Interleukin-17A Is Dispensable for Myocarditis but Essential for the Progression to Dilated Cardiomyopathy

G. Christian Baldeviano, Jobert G. Barin, Monica V. Talor, Sachin Srinivasan, Djahida Bedja, Dongfeng Zheng, Kathleen Gabrielson, Yoichiro Iwakura, Noel R. Rose, Daniela Cihakova

Rationale: One-third of myocarditis cases progresses to dilated cardiomyopathy (DCM), but the mechanisms controlling this process are largely unknown. CD4+ T helper (Th)17 cells have been implicated in the pathogenesis of autoimmune diseases, but the role of Th17-produced cytokines during inflammation-induced cardiac remodeling has not been previously studied.

Objective: We examined the importance of interleukin (IL)-17A in the progression of myocarditis to DCM using a mouse model.

Methods and Results: Immunization of mice with myocarditogenic peptide in complete Freund’s adjuvant induced the infiltration of IL-17A–producing Th17 cells into the inflamed heart. Unexpectedly, IL-17A–deficient mice developed myocarditis with similar incidence and severity compared to wild-type mice. Additionally, IL-17A deficiency did not ameliorate the severe myocarditis of interferon (IFN)γ-deficient mice, suggesting that IL-17A plays a minimal role during acute myocarditis. In contrast, IL-17A–deficient mice were protected from postmyocarditis remodeling and did not develop DCM. Flow cytometric and cytokine analysis revealed an important role for IL-17A in heart-specific upregulation of IL-6, TNFα, and IL-1β and the recruitment of CD11b+ monocyte and Gr1+ granulocyte populations into the heart. Furthermore, IL-17A–deficient mice had reduced interstitial myocardial fibrosis, downregulated expression of matrix metalloproteinase-2 and -9 and decreased gelatinase activity. Treatment of BALB/c mice with anti–IL-17A monoclonal antibody administered after the onset of myocarditis abrogated myocarditis-induced cardiac fibrosis and preserved ventricular function.

Conclusions: Our findings reveal a critical role for IL-17A in postmyocarditis cardiac remodeling and the progression to DCM. Targeting IL-17A may be an attractive therapy for patients with inflammatory dilated cardiomyopathy. (Circ Res. 2010;106:1646-1655.)

Key Words: IL-17A ■ Th17 ■ myocarditis ■ dilated cardiomyopathy ■ autoimmunity

Myocarditis, an inflammatory disease of the heart muscle, is commonly associated with cardiotropic infections, and has been linked to the development of autoimmunity.1-3 Innate immune cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-6, and IL-1β are essential for the development of experimental autoimmune myocarditis (EAM), a mouse model of myocarditis.3,5 Although the clinical manifestations of myocarditis are heterogeneous, one-third of the cases progress toward dilated cardiomyopathy (DCM). Myocarditis-induced DCM represents a significant cause of sudden death in young adults2,3; nevertheless, little is known about the mechanisms involved in postinflammatory cardiac remodeling leading to DCM.

Studies in ischemic injury models suggest that maladaptive remodeling is linked with the upregulation of transforming growth factor (TGF)β.5 It is now recognized that alterations in the myocardial extracellular matrix by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are critical during myocardial fibrosis and cardiac remodeling.6,7 However, the upstream molecules controlling these common mechanisms are yet to be fully characterized.6-7 The identification of pathways involved in the progression to cardiomyopathy is essential for the development of novel immunotherapies for patients with inflammatory heart diseases.

CD4+ T helper lymphocytes are essential for the development of autoimmune disease in various experimental models, including EAM.2 CD4+ T cells differentiate into discrete subsets that mediate unique effector functions. T-helper (Th1) cells were once considered the major pathogenic subset mediating organ-specific autoimmune diseases8; however,
genetic deficiency of critical molecules in the Th1 pathway failed to prevent autoimmunity.\(^9,10\) Th17 cells have been recently implicated in the pathogenesis of autoimmune diseases\(^11-13\); however, there is conflicting data regarding the role of Th17-secreted cytokines in the pathogenesis of autoimmune disease in various models.\(^14-17\) Furthermore, little is known about the role of IL-17A in the process of postinflammatory tissue remodeling leading to end-stage organ failure. In this report, we examine the role of IL-17A in the development of myocarditis, and the progression from acute myocarditis to DCM. We found that inflammatory myocarditis and dilated cardiomyopathy can be uncoupled by IL-17A deficiency. Although IL-17A plays a minor role in acute myocarditis, it represents a critical determinant of progression to DCM.

**Methods**

**Mice**

Six- to 10-week-old male wild-type (WT), Ifng\(^{-/-}\), and DO11.10 transgenic mice all on the BALB/cJ background were obtained from The Jackson Laboratory (Bar Harbor, ME). Il17a\(^{-/-}\) mice were previously described.\(^11\) Il17a\(^{-/-}\) Ifng\(^{-/-}\) double-deficient and Il17a\(^{-/-}\) DO11.10 transgenic mice were generated by intercrossing single mutant or transgenic mice. Procedures were approved by the Johns Hopkins Animal Care and Use Committee.

