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Fatal Eosinophilic Myocarditis Develops in the Absence of IFN-γ and IL-17A

Jobert G. Barin,*‡ G. Christian Baldeviano,*§ Monica V. Talor,† Lei Wu,‡ SuFey Ong,‡ DeLisa Fairweather,¶ Djahida Bedja,‖ Natalie R. Stickel,§ Jillian A. Fontes,‡ Ashley B. Cardamone,‖ Dongfeng Zheng,‖ Kathleen L. Gabrielson,‖ Noel R. Rose,*‡+ and Daniela Čiháková‡

CD4+ T cells play a central role in inflammatory heart disease, implicating a cytokine product associated with Th cell effector function as a necessary mediator of this pathophysiology. IFN-γ–deficient mice developed severe experimental autoimmune myocarditis (EAM), in which mice are immunized with cardiac myosin peptide, whereas IL-17A–deficient mice were protected from progression to dilated cardiomyopathy. We generated IFN-γ−/−IL-17A−/− mice to assess whether IL-17 signaling was responsible for the severe EAM of IFN-γ−/− mice. Surprisingly, IFN-γ−/−IL-17A−/− mice developed a rapidly fatal EAM. Eosinophils constituted a third of infiltrating leukocytes, qualifying this disease as eosinophilic myocarditis. We found increased thickness at end diastole; WT, wild-type.

Myocarditis encompasses a diverse family of inflammatory heart diseases associated with a large variety of infectious and pharmacologic causes (1, 2). We have published extensively on myocarditis as a paradigm of post-infectious autoimmunity, in which autoaggressive responses represent a critical intermediary in the development of lasting cardiologic sequelae, often culminating in inflammatory dilated cardiomyopathy (DCM) and heart failure (3–5). Myocarditis can take several phenotypic forms in humans, including necrotizing eosinophilic myocarditis, a variant associated with poor prognosis and rapidly fatal outcomes (6–8).

Experimental autoimmune myocarditis (EAM) is a model of human inflammatory heart disease in which animals are immunized with cardiac Ags to recapitulate autoreactive responses following viral clearance (9, 10). The disease is dependent on CD4+ T cells, implicating cytokine products of these cells as necessary signaling intermediaries in disease pathophysiology (11).

IL-17 has been described as a family of proinflammatory cytokines bridging innate and adaptive immunity (12). We recently reported a critical requirement for IL-17A in mediating profibrotic remodeling in the heart during EAM and subsequent progression to DCM; although IL-17A−/− mice are protected from fibrosis, they are not protected from inflammatory myocarditis (13). The archetypal member of this family, IL-17A, elicits neutrophilic inflammation intermediaries in disease pathophysiology (11).

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The involvement of Th17 CD4+ cells in autoimmune disease continues to be an active area of investigation. The importance of the Th17 lineage was discovered, in part, by contrasting the roles of IL-23 and IL-12 in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) (21). Adoptive transfer experiments have shown that Th17-conditioned cells induced qualitatively different, if not more severe, autoimmune disease than did Th1 cells (22–24).

Th17 lineage cells are thought to be responsible for the protective effect of IFN-γ in several animal models of autoimmune disease.
(25, 26). IFN-γ−/− mice develop more severe EAM than do wild-type (WT) controls, and progress to fibrosis and DCM at later timepoints (27–30). More severe disease may be elicited in IFN-γ−/− mice through unconstrained outgrowth of autoaggressive Th17 clones in vivo. Several investigators have reported increased Th17 responses in association with severe disease in IFN-γ−/− mice, consistent with this hypothesis (31–35). These findings led us to investigate whether the protective effect of IFN-γ in EAM is mediated through suppressing Th17 differentiation in autoaggressive CD4+ T cells. Unexpectedly, we found that mice deficient in both IFN-γ and IL-17A developed a severe, rapidly fatal form of EAM, characterized by extensive eosinophilic infiltration, cardiomyocyte necrosis, and thrombogenesis, with evidence of Th2 deviation and rapid decline of cardiac function. Comparisons to both IL-17A−/− and IFN-γ−/− mice suggest that both cytokines collaborate in the suppression of autoaggressive Th2 differentiation and eosinophilic cardiac infiltration. In this article, we demonstrate a requisite role for eosinophils in effecting the rapid mortality of eosinophilic myocarditis. We further provide evidence of specific features of autoimmune pathophysiology independently mediated by eosinophils, and the Th2 effector program, respectively.

