HHS Public Access

Author manuscript

Eur J Immunol. Author manuscript; available in PMC 2017 December 01.

Published in final edited form as:

Eur J Immunol. 2016 December ; 46(12): 2749–2760. doi:10.1002/eji.201646557.

Macrophages and cardiac fibroblasts are the main producers of eotaxins and regulate eosinophil trafficking to the heart

Nicola L. Diny[1], **Xuezhou Hou**[1], **Jobert G. Barin**[2], **Guobao Chen**[2], **Monica V. Talor**[2], **Julie Schaub**[2], **Stuart D. Russell**[3], **Karin Klingel**[4], **Noel R. Rose**[1,2,5], and **Daniela Čiháková**[1,2]

[1]W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA

[2]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[3]Department of Medicine Cardiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[4]Abteilung für Molekulare Pathologie, Eberhard Karls Universität Tübingen, Tübingen, Germany

[5]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Abstract

Cardiac manifestations are a major cause of morbidity and mortality in patients with eosinophil-associated diseases. Eosinophils are thought to play a pathogenic role in myocarditis. We investigated the pathways that recruit eosinophils to the heart using a model of eosinophilic myocarditis, in which experimental autoimmune myocarditis (EAM) is induced in IFN$\gamma^{-/-}$IL-17A$^{-/-}$ mice. Two conditions are necessary for efficient eosinophil trafficking to the heart: high eotaxin (CCL11, CCL24) expression in the heart and expression of the eotaxin receptor CCR3 by eosinophils. We identified cardiac fibroblasts as the source of CCL11 in the heart interstitium. CCL24 is produced by F4/80^{+} macrophages localized at inflammatory foci in the heart. Expression of CCL11 and CCL24 is controlled by Th2 cytokines, IL-4 and IL-13. To determine the relevance of this pathway in humans, we analyzed endomyocardial biopsy samples from myocarditis patients. Expression of CCL11 and CCL26 was significantly increased in eosinophilic myocarditis compared to chronic lymphocytic myocarditis and positively correlated with the number of eosinophils. Thus, eosinophil trafficking to the heart is dependent on the eotaxin-CCR3 pathway in a mouse model of EAM and associated with cardiac eotaxin expression in patients with eosinophilic myocarditis. Blocking this pathway may prevent eosinophil-mediated cardiac damage.

Correspondence: Dr. Daniela Čiháková, Johns Hopkins University, Department of Pathology, Division of Immunology, 720 Rutland Ave, Baltimore, MD 21205, USA, Fax: +1-410-614–3548, dcihako1@jhmi.edu.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Keywords

Cardiomyopathy; Cell trafficking; Chemokines; Eosinophils; Eotaxins; Experimental autoimmune myocarditis; Inflammation

Introduction

Cardiac manifestations are a major cause of morbidity and mortality in patients with eosinophilia. In hypereosinophilic syndrome (HES), 20–50% of patients develop fibrosis or inflammation of the endocardium, myocardium, or pericardium [1–3]. Similarly, one third of patients with eosinophilic granulomatosis with polyangiitis (EGPA) suffer from cardiovascular manifestations [4, 5]. Eosinophilic myocarditis can also develop in the absence of prolonged peripheral eosinophilia, for example, in the potentially fatal form of acute necrotizing eosinophilic myocarditis, in patients with parasitic diseases, or as hypersensitivity myocarditis in response to drugs [6, 7]. No specific biomarkers or treatments are currently available for eosinophilic myocarditis; patients usually receive immunosuppressive therapy and symptomatic treatment in later stages [7–9]. A recent report from the NIH taskforce on the Research Needs of Eosinophil-Associated Diseases underscored the need for animal models to study organ-targeted eosinophil accumulation [10].

Given the strong association of eosinophils with cardiac disease, eosinophils are believed to play a pathogenic role in the heart [2, 11, 12]. Eosinophils may be directly cytotoxic to cardiomyocytes [13–15], activate cardiac mast cells [16], or release pro-thrombotic tissue factor [17]. Eosinophil granule proteins are deposited in the myocardium during eosinophilic myocarditis [18]. They are thought to damage the endocardium, resulting in thrombosis and endocarditis, and eventually in endomyocardial fibrosis and valvular complications [1, 3]. Animal studies further strengthen the evidence that eosinophils contribute to pathology and mortality in eosinophilic myocarditis [19–21].

Despite the clear link between eosinophils and cardiac damage, the pathways that recruit eosinophils to the heart have not been described. Eosinophils develop in the bone marrow and are released into the blood in response to IL-5. From there, they migrate into tissues [22]. Eosinophils express numerous receptors for chemokines and other mediators that can direct trafficking [23]. The eotaxins are the strongest eosinophil attracting chemokines [24] and the most studied [25–28]. Whether eotaxins play a role in eosinophil trafficking to the heart, however, is not known.

To discover the signals responsible for eosinophil trafficking to the heart, we used a mouse model of eosinophilic myocarditis previously established by our group [20]. Induction of experimental autoimmune myocarditis (EAM) in mice lacking the key Th1 and Th17 cytokines IFN-γ and IL-17A results in a fatal, Th2-driven form of myocarditis that is characterized by a predominantly eosinophilic infiltrate [20]. We identified the signaling pathway controlling eosinophil trafficking to the inflamed heart and the cell types producing the required chemoattractants. We then validated our findings in patients with eosinophilic myocarditis.

Results

Eosinophils migrate to the heart in response to eotaxin-CCR3 signaling

We hypothesized that the eotaxin-CCR3 pathway is important for eosinophil trafficking to the heart based on our previous observations that *Ccl11* (eotaxin-1) and *Ccl24* (eotaxin-2) expression are substantially increased in IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ mice with eosinophilic myocarditis compared to WT mice during EAM [20]. By including additional groups, we determined that this expression pattern also occurs in the absence of eosinophils in ΔdblGATA1 and IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice (Fig. 1A). IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice showed substantially higher cardiac expression of *Ccl11* and *Ccl24* than ΔdblGATA1 mice at day 21 of EAM. This finding establishes that eosinophils are not required for eotaxin expression in the heart during EAM.

We employed adoptive cell transfer to determine the importance of the eotaxin receptor CCR3 in eosinophil trafficking to the heart (Fig. 1B). Eosinophils were isolated from IL-5 transgenic mice (IL-5Tg) because these mice develop massive eosinophilia [29]. Recipient ΔdblGATA1 and IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice deficient in eosinophils were immunized on days 0 and 7 to induce EAM. On day 20, eosinophils were isolated from peripheral blood of IL-5TgCCR3$^{+/+}$ and IL-5TgCCR3$^{-/-}$ donor mice and 10^7 donor cells were injected intravenously into recipients. The following day, recipients were sacrificed. Eosinophil infiltration in multiple organs was quantified by flow cytometry (Fig. 1C–E, Supporting Information Fig. 1A and B). All eosinophils recovered in the recipients were necessarily donor-derived because the ΔdblGATA1 mutation blocks eosinophil development in the bone marrow [30]. Only when CCR3-expressing eosinophils were injected into IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 recipients, could significant numbers of eosinophils be retrieved from the heart (Fig. 1C, D). This combination of donor and recipient alone resulted in a significant increase in cardiac eosinophils in both frequency (Fig. 1C, E) and absolute numbers (Fig. 1D). Use of bone marrow-derived eosinophils from WT and CCR3$^{-/-}$ donors for adoptive transfer yielded the same results (*data not shown*). Expression of CCR3 by eosinophils allowed for substantial eosinophil trafficking to the heart, but only in the presence of high cardiac eotaxin expression. Therefore, two conditions are required for efficient eosinophil trafficking to the heart: substantial eotaxin expression in the heart as in the IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice and expression of the CCR3 receptor on eosinophils. These data led us to conclude that the CCR3-eotaxin pathway is essential for eosinophil trafficking to the heart.

Eosinophils reach the highest frequency in the hearts of IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice

To determine whether eosinophil migration into tissues in response to the eotaxin-CCR3 pathway was intact in ΔdblGATA1 recipients, we analyzed eosinophil numbers in the small intestine in parallel with the heart. The intestine harbors eosinophils under steady-state conditions in mice and humans [27, 31]. Eosinophil trafficking to the intestine is known to be dependent on the eotaxin-CCR3 pathway [25]. CCR3-expressing eosinophils were found at significantly higher frequencies in the intestines of both recipients compared to CCR3-deficient eosinophils (Fig. 1E). Adoptively transferred eosinophils were also retrieved from peripheral blood, spleen, and bone marrow at low frequencies (Fig. 1E, Fig. 2, Supporting

Information Fig. 1B). A role for the eotaxin-CCR3 pathway has not been described for these organs, and we did not find more $CCR3^{+/+}$ eosinophils compared to $CCR3^{-/-}$ eosinophils in any of these tissues (Fig. 1E, Supporting Information Fig. 1B).

