GRAFT ANTIGEN-SPECIFIC CD4+ T CELLS REQUIRE CD154

EXPRESSION TO CLONALLY EXPAND AND DIFFERENTIATE

TO THE TH1 PHENOTYPE AND INITIATE mTOR DEPENDANT

INTIMAL HYPERPLASIA IN CARDIAC ALLOGRAFTS

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Dedication

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Abstract

A defined model system was used to address the role of minor histocompatibility antigen-specific CD4+ T cells in cardiac allograft vasculoopathy (CAV). The coronary arteries of vascularized heart grafts from Act-mOVA transgenic mice expressing the model antigen ovalbumin developed CAV in normal recipients and those lacking CD8+ T cells but not in those lacking CD4+ T cells. Furthermore, purified ovalbumin-specific CD4+ T cells from T cell antigen receptor (TCR) transgenic mice caused CAV in ovalbumin-expressing heart grafts in lymphocyte-deficient mice. This model system was used to study the role of CD154 expression, specific T helper subtype cytokine secretion, and the post CD4+ T cell-mediated injury phase of intimal hyperplasia. The graft antigen-specific CD4+ T cells only caused intimal hyperplasia when expressing CD154 and were found in the intima of the affected coronary arteries along with CD40+ cells. These results show that minor histocompatibility antigen-specific CD4+ T cells are required to cause cardiac allograft vasculopathy. They are capable of doing so without contributions from other lymphocytes, and may cause CAV by using CD154 to stimulate other non-lymphoid cells in the intima. The mechanism of CD154-dependant CD4+ T cell mediated CAV is not known. In order to determine the relevant cytokine profile involved in CD4+ T cell mediated CAV, ovalbumin-specific CD4+ T cells were differentiated in vitro into T_H1, T_H2, and T_H17 cells, which produce the prototypic cytokines, IFN-γ, IL-4, and IL-17, respectively, and transferred into lymphocyte-deficient recipients of ovalbumin-expressing heart grafts. In vitro-generated T_H1 and T_H17, but not T_H2 cells caused CAV. However, naïve ovalbumin-specific CD4+ T cells differentiated only into T_H1 cells in

vivo in recipients of ovalbumin-expressing heart grafts. Surprisingly, ovalbumin-expressing grafts transplanted into both STAT-4-deficient recipients with defective T_H1 subset generation and IFN-γ-deficient recipients developed CAV, although to a less severe degree than in wild-type recipients. Furthermore, IFN-γ-deficient ovalbumin-specific CD4+ T cells caused CAV similar to normal ovalbumin-specific CD4+ T cells. Thus, minor antigen-specific CD4+ T cells differentiate to the T_H1 subset in the presence of heart grafts expressing the relevant antigen, causing CAV in the absence of other lymphocytes. Although IFN-γ is involved, it is not the only factor produced by these cells that causes CAV. Whether a single episode of CD4+ T cell mediated injury, as opposed to multiple rejection episodes or ongoing activation, are required for the development of CAV is not known. When CD4+ T cells were depleted after the peak of clonal expansion but before the onset of intimal hyperplasia, CAV still developed in Act-mOVA grafts, suggesting that graft antigen-specific CD4+ T cells are critical for the immune injury phase of CAV but are not required once the graft is injured. Furthermore, the intimal hyperplasia phase of CAV was found to be mTOR dependant. Although graft antigen-specific CD4+ T cells initiate CAV, they are not necessary for progression of CAV.

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Section I: Introduction

Allografts may undergo different types of rejection depending on the immune response

Primarily vascularized allografts undergo different types of rejection depending on the immune response. The earliest form of rejection that may be noted after transplant is hyperacute rejection which results when preformed antibody against A/B blood group antigens or major histocompatibility complex (MHC) activates complement and the coagulation cascade within the allograft arteries and arterioles leading to immediate graft thrombosis and fulminant graft failure (1). Although rare, this may result from clerical errors and almost always necessitates explant of the graft or radical treatments such as plasmapharesis. In major histocompatibility mismatched grafts, acute rejection may also result from non-specific binding of CD4+ and CD8+ T cells to MHC on donor antigen presenting cells with activation and clonal expansion of relatively large proportions of recipient lymphocytes (2, 3). This usually results in clinical graft dysfunction and is both prevented and treated with immunosuppression. Chronic rejection is characterized by intimal hyperplasia of medium-sized graft arteries and arterioles and fibrosis of the histological units of the graft (4). For example, chronic rejection is characterized by fibrosis of the portal triads in liver allografts, sclerosis of the glomerulus in the transplant kidney, bronchiolitis obliterans in transplanted lungs, and cardiac allograft vasculopathy in cardiac allografts, and these processes often progress subclinically until the graft ultimately fails.

Recipient MHC present donor derived peptides to cognate T cells

When allografts shed antigen, the donor derived antigens are processed by recipient dendritic cells and the peptides are presented in recipient MHC class I and class II molecules to recipient CD8+ and CD4+ T cells, respectively (5-7). In the setting of costimulatory signals such as those present perioperatively, this leads to clonal expansion of graft antigen-specific lymphocytes that then migrate to the allograft and mediate rejection (8). Chronic rejection can occur in cases where the recipient and donor are matched with respect to MHC alleles. Rejection in this case is due to minor histocompatability antigens, which can be any protein that differs between donor and recipient and contains an MHC-binding peptide (9). When MHC-matched recipients and donors differ only in an MHC II-binding peptide, then the only recipient T cells that will be activated are CD4+ T cells. This may result in chronic rejection because CD4+ T cells are not capable of directly lysing graft target cells like cytotoxic CD8+ T cells.

CAV is initiated by an alloimmune response

CAV is the major cause of late heart allograft failure. This process is initiated by damage to the transplanted heart by the recipient's immune response, which in turn leads to proximal intimal hyperplasia, occlusion, and eventually loss of

cardiac function associated with decreased patient survival (4, 10-12). CAV is characterized by thickening of the intimal layer due to smooth muscle cell activation, proliferation, and deposition of excess extracellular matrix. The diffuse neointimal thickening and graft arteriosclerosis eventually lead to graft failure (13, 14). While non-immunologic factors contribute to CAV, suppression of the adaptive immune response can prevent chronic rejection, indicating that elucidation of the immune mechanism of CAV would be useful in developing effective therapies (15-17). However, the mechanism of CAV remains poorly understood in part because of the lack of informative animal models. In particular, no models exist where the lymphocytes that cause rejection can be tracked and manipulated without nonspecific effects on other cell types. Accumulating evidence (15, 16, 18-21) indicates that recipient CD4+ T cells play an essential role in chronic rejection of MHC-mismatched heart grafts, although roles for antibody and CD8+ T cells have been reported (17, 22, 23). The situation is less clear for minor histocompatibility antigens. Although Tanaka et al. found cardiac myosin-specific T cells in recipients of minor antigen-mismatched heart grafts (24), it was not established that these cells played a role in CAV. Nonetheless, this finding is consistent with accumulating evidence that recipient CD4+ T cells play an essential role in chronic rejection of both minor antigen (19) and MHC-mismatched heart grafts (15, 16, 18-20). Heeger and coworkers found that minor antigen-expressing heart grafts developed CD4+ T cell-dependent CAV in recipients that were previously primed with a skin graft (19). However,

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the fact that priming was required leaves open the question of whether or not minor antigen-specific CD4+ T cells are required for chronic rejection in immunologically naïve individuals.

CD4+ T cell expression of CD154 and development of CAV

The mechanism whereby CD4+ T cells participate in graft rejection may involve CD154, the ligand for CD40 (25). CD40 is a cell surface protein expressed on many cell types including dendritic cells, moncytes/macrophages, and B cells, and it binds CD154 on T cells (25-27). T helper cells activate B cells through CD154: CD40 binding (28) Conversely, CD154 activation by CD40 on dendritic cells and B cells is a strong costimulatory signal for T cells in developing T helper function, as well (27, 29, 30). Consistent with a critical costimulatory effect of CD154 ligation, CD4+ T cell-mediated acute rejection of MHC-mismatched grafts was prevented by blockade of CD154 (31). CD154 may also play some role in chronic rejection (32, 33), although this point is complicated by the fact that CD154 blockade only prevents chronic rejection in some cases when combined with CD28 blockade (34). This issue is further complicated by the finding of several other groups that intimal hyperplasia developed after CD154 blockade, or in CD154-deficient recipients of MHC mismatched heart grafts (35, 36). Thus, the role that CD154 plays in CD4+ T cell-mediated chronic rejection is currently unclear.

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The T helper subtype of CD4+ T cells initiating CAV

Cytokine production is another potential mechanism by which graft antigen-specific CD4+ T cells could cause CAV. Three T helper subsets characterized by distinct cytokine production have been identified, T_H1, T_H2, and T_H17 (37, 38). T_H1 helper cells produce IL-2, IFN-γ, GM-CSF, TNF-α, and IL-3 when stimulated through their TCRs, whereas T_H2 helper cells produce IL-3, IL-4, IL-5, and IL-13. The recently identified T_H17 helper cells produce IL-17, IL-6, and TNF-α. Which of these CD4+ T helper subsets causes CAV is not known.

Recently, Van Loosdregt and colleagues found an abundance of memory CD4+ T cells of the T_H1 subset in the neointima and adventitia of coronary arteries from human heart grafts with CAV compared to those without CAV (39). In a chimeric human-mouse model, IFN-γ is sufficient to cause CAV in the absence of any lymphocytes (40). Furthermore, CAV is reduced in MHC class II-disparate grafts transplanted into IFN-γ-deficient (41) or STAT-4-deficient recipients (42) that were immunosuppressed to prevent acute rejection. However, the antigenic

epitopes instigating rejection and the graft antigen-specific cells mediating

rejection were not defined in these models. Because definitive evidence for a

pathogenic role for IFN-γ in CAV in human heart transplant is lacking (43), a

model where the cells mediating CAV are known and could be manipulated

would be helpful.