Materials and Methods

Mice

IL-17A−/− founder mice were kindly provided by Yoichiro Iwakura (University of Tokyo, Tokyo, Japan). IFN-γ−/− and WT BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-17A−/− mice were crossed to IFN-γ−/− and bred to homozygosity at both loci employing established PCR genotyping (36, 37). Established IFN-γ−/− IL-17A−/− BALB/c mice were outcrossed to the ΔdbGATA1 BALB/c strain (JAX 5653), and resulting progeny were intercrossed for a minimum of two generations to generate homozygous triple-mutant founders. As a result, cross-intermediates did not include ΔdbGATA1 BALB/c strain, which may be the focus of future studies. All mice were maintained in the Johns Hopkins University School of Medicine specific pathogen–free vivarium. Experiments were conducted on 6- to 12-wk-old male mice, in compliance with the Animal Welfare Act and the principles described in the Guide for the Care and Use of Laboratory Animals (38). All methods and protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University.

Induction of EAM

For the induction of EAM, we employed the myocardiotoxic peptide MyHC614-629 (Ac-SLKMLMTLFSYASAD) (39), commercially synthesized by 9-fluorenylmethylcarbonyl chemistry and purified to a minimum of 90% by HPLC (GenScript). On days 0 and 7, mice received s.c. 2–6 μg MyHC614-629 peptide emulsified in CFA (Sigma-Aldrich) supplemented to 5 mg/ml heat-killed Mycobacterium tuberculosis strain H37Ra (Difco). On day 0, mice also received 500 ng pertussis toxin i.p. (List Biologicals).

Coxsackie viral infection

Male 6- to 8-wk-old mice were maintained under pathogen-free conditions in the animal facility at the Johns Hopkins School of Medicine. Mice were inoculated i.p. with 107 PFU heart-passaged coxsackievirus B3 (CVB3) (containing CVB3 and cardiac myosin) diluted in sterile PBS on day 0. This autoimmune model of CVB3 myocarditis closely resembles EAM (40). CVB3 (Nancy strain) was originally obtained from the American Type Culture Collection (Manassas, VA) and grown in Vero cells (American Type Culture Collection), as previously described (41). Myocarditis was assessed at day 10 post infection, during the peak of acute inflammation.

Assessment of EAM

Mice were most commonly evaluated for the development of EAM at the peak of disease on day 21. Heart tissues were fixed in SafeFix (Thermo Fisher Scientific). Tissues were embedded longitudinally, and 5-μm step sections were cut and stained with H&E, or Masson’s Trichrome Blue (Histoserv). Myocarditis severity was evaluated by microscopic approximation of the percentage of area of myocardium infiltrated with inflammatory cells, fibrosis, and cardiomyocyte necrosis, determined from five sections per heart according to the following scoring system: grade 0, no inflammation; grade 1, < 10% of the heart section is involved; grade 2, 10–30%; grade 3, 30–50%; grade 4, 50–90%; grade 5, > 90% (9). Grading was performed by a minimum of two independent, blinded investigators and averaged.

Flow cytometry

Heart-infiltrating leukocytes were isolated from animals perfused for 3 min with 1× PBS + 0.5% BSA and digested in gentleMACS C Tubes according to the manufacturer’s instructions (Miltenyi Biotec). Splenocytes were also extracted into single-cell suspension in 1× PBS + 0.5% BSA, and RBCs were lysed by < 5 min incubation in ACK lysis buffer (Biofluids). Prior to surface staining, viability was determined by LIVE/DEAD staining according to the manufacturer’s instructions (Molecular Probes). Cells were washed, and FcγR1IIB was blocked with sCD16/32 (eBioscience). Surface markers were stained with fluorochrome-conjugated mAbs (eBioscience, BD Pharmingen, BioLegend). Samples were acquired on the LSR II quad-laser cytometer running FACS Diva 6 (BD Immunocytometry). Data were analyzed with FlowJo 7.6 (TreeStar Software).