Importantly, transferred $CCR3^{+/+}$ eosinophils in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 recipients reached higher frequencies in the heart than any other organ (Fig. 2A). Eosinophil frequency in the heart was about 10- to 20-fold higher than in the blood or spleen and about twofold higher than in the intestine. In contrast, in ΔdblGATA1 recipients transferred $CCR3^{+/+}$ eosinophils were increased in the small intestine compared to all other organs, including the heart (Fig. 2B). Thus, eosinophils are specifically recruited to the heart by the Th2-driven myocarditis that develops in the absence of IFN-γ and IL-17A.

Genetic ablation of CCR3 in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ mice blocks eosinophil trafficking to the heart

To further test our hypothesis that eosinophil trafficking to the heart is mainly CCR3-dependent in eosinophilic myocarditis, we crossed IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ mice with $CCR3^{-/-}$ mice. Eosinophil frequencies in the heart were dramatically reduced from 24% in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ mice to 2% in IFN-$\gamma^{-/-}$IL-$17A^{-/-}CCR3^{-/-}$ mice on day 21 of EAM (Fig. 3A, B). Eosinophil frequencies in the spleens did not differ between the two strains (Fig. 3C). Although considerably diminished, eosinophils were not entirely absent from the hearts of IFN-$\gamma^{-/-}$IL-$17A^{-/-}CCR3^{-/-}$ mice (Fig. 3A, B) and accounted for similar frequencies in heart and spleen on day 21 of EAM (Fig. 3B, C). This suggests that there is no specific recruitment of eosinophils to the heart in the absence of CCR3.

Expression of CCR3 has also been reported on human Th2 cells [32]. We observed similar $CD4^+$ T cell frequencies in the presence or absence of CCR3 during EAM (Fig. 3D). This indicates that reduced eosinophil trafficking to the heart in IFN-$\gamma^{-/-}$IL-$17A^{-/-}CCR3^{-/-}$ mice is not a consequence of decreased T cell infiltration. Moreover, all 3 strains developed myocarditis of similar histopathologic severity (*data not shown*). Taken together, these findings demonstrate that in eosinophilic myocarditis without peripheral eosinophilia, as is observed in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ mice, eosinophil trafficking to the heart is dependent on the eotaxin-CCR3 pathway.

CCL11 and CCL24 are expressed by different cell types in the heart

Immunohistochemical staining for eotaxins on serial heart sections revealed interstitial expression of CCL11 at day 21 of EAM in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice (Fig. 4A), ΔdblGATA1 mice (Fig. 4E), and naïve mice of both strains (*data not shown*). Only single $CCL11^+$ cells were observed in inflammatory foci (Fig. 4B, F). This pattern is consistent with expression by fibroblasts. CCL24 was not detected interstitially between cardiomyocytes (Fig. 4C, G). Instead, CCL24 localized to areas of inflammation in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice (Fig. 4D), consistent with expression by heart infiltrating cells. CCL24 was not detectable in ΔdblGATA1 mice (Fig. 4G, H) or naïve mouse hearts of either genotype (*data not shown*). This demonstrates that CCL11 and CCL24 are expressed by different cell types in the heart and under different conditions.

Cardiac fibroblasts are the major CCL11 producers in the heart

Next, we sought to establish which cell types were responsible for eotaxin production. We used a *Ccl11* mRNA probe for flow cytometry to address this question in an unbiased manner. We included surface markers for the identification of tissue resident cells and infiltrating hematopoietic-lineage cells (Supporting Information Fig. 2). Out of the different cell populations in the heart at day 21 of EAM, the highest expression of *Ccl11* was detected in $CD45^{-}CD31^{-}CD140a^{+}$ cardiac fibroblasts (Fig. 4I, J). This fibroblast subset showed significantly higher *Ccl11* expression compared to total viable cells for both IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 and ΔdblGATA1 mice (Fig. 4J). Low levels of *Ccl11* expression could be detected by qPCR even in naïve hearts. The highest *Ccl11* signal in naïve heart cells was also in the $CD140a^{+}$ fibroblast subset (*data not shown*). Thus, cardiac fibroblasts are the major producer of *Ccl11* in the heart during steady-state and in myocarditis. To confirm these results with an independent method, we sorted several cell populations from ΔdblGATA1 and IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice at day 21 of EAM and analyzed eotaxin expression by qPCR. $CD31^{+}$ endothelial cells were depleted before cells were sorted by FACS (Supporting Information Fig. 3A). Again, $CD45^{-}CD140a^{+}$ fibroblasts showed the highest expression of *Ccl11* compared to all other sorted cells (Fig. 4K). In conclusion, the major producers of CCL11 are $CD140a^{+}$ cardiac fibroblasts located interstitially between myocytes. These CCL11-expressing fibroblasts were found in both ΔdblGATA1 and IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice at steady-state and in EAM.

CCL24 is produced by $F4/80^{+}$ macrophages during myocarditis

Unlike *Ccl11*, which was upregulated 4-fold during EAM in ΔdblGATA1 compared to naïve mice, *Ccl24* did not increase in EAM in ΔdblGATA1 mice (Supporting Information Fig. 3B). In the IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice, however, *Ccl24* was highly upregulated during EAM (Fig. 4L, Supporting Information Fig. 3B). The populations shown in Figure 4K did not conclusively reveal the major *Ccl24* expressing cell type (Supporting Information Fig. 3B). Therefore, we sorted 10 overlapping populations (Supporting Information Fig. 4) and assayed them for Ccl24 expression. $CD45^{+}CD11b^{+}Ly6G^{-}F4/80^{+}$ macrophages were the major *Ccl24* expressing cells in EAM in IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice (Fig. 4L). Expression of CCL24 by macrophages is consistent with localization to inflammatory foci observed by immunohistochemistry (Fig. 4D). The staining for eotaxin proteins on serial sections (Fig. 4A–H) and qPCR from different sorted cell populations (Fig. 4K, L, Supporting Information Fig. 5) clearly demonstrated that CCL11 and CCL24 were expressed by different cell types during EAM. CCL24 was produced by $F4/80^{+}$ macrophages, localized in foci of inflammation, and detected as protein only in immunized IFN-$\gamma^{-/-}$IL-$17A^{-/-}$ ΔdblGATA1 mice.

Primary cardiac fibroblasts produce eotaxins in vitro in response to IL-4 and IL-13

We isolated primary fibroblasts from the hearts of naïve adult WT mice to determine if cardiac fibroblasts could produce CCL11 in response to Th2 cytokines. In vitro culture of fibroblasts with the Th2 cytokines IL-4 and IL-13, but not IL-5, induced *Ccl11* mRNA expression (Fig. 5A) and secretion into the cell culture medium (Fig. 5B). The Th1 and Th17 cytokines IFN-γ and IL-17A did not induce CCL11 (*data not shown*). This confirms

that cardiac fibroblasts produce CCL11 and do so in response to key Th2 cytokines IL-4 and IL-13.

Surprisingly, IL-4 and IL-13 also induced CCL24 expression in cardiac fibroblasts (Fig. 5C, D) although at a much lower magnitude than CCL11. This is consistent with the observation that higher expression of CCL24 was detected in CD140a^{+} fibroblasts of IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice compared to ΔdblGATA1 mice (Supporting Information Fig. 3B).

IL-4 and IL-13 induce CCL24 secretion in macrophages

To determine if Th2 cytokines could also induce eotaxins in macrophages, we cultured bone marrow-derived macrophages with different cytokines. As expected, IFN-γ and IL-17A did not induce eotaxins in macrophages (*data not shown*). CCL11 was not induced in macrophages in response to Th2 cytokines (Fig. 5E, F). However, they secreted large amounts of CCL24 after stimulation with IL-4 and IL-13 (Fig. 5G, H). These data support macrophages as potent producers of CCL24. The increased expression of IL-4 and IL-13 from T cells in IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ compared to WT mice [20] may explain the increased eotaxin expression in these mice.

