0.29	0.80 ± 0.22

[a] Optical density values are given as mean ± SEM, and the Wilcoxon rank sum test was used to determine whether any significant differences between the experimental groups were present. Serum levels of specific IgM and IgG antibodies in animals killed on day 4 ($P < 0.01$) or 7 ($P < 0.05$) after transplantation were significantly lower in IL-6 -/- when compared to WT. No significant differences were seen on day 10 after transplantation. In both experimental groups, specific IgM and IgG levels remained stable exhibiting no increase between day 4 and day 10 aftertransplantation

no. MCA424F; Serotec), diluted 1/50 in PBS containing 4% (w/v) BSA; and fluorescein-labeled rat anti-mouse IgE heavy chain (code no. MCA419F; Serotec), diluted 1/40 in PBS containing 4% (w/v) BSA. A sheep anti-mouse C3 (code no. PC 280.U; The Binding Site, Birmingham, UK), diluted 1/100 in PBS containing 4% (w/v) BSA, followed by fluorescein-labeled rabbit anti-sheep IgG (code no. FI-6000; Vector Laboratories, Burlingame, CA), diluted 1/75 in PBS, and 2% (v/v) normal mouse serum (Dako), was used for indirect staining. The slides were mounted in PBS-glycerol and analyzed for green fluorescence in a Zeiss ultraviolet microscope.

Preparation of serum

Venous blood was sampled from the inferior vena cava of each mouse before removal of the ICC xenograft and centrifuged at 1500 rpm for 5 min at RT. Serum was collected and stored at –70 °C.

ELISA

Costar Microplates (Costar 3590, Cambridge, MA) were coated overnight at RT with 50 µl per well of ICC membrane antigens (39), diluted 1/200 in PBS. Unspecific antibody binding was blocked by incubation with 100 µl per well of 3% (w/v) BSA in test solution (PBS containing 1% [w/v] BSA and 0.1% [v/v] Tween20 [Apoteksbolaget, Stockholm, Sweden]) for 2 h at RT. Subsequent volumes added to each well consisted of 50 µl, and all incubations were followed by four washes with washing solution (0.154 M NaCl and 0.05% [v/v] Tween20 [Apoteksbolaget]). Serum samples, serially diluted in test solution, were added and incubated for 2 h at RT followed by incubation at 4 °C overnight. Rabbit anti-mouse IgM antibody (code no. 61–6800; Zymed Laboratories Inc.) and rabbit anti-mouse IgG antibody (H + L; code no. 61–6500; Zymed Laboratories Inc., South San Francisco, CA), diluted to optimal concentrations in test solution, were then added and wells were incubated for 90 min at RT. After a final incubation for 90 min at RT with alkaline phosphatase-conjugated sheep anti-rabbit IgG antibody (whole molecule; code no. A 8702; Sigma Immunochemicals), diluted 1:5000 in test solution, 50 µl of phosphatase-substrate, p-Nitrophenyl phosphate (pNPP; code no. 104–105; Sigma Diagnostics), dissolved in 1 M diethanolaminebuffer, pH 10.0, to a final concentration of 1 mg/ml, was added to each well. Absorbance was read at 405 nm (reference filter 630 nm) in a microplate autoreader. Serum dilution of 1/50 was used when comparing the different experimental groups.

Statistics

Optical density values of serum levels of IgM and IgG antibodies against ICC membrane antigens were given as mean ± SEM and the Wilcoxon rank sum test was used to determine whether any significant differences between the experimental groups were present (Table 2). *P* less than 0.05 was considered a statistically significant difference.

Results

Of the IL-6-deficient mice, 4 of 19 died 2 days after transplantation due to postoperative complications. The dead animals were found with re-opened surgical wounds where skin and muscle had previously been incised at the time of transplantation. All other animals tolerated the transplantation procedure without any signs of infirmity.

Morphology

No major differences in the kinetics of rejection between IL-6-deficient mice and wild-type controls were observed. Intact xenografts with ICCs arranged in chords and duct-like structures were seen on day 4 after transplantation. On day 7 after transplantation, xenografts were found to be heavily infiltrated with immune cells. At this time, intact ICCs were still present in some animals. Graft destruction was complete on day 10 after transplantation, and no morphologically intact endocrine cells remained at this stage of ICC xenograft rejection (Table 1).