ELISA

Supernatants from stimulated cells were stored at −80°C. For cardiac homogenates, tissues were snap frozen, stored at −80°C, homogenized in MEM + 2% tBS, and stored at −80°C until used in ELISA or Linco assays. Homogenate cytokine levels were normalized to wet heart weights prior to homogenization. Linco multiplex cytokine assays (MILLIPEDE) were performed according to the manufacturer’s instructions, and acquired on a Luminex xMAP reader. Alternatively, quantitative sandwich ELISA for supernatants was determined by colorimetric ELISA kits according to the manufacturer’s recommended protocols (R&D Systems, IBL). Total serum IgE was determined by sandwich ELISA (BD Biosciences).

Real-time PCR

Tissue mRNA was extracted in TRIzol (Invitrogen), quantitated by NanoDrop (Thermo-Fisher), DNase digested (Ambion), reverse transcribed (Fermentas), and amplified with IQ SYBR Green Mastermix (Bio-Rad) acquired on the MyiQ2 thermocycler (Bio-Rad) running iQ5 software (Bio-Rad). Synthetic oligonucleotide primers were commercially synthesized, purified, and assayed by mass spectroscopy (Integrated DNA Technologies). A total of 250 pmol of each primer template (Supplemental Table I) was used for 2 μg cDNA template for each reaction. Reactions were run as individual primer pairs for individual animals. Data were analyzed by the 2−DDCt method of Livak et al. (42), comparing threshold cycles first to Hprt expression in individual animals, then ΔCt of target genes in naive WT BALB/c controls. Certain data are pseudolinearized to depict downregulation (2−ΔΔCt < 1) as −2ΔΔCt.

Hydroxyproline determination

Cardiac samples are weighed, then hydrolyzed in 12 N HCl overnight at 120°C. Samples are dried in 96-well format plates, then incubated with 50 mm Chloramine-T (Sigma-Aldrich), followed by 1 m dimethylamino-benzaldehyde (Sigma-Aldrich) against a 1–100 μg/ml standard curve of hydroxyproline (Sigma-Aldrich). Samples are read at 570 nm, and values normalized to starting sample mass (43).

Echocardiography

Transthoracic echocardiography was performed using the Acuson Sequoia C256 ultrasonic imaging system (Siemens) with a 13 MHz transducer. Conscious, depilated, previously trained mice were gently held in a supine position. The heart was imaged in the two-dimensional mode in the parasternal short-axis view, and an M-mode cursor was positioned perpendicular to the interventricular septum and the left ventricular (LV) posterior wall at the level of the papillary muscles. From M-mode, the wall thicknesses and chamber dimensions were measured. For each mouse, three to five values for each measurement were obtained and averaged for evaluation. The LV end-diastolic dimension, LV end-systolic dimension (LVESD), interventricular septal wall thickness at end diastole (IVSTD), and LV posterior wall thickness at end diastole (LVPTED) were measured from a frozen M-mode tracing. Fractional shortening (%FS), %ejection fraction (%EF), %LV mass, and relative wall thickness were calculated from these parameters, as previously described (44).
Statistics

Multiple group comparisons were performed by ANOVA, followed by the Tukey–Kramer and Bonferroni posttest (StatPlus). Group survival was determined by Mantel–Cox log rank (GraphPad Prism). Asterisks denote statistically significant comparisons to WT.

Results

Concurrent ablation of IFN-γ and IL-17A reveals a protective effect of IL-17A in inflammatory autoimmune heart disease

To test our original hypothesis that IL-17 signaling mediates the severe disease observed in IFN-γ-deficient mice, we crossed an IL-17A-null allele to the IFN-γ-/- background, to generate IFN-γ-/-IL-17A-/- double-knockout (DKO) mice. EAM was induced in DKO mice, IFN-γ-/- single-knockout, IL-17A-/- single-knockout, and WT controls by immunization with myocarditogenic peptide (MyHCOwn, 614–629) in CFA. As we previously reported, IFN-γ-/- animals developed severe EAM, and IL-17A-/- animals developed EAM of similar severity to WT animals (13, 29).