**Induction and Assessment of Myocarditis**

Mice were subcutaneously immunized with 100 \(\mu\)g of myocarditogenic peptide (MyHC\(_{614-629}\), Ac-SLKLMATLFSTVASAD, GenScript) emulsified in complete Freund’s adjuvant (CFA) (Sigma-Aldrich, St Louis, Mo) on days 0 and 7.\(^19\) To induce dendritic cell–induced emulsified in complete Freund’s adjuvant (CFA) (Sigma-Aldrich, St Louis, Mo) on days 0 and 7.\(^19\) To induce dendritic cell–induced myocarditis, we used a previously published protocol.\(^20\) Myocarditis severity and fibrosis was evaluated by using a 1 to 5 scoring system,\(^19\) or using the Frida software (http://bui2.win.ad.jhu.edu/frida).

**Intracardiac Flow Cytometric Analysis**

Mice were anesthetized with avertin, and the aorta was cannulated to perfuse the hearts, which were then digested with collagenase IV and protease. Isolated cells were stained with fluorochrome-conjugated antibodies against CD45, CD4, CD8, CD3, CD19, CD62L, CD44, CD11b, major histocompatibility complex class II (I-A/I-E), CD11c, Gr1, Ly6C, Ly6G, CCR3, SiglecF (BD Pharmingen or eBiosciences). CD11b, major histocompatibility complex class II (I-A/I-E), CD11c, Gr1, Ly6C, Ly6G, CCR3, SiglecF (BD Pharmingen or eBiosciences).

**Lymphocyte Proliferation Assay**

T cell proliferation was assessed as previously described.\(^19\) Briefly, mice were immunized as above, and spleens collected on day 21 pi. Splenocytes were cultured with titrating amounts of MyHC\(_{614-629}\) peptide for 72 hours and pulsed with 0.5 Ci of \(^{3}H\)methyl-thymidine 16 hours before reading by a beta counter.

**Measurement of Cytokines and Chemokines**

Hearts were homogenized in media containing 2% of PBS and protease inhibitors (Sigma-Aldrich). Supernatants were collected after centrifugation and stored at \(-80^\circ\)C. The concentration of cytokines and chemokines was measured by using a Lumines XMAP-based mouse cytokine array kit (Invitrogen). In some cases, cytokine levels were confirmed by Quantikine ELISA kits (R&D Systems).

**MyHC\(_{614-629}\)–Specific ELISA**

Sera were collected on days 0 and 21 by retro-orbital venous puncture and stored at \(-80^\circ\)C. The levels of MyHC\(_{614-629}\)–reactive antibodies were determined using microtiter plates coated with 0.5 \(\mu\)g of MyHC\(_{614-629}\) peptide with phosphatase conjugated isotype-specific secondary antibodies (BD Pharmingen). Adjusted optical density measured at 450 nm (OD\(_{450}\)) was calculated by subtracting OD\(_{450}\) of negative control (nonimmunized mice) to mean of triplicate samples.\(^19\)

**Reverse Transcription–Polymerase Chain Reaction**

One microgram of RNA was digested with DNAsase (Fermentas), and converted into cDNA using M-MuLV reverse transcriptase (Fermentas). Primer sets for Col1a2, Col3a1, Mmp2, Mmp9, Timp1, and Timp4 were previously validated by the manufacturer (SA Biosciences, Frederick, Md). cDNA was amplified by SYBR green master mix using an ABI7000 Thermocycler (Applied Biosystems). Gene expression was normalized to \(\beta2\) microglobulin.

**Gel Zymography**

Heart tissues were homogenized in lysis buffer (25 mmol/L Tris-HCl, pH 7.5; 100 mmol/L NaCl; and 1% vol/vol Nonidet P-40), and cell debris removed by centrifugation. Total protein was quantified by BCA protein assay kit (Thermo scientific). Twenty-five micrograms of total protein were loaded into a precast 10% Tris-Glycine gel with 0.1% gelatin (Invitrogen). Gels were developed and stained as reported elsewhere.\(^21\)

**Transthoracic Echocardiography**

A Vevo 770 high-resolution micromaging system with a 38 MHz transducer was used (Visualsonic, Toronto, Ontario, Canada). In conscious mice, the heart was imaged in the 2D mode in the parasternal short-axis view. From the M-mode, the left ventricular wall thickness and chamber dimensions were measured.\(^19,22\)

**In Vivo Neutralization of IL-17A**

Groups of BALB/cJ mice were treated with 100 \(\mu\)g of anti-IL-17A monoclonal antibody intraperitoneally (clone: 50104), 100 \(\mu\)g of IgG2a isotype control (clone: 50104) (R & D systems) or PBS every other day starting on day 15 through 27 pi.