Immunohistochemistry

IL-6-deficient mice and wild-type controls revealed only minor differences with regard to cellular infiltration (Table 1). On day 4 after transplantation, few cells were present peripherally within the xenograft. The ma-

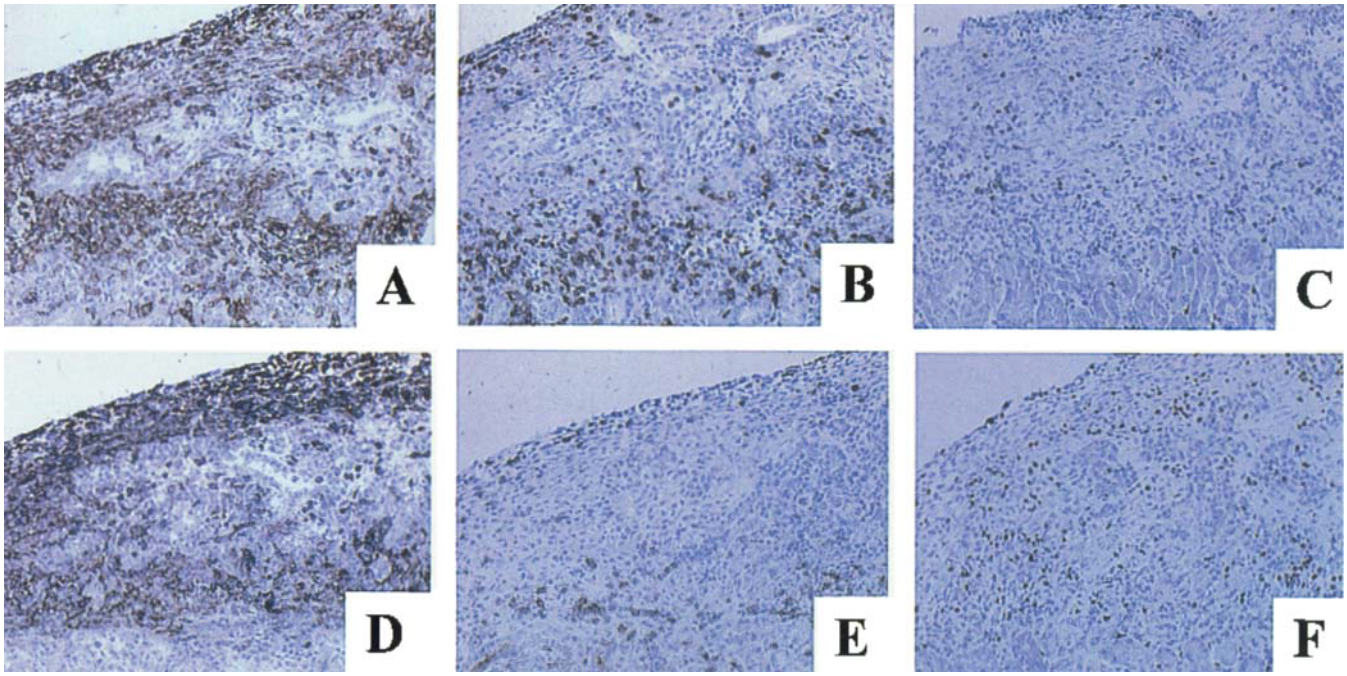

Fig. 1 Light micrographs of fetal porcine ICC xenografts after transplantation under the kidney capsule of an IL-6-deficient mouse (**A-C**) and a wild-type control animal (**D-F**). Xenografts were removed on day 7 after transplantation and immunohistochemical and histochemical stainings were performed on consecutive tissue sections. Abundant numbers of Mac-1$^+$ cells (**A** and **D**) were seen surrounding remaining ICCs. Few CD3$^+$ T cells were found accumulated peripherally (**B** and **E**). In some of the grafts (2/5) removed from IL-6-deficient animals (**B**), larger numbers of CD3$^+$ T cells were observed when compared to wild-type controls (**E**). Eosinophils were detected using a technique based on histochemical visualization of cyanide-resistant endogenous peroxidase activity. At this stage of rejection, eosinophils in small numbers started to appear within the graft (**C** and **F**). Original magnification × 400

