

XENOGENEIC EXTRACELLULAR MATRIX GRAFTS ELICIT A TH2-RESTRICTED IMMUNE RESPONSE¹

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Background. Porcine small intestinal submucosa (SIS) is an acellular, naturally derived extracellular matrix (ECM) that has been used for tissue remodeling and repair in numerous xenotransplantations. Although a vigorous immune response to xenogeneic extracellular matrix biomaterials is expected, to date there has been evidence for only normal tissue regeneration without any accompanying rejection. The purpose of this study was to determine the reason for a lack of rejection.

Methods. Mice were implanted s.c. with xenogeneic tissue, syngeneic tissue, or SIS, and the graft site analyzed histologically for rejection or acceptance. Additionally, graft site cytokine levels were determined by reverse transcriptase polymerase chain reaction and SIS-specific serum antibody isotype levels were determined by ELISA.

Results. Xenogeneically implanted mice showed an acute inflammatory response followed by chronic inflammation and ultimately graft necrosis, consistent with rejection. Syngeneically or SIS implanted mice, however, showed an acute inflammatory response that diminished such that the graft ultimately became indistinguishable from native tissue, observations that are consistent with graft acceptance. Graft site cytokine analysis showed an increase in interleukin-4 and an absence of interferon- γ . In addition, mice implanted with SIS produced a SIS-specific antibody response that was restricted to the IgG1 isotype. Reimplantation of SIS into mice led to a secondary anti-SIS antibody response that was still restricted to IgG1. Similar results were observed with porcine submucosa derived from urinary bladder. To determine if the observed immune responses were T cell dependent, T cell KO mice were implanted with SIS. These mice expressed neither interleukin-4 at the implant site nor anti-SIS-specific serum antibodies but they did accept the SIS graft.

Conclusions. Porcine extracellular matrix elicits an immune response that is predominately Th2-like, consistent with a remodeling reaction rather than rejection.

INTRODUCTION

The ability to perform successful xenotransplantation would alleviate the disparity between the large number of human organs needed for transplants compared to the small number available (1). The pig has been the donor of choice for xenotransplants because of its similar organ physiology to the human. However, most transplantations of vascularized organs from pig to human are rejected within minutes or a few hours after implantation due to hyperacute rejection that occurs when naturally occurring anti-Gal α (1, 3) Gal antibodies of the host (human) interact with the Gal α (1, 3) Gal epitope found on endothelial cells that line the blood vessels of the donor (pig) organ. This results in complement activation and subsequent rejection of the organ (2–5). Delayed xenograft rejection can also present problems in xenograft transplants. It occurs 3 to 5 days after grafting and is manifested by anti-donor antibodies binding to the endothelium of the graft. This interaction activates endothelial cells and results in the production of proinflammatory cytokines and chemokines that attract and activate host macrophages and natural killer cells (6–8).

Extracellular matrix (ECM) biomaterials of xenogeneic origin, such as porcine small intestinal submucosa (SIS), are beginning to be used as acellular, resorbable bioscaffolds for tissue repair. Because these materials are avascular, hyperacute rejection mechanisms involving endothelial activation and intravascular thrombosis of the graft are avoided. When used as xenografts, ECMs appear to induce site-specific remodeling in the organ or tissue into which they are placed. ECM biomaterials have been used successfully in many species to repair cardiovascular tissue (9), abdominal wall defects (10), urinary bladder defects (11), and ligament and tendon damage (12, 13). Despite the xenogeneic nature of the graft material and the fact that no immunosuppression is used, there has never been any clinical or histological evidence of immediate or delayed rejection. It is intuitive that the graft recipient should mount an immune response to the foreign xenograft but whether such a response occurs and its nature is presently unknown.

Several studies have analyzed the role of Th1 and Th2 lymphocytes in cell mediated immune responses to xenografts (14, 15). Th1 lymphocytes produce interleukin- (IL)2, interferon- (IFN) γ , and tumor necrosis factor- (TNF) β lead-

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ing to macrophage activation, stimulation of complement-fixing antibody isotypes (IgG2a and IgG2b in mice) and differentiation of CD8⁺ cells to a cytotoxic phenotype (16, 17). Activation of this pathway is associated with both allogeneic and xenogeneic transplant rejection (14, 15, 18). Th2 lymphocytes produce IL-4, IL-5, IL-6, and IL-10, cytokines that do not activate macrophages and lead to production of non-complement-fixing antibody isotypes (IgG1 in mice). Activation of the Th2 pathway is associated with transplant acceptance (19–21).

Our studies were designed to determine if ECM can in fact elicit an immune response and, if so, to determine the nature and kinetics of that response. The results suggest that implanted ECM elicits a vigorous immune response but this response is restricted to the Th2 pathway, which may allow acceptance and remodeling of the graft tissue.

MATERIALS AND METHODS

Animals. Female BALB/c and C57BL/6 mice 5–6 weeks of age were obtained from Charles River Laboratories (Raleigh, NC) through the National Cancer Institute (Bethesda, MD). C57BL/6 T cell KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice have gene disruptions in T cell receptor (TCR) β and δ constant regions and therefore, they lack both $\alpha\beta$ and $\gamma\delta$ T cells. Animals were maintained in a specific pathogen-free environment with food and water provided ad libitum.

ECM. Representative extracellular matrix materials, porcine SIS, and urinary bladder submucosa (UBS) (Cook Biotech, Inc., West Lafayette, IN) were prepared as previously described (22). Briefly, porcine intestine was used to prepare small intestinal submucosa. The intestine was washed free of debris, everted, and subjected to mechanical delamination to remove the superficial layers of the mucosa. Then, it was reverted to its original orientation, split open longitudinally, rinsed in water to lyse any cells present and to remove cellular debris. The SIS was cut into 1-cm³ pieces, irradiated at 3000 rads for 15 min, and stored in sterile phosphate-buffered (PBS) at 4°C until used for implantation.

Tris extracts of ECM. Soluble preparations of SIS and UBS were obtained by pulverization of frozen ECM under liquid nitrogen and suspension at 25% (w/v) in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors. This was followed by vigorous stirring for 24 hr at 4°C. The mixture was centrifuged at 12,000×g for 30 min at 4°C and the supernatants were dialyzed extensively against deionized water. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA).

Surgery. Mice were anesthetized with methoxyflurane and their abdomens clipped and prepared for sterile surgery. A 1-cm² graft of SIS, UBS, BALB/c, or C57BL/6 mouse abdominal muscle (syngeneic control), or rat abdominal muscle (xenogeneic control) was placed in a ventral midline subcutaneous pocket and secured to the underside of the skin with 6–0 prolene sutures. Sham-treated animals were subjected to surgery without implantation of tissue. Groups of four animals each were killed at days 1, 4, 7, 10, and 28 days postsurgery. A sham-operated control animal was included at each time point. At the time of sacrifice, blood was drawn from anesthetized animals by retroorbital bleeding. Animals were then euthanized by halothane inhalation and the graft site, including implanted tissue, underlying abdominal wall, and overlying skin was excised and divided longitudinally. One portion was snap-frozen in liquid nitrogen and stored at –80°C for RNA analysis and the remainder of the tissue was fixed in 10% neutral buffered formalin for histopathological evaluation.

Histopathological analysis. Paraffin-embedded tissue was sectioned to a thickness of 7 μ m and stained with hematoxylin and eosin by standard methods. Pathology at the graft site was determined in a double-blind fashion.

Cytokine RNA analysis. Total RNA was isolated from frozen implant tissue using Trizol reagent (Life Technologies, Rockville, MD) according to the manufacturer's directions. Briefly, frozen tissue was homogenized with a mortar and pestle, then transferred to a tube containing 2 ml of Trizol reagent. The samples were centrifuged at 12,000×g and the supernatants were extracted with chloroform. The RNA was precipitated with isopropanol, washed in 75% ethanol and dissolved in diethylpyrocarbonate treated water. RNA was quantitated by spectrophotometric analysis at 260 nm and determined to be intact and free of DNA contamination by electrophoresis on 1% agarose gels. Cytokine expression at the graft site at various times post implantation was determined by reverse transcriptase polymerase chain reaction (RT-PCR) using primers for IFN- γ (23), a representative Th1 cytokine, and IL-4 (23), a representative Th2 cytokine. Primers for the housekeeping gene, hypoxanthine phosphoribosyl transferase (HPRT), were included in all assays to ensure equal loading of nucleic acid. PCR amplification of 2 μ l cDNA prepared from total RNA was performed as previously described (23). The PCR products were separated on 2.5% agarose gels and visualized under UV light after staining with ethidium bromide. Real time PCR was performed as described (24).

ELISA for determination of ECM specific antibodies. Serum samples were analyzed by ELISA for IgM, IgG1, IgG2a, IgG2b, and IgG3 ECM specific antibodies. Microtiter plates were coated overnight at 4°C with 10 μ g/ml of a Tris-HCl extract of ECM. The plates were washed and blocked with PBS containing 5% fetal bovine serum and 0.3% Brij-35. Serial 2-fold dilutions of serum were added to the wells and incubated for 2 hr at room temperature (RT). The plates were washed and incubated with alkaline phosphatase conjugated goat anti-mouse isotype specific antibodies (Southern Biotechnology Associates, Birmingham, AL) for 1 hr at RT. Appropriate working dilutions and the isotype specificities of these reagents were tested using myeloma proteins of defined isotypes (Sigma, St. Louis, MO). The plates were again washed, p-nitrophenyl phosphatase substrate (Sigma) was added and absorbance was read at 405 nm on a microplate reader (Bio-Tek Instruments, Winooski, VT). Pooled serum from BALB/c mice immunized i.p. with 50 μ g Tris extract of ECM in complete Freund's adjuvant followed 28 days later by a booster injection of 50 μ g Tris extract in incomplete Freund's adjuvant was used as a positive control. Preimmune serum from implanted mice was included as a negative control.

RESULTS

Histopathology analysis of engrafted mice. The histopathological response of BALB/c mice implanted with SIS, syngeneic tissue, or xenogeneic tissue was used as a marker for acceptance or rejection. Syngeneic and xenogeneic muscle tissue were used simply as proven models for visualization of acceptance and rejection, respectively. Implantation of rat tissue caused an acute inflammatory reaction consisting mostly of polymorphonuclear leukocytes (PMNs) beginning 1 day after implantation (Fig. 1). By day 10, the inflammation was chronic and hematoxylin and eosin staining revealed neutrophils at the graft site. Immunohistochemical analysis revealed an influx of T cells based on positive staining for CD3 and negative staining for B220 (data not shown). Additionally, there was focal degeneration of the implant and evidence of giant cell formation (Fig. 2). By day 28, there was dramatic worsening of the inflammatory process both within the implant and the surrounding interface between the implant and host tissue. The implant was completely necrotic and "walled off" from the host tissue. Additionally, tissue at this time exhibited granuloma formation at the interface between the implant and host tissue (Fig. 3). Taken together,

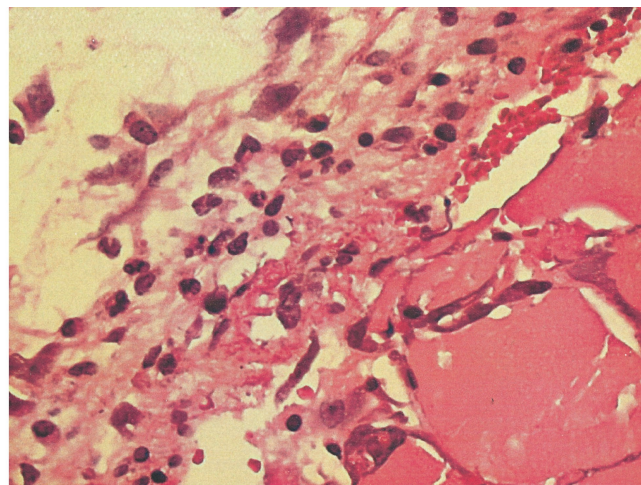
these observations are indicative of rejection of the xenogeneic implant.

