Published in final edited form as:

Exp Mol Pathol. 2017 October ; 103(2): 141–152. doi:10.1016/j.yexmp.2017.08.003.

Regulation of autoimmune myocarditis by host responses to the microbiome

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Abstract

The extensive, diverse communities that constitute the microbiome are increasingly appreciated as important regulators of human health and disease through inflammatory, immune, and metabolic pathways. We sought to elucidate pathways by which microbiota contribute to inflammatory, autoimmune cardiac disease. We employed an animal model of experimental autoimmune myocarditis (EAM), which results in inflammatory and auto-immune pathophysiology and subsequent maladaptive cardiac remodeling and heart failure. Antibiotic dysbiosis protected mice from EAM and fibrotic cardiac dysfunction. Additionally, mice derived from different sources with different microbiome colonization profiles demonstrated variable susceptibility to disease. Unexpectedly, it did not track with segmented filamentous bacteria (SFB)-driven Th17 programming of CD4⁺ T cells in the steady-state gut. Instead, we found disease susceptibility to track with presence of type 3 innate lymphoid cells (ILC3s). Ablating ILCs by antibody depletion or genetic tools in adoptive transfer variants of the EAM model demonstrated that ILCs and microbiome profiles contributed to the induction of CCL20/CCR6-mediated inflammatory chemotaxis to the diseased heart. From these data, we conclude that sensing of the microbiome by ILCs is an important checkpoint in the development of inflammatory cardiac disease processes through their ability to elicit cardiotropic chemotaxis.

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Disclosures

This work was supported by a Gilead Sciences Research Scholars in Cardiovascular Disease award to JGB. IL17RA founder animals were provided by Amgen, under an institutional MTA. The authors have no other interests to declare.

Keywords

Microbiome; Th17 cells; Autoimmune disease; Myocarditis; Segmented filamentous bacteria; Innate lymphoid cells; Chemokines

1. Introduction

The microbiome – the collective communities of viral, eubacterial, archeal, and eukaryotic organisms that mutualistically inhabit all known metazoans – is increasingly appreciated as a critical regulator of human health and disease. Rapid technical advances have enabled large-scale profiling of microbiota in large, diverse cohorts of humans across multiple complex dimensions of health, disease, age, sex, and geography (Human Microbiome Project, 2012; Yatsunenko et al., 2012). Beyond associative data, a mechanistic understanding of the interface of the microbiome with human health and disease continues to grow (Honda and Littman, 2012).

Particular interest in the microbiome has arisen from studies in autoimmune and inflammatory disease (Barin et al., 2015). Environmental factors have long been known to be major contributors to susceptibility to autoimmune and inflammatory disease, although a comprehensive understanding of these mechanisms and pathways is far from complete (Schmidt, 2011; Wang et al., 2015). If we understand the microbiome as the liminal interface between host and the microbial environment, the potential for these multitudinous organisms to regulate and influence host immune processes becomes clear.

The purpose of these investigations was to examine a role for the microbiome in regulating cardiac inflammatory disease. Our findings pointed to a role for enteric microorganisms in determining the susceptibility of mice to our model of experimental autoimmune myocarditis (EAM), and its sequela, inflammatory dilated cardiomyopathy (DCMI) (Cihakova et al., 2004; Smith, 2005). Upon setting out to determine the mechanisms by which the microbiota could regulate pathogenic inflammation in the heart, we paid particular attention to the participation of novel innate immune responses in coupling microbiota to the pathogenesis of autoimmune disease.

Recent findings point towards immune and inflammatory mechanisms centered on the cytokine interleukin-17 (IL17), as key regulators at the mucosal interface, coupling the microbiome to inflammatory and autoimmune disease. Our laboratory has previously demonstrated the importance of IL17 and IL17-producing Th17 CD4⁺ T cells in the elicitation and progression of cardiac autoimmunity, and its sequelae (Barin and Cihakova, 2013). Similarly, Th17 cells have been shown to link microbiota to animal models of autoimmune disease (Berer et al., 2011; Horai et al., 2015; Lee et al., 2011; Wen et al., 2008; Wu et al., 2010). The Th17 program bridges innate and adaptive immunity, in part through a novel class of evolutionary conserved antigen-nonspecific innate lymphocytic cells, termed the innate lymphoid cells (ILCs) (Montaldo et al., 2015). Our findings revealed a previously unreported role for ILCs in coupling microbiome sensing to the recruitment of inflammatory cells to the diseased heart via CCL20/CCR6-driven chemotaxis.

2. Materials & methods

2.1. Mice

Wild type BALB/cJ (JAX 651) and BALB/cByJ (JAX 1026) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). IFN γ ^{-/-} (JAX 2286), IFN γ RI^{-/-} (JAX 7077), CD90.1⁺ (Thy1.1⁺, JAX 5443), RAG1^{-/-} (JAX 3145), RAG2^{-/-} (JAX 8448), and RAG2^{-/-} γ c^{-/-} (JAX 14593), and IL17A-GFP^{Tg} (JAX 18472) founder animals were sourced from Jackson Labs. BALB/cAnNTac animals were sourced from Taconic Farms (Germantown, MD). IL17RA^{-/-} founder mice were kindly provided by Jay Kolls (U. of Pittsburgh), under license from Amgen. All mice were maintained in the Johns Hopkins University School of Medicine specific-pathogen free vivarium. Experiments were conducted on 6–12 week old male mice, and in compliance with the Animal Welfare Act, and the principles set forth in the *Guide for the Care and Use of Laboratory Animals*. All methods and protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University.

2.2. Induction of EAM

For the induction of EAM, we employed the myocarditogenic peptide MyHC α _{614–629} (Ac-SLKLMATLFSTYASAD) (Pummerer et al., 1996), commercially synthesized by fMOC chemistry, and purified to minimum 90% by HPLC (Genscript). On days 0 and 7, male mice received *sc* axillary installations of 100 μ g of MyHC α _{614–629} peptide emulsified in CFA (Sigma) supplemented to 5 mg/mL of heat-killed *M. tb* strain H37Ra (Difco). On day 0, mice received an additional 500 ng of pertussis toxin *ip* (List Biologicals) (Cihakova et al., 2004; Smith, 2005).