Unexpectedly, accelerated mortality was observed in IFN-γ-/-IL-17A-/- animals, beginning at day 14 post immunization (Fig. 1A). In contrast, IFN-γ-/- mice died only at later timepoints, consistent with our previously published reports (28). Across experiments, between 50 and 100% of all immunized IFN-γ-/-IL-17A-/- mice consistently died during this timeframe, in both male and female IFN-γ-/-IL-17A-/- animals (data not shown). The survival of IFN-γ-/-IL-17A-/- mice was significantly poorer than IFN-γ-/- control animals by Kaplan–Meier log-rank estimates ($p < 0.001$). Moreover, IFN-γ-/-IL-17A-/- mice had more severe cardiac inflammation at time of death than did IFN-γ-/- or other control mice (Fig. 1B).

The degree of disease severity in IFN-γ-/-IL-17A-/- mice was unprecedented for the EAM model. The majority of IFN-γ-/-IL-17A-/- animals had such severe myocarditis that the tissue resembled secondary lymphoid tissue. Infiltrating cells included numerous mononuclear cells, as well as frequent polymorphonuclear cells (Fig. 1C). We observed extensive pericardial inflammation and fibrosis, as well as extensive cardiomyocyte necrosis, endocarditis, intra-atrial thrombosis, and the formation of ectopic lymphoid follicle-like structures throughout the myocardium (Supplemental Fig. 1).

In parallel experiments, we investigated the response of IFN-γ-/-IL-17A-/- mice (and appropriate single-knockout controls) to acute infection with CVB3, a cardiotropic enterovirus that has been previously described as an important agent in the elicitation of myocarditis in humans, as well as mouse models (45–47). Similar to the EAM model, IFN-γ-deficient mice developed more severe cardiac inflammation following CVB3 infection (48). At day 10 following infection, 2 of 13 IFN-γ-/-IL-17A-/- mice had expired, and the remaining animals were moribund (Supplemental Fig. 2A), whereas all mice of the control genotypes survived to this timepoint. The severity of histopathologic cardiac inflammation was largely comparable across strains (Supplemental Fig. 2B, 2C).

IFN-γ-/-IL-17A-/- mice rapidly develop rapidly compromised cardiac function, independent of cardiac fibrosis

Echocardiographic imaging of IFN-γ-/-IL-17A-/- mice at day 14 demonstrated greatly compromised cardiac function and dramatic loss of cardiac contractility, as evidenced by diminished FS and EF in IFN-γ-/-IL-17A-/-, but not IFN-γ-/-, mice at this early timepoint (Fig. 2A). We did not observe overt signs of LV dilation (Supplemental Fig. 3); however, LV end-systolic dimensions were increased in IFN-γ-/-IL-17A-/- mice (Fig. 2B), as was the resulting calculated LV mass (Fig. 2C).

FIGURE 1. IL-17 accelerates lethal EAM on an IFN-γ-deficient background. (A) Survival of male IFN-γ-/-IL-17A-/- (violet, $n = 15$), IFN-γ-/- (red, $n = 14$), IL-17A-/- (blue, $n = 5$), and WT (gray, $n = 5$) mice during the first 28 d of EAM. (B) Cardiac histopathology of IFN-γ-/-IL-17A-/- (violet), IFN-γ-/- (red), IL-17A-/- (blue), and WT (open) mice at time of death from (A). Prematurely expired mice from (A) are represented as crosses, whereas animals that survived to endpoint are depicted as diamonds. (C) Representative histopathology from median animals of each group; staining by H&E, magnification at $×2.5$ (left) and $×100$ (right). Individual data points represent individual animals; bars indicate mean of each group.
Cardiac fibrosis was assessed at day 21 of EAM by staining with Masson’s Trichrome Blue (Fig. 3A). Total collagen content of hearts was assayed by quantitative determination of hydroxyproline content, demonstrating diminished fibrosis in IFN-γ−/−IL-17A−/− mice, compared with IFN-γ−/− mice (Fig. 3B). Transcriptional regulation of fibrotic collagens in IFN-γ−/−IL-17A−/− hearts was interrogated by real-time PCR. Similar patterns of expression were observed for collagens I and III (Fig. 3B, 3C), as well as matrix metalloproteinase 2 and matrix metalloproteinase 3 (Supplemental Fig. 3). Together, these data confirm our previous findings that IL-17A drives cardiac fibrosis, whereas IFN-γ counterregulates this remodeling process (13). However, it makes such fibrotic dilative remodeling unlikely to mediate the severe, morbid heart disease of IFN-γ−/−IL-17A−/− mice.