Implantation of syngeneic tissue resulted in an initial, acute inflammatory response both within the graft and at the interface with the host tissue (Fig. 1). By day 10, there was minimal chronic inflammation and the acute inflammation had mostly resolved (Fig. 2). By day 28, the implant was well

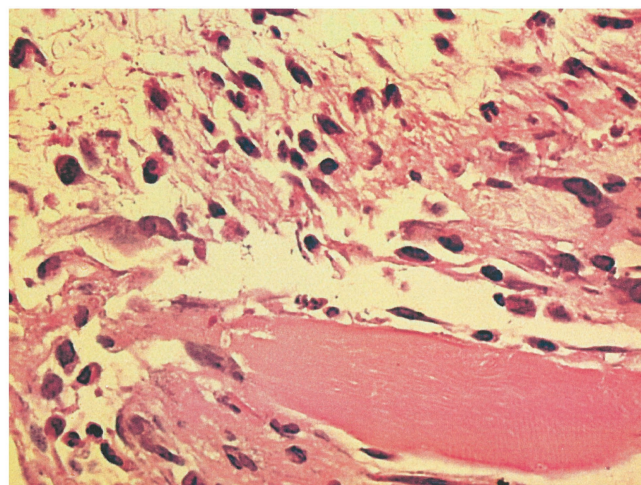
organized and all tissue degenerative changes associated with inflammation had completely resolved (Fig. 3). These observations are consistent with graft acceptance.

Implantation of SIS resulted in acute inflammation consisting of PMNs mainly at the interface of the host tissue and the SIS implant (Fig. 1). By day 10, the inflammation was greatly reduced and consisted of mononuclear cells with a

Xenogeneic



Syngeneic



SIS

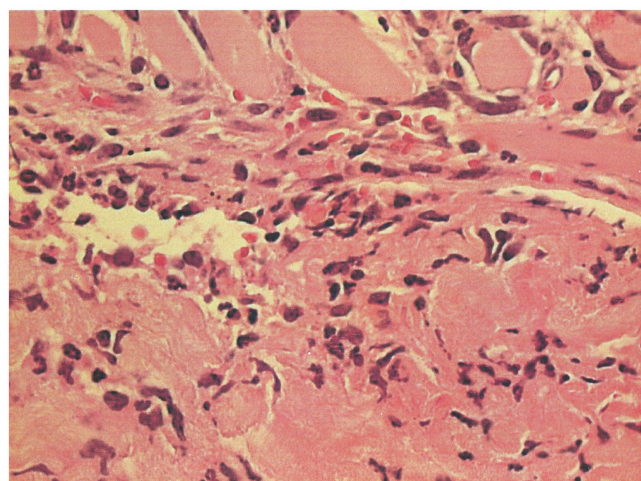


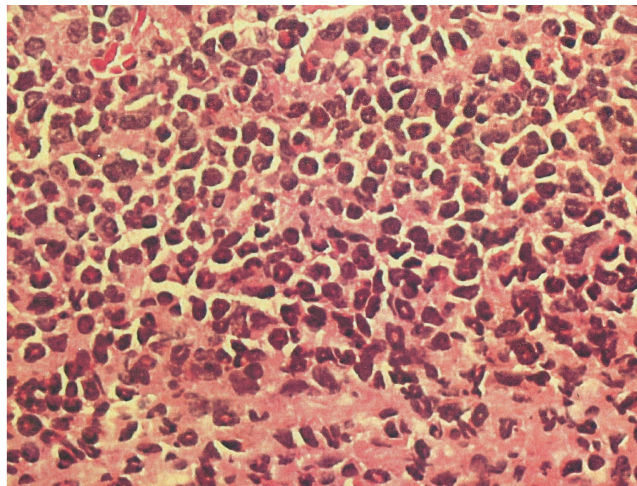
FIGURE 1. Histological analysis (40× magnification) of graft sites from BALB/c mice implanted with syngeneic muscle tissue, xenogeneic muscle tissue, or porcine SIS. Four days after tissue implantation, graft site tissue was harvested, sectioned, and stained with hematoxylin and eosin.

few scattered neutrophils (Fig. 2). The implant was surrounded by increased tissue organization and fibroblastic proliferation indicating that tissue remodeling was occurring. On day 28, the inflammatory process was completely resolved (Fig. 3). Together, these observations are indicative of acceptance. Thus, the host response to SIS was similar to that of syngeneic tissue with a transitory, acute inflamma-

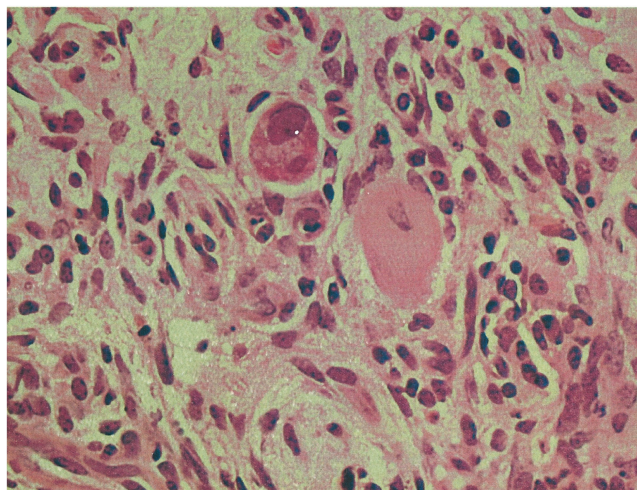
tory response, followed by resorption and remodeling by native tissue.

Cytokine mRNA analysis of SIS-grafted mice. The results of the histopathological studies were consistent with failure of the mice to reject xenogeneic SIS. Rejection of xenografts is thought to be mediated by Th1 responses involving cytotoxic T cells induced by IFN- γ (14). Therefore, we measured ex-

Xenogeneic



Syngeneic



SIS

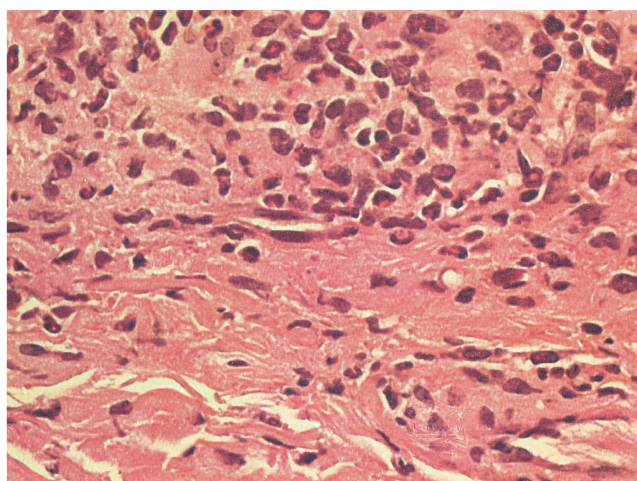


FIGURE 2. Histological analysis (40 $\times$ magnification) of graft sites from BALB/c mice implanted with syngeneic muscle tissue, xenogeneic muscle tissue, or porcine SIS. Ten days after tissue implantation, graft site tissue was harvested, sectioned, and stained with hematoxylin and eosin.

Xenogeneic

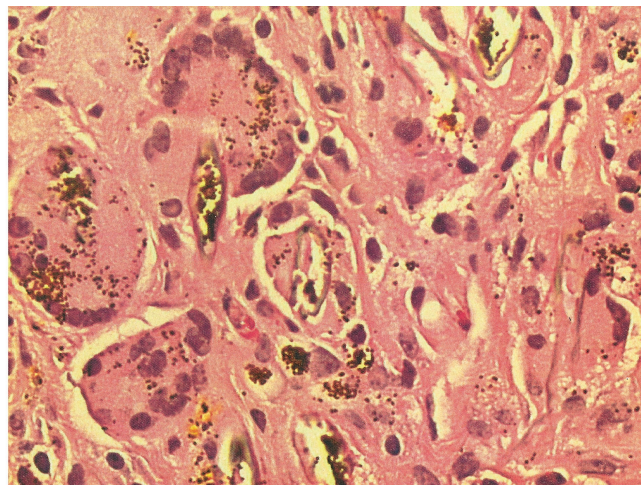
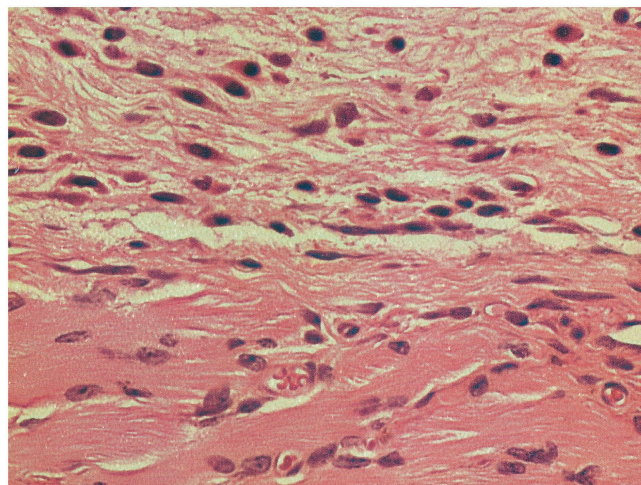
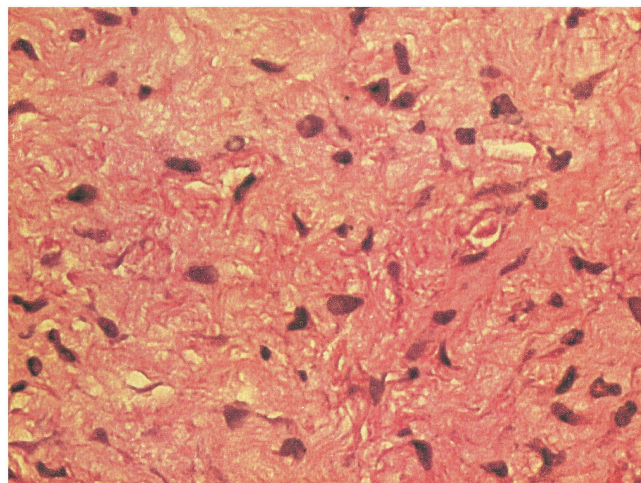


FIGURE 3. Histological analysis (40× magnification) of graft sites from BALB/c mice implanted with syngeneic muscle tissue, xenogeneic muscle tissue, or porcine SIS. Twenty-eight days after tissue implantation, graft site tissue was harvested, sectioned, and stained with hematoxylin and eosin.

Syngeneic



SIS



pression of Th1 and Th2 cytokines at the graft site after implantation into BALB/c mice. The results showed that at the time of mononuclear cell infiltration (7 days after implantation) xenografts contained both IFN- γ and IL-4 mRNA (Fig. 4). Mice implanted with syngeneic tissue expressed IL-4 mRNA, as did SIS-implanted mice, which actually demonstrated augmented IL-4 mRNA expression compared to control animals (Fig. 4). The RT-PCR results were confirmed by real-time RT-PCR which showed that SIS-implanted mice

had 100-fold fewer copies of IFN- γ mRNA at the graft site compared to mice implanted with xenogeneic tissue. Interestingly, mice implanted with SIS had 10-fold fewer copy numbers of IFN- γ compared to mice implanted with syngeneic tissue suggesting that SIS is suppressive for IFN- γ expression. The graft sites from all groups were also analyzed for IL-5, IL-6, and TGF- β . Very low levels of IL-6 were detected in some grafts although IL-5 and TGF- β were not detected (data not shown).