2.3. Adoptive transfer of EAM

For “active” adoptive transfer of EAM, male CD90.1⁺ (Thy1.1⁺) congenic BALB/c mice (JAX 5443) were immunized as above for EAM. Spleens and lymph nodes were harvested between days 12 and 14. Single cell mononuclear suspensions were prepared and restimulated for 72 h with a final concentration of 25 μ g/mL MyHC α _{614–629} + 10 ng/mL rmIL1 β + 20 ng/mL rmIL23 (R & D Systems) in complete DMEM + 10% FBS an additional 30 mM NaCl (Wu et al., 2016). Following restimulation, viable cells were enriched by gradient centrifugation on Lympholyte M (Cedarlane), followed by paramagnetic positive selection for CD4⁺ T cells (Miltenyi). 2–5 $\times$ 10⁶ viable T cells were transferred *iv* into naïve RAG1^{-/-}, RAG2^{-/-}, or RAG2^{-/-} γ c^{-/-} recipient mice (Smith, 2005).

2.4. 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 μ m serial sections were cut and stained with hematoxylin and eosin, or Masson’s Trichrome Blue (HistoServ). Myocarditis severity was evaluated by microscopic estimation of the percent 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%. Grading was performed by a minimum of two independent blinded investigators and averaged (Smith, 2005).

2.5. Polyantibiotic dysbiosis

To induce dysbiosis, automated cage watering for animals was replaced with bottled polyantibiotic cocktail, remade sterile, and replenished weekly. The cocktail consisted of 0.5 g/L ampicillin, 0.5 g/L metronidazole, 0.5 g/L neomycin, and 0.25 g/L vancomycin (Sigma-Aldrich) in a 4.0 g/L Splenda base solution to improve palatability. For EAM experiments, animals were treated with polyantibiotics for a minimum of 2 weeks prior to the induction of disease. For experiments on naïve animals, treatment proceeded for a minimum of 10 days prior to experiments.

2.6. Flow cytometry

Heart-infiltrating leukocytes were isolated from animals perfused for 3 min with 1× PBS, and digested in Gentle MACS C Tubes according to manufacturer's instructions (Miltenyi Biotec). Splenocytes were also extracted into single cell suspension in 1× PBS + 0.2% BSA, and RBCs lysed by < 5 min incubation in ACK lysis buffer (Biofluids). Prior to surface staining, viability was determined by LIVE/DEAD staining according to manufacturer's instructions (Molecular Probes). Cells were washed and FcγRII/III blocked with αCD16/32 (Affymetrix eBiosciences). Surface markers were stained with fluorochrome-conjugated mAbs (eBiosciences, BD Pharmingen, BioLegend). Intracellular and intranuclear antigens were stained following fixation and permeabilization (eBiosciences). Samples were acquired on the LSR II quad-laser cytometer running FACSDiva 6.0 (BD Immunocytometry). Data were analyzed with FlowJo 10.8 (Treestar Software).

2.7. 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% FBS, 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 (Millipore) were used according to manufacturer's instructions, and acquired on a Luminex XMAP reader. Alternately, quantitative sandwich ELISA for supernatants were determined by colorimetric ELISA kits according to manufacturers' recommended protocols (R & D Systems, IBL). Total serum immunoglobulins were determined by quantitative sandwich ELISA (BD Biosciences).

2.8. Real time PCR

Tissue mRNA was extracted in TRIZOL (Invitrogen), quantitated by Nanodrop (Thermo Fisher), DNase digested (Ambion), and amplified with iQ SYBR Green mastermix (BioRad) acquired on the MyiQ2 thermocycler (BioRad) running iQ5 software (BioRad). Synthetic oligonucleotides were commercially synthesized, purified, and assayed by mass spectroscopy (Integrated DNA Technologies). 250 pmol of each primer (Supplemental Table I) were 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^{-Ct} method of Livak and Schmittgen (2001) comparing threshold cycles first to *Hprt* expression in individual samples, then Ct of target genes in naïve, untreated controls. Certain data are pseudolinearized, to depict downregulation ($2^{-Ct} < 1$) as -2^{-Ct} .

2.9. Echocardiographic imaging

Trans-thoracic 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 (2-D) mode in the parasternal short axis view, and an M-mode cursor was positioned perpendicular to the interventricular septum at the level of the papillary muscles. From M-mode, wall thicknesses and chamber dimensions were measured. Fractional shortening (%FS), ejection fraction (%EF), LV mass, and relative wall thickness were calculated from these parameters as previously described (Cihakova et al., 2008).

2.10. Statistics

Continuous homoscedastic parametric comparisons of two groups were performed with the two-tailed Students' *t*-test (Excel). Multiple group comparisons were performed by ANOVA, followed by the Tukey-Kramer or Bonferroni post-tests (StatPlus). Discontinuous parameters (histopathology, specifically) was assessed by Mann-Whitney *U* test, or Kruskal-Wallis in the case of multiple group comparisons. Asterisks denote statistically significant comparisons: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

3. Results

3.1. Polyantibiotic treatment diminishes the severity of myocarditis in BALB/cJ mice

To test the hypothesis that the enteric microbiome contributed to host susceptibility to cardiac inflammation, autoimmunity, and maladaptive remodeling, we induced dysbiosis with a polyantibiotic cocktail, widely used to mimic germ-free animals (Round and Mazmanian, 2009), prior to the induction of EAM in BALB/cJ mice. At the peak of EAM, cardiac histopathology, enlargement, and inflammatory infiltration were significantly diminished in animals treated with antibiotics (Fig. 1a–b). We assessed the cardiac function of these animals at day 21 by M-mode echocardiography. Mice treated with antibiotics trended towards protection from cardiac dysfunction at day 21 (Fig. 1c), a relatively early timepoint in the progression of this model.

We previously demonstrated cardiac remodeling and fibrosis to be associated with IL17-driven progression to cardiac dysfunction (Baldeviano et al., 2010). Interrogation of cardiac transcripts by realtime PCR revealed diminished expression of the fibrotic collagens I and III (Fig. 1d). Importantly, we did not observe diminution of cardiac myosin-reactive autoantibodies circulating in the sera of dysbiotic animals or serum C-reactive protein (*data not shown*), arguing against the mechanism of protection being global disruption of myocarditogenic processes or systemic inflammation.

3.2. BALB/c mice sourced from Jackson and Taconic are differentially colonized with SFB

Substantive differences have been demonstrated in the microbiome profiles of inbred laboratory C57BL/6 mice sourced from different vendors, with critical consequences upon the inflammatory and immune baseline of these animals at steady-state (Ivanov et al., 2009). We sought to determine whether these differences would also be true in BALB/c mice, as this strain is susceptible to our EAM model (Cihakova et al., 2004; Smith, 2005). Using variable region 4 (V4) of the 16S ribosomal subunit for tag metagenomic profiling, we found BALB/cJ mice sourced from Jackson Labs (JAX) to have demonstrably different colonization profiles from BALB/cAnNTac mice, sourced from Taconic Farms (Tac) (Supplemental Fig. 1a) (AMOVA: $p < 0.001$). Even phylum-level abundance distributions demonstrate increased species richness and diversity in animals sourced from Taconic (Supplemental Fig. 1b); inverse Simpson's index values were greater for Tac animals, compared to JAX mice (*data not shown*).