jority of these cells stained positive for F4/80, Mac-1, and MHC class II. Scattered CD3$^+$, CD4$^+$, and CD8$^+$ T cells were found in some animals. Abundant numbers of F4/80$^+$, Mac-1$^+$ (Fig. 1 A and D), and to some extent MHC class II$^+$, cells surrounding remaining ICCs were seen on day 7 after transplantation. The number of infiltrating F4/80$^+$ and Mac-1$^+$ cells was found to be inversely related to the number of remaining ICCs. Xenografts removed from IL-6-deficient mice exhibited a somewhat larger amount of MHC class II$^+$ cells when compared to wild-type animals. Few CD3$^+$ (Fig. 1B and E), CD4$^+$, and CD8$^+$ T cells were seen accumulated peripherally. When comparing the number of CD4$^+$ and CD8$^+$ T cells invading the graft, CD4$^+$ T cells were found to predominate. In some of the grafts (2/5) removed from IL-6-deficient animals, larger numbers of CD3$^+$ T cells were observed (Fig. 1B) when compared to wild-type controls (Fig. 1E). On day 10 after transplantation, abundant numbers of cells positively stained for F4/80, Mac-1, and MHC class II were seen infiltrating the entire graft. Moderate numbers of CD3$^+$ T cells were found accumulated in the peripheral parts of the xenograft. Few CD4$^+$ and CD8$^+$ T cells were present within the same area.

Histochemistry

IL-6-deficient animals and wild-type controls exhibited identical patterns of eosinophilic infiltration (Table 1). On day 7 after transplantation, eosinophils in small numbers started to appear within the graft (Fig. 1 C and F). Moderate numbers of eosinophils were seen throughout the ICC xenograft on day 10 after transplantation.

Immunofluorescence

Similar staining patterns were seen when IL-6-deficient animals, and wild-type controls were compared with regard to antibody and complement deposition within the ICC xenograft. Although no animal displayed binding of either IgM, IgG, IgE, or C3 to xenogeneic ICCs, a diffuse staining could be seen within the area of infiltrating cells. In some animals, scattered IgM$^+$, IgG$^+$, or IgE$^+$ cells were observed. Occasionally, clusters of IgM$^+$ or IgG$^+$ cells were found within the peripheral parts of ICC xenografts removed on day 10 after trans-

plantation. These scattered Ig^+ cells might correspond to B cells rather than Ig-deposition. All animals expressed C3 on renal tubular cells.

ELISA

Serum levels of IgM and IgG antibodies against ICC membrane antigens in animals sacrificed on day 4 ($P < 0.01$) or 7 ($P < 0.05$) after transplantation were significantly lower in IL-6-deficient mice when compared to wild-type controls (Table 2). In both experimental groups, specific IgM and IgG antibody levels remained stable, exhibiting no increase between day 4 and day 10 after transplantation.

Discussion

The aim of the present study was to evaluate the role of IL-6 and eosinophils in fetal porcine ICC xenograft rejection. Earlier studies on allogeneic transplantation have suggested that IL-6 might be of major importance to allograft rejection [12, 33, 35, 36, 47, 49, 52]. Hitherto, the significance of IL-6 in xenogeneic transplantation has not been fully established. Here, we demonstrate that IL-6-deficient mice readily reject fetal porcine ICC xenografts and suggest that, in the pig-to-mouse model, the role of IL-6 in ICC xenograft rejection is minor.

Several investigators have reported that eosinophils dominate the cellular response following discordant islet xenotransplantation [20, 24, 28, 42, 43], whereas our previous studies [2, 3, 10, 17, 37, 38, 50] have shown that macrophages predominate. Using a technique based on histochemical visualization of cyanide-resistant endogenous peroxidase activity for specific detection of eosinophils [45], we show that the cellular response following fetal porcine ICC xenograft rejection is dominated by $F4/80^+$ and $Mac\text{-}1^+$ positive macrophages, and not eosinophils. Also, it is concluded that the infiltration of macrophages clearly precedes that of eosinophilic granulocytes. By day 7 after transplantation, when the rejection process is clearly initiated with massive infiltration of macrophages, only small numbers of eosinophils are present. Findings in IL-5-deficient mice implanted with fetal pig proislet xenografts further confirm that eosinophils are not required for the process of islet xenograft rejection [43].

It is still under debate whether xenoreactive antibodies are involved in the rejection process of non-vascularized xenogeneic grafts. Even though Ig-deficient mice have been proven capable of inducing complete fetal porcine ICC xenograft rejection by day 12 after transplantation, the rejection process was found to be somewhat delayed at day 6 after transplantation when compared to wild-type controls [2]. IL-6 was originally identified as a lymphokine inducing final maturation of B cells into antibody-secreting cells [8, 31, 32]. Consequently, IL-6-deficient mice exhibit reduced antibody levels and impaired antibody responses [13, 14, 15]. Measurements of serum levels of IgM- and IgG antibodies against ICC membrane antigens in our own experiments demonstrate a lack of increase in xenoreactive antibodies in IL-6-deficient mice after ICC xenotransplantation, corresponding to levels seen in wild-type controls. Nevertheless, fetal porcine ICCs are not protected against xenograft rejection in IL-6-deficient mice. Morphological evaluation of ICC xenograft rejection in IL-6-deficient animals and wild-type controls displayed similar patterns of ICC destruction and cellular infiltration with no delay in the rejection reaction as seen in Ig-deficient mice [2].