**Statistical Analysis**

Normally distributed data were analyzed by unpaired 2-tailed heteroscedastic Student’s \(t\) test (2 groups) or by 1-way ANOVA analysis (more than 2 groups). ANOVA analysis was followed by Bonferroni’s multiple comparison tests. EAM and fibrosis scores were analyzed by Mann–Whitney \(U\) test. Differences were considered significant if probability value was less than 0.05.
Results

Th17 Cells Infiltrate the Inflamed Heart During Myocarditis

To determine the frequency of lymphocyte subsets during EAM, we immunized BALB/cJ mice with myocarditogenic peptide in complete Freund’s adjuvant (MyHC614-629/CFA) and performed intracellular staining of heart-infiltrating leukocytes. On day 14 (myocarditis onset), an average of 13% of CD4⁺ T cells secreted IL-17A whereas 8% produced IFNγ (Figure 1a and 1b). By day 21 (peak of myocarditis), the percentage of IFNγ⁺ CD4⁺ T cells increased to 11% whereas the frequency of IL-17A⁺ CD4⁺ T cells remained relatively unchanged. In contrast, CD8⁺ lymphocytes mostly produced IFNγ (Figure 1a and 1b).

To gain insight into the function of Th1 and Th17 cells, we assessed their capacity to cosecrete TNFα and IFNγ. By day 21 (peak of myocarditis), the percentage of IFNγ⁺ CD4⁺ T cells increased to 11% whereas the frequency of IL-17A⁺ CD4⁺ T cells remained relatively unchanged. In contrast, CD8⁺ lymphocytes mostly produced IFNγ (Figure 1a and 1b). To gain insight into the function of Th1 and Th17 cells, we assessed their capacity to cosecrete TNFα and IFNγ. Although TNFα tended to be higher in IFNγ-secreting cells, the frequency of IL-6+ and TNFα+ IL-6+ coproducers were significantly higher in IL-17A-secreting CD4⁺ T cells compared to IFNγ-producing CD4⁺ T cells (Figure 1c). Furthermore, IL-17A⁺ CD4⁺ T cells secreted lower amounts of IL-10 compared to IFNγ⁺ CD4⁺ T cells (Figure 1d). These data suggest that both Th1 and Th17 cells infiltrate the inflamed heart but Th17 cells display a higher pathogenic phenotype.

IL-17A Is Dispensable for the Development of Myocarditis

Because Th17 cells secrete IL-17A, we examined the specific role of IL-17A in the development of EAM. We immunized groups of WT and IL-17A⁻/⁻ deficient mice (Il17a⁻⁻) with MyHCα614-629/CFA, and monitored myocarditis at various time points postimmunization (pi). Unexpectedly, II17a⁻⁻ mice developed EAM with incidence and severity similar to those of WT controls at day 21 pi (Figure 2a) and at days 14 or 35 pi (not shown). To quantitatively assess myocardial infiltration, we used Frida software to estimate the number of pixels involving cellular infiltration per area of tissue. Because CFA is a strong adjuvant, which may upregulate compensatory pathways, we determined the role of IL-17A in a CFA-free model. We induced myocarditis by transferring matured, MyHC614-629⁻ pulsed DC11c⁻ bone marrow-derived dendritic cells (MyHC614-629-BMDCs). Consistent with myocarditogenic peptide-induced myocarditis, we found no difference in the severity of myocarditis when MyHCα614-629⁻ BMDCs were transferred into syngeneic WT or II17a⁻⁻ recipients (Figure 2b). Therefore, our results suggest that IL-17A is not essential for the development of inflammatory myocarditis.

IL-17A Is Not Critical for Myosin-Specific Immune Responses During Myocarditis

Because IL-17A deficiency has been associated with impaired T cell priming, we measured the in vitro proliferative responses of splenocytes from MyHCα614-629⁻ immunized WT and II17a⁻⁻ mice. On peptide restimulation, II17a⁻⁻ splenocytes tended to proliferate less compared to WT splenocytes; however, this difference was not statistically significant (Figure 2c). To clarify the role of IL-17A in the activation/expansion of antigen-specific CD4⁺ T cells, we...
crossed Il17a−/− mice with transgenic mice expressing a major histocompatibility complex class II–restricted T cell receptor specific for a peptide from the ovalbumin protein (DO11.10 mice) to generate Il17a−/−DO11.10 mice. On transfer to WT or Il17a−/− syngeneic recipients, Il17a−/−DO11.10 CD4+ cells activated and proliferated similarly to WT DO11.10 CD4+ cells in response to OVA323 to 339/CFA immunization (Online Figure I). Thymidine incorporation assay was performed to measure MyHC614-629–specific proliferation (c). ELISA was used to measure MyHC614-629–specific IgG levels (d). Il17a−/−Ifng−/− mice was immunized as above (e). Each dot represents 1 mouse, and bars are group means (c and d) or group median (a, b, and e) of 2 to 3 independent experiments. *P<0.01 by unpaired 2-tailed t test.

**Figure 2. IL-17A is dispensable for the development of EAM.** Groups of WT and Il17a−/− BALB/c mice were immunized with MyHC614-629/CFA (a, c, and d) or injected IP with matured, MyHC614-629–pulsed BMDCs (b). EAM scores were assessed 21 days pi (a and e) or 14 days pi (b). Thymidine incorporation assay was performed to measure MyHC614-629–specific proliferation (c). ELISA was used to measure MyHC614-629–specific IgG levels (d). Il17a−/−Ifng−/− mice was immunized as above (e). Each dot represents 1 mouse, and bars are group means (c and d) or group median (a, b, and e) of 2 to 3 independent experiments. *P<0.01 by unpaired 2-tailed t test.