Cardiac eotaxin expression is increased in patients with eosinophilic myocarditis

To test whether eotaxins are also important for eosinophil trafficking in patients with eosinophilic myocarditis, we analyzed endomyocardial biopsy samples. Patients with eosinophilic myocarditis had increased numbers of heart-infiltrating eosinophils compared to patients with chronic lymphocytic myocarditis (Fig. 6A, B). Expression of *CCL11* and *CCL26* (an eotaxin specific to humans and a pseudogene in mice) was increased in eosinophilic myocarditis patients compared to chronic lymphocytic myocarditis patients (Fig. 6C). *CCL11* was detected in all samples regardless of diagnosis. In contrast, *CCL26* was detected in 75% of eosinophilic myocarditis patients but only in 50% of chronic lymphocytic myocarditis patients (Fig. 6C). *CCL24* was detected in some of the eosinophilic myocarditis biopsies but in none of the chronic lymphocytic myocarditis samples (Fig. 6C). It is possible that *CCL24* is expressed in the heart only in the context of eosinophilic myocarditis. Expression of *CCL11* and *CCL26* correlated positively with the number of infiltrating eosinophils in the biopsies (Fig. 6D). The significant increase in eotaxin expression in the hearts of patients with eosinophilic myocarditis and the correlation of eotaxin expression with eosinophil infiltration suggest that eotaxins are important for eosinophil trafficking to the human heart during eosinophilic myocarditis.

Discussion

Eosinophils can infiltrate many different organs in response to multiple local stimuli, such as chemokines and lipid mediators [23]. In this study, we present evidence that eosinophil trafficking to the heart in eosinophilic myocarditis depends on the chemokine receptor CCR3 and expression of its ligands CCL11, CCL24, and CCL26 in the heart in a mouse model and in patients with eosinophilic myocarditis. This is the first description of a precise pathway that recruits eosinophils to the heart. We identified the cell types producing eotaxins in the heart and showed that eotaxin expression in cardiac fibroblasts and

macrophages is controlled by two cytokines, IL-4 and IL-13. These findings enhance our understanding of how eosinophils contribute to cardiac pathology and provide insights into the regulation of eosinophilic heart disease and eosinophilic autoimmune diseases in general. Moreover, it opens new therapeutic avenues to prevent eosinophil-mediated heart damage.

We demonstrated that CCR3 is required for eosinophil trafficking to the heart using adoptive transfer and genetic methods. IFN-$\gamma^{-/-}$IL-17A$^{-/-}$CCR3$^{-/-}$ mice showed a dramatic reduction of eosinophils in the heart compared to IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ mice. In addition, only CCR3$^{+/+}$, but not CCR3$^{-/-}$, adoptively transferred eosinophils were recovered from the heart of eosinophil-deficient recipients with myocarditis. Using an adoptive transfer system permitted us to exclude effects of local eosinophil proliferation and positive feedback loops, such as eosinophil-derived IL-4 and IL-13 increasing local eotaxin production by other cell types. Additionally, this approach allowed us to distinguish between consequences of CCR3 expression in eosinophils versus other cell types, which is still a controversial issue in the mouse [33, 34].

While CCR3 in humans can bind ligands other than eotaxins, mouse CCR3 is only known to bind the mouse chemokines CCL11 and CCL24 [23, 24]. Together with the results from our adoptive transfer experiments, this implies that the high eotaxin expression in IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice is responsible for eosinophil accumulation in the heart. This is consistent with our previous findings that NK cell depletion results in increased eotaxin expression and eosinophil infiltration in the heart [19]. We only observed these differences between ΔdblGATA1 and IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice in the heart. The intestine showed efficient CCR3$^{+/+}$ eosinophil trafficking in both recipients. This suggests that the severe, Th2-driven EAM developing in the absence of IFN-γ and IL-17A is necessary for high cardiac eotaxin expression and eosinophilic myocarditis.

We identified the cellular sources of eotaxins in the heart. CCL11 was produced by interstitial CD140a^{+} cardiac fibroblasts and was detectable in ΔdblGATA1 and IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice. While not studied in the heart, CCL11 expression in experimental asthma was found in the peribronchial and perivascular regions, perhaps consistent with expression by fibroblasts, and in areas of severe inflammation [28]. In DSS-induced colitis, intestinal macrophages expressed CCL11 [35]. Using immunohistochemistry, we detected CCL24^{+} cells only in the hearts of immunized IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice where they localized to inflammatory foci. We identified these cells as F4/80^{+} macrophages. A previous study noticed *Ccl24* expression by microarray in CX3CR1^{+} cardiac macrophages, although other cell types were not analyzed [36]. In experimental asthma, CCL24 was expressed in the same areas as CCL11 and in bronchoalveolar lavage fluid [28]. Thus, multiple cell types are capable of CCL11 and/or CCL24 expression and differ between organs.

Our in vitro experiments showed that cardiac fibroblasts expressed CCL11 and, to a much lesser extent, CCL24 in response to IL-4 or IL-13 stimulation. Macrophages responded to IL-4 and IL-13 stimulation with CCL24 expression; CCL11 was not induced. The ability of IL-4 and IL-13 to elicit eotaxins is known [37–39], but has not been tested in cardiac

fibroblasts. We speculate that T cells and type 2 innate lymphoid cells are major producers of IL-4 and IL-13, respectively and control eotaxin production in the heart.

Analyzing endomyocardial biopsies, we found that CCL11 and CCL26 expression was increased in patients with eosinophilic myocarditis compared to chronic lymphocytic myocarditis (17-fold and 47-fold, respectively) and was positively correlated with the number of infiltrating eosinophils. We conclude that eosinophil trafficking to the heart in myocarditis patients likely occurs in response to eotaxin signaling. CCL11 and CCL26 may both recruit eosinophils to the heart. An upregulation of CCL11 and CCL26 is also observed in patients with atopic dermatitis [40–42], while only CCL26 is upregulated in eosinophilic esophagitis and asthma [43, 44].

Eotaxin expression in the heart has not been studied in the context of disease. In healthy human heart tissue CCL11 and CCL26, but not CCL24, were detected by northern blot [45–47]. CCL24 mRNA was detectable in 2/11 eosinophilic myocarditis biopsies at a low level but in none of the chronic lymphocytic myocarditis biopsies. In this aspect, the mouse model of myocarditis, where CCL11 and CCL24 are highly upregulated, differs from the patient samples. We did not determine eotaxin protein levels in endomyocardial biopsies. Follow-up studies should measure eotaxin in the heart and serum.

The cellular source and regulation of eotaxins has not been studied in the human heart. In ulcerative colitis, CCL26 was expressed in intestinal nerve ganglia [48] and CCL11 in intestinal macrophages and epithelial cells [35]. In EGPA, CCL26 was detected in endothelial cells, nasal epithelium, and other cells [49]. In asthmatics, CCL26 is expressed in bronchial epithelial cells [43]. Similar to our observations in the mouse, eotaxin production in various human cell types is induced by IL-4 and/or IL-13 [37, 38, 41, 43, 50, 51]. Depending on the cell type, this stimulation results in CCL11, CC24, or CCL26 production. This pathway likely also regulates eotaxin production in the human heart during myocarditis.

In this study, we demonstrate the importance of eotaxins and CCR3 for eosinophil localization to the heart. Several therapeutics blocking this pathway are being evaluated in allergic diseases [52–54], where they decrease tissue eosinophilia. Ongoing trials are assessing CCL11 blockade in the eosinophilic autoimmune diseases ulcerative colitis and bullous pemphigoid (ClinicalTrials.gov identifiers: NCT01671956, NCT02226146). However, none of the treatments have been studied in eosinophilic myocarditis, HES or EGPA. The potential of targeting eotaxins or CCR3 to prevent eosinophil-mediated heart damage remains to be tested.

Materials and methods

Patients

Endomyocardial biopsies were taken at multiple centers for routine diagnostic purposes to evaluate the pathologic basis of unspecified congestive heart failure. Samples were investigated in the Department of Molecular Pathology at the University Hospital of Tübingen, Germany by histology, immunohistochemistry and molecular pathology to

identify infectious agents in the myocardium as described [55]. Written informed consent for further examination was obtained from all patients. We analyzed remaining de-identified tissue. Diagnosis of myocarditis was based on established criteria [56] and all samples were negative for viral infections. Patient age and gender are summarized in Supporting Information Table 1.

Animals

Wildtype (WT, JAX 0651), ΔdblGATA1 (JAX 5653 [30]), IFN-$\gamma^{-/-}$ (JAX 2286), and CCR3$^{-/-}$ mice (JAX 5440), all on the BALB/c background, were obtained from Jackson Laboratories (Bar Harbor, ME). IL-5 transgenic (IL-5Tg) founder mice [29] were kindly provided by James Lee and Nancy Lee (Mayo Clinic Arizona, Scottsdale, AZ). IL-17A$^{-/-}$ founder mice were generously provided by Yoichiro Iwakura (University of Tokyo, Tokyo, Japan). IFN-$\gamma^{-/-}$ mice were crossed with IL-17A$^{-/-}$ mice and bred to homozygosity. Resulting IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ mice were then crossed with ΔdblGATA1 mice or with CCR3$^{-/-}$ mice and offspring were intercrossed to generate mice homozygous at all loci. All mice were maintained at the Johns Hopkins University School of Medicine specific pathogen-free animal vivarium. The number of animals used in each experiment and the number of repeats are listed in the figure legends. Experiments were performed in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the Johns Hopkins University.