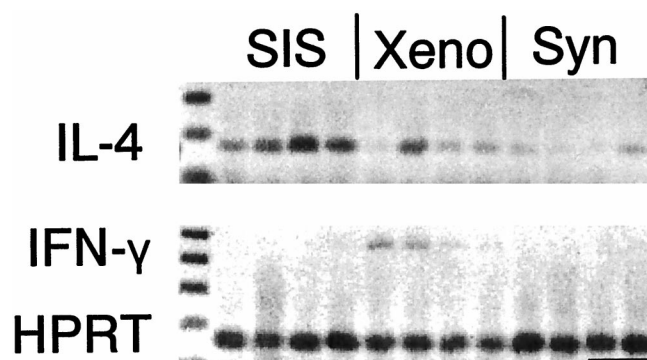


FIGURE 4. Cytokine mRNA analysis of the graft site from BALB/c mice (four per group) implanted with syngeneic muscle tissue, xenogeneic muscle tissue, or porcine SIS. Seven days postimplantation, total RNA was analyzed for the expression of IL-4 (173 bp) and IFN- γ (459 bp) by RT-PCR. HPRT (162 bp) was used as an internal control for RNA loading. Molecular size standards are shown at the left of each panel.

Anti-SIS antibody analysis of SIS-implanted mice. Induction of SIS-specific antibodies of defined Th1 isotypes (IgG2a and IgG2b) and Th2 isotypes (IgG1) was next examined as a

marker of Th subset activation. Sera from BALB/c mice immunized with a Tris extract of the SIS in complete Freund's adjuvant followed by a boost with the same material in incomplete Freund's adjuvant contained high levels of IgG1, IgG2a, and IgG2b SIS-specific antibodies (Fig. 5). This shows that mice can, under these conditions, respond to xenogeneic SIS with activation of both the Th1 and Th2 pathways. SIS implanted mice, however, expressed specific antibodies that were restricted to the IgG1 isotype. As expected, serum from normal mice did not contain SIS-reactive antibody, nor did sera from mice implanted with syngeneic or xenogeneic muscle tissue. No SIS-reactive IgM or IgG3 antibodies were detected in any of the animals (data not shown). Thus, although there was a vigorous immune response to the xenografted ECM after implantation, this response was restricted to the Th2 pathway. These results are in accordance with the pattern of cytokine expression noted at the graft site on day 7 (Fig. 4).

To confirm that the immune response to SIS was due to Th2 restriction rather than lack of sufficient antigen stimulation, antibody responses in BALB/c mice receiving two sequential SIS implants 28 days apart were measured. These animals showed a significant secondary SIS-specific antibody response but the response was still exclusively of the IgG1

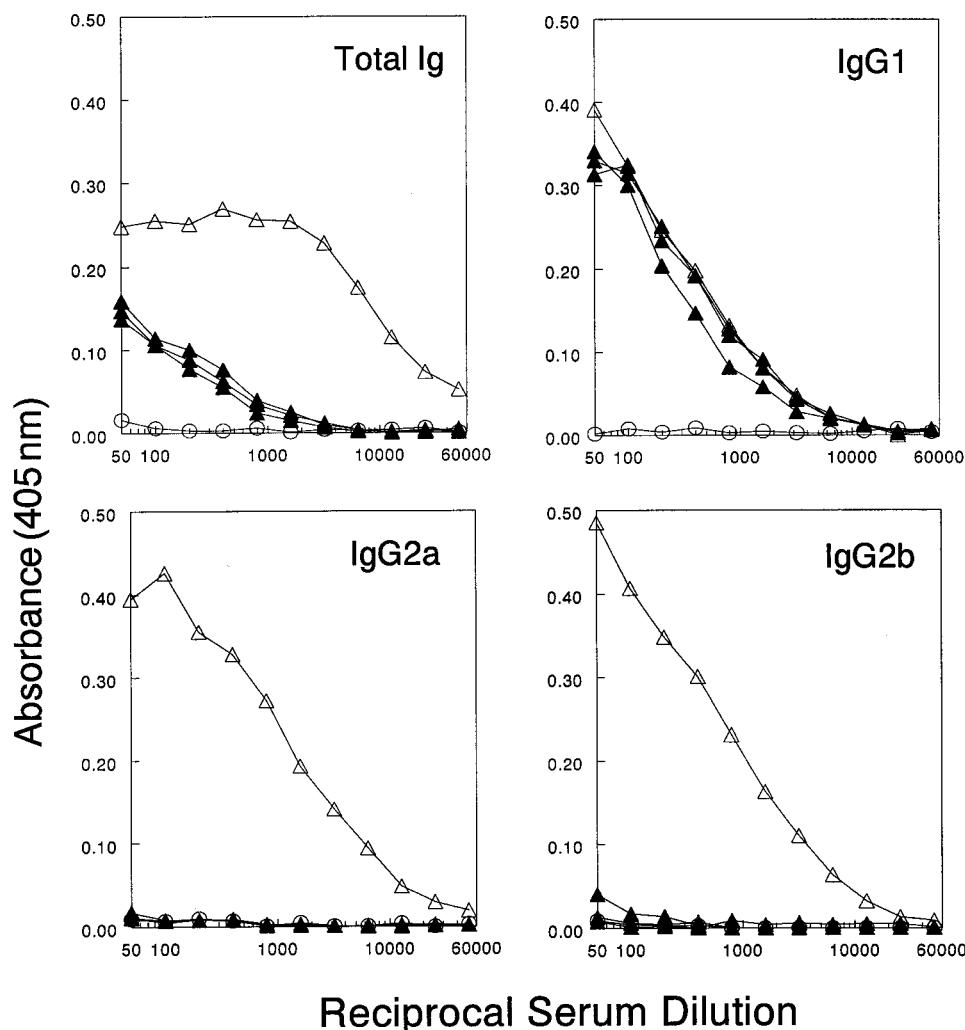


FIGURE 5. Serum antibody responses of BALB/c mice to SIS as determined by isotype-specific ELISA. Mice (three per group) were analyzed for serum anti-SIS antibody 21 days after implantation of porcine SIS (▲). Normal serum pooled from three mice served as a negative control for SIS binding (○). Serum pooled from three mice that had been deliberately immunized with solubilized SIS emulsified in complete Freund's adjuvant served as a positive control (▲).

isotype (Fig. 6). Cytokine profiles at the secondary graft site similarly showed no evidence for induction of Th1 cytokines (data not shown). Furthermore, to determine if the SIS graft was absorbing out IgG2a antibodies, antibody deposition on the SIS graft was analyzed. Immunohistochemical results showed no evidence for deposition of IgG1 or IgG2a antibodies on the graft (data not shown).

To determine if ECM derived from another source induced an immune response similar to that of SIS, UBS was implanted into BALB/c mice. As in the SIS immune response, specific antibodies were expressed by UBS implanted mice

but were restricted to the IgG1 isotype (Fig. 7). Additionally, the results demonstrated that UBS-reactive serum cross-reacted with SIS (Fig. 7). Thus, the source of ECM did not alter the restricted Th2 immune response.

The immune response to SIS is T cell dependent. To determine if the observed cytokine and antibody responses to SIS were T cell-dependent, C57BL/6 T cell KO mice were implanted with SIS and analyzed as above. Histologically, the graft sites showed a mild inflammatory response that resolved by day 10 postimplantation. By day 28 postimplantation, the graft was completely remodeled and indistinguishable from native tissue (Fig. 8). Cytokine analysis showed that T cell KO mice graft sites did not contain IL-4 mRNA (Fig. 9), demonstrating that T cells are the source of IL-4 mRNA observed in SIS implanted wild-type mice. Analysis of

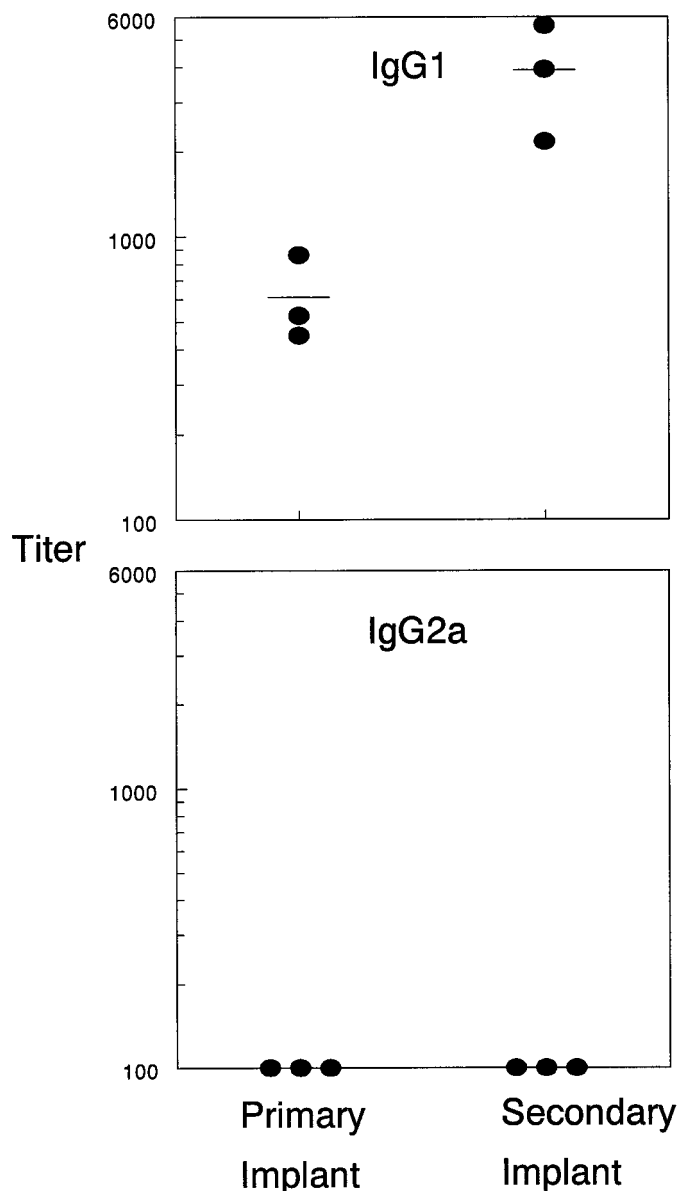


FIGURE 6. Antibody responses of BALB/c mice that received a second SIS implant as determined by isotype-specific ELISA. Mice (three per group) were implanted with SIS on days 0 and 28 and were analyzed for serum anti-SIS antibody 21 days after the primary implant and 7 days after the secondary implant. Serum titers from individual mice are represented by the symbols and the line denotes the mean titer value of each experimental group.

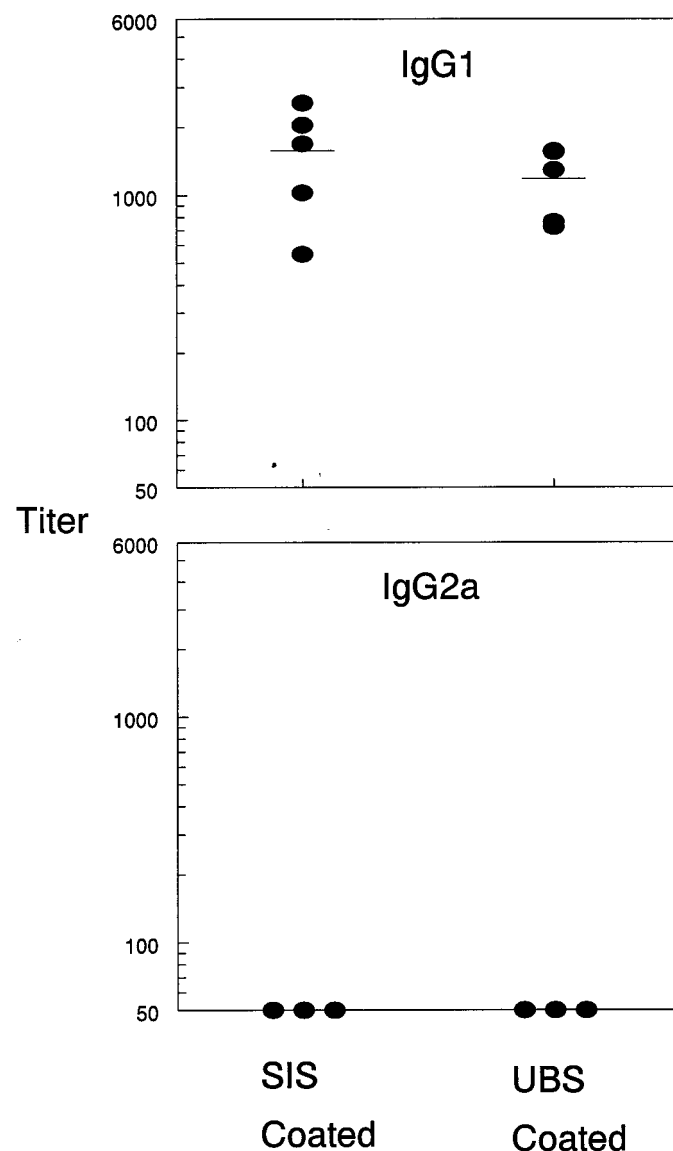


FIGURE 7. Antibody responses of BALB/c mice implanted with UBS. Sera from mice that had received UBS implants were analyzed on ELISA plates that were coated with either UBS or SIS. Serum titers from individual mice are represented by the symbols and the line denotes the mean titer value of each experimental group.