**IL-17A Does Not Contribute to Severe Myocarditis in IFNγ-Deficient Mice**

We previously found that IFNγ-deficient (Ifng−/−) mice developed uniquely severe EAM.2,9 As IL-17A was upregulated in Ifng−/− mice with EAM (data not shown), we investigated the involvement of IL-17A in the severity of EAM in Ifng−/− mice by generating IL-17A x IFNγ double-deficient mice (Il17a−/−Ifng−/−). In contrast to midgrade EAM scores in Il17a−/− and WT mice, Il17a−/−Ifng−/− mice developed severe EAM, similar to those of Ifng−/− mice (Figure 2e). Therefore, IL-17A does not contribute to the severity of EAM in Ifng−/− mice.

**IL-17A Is essential for the Progression to Dilated Cardiomyopathy**

In the chronic stage of EAM (after day 35 pi), immunized mice develop ventricular dilation and progressive impairment of ventricular function.9,29,30 To investigate the role of IL-17A in the progression to DCM, we performed longitudinal echocardiography following MyHC614-629/CFA immunization. As expected, WT mice showed progressive dilation of the left ventricle. The average left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) increased by 53% and 16% respectively on day 62 compared to day 24 pi (Figure 3a and 3b and data not shown). In sharp contrast, Il17a−/− mice were completely protected from ventricular dilation (Figure 3a and 3b). In addition, WT mice showed an average decrease of 30% in fractional shortening (FS) and 25% in ejection fraction (EF) (Figure 3c and 3d), suggesting compromised contractile performance.9,29 Conversely, Il17a−/− mice showed no deterioration in FS or EF comparing days 62 versus 24 pi (Figure 3c and 3d). Indeed, average measurements of EF and FS in Il17a−/− mice on day 62 pi were indistinguishable from those of nonimmunized mice (EF: 57% versus 52%, P=0.164; FS: 81% versus 76%, P=0.67). These findings indicate that although WT and Il17a−/− displayed similar inflammatory myocarditis, IL-17A deficiency prevented the progression of myocarditis to DCM.

**IL-17A Promotes the Production of Proinflammatory Cytokines in the Heart**

To gain new insights into the mechanism of protection from DCM, we examined whether IL-17A deficiency affected the cytokine milieu in the heart. On day 14 following MyHC614-629/CFA, Il17a−/− heart homogenates had significantly reduced levels of the proinflammatory cytokines IL1β, IL-6, TNFα, IL-18, and IFNγ (Figure 4a). No difference was found in the levels of IL-1α, IL-4, IL-9, IL-12p40, IL-10, or IL-13 (Figure 4a and data not shown). On day 21, Il17a−/− hearts showed a trend toward decreased levels of IL-1β, TNFα, IL-18 and IFNγ; but these differences were not statistically significant (Figure 4a and 4c). Notably, IL-6 remained substantially reduced at days 14 and 21 pi (Figure 4a and 4c). Furthermore, the levels of IL-17A–related cytokine IL-17F were similar between WT and Il17a−/− hearts on days 14 and 21 (Figure 4a and 4c), indicating that there was no compensatory upregulation of IL-17F in Il17a−/− mice.
TGFβ1 is critical for maladaptive fibrosis in models of myocardial infarction.5,7 Active TGFβ1 was decreased in Il17a−/− hearts on day 14 pi but not on day 21 pi (Figure 4a and 4c). Thus, protection from DCM in Il17a−/− mice is associated with tissue-specific abrogation of proinflammatory/profibrotic molecules, including IL1β, IL-6, TNFα, and TGFβ1, during myocarditis.

**IL-17A Regulates the Chemokine-Mediated Recruitment of Myeloid Populations to the Heart During Myocarditis**

Histopathology revealed similar degree of infiltration between Il17a−/− and WT hearts with myocarditis. Here, we determined the cellular composition of heart-infiltrating leukocytes. Consistent with previous findings, no significant difference was found in the absolute numbers of heart-infiltrating CD45+ leukocytes (Figure 5a). However, the frequency of myeloid populations, including CD11b+/Gr1− monocytes and Gr1− granulocytes, were significantly reduced in the hearts of Il17a−/− mice (Figure 5e and 5f). Among the granulocyte population, the percentage of Gr1intLy6GlowSiglecFhiCCR3hi eosinophils were not different (not shown). We found no decrease in the percentage of monocytes or granulocytes in the spleens or peripheral blood of Il17a−/− mice (Online Tables I and II), suggesting impaired recruitment of these populations rather than defective development or survival. In support of this argument, Il17a−/− hearts exhibited decreased amounts of the myelotropes chemokines CCL2/MCP1, CCL3/MIP1α, CXCL10/IP-10, CXCL1/KC, and CCL5/RANTES (Figure 4b and 4d). We did not find differences in CXCL2/MIP2α, CXCL9/MIG, or CXCL5/LIX (Figure 4b and 4d). Additionally, Il17a−/− hearts displayed a marked decreased in the secretion of the myelopoietic factors GM-CSF (granulocyte/macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), and M-CSF (macrophage colony-stimulating factor) (Figure 4b and 4d). There was a trend toward increased percentages of lymphocytes but these differences were not statistically significant (Figure 5b through 5d).