Among taxa reported to differentially colonize commercially-sourced mice, Th17 turnover in the mouse gut has been shown to be driven by colonization with segmented filamentous bacteria (SFB) in C57BL/6 mice from Taconic, but not Jackson (Ivanov et al., 2009). We have previously demonstrated critically important roles for Th17 cells in the pathogenesis of the EAM model (Baldeviano et al., 2010; Wu et al., 2016,2014). Using SFB-specific primers, we were able to confirm the presence of SFB in the stools of Taconic mice, but not Jackson animals, in a manner that persisted over the course of EAM (Supplemental Fig. 1c).

Following from reports from C57BL/6 mice, intracellular staining of ileal lamina propria CD4⁺ demonstrated a greater proportion of IL17A production in the guts of naïve BALB/cAnNTac mice, as compared to BALB/cJ animals (Supplemental Fig. 1d). Furthermore, this difference was specific for the ileum – CD4⁺ T cells from duodenum, lung, or spleen did not show this difference (*data not shown*). These data indicate that colonization with SFB elicits a Th17-centered defense program in the mouse hindgut.

3.3. JAX BALB/cJ animals develop more severe myocarditis than Taconic BALB/cAnNTac mice

These findings predicted that SFB-positive animals sourced from Taconic would be more susceptible to EAM, consistent with findings in other Th17-driven autoimmune disease mouse models (Lee et al., 2011; Stepankova et al., 2007; Wu et al., 2010). To further investigate the role of enteric microbiota in the pathogenesis of autoimmune heart disease, we compared EAM induced in BALB/cJ and BALB/cAnNTac animals. To our surprise, at day 21 of EAM, we observed more severe cardiac histopathology in BALB/cJ animals (Fig. 2a), in contrast to their negative SFB status. We also observed increased relative heart weights, as well as more numerous heart-infiltrating leukocytes (Fig. 2b, *left, middle*). As further confirmation of this difference, determination of cardiac hydroxyproline content as an indicator of collagenous fibrosis demonstrated greater fibrotic remodeling in the hearts of BALB/cJ animals (Fig. 2b, *right*).

These findings raised the possibility that the SFB exert a protection from myocarditis, as has been demonstrated in the type I diabetes of NOD mice (Kriegel et al., 2011). We had

included a recolonization group – where JAX mice were housed in cages previously occupied by Taconic animals, and did not observe diminution of myocarditis in this group, despite PCR confirmation of SFB (*data not shown*). Similarly, we observed no exacerbation of disease in antibiotic-treated BALB/ cAnNTac mice (*data not shown*) (Kriegel et al., 2011).

To more conclusively demonstrate that SFB did not impact the development of EAM, BALB/cJ mice were gavaged with SFB-mono-colonized feces. Recolonization was verified by realtime PCR (*data not shown*), and EAM induced in recolonized and control mice. As depicted in Supplemental Fig. 2a–b, animals recolonized with SFB developed myocardial inflammation and infiltration of comparable severity to controls. Together, these data argue against a role for the SFB in enabling the pathogenesis of autoimmune myocarditis.

Genetic and phenotypic differences have been reported among the BALB/c substrains currently employed in research (Potter, 1985). In the derivation history of these substrains, the widely used BALB/cByJ Bailey substrain of BALB mice are more closely related to BALB/ cAnNTac mice than they are to BALB/cJ animals. By virtue of the BALB/cJ and BALB/cByJ strains being both available from JAX, we employed these strains to address whether the differences we observed between BALB/cJ and BALB/cAnNTac mice might be due to drift in host genetics, irrespective of the microbiome environment. After inducing EAM, we did not observe a substantive difference in the severity of EAM in BALB/cJ and BALB/cByJ animals (Supplemental Fig. 2c). From these data, we conclude that enhanced susceptibility to myocarditis in BALB/cJ animals is not likely due to differences in host genetic variation among the BALB substrains.

3.4. Regulation of myocarditis susceptibility by the microbiota is associated with IL17-centered immune pathways

Because of the discordance of SFB status (and by extension Th17 turnover in the gut), and susceptibility to EAM, we examined immunopathologic markers in the hearts of BALB/cJ and BALB/cAnNTac mice to examine the mechanism of this differential susceptibility. By multiplex interrogation of cardiac homogenates at day 21 of EAM, we observed greater expression of the inflammatory cytokines IL1 β and IL6, as well as the myelotropic chemokines and growth factors CCL2/ MCP1, CXCL1/KC, CXCL2/MIP2, and G-CSF in BALB/cJ mice (Fig. 3a). Many of these cytokines are well-known, canonical transcriptional targets of IL17 signaling (Schmidt-Weber et al., 2007).

We also examined the infiltration of Th17 cells into the hearts of animals with respect to microbiome profiles and EAM susceptibility. In the Jackson-vs-Taconic comparison, we found more abundant ROR γ ⁺ Th17 cells in the hearts of BALB/cJ animals (Fig. 3b). We similarly found more neutrophils and Ly6C^{hi} inflammatory monocytes in BALB/ cJ mice (Fig. 3c) – cell types we have previously reported to traffic to the inflamed heart under the control of IL17-driven chemotaxis (Baldeviano et al., 2010; Wu et al., 2016). Examining dysbiotic animals, numbers of ROR γ ⁺ Th17 cells were diminished in the hearts of mice following extended treatment with oral polyantibiotics, corresponding to their protection from disease (Fig. 3d).

To further test the IL17-dependence of this effect of antibiotic treatment, we elicited EAM in antibiotic-treated mice deficient in IL17RA. Antibiotic treatment failed to suppress the development of myocarditis in IL17RA^{-/-} animals (Supplemental Fig. 3a). Mice deficient in IFN γ signaling develop a severe Th17-deviated form of disease, characterized by extensive neutrophilic infiltration, fibrotic myocardial remodeling, accelerated progression to DCMI, and death (Barin et al., 2013; Eriksson et al., 2001). We treated IFN γ ^{-/-} and IFN γ RI^{-/-} mice with antibiotics, and observed that antibiotic treated animals developed severe cardiac inflammation and infiltration, comparable to the disease of controls (Supplemental Fig. 3b). However, IFN γ ^{-/-} animals were protected from death at late phases of disease (Supplemental Fig. 3c), and had preserved fractional shortening and ejection fractions over the course of EAM (Supplemental Fig. 3d). We assessed the fibrotic remodeling associated with the severe EAM of IFN γ ^{-/-} mice by image analysis of Masson's trichrome heart sections, and found less collagenous fibrosis in the hearts of dysbiotic IFN γ ^{-/-} hearts compared to controls (Supplemental Fig. 3e). Together, these data are consistent with the microbiota contributing to autoimmune heart disease through regulating components of a Th17-associated program.