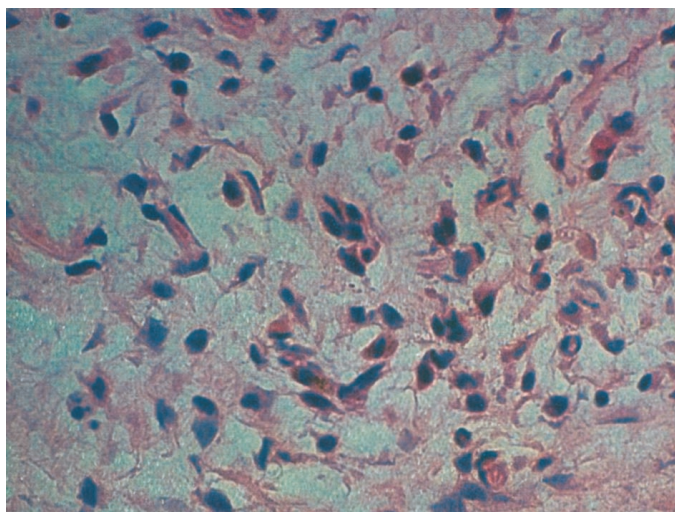


FIGURE 8. Histological analysis (40 $\times$ magnification) of graft sites from C57BL/6 T cell KO mice implanted with porcine SIS. Twenty-eight days after tissue implantation, graft site tissue was harvested, sectioned, and stained with hematoxylin and eosin.

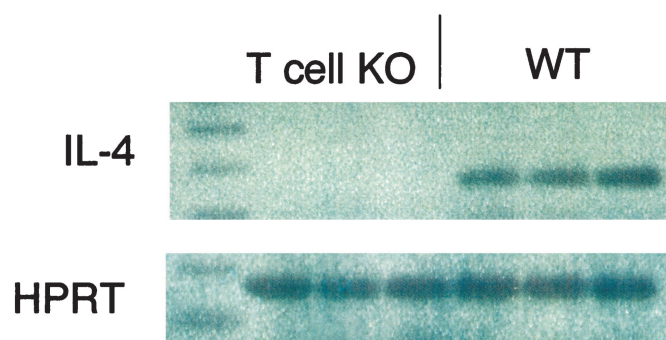


FIGURE 9. Cytokine mRNA analysis of the graft site from C57BL/6 WT and T cell KO mice (three per group) implanted with porcine SIS. Seven days postimplantation, total RNA was analyzed for the expression of IL-4 (173 bp) by RT-PCR. HPRT (162 bp) was used as an internal control for RNA loading. Molecular size standards are shown at the left of each panel.

SIS-specific antibody levels showed the absence of anti-SIS antibodies in these mice (Fig. 10). Examination of C57BL/6 μ MT KO mice, which have a targeted gene disruption in IgM that prevents B cell expression, demonstrated the absence of SIS-specific antibody responses in these mice but the presence of IL-4 mRNA (data not shown). Again, SIS was remodeled in mice lacking B cell expression. Together, these results indicate that T and B cells do indeed respond to SIS, but these cells are not necessary for SIS acceptance.

DISCUSSION

ECM grafts serve as bioscaffolds for site appropriate host tissue growth and remodeling and, in the process, are resorbed. In addition, ECM materials are acellular and avascular which makes unlikely immune-mediated destruction of tissue blood supply. The cells that populate the ECM graft are host-derived and thus nonimmunogenic. Nevertheless, it

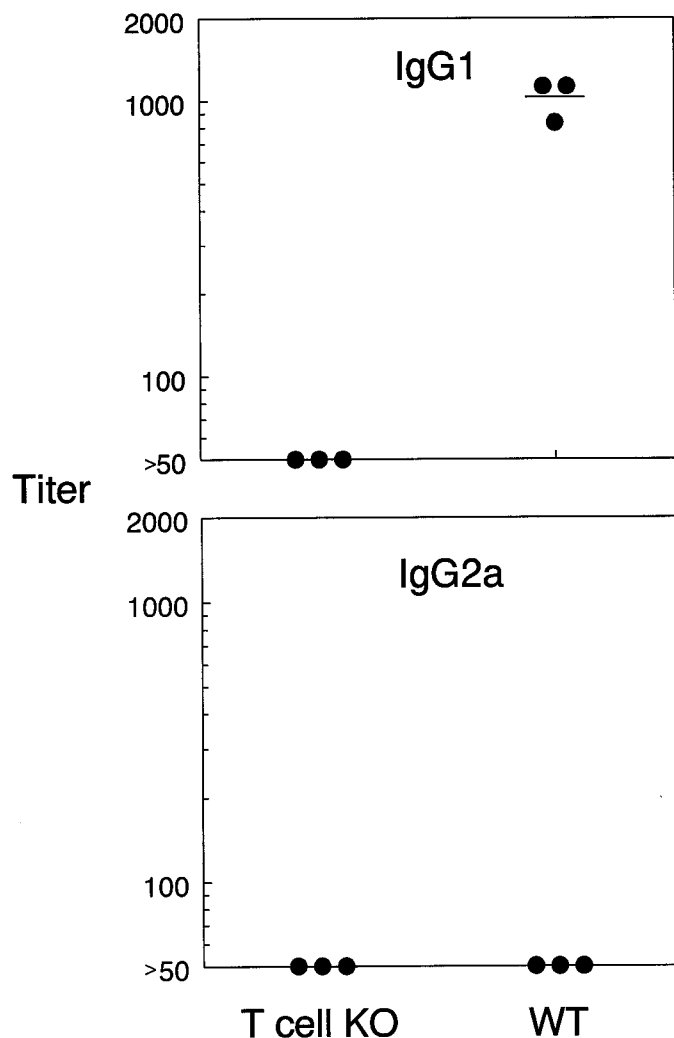


FIGURE 10. Serum antibody responses of C57BL/6 WT and T cell KO mice to SIS as determined by isotype-specific ELISA on serum. Mice (three per group) were analyzed for serum anti-SIS antibody 21 days after implantation of porcine SIS. Serum titers from individual mice are represented by the symbols and the line denotes the mean titer value of each experimental group.

has been unclear why such xenogeneic material does not appear to induce an inflammatory response that leads to rejection.

Our study shows that there is in fact a vigorous host response to implanted ECM xenografts as evidenced by an early, acute inflammatory response at the graft site that consists mostly of PMNs. Cytokine and antibody isotype analysis demonstrated the presence of a Th2 response but the absence of a Th1 response. These results differ from typical cytokine and antibody responses to xenografts, which induce a Th1 cytokine response (14). Clearly, porcine ECM is not an inert material but is associated with the production of antiinflammatory cytokines and noncomplement-fixing antibodies, which is compatible with graft acceptance. The components within SIS that elicit the immune response are not defined, but preliminary western blot analysis has shown immunoreactive bands of approximately 28 and 50 kD (Turner LA, Allman AJ, Raeder RH, Metzger DW, unpublished

observation). Interestingly, our previous studies using an SIS-specific mAb have shown the absence of SIS epitopes in remodeled tissue. Instead, host-derived and site-specific ECM is present where the graft was implanted (25).

It is possible that T and/or B cells are necessary for SIS remodeling. However, C57BL/6 T cell KO mice remodel SIS grafts in an environment devoid of IL-4 and SIS-specific antibodies. In fact, antibodies do not appear to be necessary for SIS acceptance because C57BL/6 μ MT KO mice, which have a targeted gene disruption in IgM ablating B cell expression, do not produce SIS-specific antibody responses nor do they reject SIS grafts. However, when implanted with SIS, these mice do show the presence of IL-4 and the absence of IFN- γ (Allman AJ, Badylak SF, and Metzger DW, unpublished observations). Furthermore, graft sites in wild-type mice were devoid of IgG1 and/or IgG2a antibody deposition, also indicating that antibodies are not necessary for SIS tissue remodeling.

It is possible that an immunomodulatory activity within ECM is at least partially responsible for the observed Th2 dominant response. A recent report has suggested that unknown factors in ECM can inhibit expression of IFN- γ and TNF- α from naïve T cells stimulated by anti-CD3 antibody or staphylococcal enterotoxin (26). In parallel, real time PCR data suggest that SIS suppresses IFN- γ expression based on the fact that mice implanted with SIS have significantly fewer copy numbers of IFN- γ mRNA compared to mice implanted with a syngeneic control graft.

The Th2 dominant immune response to ECM may play a pivotal role in the favorable clinical response to xenogeneic biomaterials. Bach et al. (20) found that a Th2 response was associated with acceptance of xenogeneic rat heart transplants in hamsters and a Th1-mediated response was associated with rejection. A Th2 dominant response may prevent cell-mediated inflammation of the ECM graft through the suppressive action of IL-10 on Th1 cells. The secretion of pro-inflammatory cytokines by macrophages and eosinophils is also inhibited by IL-10 (27, 28). It is noteworthy that increased levels of IL-10 mRNA were seen in all groups of ECM-implanted mice in our study. Thus, Th2-mediated anti-inflammatory activity may be an important factor in promoting ECM remodeling by modulating postsurgical inflammation. Additionally, SIS graft sites were screened for IL-5, IL-6, and TGF- β mRNA. Very low levels of IL-6 were detected and IL-5 and TGF- β were not detected.

Although there is evidence for an association between graft acceptance and a Th2 immune response, the relative roles of Th1 and Th2 cells in graft rejection remain controversial. For example, rejection of cardiac allografts has been reported to be exacerbated in mice treated with antibody to IL-12 to inhibit Th1 differentiation (29). In addition, humans receiving liver allografts have elevated levels of IL-10 and IL-4 locally at the graft rejection site but systemic levels of Th1 cytokines such as IL-2 and IFN- γ are elevated (30). Furthermore, there is evidence that graft rejection can still occur in mice with genetic defects in the IFN- γ gene (31). Experiments in progress are designed to investigate whether Th1 activation and/or Th2 inactivation leads to ECM rejection.

In summary, the mouse immune response to porcine ECM is restricted to the Th2 pathway both locally and systemically. An understanding of this immune response and of the

potential immunomodulatory activity of the ECM itself may prove useful in future clinical applications of xenotransplantation in humans.

