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INTERLEUKIN-9 PROMOTES EOSINOPHILIC REJECTION OF MOUSE HEART ALLOGRAFTS

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Background. **Eosinophils participate in allograft rejection when donor-reactive helper T lymphocytes are T-helper type 2 (Th2)-biased. Whereas the involvement of interleukin (IL)-4 and IL-5 in these forms of rejection is well established, the role of IL-9, another Th2-type cytokine promoting eosinophilia, has not been determined.**

Methods. **We first used real-time polymerase chain reaction to quantify IL-9 mRNA in rejected allografts in a mouse model of fully mismatched heart transplantation in which recipients were devoid of CD8 T cells and developed a Th2 alloimmune response. We then compared allograft survival in wild-type versus IL-9–deficient mice depleted of CD8 T cells. Finally, we compared the fate of major histocompatibility complex class II-mismatched cardiac transplants from wild-type versus IL-9 transgenic donors to determine the influence of IL-9 overexpression within the graft.**

Results. **The Th2 alloimmune response in CD8-deficient mice was associated with the accumulation of IL-9 mRNA in the rejected graft. In IL-9–deficient recipients depleted of CD8 T cells, eosinophil infiltration of heart allografts did not develop, but rejection still occurred. In the major histocompatibility complex class II disparate model, heart allografts from IL-9 transgenic donors were acutely rejected, whereas grafts from wild-type donors did not develop rejection. Acute rejection of IL-9 transgenic hearts was associated with massive eosinophil infiltration and prevented by neutralization of either IL-4 or IL-5.**

Conclusion. **IL-9 is critically involved in heart transplant eosinophilia in conjunction with IL-4 and IL-5.**

Allograft rejection depends on the recognition of foreign histocompatibility antigens by the recipient's immune system. In the absence of immunosuppressive therapy, the resulting inflammatory reaction eventually leads to the rapid destruction of the transplanted tissue. The critical role played by alloreactive $CD4^+$ T cells in promoting acute allograft rejection is well established. Whereas T-helper type 1 (Th1) cells have been shown to be important effectors of the rejection process, the contribution of Th2-type responses is still a matter of debate. Indeed, Th2 cells were found to trigger alternate pathways of rejection in several models involving tumor (*1*) or transplantation antigens (*2–6*). The main features of Th2-type rejection are its dependency on the secretion of interleukin (IL)-4 and IL-5, its recruitment of eosinophils at the site of rejection, and its inhibition by alloreactive $CD8^+$ T cells (*1*, *3–6*).

IL-9 is a T-cell–derived cytokine that plays an important role in the effector arm of Th2 responses. Analysis of transgenic mice overexpressing IL-9 has disclosed its involvement in the induction of bronchial hyperresponsiveness and airway eosinophilia (*7–9*), and allergen-induced IL-9 directly stimulates mucin production by respiratory epithelial cells in asthma (*10*, *11*). In vivo inhibition of IL-9 activity has been shown to prevent Th2-dependent expulsion of parasite and blood eosinophilia in mice infected with *Trichuris muris* (*12*). Other features of IL-9 activity include induction of mast cell tissue infiltration (*7*, *13*, *14*) and expansion of antibody-secreting B-1 cells (*15*). IL-9 is produced in vitro by Th2 $CD4^+$ T-cell clones and by immunoglobulin (Ig)E-stimulated mast cells (*16–18*). This study analyzes the role played by IL-9 in Th2-type rejection of mouse cardiac allografts. Because functional redundancy is highly prevalent among the Th2 ILs (*19*), we also investigate the relative importance of Th2 cytokines IL-4 and IL-5 in IL-9–induced rejection.