Cellular mediators of severe EAM in IFN-γ−/−IL-17A−/− mice

To address mechanisms by which IL-17A could protect mice from eosinophilic myocarditis, we performed comprehensive cytometric profiling of the cardiac infiltrate at the peak of EAM in surviving mice (Fig. 4A). Among the IFN-γ−/−IL-17A−/− mice that survived to day 21, the total number of CD45+ leukocytes was similar to that seen in IFN-γ−/− animals (Fig. 4B, left). Notably, increased neutrophil infiltration was observed in IFN-γ−/− hearts at day 21 of EAM; however, this recruitment was abolished in IFN-γ−/−IL-17A−/− mice (Fig. 4B–D). Eosinophils were most abundant in IFN-γ−/−IL-17A−/− hearts at day 21 (Fig. 4B–E). By examining the loss of light scattering of heart-infiltrating eosinophils as an indicator of degranulation (49), we observed more numerous degranulated eosinophils at day 12 of EAM in IFN-γ−/−IL-17A−/− mice (data not shown). Together, these data led us to conclude that concurrent ablation of IL-17A replaced the predominantly neutrophilic infiltrate of IFN-γ−/− mice with eosinophils.

Quantitatively, the most substantial differences in cellular infiltration were observed among granuloid cell types, although
we also found differences between IFN-γ−/− IL-17A−/− mice and IFN-γ−/− animals in infiltration with other cell types. IFN-γ−/− IL-17A−/− hearts tended to be more infiltrated with F4/80+ CD11b+ macrophages, as well as FcεRIa+ mast cells (Fig. 4B, data not shown).

We examined the spleens and blood of naive IFN-γ−/− IL-17A−/− mice to determine whether these differences in infiltrating leukocyte populations represent pre-existing disparities in the frequencies of these cell subsets prior to the induction of EAM. Comparing the spleens of unimmunized IFN-γ−/− IL-17A−/− and IFN-γ−/− mice, we did not observe differences in the number of eosinophils, regulatory T cells, or dendritic cells (data not shown). We also did not find differences in numbers of effector T cells, NK cells, γδ T cells, monocytes, or B cells (data not shown). In the peripheral blood of naive IFN-γ−/− IL-17A−/− mice, we observed a modest increase in the proportion of eosinophils. Upon mock immunization with PBS/CFA, IFN-γ−/− IL-17A−/− mice did not develop peripheral eosinophilia or eosinophilic cardiac infiltrates (data not shown). These data indicate that IFN-γ−/− IL-17A−/− mice do not exhibit signs of general systemic eosinophilia. From
this, we conclude that eosinophilic infiltration of the hearts of IFN-γ−/−IL-17A−/− mice during EAM is specifically autoimmune, and not a generalized inflammatory disease of the IFN-γ−/−IL-17A−/− strain.

**Eosinophil mediators of severe EAM in IFN-γ−/−IL-17A−/− mice**

To understand the differences in recruitment of granulocytic effectors under coordinate control by IFN-γ and IL-17A, we examined the expression of a variety of cytokines, chemokines, and growth factors in the hearts and autoreactive T cells of IFN-γ−/−IL-17A−/− and IFN-γ−/− mice with EAM. As expected, chemokines, cytokines, and growth factors associated with Th17-dependent recruitment of neutrophils were largely downregulated in the absence of IL-17, in a manner that did not track with cardiac disease (data not shown).