Therefore, we employed the Act-mOVA heterotopic mouse heart transplant

model to demonstrate that, while both T_H1 and T_H17 CD4+ T cells can cause

CAV, naïve graft antigen-specific CD4+ T cells differentiate to the T_H1 subset,

and while IFN-γ may exacerbate CAV, graft antigen-specific CD4+ T cells cause

CAV through an IFN-γ independent mechanism.

The immune phase instigates the intimal hyperplasia phase of CAV

Whether a single episode of CD4+ T cell-mediated rejection is sufficient for the

development of CAV is not known. One possibility is that ongoing CD4+ T cell

activation is required to propagate CAV. Although some animal studies suggest

that ongoing lymphocyte activation must be blocked to slow the progression of

CAV (33), no model exists where the immune cells that cause CAV can be

eliminated after activation but before the development of CAV. This is important

since evidence from kidney transplant suggests that chronic rejection is much

more likely to develop after even a single episode of acute rejection, suggesting

that ongoing graft antigen-specific CD4+ T cell activation is not required for graft

fibrosis to progress (44). Alternatively, multiple episodes of acute cellular rejection may have a cumulative effect on the worsening of CAV (45). However, a model where the CD4+ T cells causing rejection can be tracked is required to understand the relationship of cellular rejection to the development of CAV because the effects of lymphocytes cannot be distinguished from the roles of other involved effector and target cells such as endothelial cells and myofibroblasts when all are present simultaneously in the graft.

mTOR signaling is required in the intimal hyperplasia phase of CAV after the CD4+ T cell mediated injury occurs

Although accumulating evidence suggests that CAV is primarily immune mediated, the exact mechanism of CAV resulting from immune injury remains elusive. One possibility is that graft antigen-specific CD4+ T cells migrate to the graft and initiate a program of cellular proliferation and graft fibrosis through direct activation of endothelial and myofibroblast precursors. Such target cells express proliferation signal mediators such as mTOR (molecular target of rapamycin) that may be activated by the effects of CD4+ T cell-mediated injury contributing to CAV (46, 47). Rapamycin inhibition of mTOR has been shown to inhibit vascular remodeling in an aortic MHC-mismatched allograft model (48). However, the effect of T cell proliferation could not be distinguished from the effect on proliferating intimal cells in this model.

Section II: Materials and Methods

Mice

C57BL/6 (B6) mice used as graft donors were purchased from the National Cancer Institute (Frederick, MD). Act-mOVA B6 mice used as graft donors were generated in our laboratory as previously described (49). B6 and recombinase activating gene (RAG)-deficient B6 mice used as recipients were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-II TCR transgenic mice (50) on the B6 background were used to produce CD90.1+ OT-II, RAG-deficient OT-II, and CD154-deficient OT-II mice by breeding.

For experiments investigating the T helper subtype of graft antigen-specific CD4+ T cells and cytokine secretion, BALB/c, BALB/c RAG-deficient, and STAT-4-deficient BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Act-mOVA B6 mice were also backcrossed at least 13 generations with BALB/c mice to produce a BALB/c Act-mOVA congenic strain. OT-II TCR transgenic mice (50) on the B6 background were used to produce CD90.1+ IFN-γ-deficient OT-II mice by breeding, as well as CD90.1+ OT-II mice. BALB/c DO11.10 TCR transgenic mice(51) were bred in our colony.

In all instances, mice were housed in the University of Minnesota Research Animal Resources facility, which is accredited by the Association for Assessment

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and Accreditation of Laboratory Animal Care. All protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Adoptive transfer

OT-II mice on the normal B6 or CD154-deficient B6 background were used as donors of CD4+ T cells in experiments investigating the role of CD154 expression on graft antigen-specific CD4+ T cells. The OT-II cells were purified before transfer because these mice have some CD8+ T cells and a normal population of B cells (50). CD4+ T cells were purified by negative selection from spleen and lymph node preparations using a CD4+ T cell Isolation Kit (Miltenyi Biotech, Auburn, CA) or by treatment with biotinylated anti-CD8 (2 µg/ml) and anti-B220 (8 µg/ml) antibodies followed by treatment with streptavidin-coated microbeads (Miltenyi Biotech) and then purification on LS columns (Miltenyi Biotech). In a few cases, spleen and lymph nodes from OT-II mice on a RAG-deficient background were used as a source of CD4+ T cells. No purification was done in this case because these mice do not contain any other lymphocytes. In both cases, the number of OT-II cells to be transferred was calculated from flow cytometric analysis of a sample of the donor lymph node and spleen cell suspension stained with phycoerythrin (PE)-labeled anti-CD4 and fluorescein isothiocynate (FITC)-labeled anti-Vα2 antibodies (eBioscience, San Diego, CA) as described (49). Wild-type OT-II cells (2×10^6) or RAG-deficient OT-II cells (5×10^5) cells were injected intravenously into RAG-deficient B6 mice.

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In experiments involving T helper skewed OT-II cells or cytokine secretion, OT-II mice on the normal B6 background or on the IFN-γ-deficient background were used as donors of naïve CD4+ T cells. The OT-II cells were purified before transfer because these mice have some CD8+ T cells and a normal population of B cells (50). CD4+ T cells were purified by negative selection from spleen and lymph node preparations as described above. In both cases, the number of OT-II cells to be transferred was calculated from flow cytometric analysis of a sample of the donor lymph node and spleen cell suspension stained with phycoerythrin (PE)-labeled anti-CD4 and fluorescein isothiocynate (FITC)-labeled anti-Vα2 antibodies (eBioscience) as described (49). Two million wild-type naïve OT-II cells or IFN-γ-deficient OT-II cells or 5-10 x 10^5 T_H1, T_H2, or T_H17 OT-II cells were injected intravenously into RAG-deficient B6 mice. In some experiments, DO11.10 TCR transgenic CD4+ T cells were identified by staining with fluorescein isothiocynate (FITC)-labeled anti-KJ1-26 (Caltag laboratories, Burlingame, CA) and phycoerythrin (PE)-labeled anti-CD4 (eBioscience). Two million naïve DO11.10 CD4+ T cells were injected intravenously into RAG-deficient BALB/c mice.

For adoptive transfer of low numbers of OT-II and for depletion experiments, TCR transgenic OT-II CD4+ T cells from wild-type OT-II mice or CD90.1+, RAG-deficient OT-II mice were purified by magnetic bead-based negative selection

from spleen and lymph node preparations using a CD4+ T cell Isolation Kit and LS columns (Miltenyi Biotech, Auburn, CA). The number of OT-II cells to be transferred was calculated from flow cytometric analysis of a sample of the donor lymph node and spleen cell suspension stained with phycoerythrin (PE)-labeled anti-CD4 and fluorescein isothiocynate (FITC)-labeled anti-Vα2 antibodies (eBioscience) as previously described (49). One thousand naïve OT-II were transferred into wild-type recipients and two million naïve OT-II cells were transferred into RAG-deficient recipients.

Heart grafting and in vivo treatments

Heterotopic mouse heart transplantation was performed as previously described according to the method of Corry et al. (52). Cardiac allograft function was assessed by weekly abdominal palpation. For OT-II depletion experiments, the hybridoma 1A14 (IgG2a anti-moue CD90.1) was obtained as a gift from A. Khoruts (University of Minnesota, Minneapolis, MN) and a cohort of RAG-deficient recipients was treated with 400μg i.p. in 50μl PBS weekly starting at 3, 15, or 40 days after transplant. Rapamycin was obtained from A. Khoruts. A stock solution of 1mg/mL rapamycin was prepared in 100% ethanol and doses were suspended in 0.2% carboxymethylcellulose (CMC), as previously described (53). A dose of 1mg/kg delivered i.p was given every other day starting at day 15 in a cohort of recipients that underwent OT-II depletion with the anti-CD90.1 antibody clone 1A14 starting on day 15 after transplant.

In vitro production of T_H1, T_H2, and T_H17 subset CD4+ T cells

TCR transgenic OT-II CD4+ T cells from CD90.1+, RAG-deficient OT-II mice were purified by magnetic bead-based negative selection from spleen and lymph node preparations using a CD4+ T cell Isolation Kit and LS columns (Miltenyi Biotech, Auburn, CA). Purified OT-II cells were then skewed in vitro to the T_H1, T_H2, (54-56) or T_H17 (57) subsets using previously described conditions. Specifically, 2×10^5 purified OT-II cells and 5×10^6 irradiated B6 splenocytes (3,000 rad) were treated with either IL-12 (10 U/mL, eBioscience, San Diego, CA) and anti-IL-4 (10μg/mL, BD Pharmingen, San Diego, CA) for T_H1 skewing(54), IL-4 (200 U/mL, eBioscience) and anti-IL-12 (3μg/mL, BD Pharmingen) for T_H2 skewing(54, 56), or IL-23 (10 ng/mL), anti-IFN-g (10 μg/mL), rhTGF- Beta1 (1 ng/mL), and IL-6 (10 ng/mL) for T_H17 skewing(57) (all from eBioscience). All but one culture for each subset also contained ovalbumin (OVA) peptide (10 μg/mL). After the initial stimulation for 7 days in vitro, the OVA peptide stimulated lymphocytes were collected and purified by density gradient centrifugation using Lympholyte (Cedarlane Laboratories Ltd., Burlington, Ontario, Canada), and then restimulated with OVA peptide and 5×10^6 irradiated B6 splenocytes (3,000 rad) for 3 days in vitro. The collection, purification, and restimulation were then repeated a third time for 3 days.