MATERIALS AND METHODS

Mice and Grafting

C57BL/6 (B6) ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice were purchased from Harlan Netherland (Horst, The Netherlands). $B6.CD8^{-/-}$ ($H\text{-}2^b$) and bm12 ($H\text{-}2^{bm12}$) mice were obtained from Jackson Laboratory (Bar Harbor, MA). $B6.IL\text{-}9^{-/-}$ mice were described previously and were backcrossed for nine successive generations onto B6 background (*14*). FVB ($H\text{-}2^q$) mice expressing transgenic mouse IL-9 have been described (*13*). IL-9 is produced ubiquitously in these animals. The transgene consists of an IL-9 genomic fragment linked to the promoter of the murine pim-1 gene, including the TATA box and the cap site, followed by two copies of the Eμ enhancer and one copy of the Moloney murine leukemia virus long terminal repeat. (B6xFVB)F1 mice ($H\text{-}2^{b,q}$) were bred and used as recipients of major histocompatibility complex (MHC) class II antigen-disparate heart allografts. Normal and IL-9 transgenic (bm12xFVB)F1 mice ($H\text{-}2^{bm12,q}$) were used as donors. Heterotopic vascularized heart transplantation was performed as previously described (*5*). Transplant function was assessed daily by abdominal palpation. Rejection was defined as the complete absence of a palpable beat. Statistical significance was

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determined by using the log-rank test. All in vivo experiments were performed in compliance with the relevant laws and institutional guidelines. In some experiments, depletion of $CD8^+$ was performed by antibody treatment as described (*5*). Briefly, mice were injected intraperitoneally with 100 μg of rat IgG2a Ab to mouse CD8 (clone H35–17.2) on days −4, 1, and 5.

Quantification of Lymphokine Transcripts

Organs were frozen in liquid nitrogen after collection, and total RNA was extracted by the Tripure procedure (Roche Diagnostics, Brussels, Belgium). Samples were then treated with DNAase (Promega Benelux, Leiden, The Netherlands) to remove any contaminating genomic DNA. Reverse transcription was performed as follows. Eight microliters of H_2O containing 500 ng of total RNA were mixed with 2 μL of oligo-dT primer (0.5 μg/μL) and incubated for 10 min at 65°C. Samples were chilled on ice, and 10 μl of reverse transcriptase mix containing the following components were added: 4 μL of 5× reverse transcriptase buffer (250 mM Tris HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$); 2 μL of dNTP mix (10 mM each); 0.2 μL of bovine serum albumin (1 mg/mL); 0.5 μL (25 IU) of RNAguard (Pharmacia Biotech, Uppsala, Sweden); 1 μL (200 IU) of M-MLV reverse transcriptase; and 0.3 μL of H_2O. The mixture was then incubated for 60 min at 37°C. Quantitative polymerase chain reaction (PCR) was performed by using real-time fluorogenic PCR. Amplification of cDNA was performed with forward and reverse specific primers (Life Technologies, Merelbeke, Belgium). Fluorogenic probes were used for the detection of amplified products (Applied Biosystems, Foster City, CA). Amplification was performed on a LightCycler (Roche Diagnostics). A total of 45 cycles were performed. mRNA levels were expressed in absolute copy number, normalized against fixed numbers of copies of β-actin mRNA. Copy numbers were calculated for each sample from standard curves constructed from serial dilutions of purified plasmids for IL-9 (*16*) and β-actin (ATCC clone number 1150349) (ATCC, Manassas, VA) and from standard curves constructed from serial dilutions of purified PCR products amplified with specific primers spanning the products of real-time PCR for IL-4, IL-5, and interferon (IFN)-γ. The sequence of primers and probes for the real-time PCR reactions included the following: β-actin sense 5'-CTAAGGCCAACCGTGAAAAG-3', β−actin anti-sense 5'-AGCCTGGATGGCTACGTACAT-3', β-actin probe 5'-(6-Fam)TGACCCAGATCATGTTTGAGACCTTCA(Tamra)(phosphate)-3'; IL-9 sense 5'-CTCTCCGTCCCAACTGATGAT-3', IL-9 anti-sense 5'-GTGGCATTGGTCAGCTGTAAC-3', IL-9 probe 5'-(6-Fam) CCACACCGTGCTACAGGGAGGG(Tamra)(phosphate)-3'. IL-4, IL-5, and IFN-γ mRNA were quantified using the PreDeveloped TaqMan Assay Reagents (PE Biosystems, Foster City, CA).