Experimental autoimmune myocarditis

To induce myocarditis, male mice age 6–9 weeks were immunized on days 0 and 7 with 100 μg MyHC$\alpha_{614–629}$ peptide emulsified in Complete Freund's Adjuvant (Sigma-Aldrich) supplemented to 5 mg/ml with heat-killed *Mycobacterium tuberculosis* strain H37Ra (Difco). Mice were also administered 500 ng pertussis toxin (List Biologicals) *ip* on day 0.

Eosinophil isolation

Blood from female IL-5Tg and CCR3$^{-/-}$IL-5Tg donors was collected into phosphate buffered saline (PBS) with 100 U/ml Heparin, layered over Histopaque-1119 (Sigma-Aldrich), and centrifuged. Interphase cells were collected and washed in PBS. Red blood cells were lysed with ACK buffer (Quality Biologicals). Eosinophils were enriched by negative MACS selection with anti-CD90.2 and anti-CD45R beads (Miltenyi Biotech). Eosinophil purity (87%–93%) was determined by flow cytometry.

Flow cytometry and cell sorting

Hearts were perfused through the ventricles with PBS for 3 min. Single-cell suspensions were generated by digestion with Collagenase II and DNase I (Worthington) in HBSS in GentleMACS C Tubes (Miltenyi Biotech). Splenocytes were isolated into single-cell suspension, and bone marrow cells were collected from femurs followed by red blood cell lysis with ACK buffer. Intestinal lamina propria cells were isolated from the distal 5 cm of the small intestine. Intestines were flushed, cut into <1 cm pieces, and incubated twice in HBSS, 5% fetal bovine serum (FBS), 5mM EDTA, 10 mM HEPES, and Penicillin/

Streptomycin for 20 min at 37°C. Supernatants were discarded between incubations. Tissues were incubated for 60 min at 37°C in HBSS with Ca^{2+} and Mg^{2+}, 10 mM HEPES, 5% FBS, 1 mg/ml collagenase D, 0.1 mg/ml DNase I, and 3 mg/mL Dispase II (Roche). Using GentleMACS C Tubes, cells were mechanically separated according to the manufacturer's instructions (Miltenyi Biotech). Following filtration through 70 μm filters, single-cell suspensions were stained with LIVE/DEAD stain (Molecular Probes), washed, FcγRII/III blocked with a-CD16/CD32, and stained with fluorochrome-conjugated antibodies (eBioscience, BioLegend, BD Pharmingen). For detection of *Ccl11* mRNA, samples were processed using the FlowRNA II Assay kit (Affymetrix eBioscience). In brief, samples were stained with fluorochrome-conjugated antibodies, fixed and permeabilized, and hybridized with a type 1 *Ccl11* probe or *Actb* control probe. A signal-amplifying branched DNA structure was then hybridized to a probe conjugated to Alexa Fluor647. Samples were acquired on a LSRII 4-laser cytometer running FACSDiva 6.0 (BD Immunocytometry) and analyzed using FlowJo 10.0.8 (TreeStar Software). For cell sorting, single cell suspensions were layered over a Histopaque-1119 gradient, stained, and sorted on an Ari-aIIu cell sorter.

Primary adult cardiac fibroblast isolation and culture

Hearts were cannulated through the aorta and perfused for 3 min at 37°C and 4 mL/min with perfusion buffer: 7.03g/L NaCl, 1.1 g/L KCl, 0.082 g/L KH_2PO_4, 0.085 g/L Na_2HPO_4, 0.144 g/L $MgSO_4$, 2.38 g/L HEPES, 0.39 g/L $NaHCO_3$, 1 g/L glucose, 3.74 g/L Taurine, 2 g/L 2,3-Butanedione monoxime (all Sigma). Next, hearts were perfused for 4 min with perfusion buffer supplemented with 12 g/L Collagenase II and 0.5 g/L Protease XIV (Sigma-Aldrich), and for 8 min with addition of digestion enzymes and 0.03M $CaCl_2$. Hearts were cut into small pieces and cells separated by repeated pipetting for 3 min. Cells were filtered through a 70 μm filter and washed in DMEM (Gibco). Cells were plated in DMEM with 20% FBS (GE Healthcare Life Sciences), nonessential amino acids (Sigma), Penicillin/Streptomycin, 2mM L-Glutamine, and 25mM HEPES (all Quality Biological). Non-adherent cells were washed away after 1h. Fibroblasts of passage 2–3 were used in experiments. Cells and supernatants were harvested at the indicated time points after addition of recombinant mouse cytokines at 100 ng/mL (R&D Systems).

Bone marrow-derived macrophage culture

Bone marrow was isolated from adult WT male mice, red blood cells lysed with ACK buffer, and cells cultured in complete DMEM as above with 20 ng/mL M-CSF and 10 ng/mL GM-CSF (R&D Systems) for 10 days. Media was replaced on days 3 and 6. On day 10, cells were seeded in media without cytokines. Cells and supernatants were harvested 24 h after stimulation with cytokines at 100 ng/mL on day 11 (R&D Systems).

Quantitative real-time PCR

Mouse tissue and cell mRNA was extracted in TRIzol (Invitrogen), quantitated by spectrophotometry, reverse transcribed (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems), and amplified with Power SYBR Green PCR Master Mix (Bio-Rad) on a MyiQ2 thermocycler running iQ5 software (Bio-Rad). Primers for mouse genes (*Gapdh*: 5′-TCCTCCTCAGACCGCTTTT-3 and 5′-TCTGCTGGAGTCCCCTTG-3′, *Ccl11*: 5′-GAATCACCAACAACAGATGCAC-3′ and 5′-

TCCTGGACCCACTTCTTCTT-3′, *Ccl24*: 5′-TCTTAGGGCCCTTCTTGGTG-3′ and 5′-AATTCCAGAAAACCGAGTGG-3′) were commercially synthesized (Integrated DNA Technologies). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method by normalizing threshold cycles to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and controls. RNA from endomyocardial biopsies was isolated using the PureLink FFPE RNA Isolation Kit (Invitrogen) from three to five 5-μm-thick sections per sample, reverse transcribed, and amplified using the iTaq Universal Probes Supermix (Bio-Rad) with TaqMan Gene Expression Assay (Applied Biosystems) for *HPRT*, *CCL11*, *CCL24*, and *CCL26*. Threshold cycles were normalized to HPRT.

ELISA

Cell culture supernatants were assayed for CCL11 using the Mouse CCL11/Eotaxin Quantikine ELISA Kit (R&D systems) and for CCL24 using the Eotaxin-2 (CCL24) Mouse ELISA Kit (Abcam).

Histology and immunohistochemistry

Mouse hearts were cut in half, fixed in SafeFix (Thermo Fisher Scientific), paraffin embedded, and cut into 5-μm-thick sections (Histoserv). Serial sections were stained with 5 μg/mL polyclonal goat anti-mouse CCL11 or CCL24 antibodies (R&D Systems) using the Anti-Goat HRP-DAB Cell & Tissue Staining Kit (R&D Systems) and counterstained with Hematoxylin. The number of infiltrating eosinophils was determined by counting eosinophils on 40×images of H&E-stained biopsy sections. The number of eosinophils was averaged over the area observed.

Statistics

Two groups were analyzed using Student's *t*-test for normally distributed data or Mann–Whitney test for nonparametric data. Multiple group analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test or Dunnett's multiple comparisons test. Calculations were performed in Prism 6 (GraphPad Software Inc.). p values < 0.05 were considered statistically significant and are denoted by asterisks: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Xiaoling Zhang, Ross Flow Cytometry Core, for cell sorting, Sean Doughty for manuscript editing, Elizabeth Gebremariam for mouse breeding, and Elizabeth Jacobsen for an eosinophil isolation protocol.

This work was supported by NIH/NHLBI grants R01HL118183 and R01HL113008 to Daniela Čiháková, Matthew Poyner MVP Memorial Myocarditis Research Fund to Daniela Čiháková and Stuart Russell, American Heart Association Predoctoral Fellowship 15PRE25400010 and Johns Hopkins Bloomberg School of Public Health Richard J and Margaret Conn Himelfarb Student Support fund to Nicola Diny, Johns Hopkins Autoimmune Disease Research Center O'Leary-Wilson Fellowship to Xuezhou Hou, Gilead Sciences Research Scholars Program and American Autoimmune Related Diseases Association grant to Jobert Barin, and Deutsche Forschungsgemeinschaft grant KL595/2-3 to Karin Klingel.

