Neutrophils Sustain Pathogenic CD8⁺ T Cell Responses in the Heart

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This study explores the influence of innate immunity on CD8⁺ T-cell responses against heart tissue. Adoptive transfer of ovalbumin-specific CD8⁺ effector T cells into CMY-mOva mice, which express ovalbumin in cardiac myocytes, results in a lethal acute myocarditis. The inflammatory infiltrate in the heart includes neutrophils as well as T cells. We used anti-Ly6G antibody to transiently deplete neutrophils at the time of onset of disease. By day 7 after receiving 5×10^5 CD8⁺ effector T cells, 100% of control Ig-treated CMY-mOva mice had died, while 85% of anti-Ly6G-treated mice survived indefinitely. CD8⁺ T-cell infiltration and tissue damage were present in both groups, but the disease was limited in the anti-Ly6G-treated mice, with a rapid disappearance of the adoptively transferred CD8⁺ T cells within 11 days. Recovery occurred even though blood neutrophil counts began to rise 48 hours after the last anti-Ly6G treatment. Recovery was associated with a chronic CD4⁺ cell infiltrate, and a rapid decline in expression of IFN- γ and IP-10 mRNA in the myocardium. Neutrophil depletion did not effect survival of CMY-mOva mice that received 3×10^6 CD8⁺ T cells. These data show that granulocytic inflammation sustains CD8⁺ T-cell-mediated heart disease, which has important implications for the pathogenesis and treatment of acute myocarditis and allograft rejection. (*Am J Pathol* 2003, 163:2413–2420)

Innate immune responses are understood to promote T-cell-mediated adaptive immunity by activating antigen-presenting cells involved in the priming of naïve antigen-specific T cells. It is also possible that the inflammatory events associated with innate immunity may regulate the function of differentiated effector T cells. Recent evidence suggests that this is the case for T-cell responses to infections¹ and to allografts.² CD8⁺ T-lymphocyte damage to tissue cells plays a prominent role in various disease processes, including allograft rejection, viral diseases, and organ-specific autoimmunity. Acute inflammatory responses may occur simultaneously with CD8⁺

T-cell responses in various settings. For example, in organ allografts, ischemia and trauma associated with the transplantation procedure is accompanied by acute neutrophilic inflammation, and with recruitment and activation of alloreactive CD8⁺ T cells. Furthermore, effector CD8⁺ T cells can promote acute inflammation by secreting cytokines, such as interferon- γ (IFN- γ) and TNF- α , which can secondarily induce expression of endothelial adhesion molecules and chemokines that promote neutrophil recruitment.

We have recently developed a model of CD8⁺ T-cell-mediated myocarditis which involves a transgenic mouse strain (CMY-mOva) that expresses ovalbumin (Ova) in cardiac myocytes.³ Adoptive transfer of TCR transgenic Ova peptide (SIINFEKL)-specific CD8⁺ T cells in CMY-mOva mice induces a progressive, lethal myocarditis. In this study, we examine the contributory role of circulating Ly6G⁺ neutrophils in the progression of myocarditis in CMY-mOva mice. Our findings indicate that neutrophils profoundly influence the severity of CD8⁺ T-cell-dependent disease, independent of initial T-cell recruitment to the heart.

Materials and Methods

Antibodies and Reagents

The hybridoma-producing anti-mouse Ly6G mAb (clone RB6–8C5, Rat IgG2b)^{4,5} was obtained from DNAX; Ig from culture supernatants was purified using Protein-G Sepharose. Control rat IgG was purchased from Sigma (St. Louis, MO).