In Vivo Neutralization of Lymphokine Activity

IL-9 activity was neutralized by repeated injection of purified mouse anti-mouse IL-9 monoclonal antibodies (clones MM9A1 and MM9C1) (*12*). Isotype control monoclonal antibodies (IgG2a) to trinitrophenol were produced and purified on affinity columns. Anti-IL-9 and control monoclonal antibodies (0.5 mg per injection) were given intraperitoneally on days −4 and 0, and every 4 days thereafter.

IL-4 and IL-5 were neutralized in vivo by repeated injection of ascites of rat anti-mouse specific antibodies (clones 11B11 and TRFK-5, respectively) (ATCC, Manassas, VA). Ascites of isotype (IgG1)-matched control antibodies were obtained from LO-Imex (Brussels, Belgium). Antibody concentration in ascites was determined by rat Ig isotype-specific enzyme-linked immunosorbent assay (LO-Immex). Antibodies were administered intraperitoneally at 0.5 mg per injection on days −4 and 0, and every 4 days thereafter.

Histology

Organs were fixed by immersion for 24 hr in 10% neutral formalin fixative before embedding in paraffin. Two-micron sections were prepared and stained with hematoxylin-eosin or hematoxylin-Goldner's Trichrome. Tissue sections were observed using an Axioscope light microscope from Carl Zeiss (Zaventem, Belgium). Eosinophilic inflammation was quantified by counting the number of eosinophils present in distinct high-power fields across graft sections.

RESULTS

Interleukin-9 Is Involved in Eosinophil Infiltration of Cardiac Allografts

We have shown that the rejection of fully mismatched BALB/c cardiac allografts by CD8 gene-mutated B6 recipients is characterized by the recruitment of eosinophils in the rejected organ (*5*). Here, we first determined whether eosinophil infiltration of the graft in this model was associated with the production of IL-9. As shown in Figure 1A, the absence of $CD8^+$ T cells in recipients of fully histoincompatible heart allograft resulted in a remarkable increased expression of Th2 cytokines, including IL-4, IL-5, and IL-9, within the rejected organs. In contrast, the TH1 lymphokine IFN-γ was significantly less expressed in cardiac grafts rejected by CD8-deficient recipients compared with wild-type controls.

To determine the effect of IL-9 on allograft rejection, wild-type and IL-9–deficient B6 mice were compared for their ability to reject fully histoincompatible BALB/c cardiac grafts after $CD8^+$ T-cell depletion. Both types of recipients swiftly rejected their grafts, with a mean graft survival of 9 days in both groups. However, histologic analysis of rejected allografts revealed a marked difference between wild-type and IL-9–deficient recipients. Indeed, typical eosinophil infiltrates in the myocardium were found in the former but were virtually absent in the latter (Fig. 1B and C). Thus, IL-9 seems to be required for the development of eosinophil infiltrates in cardiac allografts but not for the occurrence of rejection, indicating that in the absence of IL-9, an eosinophil-independent pathway of rejection is operative. Indeed, B6 mice are known to develop strong anti-BALB/c natural killer (NK) cell activity (*20*), and $H\text{-}2^d$–directed NK cytotoxicity may represent the main effector pathway for the rejection of BALB/c cardiac transplant in $CD8^+$ cell-depleted IL-9–deficient B6 recipients.

Overexpression of Interleukin-9 in Major Histocompatibility Complex Class II-Incompatible Donor Hearts Elicits Acute Rejection

To further delineate the significance of IL-9 in allograft rejection, we decided to use a model in which rejection occurred independently of MHC class I disparities to avoid NK cell activation. The bm12 mouse strain, derived from C57BL/6 (B6), possesses a naturally occurring mutation in its MHC class II Aβ gene conferring the allogenicity of the molecule $I\text{-}A^{bm12}$ in B6 mice. In the setting of cardiac transplantation, this incompatibility in not sufficient to elicit acute rejection but results in chronic rejection that develops beyond 60 days posttransplantation (*21*, *22*). Experiments were designed to determine whether intragraft expression of IL-9 could modify the survival of cardiac grafts expressing bm12 alloantigens. For this purpose, we constructed (bm12xFVB)F1 transgenic mice ($H\text{-}2^{bm12,q}$) overexpressing IL-9 by crossing normal bm12 mice and IL-9 transgenic FVB animals. Hearts from either IL-9 transgenic or wild-type