Abbreviations

EAM	experimental autoimmune myocarditis
EDTA	Ethylenediaminetetraacetic acid
EGPA	eosinophilic granulomatosis with polyangiitis
FBS	fetal bovine serum
HBSS	Hanks Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES	hypereosinophilic Syndrome
H&E	Hematoxylin & Eosin
PBS	phosphate buffered saline
WT	wildtype

References

1. Weller PF, Bubley GJ. The idiopathic hypereosinophilic syndrome. Blood. 1994; 83:2759–2779. [PubMed: 8180373]
2. Parrillo JE, Borer JS, Henry WL, Wolff SM, Fauci AS. The cardiovascular manifestations of the hypereosinophilic syndrome. Prospective study of 26 patients, with review of the literature. Am J Med. 1979; 67:572–582. [PubMed: 495628]
3. Ogbogu PU, Rosing DR, Horne R, McDonald K. Cardiovascular manifestations of hypereosinophilic syndromes. Immunol Allergy Clin North Am. 2007; 27:457–475. [PubMed: 17868859]
4. Comarmond C, Pagnoux C, Khellaf M, Cordier JF, Hamidou M, Viallard JF, Maurier F, et al. Eosinophilic granulomatosis with polyangiitis (Churg-Strauss): clinical characteristics and long-term followup of the 383 patients enrolled in the French Vasculitis Study Group cohort. Arthritis Rheum. 2013; 65:270–281. [PubMed: 23044708]
5. Moosig F, Bremer JP, Hellmich B, Holle JU, Holl-Ulrich K, Laudien M, Matthis C, et al. A vasculitis centre based management strategy leads to improved outcome in eosinophilic granulomatosis and polyangiitis (Churg-Strauss, EGPA): monocentric experiences in 150 patients. Ann Rheum Dis. 2013; 72:1011–1017. [PubMed: 22887848]
6. Ginsberg F, Parrillo JE. Eosinophilic myocarditis. Heart Fail Clin. 2005; 1:419–429. [PubMed: 17386864]
7. Baandrup U. Eosinophilic myocarditis. Herz. 2012; 37:849–852. [PubMed: 23179050]
8. Klion AD. How I treat hypereosinophilic syndromes. Blood. 2015; 126:1069–1077. [PubMed: 25964669]
9. Fung G, Luo H, Qiu Y, Yang D, McManus B. Myocarditis. Circ Res. 2016; 118:496–514. [PubMed: 26846643]
10. Bochner BS, Book W, Busse WW, Butterfield J, Furuta GT, Gleich GJ, Klion AD, et al. Workshop report from the National Institutes of Health Taskforce on the Research Needs of Eosinophil-Associated Diseases (TREAD). J Allergy Clin Immunol. 2012; 130:587–596. [PubMed: 22935587]
11. Fauci AS, Harley JB, Roberts WC, Ferrans VJ, Gralnick HR, Bjornson BH. NIH conference. The idiopathic hypereosinophilic syndrome. Clinical, pathophysiologic, and therapeutic considerations. Ann Intern Med. 1982; 97:78–92. [PubMed: 7046556]

12. Parrillo JE. Heart disease and the eosinophil. N Engl J Med. 1990; 323:1560–1561. [PubMed: 2233938]
13. Spry CJ, Tai PC, Davies J. The cardiotoxicity of eosinophils. Postgrad Med J. 1983; 59:147–153. [PubMed: 6844197]
14. Tai PC, Hayes DJ, Clark JB, Spry CJ. Toxic effects of human eosinophil products on isolated rat heart cells in vitro. Biochem J. 1982; 204:75–80. [PubMed: 7115333]
15. Corradi D, Vaglio A, Maestri R, Legname V, Leonardi G, Bartoloni G, Buzio C. Eosinophilic myocarditis in a patient with idiopathic hypereosinophilic syndrome: insights into mechanisms of myocardial cell death. Hum Pathol. 2004; 35:1160–1163. [PubMed: 15343520]
16. Patella V, de Crescenzo G, Marinò I, Genovese A, Adt M, Gleich GJ, Marone G. Eosinophil granule proteins activate human heart mast cells. J Immunol. 1996; 157:1219–1225. [PubMed: 8757629]
17. Cugno M, Marzano AV, Lorini M, Carbonelli V, Tedeschi A. Enhanced tissue factor expression by blood eosinophils from patients with hypereosinophilia: a possible link with thrombosis. PLoS One. 2014; 9:e111862. [PubMed: 25375118]
18. deMello DE, Liapis H, Jureidini S, Nouri S, Kephart GM, Gleich GJ. Cardiac localization of eosinophil-granule major basic protein in acute necrotizing myocarditis. N Engl J Med. 1990; 323:1542–1545. [PubMed: 2233934]
19. Ong S, Ligons DL, Barin JG, Wu L, Talor MV, Diny N, Fontes JA, et al. Natural killer cells limit cardiac inflammation and fibrosis by halting eosinophil infiltration. Am J Pathol. 2015; 185:847–861. [PubMed: 25622543]
20. Barin JG, Baldeviano GC, Talor MV, Wu L, Ong S, Fairweather D, Bedja D, et al. Fatal eosinophilic myocarditis develops in the absence of IFN-g and IL-17A. J Immunol. 2013; 191:4038–4047. [PubMed: 24048893]
21. Abston ED, Barin JG, Cihakova D, Bucek A, Coronado MJ, Brandt JE, Bedja D, et al. IL-33 independently induces eosinophilic pericarditis and cardiac dilation: ST2 improves cardiac function. Circ Heart Fail. 2012; 5:366–375. [PubMed: 22454393]
22. Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J Exp Med. 1995; 182:1169–1174. [PubMed: 7561691]
23. Zhu, X., Zimmermann, N. Eosinophil trafficking. In: Lee, J., Rosenberg, H., editors. Eosinophils in Health and Disease. Burlington: Elsevier Science; 2012. p. 121-228.
24. Borchers MT, Ansay T, DeSalle R, Daugherty BL, Shen H, Metzger M, Lee NA, et al. In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration. J Leukoc Biol. 2002; 71:1033–1041. [PubMed: 12050190]
25. Matthews AN, Friend DS, Zimmermann N, Sarafi MN, Luster AD, Pearlman E, et al. Eotaxin is required for the baseline level of tissue eosinophils. Proc Natl Acad Sci U S A. 1998; 95:6273–6278. [PubMed: 9600955]
26. Gouon-Evans V, Pollard JW. Eotaxin is required for eosinophil homing into the stroma of the pubertal and cycling uterus. Endocrinology. 2001; 142:4515–4521.
27. Mishra A, Hogan SP, Lee JJ, Foster PS, Rothenberg ME. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. J Clin Invest. 1999; 103:1719–1727. [PubMed: 10377178]
28. Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J Immunol. 2005; 175:5341–5350. [PubMed: 16210640]
29. Lee NA, McGarry MP, Larson KA, Horton MA, Kristensen AB, Lee JJ. Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. J Immunol. 1997; 158:1332–1344. [PubMed: 9013977]
30. Yu C, Cantor AB, Yang H, Browne C, Wells RA, Fujiwara Y, Orkin SH. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J Exp Med. 2002; 195:1387–1395. [PubMed: 12045237]