3.5. Host responses to microbiota are regulated by ILC3-associated pathways

SFB colonization elicits Th17 differentiation by ileal CD4⁺ T cells (Ivanov et al., 2009), possibly accounting for the increased susceptibility of SFB-positive animals to some autoimmune disease models (Lee et al., 2011; Stepankova et al., 2007; Wu et al., 2010), but not others (Dijkstra et al., 2007; Kriegel et al., 2011). In our myocarditis model, SFB colonization and steady-state ileal Th17 differentiation in naïve animals (Supplemental Fig. 1) did not track with susceptibility to inflammatory cardiac disease (Fig. 2, Supplemental Fig. 2). However, our findings from dysbiosis and vendor comparisons supported a role for microbiome in promoting cardiac inflammation and autoimmune pathology. Moreover, we found evidence in diseased hearts for a cytokine and cellular profile that was consistent with a Th17-centered immune process tracking with this differential disease susceptibility. To reconcile this disparity, we considered CD4⁺ T cell-independent mechanisms that contributed to and regulated Th17 responses.

Employing both the dysbiosis and vendor comparison models, we extensively profiled antigen-presenting cells (CD103⁺ dendritic cells and CX3CR1^{hi} macrophages), innate myeloid populations (neutrophils, monocytes), and other T cells including CD8⁺ and $\gamma\delta$ populations, with particular regard for their expression of proinflammatory Th17-associated cytokines. In summary, these populations either showed no differences in comparison, or demonstrated differences discordant with disease susceptibility (*data not shown*).

We further examined a newly described class of antigen-nonspecific innate lymphoid cells (ILCs). This expanding family of cells expresses markers shared with the lymphoid lineages, such as CD90/Thy1 and IL7R α . The subtypes of these cells that have been characterized bear functional symmetry with CD4⁺ T cells – the ILC1, ILC2, and ILC3 populations produce cytokine profiles analogous to Th1, Th2, and Th17 cells, respectively (Diefenbach et al., 2014; Spits et al., 2013). Most importantly, they lack the canonical antigen receptors which functionally define the mainline lymphoid lineages – the T cell or B cell antigen

receptors (Artis and Spits, 2015). Having been subsumed into the ILC paradigm, NK cells can be considered the prototypic lineage for understanding ILC function (Geiger and Sun, 2016). ILC subsets are found in numerous compartments, but are relatively enriched at mucosal surfaces. In the lower GI tract, they are important for maintenance of the enteric barrier, and coordinate tuning of immune and inflammatory responses (Kim et al., 2015; Sonnenberg et al., 2012).

With this in mind, we examined subpopulations of ILCs in the distal ileal lamina propria of naïve BALB/cJ and BALB/cAnNTac mice. By staining for markers associated with ILC1, ILC2, and ILC3 populations, we observed a greater proportion of ILCs expressing ROR γ t, a critical defining transcription factor of the ILC3 lineage, in the ileal lamina propria of BALB/cJ mice (Fig. 4a). The ILC3 lineage was of particular interest, as these cells produce cytokines associated with Th17 responses, particularly IL17A and IL22 (Sonnenberg and Artis, 2015). Furthermore, this difference in ILC3 differentiation did not appear to extend to the ILC1 and ILC2 subsets. Similarly, this pattern was not observed among ILCs assayed from the lung or spleen (*data not shown*). Realtime qPCR interrogation of net transcriptional profiles in the distal ilea were consistent with an ILC3 program in BALB/cJ mice – increased expression of Th17/ILC3-associated transcripts like *Il17a*, *Reg3g*, and *Saa3*, compared to BALB/cAnNTac mice (Fig. 4b). It was notable that these, and other related transcripts, associated with the increased ILC3 population and myocarditis susceptibility in BALB/cJ mice, rather than Th17 CD4⁺ T cells and SFB colonization in BALB/cAnNTac animals. From these data, we concluded the net balance of Th17/ILC3-associated gene expression in the gut tracked with microbiome-sensitive ILC3 cells rather than SFB-driven Th17 differentiation.

As further confirmation of the effect of microbiome on lymphocyte differentiation in the mouse gut, we turned to the polyantibiotic dysbiosis system. Using fluorogenic reporter IL17A-GFP^{Tg} mice (Lee et al., 2012), we observed fewer $\alpha\beta$ T cells expressing IL17A reporter in the ileal lamina propria of naïve mice following treatment with poly-antibiotic (Fig. 4c), consistent with published reports. However, we also observed diminished ILC3 proportions in the gut of animals treated with antibiotics (Fig. 4d), consistent with our findings in the JAX v Tac comparison. Together, these data indicate that ILC3 differentiation at steady-state is associated with susceptibility to subsequent EAM.

3.6. Innate lymphoid cells are critical for leukocyte recruitment to the heart

The diminution of gastrointestinal ILC3 in dysbiotic animals implicated this innate lymphoid lineage in coupling sensing of the enteric microbiome to susceptibility to cardiac inflammation. To address this possibility, we paired adoptive transfer models of myocarditis (AT-EAM) (Smith, 2005) to one of two systems for depleting ILCs. In the first, allotypic markers were used to distinguish recipient from donor-derived lymphocytes, allowing for monoclonal antibody depletion of resident-derived ILCs (Sonnenberg et al., 2012). In the second, critical signaling receptors for cytokines essential for lymphoid development were genetically ablated – namely the common gamma chain (γ c, CD132), a shared signaling subunit for essential lymphoid growth factors (Crellin et al., 2010).