Eosinophil recruitment to sites of inflammation is largely mediated by chemokine utilization of the receptor CCR3 by the ligand CCL11/eotaxin-1 (50). Interrogation of cardiac homogenates of IFN-γ−/−IL-17A−/− hearts during EAM demonstrated substantially greater production of CCL11/eotaxin-1 at day 12, and CCL24/eotaxin-2, compared with WT controls (p < 0.001), at day 21 (Fig. 5A). No differences were detected in CCL26/eotaxin-3 expression at day 12; no expression was detectable at day 21 (data not shown). Altogether, these data indicate that IFN-γ and IL-17A collaborated in suppressing eosinophilic recruitment to the heart, via suppression of CCL11/eotaxin-1 and CCL24/eotaxin-2 production. We did not detect production of CCL11/eotaxin-1 from restimulated splenocyte cultures (data not shown), indicating its likely production by a nonhematopoietic, heart-resident cell type.

**Autoaggressive Th2 deviation in severe EAM in IFN-γ−/−IL-17A−/− mice**

Elevations in the expression of eotaxin CCR3 ligands implied deviation of autoreactive CD4+ T cells to a Th2 phenotype. To further examine whether autoreactive IFN-γ−/−IL-17A−/− CD4+ cells were preferentially differentiating into Th2 cells at the single-cell level, we performed intracellular staining for cytokines of heart-infiltrating CD4+ T cells at day 12 of EAM. CD4+ T cells staining for both IL-4 and IL-13 were increased in the hearts of IFN-γ−/−IL-17A−/− mice, compared with the single-knockout controls, further confirming the expansion of autoaggressive CD4+ cells with a Th2 phenotype in the combined absence of IFN-γ and IL-17A (Fig. 5B, 5C). Together, these data indicate that autoreactive CD4+ cells are constrained from Th2 differentiation by a synergistic action of IFN-γ and IL-17. Intriguingly, this suppressive function appears to be preferentially targeted toward eosinophil-tropic functions of Th2 cells. It further implicates this eosinophilic Th2 deviation in the early mortality of IFN-γ−/−IL-17A−/− EAM. In contrast, IL-5 production in cardiac homogenates, cardiac transcripts, and restimulated splenocytes did not track with eosinophilia or disease in a cognate manner (data not shown). From these findings, we conclude that the collaborative suppression of cardiac eosinophilia by IFN-γ and IL-17A is mediated through eosinotropic chemokines that may be Th2 dependent, rather than through local or systemic expansion of eosinophils by IL-5.

**Genetic ablation of eosinophils reverses the mortality of IFN-γ−/−IL-17A−/− EAM**

To demonstrate conclusively that eosinophils acted as requisite effectors of the severe, fatal cardiac disease in the EAM of IFN-γ−/−IL-17A−/− mice, we crossed onto this background a mutated allele bearing a deletion of the high-affinity double-GATA binding site in the promoter of GATA1. This mutation selectively ablates differentiation of eosinophils, while leaving erythroid lineages unaffected (51). The resulting triple-mutant ΔdblGATA1 × IFN-γ−/−IL-17A−/− mice showed fewer Siglec F+Ly6Gint eosinophils in peripheral blood (Fig. 6A).

Upon induction of EAM, eosinophil-deficient triple-mutant mice were protected from the fatal heart failure observed in IFN-γ−/−IL-17A−/− mice (Fig. 6B). This protection was incomplete, as 5 of 27 triple-mutant animals died before the termination of the experiment on day 21; this survival rate approximately matches that of IFN-γ−/− animals (Fig. 1A, data not shown). Statistically comparing ΔdblGATA1 × IFN-γ−/−IL-17A−/− and IFN-γ−/−IL-17A−/− mice, Mantel–Cox log-rank survival estimates were significantly different (p = 0.0039). We observed similar low rates of mortality in either male or female ΔdblGATA1 × IFN-γ−/−IL-17A−/− mice (data not shown). Surprisingly, the severity of cardiac enlargement or inflammation did not differ between IFN-γ−/−IL-17A−/− and ΔdblGATA1 × IFN-γ−/−IL-17A−/− mice (Fig. 6C, 6D).