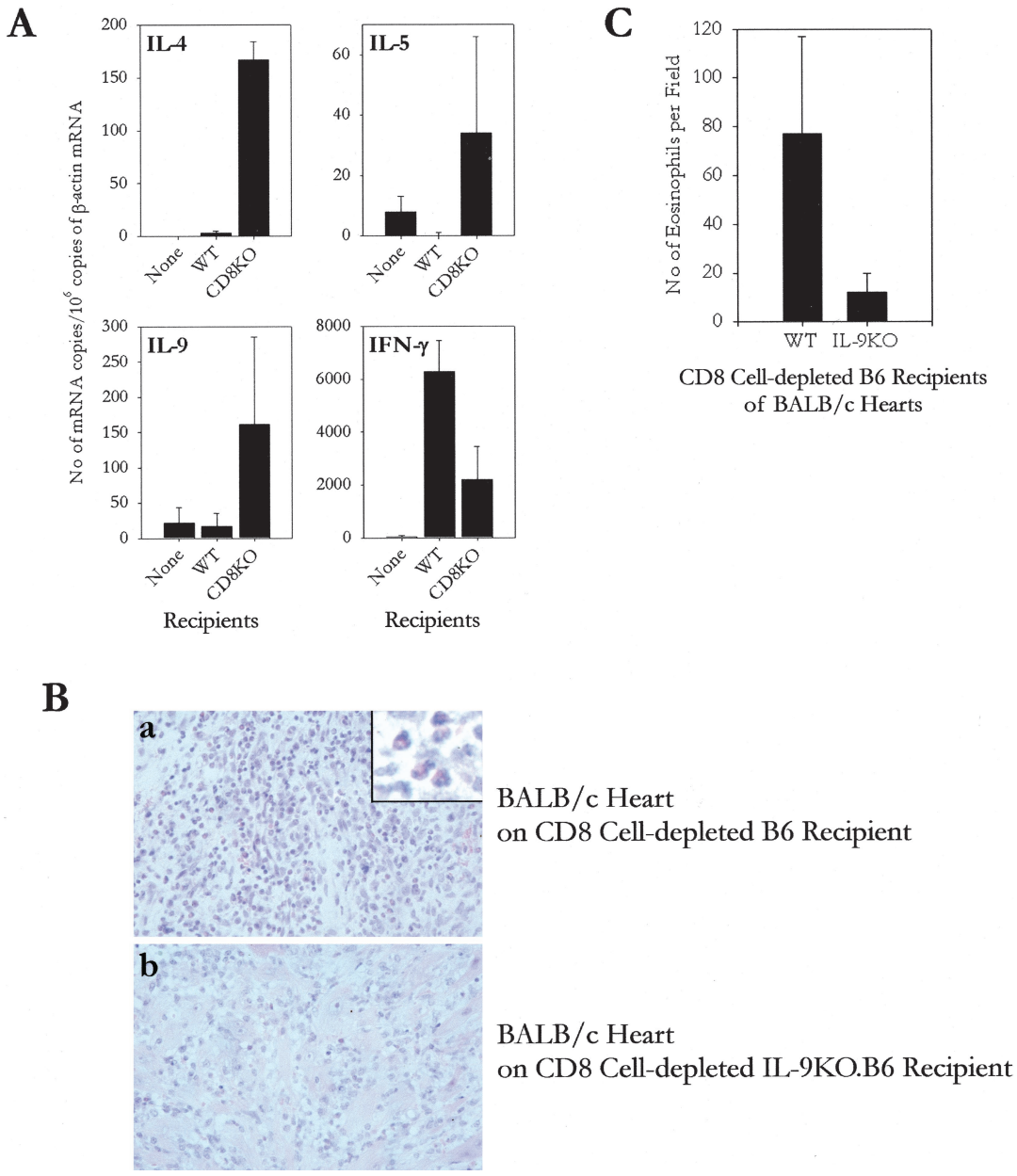

FIGURE 1. Eosinophilic inflammation of major histocompatibility complex (MHC) class II and I antigen-disparate cardiac allograft requires the production of interleukin (IL)-9. (A) Cardiac allografts rejected by CD8-deficient recipients contain mRNA for T-helper type 2 (Th2) cytokines, including IL-4, IL-5, and IL-9. (B) Eosinophils do not infiltrate cardiac grafts in the absence of IL-9 biologic activity. $CD8^+$ cell-depleted normal B6 (A) or IL-$9^{-/-}$.B6 (B) recipients were transplanted with BALB/c hearts, and the effect of IL-9 neutralization on eosinophilic inflammation of the graft was observed. (C) Eosinophil count in BALB/c hearts rejected by $CD8^+$ cell-depleted normal (wild-type) and IL-9–deficient B6 recipients (IL-9KO). Number of cells in five high-power magnification fields per section (0.0125 mm^2) is given. Significance was calculated by the Mann-Whitney *U* test ($P<0.01$).

(bm12xFVB)F1 donors were then transplanted heterotopically into (B6xFVB)F1 recipients ($H\text{-}2^{b,q}$). Whereas, as expected, all wild-type (bm12xFVB)F1 allografts remained functional for more than 50 days, all IL-9 transgenic (bm12xFVB)F1 hearts were acutely rejected in less than 30 days (Fig. 2A). In vivo administration of neutralizing anti-IL-9 monoclonal antibodies to recipients of IL-9 transgenic allografts prevented acute rejection, indicating that accelerated rejection was indeed induced by IL-9 produced at the graft level (Fig. 2A). Furthermore, acute graft damage was not solely the result of IL-9 activity but also depended on the presence of alloreactive T cells, because transgenic hearts grafted in syngeneic recipients did not experience rejection and survived indefinitely (Fig. 2A).

Histologic analysis of rejected IL-9 transgenic cardiac allografts revealed a dense cellular infiltration of the myocardium. The striking feature was the presence of numerous eosinophils and lymphocytes in the infiltrates (Fig. 2C and 3B). Allografts from wild-type donors did not exhibit such leukocyte infiltration when analyzed 30 days posttransplan-

