Attenuated Cardiac Allograft Vasculopathy in Mice With Targeted Deletion of the Transcription Factor STAT4

Jörg Koglin, MD; Troels Glysing-Jensen, BS; Silpa Gadiraju; Mary E. Russell, MD

Background—To study transcription factor signaling pathways that mediate cardiac allograft vasculopathy, we used mice with targeted gene deletion of signal transducer and activator of transcription (STAT)4 and STAT6 as recipients in our mouse cardiac transplant model of chronic rejection.

Methods and Results—At day 55 after transplantation, cardiac grafts placed into STAT4 −/− (n=10) had reduced frequency (24±2%) and severity (9±4%) of vascular occlusion compared with wild-type controls (n=7, frequency 70±12% [P<0.001], severity 25±6% [P<0.05]). This decrease was associated with reduced intragraft expression (90P RT-PCR and immunohistochemistry) of the Th1 signature cytokines interferon-γ (P<0.001) and interleukin (IL)-2 (P<0.001). Furthermore, cardiac grafts in STAT4 −/− had fewer infiltrating CD45+ mononuclear cells (99±27 cells/mm³ compared with 551±168 cells/mm³ in wild-type controls [P<0.05]) and reduced expression of P-selectin (P<0.001) and E-selectin (P<0.001) ligand, recently shown to regulate Th1 cell recruitment. In contrast, in grafts placed into STAT6 −/− (n=11), the development of cardiac allograft vasculopathy (frequency 62±8%, severity 28±6%) and Th2 cytokine profiles (IL-4, IL-10) were comparable to those in wild-type controls.

Conclusions—Hence, we show that immune responses mediated by STAT4, but not STAT6, contribute to the development of cardiac allograft vasculopathy. We speculate that when present, STAT4-mediated signaling pathways may promote cardiac allograft vasculopathy by directing Th1-specific lymphocyte recruitment, activation, and effector functions.

Key Words: lymphocytes ■ transplantation ■ immune system ■ cytokines ■ cell adhesion molecules

A fter solid organ transplantation, chronic rejection with the development of cardiac allograft vasculopathy is the major impediment to long-term graft survival. Experimental models have helped us to begin to understand the pathogenesis of this form of vascular occlusion. The regulation of the pathogenesis has been hypothesized as a coordinate and interactive event involving various inflammatory cell types. In addition to B lymphocytes and macrophages, T lymphocytes have been identified to play a crucial role.1–3 On activation, T lymphocytes differentiate into Th1 and Th2 subsets, characterized by differences in their cytokine repertoire,4,5 adhesion mechanisms,6,7 effector function profile, and character of local immune response.5,5 Transcription factors have been identified that regulate these Th1 and Th2 cell differentiation programs in a proximal manner. The signal transducer and activator of transcription (STAT) protein family includes STAT4, which controls interleukin (IL)-12–mediated Th1 differentiation.8,9 Mice with targeted deletion of STAT4 signaling show impaired differentiation of Th1 cells in response to either IL-12 or Listeria monocytogenes,8,9 whereas targeted deletion of STAT6 interrupts Th2 differentiation.10–12 Lymphocytes from STAT6-knockout mice have defective IL-4–mediated Th2 differentiation and proliferation.10–12 These mice provide the opportunity to examine the role of transcription factor pathways regulating the Th1 or Th2 pathway.

To study the impact of these transcription factor programs on graft vasculopathy, we used STAT4− and STAT6− knockout mice as recipients in our heterotopic cardiac transplant model of chronic rejection.2,13,14 We assessed the effect of recipient STAT4 or STAT6 deficiency on intragraft cytokine profiles, arteriosclerotic lesion development, Th1/Th2-specific cytoadhesion signaling, and leukocyte recruitment.