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CD4+ T CELL RECOGNITION OF A SINGLE DISCORDANT HLA-A2-TRANSGENIC MOLECULE THROUGH THE INDIRECT ANTIGEN PRESENTATION PATHWAY INDUCES ACUTE REJECTION OF MURINE CARDIAC ALLOGRAFTS¹

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To further define the role of indirect allorecognition, cardiac allografts from HLA-A2-transgenic (HLA-A2+) C57BL/6 mice were heterotopically transplanted into normal C57BL/6, CD4 T cell-knockout (KO) C57BL/6 mice, CD8 T cell-KO C57BL/6 mice, fully MHC-discordant BALB/c mice (allogeneic control), and HLA-A2+ C57BL/6 mice (syngeneic control). HLA-A2+ grafts were acutely rejected when transplanted into BALB/c mice (mean survival time: 10±0.8 days), normal C57BL/6 mice (mean survival time: 16.5±2.1 days) as well as CD8-KO mice (mean survival time: 12.8±1.3 days). Histopathological analysis revealed classical acute cellular rejection with moderate to severe diffuse interstitial CD4+ and CD8+ cellular infiltrates and significant intra-graft deposition of IgG and complement. In contrast, HLA-A2+ grafts were not rejected when transplanted into CD4-KO mice or HLA-A2+ mice. CD8-KO recipients treated with an anti-CD4 monoclonal antibody, but not with an anti-NK monoclonal antibody, failed to reject their allografts with prolonged administration of antibody (30 days). Spleen cells from mice rejecting HLA-A2+ allografts failed to lyse HLA-A2+ target cells indicating a lack of involvement of CD8+ T cells in the rejection process.

In contrast, spleen cells from rejecting animals proliferated significantly to both HLA-A2+ cells and to a peptide derived from the HLA-A2 molecule. Development of anti-HLA-A2 antibodies was observed in all animals rejecting HLA-A2+ allografts. These results suggest that indirect allorecognition of donor MHC class I molecules leads to rejection of cardiac allografts and development of alloantibodies in this unique transplant model in which there is a single MHC discordance between donor and recipient.

INTRODUCTION

T cells are known to play a critical role in allograft rejection. It is well established that MHC class I-incompatible allografts stimulate immune responses in which both CD8+ and CD4+ T cells participate (1,2). However, because CD4+ T cells generally react to MHC class II molecules, the contribution of such cells to rejection of MHC class I disparate grafts is attributed to recognition of allogeneic MHC class I differences by the indirect pathway of allorecognition in which a class I molecule has been processed and presented in the context of MHC class II molecules on the surface of self antigen presenting cells (3). It has been suggested that the direct recognition pathway may be preferentially involved in acute rejection and indirect recognition may be more pertinent to chronic rejection (4–6). In this regard, recent studies in rodent models (7,8) and in human transplant recipients (9–11) have indicated that CD4+ T cells recognizing either MHC class I or class II-derived peptides through the indirect antigen presentation pathway may play an important role in chronic allograft rejection. These indirect alloresponses can

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provide T cell help for induction of CD8⁺ T cells as well as for the production of specific alloantibodies by B cells (12).

Recent studies in murine models of skin allograft rejection have supported the notion that indirect T cell allorecognition is sufficient to cause acute rejection (13,14). However, the T cells responding via the indirect pathway against the allopeptides derived from discordant MHC class I often constituted only a small proportion of the total alloreactive T cells. Additionally, the characterization of these T cells responding to antigen presented via indirect allorecognition has been very difficult. Recent studies using a skin allograft model have found that during acute rejection, more than 90% of the response was directed toward intact donor MHC molecules although T cells recognizing indirectly presented donor-derived peptides, accounted for less than 10% (15). Further, it was noted that this indirect response was comprised of reactivity toward both MHC-derived epitopes, and to a lesser extent, minor histocompatibility antigens. In early acute rejection, this indirect response is confined to either a single or a few dominant determinants, although other determinants remain cryptic. However, at later time points after transplantation, spreading of T cell alloresponses to formerly cryptic donor MHC determinants can take place suggesting that initially cryptic donor MHC peptides might contribute to the process of chronic allograft rejection. Because skin allograft transplantation is well known to elicit an unusually strong immune response, it is generally not considered comparable to vascularized solid organ allografts. Therefore, it is important to determine whether indirect antigen presentation of discordant MHC class I molecules can also lead to acute rejection of vascularized solid organs. Toward this goal, we transplanted hearts from HLA-A2-transgenic (HLA-A2⁺) C57BL/6 mice into normal C57BL/6 recipients. This unique allograft model provides an opportunity to dissect out the role of indirect antigen presentation of discordant MHC class I molecules where there is a single MHC class I disparity between the donor and recipient. Further, the role of minor histocompatibility antigens in the rejection process is also eliminated. In the study described herein, we demonstrate an important role for the indirect CD4⁺ T cell allorecognition in the process of acute rejection of vascularized solid organ allografts.

MATERIALS AND METHODS

Animals. HLA-A2⁺ C57BL/6 mice were a kind gift from Dr. Victor H. Englehard (University of Virginia, Charlottesville, VA). CD4 T cell-knockout (KO) C57BL/6 mice, CD8 T cell-KO C57BL/6 mice, normal C57BL/6 mice, and BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, MA) and housed in our pathogen-free facility.

Peptides. Peptides corresponding to the hyper-variable region in the $\alpha 1$ domain of HLA-A1, HLA-A2, HLA-A3, and HLA-B44 were synthesized using an Applied Biosystems Synergy peptide synthesizer (model 432A, Foster City, CA). Purity was determined by high-performance liquid chromatography (HPLC) and mass spectrometry. The peptide sequences are given in Table 1.

Heterotopic cardiac transplantation. Cardiac allografts were transplanted heterotopically using previously described techniques (16). Briefly, donor ascending aorta and the pulmonary trunk from the heart graft were anastomosed end-to-side to the recipient infrarenal abdominal aorta and inferior vena cava, respectively, using 10–0 sutures. Cold ischemic times were less than 30 min. Rejection was defined by cessation of a palpable heartbeat and was confirmed

histologically. Cardiac grafts, spleens, and serum were harvested at the time of rejection. Cardiac grafts surviving 30 days or longer were considered nonrejected. The lack of rejection at 30 days or longer after transplantation was also confirmed histologically.

Monoclonal antibody treatment protocol. The GK-1.5 anti-CD4 monoclonal antibody, the 53–6.72 anti-CD8 monoclonal antibody, and the PK-136 anti-NK monoclonal antibody were obtained as hybridoma cell lines from the American Type Culture Collection (Manassas, VA) and cultured in RPMI medium supplemented with 5% heat-inactivated fetal bovine serum. Precipitation of the antibodies was performed using 45–50% ammonium sulfate. Then, the antibodies were dialyzed overnight in phosphate-buffered saline and spectrophotometry was used to assess protein concentration while purity was determined by means of 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. In some experiments, anti-CD4 (500 μ g), anti-NK (300 μ g), or anti-CD8 (500 μ g) (negative control) monoclonal antibodies were injected intra-peritoneally into CD8-KO recipients at days –6, –1, +5, +10, +15, +20, and +25. Depletion of each spleen cell subpopulation by the corresponding monoclonal antibody was tested in naïve mice prior to use (data not shown).

Histological analysis. For conventional histology, tissues were fixed in 10% paraformaldehyde, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin or Trichrome. For immunohistochemistry, tissues were frozen fresh and sectioned at 5 μ m on a cryostat. Endogenous peroxidase activity was quenched with 3% H₂O₂ for 10 min before staining. The sections were then blocked for 30 min with phosphate-buffered saline supplemented with 0.5% blocking reagent (TSA-indirect kit, NEN Life Science Products, Boston, MA), 2% normal goat serum (Gibco BRL), and 0.1% Tween-20 (Sigma, St Louis, MO). Then, the sections were incubated overnight at 4°C with the H129.19 rat anti-mouse CD4 or the 53–6.7 rat anti-mouse CD8 monoclonal antibodies (BD Pharmingen, San Diego, CA) diluted at 2.5 μ g/ml in blocking buffer. The sections were incubated for 1 hr at 25°C with biotin-conjugated F(ab')₂ goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in blocking buffer. The presence of CD4⁺ and CD8⁺ T cells infiltrating the grafts were detected by means of the TSA-indirect kit (NEN Life Science Products) following the manufacturer's instructions. Briefly, the sections were incubated with horseradish peroxidase-conjugated streptavidin for 30 min. The reaction was then amplified by incubation with biotin-conjugated tyramide for 5 min followed by incubation with horseradish peroxidase-conjugated streptavidin for 30 min. The immune complexes were detected with 3,3'-diaminobenzidine (ICN Pharmaceuticals, Aurora, OH). In parallel experiments, the sections were incubated overnight at 4°C with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-mouse complement C3 (Cappel, ICN Pharmaceuticals) diluted 1:100 in blocking buffer. The IgG and C3 deposition in the grafts was detected by means of the TSA-indirect kit (NEN Life Science Products) following the manufacturer's instructions. Briefly, the reaction was amplified by incubation with biotin-conjugated tyramide for 5 min followed by incubation with horseradish peroxidase-conjugated streptavidin for 30 min. The immune complexes were detected with 3,3'-diaminobenzidine (ICN Pharmaceuticals) and the sections were counterstained with Gill's hematoxylin (Sigma).

Detection of anti-HLA-A2 antibodies. The presence of anti-HLA-A2 antibody in serum samples was detected by means of flow cytometric analysis. Briefly, HLA-A2⁺ spleen cells (1×10^6) were incubated for 30 min at 4°C with sera from naïve C57BL/6 mice or HLA-A2⁺ cardiac allograft recipients diluted 1:2 in phosphate-buffered saline supplemented with 2% bovine serum albumin, 25 mM HEPES buffer, and 0.02% sodium azide. The cells were incubated for 30 min at 4°C with fluorescein-conjugated F(ab')₂ goat anti-mouse IgG (1:50) (Becton Dickinson, Franklin Lakes, NJ). The cells were then fixed in 1% paraformaldehyde and single-color flow cytometric analysis was performed using a FACScan flow cytometer (Becton

TABLE 1. HLA class I allopeptide sequences

HLA allele	Peptide sequence	Amino acid position	HLA region
HLA-A1	WDQETRNMKAHSQTDRLNGLTGRGY	60–84	α 1 domain
HLA-A2	WDGETRQVKAHSQTHRVLDLGLTGRGY	60–84	α 1 domain
HLA-A3	WDQETRNVKAQSQTDRVDLGLTGRGY	60–84	α 1 domain
HLA-B44	WDRETQISKTNLTQTYRENLRTAARY	60–84	α 1 domain

Dickinson). The results of the test sera are expressed as the mean fluorescence channel shift over the mean fluorescence channel obtained with sera from naïve C57BL/6 mice (negative control).

Mixed lymphocyte reaction. Spleen cells from HLA-A2+ allograft recipients were seeded in quadruplicate cultures at a concentration of 1×10^5 cells/well in round-bottom 96-well plates in the presence or absence of irradiated (3×10^3 rad) HLA-A2+ spleen cells (1×10^5 cells/well). The cells were cultured in Dulbecco's modified eagle's medium (Cellgro, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 25 mM HEPES buffer, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 125 ng/ml fungizone, and 50 μ M 2-mercaptoethanol (Gibco BRL, Life Technologies). After 3 days, 1 μ Ci/well [^{3} H]-thymidine was added to each well. After 24 hr, [^{3} H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results expressed as stimulation indices corresponded to the ratio of the counts per minute of the cultures in the presence of HLA-A2+ cells to the mean counts per minute of the cultures in the absence of HLA-A2+ cells (negative control).

Proliferation assay. Spleen cells from naïve mice or HLA-A2+ cardiac allograft recipients were seeded at a concentration of 1×10^5 cells/well in round-bottom 96-well plates in the presence of a synthetic peptide derived from the HLA-A2 molecule (50 μ g/ml), a pool of synthetic peptides derived from the HLA-A1, HLA-A3, and HLA-B44 molecules (50 μ g/ml), or bovine serum albumin (50 μ g/ml). The cells were cultured in Dulbecco's modified eagle's medium supplemented as described above. After 5 days, 1 μ Ci/well [^{3} H]-thymidine was added to each well. After 24 hr, [^{3} H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results expressed as stimulation indices corresponded to the ratio of the counts per minute of the cultures in the presence of HLA-derived peptides or bovine serum albumin to the mean counts per minute of the cultures in the absence of peptides or bovine serum albumin (negative control).

Statistical analysis. Differences in graft survival were assessed using the two-tailed Student *t* test with the alpha set a priori at $P < 0.05$.