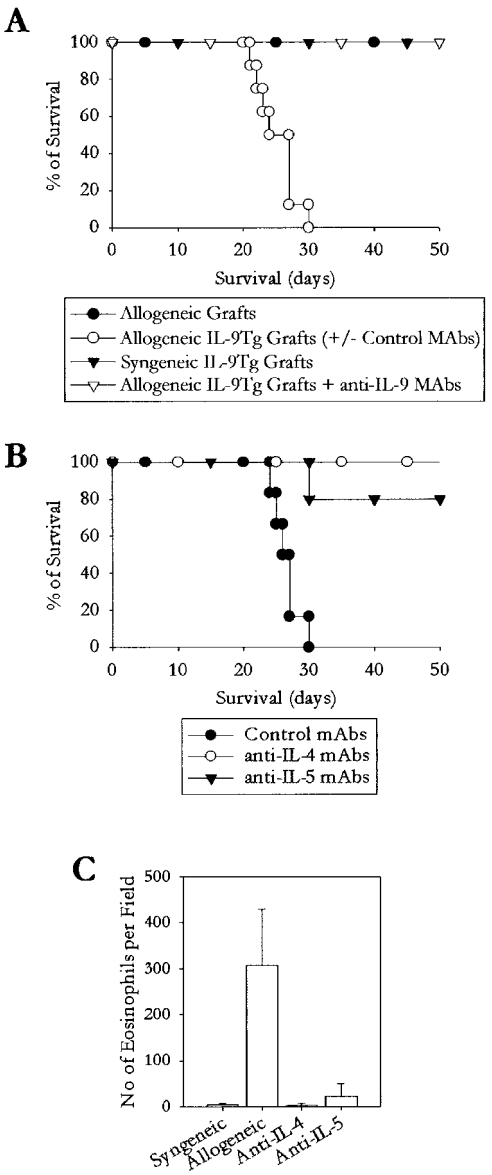

FIGURE 2. Intragraft expression of IL-9 triggers eosinophilic rejection of MHC class II antigen-disparate cardiac allografts. (A) Recipient mice underwent transplantation with normal (●) (n=6) or IL-9 transgenic (○) (n=5) MHC class II antigen-disparate heart allografts, and graft survival was assessed daily by abdominal palpation. Control groups included recipients undergoing transplantation with IL-9 transgenic syngeneic hearts (▾) (n=4) and recipients undergoing transplantation with IL-9 transgenic heart allografts and given mouse anti-mouse IL-9 monoclonal antibody (mAb) (▽) (n=3) or isotype control mouse mAb (○) (n=3; $P<0.01$). (B) Recipients of IL-9 transgenic heart allografts were given a regimen of anti-mouse IL-4 mAb (○) (n=4), anti-mouse IL-5 mAb (▾) (n=5), or control isotype IgG1 mAb (●) (n=6). Graft survival was assessed daily ($P<0.01$). Experiments presented in A and B were performed independently. (C) After staining with hematoxylin-eosin, eosinophils were counted on tissue sections from IL-9 transgenic hearts grafted in syngeneic or allogeneic recipients that received control antibodies or anti-IL-4 or anti-IL-5 neutralizing antibodies. Eosinophil counts were performed by light microscopy in 10 high-power magnification fields per section (0.025 mm^2) randomly chosen. Significance was calculated by the Mann-Whitney *U* test ($P<0.01$).

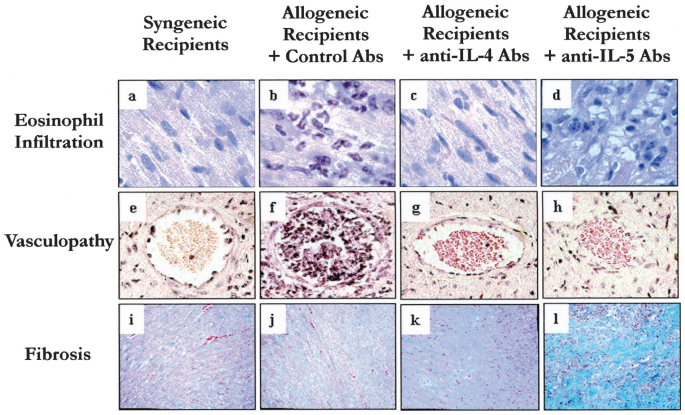

FIGURE 3. Allograft expression of IL-9 induces the recruitment of eosinophils, graft arteriopathy, and tissue fibrosis. IL-9 transgenic cardiac grafts were transplanted into syngeneic (a, e, i) or MHC class II antigen-disparate (b–d, f–h, j–l) recipients. Recipients were injected with control IgG1 isotype mAb (b, f, j) or with neutralizing anti-IL-4 (c, g, k) or anti-IL-5 (d, h, l) monoclonal antibodies. Grafts were taken for histology at rejection (b, f, j) or 50 days after transplantation (a, c–e, g–i, k, l). After fixation, tissue sections were stained with hematoxylin-eosin or Goldner's trichrome (i–l). IL-9 transgenic heart allografts showed eosinophil infiltrates (b) that were not observed in organs grafted in syngeneic (a) or anti-IL-4–treated (c) or anti-IL-5–treated (d) allogenic recipients. Arterial lesions were observed in IL-9 transgenic allografts (f). These lesions were absent from IL-9 transgenic grafts transplanted into syngeneic (e) or anti-IL-4 mAb-treated allogenic (g) recipients. Many arteries in transgenic hearts transplanted into anti-IL-5 mAb-treated recipients did not show lesions (h). Fibrosis was observed in long-term surviving grafts transplanted into anti-IL-5–treated allogenic recipients (l). Fibrotic structures did not develop in acutely rejected IL-9 transgenic hearts (j) or in organs grafted in syngeneic (i) and IL-4–treated allogenic (k) recipients. Magnification ×1000 (a–d); ×400 (e–h); ×200 (i–l).