31. Kato M, Kephart GM, Morikawa A, Gleich GJ. Eosinophil infiltration and degranulation in normal human tissues: evidence for eosinophil degranulation in normal gastrointestinal tract. Int Arch Allergy Immunol. 2001; 125(Suppl 1):55–58. [PubMed: 11408775]
32. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science. 1997; 277:2005–2007. [PubMed: 9302298]
33. Humbles AA, Lu B, Friend DS, Okinaga S, Lora J, Al-Garawi A, Martin TR, et al. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. Proc Natl Acad Sci U S A. 2002; 99:1479–1484. [PubMed: 11830666]
34. Collington SJ, Westwick J, Williams TJ, Weller CL. The function of CCR3 on mouse bone marrow-derived mast cells in vitro. Immunology. 2010; 129:115–124. [PubMed: 20050333]
35. Ahrens R, Waddell A, Seidu L, Blanchard C, Carey R, Forbes E, Lampinen M, et al. Intestinal macrophage/epithelial cell-derived CCL11/eotaxin-1 mediates eosinophil recruitment and function in pediatric ulcerative colitis. J Immunol. 2008; 181:7390–7399. [PubMed: 18981162]
36. Pinto AR, Godwin JW, Chandran A, Hersey L, Ilinykh A, Debuque R, Wang L, et al. Age-related changes in tissue macrophages precede cardiac functional impairment. Aging (Albany NY). 2014; 6:399–413. [PubMed: 24861132]
37. Mochizuki M, Bartels J, Mallet AI, Christophers E, Schröder JM. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. J Immunol. 1998; 160:60–68. [PubMed: 9551956]
38. Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, Lo D. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. J Immunol. 1999; 162:2477–2487. [PubMed: 10072486]
39. Takahashi K, Imaeda H, Fujimoto T, Ban H, Bamba S, Tsujikawa T, Sasaki M, et al. Regulation of eotaxin-3/CCL26 expression by Th2 cytokines in human colonic myofibroblasts. Clin Exp Immunol. 2013; 173:323–331. [PubMed: 23607908]
40. Yawalkar N, Uguccioni M, Schaerer J, Braunwalder J, Karlen S, Dewald B, Braathen LR, et al. Enhanced expression of eotaxin and CCR3 in atopic dermatitis. J Invest Dermatol. 1999; 113:43–48. [PubMed: 10417617]
41. Kaburagi Y, Shimada Y, Nagaoka T, Hasegawa M, Takehara K, Sato S. Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-1alpha, MIP-1beta, and eotaxin) in patients with atopic dermatitis. Arch Dermatol Res. 2001; 293:350–355. [PubMed: 11550808]
42. Kagami S, Kakinuma T, Saeki H, Tsunemi Y, Fujita H, Nakamura K, Takekoshi T, et al. Significant elevation of serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, in patients with atopic dermatitis: serum eotaxin-3/CCL26 levels reflect the disease activity of atopic dermatitis. Clin Exp Immunol. 2003; 134:309–313. [PubMed: 14616792]
43. Blanchard C, Wang N, Stringer KF, Mishra A, Fulkerson PC, Abonia JP, Jameson SC, et al. Eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis. J Clin Invest. 2006; 116:536–547. [PubMed: 16453027]
44. Larose MC, Chakir J, Archambault AS, Joubert P, Provost VER, Laviolette M, Flamand N. Correlation between CCL26 production by human bronchial epithelial cells and airway eosinophils: Involvement in patients with severe eosinophilic asthma. J Allergy Clin Immunol. 2015; 136:904–913. [PubMed: 25936567]
45. Kitaura M, Nakajima T, Imai T, Harada S, Combadiere C, Tiffany HL, Murphy PM, et al. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. J Biol Chem. 1996; 271:7725–7730. [PubMed: 8631813]
46. Patel VP, Kreider BL, Li Y, Li H, Leung K, Salcedo T, Nardelli B, et al. Molecular and functional characterization of two novel human C–C chemokines as inhibitors of two distinct classes of myeloid progenitors. J Exp Med. 1997; 185:1163–1172. [PubMed: 9104803]
47. Kitaura M, Suzuki N, Imai T, Takagi S, Suzuki R, Nakajima T, Hirai K, et al. Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. J Biol Chem. 1999; 274:27975–27980. [PubMed: 10488147]

48. Smyth CM, Akasheh N, Woods S, Kay E, Morgan RK, Thornton MA, O'Grady A, et al. Activated eosinophils in association with enteric nerves in inflammatory bowel disease. PLoS One. 2013; 8:e64216. [PubMed: 23717571]

49. Polzer K, Karonitsch T, Neumann T, Eger G, Haberler C, Soleiman A, Hellmich B, et al. Eotaxin-3 is involved in Churg-Strauss syndrome– a serum marker closely correlating with disease activity. Rheumatology (Oxford). 2008; 47:804–808. [PubMed: 18397958]

50. Takahashi K, Imaeda H, Fujimoto T, Ban H, Bamba S, Tsujikawa T, Sasaki M, et al. Regulation of eotaxin-3/CCL26 expression by Th2 cytokines in human colonic myofibroblasts. Clin Exp Immunol. 2013; 173:323–331. [PubMed: 23607908]

51. Lezcano-Meza D, Davila-Davila B, Vega-Miranda A, Negrete-Garcia MC, Teran LM. Interleukin (IL)-4 and to a lesser extent either IL-13 or interferon-gamma regulate the production of eotaxin-2/CCL24 in nasal polyps. Allergy. 2003; 58:1011–1017. [PubMed: 14510718]

52. Gauvreau GM, Boulet LP, Cockcroft DW, Baatjes A, Cote J, Deschesnes F, Davis B, Strinich T, et al. Antisense therapy against CCR3 and the common beta chain attenuates allergen-induced eosinophilic responses. Am J Respir Crit Care Med. 2008; 177:952–958. [PubMed: 18244953]

53. Ding C, Li J, Zhang X. Bertilimumab Cambridge Antibody Technology Group. Curr Opin Investig Drugs. 2004; 5:1213–1218.

54. Gauvreau GM, Pageau R, Seguin R, Carballo D, Gauthier J, D'Anjou H, Campbell H, et al. Dose-response effects of TPI ASM8 in asthmatics after allergen. Allergy. 2011; 66:1242–1248. [PubMed: 21605124]

55. Zuern CS, Muller KAL, Seizer P, Geisler T, Banya W, Klingel K, Kandolf R, et al. Cyclophilin A predicts clinical outcome in patients with congestive heart failure undergoing endomyocardial biopsy. Eur J Heart Fail. 2013; 15:176–184. [PubMed: 23243067]

56. Caforio ALP, Pankuweit S, Arbustini E, Basso C, Gimeno-Blanes J, Felix SB, Fu M, et al. Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. Eur Heart J. 2013; 34:2636–2648. [PubMed: 23824828]

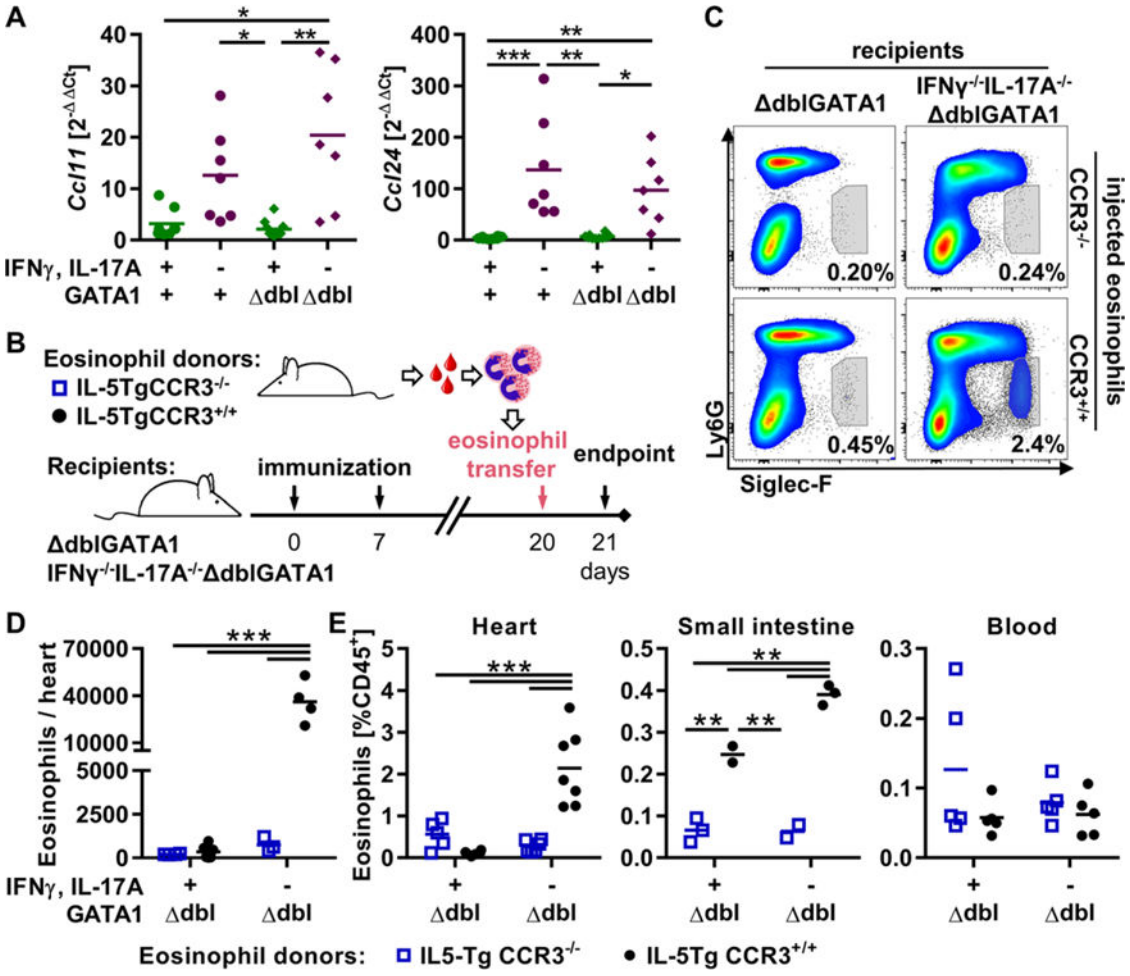

Figure 1.