Methods

Mice

As heart donors, inbred male CBA/CaJ (H-2k) mice 6 to 8 weeks old were used. As recipients, male C57BL/6J×129Sv (H-2b) mice with targeted deletion of STAT4 (STAT4 −/−, n=10) or STAT6 (STAT6 −/−, n=11) were compared with C57BL/6J×129Sv wild-type controls (n=7, hereafter referred to as wild-type controls). The genotype of the knockout mice was confirmed by polymerase chain reaction (PCR) using genomic mouse-tail DNA. The knockout mice were all backcrossed 6 times to C57BL/6J (F6). F2 hybrids
were used as physiological wild-type controls. Because these F2 mice provide only an approximate genetic match, we had to confirm that potential differences were not due to a more dominant 129Sv influence in this group. Therefore, inbred wild-type mice on a pure C57BL/6J background (n=8, hereafter referred to as inbred controls) were used as a second comparative control group. STAT4- and STAT6-deficient mice were generously supplied by Dr James N. Ihle (St Jude Children’s Research Hospital, Memphis, Tenn). CBA/Caj (stock No. 000654), C57BL/6J×129 Sv (stock No. 101045), and C57BL/6J mice (stock No. 000664) were obtained from Jackson Laboratory (Bar Harbor, Maine).

Heterotopic Mouse Transplant Model of Chronic Rejection

Vascularized, heterotopic abdominal transplantation of the donor heart was performed, monitored, and harvested at day 55 as previously described.2,13–16 Immunosuppressive therapy to prevent acute parenchymal rejection and produce grafts undergoing chronic rejection consisted of anti-CD4 and anti-CD8 (GK1.5 and 2.43, 2 mg IP on days 1 to 4 and then weekly to day 28).2,13,14

Cytokine Expression

Relative gene transcript levels were measured by reverse transcription (RT)-PCR from graft cDNA panels as published previously.2,13–16 In this model, in which the small size of allograft tissue is limiting, 32P RT-PCR offers the advantage of studying a large number of factors in replicate in a semiquantitative manner. PCR primers were designed with MacVector 5.0 (Oxford Molecular Scientific), and specificity of the primer sequence was confirmed by basic local alignment search tool (BLAST) analysis. For each individual primer pair, specific annealing temperature and cycle number were optimized by serial annealing studies, PCR cycle studies, and cDNA dilution studies. The logarithmic ranges of amplification were established as previously described to ensure that the amplified PCR product reflects the original levels of transcription. Primer sequences, sequence accession numbers, optimized annealing temperatures, and cycle numbers are listed in the Table.

Triplicate samples were amplified with AmpliTaq Gold DNA polymerase (Perkin-Elmer). [32P]dCTP (150 000 cpm per reaction) was included to quantify the amplified product. Incorporated [32P]dCTP in amplified products from dried agarose gels was used for determining the amplified product value by mean that value for the control gene G3PDH in the respective sample and reported as mean±SEM for all grafts in each recipient group.

To confirm the presence of cytokine gene products shown to be regulated at the transcript level, we performed immunostaining in frozen sections from 4 representative grafts from each recipient group as published previously.15 The primary antibodies used were anti–interferon (IFN)-γ (clone R4-6A2, 1:100, Pharmingen), anti–IL-2 (clone S4B6, 1:100, Pharmingen), and anti–IL-10 (clone JES5-12d7, 1:100, Pharmingen).

Morphometric Vessel Analysis

Vascular analysis was performed in paraffin sections stained with Verhoeff’s elastin as previously described.14 Microscopic images of all elastin-positive vessel cross sections were used to trace the lumen and the internal elastic lamina (NIH Image 1.6) and derive the percentage of luminal occlusion.14 Values were calculated as the mean from all captured vessels per heart and reported as mean±SEM for all grafts in each recipient group.

Leukocyte Infiltration

Immunostaining for the leukocyte common antigen CD45 (clone 30F11.1, 1:1000, 1 hour at 37°C, Pharmingen) was performed in paraffin sections from 5 representative grafts in each group. Microscopic images over the entire sagittal cross section of all grafts were captured with NIH Image 1.6 (NIH). The number of CD45-positive cells/mm² was counted and reported as mean±SEM for all grafts in each group.

Statistics

For comparisons between the average measurements of 2 groups, unpaired t tests were used. For comparison between >2 groups, ANOVA and the Bonferroni-Dunn procedure were used for post hoc testing. A probability value <0.05 was considered significant.