Mice

The CMY-mOva transgenic mouse line,³ which expresses membrane-bound ovalbumin (mOva) exclusively on cardiac myocytes, was maintained on a C57BL/6-Thy 1.2 (CD90) background. All CMY-mOva transgenic mice used were heterozygous for the mOva transgene. Both male and female mice were used at between 6 and 10 weeks of age (approximately 50% of each sex), and the

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ratio of males to females was matched between experimental groups. The TCR transgenic OT-I mouse strain⁶ was kindly provided by W.R. Heath and F. Carbone (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and was maintained on a C57BL/6-Thy 1.1 (CD90.1) background. The OT-I TCR is expressed on CD8⁺ T cells and is specific for Ova peptide 257–264 (SIINFEKL) bound to H-2K^b.⁷ Wild-type female C57BL/6 mice used in the study were purchased from Jackson Lab, and used at 6 to 8 weeks of age. All mice were bred in the pathogen-free facility at the Braunwald Medical Research Center, in accordance with the guidelines of the Committee of Animal Research at the Harvard Medical School and the NIH animal research guidelines.

Depletion of Ly6G⁺ Cells

Systemic depletion of Ly6G positive cells was performed as described.^{4,5} Briefly, mice (body weight 20 to 24 g) were injected intraperitoneally with 200 μ g RB6–8C5 mAb dissolved in sterile PBS, at 1, 3, 5, and 7 days after adoptive transfer of OT-I T cells. The control animals for this treatment were injected with equal amounts of rat IgG at the same times. The effectiveness of the antibody-mediated deletion was assessed by counting polymorphonuclear leukocytes on Wright-Giemsa-stained tail blood smears on glass slides (HEMA-3; Biochemical Sciences, Swedesboro, NJ).

Cell Preparations

All cell cultures were in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO), 2 mmol/L Na-pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mmol/L HEPES (Invitrogen). Spleen and lymph nodes (LN) were harvested from OT-I TCR transgenic mice and single-cell suspensions were prepared by mechanically crushing the lymphoid organs on a stainless-steel mesh and passing the cells through a 70- μ m nylon cell strainer (BD Biosciences, Palo Alto, CA). The resulting suspension was treated with Tris-ammonium chloride (TAC) buffer to lyse red blood cells and CD8⁺ cells were purified using MACS CD8a (Ly-2) MicroBeads kit (Miltenyi Biotec, Auburn, CA). This protocol typically yielded >95% CD44 low CD8⁺ CD4⁺ cells, as assessed by flow cytometry. Preparation of OT-I CD8⁺ effector T cells was performed as described.³ Briefly, naïve CD8⁺ OT-I cells were placed in culture with mitomycin-C (Sigma) treated antigen-presenting cells prepared from spleens of C57BL/6 mice at a T cell:APC ratio of 1:10, and Ova peptide antigen (SIINFEKL) was added at a final concentration of 1 μ mol/L. These cultures were supplemented with 2 μ g/ml anti-CD28 (BD Pharmingen, Palo Alto, CA), 50 U/ml recombinant mouse IL-2 (R&D Systems, Cambridge, MA), and 10 ng/ml recombinant mouse IL-12 (R&D Systems). The cultures were placed in 75 cm² flasks and incubated at 37°C, 5% CO₂. After 3 days of stimulation, all cultures were diluted 1:1 with fresh me-

dium containing 40 U/ml IL-2 (R & D Systems) and OT-I CTL effectors were harvested for use at day 5.

Serum Troponin Determination

Serum levels of cardiac troponin-T (cTnT) were measured by a clinical quantitative immunoassay technique, which cross reacts with mouse troponin (Troponin T STAT; Roche Diagnostics, Basel, Switzerland) on the 1010 Elys Immunoanalyzer (Roche Diagnostics).

