Expansion of CD25+ Innate Lymphoid Cells Reduces Atherosclerosis

Daniel Engelbertsen, Amanda C. Foks, Noah Alberts-Grill, Felicia Kuperwaser, Tao Chen, James A. Lederer, Petr Jarolim, Nir Grabie, Andrew H. Lichtman

Objective—Innate lymphoid cells (ILCs) are a newly discovered subset of immune cells that promote tissue homeostasis and protect against pathogens. ILCs produce cytokines also produced by T lymphocytes that have been shown to affect atherosclerosis, but the influence of ILCs on atherosclerosis has not been explored.

Approach and Results—We demonstrate that CD25+ ILCs that produce type 2 cytokines (ILC2s) are present in the aorta of atherosclerotic immunodeficient ldlr<sup>−/−</sup>rag1<sup>−/−</sup> mice. To investigate the role of ILCs in atherosclerosis, ldlr<sup>−/−</sup>rag1<sup>−/−</sup> mice were concurrently fed an atherogenic diet and treated with either ILC-depleting anti-CD90.2 antibodies or IL-2/anti-IL-2 complexes that expand CD25+ ILCs. Lesion development was not affected by anti-CD90.2 treatment, but was reduced in IL-2/anti-IL-2-treated mice. These IL-2-treated mice had reduced very low–density lipoprotein cholesterol and increased triglycerides compared with controls and reduced apolipoprotein B100 gene expression in the liver. IL-2/anti-IL-2 treatment caused expansion of ILC2s in aorta and other tissues, elevated levels of IL-5, systemic eosinophilia, and hepatic eosinophilic inflammation. Blockade of IL-5 reversed the IL-2 complex–induced eosinophilia but did not change lesion size.

Conclusions—This study demonstrates that expansion of CD25-expressing ILCs by IL-2/anti-IL-2 complexes leads to a reduction in very low–density lipoprotein cholesterol and atherosclerosis. Global depletion of ILCs by anti-CD90.2 did not significantly affect lesion size indicating that different ILC subsets may have divergent effects on atherosclerosis. (Arterioscler Thromb Vasc Biol. 2015;35:2526-2535. DOI: 10.1161/ATVBAHA.115.306048.)

Key Words: atherosclerosis ■ cytokines ■ eosinophils ■ interleukin ■ triglycerides

The development of atherosclerotic lesions and the destabilization of established lesions are promoted by local arterial and systemic inflammation driven by innate and adaptive immune responses. Interferon-γ IFN-γ-secreting Th1 cells enhance lesion development, but the influence of Th2 or Th17 cells is uncertain.1 Innate lymphoid cells (ILCs) have emerged as important effector cells in both protective immunity against pathogens and immune/inflammatory diseases.2,3 Each subset of ILCs, types 1, 2, or 3, secretes a distinct group of cytokines. The pattern of cytokine production corresponds approximately to that of its T-cell counterpart: ILC1s secrete Th1-related cytokines, ILC2s secrete Th2-related cytokines, and ILC3s secrete Th17-related cytokines. The influence of each of these ILC subsets to atherosclerosis is largely unknown. Natural killer cells, which are cytotoxic and IFN-γ-secreting innate cells that are phenotypically related to the ILC1 subset, seem to promote atherosclerotic lesion development in mice.4 The contribution of noncytotoxic IFN-γ-secreting ILC1s has not been addressed. Group 2 ILCs secrete IL-4, IL-5, IL-9, and IL-13 in response to IL-25, IL-33, and TSLP (thymic stromal lymphopoietin). These cells play a role in metabolic homeostasis by reducing adiposity, a function mediated in part by IL-5-dependent eosinophil activation.5–7 As of yet, there is no ILC-specific knockout mouse line that does not also have deficiencies in other lymphocyte populations.8 However, injections of anti-CD90 antibodies selectively deplete CD90-expressing ILCs in V-j-J recombinase-1 or recombinase-2 (rag1 or rag2)-deficient mice that lack all lymphocytes, and this strategy has been used to assess the role of ILCs in health and disease.9,10

It was recently shown that administration of IL-2 complexed with a particular anti-IL-2 antibody (clone JES6-1) to rag1<sup>−/−</sup> mice selectively allows expansion of CD25<sup>+</sup> ILC2s.11 In immunocompetent mice, treatment of mice with IL-2/anti-IL-2 complexes expands regulatory T cells,12,13 and it has been proposed as a therapeutic approach for autoimmunity, graft versus host disease, and allograft rejection.14 IL-2/anti-IL-2 complex therapy reduces atherosclerotic lesion development in mice,15,16 but the effect of this therapy on ILC expansion in atherosclerosis models is not known.