Results

Intragraft Cytokine Activation

Cardiac grafts placed into STAT4 −/− recipients had reduced expression of Th1 cytokines. As shown in Figure 1A, corrected transcript levels for IFN-γ (0.03±0.01 relative units [RU]) and IL-2 (0.06±0.01 RU) were significantly

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**T-Cell Differentiation and Graft Vasculopathy**

**Table 1: Primer Sequences, Sequence Accession Numbers, Annealing Temperatures, and Cycle Numbers**

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As shown in Figure 2, grafts in STAT4−/− recipients show significantly reduced transcript levels for Th1 cytokines IFN-γ and IL-2 compared with those from wild-type controls (A). 32P RT-PCR amplification was normalized against G3PDH and is presented as mean corrected transcript levels ± SEM in RU of triplicate analysis of all grafts in each group. Representative photomicrographs show IFN-γ (B) and IL-2 staining (C) in mononuclear cells infiltrating perivascular and myocardial spaces. Photomicrography is of representative sections in allografts from wild-type recipients (original magnification ×200).

lower than those in grafts from wild-type control recipients (IFN-γ 0.50±0.12 RU, P<0.001; IL-2 0.60±0.11 RU, P<0.001). In grafts from wild-type control recipients, IFN-γ antigen typically localized within the thin cytoplasmic rim of mononuclear cells (Figure 1B). These positive cells were diffusely distributed throughout the perivascular spaces and the myocardium. In contrast, grafts from STAT4−/− had occasional but rare IFN-γ-positive cells. IL-2 antigen also localized to mononuclear cells (Figure 1C). IL-2 was expressed in disseminated clusters of mononuclear cells throughout the entire graft cross section. In contrast, grafts from STAT4−/− only had far fewer IL-2-positive mononuclear cells. The deficiency in Th1 cytokines was associated with modest but significant reductions in the Th2 cytokines IL-4 (0.02±0.01 RU, P<0.05) and IL-10 (0.10±0.02 RU, P<0.05).

In contrast, grafts placed into STAT6−/− recipients had expression levels of Th2 cytokines comparable to those of wild-type controls. Corrected transcript levels for IL-4 were expressed at baseline levels in both groups (STAT6−/− 0.09±0.02 RU, controls 0.07±0.02 RU). IL-10 transcript levels were unchanged in grafts from STAT6−/− (0.35±0.07 RU) compared with control recipients (0.29±0.07 RU). The presence of the IL-10 gene product was confirmed by immunostaining in grafts from both groups. In both STAT6−/− and wild-type control recipients, IL-10 antigen was present in the thin cytoplasmatic rim of mononuclear cells, which were distributed throughout the perivascular myocardium (data not shown). Transcript levels for the Th1 cytokines IFN-γ (0.42±0.16 RU) and IL-2 (0.57±0.11 RU) were not significantly changed.

Hence, transplantation into STAT4-deficient recipients ablated the Th1 response compared with wild-type controls, whereas STAT6-deficient recipients had a profile of signature cytokines resembling that of wild-type controls.

**Graft Vasculopathy**

As shown in Figure 2, grafts in STAT4−/− recipients had less prominent vascular thickening than those in wild-type controls. Grafts from wild-type controls and STAT6-deficient recipients had circumferential thickening of the vessels with perivascular mononuclear cell infiltration. The expanded neointima was composed of cells with features of vascular smooth muscle cells and mononuclear cells. In marked contrast, grafts from STAT4-deficient recipients had only a small amount of neointimal formation, with markedly less perivascular mononuclear cell infiltration. Because of the small amount of neointimal formation in the majority of the vessels, the composition of the expanded neointima was difficult to discern.

Quantitative analysis of all elastin-positive vessels showed that grafts placed into STAT4−/− recipients had significant reductions in frequency (24±2% of all vessels [n=113] affected) and severity (9±4% mean luminal occlusion) of cardiac allograft vasculopathy compared with those placed into wild-type controls (70±12% of all vessels [n=56], 25±6% mean luminal occlusion). In contrast, in grafts placed into STAT6−/− recipients, the development of cardiac allograft vasculopathy was comparable to that in wild-type controls (62±8% of all vessels affected, with a mean luminal occlusion of 28±6%). Frequency and severity of allograft vasculopathy were comparable in grafts placed into C57BL/6J×129Sv wild-type controls and those placed into C57BL/6J inbred controls (61±10% of all vessels [n=101], 29±7% mean luminal occlusion). This clearly argues that the significant reduction in vascular occlusion in grafts from STAT4−/− recipients can be attributed to the deletion of the transcription factors rather than potential differences in the genetic background. Taken together, STAT4, but not STAT6, plays an essential role in the development of cardiac allograft vasculopathy.
Mononuclear Cell Infiltration