Flow Cytometry Analyses

Before flow-cytometry analysis, all cell preparations were washed twice in staining buffer (DPBS with 1% BSA). For phenotypic analysis of surface markers, 0.5×10^6 cells were suspended in 100 μ l staining buffer containing 1 μ g of each specific antibody, purchased from BD Pharmingen, and incubated on ice for 20 minutes followed by washing and fixation with 0.5% paraformaldehyde. Stained cell preparations were then analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (BD Biosciences). Expression of the OT-I TCR was verified by combined stain with anti-V α 2 and anti-V β 5 antibodies (BD Pharmingen). Other specific antibodies used for flow cytometry were as follows: PE-conjugated anti-mouse CD90.1/Thy 1.1 (clone OX-7); FITC-conjugated anti-mouse CD44 (clone IM7); FITC-conjugated anti-mouse CD25 (IL-2R α chain) (clone 7D4). *In vivo* proliferation of T cells was followed by flow cytometric analysis of carboxyfluorescein-succinimidyl-ester (CFSE) (Molecular Probes, Eugene, OR) stained cells, as described.³

Generation of cDNA and Quantitative Real-Time PCR

Isolation of total RNA from heart tissue, cDNA synthesis, and real-time polymerase chain reaction (PCR) analysis were performed as we previously described.³ Briefly, total RNA was isolated from approximately 10 mg of a biventricular section of heart tissue, by homogenization in TRIzol reagent (Invitrogen). Residual traces of DNA were eliminated by DNaseI (Invitrogen). Total RNA was quantified by absorbance at 260 nm and used as templates for reverse transcription (RT) of first-strand cDNA using the ThermoScript RT-PCR system and random hexamer primers (Invitrogen) according to the manufacturer instructions. Quantitative real-time RT-PCR was performed with SYBR Green PCR mix (Applied Biosystems, Foster City, CA) and specific oligonucleotide primers designed using PrimerExpress software (Applied Biosystems). 50 ng of cDNA specific oligonucleotide primers (450 nmol/L each) were placed into reaction-wells in a 96-well optical reaction plates (Applied Biosystems). The thermal cycle conditions were: activation, 50°C 2 minutes; denaturation, 95°C 10 minutes; and cycle (95°C 15 seconds to 60°C 1 minute) 40 times. Specific primers used for sequence detection were as follows: For detection of message for IFN- γ , 5'-AACGCTACACACTGCATCTTGG (sense) and

5'-GCCGTGGCAGTAACAGCC (antisense); for IP10 5'-GCCGTCATTTCTGCCTCA -3' (sense) and 5'-CGTCCTTGCGAGAGGGATC -3' (antisense); for RANTES 5'-CAAGTGCTCCAATCTTGCAGTC -3' (sense) and 5'-TTCTCTGGGTGGCACACAC -3' (antisense); for GAPDH, 5'-GGCAAATTCACGGCACAGT (sense) and 5'-AGATGGTGATGGGCTTCCC (antisense). All real-time reactions were carried out on an ABI 5700 Sequence Detection System (Applied Biosystems) and analysis was done with accompanying software. The presence of single amplicons resulting from real-time RT-PCR was verified by dissociation curve analysis and the specific identity of the amplicons was confirmed by sequencing. Levels of specific gene expression in tissue samples presented as relative to endogenous levels of GAPDH expression in the same sample. All heart specimens examined in this study had nearly equal levels of the GAPDH gene expression that were not influenced by the inflammatory conditions, nor by the treatments applied.

Immunohistochemistry

Five- μ m-thick cryostat sections of heart were fixed in acetone, blocked with 1% bovine serum albumin in PBS at room temperature, incubated with unlabeled primary antibodies (each at 1 to 10 μ g/ml) at room temperature, followed by PBS wash, and then incubated with biotinylated secondary antibodies (each at 2.5 or 5 μ g/ml) at room temperature.⁸ Primary rat anti-mouse antibodies included anti-CD4, anti-CD8, anti-Ly6G (GR-1), and anti-CD11b, (all from BD Pharmingen). Isotype-matched antibodies were used as controls. The sections were then incubated with biotinylated goat anti-rat Ig, 1:200 (Jackson ImmunoResearch, West Grove, PA) at room temperature. CD90.1 (Thy 1.1) positive cells were detected in frozen sections using a biotinylated antibody (BD Pharmingen). Sections were then blocked with 0.3% hydrogen peroxide/PBS at room temperature, and then incubated with horseradish peroxidase (HRP)-avidin-biotin complex solutions at 1:1:100 dilution (Vector Laboratories, Burlingame, CA). Specific antibody binding was detected with 3-amino-9-ethylcarbazole (Vector Laboratories) and counterstained with Gill's number 2 hematoxylin solution (Polysciences, Warrington, PA).