Immunohistological findings obtained in our previous studies on fetal porcine ICC xenograft rejection in rodents [2, 3, 10, 37, 38, 50] have displayed a morphological pattern of cellular infiltration much like the immune response associated with intracellular bacterial infections [26]. Further, the morphology seen in pre-immunized rats has been shown to exhibit certain similarities with the DTH-reaction [17]. IL-6-deficient mice fail to control infection with intracellular bacteria such as *Listeria monocytogenes* [4, 13] and *Mycobacterium tuberculosis* [21]. However, even though these animals exhibit impaired immune responses strongly associated with a T_H1-like cytokine profile, IL-6 is characterized as a T_H2 cytokine [29, 30]. Although IL-10-deficient animals have not yet been studied in a fetal porcine ICC xenograft transplantation model, prior work using anti-IL-3 mAb-treated or IL-4- or IL-5-deficient mice has failed to demonstrate a central role of a T_H2 response in islet xenograft rejection [43]. Analyses of intragraft cytokine mRNA expression in acute pig proislet xenograft rejection have shown that IL-2, interferon-γ, IL-3, IL-4, IL-5, and IL-10 transcripts are induced in response to the xenograft by 4 days after transplantation, whereas T_H2-associated cytokines transcripts (IL-3, IL-4, IL-5, and IL-10) remain selectively enhanced in the xenografts from day 5 [28]. These results are consistent with histochemical findings in the present work. Here, we show that by day 7 after transplantation, when collapsing ICC xenografts are heavily infiltrated by immune cells, and the acute rejection process is clearly initiated, only small numbers of eosinophils are present within the xenograft. On day 10 after transplantation, when the T_H2-associated cytokine IL-4 is the only cytokine significantly enhanced [28], an increased amount of eosinophils are seen throughout the rejected xenograft. An identical pattern of cellular infiltration with abundant numbers of macrophages and small amounts of eosinophils was found in BALB/c mice transplanted with fetal porcine ICCs under the kidney capsule (data not shown). BALB/c mice are known as

strong T_H2 responders to parasite [7, 27] and autoimmune [44] antigens, further establishing ICC xenograft rejection as a T_H1-dependent process characterized by massive macrophage infiltration [2, 3, 10, 37, 38, 50] critically dependent on $CD4^+$ T cells bearing T cell receptor $\alpha\beta$ chains [3, 6, 41, 42, 51]. Nevertheless, recent data obtained in our laboratory have shown that animals in which major parts of the T_H1-associated cytokine pathway, namely interferon-γ and IL-2 [38] or tumor necrosis factor-α [3], have been blocked, readily reject fetal porcine ICC xenografts. Taken together, it might be speculated that the rejection process of an ICC xenograft induces a vast immune response consisting of both T_H1- and T_H2-related components. Hence, the acute cellular rejection reaction following ICC xenograft transplantation does not seem to be crucially dependent on any one of these cytokines associated with T_H1- or T_H2-immunity. However, analyses on T_H cell subset differentiation have indicated that IL-12 promotes the *in vitro* differentiation of T_H lymphocytes into cells showing a T_H1-like profile of cytokine production [22]. Interestingly, a recent study on liver allograft rejection [46] has shown that administration of IL-12 to otherwise spontaneously accepting mouse liver allograft recipients was associated with a suppression of allospecific cytotoxic killer, natural killer, and lymphokine-activated killer activity, but an enhanced infiltration of F4/80^+ macrophages and higher complement-directed cytotoxic antibody titers (cf. xenograft rejection). IL-2-treatment, on the other hand, was accompanied by an increased infiltration of cytotoxic T lymphocytes, natural killer cells, and lymphokine-activated killer cells (cf. allograft rejection). Thus, even though both cytokines induced liver allograft rejection by day 5–7 after transplantation, rejection of mouse liver allografts mediated by IL-12 involves macrophages and cytotoxic antibodies largely resembling a DTH-reaction (and ICC xenograft rejection), whereas IL-2 appears to favor alloreactive cytotoxic killer activity.

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