Intracellular cytokine staining and flow cytometry

The number of OT-II cells in the spleens of heart graft recipients was determined by flow cytometry as described by Ehst et al. (49) following staining with PerCP-labeled anti-CD90.1 (BD Pharmingen, San Diego, CA) and FITC-labeled anti-TCR Vα2 antibodies (eBioscience). The percentage of CD90.1+, TCR Va2+ cells was used together with a viable cell count to calculate the total number of CD90.1+, TCR Va2+ cells present in the original sample. In some experiments, CD154-deficient or wild-type OT-II cells were identified by staining with PE-labeled anti-TCR Vα2 (BD Pharmingen), FITC-labeled anti-CD90.1 (eBioscience), and PerCP-labeled anti-CD4 antibodies (BD Pharmingen).

For intracellular cytokine staining, heart graft recipients received either 100 μg of OVA peptide intravenously or no injection and after 2 hours, the heart grafts and spleens were collected, minced into a single cell suspension, fixed in 2% formaldehyde, and permeabilized for intracellular cytokine staining (58). In cases where DO11.10 mice were used for adoptive transfer, the transgenic T cells were identified in cell suspensions from the heart grafts and spleens using APC-labeled KJ1-26 (Caltag) and PerCP Cy5.5-labeled anti-CD4 (BD Pharmingen), after excluding all Pacific Blue-labeled anti-CD11b, -F4/80 (Caltag), -CD11c, -Gr-1 (eBioscience), and -NK1.1 (BioLegend, San Diego, CA) (dump)-positive events. Intracellular cytokine staining was performed using FITC-labeled anti-IFN-γ (eBioscience), PE-labeled anti-IL-5 (eBioscience), or PE-labeled anti-IL-17

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(BD Pharmingen). In vitro skewed OT-II cells from the spleens of heart graft recipients were identified by flow cytometry as described by Ehst et al. (49). Staining of fixed and permeabilized cells with PE Cy7-labeled anti-CD90.1 and PerCP Cy5.5-labeled anti-CD4 (BD Pharmingen) was used to identify OT-II cells. Cytokine staining in these cases was performed with PE-labeled anti-IL-5, PE-labeled anti-IL-17, APC-labeled anti-IFN-γ (eBioscience), and FITC-labeled anti-TNF-α (eBioscience).

In wild-type recipients transferred with a low number of naïve OT-II cells, the OT-II cells were identified using a modification of the magnetic bead-based positive selection method that has been previously described (59). Biotin-labeled CD90.1+ OT-II cells were positively selected using MACS columns (Miltenyi Biotec, Auburn, CA) and staining of fixed cells with PE Cy5-labeled anti-Vα2 and FITC-labeled anti-CD4 (eBioscience, San Diego, CA) was used to identify OT-II cells for quantification of the total number of splenic. In RAG-deficient recipients in OT-II depletion experiments, splenic OT-II were stained with a Pacific Orange-labeled dump (anti-CD11b, -CD11c, -Gr-1, and -NK1.1), PE-labeled anti-CD90.1, PerCP Cy5.5-labeled anti-CD4, and FITC-labeled anti-Vα2 in order to quantitate the total number of OT-II. In some cases, staining was performed with APC-labeled anti-CD90.1, PerCP Cy5.5-labeled anti-CD4, and FITC-labeled anti-Vα2, and PE-labeled anti-Vβ5 (eBioscience).

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Histology and immunohistology

Heart grafts were divided in half by axial sectioning to separate the top and the bottom. Eight micron sections were cut through each half. Coronary arteries were identified as vessels with a visible internal and external elastic lamina. Distal coronary arteries were identified by their position in the epicardium or muscle of the bottom half of the heart. Proximal coronary arteries were identified by their position in the top half of the heart near the aorta. 20x images of at least three sections from each heart and 1-3 vessels per section were analyzed. All coronary arteries in a given section with an intact internal and external elastic lamina were included in the analysis. Elastin was detected by staining heart graft sections sequentially with rabbit anti-mouse tropoelastin (Elastin Products Corp., St. Joseph, MO), biotin-labeled goat anti-rabbit IgG (eBioscience), streptavidin-labeled horseradish peroxidase, and Cy3-labeled tyramide. Nuclei were labeled with Sytox nucleic acid stain (Molecular Probes, Eugene, OR). Images covering the entire area of each section were acquired at 10x magnification using a Bio-Rad MRC-1000 or 1024 confocal microscope, and then assembled into a single composite image using Photoshop 7.0 software (Adobe). A section of an Act-mOVA heart graft section stained with a biotin-labeled isotype control antibody or with control rabbit polyclonal IgG (for elastin) was used to set the threshold so that no signal was detected in the Cy3 channel. These settings were then applied to the heart graft sections stained with anti-elastin antibody and biotin-labeled

goat anti-rabbit IgG. Verhoeff's elastin staining was also performed by the University of Minnesota Cancer Center Histopathology Shared Resource Core.

The area of the intima and the media was determined using ImageJ software (NIH). The value of the area of the lumen was subtracted from the area within the internal elastic lamina to determine the area of the intima. The area within the internal elastic lamina was subtracted from the area within the external elastic lamina to determine the area of the media. The ratio of the intima to the media was determined by dividing the area of the intima by the area of the media.

Perivascular cells expressing CD90.1, CD11b, CD11c, Gr-1, or CD40 were detected by staining 8-micron heart cross-sections sequentially with biotin-labeled antibodies specific for these molecules, streptavidin-labeled horseradish peroxidase, and Cy3-labeled tyramide. In the case of CD40, indirect labeling with biotinylated tyramide was performed before labeling with Cy3-labeled streptavidin. After blocking residual horse radish peroxidase and free biotin sites with 3% hydrogen peroxide with 0.1% sodium azide and avidin and biotin blocks (Vector Laboratories, Burlingame, CA), sections stained with only one of the above primary antibodies were also stained with biotin-labeled rat anti-mouse CD31 (Southern Biotech, Birmingham, AL) or rabbit anti-mouse tropoelastin (Elastin Products Corp., St. Joseph, MO) and then sequentially with biotin-labeled goat anti-rabbit IgG (eBioscience), streptavidin-labeled horse radish

peroxidase, and Cy5-labeled tyramide. Nuclei were labeled with Sytox. An isotype control was used to set the signal in the Cy3 channel so that no pixels were detected. The number of Cy3+ cells per perivascular section was determined using Photoshop 7.0 (Adobe) using a modification of our published method (60). The Cy3 images were converted to the grayscale mode. Under the Image tab, Adjustments/Levels were opened, and the left (black) slider under the Input Levels histogram was adjusted to a value of 250 to exclude background. Under the Select tab, Color Range was opened, and Highlights were selected under the Sampled Colors command. Under the Select tab, the Expand function of the Modify feature was used to expand the selected pixels by 6. Under the Select tab, the Contract function of the Modify feature was used to contract the selected pixels by 3. This Expand/Contract sequence had the effect of converting irregularly stained cells to uniform spheres, one sphere/cell. Under the Edit tab, the Fill function was selected and the Contents function was used to designate White as the Fill color. The Blending mode was Normal and the Opacity 100%. For each image, 10 individual cells were selected and under the Select tab, Color Range was opened, and Highlights were selected under the Sampled Colors command. The Histogram function was used to determine the number of pixels per cell. Under the Select tab, Color Range was opened, and Highlights were selected under the Sampled Colors command for the entire image to obtain the total number of pixels in the image. This value was divided by the number of pixels/cell to determine the number of cells per section. This operation allowed

measurement of cells that were clumped together in the image. Cells were counted in 1-3 coronary arteries per section from 1-3 sections per graft from 1-3 mice per group.

For experiments involving T helper subtype and cytokine expression, heart grafts were prepared and stained for elastin as described above. Intimal hyperplasia was assessed using the same method after acquiring images of coronary arteries with a visible internal and external elastic lamina at 10x or 20x magnification using a Bio-Rad MRC-1000, 1024 confocal, or Leica AF6000 microscope.

Intragraft cells expressing CD90.1 were detected by staining 8 μm heart cross-sections sequentially with biotin-labeled anti- CD90.1, streptavidin-labeled horseradish peroxidase, and Cy5-labeled tyramide. Sections stained with anti-CD90.1 were also stained with rabbit anti-mouse tropoelastin (Elastin Products Corp., St. Joseph, MO), as described above, after blocking residual horse radish peroxidase and free biotin sites with 3% hydrogen peroxide with 0.1% sodium azide and avidin and biotin blocks (Vector Laboratories, Burlingame, CA). Nuclei were labeled with Sytox. An isotype control was used to set the signal in the Cy5 channel so that no pixels were detected. For quantification of intragraft CD90.1+ cells, 20x-magnification images were obtained, and the number of Cy5+ cells per section was determined using Photoshop 7.0 (Adobe) using a modification of our published method (60). The Histogram function was used to determine the total

number of Cy5+ pixels and the number of pixels per cell. For Cy5 images, under the Image tab, the RGB mode was selected. Under the Adjusments section of the Image tab, Curves was selected and the blue and green curve outputs were reduced to zero. Then, under the Select tab, Color Range was opened, and Red was selected under the Sampled Colors command for the entire image to obtain the total number of red pixels in the image. This value was divided by the number of red pixels per cell in order to determine the number of cells per section. This operation allowed measurement of cells that were clumped together in the image.

For experiments involving depletion of OT-II cells and treatment with rapamycin, grafts were prepared as described above and stained for elatin and CD90.1. Images were obtained on a Leica AF6000 microscope covering the entire axial section of each graft and intimal hyperplasia was assessed by measuring every artery in the section with a visible internal and external elastic lamina.

Statistical method

The 2-tailed Student's T Test with unequal variance was used to determine the p-value for each group of measurements.