RESULTS

Rejection of HLA-A2+ allografts by CD4+ T cells. To determine whether a single human MHC class I disparity between the donor and recipient will result in solid allograft rejection, we transplanted HLA-A2+ cardiac allografts into normal C57BL/6 recipients. As shown in Table 2, these HLA-A2+ allografts were rejected after 16.5 ± 2.1 days after transplantation. In contrast, HLA-A2+ isografts transplanted into HLA-A2+ mice did not show any signs of rejection by day 30 after transplantation. Fully discordant BALB/c mice rejected HLA-A2+ allografts significantly earlier than normal C57BL/6 mice (mean survival time: 10.0 ± 0.8 days) and rejected normal C57BL/6 allografts in 8.5 ± 0.6 days (data not shown). HLA-A2+ allografts transplanted into CD4-KO mice did not show any signs of rejection by day 30 after transplantation. In contrast, CD8-KO mice rejected HLA-A2+ allografts significantly earlier than normal C57BL/6 mice (mean survival time: 12.8 ± 1.4 days) indi-

cating 1) a primary role for CD4+ T cells in this rejection process and 2) that CD8+ T cells are not necessary in the rejection process of HLA-A2+ cardiac allografts in this model. Further, early rejection of HLA-A2+ allografts by CD8-KO mice in comparison with rejection by normal C57BL/6 mice strongly suggest a possible role for CD8+ T cells in the immunoregulation of the rejection process.

As shown in Figure 1, the histopathology of HLA-A2+ cardiac allograft rejection observed in fully discordant BALB/c recipients (Fig. 1A) was similar to that one observed in normal C57BL/6 recipients (Figs. 1B and C). Moderate to severe diffuse interstitial infiltrates were noted in both groups rejecting allografts. In the region of lymphocytic infiltrate, multi-focal myocyte necrosis was seen along with interstitial vasculitis and edema. Vasculitis was mainly arteriolitis that was often moderate to severe. In contrast, HLA-A2+ isografts transplanted into HLA-A2+ mice and obtained 30 days after the transplant did not show any histopathologic signs of acute rejection (Fig. 1D). These data suggest that a single MHC class I disparity can induce solid organ allograft rejection, although delayed (mean survival time: 16.5 ± 2.1 days) in comparison to fully discordant solid organ allografts (mean survival time: 10.0 ± 0.8 days) (Table 2). Several studies have shown that murine CD8 cannot interact with HLA class I molecules (17–19). However, the possibility of direct antigen recognition of HLA-A2 molecules on the cardiac allografts by CD8+ T cells could not be ruled out from these experiments. To address this question, splenic CD8+ T cells isolated from rejecting C57BL/6 mice were tested for their ability to lyse HLA-A2+ targets in a standard chromium release assay. No detectable T cell-mediated cytotoxic activity was observed from any of the C57BL/6 mice rejecting HLA-A2+ allografts (data not shown). These data indicated that CD8+ T cells do not play a significant role in the rejection process observed in this model. To completely rule out the possibility of direct recognition of the HLA-A2

TABLE 2. HLA-A2+ cardiac graft survival

Group	Recipient	n	Graft survival	MST (days $\pm$ SD)
1	C57BL/6	4	14, 17, 19, 16	16.5 ± 2.1
2 ^a	HLA-A2+	4	30 $\times$ 4	30
3 ^b	BALB/c	4	10, 11, 9, 10	10.0 ± 0.8
4 ^c	CD4-KO	3	30 $\times$ 3	30
5 ^d	CD8-KO	4	11 $\times$ 2, 14, 12	12.0 ± 1.4
6 ^e	CD8-KO + anti-CD4	4	30 $\times$ 4	30
7	CD8-KO + anti-NK	4	11, 13, 14, 13	12.8 ± 1.3
8	CD8-KO + anti-CD8	4	11, 14, 14, 15	13.5 ± 1.7

^a Group 2 vs. 1, $P = 0.001$.

^b Group 3 vs. 1, $P = 0.008$.

^c Group 4 vs. 1, $P = 0.001$.

^d Group 5 vs. 1, $P = 0.006$.

^e Group 6 vs. 5, $P = 0.0001$.

molecule expressed on cardiac allografts by CD8⁺ T cells as well as to eliminate a possible bystander role for CD8⁺ T cells in the rejection of the HLA-A2⁺ allografts, we transplanted HLA-A2⁺ allografts into CD8-KO mice. All of the CD8-KO recipients rejected their allografts in 12.0 ± 1.4 days (Table 2). Histopathological analysis confirmed rejection to be of an acute/subacute nature (Fig. 1E and F). Severe mixed inflammatory infiltrates including lymphocytes and neutrophils at a 2:1 ratio were noted. Additionally, myocyte necrosis in the area of inflammatory cellular infiltrates, perivascularitis, endothelitis, and mild to moderate interstitial fibrosis was present. To confirm the primary role of CD4⁺ T cells during the rejection process in this model for indirect allograft recognition, we transplanted HLA-A2⁺ allografts into CD4-KO mice. None of these animals rejected their cardiac allografts at 30 days after transplantation (Table 2). HLA-A2⁺ cardiac allografts transplanted into these CD4-KO mice did not show any histopathological signs of acute rejection (Fig. 1G). In addition, CD8-KO mice receiving anti-CD4 monoclonal antibody treatment did not reject their grafts as long as administration of the antibody was continued (30 days) (Table 2). Histopathological analysis of these allografts confirmed the lack of rejection in these animals (Fig. 1H). To rule out the possible role for NK cells in the rejection process in CD8-KO allograft recipients, 4 HLA-A2⁺ allograft recipients were treated with an anti-NK monoclonal antibody as described above. All of these animals rejected their cardiac allografts in 12.8 ± 1.1 days (Table 2). Histopathological analysis of these allografts confirmed a process of acute rejection with similar characteristics observed in the HLA-A2⁺ allografts rejected by normal as well as CD8-KO mice (data not shown). As control group for the *in vivo* monoclonal antibody treatment, CD8-KO mice were injected with anti-CD8 monoclonal antibody as described above. As shown in Table 2, this treatment protocol did not have any beneficial effect in the mean survival time of these HLA-A2⁺ allografts (mean survival time: 13.5 ± 1.7) when compared to HLA-A2⁺ allografts transplanted into CD8-KO (mean survival time: 12.0 ± 1.4). In addition, histopathological analysis of these allografts confirmed a process of acute rejection with similar characteristics observed in the HLA-A2⁺ allografts rejected by normal as well as CD8-KO mice (data not shown).

To further characterize the acute rejection observed in HLA-A2⁺ allografts transplanted into normal C57BL/6 mice, we performed immunohistochemical analysis of HLA-A2⁺ cardiac grafts transplanted into HLA-A2⁺ mice (isografts), BALB/c mice (fully discordant allografts), and normal C57BL/6 mice for infiltrating CD4⁺ and CD8⁺ T cells as well as deposition of both IgG and complement C3. As shown in Figure 2, HLA-A2 isografts transplanted into HLA-A2 mice did not show any CD4⁺ or CD8⁺ T cell infiltration (Figs. 2A and D, respectively) as well as any IgG or complement (C3) deposition (Figs. 2G and J, respectively). As expected, a significant infiltration by CD4⁺ and CD8⁺ T cells was observed in HLA-A2 allografts transplanted into BALB/c mice (Figs. 2B and E, respectively). In addition, a significant deposition of both IgG and complement (C3) was observed in these rejected allografts (Figs. 2H and K, respectively). A similar immunohistopathology was observed in HLA-A2 allografts transplanted into C57BL/6 mice. A significant infiltration by CD4⁺ T cells was observed in these allografts in numbers comparable to allografts rejected by BALB/c mice (Fig. 2C). It

is noteworthy that a significant infiltration by CD8⁺ T cells was also observed in these allografts (Fig. 2F). This was an unexpected result based on the fact that there is no direct recognition of murine CD8⁺ T cells of the HLA-A2 molecule (17–19) and no CD8⁺ T cell-mediated cytotoxic reactivity was observed against HLA-A2⁺ cells (data not shown). Comparable to the observation made in BALB/c recipients, a significant deposition of both IgG and complement C3 was also observed in these rejected allografts (Figs. 2I and L, respectively). Overall, the data presented above suggest that the immunopathology of HLA-A2⁺ allograft rejection in C57BL/6 mice is dependent on the activation of alloreactive CD4⁺ T cells that leads to the activation of complement by IgG alloantibodies.

Positive mixed lymphocyte reaction in HLA-A2+ allograft recipients

To determine whether specific CD4⁺ T cell activation occurred against the HLA-A2 molecule during the process of cardiac allograft rejection, spleen cells from rejecting recipients were used in lymphoproliferation studies. As shown in Figure 3, spleen cells from BALB/c, normal C57BL/6, and CD8-KO recipients demonstrated augmented proliferation to HLA-A2⁺ spleen cells. In contrast, CD4-KO and HLA-A2⁺ recipients did not show any reactivity against HLA-A2⁺ spleen cells. As expected, fully discordant BALB/c mice showed a significantly higher proliferative response against HLA-A2⁺ spleen cells as compared to normal C57BL/6 or CD8-KO mice that are discordant only at the HLA-A2 molecule. These results suggest that HLA-A2-reactive CD4⁺ T cells were expanded *in vivo* during the rejection process of the HLA-A2⁺ allografts and suggest that indirect antigen presentation of HLA-A2-derived peptides may have occurred leading to the rejection.

Development of anti-HLA antibodies in HLA-A2+ allograft recipients

As shown above, a significant deposition of IgG and complement C3 was observed in rejected HLA-A2 allografts by normal C57BL/6 mice (Figs. 2I and L, respectively). To determine whether development of anti-HLA-A2 alloantibodies indeed occurs in these recipients, we analyzed sera collected from normal C57BL/6 and CD8-KO that had rejected their HLA-A2⁺ allografts as well as sera from CD4-KO and HLA-A2⁺ mice that did not reject their HLA-A2⁺ grafts (collected 30 days after transplantation). Flow cytometric analysis using HLA-A2⁺ spleen cells demonstrated *in vivo* production of IgG anti-HLA-A2 antibodies in normal C57BL/6 and in CD8-KO mice rejecting HLA-A2⁺ allografts (Fig. 4). These same samples did not show any reactivity to spleen cells obtained from normal C57BL/6 mice (data not shown). In addition, sera from nonrejecting CD4-KO and HLA-A2⁺ mice transplanted with HLA-A2⁺ isografts did not show any anti-HLA-A2 reactivity (Fig. 4). Taken together, these results suggest that indirect antigen presentation of HLA-A2 molecules occurs during the process of acute rejection of HLA-A2⁺ allografts and that the activation of HLA-A2-reactive CD4⁺ T cells leads to development of a humoral anti-HLA-A2 alloreactivity in recipients that reject their HLA-A2⁺ allografts. In addition, the results obtained from CD8-KO mice are in agreement with the concept that rejec-