CCR3 is required for eosinophil trafficking to the heart in eosinophilic myocarditis. Mice were analyzed at day 21 of experimental autoimmune myocarditis (EAM). (A) Expression of *Ccl11* and *Ccl24* in heart homogenates was analyzed by qPCR ($2^{-\Delta\Delta Ct}$ values relative to *Gapdh* and naïve WT mice). (B) Schematic of adoptive eosinophil transfer into immunized recipient mice for data in (C–E). (C) Representative bivariate flow cytometry plots showing $SiglecF^{+}Ly6G^{lo}$ eosinophils from 3–7 mice/group as a proportion of viable $CD45^{+}$ heart-infiltrating cells following transfer. (D) Total number of heart-infiltrating eosinophils after eosinophil transfer. (E) Frequency of eosinophils in heart, small intestine and blood following adoptive transfer. Data points represent individual animals, bars indicate means. Data are representative of 2–4 independent experiments, with 3–7 mice/group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; one-way ANOVA followed by Tukey's multiple comparison.

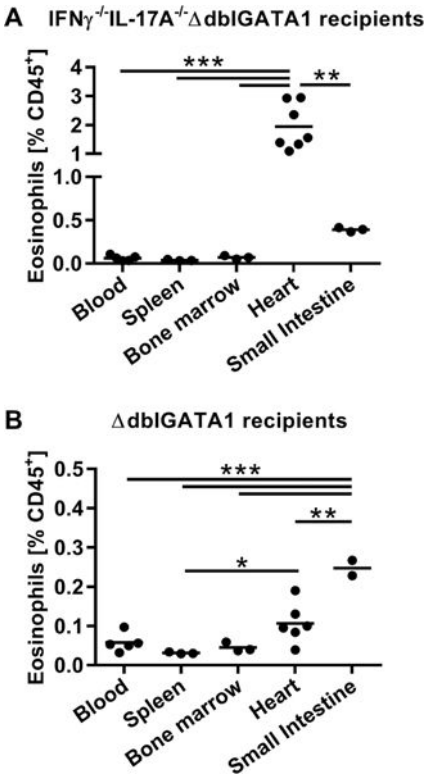

Figure 2.

Adoptively transferred eosinophils reach highest frequency in the heart. (A and B) Eosinophils were adoptively transferred into (A) IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 and (B) ΔdblGATA1 recipients (3–7 mice/group) on day 20 of EAM. Eosinophil frequency in indicated organs one day following adoptive transfer is shown. The same experiment as in Figure 1E is shown. Data points represent individual animals, bars indicate means. Data are representative of 2 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; one-way ANOVA followed by Tukey's multiple comparison.

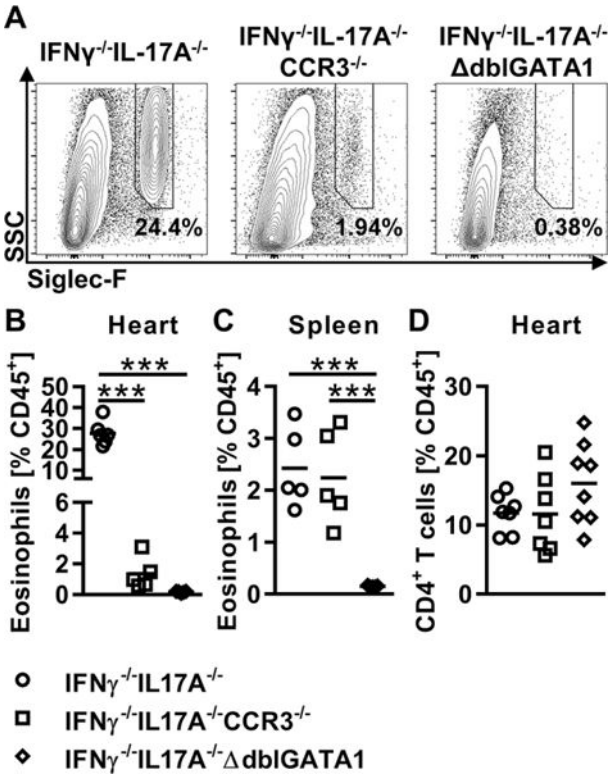

Figure 3.

Genetic ablation of CCR3 in IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ mice reduces eosinophil frequency in the heart. Mice were analyzed at day 21 of EAM. (A) Representative bivariate flow cytometry plots of heart-infiltrating CD45^{+} cells. Gates show frequency of SiglecF^{+}SSC^{hi} eosinophils. (B and C) Eosinophil and (D) T helper cell frequency in heart and spleen in the indicated mouse strains. Data points represent individual animals, bars indicate means. Data are representative of 2 independent experiments with 5–8 mice/group. ***$p < 0.001$; one-way ANOVA followed by Tukey's multiple comparison.

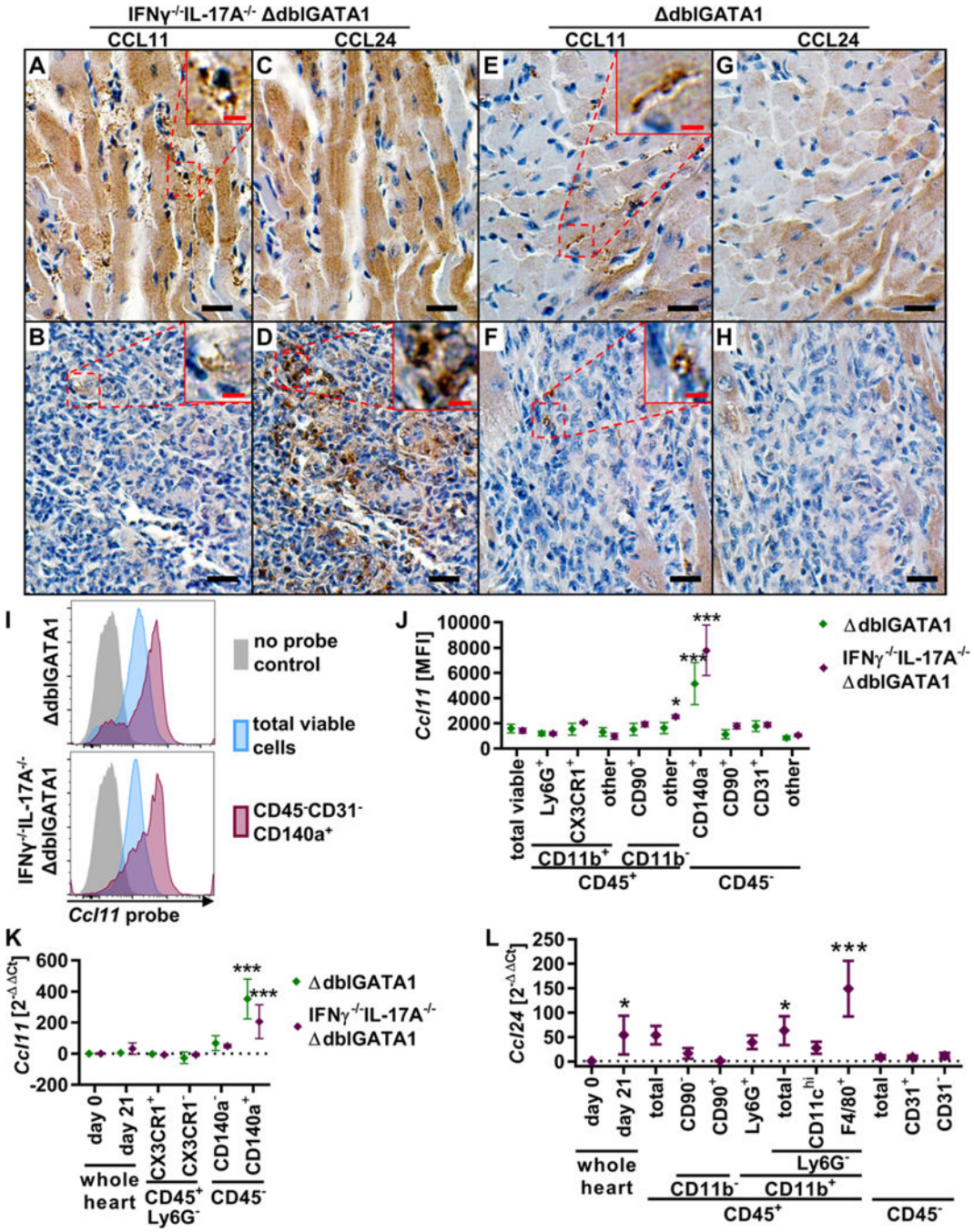

Figure 4.