Section III: Results

We addressed the roles of graft antigen-specific CD4+ T cells in CAV using a transgenic B6 mouse strain, designated Act-mOVA, which expresses chicken ovalbumin (OVA) on the surface of most cells of the body (49). Since Act-mOVA mice differ genetically from B6 mice only with respect to OVA expression, rejection of Act-mOVA tissues by B6 recipients is due to an immune response to this single, defined minor histocompatability antigen (49). We used a vascularized heart transplant model to investigate the requirement for graft antigen-specific CD4+ T cells in CAV in the absence of other lymphocytes. This model was used to examine the relationship between the kinetics of graft antigen-specific CD4+ T cell clonal expansion and contraction and the development of CAV. In addition, this model was employed to investigate the role of CD154 expression of graft antigen specific CD4 + T cells, as well as the specific T helper differentiation and cytokine secretion of the lymphocytes that cause CAV. This model also allows the tracking and manipulation of the graft antigen specific CD4+ T cells during specific phases of CAV in order to determine whether a single episode of CD4+ T cell-mediated rejection may cause CAV, as opposed to ongoing CD4+ T cell activation. Using this model where the CD4+ T cells causing the CAV can be tracked and eliminated, we investigated the role of mTOR activation during the intimal hyperplasia phase of CAV, independent of the immune response.

Polyclonal CD4+ T cells are required for the development of CAV

In a previous report, work in the Jenkins laboratory demonstrated that wild-type

B6 and B cell-deficient B6 mice reject full-thickness Act-mOVA tail skin grafts in

about 4 weeks (49). However, Act-mOVA skin grafts were not rejected by

recipients lacking $\alpha\beta$-T cells, and slowly by recipients lacking CD4+ T cells. In

contrast, Act-mOVA skin grafts were damaged by recipients lacking CD8+ T cells

as evidenced by 80% shrinkage, but were retained indefinitely. These results

indicated that CD8+ T cells are required for complete rejection of Act-mOVA skin

grafts, whereas CD4+ T cells cause a more limited form of rejection.

The skin graft results raised the possibility that CD4+ T cells may cause CAV in

Act-mOVA allografts. This possibility was assessed with heterotopic heart

transplants, which are often used to study CAV (4). Act-mOVA hearts were

grafted either into normal B6 recipients, CD8-deficient recipients, which lack

CD8+ T cells (61), or MHC II-deficient recipients, which lack CD4+ T cells (61).

B6 grafts transplanted into B6 recipients served as negative controls. All of the

transplanted Act-mOVA hearts as well as control B6 hearts were still beating at

50 days, indicating that acute rejection had not occurred. However,

immunohistology revealed significant thickening of the intimal layer of the

proximal coronary arteries of Act-mOVA (Figure 1A, C) but not B6 grafts (Figure

1A, B) in normal B6 recipients. Intimal thickening also occurred in the distal

coronary arteries of Act-mOVA grafts in B6 recipients but was more heterogeneous (Figure 1A) than that observed in the proximal vessels. Significant intimal hyperplasia also developed in the proximal coronary arteries of Act-mOVA heart grafts in recipients lacking CD8+ T cells (Figure 1A, D), although to a lesser degree than in normal B6 recipients. In contrast, significant intimal hyperplasia did not develop in Act-mOVA heart grafts in recipients lacking CD4+ T cells (Figure 1A, E). These results demonstrate that CD4+ T cells are required for CAV of this minor antigen-disparate graft. CD8+ T cells are not required, but may contribute in recipients that also contain CD4+ T cells.

CAV develops in minor antigen disparate grafts in recipients containing only graft antigen-specific CD4+ T cells

To determine if graft antigen-specific CD4+ T cells alone were capable of causing CAV, RAG-deficient B6 mice, which lack lymphocytes,were reconstituted with purified CD4+ T cells from OVA-specific OT-II TCR transgenic mice and then transplanted with Act-mOVA or control B6 hearts. Intimal hyperplasia was detected by elastin staining as shown in Figure 1, which was confirmed by the conventional Verhoeff's stain (62) (Figure 2B, C). Again, Act-mOVA hearts were not acutely rejected by RAG-deficient B6 mice containing OT-II cells, and were still beating 80 days after transplantation. However, the proximal coronary arteries of Act-mOVA grafts (Figure 2A, C) but not B6 grafts (Figure 2A, B) in this

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type of recipient contained intimal hyperplasia. In contrast, the distal portions of the coronary arteries of Act-mOVA hearts were normal (Figure 2A). Intimal hyperplasia within the proximal coronary arteries of Act-mOVA hearts in RAG-deficient B6 mice containing OT-II cells was not present before 50 days after grafting (Figure 2D), indicative of a slow process.

In order to demonstrate that the CAV observed after transfer of purified wild-type OT-II cells was not due to a small number of contaminating B cells or CD8+ T cells, separate RAG-deficient recipients were transferred with 5×10^5 CD4+ T cells from a RAG-deficient OT-II cell donor, which did not contain any lymphocytes other than the OT-II cells. The recipients of these monoclonal OT-II cells also developed intimal hyperplasia of the proximal but not the distal coronary arteries (Figure 2A). Therefore, graft antigen-specific CD4+ T cells can cause CAV of the main, proximal coronary arteries in the absence of all other lymphocyte subsets.

Graft antigen-specific CD4+ T cells undergo clonal expansion before the onset of CAV and then contract as CAV develops

In order to establish the kinetics of CD4+ T cell activation with respect to the development of CAV, B6 recipients underwent heterotopic mouse heart transplant with Act-mOVA grafts after adoptive transfer with a near-physiologic (59), low number of graft antigen-specific transgenic CD4+ T cells (CD90.1+ OT-

II cells). Recipients were then sacrificed weekly and OT-II cells were purified from recipient spleens and quantified after positive selection on magnetic beads (Figure 3A). While OT-II cells were not detectable in un-transferred recipients (Figure 3A, left panel), a small number of OT-II cells were detected in transferred recipients before transplant (Figure 3A, middle panel and 3C), and OT-II cells underwent a 500-fold clonal expansion by week 2 after transplant (Figure 3A, right panel and 3C). Recipient hearts from each time point were examined in axial section for the presence of CAV and this was quantified using the ratio of the area of the intima to the media (Figure 3B and 3C). While CAV was not present before the clonal expansion of OT-II cells (Figure 3B, left panel and 3C), CAV was found in some Act-mOVA grafts within 1 week of the time of maximum clonal expansion of OT-II cells (Figure 3B, middle panel and 3C). Of interest, while the number of graft antigen-specific CD4+ T cells decreased dramatically after the point of maximal clonal expansion and before the onset of CAV, CAV continued to progress as the number of graft antigen-specific CD4+ T cells plummeted (Figure 3B right panel and 3C). This finding suggests that once immune injury has occurred, ongoing graft-antigen-specific CD4+ T cell activation is not required for the progression of CAV.

Graft antigen-specific CD4+ T cells, monocytes, macrophages, and dendritic cells accumulate in Act-mOVA heart grafts

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The number and anatomic distribution of OT-II T cells in Act-mOVA heart graft

recipients was measured to gain clues about the mechanism of CAV. The

transferred OT-II cells were easily detected in the spleens of RAG-deficient

recipients by expression of CD90.1 and CD4 (Figure 4A). The specificity of this

staining was confirmed by the finding that CD90.1+, CD4+ cells were not

detected in the spleens of RAG-deficient B6 mice that did not receive OT-II cells

(Figure 4B). OT-II cells increased to a greater extent in the spleens of Act-mOVA

graft recipients than in B6 graft recipients (Figure 4C). In addition, OT-II cells

accumulated in Act-mOVA (Figure 5A), but not B6 heart grafts (Figure 5B), to a

maximum level by14 days after grafting, and they remained elevated above the

level in B6 grafts at 80 days (Figure 5C). The OT-II cells were concentrated near

the proximal coronary arteries of Act-mOVA heart grafts (Figure 5A), and many

OT-II cells were present in the thickened intimal layer (Figure 5A inset). The

increased number of OT-II cells in the spleens and transplanted hearts of Act-

mOVA but not B6 heart graft recipients was strong evidence of OVA-dependent

activation.

Although graft antigen-specific CD4+ T cells were the only lymphocytes required

to initiate CAV in this system, it was likely that other inflammatory cells were also

involved. This possibility was tested by determining whether or not monocytes

(CD11b+, Gr-1+) (63), macrophages (CD11b+, Gr-1-), and/or dendritic cells

(CD11c+) were present in the thickened intimal layers of Act-mOVA grafts. At

day 80 after transplantation, when intimal hyperplasia was present (Figure 2D), CD11b+, Gr-1+ cells surrounded, or were within the intimal layer of the proximal coronary arteries in Act-mOVA (Figure 6A, yellow cells) but not B6 grafts (Figure 6B). CD11b+ Gr-1- cells (Figure 6A, red cells), CD11c+ cells (Figure 6C), and CD40+ cells (Figure 6D) were also present in the intimal layer at this time. Thus, the hyperplastic intima of Act-mOVA grafts contained CD11b+, Gr-1+ monocytes (63), CD11b+, Gr-1- macrophages, and CD11c+ dendritic cells. Many of these populations are known to express CD40 (26, 64-66).

CD40 ligand expression is required by minor graft antigen-specific CD4+ T cells to cause intimal hyperplasia

Because CD40+ inflammatory cells were present in the grafts with CAV, it was of interest to determine if the T cell ligand for this molecule, CD154 was critical for the ability of minor antigen-specific CD4+ T cells to cause CAV. This possibility was tested by assessing the capacity of CD154-deficient OT-II cells to cause CAV in Act-mOVA hearts grafted into RAG-deficient B6 mice. Unlike Act-mOVA grafts from recipients of wild-type OT-II, which developed intimal hyperplasia, Act-mOVA grafts from recipients of CD154-deficient OT-II cells looked completely normal (Figure 2A). Thus, CD154 expression by OT-II cells was essential for their ability to cause CAV.