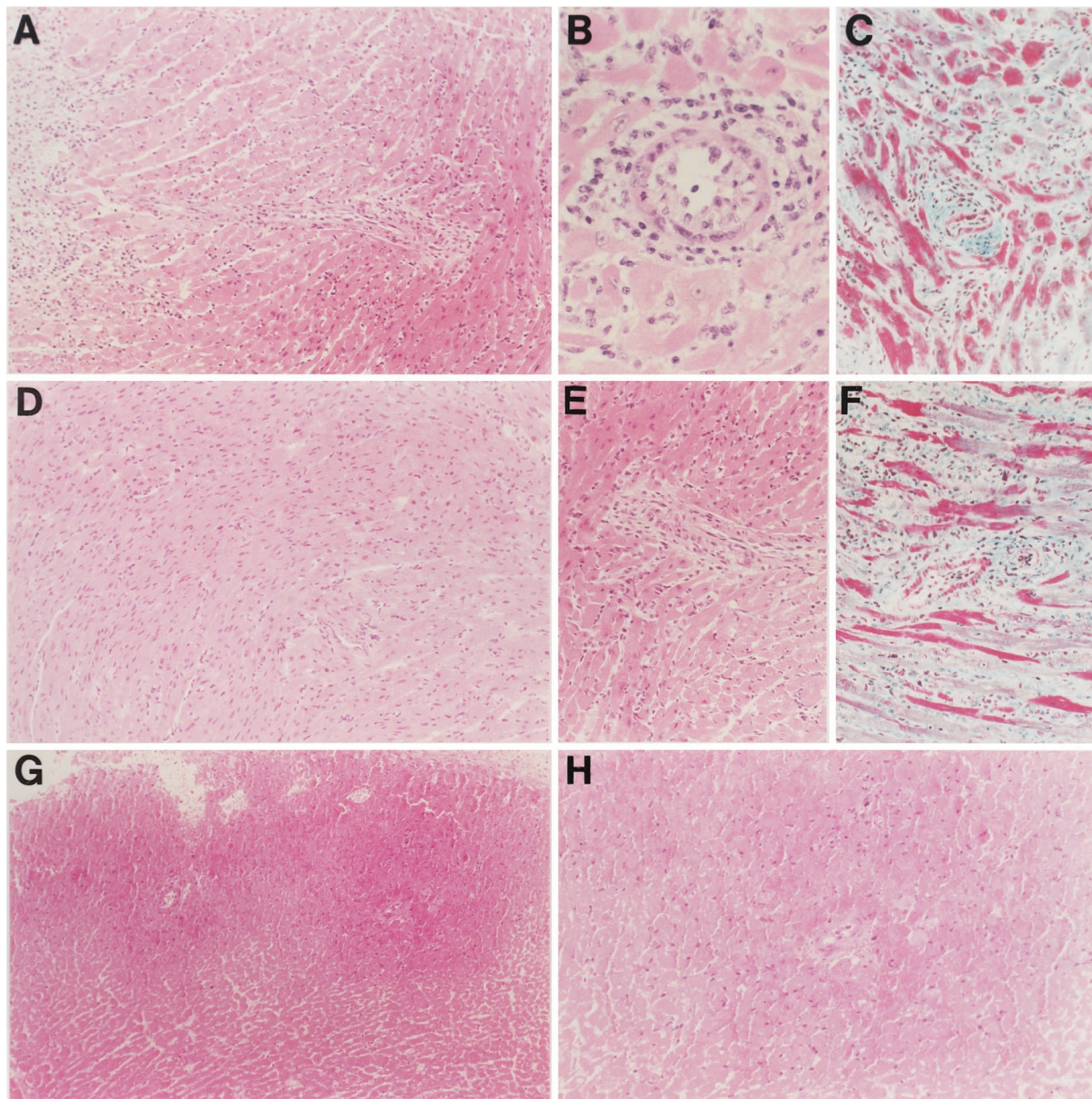


FIGURE 1. Histological analysis of HLA-A2+ allograft rejection. HLA-A2+ grafts were fixed in 10% paraformaldehyde, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin (A, B, D, E, G, and H) or Trichrome (C and F). Rejected grafts were harvested after clinical signs of rejection. Not rejected grafts were harvested 30 days after transplantation. HLA-A2+ grafts were harvested from BALB/c mice (A), normal C57BL/6 mice (B and C) HLA-A2+ mice (D), CD8-KO mice (E and F), CD4-KO mice (G), and CD8-KO mice receiving anti-CD4 monoclonal antibody treatment (H). Original magnification $\times 100$.

tion of HLA-A2+ cardiac allografts by C57BL/6 mice is mediated by CD4+ T cells primed against HLA-A2 molecules through the indirect pathway of antigen recognition.

HLA-A2-specific T cell proliferation in HLA-A2+ allograft recipients

To further confirm that processing and presentation of HLA-A2 molecules takes place during the process of HLA-

A2+ allograft rejection, we analyzed the ability of spleen cells from rejecting C57BL/6 mice to respond to relevant and irrelevant HLA-derived peptides. As shown in Figure 5, a significant proliferative response was observed when spleen cells from rejecting C57BL/6 mice were cultured in the presence of a peptide derived from the HLA-A2 molecule. As also shown in Figure 5, no response was observed when spleen cells from rejecting C57BL/6 mice were culture in the pres-

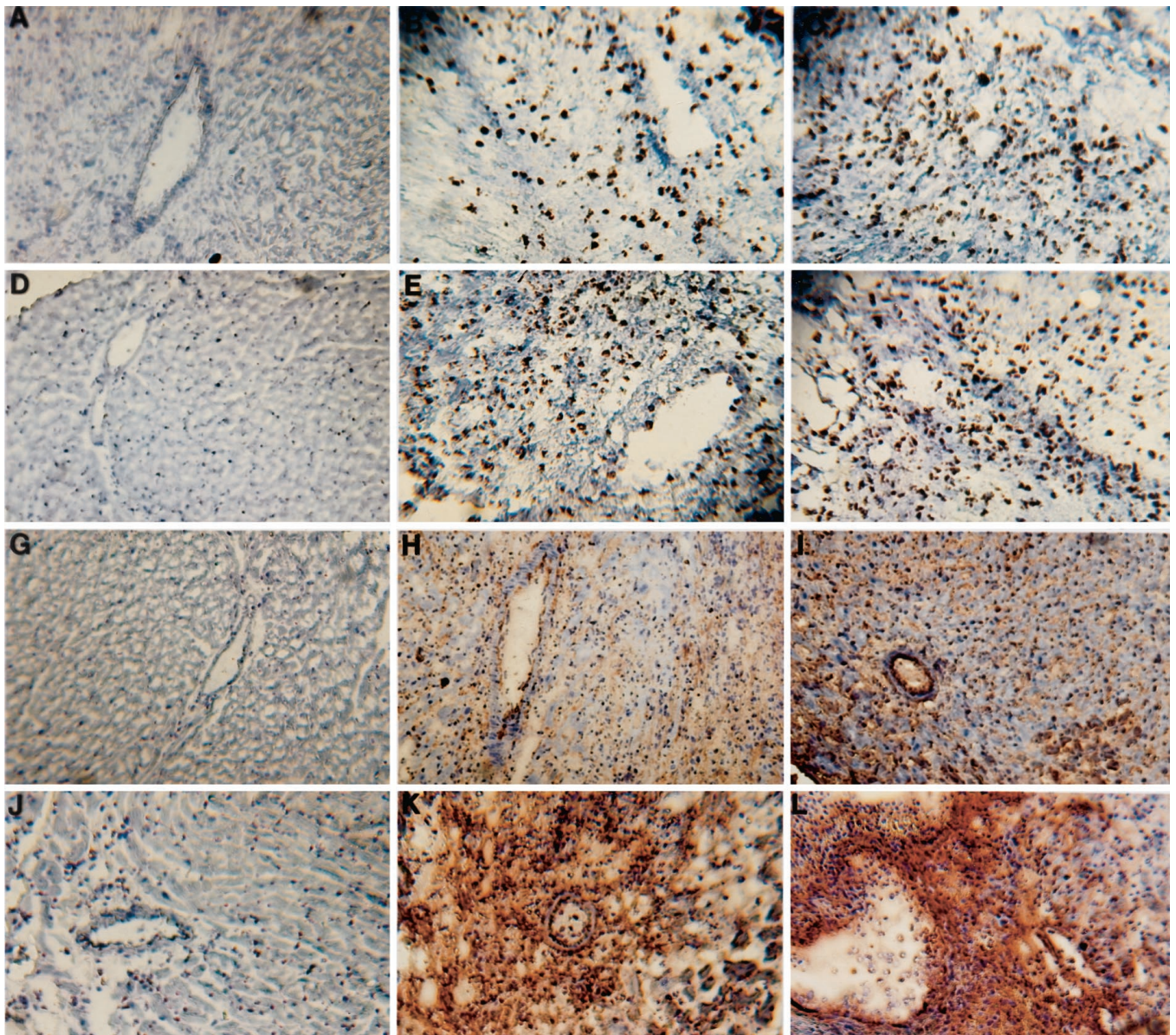


FIGURE 2. Immunohistological analysis of HLA-A2+ allograft rejection. Frozen HLA-A2+ grafts were cut into 5- μ m sections and stained with the H129.19 anti-CD4 monoclonal antibody (A, B, and C), the 53-6.7 anti-CD8 monoclonal antibody (D, E, and F), a polyclonal goat anti-mouse IgG antiserum (G, H, and I), and a polyclonal goat anti-complement C3 antiserum (J, K, and L). Rejected grafts were harvested after clinical signs of rejection. Not rejected grafts were harvested 30 days after transplantation. HLA-A2+ grafts were harvested from HLA-A2+ mice (A, D, G, and J), BALB/c mice (B, E, H, and K), and normal C57BL/6 mice (C, F, I, and L). Original magnification x100.

ence of a pool of irrelevant peptides derived from the HLA-A1, HLA-A3, or HLA-B44 molecules. In addition, no response was observed when spleen cells from naive C57BL/6 mice were culture in the presence of the same peptides derived from HLA-A2, HLA-A1, HLA-A3, or HLA-B44 molecules (Fig. 5). These results suggest that indirect antigen presentation of peptides derived from HLA-A2 molecules takes place in animals rejecting HLA-A2+ allografts.

DISCUSSION

Allograft rejection of MHC class I disparate organs is thought to be mediated primarily by CD8+ T cells via direct recognition of allogeneic MHC class I antigens expressed on

the graft tissue. Recent reports, including the use of gene knockout animals by Krieger et al. (20) have demonstrated that CD4+ T cells, but not CD8+ T cells, are absolutely required to initiate allograft rejection. MHC allorecognition by CD4+ T cells can occur in two different ways: 1) through the direct pathway in which T cells recognize intact allo-MHC class II molecules expressed by passenger donor bone marrow-derived cells and/or parenchymal cells of the allograft, or 2) through the indirect pathway in which T cells recognize processed allo-MHC class I or class II molecules presented as peptides by self antigen-presenting cells on MHC class II molecules (21). Several recent reports in liver, kidney, and lung transplant recipients have shown that do-

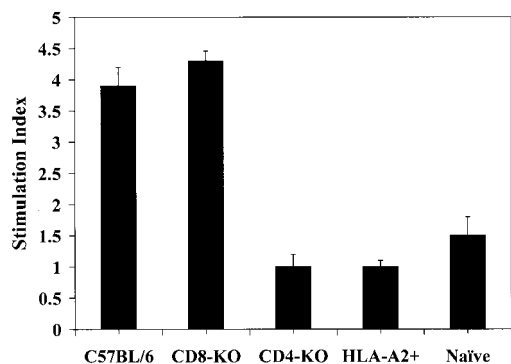


FIGURE 3. Mixed lymphocyte reaction in HLA-A2+ cardiac allograft recipients. Spleen cells from naïve C57BL/6 mice or HLA-A2+ cardiac allograft recipients (C57BL/6, CD8-KO, CD4-KO, and HLA-A2+) were cultured in the presence or absence of irradiated HLA-A2+ spleen cells. After 3 days, [^{3} H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results expressed as stimulation indices corresponded to the ratio of the counts per minute of the cultures in the presence of HLA-A2+ cells to the mean counts per minute of the cultures in the absence of HLA-A2+ cells (negative control). Results shown are representative of three different experiments.