Grafts in STAT4-deficient recipients had fewer infiltrating CD45+ mononuclear cells, as shown in the representative photographs in Figures 3A and 3C. Analysis of 410 high-power fields showed that grafts placed into STAT4 −/− recipients had significantly lower numbers of graft-infiltrating CD45-positive cells (99 ± 27 cells/mm²) compared with wild-type controls (551 ± 68 cells/mm², P < 0.05; Figure 3B).

For quantification of CD4, CD8, and Mac-1 expression to estimate the contribution of CD4- and CD8-positive lymphocytes and macrophages, we elected to measure corrected transcript levels because the resolution of these cell surface markers after immunostaining of frozen sections is not sufficient to perform quantification. Grafts placed into STAT4 −/− recipients had significantly lower transcript levels for CD4 (0.24 ± 0.04 RU) and CD8 (0.10 ± 0.04 RU) than those placed into wild-type controls (CD4 0.85 ± 0.12 RU, P < 0.001; CD8 0.68 ± 0.24 RU, P < 0.05). There was no significant difference in corrected transcript levels for Mac-1 (STAT4 −/− 0.35 ± 0.02 RU, wild-type controls 0.49 ± 0.09 RU). Taken together, there is reduced mononuclear cell infiltration in grafts from STAT4-deficient recipients, which may be related, in part, to differential recruitment of CD4- and CD8-positive lymphocytes.

Cytoadhesion Pattern

Because recruitment of Th1 lymphocytes has been shown to be regulated by P- and E-selectin cytoadhesion pathways, we measured intragraft transcript levels (Figure 4). Corrected transcript levels for P- and E-selectin, expressed by the donor-derived endothelium, were comparable in grafts from STAT4 −/− (P-selectin 0.37 ± 0.02 RU, E-selectin 0.20 ± 0.05 RU) and wild-type control recipients (P-selectin 0.35 ± 0.04 RU, E-selectin 0.33 ± 0.03 RU). In contrast, transcript levels for P- and E-selectin glycoprotein ligands (PSGL and ESGL), expressed by recipient-derived lymphocytes, were significantly lower in grafts placed into STAT4 −/− recipients (PSGL 0.14 ± 0.02 RU, ESGL 0.30 ± 0.03 RU) than in those placed into wild-type controls (PSGL 0.62 ± 0.11 RU, P < 0.001; ESGL 0.71 ± 0.17 RU, P < 0.01). Hence, STAT4-deficient recipients had reduced expression of glycoprotein ligands, which are responsible for selective tissue recruitment of Th1 cells.

Discussion

Here, we show that immune responses mediated by STAT4, but not STAT6, contribute to the development of cardiac allograft vasculopathy. Cardiac allografts placed into immunosuppressed recipient mice with targeted deletion of STAT4 had marked reductions in frequency and severity of vascular occlusion. The decrease in intimal thickening in the STAT4-deficient microenvironment was associated with low intragraft expression of Th1 signature cytokines (IFN-γ and IL-2), reduced expression of Th1-specific cytoadhesion markers (PSGL and ESGL), and decreased infiltration of CD45-positive cells within the graft. Hence, when present, STAT4-mediated pathways contribute to the alloimmune response that culminates in cardiac allograft vasculopathy by directing Th1-specific effector functions.

STAT4-Mediated Th1 Forces Promote Cardiac Allograft Vasculopathy

This study is the first to exploit mice with targeted deletion of STAT4 to characterize the role of Th1 cytokine programs in vivo. As opposed to single cytokine knockouts used in the past, this approach offers the advantage of presumably disrupting an entire differentiation program, resulting in deficiencies of multiple cytokines and effector pathways. In these mice, our results indicate that Th1-type immune activation may be an essential driving force in the development of chronic rejection.