Histological Grading of Myocarditis

Myocarditis was graded by microscopic examination of hematoxylin and eosin (H&E)-stained sections of formalin-fixed and paraffin-embedded heart tissue. Grading was performed in a blinded fashion, by a trained pathologist after microscopically examining the entire area of three sections with a grid ocular, using a 0–4 scale, modified from⁹ as follows: grade 0, no disease; grade 1, up to 5% of the cross-sectional area of the heart section; grade 2, 6% to 20%; grade 3, 21% to 50%; grade 4, >50%.

Results

Neutrophils Are Present in Myocardium of CMY-mOva Mice with OT-I-Mediated Myocarditis

As previously reported, adoptive transfer of CD8⁺ OT-I effectors into CMY-mOva mice causes a progressive myocarditis, with severity proportional to T-cell dose.³ Clinical and pathological onset of disease occurs by day 5 after 0.5×10^5 T-cell transfer.³ Examination of H&E-stained sections of hearts taken from CMY-mOva mice at day 5 indicates the presence of neutrophils in addition to abundant lymphoblasts (Figure 1, A and B). The presence of both OT-I T cells and neutrophils in these sections is also evident from immunohistochemically stained sections, using antibodies specific for Thy 1.1 (CD90.1) marking the adoptively transferred T cells (Figure 1C), and for anti-Ly6G, which stains granulocytes (Figure 1D). It should be noted that there was no inflammation or immunohistochemically identifiable Thy 1.1⁺ T cells in the hearts of wild-type C57BL/6 mice that received OT-I transfers, as previously described.³

Transient Depletion of Ly6G⁺ Cells Alters the Course of CTL-Mediated Disease and Results in Long-Term Recovery

To evaluate the significance of neutrophils in the pathological process of CD8⁺ T-cell-dependent myocarditis in CMY-mOva mice, Ly6G⁺ cells were depleted by administration of RB6-8C5 mAb.^{4,5} This treatment was rapidly effective in reducing neutrophil counts by 95% to 98%, as determined by blood smears (Figure 2). Histological sections of hearts of experimental animals taken at day 5 show that Ly6G⁺ cell depletion substantially reduces the formation of necrotic foci and neutrophil accumulation in the heart, without appreciably altering of the presence of pathogenic Thy1.1⁺(OT-I) T cells (Figure 3). Scattered CD4⁺ cells were detected in hearts from anti-Ly6G⁺-treated animals at day 5 after CD8⁺ T-cell transfer, but CD4⁺ cells were very infrequent in hearts from control-Ig-treated animals.

The histological grade of myocarditis in hearts from neutrophil-depleted animals was significantly reduced compared to control-Ig-treated animals (Figure 4), but there still was a considerable amount of histological disease in both groups. Furthermore, elevated serum cardiac troponin in both Ly6G-depleted and control groups indicates that the initial tissue damage caused by CTL was substantial, independent of neutrophils (Figure 5).