We employed an “active” AT-EAM model, in which CD90.1⁺ (Thy1.1⁺) T cell donors were immunized with myosin peptide, and spleen CD4⁺ T cells harvested and transferred to lymphopenic recipients (Smith, 2005; Wu et al., 2016), in which ILCs were depleted by mAb specific for the recipient CD90.2 (Thy1.2) allotype (Fig. 5a). By targeting the RAG-independent recipient-derived CD90.2⁺ population, innate lymphoid cells are depleted (Fig. 5b) (Sonnenberg et al., 2012). To validate the effectiveness of depletion, making sure depleting antibodies did not simply mask assay antibodies, NKp46⁺ NK cells were depleted by proportionally equivalent numbers, irrespective of the CD90.2/Thy1.2 marker (*data not shown*). When ILCs were depleted in this manner, net recruitment of leukocytes to the heart was diminished (Fig. 5c, *left*). To verify that this effect was not solely due to the depletion of host innate lymphoid cell types, we ascertained that this diminution was true of Ly6G^{hi} neutrophils (Fig. 5c, *middle*), as well as donor-derived CD90.1⁺CD4⁺ T cells (Fig. 5c, *right*). This depletion effect was not true in the spleens of α -CD90.2 treated animals (*data not shown*), indicating this was a specific defect of inflammatory cardiotropic chemotaxis.

As further confirmation of this effect, we replaced the allotypic mAb depletion strategy with a complementary genetic model of defective ILC development. ILCs are known to be dependent on signaling by common gamma-chain (γ c) cytokines, particularly IL7 and IL15, for development and survival (Serafini et al., 2015). Primed myocarditogenic CD4⁺ T cells were transferred into lymphopenic RAG2^{-/-} or RAG2^{-/-} γ c^{-/-} animals (Fig. 5d), the latter of which demonstrate a clear defect in innate lymphoid cell development (Fig. 5e). As with the mAb depletion strategy, fewer inflammatory leukocytes were found in the hearts of RAG2^{-/-} γ c^{-/-} mice following transfer, including neutrophils and CD4⁺ T cells (Fig. 5f). Together, these data supported an important role for the ILC lineages in governing the chemotactic recruitment of leukocytes to the inflamed heart.

To address a possible role for ILCs in the priming of autoaggressive myocarditogenic T cells, we switched from “active” AT-EAM to a “parking” model: CD4⁺ T cells were transferred from naïve donors into lymphopenic RAG knockout animals prior to the induction of EAM. Whether recipient ILCs were depleted by allotypic mAbs (Supplemental Fig. 4a–c) or ablation of the common-gamma chain (Supplemental Fig. 4d–f), the development of EAM in recipients was comparable to controls (Supplemental Fig. 4c,f), arguing against a role for ILCs in the initial priming and activation of myocarditogenic T cells.

3.7. Microbiota and innate lymphoid cells regulate CCL20/CCR6-driven chemotaxis to the inflamed heart

Thus far, our data pointed to a role for the microbiome in regulating cardiac inflammation, through the maintenance of ILC programming. While we have demonstrated a role for ILCs in susceptibility to myocarditis, it was not clear from these findings how ILCs transduce microbiome-derived signals into cardiac inflammatory processes. Transcripts from the distal ilea of dysbiotic mice at day 9 of EAM (an early timepoint in the disease model) were interrogated by qRT-PCR to assess the dependence of chemokine and trafficking molecule expression on microbiome-derived signals. The majority of these transcripts were modestly downregulated in dysbiotic animals (*data not shown*). Expression of the chemokine CCL20/

MIP3 α was the most significantly downregulated transcript resulting from antibiotic dysbiosis in the early phase of EAM (Fig. 6a).

Because CCL20/MIP3 α -driven chemotactic recruitment is preferentially associated with Th17-associated effector responses, this pathway was particularly attractive as it could serve to tie gut immune processes to Th17-driven cardiac pathophysiology (Ito et al., 2011; Lee and Korner, 2014). Microbiome regulation of ILC3-mediated CCL20-centered chemotaxis might further reconcile the microbiome-sensitivity of cardiac disease bearing a Th17 signature, against the steady-state SFB-driven differentiation of canonical Th17 cells themselves.

We examined CCL20/MIP3 α expression in the hearts of diseased animals by intracellular staining. Of the cell types reported to express CCL20/MIP3 α , we found CD31⁺ endothelia to be the most significant producers of this chemokine in hearts at peak EAM, on a per-cell basis (Fig. 6b). Comparing the hearts of dysbiotic animals with controls during the course of EAM, we observed that antibiotic treatment diminished the expression of CCL20/MIP3 α from cardiac endothelial cells (Fig. 6c). Decreased CCL20/MIP3 α expression was observed in other less-numerous CCL20⁺ cell populations following antibiotic treatment, including CD11c^{hi} dendritic cells, CD64^{+/lo}CD11b⁺ macrophages and monocytes, as well as gp38/podoplanin-1⁺ and CD140a/PDGF-R α ⁺ cardiac fibroblasts (Fig. 6d).

We further found evidence that ILC depletion also affected CCL20 expression in the heart. Cardiac CCL20/MIP3 α trended towards being diminished when ILCs were depleted by allotypic mAb in AT-EAM recipients (Fig. 6e), as well as in ILC-deficient RAG2^{-/-} γ c^{-/-} AT-EAM recipients (Fig. 6f). Together, these data were consistent with the notion that microbiota are important for the maintenance of CCL20/MIP3 α expression in the gut at steady-state, and in the inflamed heart.

3.8. Antibiotic dysbiosis diminished CCL20/CCR6 chemotaxis

The cognate receptor for CCL20/MIP3 α – CCR6 – was also of interest, as its expression is highly enriched on Th17 cells, as well as other cell types associated with IL17-centered immunity (Hirota et al., 2007; Yamazaki et al., 2008). Therefore, we sought to examine the dependence of CCL20/CCR6-driven chemotactic recruitment in our experimental models of microbiome and ILC regulation of myocarditis.

Our data above demonstrate fewer ROR γ t⁺ Th17 cells infiltrating the hearts of antibiotic-treated animals at the peak of EAM (Fig. 3d). More comprehensive analysis indicated other CCR6-expressing lymphoid populations, including γ δ T cells and ILC3s, were also less numerous in the hearts of dysbiotic animals (Fig. 7a). Assessing the expression of CCR6 on heart-infiltrating cells further demonstrated diminution of CCR6-expressing ROR γ t⁺ Th17 cells following antibiotic treatment (Fig. 7b). Examining the expression of CCR6 on relevant populations in the guts of naïve, unimmunized animals treated with polyantibiotics demonstrated defective CCR6 expression on γ δ T cells and ILC3s in the distal ileal lamina propria – as well as on the mucosa-specialized antigen-presenting CX3CR1^{hi} macrophage subset (Supplemental Fig. 5). This latter finding was especially intriguing as these APCs are

believed to be critically important in the licensing of Th17 responses to enteric pathobionts and microbiota (Farache et al., 2013; Rescigno and Di Sabatino, 2009).