These findings complement previous studies showing reduced vascular occlusion if mice with targeted deletion of the Th1 cytokine IFN-γ are used as recipients in immunosuppressed models producing chronic graft survival. However, depending on the immunosuppressive program, recipient IFN-γ deficiency may also accelerate late forms of parenchymal rejection, which supports the argument that IFN-γ may be protective. For the Th2 cytokine IL-4, targeted deletion of recipient sources did not alter survival times or
development of vascular thickening in mouse transplant models with various immunosuppressive programs.\textsuperscript{15,16,20}

Recently, migration studies have shown that Th1 cells are selectively recruited into inflamed sites in Th1- but not in Th2-dominated disease models by distinct cytoadhesion pathways.\textsuperscript{6,21} Only Th1 cells accumulated in sites of a delayed-type hypersensitivity reaction of the skin or arthritic joints. Th1 cell migration was blocked by antibodies against P- and E-selectin. Hence, STAT4-mediated pathways, when present, might upregulate surface expression of ESGL and PSGL (and potentially other adhesion mechanisms), thereby controlling infiltration of activated Th1 cells. In response to alloimmune stimulation, these graft-infiltrating cells would express Th1-type cytokines, initiating downstream effects promoting arteriosclerotic thickening. IFN-\(\gamma\), for example, has been shown to promote cardiac allograft vasculopathy through increased expansion of vascular smooth muscle cells within the neointima.\textsuperscript{13} Alternatively, STAT4-mediated expression of Th1 cytokines might also amplify the arteriosclerotic cascade by inducing expression of ESGL and PSGL in an autocrine fashion. Studies in IFN-\(\gamma\)-knockout recipients have shown reduced expression of leukocyte adhesion molecules associated with attenuated lesion development.\textsuperscript{18} In the present study design, we were not able to determine whether decreased PSGL and ESGL expression causes the reduced Th1 cell infiltration or whether it just reflects the reduction in glycoprotein-bearing Th1 cells. At this point, the transcriptional control of the expression of the respective glycoprotein ligands in T cells has not yet been studied. Future studies will help to dissect the interrelated roles of cytokines and cytoadhesion molecules contributing to Th1-mediated effector functions.

Cardiac Allograft Vasculopathy Develops Independently of STAT6

Evidence that STAT6 is essential for Th2 responses has been established in Th2-dominated disease models. Lymphocytes from STAT6-deficient mice infected with \textit{Nippostrongylus brasiliensis} produced profoundly reduced IgE and IgG1 responses\textsuperscript{12} and failed to expel the parasite.\textsuperscript{22} STAT6-deficient mice infected with \textit{Schistosoma mansoni} developed smaller hepatic granulomas associated with reduced amounts of Th2 cytokines.\textsuperscript{20} Finally, STAT6-deficient mice showed markedly reduced airway reactivity, peribronchial inflammation, and eosinophilia in allergen-induced airway models.\textsuperscript{24,25} Hence, targeted deletion of STAT6 proved to be sufficient to disrupt Th2 responses in these experimental model systems.

In contrast, in our model system, disruption of recipient sources of STAT6 did not result in any phenotypic changes in the graft vasculature or cytokine activation. As described above, IL-4 transcripts were hard to detect in grafts placed into both wild-type and STAT6-deficient recipients. Gift expression of IL-10, IFN-\(\gamma\), and IL-2 was comparable in grafts from both groups. One interpretation is that cardiac allograft vasculopathy in chronically rejecting hearts develops independently of STAT6-mediated signaling pathways. This would extend previous studies in IL-4 knockout recipients showing no differences in parenchymal and vascular outcome.\textsuperscript{15,16,20} Taken together, these findings argue against a role for Th2 forces in promoting chronic rejection.

Conclusions

We used recipient mice with targeted gene deletion to demonstrate a significant role for STAT4-mediated Th1 forces in the pathogenesis of cardiac allograft vasculopathy. When present, STAT4 might promote the development of cardiac allograft vasculopathy by directing P- and E-selectin-mediated recruitment of activated Th1 cells into the graft. This would result in increased inflammatory activation and graft-specific secretion of the proatherosclerotic Th1 effector cytokines IFN-\(\gamma\) and IL-2. In the future, this model system can be used to identify the downstream molecular pathways regulated by Th1 forces that contribute to cardiac allograft vasculopathy.

Acknowledgments

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References


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