tation (data not shown). Likewise, IL-9 transgenic hearts transplanted into syngeneic recipients were devoid of cellular infiltrates (Fig. 2C and 3A). Acutely rejected IL-9 cardiac allografts also exhibited substantial arteriopathy (Fig. 3F). The arterial lesions consisted of dense accumulations of mononuclear cells and fibrotic structures resulting in virtually complete occlusion of the coronary arteries. These lesions, however, did not involve proliferation of smooth muscle cells. They resembled arteriopathy seen in cardiovascular disease associated with the hypereosinophilic syndrome (*23*), involving cytolytic lesions of the arterial endothelium and thrombus formation. This graft arteriopathy was absent in allografts from wild-type donors (data not shown) and after transplantation of IL-9 transgenic hearts in syngeneic recipients (Fig. 3E).

Interleukin-4 and -5 Are Involved in Acute Rejection of Cardiac Allografts Overexpressing Interleukin-9

Th2-derived cytokines, such as IL-4, IL-5, and IL-9, have all been implicated in diseases such as asthma, allergy, parasite infection, and autoimmunity. However, their relative importance in specific diseases often remains obscure as the result of functional redundancy. Moreover, because of the

complexity of cytokine networks, compensatory mechanisms often prevail in the induction of Th2 cytokine-regulated immune activation. To study the contributions of two other major Th2 cytokines, IL-4 and IL-5, in the rejection of IL-9 transgenic hearts, we blocked the activity of these cytokines in vivo by administering neutralizing monoclonal antibodies. As shown in Figure 2B, IL-4 neutralization prevented acute rejection of IL-9 transgenic cardiac allografts. Histologically, these grafts did not exhibit leukocyte infiltration or arteriopathy (Fig. 2C and 3G) and could not be distinguished from IL-9 transgenic grafts transplanted into syngeneic recipients.

IL-5 is a key cytokine for the differentiation, activation, and tissue recruitment of eosinophils. In this model, IL-5 neutralization extended the survival of most allografts (Fig. 2B). Although it prevented eosinophil influx in the graft, it did not abrogate leukocyte infiltration (Fig. 2C and 3D). Anti-IL-5 treatment also substantially inhibited the arteriopathy affecting rejected IL-9 transgenic hearts, and many coronary vessels did not develop lesions (Fig. 3H). Notably, IL-9 transgenic hearts that survived indefinitely after anti-IL-5 treatment exhibited substantial fibrosis (Fig. 3L). Such fibrotic lesions were not found in IL-9–overexpressing cardiac allografts transplanted into IL-4–deficient recipients (Fig. 3K) or in syngeneic IL-9 transgenic grafts (Fig. 3I).

DISCUSSION

Our results highlight the potential role played by IL-9 in the process of allograft rejection mediated by Th2 $CD4^+$ T cells. We observed the following: (1) IL-9 mRNA transcripts are specifically present within cardiac allografts undergoing Th2-mediated rejection, (2) failure to produce IL-9 prevents eosinophilic inflammation of cardiac allografts, (3) transgenic expression of IL-9 within MHC class II antigen-disparate cardiac allografts selectively triggers acute rejection together with infiltration of eosinophils, and (4) blockade of IL-4 or IL-5 abolishes or reduces rejection of IL-9–expressing cardiac grafts. Taken together, these findings demonstrate that IL-9 can promote allograft rejection in conjunction with IL-4 and IL-5.