Switching to the JAX v Tac comparison, we again observed more numerous CCR6-positive CD4⁺ and $\gamma\delta$ T cells infiltrating the hearts of BALB/cJ animals (Fig. 7c), although this did not appear to be as significant as the dysbiosis system in terms of proportional expression of the CCR6 receptor. Lastly, we examined cardiac T cells in ILC-defective RAG2^{-/-} γ c^{-/-} active AT-EAM recipient mice (as above, in Fig. 5d–f). We found fewer CCR6-expressing CD4⁺ cells and ROR γ t⁺ Th17 cells in the hearts of RAG2^{-/-} γ c^{-/-} animals (Fig. 7d), mirroring the diminution of CCR6⁺ lymphocytes and Th17 cells in the hearts of dys-biotic and Taconic-sourced animals. Together, we conclude from these data that the CCL20/CCR6 chemotactic axis is a shared, convergent pathway that is sensitive to inputs from the enteric microbiome and the ILC compartment in a manner that tracks with susceptibility to myocarditis.

4. Discussion

In these experiments, we report evidence for a role for microbiome-regulated innate immune signals mediating inflammatory recruitment of leukocytes to the diseased heart. Microbiome research in humans and animal models is flourishing, as techniques and tools for profiling these vast, complex communities disseminate. Coupled to this, is an increasing understanding of the network of inflammatory, immune, and metabolic pathways by which the microbiome interfaces with human health and disease (Belkaid and Hand, 2014; Brestoff and Artis, 2013). Evidence in patients and animal models delineate some of these pathways by which microbiota influence susceptibility of the host to auto-immune and inflammatory disease (Abdollahi-Roodsaz et al., 2016; Barin et al., 2015).

Substantially less is known about the contributions of microbiome-coupled pathways to cardiovascular disease (Ferguson et al., 2016; Kitai et al., 2016). Key translational findings describe microbiome-derived metabolites, specifically cholinergic trimethylamine derivatives, in the susceptibility of humans and animal models to atherosclerotic disease (Senthong et al., 2016; Tang and Hazen, 2014; Wang et al., 2011). Patients with chronic heart failure were found to have increased plasma LPS levels, suggestive of increased permeability of the enteric barrier (Sandek et al., 2012). Additional evidence comes from putative causative associations of periodontal disease with atherogenesis (Desvarieux et al., 2005), although the mechanistic details and clinical relevance of these findings remain controversial (Lockhart et al., 2012).

While these findings point to metabolomic roles for the microbiome in regulating the development of vascular atherogenesis, less is known how inflammatory and immunologic pathways couple microbiota to cardiac health and disease. To begin to understand the mechanisms underlying this relationship, we inferred some of these pathways from a growing body of investigation into the interface of the immune system with the microbiome, in line with our laboratory's long-standing interest in autoimmunity (Barin et al., 2015). We treated susceptible BALB/cJ mice with a polyantibiotic cocktail, commonly employed for experimental dysbiosis (Morgun et al., 2015), to mimic germ-free or gnotobiotic conditions,

and found antibiotic treatment protected mice from the development of EAM, cardiac remodeling, and progression to heart failure. This finding supported a role for the microbiome in modulating the severity of inflammatory cardiac disease in host animals.

Some of the proposed mechanisms of microbiome-dependent regulation of host inflammatory disease revolve around the ability of specific microbial taxa to evoke Th17-associated immunity (Lee et al., 2011; Stepankova et al., 2007; Wu et al., 2010). Our laboratory has established a critical role for Th17-centered immunity in the development of cardiac inflammatory disease and subsequent heart failure, eliciting our immediate interest in examining Th17-associated pathways in coupling microbiota to heart disease. An important role for segmented filamentous bacteria (SFB) in eliciting Th17 responses from intestinal T cells has been well-described (Goto et al., 2014; Ivanov et al., 2009; Ivanov et al., 2008). This pathway has been shown to be shared by microbes that occupy similar habitats, attaching or adhering to intestinal epithelia (Atarashi et al., 2015; Tan et al., 2016). These data predict that SFB colonization contributes to susceptibility to autoimmune disease through the induction of Th17 cells (Lee et al., 2011; Wu et al., 2010). Intriguingly, SFB colonization has been shown to be protective in the spontaneous type 1 diabetes model that arises in the NOD mouse strain (Kriegel et al., 2011; Yurkovetskiy et al., 2013).

We first employed the differential SFB colonization profiles of BALB/c mice sourced from Jackson Labs and Taconic Farms, described by Ivanov et al. (2009). In contrast to their findings, we observed SFB colonization tracked against the severity of resulting EAM. From specific experimental SFB colonization, we concluded that SFB had neither protective nor pathogenic effects on resulting disease. The CD4⁺ T cell response to SFB has been recently described to be directed specifically against SFB-derived antigens (Goto et al., 2014; Yang et al., 2014). BLAST alignment of the SFB genome against known cardiac autoantigens demonstrated no credible similarity or mimics (*data not shown*), consistent with the lack of effect in our system. From these data, we concluded that susceptibility to EAM was not driven by SFB colonization.

This finding made problematic the notion that Th17-associated pathways were responsible for coupling the microbiome to regulation of cardiac autoimmunity and remodeling. In spite of this, we observed a Th17 signature associated with differential susceptibility to EAM, including Th17-associated cytokines and chemokines, as well as cardiac recruitment of ROR γ ^t Th17 cells, neutrophils, and Ly6C^{hi} inflammatory monocytes. The importance and involvement of Th17-associated pathways was consistent with our findings modulating microbiota in Th17-defective or -deviated models of EAM. Antibiotic treatment exerted no effect on the development of EAM in IL17RA^{-/-} mice (Wu et al., 2014), whereas dysbiosis protected highly Th17-deviated IFN γ ^{-/-} animals from maladaptive cardiac remodeling, heart failure, and death (Eriksson et al., 2001).

This conceptual disparity could be resolved by examining cells and pathways epistatically peripheral to the Th17 program – a cell type other than a CD4⁺ T cell that still contributed to Th17 responses. Our attention was drawn to cells of the innate lymphoid (ILC) lineage, as these cells bear lineage relationships paralleling the effector differentiation programs undertaken by CD4⁺ T cells with key differences; instead of Th1, Th2, and Th17 cells which

acquire effector differentiation during activation, ILCs can already be found in tissues as ILC1, ILC2, and ILC3 cells, respectively mirroring T cell subsets (Diefenbach et al., 2014; Spits et al., 2013). We found that ILC3s correlated closely with disease susceptibility in steady-state ilea of both the JAX v Tac, as well as the antibiotic dysbiosis systems. In the JAX v Tac comparison, we found more ROR γ t⁺ ILC3s in the ilea of BALB/cJ mice, in contrast to their SFB status and steady-state IL17A⁺ Th17 turnover.