CD4+ T cells in Il17a−/− hearts displayed a higher activation status (Figure 5h); however, the frequency of Foxp3+CD4+ T cells, representing regulatory T cells (Tregs), was not different in the hearts or spleens of Il17a−/− at days 14 or 21 pi (Figure 5i), suggesting that the numbers of Tregs are not regulated by IL-17A. Therefore, IL-17A modulates the chemokine-dependent recruitment of myeloid populations into the heart during the progression of EAM to DCM.

**Protection From DCM in IL-17A-Deficient Mice Is Associated With Decreased Myocardial Fibrosis**

Increased extracellular matrix turnover and dysregulation of matrix metalloproteinases are hallmarks of maladaptive remodeling.5,30 Masson’s trichrome staining revealed greatly reduced interstitial collagen deposits in Il17a−/− hearts compared to WT hearts (Figure 6a). Moreover, gene expression analysis indicated a significant decrease in the expression of col1a2 (type I) and col3a2 (type III collagen) in Il17a−/− hearts (Figure 6b). Notably, transcripts for Mmp2 (gelatinase A) and Mmp9 (gelatinase B) were also substantially diminished. Conversely, the expression of Timp1 and Timp4 were increased in Il17a−/− hearts (Figure 6b). Because MMPs are also post-transcriptionally regulated,6,7,31 we performed gel zymography studies. Myocardial tissues from Il17a−/− mice displayed severely reduced gelatinolytic activity (Figure 6c and 6d), including reduced activity of both MMP-2 and MMP-9. Therefore, decreased myocardial fibrosis in IL-17A–deficient mice correlated with decreased expression and activity of MMP-9 and MMP-2.

**Neutralization of Endogenous IL-17A Preserves Systolic Function in Mice With Established Myocarditis**

We examined weather treatment with an IL-17A–neutralizing antibody (αIL-17A monoclonal antibody [mAb]) after the onset of myocarditis prevented DCM. We treated diseased WT BALB/cJ mice (EAM, day 14 pi) with αIL-17A mAb, IgG2a isotype control (IgG2a), or vehicle (PBS) from days 15 through 27 pi and performed echocardiography on day 36 pi. Treatment with αIL-17A mAb significantly reduced both LVEFs (Online Figure III, a) and LVEDd compared to PBS group or IgG2a group (ANOVA: F2,21=5.34, P=0.013) (Figure 7a). In addition, αIL-17A mAb significantly im-
proved FS (ANOVA: $F_{2,21} = 5.64, P = 0.011$) (Figure 7b), EF (Online Figure III, b), and reduced cardiac hypertrophy (Online Figure III, c and d). Furthermore, Masson’s trichrome staining revealed abrogation of interstitial fibrosis following treatment with anti-IL-17A mAb (ANOVA: $F_{2,21} = 14.33, P = 0.0001$) (Figure 7c and 7d). We performed a follow-up measurement at day 65 pi. Even at this time point, we observed an increase in EF in the anti-IL-17A mAb group (PBS: 65.7±5.9%, IgG2a: 66.7±2.9%, anti-IL-17A: 76.18±4.9%); however, this difference did not attain statistical significance (ANOVA: $F_{2,21} = 1.97, P = 0.165$) because 2 mice from the anti-IL-17A mAb treatment group reversed to DCM. In summary, anti-IL-17A antibodies administered in mice with established myocarditis abrogated myocardial fibrosis, decreased ventricular dilation and preserved cardiac function.

Discussion
Th17 cells are implicated in the pathogenesis of autoimmune diseases in mice, as well as humans.11–13,32 Although Th17 cells secrete IL-17A, conflicting results have been published.
Figure 5. IL-17A controls the recruitment of myeloid cells into the heart. Frequency of various heart-infiltrating leukocytes populations were determined by flow cytometry at peak of myocarditis (21 days pi). Absolute numbers (a) or relative numbers (b through i) are depicted, where each dot represents an individual mouse and bars indicate group means. Statistics were obtained by unpaired 2-tailed t test (*P<0.01).

Regarding the role of this cytokine in various models of autoimmune inflammation, here, we show that IL-17A is not essential for the development of autoimmune myocarditis. In contrast, our results demonstrate a critical role for IL-17A in the development of myocardial fibrosis and cardiac remodeling leading to end-stage DCM.