Cardiac function was assessed at day 15 by echocardiographic imaging. Consistent with their greater survival, ΔdblGATA1 ×
IFN-$\gamma^{-/-}$ IL-17A$^{-/-}$ mice had substantially improved cardiac function at this timepoint (Fig. 6E). Functional improvement of FS and EF were largely due to diminished wall thickness parameters (IVSD, LVPWTED, LV mass, and relative wall thickness), rather than signs of ventricular dilation. Consistent with this observation, among the primary measurements in M-mode echocardiography (LV end-diastolic dimension, LVESD, IVSD, LVPWTED, and heart rate), the thickness of the LV posterior wall correlated best with the improved survival of $\text{dblGATA1}^{-/-} \times \text{IFN-$\gamma^{-/-}$ IL-17A$^{-/-}$}$ mice (Fig. 6F). Together, these data demonstrate that eosinophils are requisite mediators of the fatally severe myocarditis of IFN-$\gamma^{-/-}$ IL-17A$^{-/-}$ mice, by driving inflammatory hypertrophic cardiomyopathy.

Discussion
We report in this article that in concert with IFN-$\gamma$, IL-17A exerts a protective effect in eosinophilic heart disease. IL-17A has been ascribed protective functions in various other inflammatory diseases, including CD45RB$^{hi}$ T cell transfer colitis (52), dextran...
sulfate sodium colitis (53), and uveitis secondary to spondyloarthritis (54). These results indicate that, in addition to anatomic considerations, IL-17A has divergent pathophysiologic functions, depending on the context of other cytokines.

The paradoxical protective effect of IFN-γ in several models of autoimmune disease provides evidence that IFN-γ can suppress disease, contrary to the expected proinflammatory role of Th1 cells. This paradox had seemingly been resolved by the discovery of the Th17 lineage. IL-23p19-deficient animals were protected from EAE, whereas IL-12p35-deficient animals developed disease comparable to that of controls (21).

We conclude from our prior experiments that the Th17 lineage prompts the pathogenesis of EAM. IL-17A is the prototypic product of Th17 cells, which differentiate through a process requiring TGF-β, IL-6, and IL-23 (17, 18, 55). Although IL-17A is not essential for the inflammatory process of EAM, it instead drives cardiac remodeling, fibrosis, and DCM development (13).

In WT mice, both Th1 and Th17 cells infiltrate the heart during EAM, but Th17 cells produce higher levels of inflammatory cytokines, such as TNF-α, that have previously been shown to drive cardiac inflammation (13). In addition, we have found that Th17-polarized cells are sufficient to transfer myocarditis (G.C. Baldeviano and D. Číháková, unpublished observations). Our data remain consistent with the idea that Th17 cells mediate the severe disease of IFN-γ−/− animals, as evidenced by enhanced neutrophil infiltration, as well as increased cardiac production of G-CSF and IL-6 in IFN-γ−/− mice (data not shown).

The myocarditic phenotype observed in IFN-γ−/− IL-17A−/− mice is the most severe yet reported, even more so than the EAM of IFN-γ−/−, IFN-γRI−/−, or IL-13−/− animals (28, 44, 56). The rapid mortality begins at approximately day 14, shortly after the emergence of inflammatory cardiac lesions in WT mice, consistent with the myocarditic process being the cause of death in IFN-γ−/− IL-17A−/− animals. At this timepoint, we observed a greater proportion of degranulating eosinophils, continuing to extensive eosinophilic infiltrates by day 21, at which point eosinophils constitute roughly a third of infiltrating cells. Most importantly, concurrent developmental ablation of eosinophils improved survival of IFN-γ−/− IL-17A−/− mice.

Upstream of this fatal eosinophilic recruitment, we observed enhanced Th2 deviation in IFN-γ−/− IL-17A−/− mice. Th2 deviation in IFN-γ−/− mice corresponds to the classical Th1/Th2 model (57). We have found novel evidence that IL-17 signaling collaborates with IFN-γ in the suppression of production of IL-4 and IL-13 by autogressive Th2 cells. Although other Th2 and eosinophilic autoimmune disease models have been described (58–65), to our knowledge this is the first that has been elicited by an intrinsically deviated response. Moreover, these data conclusively demonstrate a primary role for eosinophils in eliciting the rapid mortality of this deviated autoimmune response. The transcription factor eomesodermin has recently been reported to be a critical regulator of this regulatory hierarchy among the Th2 cytokines, by selectively suppressing targeting of the Th2 transcription GATA3 to the Il5 locus in memory Th2 cells (66). These data provide novel evidence that IFN-γ and IL-17A are selectively collaborative in controlling eosinophilic effector functions of the Th2 response.