To investigate the role ILCs could play in EAM, we coupled two methods of ILC depletion (allotypic mAb depletion or γ c deletion) to two variants of adoptive transfer models of EAM. Applying the “parking” AT-EAM variant, in which naïve CD4⁺ T cells were transferred and recipients immunized demonstrated no difference in resulting disease. However, in “active” AT-EAM, in which primed CD4⁺ T cells were transferred from immunized donors, we found ILC deficiency limited the recruitment of inflammatory leukocytes to the heart. This was true whether ILCs were depleted by allotypic mAb or developmentally ablated. Together, we conclude from these experiments that ILCs were important in recruiting leukocytes to the inflamed heart, but were dispensable for initial activation of autoaggressive CD4⁺ T cells.

From these data, it is not clear to us where local ILC3s originate from in the inflamed heart. While some tissues support local differentiation of ILC3s from common progenitors in situ at steady-state, it may also be possible that additional differentiation is driven from precursors recruited during chronic inflammatory processes. Murine ILC3s have been shown to recruit to injured skin in response to Notch-driven CCL20 (Li et al., 2016). It may also be possible that differentiated tissue ILCs bear a measure of plasticity, depending on local microenvironment conditions (Nussbaum et al., 2017).

Our next question was to examine the mechanisms by which ILC3s mediated inflammatory recruitment of leukocytes to the heart in a manner that was regulated by signals derived from the microbiome. We interrogated a panel of 20 chemokines and adhesion molecules, among which, the Th17-associated chemokine CCL20/MIP3 α was the only significantly downregulated analyte. CCL20/MIP3 α , has been shown to be important for trafficking of Th17 cells to the enteric mucosa, as well as sites of Th17-directed inflammation (Esplugues et al., 2011; Hirota et al., 2007). The chemokine receptor for CCL20 – CCR6, is highly enriched on Th17 cells and γ δ T cells, among others (Mabuchi et al., 2013; Yamazaki et al., 2008). ILCs themselves are not known to be a significant source of CCL20 in mice (*ImmGen data not shown*). We found CD31⁺ endothelia to be the most significant cardiac source of CCL20 in mice with EAM. CCL20 expression by cardiac endothelia, as well as by macrophages, dendritic cells, and fibroblasts was diminished by antibiotic dysbiosis. Importantly, cardiac CCL20 expression was also curtailed in the ILC-depletion experimental systems, as well.

Corollary to this decrease in CCL20 expression, we found diminished recruitment of CCR6-expressing cell types in the hearts of dys-biotic mice, including ROR γ t⁺ Th17 cells, γ δ T cells, and ILC3s. Similar CCR6-centered defects of recruitment were also observed in EAM-resistant BALB/cAnNTac mice; and ILC-deficient animals. Datamining existing transcriptional profiling datasets from germ-free mice is consistent with microbiome-

dependent maintenance of CCR6 expression in the enteric compartment (*GEO data not shown*) (El Aidy et al., 2012; Gaboriau-Routhiau et al., 2009; Reikvam et al., 2011). Our findings point to a novel role for microbiome-sensitive ILCs in directing CCL20/CCR6-mediated inflammatory chemotaxis to the diseased heart. How ILCs direct endothelia (and other CCL20-expressing cells) to regulate the CCL20/CCR6 chemotactic axis is also unanswered by these experiments.

There may exist additional non-synonymous, non-exclusive pathways unaddressed by these experiments. A host of metabolomic defects occur in prolonged polyantibiotic treatment, many of which may be dependent on enteric microbes. We did not observe differences in autoantibody or serum C-reactive protein in dysbiotic animals (*data not shown*), arguing against antibiotics eliciting global immunosuppression or defects of systemic inflammatory processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding statement

This work was supported by a Gilead Research Scholar and AARDA awards to Jobert G. Barin, NIH/NHLBI grants R01HL118183 and R01HL113008 to Daniela Miháková. Nicola L. Diny, and Hee Sun Choi are recipients of AHA Predoctoral Fellowships.

The authors wish to extend their gratitude to Jay Kolls and Amgen for the generous provision of IL17RA^{-/-} founder mice; to Xiaoling Liang for expert assistance with flow cytometric analysis; to Linda Orzolek and Haiping Hao of the JHU High-Throughput Biology Center for expert assistance with the MiSeq next-generation sequencing platform; to David A. Kass and Peter Rainer for guidance and assistance with cardiologic disease modelling; and to Inés Martínez, Jens Walter, and Maria-Elisa Perez-Muñoz for expert assistance, guidance, and training with microbiome profiling methods.

Abbreviations

EAM	experimental autoimmune myocarditis
DCMI	inflammatory dilated cardiomyopathy
SFB	segmented filamentous bacteria
ILC	innate lymphoid cell
MyHCα	cardiac myosin heavy chain, alpha
γc	common gamma chain
AMOVA	analysis of molecular variance

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yexmp.2017.08.003>.

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Fig. 1. Polyantibiotic treatment of BALB/cJ mice limits EAM, and progression to DCMI

A) Cardiac pathology of BALB/cJ mice fed polyantibiotic cocktail or control water at day 21 of EAM. Representative cardiac histopathology micrographs depict median individuals of each group, stained by H & E.

B) Relative heart weight, normalized to body weight (left); total viable CD45⁺ cells infiltrating the hearts of BALB/cJ mice, determined cytometrically (right) at day 21 of EAM.

C) Determination of cardiac function by M-mode echocardiographic imaging of mice at day 21 of EAM. Calculated parameters portray cardiac function: ejection fraction (left) and fractional shortening (right).

D) Cardiac expression of fibrotic genes determined by realtime qPCR from total mRNA of hearts at day 21 of EAM.

Data are representative of five independent experiments. Diamonds and circles depict individual animals, bars represent arithmetic means of each group.

Fig. 2. BALB/cJ mice develop more severe EAM than BALB/cAnNTac animals

A) Cardiac histopathology at day 21 of EAM in BALB/cJ (blue, JAX) and BALB/cAnNTac (red, Tac) mice. Representative cardiac histopathology micrographs depict median individuals of each group, stained by H & E.

B) Relative heart weight, normalized to body weight (left); total viable CD45⁺ cells infiltrating the hearts of BALB/cJ and BALB/cAnNTac mice, determined cytometrically (middle); and cardiac hydroxyproline content (right) at day 21 of EAM.