A previous study showed that neutralization of IL-17A reduced myocarditis severity. Our results, however, clearly
demonstrate that genetic ablation of IL-17A had no significant impact on the incidence or severity of myocarditogenic peptide- or dendritic cell-induced myocarditis. In line with our results, others have found that Il17a−/− mice developed experimental autoimmune uveitis with similar severity to WT mice.14,15 Additionally, Haak et al reported that IL-17A deficiency or IL-17A overexpression did not significantly impact the incidence or severity of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis.17

Autoimmune diseases have so far been associated with Th1 and Th17 cells.11–15 Here, we show for the first time that a severe autoimmune disease can be elicited in the absence of IFNγ and IL-17A, the prototypic Th1 and Th17 cytokines. These results also suggest that upregulation of IL-17A does not contribute to the severity of disease in Ifng−/− mice. In contrast to our findings, a previous report proposed that IL-17A was a critical mediator of the severity of EAM in T-bet-deficient mice.20 It is possible that T-bet may play a more dominant role in regulating pathogenic Th17 cells in comparison to IFNγ. We are currently investigating the mechanisms that drive myocarditis in Ifng−/−Il17a−/− double-deficient mice, which may identify novel pathways independent of Th1/Th17 effector function.

What drives EAM in the absence of IL-17A? First, deficiency in IL-17A does not affect the differentiation of Th17 cells, or the secretion of other effector cytokines by these cells (eg, TNFα, IL-22 or IL-17F).35 Because both IL-17A and IL-17F signal through the same heteromeric receptor,36 it is possible that receptor ligation by IL-17F may be sufficient to induce significant proinflammatory signals in Il17a−/− mice, resulting in significant myocarditis inflammation. Alternatively, it is possible that secretion of IL-17A by Th17 cells is simply not required for their pathogenic properties. This idea is further supported by a report, wherein Th17 cells elicited in the presence of TGFβ and IL-6 but in the absence of IL-23 were not able to induce EAE, regardless of their abundant secretion of IL-17A.37

We show that IL-17A was essential for the progression of myocarditis to DCM. IL-17A was required for the sustained expression of IL-6 during myocarditis, and reduced cardiac IL-6 was associated with arrested DCM development. It is plausible that the upregulation of IL-6 represents a mechanism downstream of IL-17A contributing to the development of DCM, possibly by promoting myocardial fibrosis. In a previous study, neutralization of IL-6 ameliorated fibrosis and improved heart function during chronic cardiac allograft rejection.38 Further experiments are needed to confirm the specific role of IL-6 in the progression to DCM. Additionally, we show that IL-17A is important for the recruitment of Gr1+ granulocytes into the heart. We have previously observed that the proportion of granulocytes positively correlated with deterioration of cardiac systolic function,29,39 highlighting a potential contribution of these cells during cardiac remodeling leading to DCM. We also explored a potential role for regulatory mechanisms. Neither the levels of IL-10 nor the frequency of regulatory T cells was increased in the hearts or spleens of IL-17A−/− mice. Therefore, it is unlikely that protection from DCM could be explained by dysregulation of these pathways; although we cannot rule out a potential contribution for highly-localized or cell-specific differences in these regulatory pathways.

Protection from DCM in IL-17A deficient mice was also associated with reduced gene transcription and gelatinase activity of MMP-2 and MMP-9. Elevated amounts of Mmp2 and Mmp9 have been found in left ventricular samples of patients with nonischemic cardiomyopathy.40 Additionally, treatment with a specific inhibitor of MMP-9 was able to suppress myocarditis and DCM in a rat model of experimental autoimmune carditis.41 These observations suggest that IL-17A may contribute to fibrosis by directly signaling through heart-resident cells to promote remodeling of the myocardial extracellular matrix. Supporting this hypothesis, IL-17A has been shown to induce the secretion of MMPs from cardiac fibroblasts.42 However, it is unknown whether IL-17A exerts direct effects on cardiomyocytes. We are currently studying the functional consequences of IL-17A signaling on these cells.

While this work was under review, Wilson et al reported similar findings in experimental models of lung fibrosis.43 Interestingly, their study provided further evidence that IL-17A–dependent mechanisms including lung inflammation (secretion of TNFα and IL-1β), recruitment of neutrophils into the airways, and activation of MMP-2 and MMP-9 all critically mediate pulmonary fibrosis.43 This study, together with ours, makes a compelling case that IL-17A controls fundamental processes underlying tissue fibrosis and remodeling during various fibrotic pathologies.

In contrast to other autoimmune diseases such as rheumatoid arthritis or psoriasis, there is no immunomodulatory therapy for autoimmune myocarditis. Myocarditis in humans...
is highly heterogeneous in etiology; however, recent reports indicate that a subgroup of myocarditis patients may benefit from immune-targeted therapies. Patients with DCM have increased levels of Th17 cytokines in their blood, including increased IL-17A, IL-6, and IL-23. Our data suggest that a treatment with IL-17A—antagonizing agents may prevent or slow down the progression to DCM in patients with established myocarditis.

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**Disclosures**

None.

**References**


Novelty and Significance

**What Is Known?**

- Th17 cells have been implicated in the pathogenesis of certain inflammatory autoimmune diseases in animal models as well as in humans.
- Patients with dilated cardiomyopathy have elevated levels of Th17 cytokines in their blood, including increased IL-17A, IL-6 and IL-23.
- Little is known about the steps leading from cardiac inflammation to dilated cardiomyopathy.