In other systems, CD154 plays a role in the clonal expansion of antigen activated CD4+ T cells (30, 67). Indeed, CD154-deficient OT-II cells accumulated poorly compared to wild-type OT-II cells in the spleens of Act-mOVA heart recipients (Figure 4C) and failed to accumulate at significant levels in the Act-mOVA heart grafts themselves (Figure 5C). Thus, suboptimal graft accumulation due to poor clonal expansion in the spleen is the likely explanation for the inability of CD154-deficient OT-II cells to cause CAV.

T_H1 and T_H17, but not T_H2 skewed graft antigen-specific CD4+ T cells can cause CAV in the absence of other lymphocytes

In order to determine whether the graft antigen-specific CD4+ T cells responsible for CAV belonged to a specific T helper subset, RAG-deficient B6 mice were adoptively transferred with in vitro skewed T_H1, T_H2, or T_H17 OT-II cells. T helper subset-specific cytokine production was confirmed by intracellular staining of samples from each group at the time of graft harvest at 30 days (Figure 7, Table 1), 2 hours after intravenous injection of 100 μg OVA peptide intravenously (Figure 7C, E) or no injection as a negative control (Figure 7B, D). As expected, T_H1 skewed splenic OT-II cells were found to produce IFN-γ, but not IL-5 or IL-17, while T_H2 skewed OT-II produced IL-5, but not IFN-γ or IL-17 (Figure 7B-E). Again as expected, T_H17 skewed OT-II cells produced IL-17, as well as a small amount of IFN-γ and no IL-5 (Figure 7B-E). None of the OT-II populations

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produced cytokines in the absence of the in vivo challenge with OVA peptide (Figure 7B, D).

Having determined that the skewed T helper subsets produced the expected cytokines, the intima to media ratio within the heart grafts was measured in order to determine whether different T helper subsets were capable of causing CAV (Figure 8 and Table 1). While CAV was not found in the coronary arteries of B6 Act-mOVA grafts from recipients of T_H2 OT-II cells (N=3, Figure 8B, D), CAV was found in the coronary arteries of B6 Act-mOVA grafts from 4 of 6 recipients of T_H1 OT-II cells (Figure 8A, D) and in 4 of 5 recipients of T_H17 cells (Figure 8C, D).

The preceding findings suggested that both T_H1 and T_H17 graft antigen-specific CD4+ T cells can cause CAV, while T_H2 CD4+ T cells cannot. This possibility was investigated further by comparing the levels of recall cytokine production by T_H1 or T_H17 cells to the degree of CAV in individual graft recipients (Figure 9). No positive correlation was found between the amount of IFN-γ or IL-17 production by either T_H1 (Figure 9A) or T_H17 (Figure 9B) OT-II cells and the development of CAV. Thus, either very small amounts of either cytokine are sufficient to cause CAV, or these cytokines are not involved.

Naïve graft antigen-specific CD4+ T cells differentiate to the T$_H$1 subset and infiltrate the graft.

Recent findings demonstrated that T$_H$1 CD4+ T cells predominate in the adventitia and intima of graft coronary arteries afflicted with CAV (39). Therefore, we sought to determine whether naïve graft-antigen-specific CD4+ T cells differentiate to the T$_H$1, T$_H$2, or T$_H$17 subsets in our system. Naïve DO11.10 TCR transgenic CD4+ T cells specific for the OVA peptide 323-339 presented by the BALB/c I-A^d MHC II molecule were transferred into RAG-deficient BALB/c recipients that then received either wild-type BALB/c or BALB/c Act-mOVA heart grafts. After the peak of DO11.10 cell clonal expansion at 14 days (21), BALB/c Act-mOVA recipients were restimulated with intravenous OVA peptide or no injection, and recipient spleens and heart grafts were harvested for intracellular staining for IFN-γ, IL-5, and IL-17.

DO11.10 cells in the spleens and grafts were identified in adoptively transferred mice, but not in untransferred mice, by staining with anti-CD4 and the clonotypic antibody KJ1-26 specific for the DO11.10 T cell receptor (Figure 10A). No cytokine production was detected by direct ex vivo intracellular cytokine staining of splenic (Figure 10B) or intragraft (Figure 10C) DO11.10 cells from mice that received BALB/c Act-mOVA grafts compared to those that received BALB/c grafts (data not shown), suggesting that the level of antigen presented to splenic

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or intragraft antigen-specific CD4+ T cells was very low at this time point. However, after OVA peptide restimulation, DO11.10 cells in both the spleen (Figure 10B) and the graft (Figure 10C) produced IFN-γ, but not IL-5 or IL-17. As a positive control for the detection of IFN-γ, IL-5, and IL-17, in vitro skewed DO11.10 cells were used to demonstrate that DO11.10 cells have the potential to become T_H1, T_H2, or T_H17 subsets, as do OT-II cells, and subset specific cytokines can be detected by intracellular cytokine staining (Figure 7 and data not shown). Therefore, these findings demonstrate that naïve graft antigen-specific CD4+ T cells differentiate to the T_H1 subset and infiltrate the graft.

CAV develops in the coronary arteries of minor antigen disparate grafts transplanted into STAT-4-deficient recipients.

The finding that graft antigen-specific CD4+ T cells differentiate to the T_H1 subset and infiltrate the graft is consistent with the predominance of T_H1 CD4+ T cells of unknown antigen specificity in the adventitia and intima of human coronary arteries afflicted with CAV(39). In order to determine whether T_H1 cytokine production is required for the development of CAV, BALB/c Act-mOVA heart grafts were transplanted into either wild-type BALB/c (Figure 11B, D) or STAT-4 deficient recipients (Figure 11C, D), in which CD4+ T cell differentiation to the T_H1 subset is greatly reduced (56). As a negative control, BALB/c grafts were transplanted into wild-type BALB/c recipients (Figure 11A, D). Intimal hyperplasia

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did not develop in negative control isografts. However, CAV developed in the coronary arteries of STAT-4 deficient recipients, although to a somewhat lesser degree than in wild-type recipients (p=0.03). These findings suggest that STAT-4 dependent cytokine production may contribute to CAV, but it is not required.

IFN-γ production is not required for the development of CAV

However, it was still possible that IFN-γ production by graft antigen-specific CD4+ T cells was required for CAV since T_H1 cells can differentiate through a minor STAT-4-independent pathway in certain cases (68). Therefore, B6 Act-mOVA grafts were transplanted into either wild-type B6 (Figure 12B, D) or IFN-γ-deficient B6 recipients (Figure 12C, D), and B6 isografts were transplanted into B6 recipients as negative controls (Figure 12A, D). Consistent with the finding in STAT-4 deficient recipients, IFN-γ was not required for CAV to develop in B6 Act-mOVA grafts, although CAV in Act-mOVA grafts from IFN-γ-deficient recipients was attenuated when compared to CAV in Act-mOVA grafts from wild-type recipients (Figure 12B-D, p=0.0004). Because we recently demonstrated that CD4+ T cells are required for the development of CAV in this model(21), this finding suggested that CD4+ T cells cause CAV through an IFN-γ independent mechanism.

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Graft antigen-specific CD4+ T cells cause CAV through an IFN-γ independent mechanism

In order to test the hypothesis that CD4+ T cells cause CAV through an IFN-γ independent mechanism, B6 Act-mOVA grafts were transplanted into RAG-deficient recipients after adoptive transfer with either purified wild-type naïve OT-II cells (Figure 13B, D) or IFN-γ-deficient naïve OT-II cells (Figure 13C, D). Negative control wild-type B6 grafts transplanted into RAG-deficient recipients transferred with purified wild-type OT-II cells did not develop CAV (Figure 13A, D), while B6 Act-mOVA grafts did. Also, CAV developed in Act-mOVA grafts transplanted into recipients transferred with IFN-γ-deficient OT-II cells to a similar degree compared to those transferred with wild-type OT-II (p=0.11), demonstrating that IFN-γ production is not required by graft antigen-specific CD4+ T cells to cause CAV. Quantification of CD90.1+ cells by immunohistology (Figure 13E) demonstrated similar numbers of perivascular wild-type OT-II CD4+ T cells compared to IFN-γ-deficient OT-II CD4+ T cells in the transplanted hearts demonstrating that graft antigen-specific CD4+ T cells traffic to coronary arteries and cause intimal hyperplasia independent of IFN-γ production.

Graft antigen-specific CD4+ T cells may be eliminated once they have undergone clonal expansion

The model of CAV using lymphocyte deficient recipients of a pure monoclonal population of graft antigen-specific CD4+ T cells allows definitive manipulation of the instigating antigen-specific CD4+ T cell response. In order to determine the exact time during the immune response at which CD4+ T cells are required for the development of CAV, OT-II cells were adoptively transferred into lymphocyte-deficient B6 mice and the recipients then underwent heterotopic mouse heart transplant with Act-mOVA B6 allografts. Recipients were then sacrificed at regular intervals and the number of splenic OT-II cells was quantified using flow cytometry. While no OT-II cells were detectable in un-transferred animals (Figure 14B, left panel), OT-II cells were easily detected after adoptive transfer and grafting with Act-mOVA hearts (Figure 14B, middle panel). Of note, the kinetics of T cell clonal expansion and contraction in this model (Figure 14C) are very similar to those in a model where a physiologic number of naïve antigen-specific CD4+ T cells experience graft antigen in a polyclonal recipient (Figure 3C). In order to determine if the OT-II cells could be eliminated after clonal expansion, a group of these recipients were treated with a depleting antibody specific for the CD90.1 congenic marker on the OT-II cells (Figure 14B, right panel and 14C). Indeed, shortly after treatment with depleting antibody, OT-II cells were no longer detectable in the spleens of Act-mOVA recipients.