CCL11 is expressed by cardiac fibroblasts while CCL24 is expressed by F4/80$^+$ macrophages during eosinophilic myocarditis. Mice were analyzed at day 21 of EAM. (A–H) Immunohistochemistry staining for CCL11 (A–B, E–F) and CCL24 (C–D, G–H) of heart sections from day 21 of EAM in IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice (A–D) and ΔdblGATA1 mice (E–H) showing areas without (A, C, E, G) and with inflammation (B, D, F, H). (A–H) Images are representative of two to three mice per group from a single experiment. Black scale bars: 20 μm, red scale bars: 5 μm. (I–J) *Ccl11* expression was

detected using a probe against *Ccl11* mRNA in the FlowRNA II Assay on day 21 of EAM in 5–6 mice per group from one experiment. (I) *Ccl11* expression in total viable cells (blue) and CD140a^{+} fibroblasts (red) from concatenated samples. (J) Mean fluorescence intensity (MFI) for *Ccl11* probe in total viable cells and indicated cell populations. (K) *Ccl11* expression in cells sorted as shown in Supporting Information Fig. 3 from 3 ΔdblGATA1 and 3 IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice. (L) *Ccl24* expression in cells sorted as shown in Supporting Information Fig. 4 from 4 individual IFN-$\gamma^{-/-}$IL-17A$^{-/-}$ ΔdblGATA1 mice. (K, L) Eotaxin expression was analyzed by qPCR and normalized to *Gapdh* and naïve mouse heart homogenates. Naïve and immunized mice are included for comparison. (J–L) Data are shown as mean ± SD from a single experiment. *$p < 0.05$, ***$p < 0.001$; one-way ANOVA followed by Dunnett's multiple comparison test comparing all subsets to total viable cells (J) or to naïve heart (K, L) for each genotype.

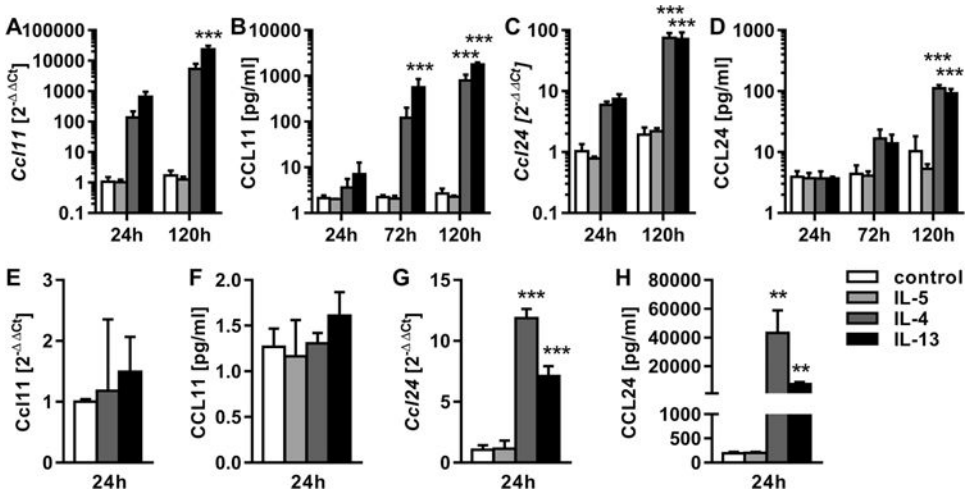

Figure 5.

IL-4 and IL-13 induce CCL11 in cardiac fibroblasts and CCL24 in macrophages. (A–D) Adult mouse cardiac fibroblasts and (E–H) bone marrow-derived macrophages from naïve WT mice were cultured with IL-5, IL-4, IL-13 or without (control). (A, C, E, G) *Ccl11* and *Ccl24* mRNA expression was measured by qPCR and normalized to *Gapdh* and controls. (B, D, F, H) Eotaxin concentrations in cell culture supernatants were measured by ELISA at indicated time points. All groups were compared to controls at day 1. (A–H) Data are shown as mean + SD (technical triplicates) and are representative of two to three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA followed by Dunnett's multiple comparison.

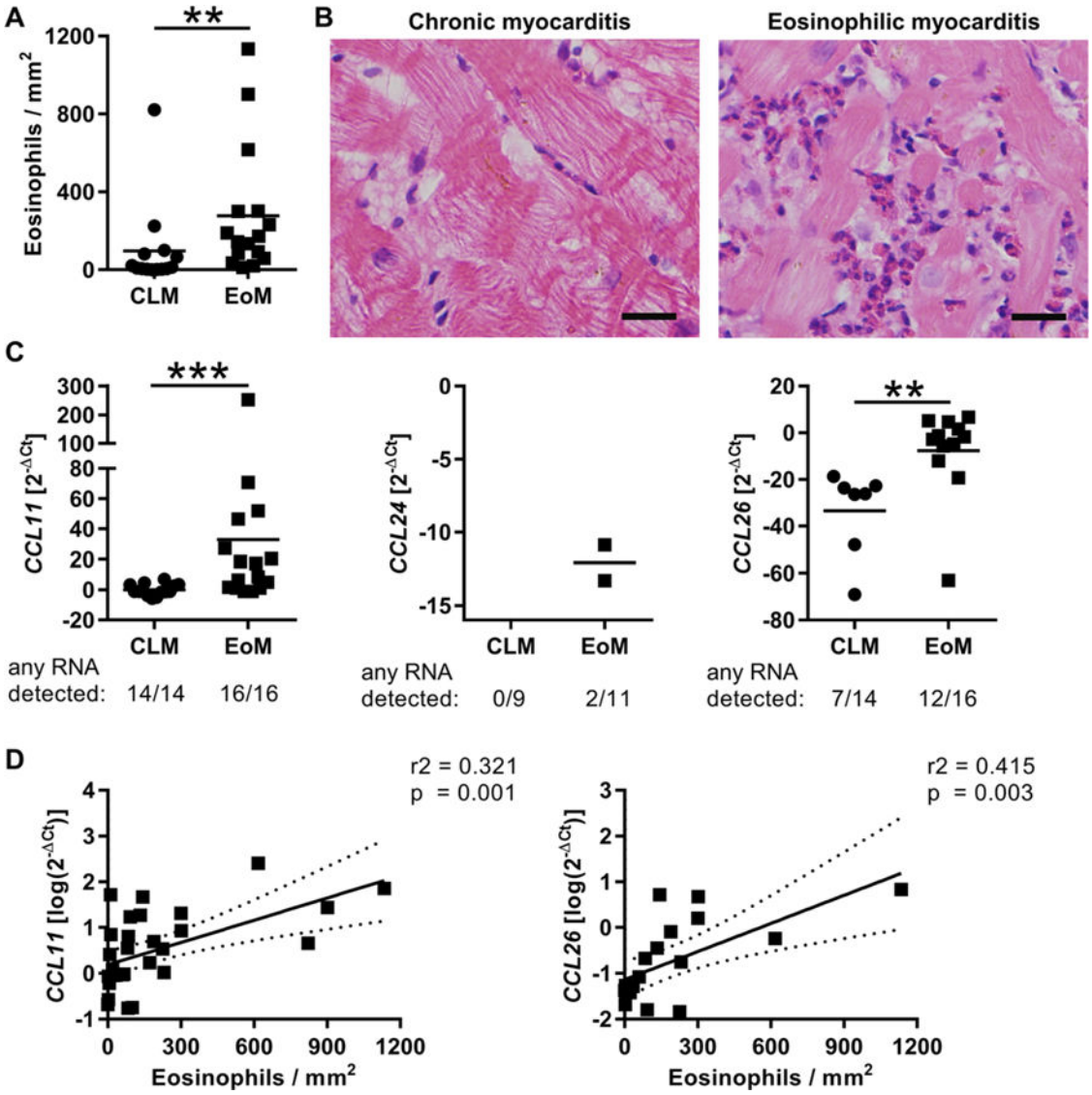

Figure 6.

Eotaxins are increased in the hearts of patients with eosinophilic myocarditis. (A) Eosinophil infiltration was quantified on H&E-stained biopsy sections from 14 chronic lymphocytic myocarditis (CLM) and 16 eosinophilic myocarditis (EoM) patients. (B) Representative images of (A). Scale bars: 20 μm. (C) Eotaxin mRNA expression in endomyocardial biopsy samples is shown as fold expression relative to HPRT ($2^{-\Delta Ct}$). (D) Linear regression of eotaxin mRNA expression on the number of infiltrating eosinophils. Dashed lines indicate 95% confidence intervals. $^{**}p < 0.01$, $^{***}p < 0.001$; Mann–Whitney *U*-test.