**What New Information Does This Article Contribute?**

- Using a mouse model, we found that IL-17A is not required for the development of inflammatory autoimmune myocarditis.
- This study shows for the first time that IL-17A is critical for the progression from myocarditis to dilated cardiomyopathy.
- We report a novel pro-fibrotic role for IL-17A in promoting myocardial fibrosis by the upregulation of MMP-2 and MMP-9.

Dilated cardiomyopathy (DCM) is a major cause of heart failure in individuals below the age of 40. It is associated with high mortality and is, therefore, a major indication for cardiac transplantation. Although there is likely a causal relationship between myocarditis and some cases of subsequent DCM, little is known about the pathogenesis of post-myocarditis DCM. We report here that mice genetically deficient in IL-17A developed autoimmune myocarditis comparable in severity to control mice, but surprisingly IL-17A–deficient mice were protected from developing DCM. IL-17A promotes DCM by mechanisms including: (1) secretion of proinflammatory cytokines such as IL-6; (2) migration of granulocytes and monocytes to the heart; and (3) induction of myocardial fibrosis and upregulation of MMP-2 and MMP-9. Finally, we were able to decrease the progression of established myocarditis to DCM in mice by inhibiting IL-17A. These findings reveal a novel role for IL-17A in cardiac remodeling and fibrosis and suggest that targeting IL-17A may minimize postmyocarditis fibrotic changes and preserve cardiac function in patients with established myocarditis.
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Supplement material

(a) CFSE-labeled WT DO11.10 CD4+ T cells
(2 x 10^9)

WT BALB/c

day-3

Day 0

Day 6

CFSE-labeled Il17α-/- DO11.10 CD4+ T cells
(2 x 10^9)

Il17α-/- BALB/c

CFSE

Ova

CFA

Activation

Proliferation

Apoptosis

(b) Inguinal LN

WT

Il17α-/-

Unimmunized

0.09 %

0.09 %

Peripheral blood

WT

Il17α-/-

Ova

CFA

0.83 %

1.83 %

K1264

CD4+

1.4 %

2.43 %

(c) CD2L1 (CD40L) (%CD4+KJ126+)

WT

Il17α-/-

p = 0.036

(d) CFSE

(%CD4+KJ126+)

WT

Il17α-/-

p = 0.062

(e) TAD-AnnV

(%CD4+KJ126+)

WT

Il17α-/-

p = 0.073

Online Figure I; Baldeviano GC et al.
Online Figure I. IL-17A deficiency does not affect the activation, proliferation or expansion of DO11.10 CD4+ T cells in vivo. Purified CFSE-labeled Il17a−/− or WT DO11.10 CD4+ T cells were intravenously transferred into Il17a−/− or WT BALB/c mice (n=4-6 mice per group). After three days, both groups were immunized with OVA323-339/CFA (a). The expansion of KJ1-26+CD4+ cells before and 6 days after immunization with OVA323-339/CFA was monitored by flow cytometry (b). In addition, flow cytometry analysis was used to determine activation status, as indicated by expression of CD44 and L-selectin CD62L (c); proliferation, as assessed by dilution of CFSE (d); and early apoptosis, as indicated by gating on 7AAD−AnnexinVhi (e). Bar graphs show group mean ± SEM, p-value was obtained by two-tailed heteroscedastic student t test.
Online Figure II. Myosin-specific humoral response is altered in II17a−/− mice following immunization. Groups of WT and II17a−/− mice were immunized with MyHC_{614-629}/CFA, sera was collected on day 21 pi, and MyHC_{614-629}-specific IgG1 (a), IgG2a (b), IgG2b (c) and IgG3 (d) autoantibodies were assessed by ELISA (see methods). Plots show individual mouse data and group mean. p-value was obtained by two-tailed heteroscedastic student t test.
Online Figure III. Neutralization of IL-17A after the onset of myocarditis prevents ventricular dilation and preserves systolic function. Groups of BALB/cJ mice with established myocarditis (by day 14 pi) were treated with αIL-17A mAb (closed diamonds), IgG2a isotype control mAb (grey diamonds) or PBS vehicle control (open diamonds). At day 36 pi, left-ventricular end-diastolic diameter (LVESd) (a), ejection fraction (EF) (b), left ventricular mass (c), and relative wall thickness (d) were estimated by echocardiographic analysis. p-value was calculated by one-way ANOVA followed by Bonferroni’s multiple comparison test.
Online Table I: Percentages of leukocyte populations in the spleen of WT and $I{l17a}^{-/-}$ mice on day 21 of EAM