Splenic graft antigen-specific CD4+ T cells are not required for the development of CAV once they have undergone clonal expansion

In order to determine whether OT-II cells are required for the development of CAV after maximum clonal expansion, grafts were examined for CAV at various time points (Figure 15). CAV was found as early as 3 weeks after grafting and progressed to involve all grafts by 65 days in recipients with persistent OT-II (Figure 15A middle panel and 15B). In contrast, CAV did not develop if OT-II cells were depleted 2 days after Act-mOVA heart transplant (Figure 15A right panel and 15B), indicating that more than day of OT-II cell activation were required for CAV to develop. In order to determine if OT-II cells were required for the progression of CAV after the time of maximum clonal expansion, grafts were examined from recipients that were depleted of OT-II 15 days after grafting (Figure 15A right panel and 15B). Despite the absence of the OT-II cells that cause the CAV after maximum clonal expansion, CAV was present 35 days after grafting and continued to progress.

Progression of CAV after immune-mediated injury does not require intragraft antigen-specific CD4+ T cells

Although these findings suggest that splenic graft antigen-specific CD4+ T cells are not required for the progression of CAV once they have undergone clonal

expansion, residual OT-II in the graft could still propagate CAV. In order to address this possibility, the depletion of intragraft OT-II was assessed using immunohistology. While OT-II depletion was greater than 90% (Figure 16B and C) compared to untreated recipients (Figure 16A and C) CAV still progressed in Act-mOVA grafts. The development of CAV despite the depletion of intragraft OT-II cells demonstrates that graft antigen-specific CD4+ T cells are not required in the spleen or the graft once they have undergone clonal expansion. This finding suggests that there are two phases of CAV: a graft antigen-specific CD4+ T cell-dependent phase characterized by graft antigen-specific CD4+ T cell activation and trafficking to the graft and an injury response phase characterized by intimal hyperplasia and independent of ongoing graft antigen-specific CD4+ T cell activation.

The injury response of intimal hyperplasia to CD4+ T cell mediated injury is mTOR dependent

The mTOR pathway is known to be critical to cellular proliferation (69) and mTOR inhibition by everolimus reduces the severity and incidence of CAV after heart transplant in humans (70). In order to determine whether activation of the mTOR pathway is required for the injury response phase of CAV that follows graft antigen-specific CD4+ T cell mediated injury, a cohort of Act-mOVA heart recipients were treated with rapamycin after depletion of OT-II at the height of

clonal expansion (Figure 14C and 17). While CAV did not develop in Act-mOVA

grafts before 15 days or if OT-II cells were depleted before clonal expansion,

CAV progressed in all Act-mOVA grafts if OT-II were not depleted and in Act-

mOVA grafts of recipients even if OT-II were depleted at the peak of clonal

expansion or well after CAV had already begun (Figure 17). In contrast to Act-

mOVA grafts that developed CAV despite the depletion of OT-II at the maximum

of clonal expansion, CAV was completely ameliorated in graft antigen-specific

CD4+ T cell damaged hearts after treatment with rapamycin. This finding

suggests that the mTOR pathway is critical to the injury response phase of CAV

when CD4+ T cell injury is no longer required to propagate CAV.

Section IV DISCUSSION

The role of graft antigen-specific CD4+ T cells in CAV

The finding of CAV in Act-mOVA heart grafts in recipients whose only

lymphocytes were graft antigen-specific CD4+ T cells demonstrated that this

subset plays an important role in CAV induced by minor histocompatibility

antigens. Although results from CD8-deficient recipients indicate that CD4+ T

cells can also cause CAV in MHC-mismatched heart grafts (15, 16, 18, 20), other

results from mice lacking only CD4+ T cells indicate that CD8+ T cells also have

this potential (22, 23, 71). Thus, our finding that CAV did not develop in Act-

mOVA heart grafts in recipients containing CD8+ T cells but lacking CD4+ T cells

indicates that CD4+ T cells play a more unique role in chronic rejection of grafts

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that differ from the recipient only with respect to minor antigens. CD8+ T cells may play a minor role in chronic rejection of Act-mOVA grafts, as evidenced by the slightly more severe CAV that developed in normal B6 recipients, which contain CD4+ and CD8+ T cells, compared to CD8-deficient recipients, which contain only CD4+ T cells. However, if CD8+ T cells do participate in chronic rejection in this system, they only do so when CD4+ T cells are also present.

B cells and antibodies were also not required for CAV in Act-mOVA grafts as evidenced by the fact that it developed in RAG-deficient recipients of RAG-deficient OT-II cells. However, as in the case of CD8+ T cells, graft antigen-specific B cells and antibodies may play an augmenting role. However, the result that MHC II-deficient mice lacking CD4+ cells but containing B cells did not develop CAV demonstrates that even this potential minor role from B cells must be completely dependent on CD4+ T cells.

Graft antigen-specific CD4+ T cells must express CD154 in order to cause CAV

Our results also showed that CD154 expression by graft antigen-specific CD4+ T cells is essential for CAV stimulated by a minor histocompatibility antigen. In contrast, previous studies demonstrated that CD154 is not required for CAV stimulated by fully- (35, 36) or MHC I only- (72) disparate heart grafts. The

reduced requirement for CD154 in these cases may relate to the participation of

CD8+ T cells that do not utilize CD154 to mediate rejection (73). The finding that

CD154 is required for acute rejection of MHC-mismatched grafts where CD4+ T

cells are the major mediators of rejection is consistent with this possibility (72).

The failure of CD154-deficient OT-II cells to accumulate in the proximal coronary

arteries is a likely explanation for the lack of CAV in Act-mOVA grafts. The poor

accumulation of CD154-deficient OT-II cells in the proximal coronary arteries of

Act-mOVA grafts correlated with an expansion defect in the spleen. This finding

is consistent with other studies showing that CD154 expression is required for

CD4+ T cells to proliferate maximally in response to antigenic stimulation in the

secondary lymphoid organs (30, 74). CD154 may be important in this regard as a

stimulus for the induction of CD28 ligands on CD40+ antigen-presenting cells

(75), which are critical for maximal T cell production of the growth factor IL-2 (76).

It is also possible that CD154 expression is required for CD4+ T cells to activate

CD40+ cells once both populations migrate to the intimal layer. The co-

localization of graft antigen-specific CD4+ T cells, with myeloid cells and dendritic

cells in the thickened intimal layers of Act-mOVA grafts is consistent with this

possibility. The CD11c+ dendritic cells, which were probably derived from the

recipient (20, 77), or the CD11b+, Gr-1- macrophages may have been presenting

OVA peptide-MHC II complexes to the CD4+ T cells. This presentation may in

turn have activated the CD4+ T cells to express CD154, which could then bind to

CD40 on the antigen-presenting cells, stimulating them to produce profibrotic

cytokines. Although this scenario could also apply to the CD11b+, Gr-1+ cells,

which were probably immature monocytes (63), it is less likely because such

cells do not express MHC II molecules (78). However, it is still possible that

CD4+ T cells express CD154 in response to antigen presentation in the intima,

and then use it to stimulate bystander monocytes. Resolution of this issue will

required targeted elimination of the various CD40+ cells within the intima.

CAV if mediated by graft antigen-specific CD4+ T cells of the T_H1 subtype but IFN-γ expression is not required

Accumulating evidence from studies of human and animal heart grafts suggests

that IFN-γ is involved in the development of intimal hyperplasia(79). For example,

it has been reported that IFN-γ is sufficient to cause CAV in the absence of other

leukocytes (40). Since we had shown that graft antigen-specific CD4+ T cells

also have this capability it was reasonable to hypothesize that IFN-γ production

by graft antigen-specific CD4+ T cells is necessary for intimal hyperplasia.

Consistent with this model, we found that naïve, minor graft antigen-specific

CD4+ T cells differentiate naturally to the T_H1 subset in vivo and migrate to the

heart graft. In addition, we found that CAV in minor antigen disparate grafts from

recipients that lacked STAT-4 or IFN-γ was slightly reduced in severity,

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suggesting that IFN-γ may contribute to CAV in this model. IFN-γ may cause CAV

by induction of iNOS in inflammatory monocytes or in bystander T cells(80, 81).

Alternatively, IFN-γ may act directly to induce vascular smooth muscle cell

proliferation through the mammalian mTOR/raptor complex signaling pathway

(82). IFN-γ may also act directly on vascular smooth muscle cells to upregulate

Fas trafficking to the cell surface and potentiate apoptosis, initiating the abnormal

proliferation of fibroblasts observed in CAV (83). Whether IFN-γ effects on

vascular smooth muscle cells are direct or indirect, and whether a single,

predominant pathway or multiple redundant pathways are required, has not been

established.

Although IFN-γ may contribute, the development of CAV in OVA-expressing

grafts despite the lack of IFN-γ production by OT-II cells suggests that minor graft

antigen-specific CD4+ T cells can cause CAV through an IFN-γ-independent

mechanism. The finding that T_H17 CD4+ T cells were able to cause CAV raises

the possibility that a common cytokine produced by both T_H1 and T_H17 CD4+ T

cells may cause CAV. Although TNF-α was produced by both T_H1 and T_H17

CD4+ T cells (data not shown), it was also produced by T_H2 CD4+ T cells that did

not cause CAV, consistent with previous data suggesting that it is not likely the

causative cytokine (84). In addition, our finding that even highly skewed OT-II

cells produce a small amount of IFN-γ upon antigen re-stimulation leaves open

the possibility that CAV in recipients transferred with T_H17 OT-II cells was

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actually caused by a contaminating population of T_H1 cells. On the other hand, a

small population of contaminating T_H1 OT-II cells in recipients transferred with

T_H2 OT-II cells was not sufficient to cause CAV, again favoring a common

mechanism shared by T_H1 and T_H17 CD4+ T cells. Also, the fact that highly

skewed T_H1 CD4+ T cells did not produce IL-17 and still caused CAV suggests

that IL-17 is not the causative cytokine in CAV. Future studies directed at

characterization of other cytokines and effector molecules of graft antigen-

specific T_H1 CD4+ T cells will be required to elucidate the mechanism of IFN-γ

independent CD4+ T cell mediated CAV.