Survival studies indicated that neutrophil depletion provided significant protection of CMY-mOva mice from the lethal pathological effects of Ova-specific CTL. All control antibody-treated CMY-mOva mice typically died within 7 days after transfer of 0.5×10^6 OT-I effector CTL (Figure 6). In contrast, 85% of anti-Ly6G-treated mice survived indefinitely (Figure 6), even though blood neutrophil counts began to rise by day 8 and were at pretreatment levels at day 13 (Figure 2). These surviving mice showed evidence of slight weakness at 5 days, but

Figure 1. Neutrophils are present in CMY-mOva hearts following adoptive transfer of OT-I CD8⁺ effector T cells. Routine H&E-stained histological sections (**A** and **B**) were prepared from hearts taken from Thy 1.1 CMY-mOva mice 5 days after transfer of 0.5×10^6 effector OT-I cells, as described in Materials and Methods. Representative neutrophils are identified by an arrow. Frozen sections from the same hearts shown in **A** and **B** were immunohistochemically stained for Thy1.1 (CD90.1), which identifies the adoptively transferred OT-I cells (**C**) or for Ly6G, which identifies neutrophils (**D**). Original magnifications: $\times 100$ (**A**); $\times 1000$ (**B**); $\times 400$ (**C** and **D**).

recovered by day 11 and appeared normal until euthanasia up to 3 months later. To confirm that the OT-I CTL were capable of causing lethal cardiac damage independent of neutrophils, the effect of neutrophil depletion was assessed after transfer of very high numbers of T cells.

Anti-Ly6G treatment had no effect on survival of CMY-mOva mice after transfer of 3×10^6 OT-I CTL effectors; all mice in treated and control groups were dead by 4 days, and the majority died within 72 to 90 hours.

Figure 2. Time-course of neutrophil depletion and reconstitution in anti-Ly6G-treated CMY-mOva mice. CMY-mOva mice were adoptively transferred with 0.5×10^6 OT-I CTL effectors. RB6-8C5 mAb or control rat IgG (200 mg) was administered i.p. four times, as indicated by arrows. Sequential tail vein blood smears were prepared and differential white blood cell counts were determined. Clinical onset of myocarditis is marked by the gray area. Data points represent mean percent neutrophils $\pm$ SEM from three consecutive experiments each with four mice per treatment group. **a:** No survival in control group beyond day 7. **b:** Data from surviving mice (85% of total).

CD4⁺ but Not CD8⁺ T Cells Persist in Hearts of Recovered Anti-Ly6G⁺-Treated CMY-mOva Mice

Histological examination of hearts from anti-Ly6G-treated mice that recovered from myocarditis revealed the presence of small foci of tissue repair, with fibrosis and chronic inflammation, but no neutrophils (Figure 7, A and B). Immunohistochemical stains indicate that CD4⁺ cells comprise part of the persistent infiltrate in the recovered hearts (Figure 7, C and D). Therefore, neutrophil depletion at the time of CD8⁺ T-cell damage to the heart results in the early (Figure 3) and persistent (Figure 7) presence of CD4⁺ cells. In contrast to CD4⁺ cells, there were no detectable OT-I cells left in the hearts or draining mediastinal lymph nodes of anti-Ly6G-treated mice by day 11 (not shown).

To assess if the chronic infiltrates seen in the hearts of anti-Ly6G-treated mice were causing myocyte damage, we measured serum troponin and found it was reduced

Figure 3. T-cell infiltration into CMY-mOva hearts with or without neutrophil depletion. CMY-mOva mice received OT-I adoptive transfers and anti-Ly6G or control Ig, as described in the Figure 2 legend, and hearts were removed at day 5 after T-cell transfer. Routine H&E-stained histological sections were prepared from control Ig-treated (A) or anti-Ly6G-treated (B) mice. Frozen sections of hearts from control Ig-treated (C) or anti-Ly6G-treated (D) animals were stained for Thy 1.1 to detect the adoptively transferred OT-I cells. Sections of hearts from control Ig-treated (E) or anti-Ly6G-treated (F) animals were also stained for CD4. Sections were prepared from all mice in the study; representative sections are shown. Original magnification: $\times 100$.

to undetectable levels by day 11 after OT-I transfer, and remained undetectable at day 19, despite the persistence of these CD4⁺ cells (data not shown). Real-time RT-PCR analysis of inflammatory gene mRNA expression show comparable expression of IFN- γ , IP-10, and RANTES mRNA in hearts from control Ig and anti-Ly6G-treated animals at day 5 after T-cell transfer (Figure 8). No expression of these genes was detected in animals that did not receive T-cell transfers (data not shown) consistent with our previously published IFN- γ mRNA analyses.³ The level of IFN- γ and IP-10 mRNA in hearts from the surviving anti-Ly6G-treated mice rapidly declined

and approached undetectable levels by day 19. RANTES mRNA levels also declined, but remained detectable at day 19.