<table>
<thead>
<tr>
<th></th>
<th>WT BALB/c</th>
<th></th>
<th>$I{l17a}^{-/-}$ BALB/c</th>
<th></th>
<th>$p$-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (%)</td>
<td>SEM</td>
<td>Mean (%)</td>
<td>SEM</td>
<td></td>
</tr>
<tr>
<td>Percentage of CD45+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>7.28</td>
<td>0.80</td>
<td>8.32</td>
<td>0.84</td>
<td>0.391</td>
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<tr>
<td>CD8+ T cells</td>
<td>2.87</td>
<td>0.28</td>
<td>3.11</td>
<td>0.40</td>
<td>0.632</td>
</tr>
<tr>
<td>CD19+B220+ B cells</td>
<td>17.38</td>
<td>1.60</td>
<td>23.12</td>
<td>1.39</td>
<td>0.027</td>
</tr>
<tr>
<td>CD11b+ monocytes</td>
<td>57.13</td>
<td>4.18</td>
<td>50.27</td>
<td>2.81</td>
<td>0.216</td>
</tr>
<tr>
<td>CD11b+F480+ Mφ</td>
<td>3.38</td>
<td>0.22</td>
<td>4.23</td>
<td>0.16</td>
<td>0.016</td>
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<tr>
<td>Gr1+ granulocytes</td>
<td>30.34</td>
<td>5.49</td>
<td>29.91</td>
<td>1.70</td>
<td>0.942</td>
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<tr>
<td>CD11c$^{hi}$ dendritic cells</td>
<td>1.43</td>
<td>0.12</td>
<td>2.37</td>
<td>0.12</td>
<td>0.001</td>
</tr>
<tr>
<td>Percentage of lymphoid gate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>21.90</td>
<td>1.58</td>
<td>21.84</td>
<td>0.62</td>
<td>0.972</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>7.36</td>
<td>0.66</td>
<td>6.76</td>
<td>0.47</td>
<td>0.482</td>
</tr>
<tr>
<td>Percentage of CD4+ lymphocytes</td>
<td></td>
<td></td>
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<tr>
<td>CD4+CD44$^{hi}$</td>
<td>15.12</td>
<td>0.59</td>
<td>15.92</td>
<td>0.32</td>
<td>0.321</td>
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<tr>
<td>CD4+CD45RB$^{low}$</td>
<td>57.19</td>
<td>3.05</td>
<td>57.88</td>
<td>1.80</td>
<td>0.851</td>
</tr>
<tr>
<td>CD4+CD44$^{hi}$CD45RB$^{low}$</td>
<td>15.13</td>
<td>0.66</td>
<td>16.44</td>
<td>0.49</td>
<td>0.151</td>
</tr>
<tr>
<td>CD4+CD44$^{hi}$CD62L$^{low}$</td>
<td>10.30</td>
<td>0.58</td>
<td>11.34</td>
<td>0.61</td>
<td>0.251</td>
</tr>
<tr>
<td>CD4+CD44$^{hi}$CD62L$^{hi}$</td>
<td>4.81</td>
<td>0.29</td>
<td>4.55</td>
<td>0.27</td>
<td>0.535</td>
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<tr>
<td>CD4+CD25+foxp3+</td>
<td>18.06</td>
<td>0.82</td>
<td>13.63</td>
<td>0.72</td>
<td>0.004</td>
</tr>
</tbody>
</table>

† Statistics by unpaired two-tailed heteroscedastic student t test
5 - 6 mice per group
Online Table II: Percentages of leukocyte populations in the peripheral blood of WT and *Il17a*−/− mice on day 21 of EAM

<table>
<thead>
<tr>
<th></th>
<th>WT BALB/c</th>
<th></th>
<th>Il17a−/− BALB/c</th>
<th></th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td></td>
</tr>
<tr>
<td><strong>Percentage of CD45+ cells</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CD3+ T cells</td>
<td>9.46</td>
<td>0.89</td>
<td>13.81</td>
<td>2.91</td>
<td>0.190</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>7.65</td>
<td>0.69</td>
<td>11.80</td>
<td>2.52</td>
<td>0.151</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>2.36</td>
<td>0.17</td>
<td>2.63</td>
<td>0.50</td>
<td>0.625</td>
</tr>
<tr>
<td>CD19+B220+ B cells</td>
<td>0.27</td>
<td>0.13</td>
<td>0.21</td>
<td>0.03</td>
<td>0.230</td>
</tr>
<tr>
<td>CD11c&lt;sup&gt;hi&lt;/sup&gt; dendritic cells</td>
<td>0.22</td>
<td>0.02</td>
<td>0.68</td>
<td>0.14</td>
<td>0.008</td>
</tr>
<tr>
<td>CD11b+ monocytes</td>
<td>52.30</td>
<td>3.51</td>
<td>48.62</td>
<td>4.28</td>
<td>0.537</td>
</tr>
<tr>
<td>CD11b+Ly6C+ inflammatory φ</td>
<td>3.47</td>
<td>0.46</td>
<td>5.85</td>
<td>5.85</td>
<td>0.297</td>
</tr>
</tbody>
</table>

**Percentage of CD4+ lymphocytes**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD44&lt;sup&gt;hi&lt;/sup&gt;CD62L&lt;sup&gt;low&lt;/sup&gt;</td>
<td>8.24</td>
<td>6.87</td>
<td>12.34</td>
<td>12.34</td>
<td>0.032</td>
</tr>
<tr>
<td>CD4+CD44&lt;sup&gt;hi&lt;/sup&gt;CD62L&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>11.18</td>
<td>0.93</td>
<td>12.99</td>
<td>1.44</td>
<td>0.322</td>
</tr>
</tbody>
</table>

† Statistics by unpaired two-tailed heteroscedastic student t test
n= 5 - 6 mice per group.