CD4+ T cells initiate an intimal hyperplasia phase of CAV that does not require ongoing immune activation

Although elimination of the immune response prevents CAV and despite

improved immunosupression, CAV continues to plague the long-term function of

cardiac allografts. This may be due to the fact that a single episode of rejection

may lead to CAV. Therefore, sustained or more intense immunosuppression

alone will not be sufficient to halt CAV once an episode of rejection has occurred.

In order to determine whether CAV is a manifestation of a single defined

immune-mediated injury, such as an episode of acute rejection, or whether

ongoing lymphocyte activation or multiple rejection episodes are required, we

developed a model of CAV allowing the graft antigen-specific CD4+ T cells

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causing the rejection to be eliminated, leaving no other lymphocytes. We found that CAV developed and progressed despite the absence of the graft antigen-specific CD4+ T cells that had undergone activation and clonal expansion. This finding suggests that once the graft antigen-specific CD4+ T cells injure the graft, their presence is no longer required for the propagation of a response to injury involving proliferation of intimal cells.

The intimal hyperplasia phase of CAV initiated by CD4+ T cell injury is mTOR dependant

Rapamycin has proven to slow the progression of CAV, but the exact mechanism of this inhibition is not known (70, 85). Expression of extracellular matrix proteins associated with fibrosis and intimal hyperplasia is diminished after treatment with rapamycin in a rat aortic allograft model of intimal hyperplasia (48). However, rapamycin impacts many different proliferating cell types through the mTOR pathway. Therefore, in this model effects on both immune cells such as lymphocytes and non-immune cells may lead to less fibrosis and intimal hypeplasia (46, 47). We have defined two phases of CAV, the CD4+ T cell-mediated injury phase, and the intimal hyperplasia phase that progresses completely independent of any subsequent lymphocyte-mediated injury. This allowed us to determine what signaling pathways might be critical to the intimal hyperplasia phase separate from the immune response. We found that mTOR

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activation is required for the intimal hyperplasia phase of CAV. This implies that once CD4+ T cell mediated rejection has occurred, proliferation signal inhibitors must be used to prevent progression of CAV. Further definition of the cell types participating in this phase of CAV may provide additional targets for discrete inhibition of the response to injury in order to salvage immune injured grafts and provide additional potential targets separate from additional immunosuppression.

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Appendices-List of Figures
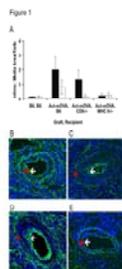
Figure 1: Intimal hyperplasia in Act-mOVA versus B6 heart grafts from CD8+ T cell-deficient recipients but not CD4+ T cell-deficient recipients.

Normal B6 recipients (C, n=3), CD8-/- (CD8+ T cell-deficient) recipients (D, n=3), or MHC II -/- (CD4+ T cell-deficient) recipients (E, n=3) were grafted with Act-mOVA heterotopic heart grafts. Normal B6 recipients were grafted with B6 heterotopic heart grafts as negative controls (B, n=2). Animals were sacrificed at 50 days and sections from the proximal and distal coronary arteries were stained with anti-elastin (green) and Sytox nuclear stain (blue). The area of the intima (defined as the space between the lumen (white arrow) and the internal elastic lamina (red arrow)) and the media were measured and the ratio of the intima to the media was determined in proximal (■) and distal (□) coronary arteries (A) on 20x images. Error bars represent the standard deviation.
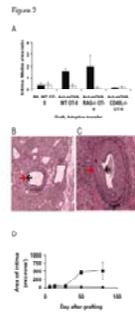
Figure 2: OT-II cells require CD154 expression to cause intimal hyperplasia in the proximal coronary arteries of Act-mOVA grafts. B6 RAG-deficient recipients were injected intravenously with $2x10^6$ purified wild-type OT-II cells and received either a B6 (B, n=4) or an Act-mOVA (C, n=4) heart graft, or they were injected intravenously with $5x10^5$ RAG-deficient OT-II (n=3) or CD154-deficient OT-II cells (n=4) and received Act-mOVA heart grafts. Grafts were harvested at 80 days and axial sections were cut through the base (proximal coronary arteries) and apex (distal coronary arteries) and stained with anti-mouse tropoelastin and Sytox as in Figure 1 or Verhoeff's Elastin (B, C, 10x). The intima:media area ratio (A) was determined in proximal (■) and distal (□) coronary arteries from elastin stained sections as by identifying the internal elastic lamina (red arrow) and lumen (black arrow) as described in Figure 1. In addition, the area of the intima was measured in 1-2 grafts from B6 (λ) and Act-mOVA (ν) grafts from recipients reconstituted with purified OT-II cells at various time points up to 80 days. Error bars represent the standard deviation.

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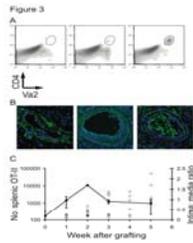
Figure 3. Graft antigen-specific CD4+ T cell kinetics after heterotopic mouse heart transplant with a single minor antigen-disparate graft. Wild-type B6 recipients underwent adoptive transfer with one thousand naïve, CD90.1+ OT-II cells and then underwent heterotopic mouse heart transplant with Act-moVA hart grafts. Recipients were sacrificed at weekly intervals (2-3 per time point) and spleens were harvested to determine the total number of splenic OT-II cells (A and C, solid circles and line) by flow cytometry after staining for CD90.1, CD4, and Vα2 (left panel-no transfer, middle panel-transfer only, right panel-2 weeks after transfer and grafting). The development of CAV was quantified by determining the ratio of the area of the intima to the media (open circles) from immunohistology on axial heart sections stained for elastin (green) and nuclei (blue).
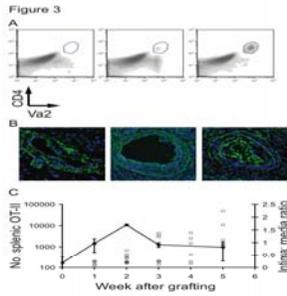
Figure 3

Figure 4: Activation of OT-II cells by Act-mOVA but not B6 grafts after heterotopic mouse heart transplant requires CD154 expression. B6 RAG-deficient mice were injected intravenously with 2×10^6 purified OT-II cells (λ, ν) or 2×10^6 CD154-deficient OT-II cells (σ) and received either a B6 (λ) or an Act-mOVA (ν, σ) heart graft. One-4 animals were sacrificed at each time point and OT-II cells were identified in the spleens of graft recipients (A) by staining with PerCP-labeled anti-CD90.1 and PE-labeled anti-CD4 antibodies. Similarly stained spleen cells from a B6 RAG-deficient mouse that did not receive OT-II cells are shown as a negative control (B). In some experiments, staining was performed with FITC-labeled anti-Vα2 antibodies, in addition to PerCP-labeled anti-CD90.1, for identification of OT-II cells with similar results. The absolute number of OT-II cells in the spleen for each sample from two separate experiments is shown in (C). Error bars represent the standard deviation.

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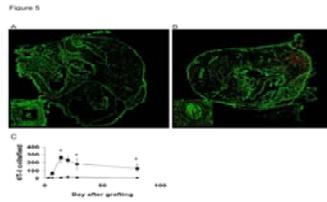
Figure 5: OT-II cell infiltration of Act-mOVA grafts. Sections through day 80

Act-mOVA (A) or B6 (B) grafts from B6 RAG-deficient recipients injected

intravenously with $2x10^6$ wild-type OT-II cells were stained with anti-elastin

(green) and anti-CD90.1 (red). Images (10x) covering each entire section and

were obtained and assembled into single composite images (A, B), in which the

size of the red CD90.1+ cells was expanded 3-fold so as to be visible at this low

magnification. Higher power views of the proximal coronary arteries in the

composite images (white boxes) are shown in the lower left corner or each image

with the red CD90.1+ cells shown at their normal size. The internal elastic lamina

is identified by a red arrow and lumen by a white arrow. The number of CD90.1+

cells in the higher power fields containing the proximal coronary arteries (20x,

insets in A and B) from B6 (λ) or Act-mOVA (ν) grafts are shown in (C) along with

values derived from similarly stained sections (data not shown) of Act-mOVA ( )

heart grafts from RAG-deficient B6 recipients of $2x10^6$ CD154-deficient OT-II

cells. The values with asterisks were significantly greater (P<0.05, one-tailed

Student's t-test with unequal variance) than the values from B6 heart graft

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recipients at the same time point. Error bars represent the standard error of the

mean.
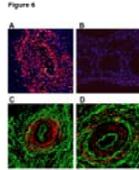
Figure 6: Infiltration of B6 and Act-mOVA grafts by CD11b+ cells,

CD11b+/Gr-1+ cells, CD40+ cells, and CD11c+ cells. Axial sections through

the base of day 80 Act-mOVA (A, C, D) or B6 (B) grafts were stained with

antibodies specific for Gr-1(A, B), CD11b (A, B), CD11c (C) and CD40 (D).