Activated eosinophils exert cytotoxic activity through the release of several molecules such as eosinophil-derived neurotoxin, eosinophil cationic protein, major basic protein, and eosinophil peroxidase (*24*). These molecules are most likely responsible for the ability of eosinophils to affect microvascular permeability and induce acute tissue damage in rejected organs. The observation that neutralization of IL-5 inhibits the rejection of IL-9–expressing hearts argues in favor of eosinophils being the prime effectors in IL-9–dependent rejection. However, the mechanism of activity of IL-9 on eosinophils remains ill defined. By increasing the expression of the IL-5 receptor α chain at the surface of eosinophils and by enhancing eosinophil resistance to apoptosis (*25*), IL-9 could favor the development of eosinophilic infiltrates into the rejected graft. Because it has also been shown to induce the expression of chemotactic factors (*9*), IL-9 might as well act indirectly on leukocyte recruitment inside the graft. We observed that IL-9 transgenic hearts undergoing rejection specifically exhibited higher levels of CCL-7 (MCP-3), CCL-12 (MCP-5), and CCL-24 (eotaxin-2) (data not shown). These chemokines have been reported to have a strong chemotactic activity on Th2 $CD4^+$ T cells and eosinophils, possibly through the engagement of CCR-1 or CCR-3 at the cell surface (*26*). Up-regulation of MHC class II molecule expression on graft cells by T-cell–derived cytokines would then ensure the sustained stimulation of alloreactive T cells and the development of full-scale Th2-type inflammation leading to rejection.

The observation that neutralization of IL-4 prevented the rejection of IL-9 transgenic cardiac grafts demonstrates that IL-9–dependent mechanisms of rejection need IL-4 to develop. Although IL-4 is presumed to be necessary for the generation of IL-9–secreting Th2 cells, it might also regulate the expression of molecules required for the development of IL-9–dependent inflammatory processes leading to graft rejection. For example, the expression level of the vascular cell adhesion molecule-1 (CD106) (*27*), which is normally associated with inflammatory processes, is up-regulated by IL-4. Moreover, the production of other molecules involved in the regulation of Th2-type inflammation, such as chemokines, can also be controlled by IL-4 (*26*).

IL-9, earlier described as a T-cell growth factor (*16*), could also participate in the ontogeny of T-cell response and in the development of alloreactive Th2 cell clones. However, recent data from studies using mice deficient for the IL-9 gene indicate that IL-9, although it might modulate the process, is not required for T-cell development and generation of Th2 cells (*14*). Thus, if IL-9 does play a role in the development of alloreactive Th2 cells, it seems that alternative pathways would probably work in its absence. Nonetheless, IL-9 has been described as a potent anti-inflammatory factor capable of inhibiting IL-12 production and septic shock associated with infection by bacteria (*28*). Whether one of the effects of IL-9 in Th2-mediated rejection is to inhibit the development of Th1 T cells is not known.

Our observation that IL-9 mRNAs are detected in rejected fully MHC-mismatched allografts is in agreement with Van Hoffen and colleagues' results in transplanted human hearts (*29*). This observation, however, is in direct contrast with the report made by Li et al., who failed to observe RNA transcripts for IL-9 in mouse islet or human renal allografts undergoing acute rejection (*30*). Moreover, our study showed that the absence of systemic IL-9 prevents eosinophil recruitment to the graft but does not affect rejection of full MHC-disparate allograft. Such data argue against the importance of this phenomenon in rejection. However, we and other investigators have shown that the mechanisms of rejection associated with eosinophilic infiltration are only operational in the absence of MHC class I-dependent cell activation (*4–6*). It is therefore possible that in donor and recipient combinations involving full MHC disparities, rejection mechanisms promoted by IL-9 are masked by more prevalent effector pathways, such as cytotoxic T lymphocyte- or NK-mediated cytotoxicity. On the other hand, one can also propose that applying immunosuppressive therapies targeting the classical pathways of rejection might favor the emergence of alternate effector pathways in which the production of IL-9 could represent a major event (*29*). The fact that transgenic expression of IL-9 in donor MHC class II-disparate heart-triggered rejection indicates that the local production, rather than systemic, determines the ability of the lymphokine to mediate the rejection process.

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