Discussion

This study demonstrates that Ly6G⁺ granulocytes, which are largely neutrophils, profoundly influence the progression of a disease process initiated by, and dependent on tissue-antigen-specific CD8⁺ effector T cells. The findings lend support to the emerging idea that innate im-

Figure 4. Histological grade of myocarditis in anti-Ly6G and control Ig-treated CMY-mOva mice. Myocarditis score was determined on sections of the same hearts shown in Figure 3, as described in Materials and Methods. Data represent mean score $\pm$ SEM from each experimental group ($n = 7$). * $P = 0.02$, unpaired two-tailed t -test.

mune system can modulate T-cell function in the effector phase of an adaptive immune response.

The data show that Ly6G⁺ cells are required to sustain a rapidly progressive and fatal myocarditis initiated by heart-antigen-specific CTL. Despite significant T-cell infiltration in both anti-Ly6G⁺ and control animals at day 5

Figure 5. Serum cardiac troponin T in anti-Ly6G and control Ig-treated CMY-mOva mice. Data represent mean $\pm$ SD serum troponin levels of 10 anti-Ly6G and eight control Ig-treated mice described in legend to Figure 3. The serum level of troponin T in CMY-mOva mice without OT-I transfer is below the detection limit of the assay, indicated by dotted line. * $P > 0.005$.

Figure 6. Depletion of neutrophils enhances survival of CMY-mOva mice from myocarditis. CMY-mOva mice received adoptive transfers of 0.5×10^6 (A) or 3×10^6 (B) OT-I cells, and were treated with anti-Ly6G antibody or control Ig, as described in Figure 2. The data are pooled from six identically performed experiments, each with 5 to 6 mice per treatment group. Surviving mice in the control Ig group were sacrificed after day 70.

after T-cell transfer, survival was significantly greater in the treated group. It is possible that granulocyte-mediated damage to myocytes may enhance the release of ovalbumin, which would then be available for cross-presentation to the T cells by other antigen-presenting cells in both draining lymph node and heart. Cross-presentation of Ova to naïve OT-I cells has been shown to be critical for the initiation of responses that cause diabetes in mice that express Ova in pancreatic islets.¹⁰ However release of antigen by granulocyte-mediated damage to myocytes is not likely to be the major reason why granulocytes are essential to sustain the T-cell response in CMY-mOva mice. In our model, differentiation of effector OT-I cells is accomplished *in vitro*, and there is likely to be ample Ova-peptide presented by class I MHC on the surface of myocytes to activate the effector T cells in the heart, without the need for cross-presentation. In support of this assumption, we have observed spontaneous activation of OT-I effectors *in vitro* with freshly prepared suspensions of heart cells from previously unmanipulated CMY-mOva mice (unpublished data).

The contribution of granulocytes to myocarditic disease in our model appears to go beyond direct tissue damage, since transient Ly6G⁺ cell depletion at a critical time during onset of T-cell-induced disease has long term consequences, including the disappearance of CD8⁺ effectors from both heart and draining lymph node, and the establishment of a chronic CD4⁺ T-cell infiltrate. One

Figure 7. Anti-Ly6G therapy is associated with persistent infiltration of the heart with CD4⁺ cells. CMy-mOva mice that received adoptive transfer of 0.5×10^6 OT-I cells and anti-Ly6G therapy, as described in Figure 2, were sacrificed at day 11 (A and C) and 19 (B and D) after T-cell transfer, and heart sections were prepared. The figure shows routine H&E-stained histological sections (A and B) and frozen sections stained for CD4 (C and D). Typical sections are shown from each group ($n = 4$ per group). Original magnification: $\times 400$.

interpretation of these findings is that regulatory mechanisms, which serve to protect the heart from effector CD8⁺ T cells, are turned off in the setting of pathogen-driven innate responses with associated granulocytic inflammation. In the studies described here, we have experimentally isolated and focused on the effector phase of the T-cell response by providing pre-armed CTL effectors generated *in vitro* in the presence of IL-12.