Images (20x) around coronary arteries are shown.
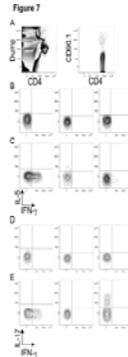
Figure 7. Graft antigen-specific CD4+ T cells can be skewed to the T$_H$1, T$_H$2, or T$_H$17 subset in vitro. OT-II CD4+ T cells were skewed in vitro to the T$_H$1 (left panels B-E), T$_H$2 (middle panels B-E), or T$_H$17 (right panels B-E) subset and then 5-10 x 10^5 T$_H$ skewed OT-II CD4+ T cells were adoptively transferred into B6 RAG-deficient recipients that then received a B6 Act-mOVA heterotopic heart transplant. After 30 days, animals either received 100 μg OVA peptide by intravenous injection 2 hours before harvesting the splenocytes (C and E, N=3-4 per group) or no peptide (B and D, N=2-3 per group). Splenic OT-II CD4+ T cells were identified by gating on dump negative, CD4+, CD90.1+ events (A). Intracellular staining was performed for IL-5 (B and C), IFN-γ (B-E), and IL-17 (D and E). Data are from 2 pooled experiments.
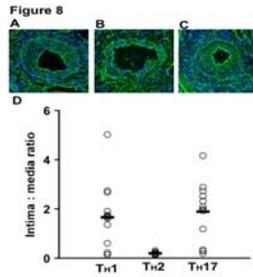
Figure 8. Graft antigen-specific CD4+ T_H1 and T_H17, but not T_H2 cells can cause CAV in the absence of other lymphocytes. B6 RAG-deficient recipients received a B6 Act-mOVA heterotopic mouse heart transplant after adoptive transfer of 5-10 x 10^5 T_H1 (A, N=5), T_H2 (B, N=3), or T_H17 (C, N=5) skewed OT-II CD4+ T cells. Grafts were harvested on day 30 for immunohistology for elastin (green) and nuclei (blue). The intima: media ratio (open circles) was determined from 1-2 measurements of each coronary artery from each graft (D) and the mean for all measurements in each group is shown (solid bars).
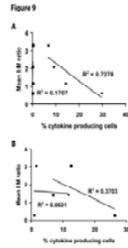
Figure 9. Neither T$_H$1, nor T$_H$17 specific cytokine production by graft antigen-specific CD4+ T cells correlates with the development of CAV. After determining cytokine production of OT-II cells as described in Figure 1 and the intima to media ratio as described in Figure 2, the mean intima to media (I:M) ratio measured for each Act-mOVA heart was plotted against the percentage of IFN-γ ( ) or IL-17( ) produced by the OT-II cells from that specific recipient after restimulation with OVA peptide as described in Figure 1.
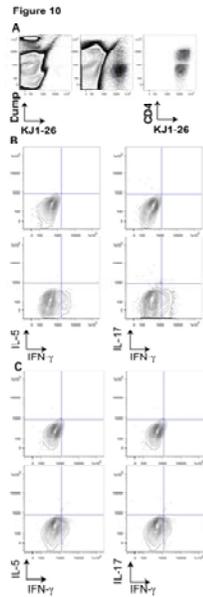
Figure 10. Naïve graft antigen specific CD4+ T cells differentiate to the T_H1 subset and migrate to the heart graft. RAG-deficient BALB/c recipients were adoptively transferred with 5 x 10^5 DO11.10 CD4+ T cells and then received a BALB/c Act-mOVA heart graft. After 14 days, recipients either received no injection (upper panels B, C) or 100 μg OVA peptide (lower panels B, C) by intravenous injection. After two hours, spleens and grafts were harvested and DO11.10 cells identified in transferred (A, right 2 panels) but not untransferred (A, left panel) recipients. Intracellular staining was performed for IL-5, IFN-γ, and IL-17 in the spleen (B) and heart (C). Data are representative of 3 experiments with 1-3 animals per group.
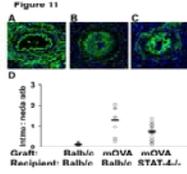
Figure 11. STAT-4 is not required for the development of CAV in Act-mOVA heart grafts. Balb/c (A) or Balb/c Act-mOVA grafts (B, C) were transplanted into either wild type (A, B, N=3) or STAT-4-deficient (C, N=3) Balb/c recipients and grafts were harvested on day 30 for immunhistology for elastin (green) and nuclei (blue) in order to determine the intima: media ratio (D). The mean (solid bars) for each group was determined from individual measurements of each coronary artery (open circles).

Figure 12. IFN-γ production is not required for the development of CAV in Act-mOVA heart grafts. B6 (A, N=3) or B6 Act-mOVA grafts (B, C, N=4 each) were transplanted into either wild type (A, B) or IFN-γ-deficient (C) B6 recipients and grafts were harvested on day 30 for immunhistology for elastin (green) and nuclei (blue) in order to determine the intima: media ratio (D). The mean (solid bars) for each group was determined from individual measurements of each coronary artery (open circles).
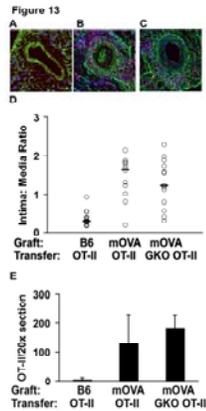
Figure 13. IFN-γ production by graft antigen specific CD4+ T cells is not required for the development of CAV in Act-mOVA heart grafts. B6 RAG-deficient recipients were adoptively transferred with either purified, wild type OT-II CD4+ T cells (A, B) or IFN-γ-deficient OT-II CD4+ T cells and then received either a wild type B6 graft (A) or a B6 Act-mOVA graft (B, C). Grafts were harvested at 65 days and immunohistology was performed for elastin (green), CD90.1 (red) and nuclei (blue) in order to determine the intima: media ratio (D) and the number of perivascular OT-II cells (E). The mean (solid bars) for each group was determined from individual measurements of each coronary artery (open circles). N=3-4 animals per group.

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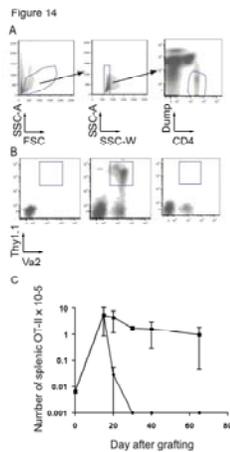
Figure 14. Elimination of splenic graft-antigen specific CD4+ T cells after clonal expansion. RAG-deficient recipients underwent adoptive transfer with 2 x 10^6 CD90.1+ OT-II cells followed by grafting with Act-mOVA hearts. One cohort of mice underwent treatment with anti-CD90.1+ mAb IgG2a (1A14) 400μg i.p. weekly starting on day 15 after grafting (C,) while the other group underwent no treatment (C,). Animals (3-5 per time point for each group) were sacrificed at regular intervals and spleens were harvested for flow cytometry after staining with CD90.1, CD4, and Vα2 in order to determine the total number of splenic OT-II cells. The gating strategy is shown (A), as well as representative samples from an untransferred mouse (B, right panel), a transferred and grafted mouse (B, middle panel), and transferred and grafted recipient after treatment with anti-CD90.1 mAb.

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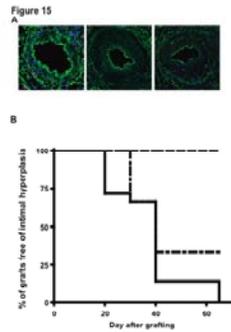
Figure 15. Progression of CAV after CD4+ T cell mediated injury. RAG-deficient recipients underwent adoptive transfer with 1 x 106 CD90.1+ OT-II and after grafting with Act-mOVA hearts one group was treated with anti-CD90.1 mAb on day 2 (Left panel A and B, ––), one group was treated with anti-CD90.1 mAb on day 15 at the height of OT-II clonal expansion (Middle panel A and B, — – —) and another group was not treated (Right panel, A and B, —). Mice were sacrificed at regular intervals and heart grafts (3-5 per time point) were examined by immunohistology for the presence of CAV as determined by the intima: media ratio.
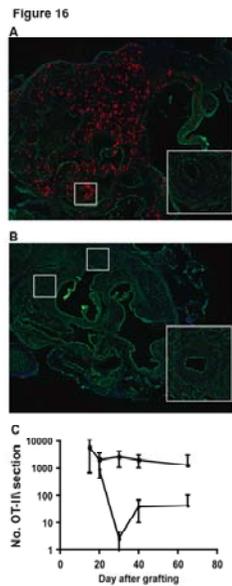
Figure 16. Intragraft antigen-specific CD4+ T cells. RAG-deficient recipients underwent adoptive transfer with 2 x 10^6 CD90.1+ OT-II and after grafting with Act-mOVA hearts one group was treated with anti-CD90.1 (B, C) and while the control group was not treated (A, C). Immunohistology for elastin (green), CD90.1 (red), and nuclei (blue) was performed and the number of OT-II was calculated in each axial section (3-5 mice per time point).

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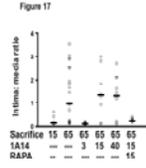
Figure 17. CD4+ T cells are not required for the mTOR-dependant intimal hyperplasia phase of CAV. RAG-deficient recipients underwent adoptive transfer with 2 x 10^6 CD90.1+ OT-II and after grafting were sacrificed at 15 or 65 days (left 2 columns) or treated with anti-CD90.1 mAb starting before clonal expansion (3 days), at the peak of clonal expansion (15 days) or after OT-II conraction and the onset of CAV (40 days). One cohort was treated with rapamycin starting at day 15 in addition to anti-CD90.1 depleting mAb.

Appendices-List of Tables

Table 1

T$_H$ skewing	%IL-5+	%IL-17+	%IFN-g+	Mean I:M ratio
1	0	0	9.09	2.1
1	0.65	0.14	14.2	1.21
1	0	0.079	29.7	0.63
1	0.045	0.2	6.74	3.35
2	2.92	0	0.81	0.16
2	4.07	0	0.58	0.22
2	2.6	2.17	19.5	0.76
2	10.5	0.53	14	0.32
17	0.34	12.5	1.52	3.07
17	1.52	26.3	0.89	0.29
17	0.64	6.87	11.7	1.42

Table 1 Cytokine production by graft antigen specific CD4+ T$_H$1, T$_H$2 and T$_H$17 after in vitro skewing and restimulation in vivo.