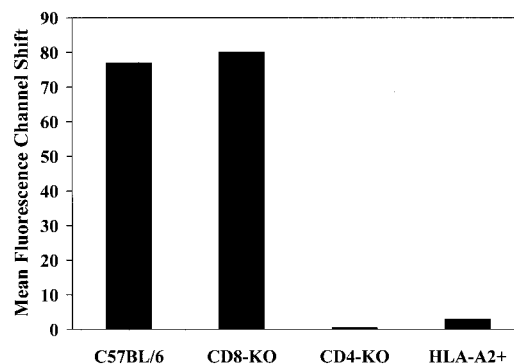


FIGURE 4. Development of anti-HLA antibodies in HLA-A2+ cardiac allograft recipients. HLA-A2+ C57BL/6 spleen cells were incubated with sera from naïve C57BL/6 mice or HLA-A2+ cardiac allograft recipients (C57BL/6, CD8-KO, CD4-KO, and HLA-A2+). The cells were then incubated with fluorescein-conjugated F(ab')₂ goat anti-mouse Ig and analyzed by single-color flow cytometric analysis. The results are expressed as the mean fluorescent channel shift over the mean fluorescent channel obtained with sera from naïve C57BL/6 mice (negative control). Number of cells analyzed: 3×10^3 . Results shown are representative of three different experiments.

nor allo-antigen are shed from the donor organs and are thus available for processing and presentation by self antigen-presenting cells (22, 10–11). These findings, along with the fact that, both in mice and humans, some of the peptides eluted from the MHC class II molecules are derived from MHC molecules, strongly favor an important role for indirect antigen presentation pathways in allograft rejection. In support of indirect allorecognition, earlier studies in the mouse model have shown that adoptive transfer of syngeneic antigen-presenting cells isolated from the spleen of primed animals could sensitize a recipient to reject skin allografts. Several reports in both mouse and rat models have shown that synthetic peptides derived from either MHC class I or class II molecules can prime animals to reject skin allografts (23). In addition, T cells capable of indirect recognition of these MHC-derived peptides could also be identified during the process of rejection of these skin allografts.

It is generally believed that CD4+ T cells primed by donor allopeptides via the indirect pathway facilitate delayed-type hypersensitivity responses and production of anti-donor antibody leading to rejection of the allograft. These primed CD4+ T cells could also provide help during the activation of alloreactive CD8+ T cells that could contribute to allograft destruction by cytotoxicity through direct allorecognition. However, the characterization of CD4+ T cells responding to antigens presented by the indirect allorecognition pathway has been limited due to the low frequency of cells responding via the indirect pathway of antigen presentation as well as multiple antigens on the cells that are the source of peptides for presentation. A recent report by Benichou et al. (15) has clearly pointed out these difficulties. In his studies using a skin allograft model, T cells recognizing indirectly presented donor-derived peptides accounted for less than 10% of total lymphocytes. Further, this response was comprised of reactivity toward peptide determinants derived from both MHC and minor histocompatibility antigens, indicating the com-

plexity of responses when there are multiple antigen disparities between donor and recipient. Our results presented herein clearly overcome these difficulties because the unique model described herein has only one MHC class I disparity with no minor histocompatibility antigen disparities between donor and recipient. Therefore, in this model, T cells responding to peptides derived from HLA-A2 molecules can be easily

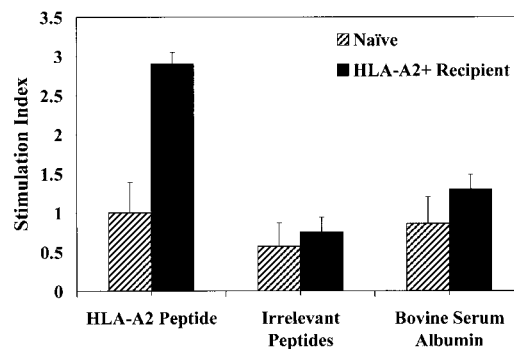


FIGURE 5. HLA-A2-specific T cell proliferation in HLA-A2+ cardiac allograft recipients. Spleen cells from naïve or HLA-A2+ cardiac allograft recipients (C57BL/6) were cultured in the presence or absence of a synthetic peptide derived from the HLA-A2 molecule, a pool of irrelevant synthetic peptides derived from the HLA-A1, HLA-A3, and HLA-B44 molecules, or bovine serum albumin. After 5 days, [^{3} H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results expressed as stimulation indices corresponded to the ratio of the counts per minute of the cultures in the presence of HLA-derived peptides or bovine serum albumin to the mean counts per minute of the cultures in the absence of peptides or bovine serum albumin (negative control). Results shown are representative of three different experiments. Results shown are representative of three different experiments.

identified for further characterization as well as their role in mediating rejection.

Normal C57BL/6 and CD8-KO mice rejecting HLA-A2+ allografts demonstrated histopathology similar to the lesions observed in fully discordant HLA-A2+ allografts in BALB/c mice. In addition, spleen cells from rejecting animals proliferated specifically to HLA-A2-expressing cells and failed to show detectable CD8+ T cell cytotoxic reactivity *in vitro* (data not shown) suggesting priming of CD4+ but not CD8+ T cells. Further, a significantly higher proliferative response was observed when spleen cells from rejecting C57BL/6 mice were cultured in the presence of a relevant HLA-A2-derived peptide as compared to spleen cell cultured in the presence of irrelevant peptides derived from other HLA molecules. These results indicate that the T cell proliferation is mediated by indirect antigen presentation of the HLA-A2 molecule. During these experiments, we used a peptide corresponding to the hypervariable region of the $\alpha 1$ domain of the HLA-A2 molecule. Studies are currently underway to determine the dominant peptide epitopes of the HLA-A2 molecule as well as possible epitope spreading during the process of HLA-A2+ allograft rejection. In this regard, Benichou et al. (15) have shown that the T cell response to a single dominant donor MHC peptide represented 10–30% of the initial indirect alloresponse and accounted for most of the memory T cell response after rejection of C57/B10.A skin grafts by BALB/c mice.

To rule out any involvement of CD8+ T cells in the rejection process of HLA-A2+ cardiac allografts, we performed transplants into CD8-KO mice. As shown in Table 2, all of these animals rejected the HLA-A2+ allografts. In addition, histological analysis of rejected HLA-A2+ allografts by CD8-KO mice demonstrated similar pathology as that observed in HLA-A2+ allografts transplanted into C57BL/6 mice. Further, treatment of CD8-KO mice with a monoclonal antibody specific for CD4+ T cells completely abrogated rejection as long as the administration of the antibodies was continued. Anti-NK cell monoclonal antibody treatment had no effect in rejection of HLA-A2+ allografts in the CD8-KO animals indicating that NK cells did not play any significant role in the rejection process. As shown in Figure 3, spleen cells from CD8-KO mice that rejected HLA-A2+ allografts also proliferated specifically to HLA-A2+ cells. These results suggest that CD4+ T cells alone can initiate cardiac allograft rejection when there is a single MHC class I antigen discordance between the donor and recipient. Interestingly, HLA-A2 allografts rejected by normal C57BL/6 mice presented an infiltration by both CD4+ and CD8+ T cells comparable to the one observed in fully discordant BALB/c mice. This was an unexpected result based on the fact that there is no direct recognition of murine CD8+ T cells of the HLA-A2 molecule (17–19) and no CD8+ T cell-mediated cytotoxic reactivity was observed against HLA-A2+ cells (data not shown). This unexpected CD8+ T cell infiltration observed in HLA-A2 allografts rejected by normal C57BL/6 mice may be due to low affinity HLA-A2-reactive CD8+ T cells that do not present detectable cytotoxic reactivity. This is further indicated by the fact that CD4-KO mice do not reject their HLA-A2 allografts. As shown in Table 2, CD8-KO mice rejected HLA-A2+ allografts significantly earlier than normal C57BL/6 mice. This raises the possibility that CD8+ T cells have a regulatory activity that is lacking in the CD8-KO mice

leading to earlier rejection. Studies are currently underway to determine the phenotype and functional characteristics of these cells and to determine their regulatory properties. Recently it has been reported that a subpopulation of CD8+ T cells of CD8+CD28– phenotype can regulate xeno-reactivity *in vitro* by blocking the CD154/CD40 interaction required for efficient costimulation of activated T cells (24–26). The rejection model presented herein provides a unique opportunity to determine whether CD8+ T cells of regulatory phenotype play a significant role in induction of allograft tolerance *in vivo*.

Another important finding in our study is the development of anti-HLA-A2 antibodies both in normal C57BL/6 and CD8-KO recipients that rejected HLA-A2+ allografts. In contrast, CD4-KO recipients did not develop anti-HLA-A2 antibodies. These results strongly indicate that CD4+ T cells recognizing HLA-A2 allo-antigen by the indirect pathway are providing help to B cells to produce anti-HLA-A2 antibodies. Recent studies by Pettigrew et al. (12) have shown that immunization of PVG.RT1 u rats with soluble class I MHC allo-antigen stimulated a strong CD4+ T cell-dependent anti-MHC class I humoral response and caused markedly accelerated rejection of MHC class I discordant cardiac allografts. Therefore, it is likely that antibody-dependent effector mechanisms may be involved in the rejection of HLA-A2+ cardiac allografts after transplantation into normal C57BL/6 and CD8-KO recipients. In fact, our results show that anti-HLA-A2 IgG antibodies are binding to the HLA-A2 molecule which may be involved in the activation of the complement cascade.

Taken together, our results suggest an important role by CD4+ T cells in the indirect pathway of allorecognition. In our experiments, both donors and recipients express the same H-2^b class I and class II molecules. Thus, HLA-A2-derived peptides could have been presented through the exogenous pathway of antigen presentation by MHC class II positive cells on the graft (i.e., endothelial cells). Therefore, the possibility exists that donor-reactive CD4+ T cells generated during the acute rejection process may have recognized HLA-A2-derived peptides directly on the graft and hence directly induced cytolysis of graft cells. However, negative results from T cell-mediated cytotoxicity assays performed with spleen cells from rejecting mice indicate a lack of direct recognition of HLA-A2-derived peptides presented in the context of MHC class II molecules on graft parenchymal cells. In addition, we have observed that discordance at the H3b and H13 minor histocompatibility antigens does not induce acute rejection of murine cardiac allografts (unpublished results). It is important to note that H3b and H13 minor histocompatibility antigens stimulate MHC class II-restricted CD4+ T cells and MHC class I-restricted CD8+ T cells, respectively (27). Therefore, it is unlikely that the rapid rejection of HLA-A2+ cardiac allografts observed in CD8-KO mice (12.0 ± 1.4 days) had been caused by a direct recognition of HLA-A2-derived peptides by CD4+ T cells in the context of H-2^b class II molecules expressed by parenchymal cells of the graft. It is yet not clear how CD4+ T cells activated through the indirect pathway in our model may be exerting acute allograft rejection. Experiments are under way in our laboratory using MHC class II-KO mice both as recipients as well as donors of HLA-A2+ cardiac allografts to address these issues.

The clinical importance of indirect antigen presentation as well as the possible role of allo-antibodies developed after transplantation in the immunopathogenesis of cardiac allograft rejection is beginning to be elucidated. Liu et al. (9) demonstrated donor-specific HLA class II allo-peptide T cell reactivity in human cardiac allograft recipients that experienced episodes of rejection and that spreading of epitopes within the peptide occurs during the post transplant period. They noted that indirectly primed T cells are 100- to 1000-fold lower in number and postulated that a small number of peptide specific T cells are mediating chronic rejection. In addition, Vella et al. (10) demonstrated that T cells of patients undergoing renal chronic allograft rejection also recognize and respond to donor-derived HLA class II peptides. Our laboratory, for the first time, also reported that T cells recognizing discordant donor HLA class I peptides are increased in frequency in patients with bronchiolitis obliterans syndrome after lung transplantation (11). Our group also noted a strong correlation between the development of anti-HLA antibodies and development of bronchiolitis obliterans syndrome after lung transplantation suggesting a possible role for primed CD4⁺ Th cells recognizing donor HLA class I peptides by indirect antigen presentation pathway (28). All of these clinical reports in cardiac, renal, and lung transplantation indicate that indirect allorecognition by CD4⁺ T cells may play a key role during the process of clinical chronic allograft rejection.

Current immunosuppressive therapy regimens do not seem to block the indirect allorecognition pathway. To prevent chronic rejection, it is imperative that methods and agents that block the indirect pathway be instituted. We have developed a model of cardiac allograft rejection mediated by the indirect allorecognition pathway of a single discordant MHC class I antigen that should enable us to further our understanding of the mechanisms of CD4⁺ T cell-mediated as well as antibody-mediated damage to the allograft and how to overcome these immune mechanisms.

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