The presence of CD4⁺ cells in CMy-mOva hearts of anti-Ly6G⁺-treated CMy-mOva mice during and after recovery from CD8⁺-mediated myocarditis suggests that regulatory T cells (Treg) are responsible for the suppression and disappearance of the CTL response when neutrophils are absent. There is evidence that tissue-resident Treg cells may be induced by autoantigens and suppress ongoing inflammatory processes.¹¹ The way in which such Treg suppress responses may involve cytokines such as IL-10, or cell:cell contact-dependent mechanisms. We have not been able to detect IL-10 mRNA by real-time RT-PCR in hearts of anti-Ly6G-treated CMy-mOva mice (data not shown). Additional studies will be required to address the question of long-term suppression of CD8⁺ T-cell responses in anti-Ly6G-treated animals and the role of Treg in this process.

In addition to neutrophils, Ly6G is expressed on eosinophils,⁵ and on a subset of plasmacytoid dendritic

Figure 8. Decline in inflammatory gene expression in hearts from anti-Ly6G-treated CMy-mOva mice. Mice that received adoptive transfer of 0.5×10^6 OT-I cells and anti-Ly6G or control Ig therapy, as described in Figure 2, were sacrificed at day 5, 11, and 19 after T-cell transfer, as described in Figure 7. RNA was prepared from hearts and real-time RT-PCR analysis of IFN- γ , IP-10, and RANTES mRNA was performed as described in Materials and Methods. Typical results of one of three experiments are shown.

cells.¹² Therefore, the effects of antibody-mediated depletion of Ly6G⁺ cells in our study may reflect, in part, the role of each of these cell types. There are no reliable ways to definitively identify eosinophils and distinguish them from neutrophils in histological sections of mouse tissues. There are very few leukocytes with doughnut-shaped nuclei and eosinophilic cytoplasm, which is typical of eosinophils, in the inflamed hearts in this study. Furthermore, it is unlikely that eosinophils are a major component of the acute inflammatory infiltrate that accompanies the T-cell infiltrate in our model of myocarditis, because the cytokines and chemokines that are expressed, including IFN- γ , IP-10, and RANTES are typical of "type 1", eosinophil-poor, inflammatory processes. Ly6G⁺ plasmacytoid dendritic cells produce IFN- α in response to certain viral infections, and may play a critical role in the priming of CD8⁺ T-cell responses.^{13,14} In the studies described here, naïve CD8⁺ T-cell priming was performed *in vitro* in the presence of IL-12 before transfer to CMy-mOva mice, and the absence of Ly6G⁺ dendritic cells is not likely to have an impact on the function of the adoptively transferred effector CD8⁺ T cells.

The contribution of different types of effector cells that cause myocardial damage in acute allograft rejection or in viral and autoimmune myocarditis in humans is poorly understood.¹⁵ CD8⁺ T cells are clearly implicated in mouse models of these diseases.^{16,17} Neutrophils are also often present in mouse cardiac allografts.¹⁸ Their presence likely reflects both ischemic injury to the myocardium associated with transplantation, and pro-inflammatory cascades initiated by alloreactive T cells. The protective benefit of neutrophil depletion in our mouse model suggests that therapeutic interventions designed to transiently reduce granulocyte infiltration may have long-term benefits in patients with active CD8⁺ T-cell damage to the heart.

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