Importantly, we found evidence for pathophysiologic contributions of eosinophils that directly affect survival, which segregate away from effects on net cardiac inflammation. Eosinophil-deficient ΔdblGATA1 × IFN-γ−/− IL-17A−/− mice developed severe cardiac infiltrates and inflammation, comparable to that in IFN-γ−/− IL-17A−/− mice, but did not die. Importantly, this protection observed in the triple-mutant genotype was associated with largely restored cardiac function, underscoring the important role of the eosinophil in mediating the death of IFN-γ−/− IL-17A−/− mice. We are further undertaking studies using triple-mutant mice to examine specific eosinophil chemotactic and effector pathways in mediating cardiotoxic mortality.

We interrogated a number of biomarkers to assess alternative deviation pathways that may contribute to the severe EAM of IFN-γ−/− IL-17A−/− mice. We did not find compelling evidence for Tregs, Th9, nonclassical Th17, or Th22 cells contributing to the disease phenotype of DKO mice (data not shown). We also did not find persuasive evidence of mast cell involvement in the disease of IFN-γ−/− IL-17A−/− mice. Despite the fact that more mast cells were detected in IFN-γ−/− IL-17A−/− hearts, levels of intracardiac histamine and serum IgE did not track with disease (data not shown). Although these data do not definitively rule out the participation of these pathways, they emphasize that eosinophilic Th2 deviation is primarily responsible for the severe, fatal disease of IFN-γ−/− IL-17A−/− mice.

Among the types of myocarditis seen in patients, eosinophilic necrotizing myocarditis has one of the worst prognoses, often leading to rapid cardiac failure and death (6, 67, 68). Most often, eosinophilic myocarditis in patients is seen as a sequela of eosinophilia-associated systemic disease, in which infiltration is typically perivascular, with less profound damage to cardiomyocytes and better prognosis (69). However, necrotizing eosinophilic myocarditis may not be associated with increased numbers of peripheral eosinophils in the periphery. It progresses quickly, with severe eosinophilic infiltration in the myocardium, severe cardiomyocyte necrosis, intraventricular thrombi, and poor prognosis (69–71). All of these features were observed as part of the necrotizing eosinophilic myocarditis in IFN-γ−/− IL-17A−/− mice.

In a broader context, we can further envision that autoimmune diseases, as a whole, may be more heterogeneous than the Th17-centered view popularized since the discovery of the cell lineage. Th1 cells have been described as potentiating autoimmune effector functions in several models, albeit to a generally lesser degree than Th17 cells (22, 24, 72). Clearly, the pathogenicity of various cell lineages is linked to the location and inflammatory context of the model system in question. Our data argue for substantially greater redundancy among autoaggressive effector T cell subsets than has been previously appreciated.

It remains to be seen whether this redundancy is true for other autoimmune disease models. IFN-γ−/− IL-17A−/− mice were not protected from EAE, nor did they develop accelerated disease (73). This fundamental disjoint between EAE and EAM may reside in the fact that the CNS lies behind the blood–brain barrier and is historically regarded as a site of immune privilege. It is evident that different anatomic compartments possess different mechanisms to suppress inflammatory responses. Our data suggest that what we view as a predominantly Th1/Th17 response may be beneficial, insofar as it suppresses a potentially more devastating outcome. Because eosinophilic myocarditis is recognized as a distinct clinical entity with a particularly poor prognosis, our findings indicate that shared etiologic mechanisms may result in a qualitatively diverse spectrum of disease outcomes, depending on T cell polarization and the downstream effector mechanisms involved.

These findings may have further implications for the deviation strategies entering clinical use as the first generation of IL-12/23p40 antagonists are deployed for use in treating autoimmune disease. Caution may be warranted in the investigational use of these drugs in diseases with the potential for eosinophilic deviation.
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Disclosures

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References