Data are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. A Th17 signature is associated with microbiome-sensitive cardiac inflammation

A) Cardiac expression of inflammatory cytokines and chemokines, determined by Lincos multiplex interrogation of cardiac homogenates. Data are normalized to wet starting weight of sample.

B) Absolute numbers of ROR γ ⁺CD4⁺ Th17 cells in the hearts of BALB/cJ and BALB/cAnNTac animals at day 21 of EAM. Representative bivariate plots depict viable TCR β ⁺CD45⁺-gated lymphocytes from concatenates of each group.

C) Absolute numbers of Ly6G^{hi}CD11b⁺ neutrophils and Ly6C^{hi}CD11b⁺Ly6G⁻ inflammatory monocytes in the hearts of BALB/cJ and BALB/cAnNTac mice.

D) Absolute numbers of ROR γ ⁺CD4⁺ Th17 cells in the hearts of polyantibiotic or control BALB/cJ animals at day 21 of EAM. Representative bivariate plots depict viable CD3 ϵ ⁺CD45⁺-gated lymphocytes from concatenates of each group.

Fig. 4. Innate lymphoid cell (ILC) populations in the steady-state ileal lamina propria associate with susceptibility to EAM

A) Representative gating for innate lymphoid cell (ILC) populations from the lamina propria of the distal ilea in naïve, unimmunized BALB/cJ and BALB/cAnNTac mice; enumerated as T-bet⁺ ILC1, ST2/IL33R⁺ ILC2, and RORγt⁺ ILC3 subpopulations (right). *Lineage* cocktail includes CD3e, CD11b, CD11c, CD19, CD45R/B220, CD49b/DX5, Gr1 (Ly6G/Ly6C), TCRγδ, and Ter119. Representative bivariate plots depict viable CD45⁺-gated lymphocytes from concatenates of each group.

B) Realtime qPCR profiling of Th17/ILC3-associated transcripts from the distal lamina propria of naïve, unimmunized BALB/c mice. Data are represented as linearized fold difference values of BALB/cJ mice, relative to mean values from BALB/cAnNTac animals, normalized to *Hprt*.

C) Expression of IL17A by viable CD45⁺-gated αβ T cells in the ileal lamina propria of naïve, unimmunized IL17A-GFP reporter mice or nontransgenic littermates treated with polyantibiotic or control water in representative bivariate plots of concatenates of (*n* = 3) groups.

D) RORγt⁺ ILC3s in the ileal lamina propria of naïve, unimmunized polyantibiotic-treated or control BALB/cJ animals, expressed as a proportion of *Lineage*⁺CD90⁺IL7Rα⁺-gated ILCs. Representative bivariate plots depict viable ILC-gated lymphocytes from concatenates of each group.

Fig. 5. ILCs are critical for the recruitment of leukocytes to the inflamed heart

A) Schematic representation of depletion of ILCs from active AT-EAM recipients by allo-mAbs to CD90.2 (Thy1.2), clone 30H12.

B, E) Representative bivariate plots depict donor and recipient-derived viable CD11b⁻CD45⁺-gated concatenates of each group from A) mAb depletion of ILCs or D) genetically defective development of ILCs at day 14 post-transfer.

C, F) Enumerative cardiac cytometry of total CD45⁺ leukocytes, Ly6G^{hi}CD11b⁺ neutrophils, and CD90.1⁺CD4⁺TCRβ⁺ T cells from A) mAb depletion of ILCs or D) genetically defective development of ILCs at day 14 post transfer.

D) Schematic representation of deletion of ILCs from RAG2^{-/-}γc^{-/-} recipients in active AT-EAM. Data are representative of two independent experiments.

Fig. 6. Microbiome profiles and ILCs regulate expression of CCL20/MIP3α in the ilea at steady-state, and in the inflamed heart

A) CCL20/MIP3α expression in the distal ilea of polyantibiotic-treated or control BALB/cJ mice at day 9 of EAM, as interrogated by realtime qPCR. Data are represented as fold change, relative to control animals, normalized to *Hprt* housekeeper.

B) Proportional distribution of CCL20/MIP3α⁺ populations in the hearts of untreated BALB/cJ mice at day 21 of EAM, as determined by backcalculated values from cytometry in C–D. Slices indicate arithmetic mean of each population across the group.

C) CCL20/MIP3α expression in viable CD31⁺CD45⁻ cardiac endothelial cells in dys-biotic or control BALB/cJ mice over the course of EAM. Representative histogram overlays depict concatenates of each group.

D) CCL20/MIP3α expression, as a proportion of: CD64⁺CD11b⁺CD45⁺ macrophages, CD11c^{hi}CD45⁺ dendritic cells, and gp38/podoplanin-1⁺ and CD140a/PDGF-Rα⁺ fibroblasts at day 21 of EAM in dysbiotic or control BALB/cJ mice.

E–F) CCL20/MIP3 α expression, determined by quantitative sandwich ELISA interrogation of cardiac homogenates from E) α -CD90.2 mAb parking AT-EAM or F) RAG2^{-/-} γ c^{-/-}ILC-deficient active AT-EAM recipients. Data are normalized to wet starting weight of sample.

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Fig. 7. Microbiome profiles and ILCs are regulators of CCR6-driven recruitment of Th17-associated lymphocytes to the inflamed heart

A) Cytometric enumeration of ROR γ t-expressing $\gamma\delta$ T cells and *Lineage*⁻CD90⁺IL7R α ⁺ ILC3s in the hearts of polyantibiotic-treated and control BALB/cJ mice at day 21 of EAM. Representative bivariate plots depict viable CD45⁺ lymphoid populations from concatenates of each group.

B) CCR6 expression on cardiac ROR γ t⁺CD4⁺ Th17 cells in polyantibiotic-treated and control BALB/cJ mice at day 21 of EAM. Representative bivariate plots depict viable CD4⁺CD3 ϵ ⁺CD45⁺-gated lymphocyte populations from concatenates of each group.

C) CCR6 expression on CD4⁺ and $\gamma\delta$ T cells in the hearts of BALB/cJ (blue) and BALB/cAnNTac (red) mice at day 21 of EAM. Representative bivariate plots depict viable CD45⁺-gated lymphocyte subpopulations from concatenates of each group.

D) CCR6 and ROR γ t expression on cardiac CD4⁺ T cells in ILC-deficient RAG2^{-/-} γ c^{-/-} and control RAG2^{-/-} active AT-EAM recipients at day 14 post-transfer. Representative bivariate plots depict viable CD4⁺TCR β ⁺CD45⁺-gated T cells from concatenates each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)