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In this study, we investigated the influence of ILCs on atherosclerotic lesion development. We used antibody-mediated global ILC depletion and IL-2/anti–IL-2-driven expansion in ldlr<sup>−/−</sup> rag1<sup>−/−</sup> mice, which are atherosclerosis prone but lack adaptive immune cells. We show that IL-5-producing ILCs are present in atherosclerotic aortas. Global depletion of all CD90<sup>+</sup> ILCs, which include the majority of all 3 groups of ILCs, leads to a reduction in types 1, 2, and 3 cytokine production in the spleen, with no net effect on atherosclerotic lesion development. However, IL-2/anti–IL-2 treatment results in a marked increase in ILC2s, eosinophilia, reduced very low-density lipoprotein (VLDL) cholesterol levels and protection against atherosclerotic lesion development. The results highlight the potential role of therapeutic expansion of type 2 ILCs for the treatment of atherosclerotic vascular disease.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**Aortic CD90<sup>+</sup>CD127<sup>+</sup>CD25<sup>+</sup> ILCs Produce Type 2 Cytokines**

First, we tested if hypercholesterolemia would influence levels of ILCs in the aorta. Mice were fed either high-fat diet (HFD) or Chow diet for 10 weeks after which the aorta was digested and stained for the presence of ILCs, defined as CD45<sup>+</sup> lineage<sup>−</sup> (lin: CD11b, B220, Gr-1, CD3, CD5) CD90<sup>−</sup>CD127<sup>−</sup> (Figure IA in the online-only Data Supplement). As expected, the number of aortic CD45<sup>+</sup> leukocytes was increased in atherosclerotic HFD-fed ldlr<sup>−/−</sup> mice (Figure IB in the online-only Data Supplement). However, the number of CD90<sup>−</sup>CD127<sup>−</sup> ILCs (Figure IC in the online-only Data Supplement) or CD90<sup>−</sup>CD127<sup>−</sup>CD25<sup>+</sup> ILCs (Figure ID in the online-only Data Supplement) were not increased in the aorta of atherosclerotic HFD-fed ldlr<sup>−/−</sup> mice compared with Chow-fed ldlr<sup>−/−</sup> mice or C57BL/6 control mice. To further characterize these ILCs, we digested aortas from HFD-fed ldlr<sup>−/−</sup>rag1<sup>−/−</sup> mice, which are atherosclerosis prone but lack adaptive immune cells. We show that IL-5-producing ILCs are present in atherosclerotic aortas. Global depletion of all CD90<sup>+</sup> ILCs, which include the majority of all 3 groups of ILCs, leads to a reduction in types 1, 2, and 3 cytokine production in the spleen, with no net effect on atherosclerotic lesion development. However, IL-2/anti–IL-2 treatment results in a marked increase in ILC2s, eosinophilia, reduced very low-density lipoprotein (VLDL) cholesterol levels and protection against atherosclerotic lesion development. The results highlight the potential role of therapeutic expansion of type 2 ILCs for the treatment of atherosclerotic vascular disease.

**Treatment With IL-2/Anti-IL-2 Complexes Increases ILC2s in Perivascular Adipose Tissue**

Previous studies have shown that dermal ILC2s are resistant to depletion by anti-CD90.2 treatment. To determine if we were able to deplete ILCs in the aorta, in a separate cohort, we injected HFD-fed ldlr<sup>−/−</sup>rag1<sup>−/−</sup> mice with phosphate-buffered saline, anti-CD90.2, or IL-2/JES6-1 for 2 weeks and analyzed cell in the aorta by flow cytometry. The percentage of aortic CD127<sup>−</sup>CD25<sup>+</sup> ILC2s was only slightly reduced by anti-CD90.2 treatment (29% versus 23% CD127<sup>−</sup>CD25<sup>+</sup> of lin<sup>−</sup> cells; Figure 3A). The CD127<sup>−</sup>CD25<sup>+</sup> cells isolated from anti–CD90.2-treated mice displayed reduced levels of CD90 expression (Figure IVA in the online-only Data Supplement). Aortic CD90<sup>−</sup>CD127<sup>−</sup>CD25<sup>+</sup> cells from anti–CD90.2-treated mice were sorted and stimulated with phorbol 12-myristate 13-acetate/ionomycin. After 24 hours of stimulation, cytokines in supernatant were measured. These cells produced IL-5 and IL-4, but no detectable levels of IFN-γ or IL-17, indicating an ILC2 phenotype (Figure IVB–IVE in the online-only Data Supplement). This suggests that anti-CD90.2 treatment reduces but does not completely deplete ILC2s in the aortic tissue.
Notably, IL-2/JES6-1 injections increased the proportion of CD127+CD25+ ILCs in the aorta (Figure 3A and 3C). Next, we investigated localization of expanded ILC2s in the tissue. Cell suspensions of aortas with or perivascular adipose tissue (PVAT) removed were stained. ILC2s (lin−CD90+CD25+ST2+) were primarily found in aortic PVAT, although we observed a minor increase of ILC2s in aorta stripped from PVAT after treatment with IL-2/JES6-1 (Figure 3C).

IL-2/Anti-IL-2 Complex Treatment Reduces Atherosclerosis

Aortic roots from mice fed HFD for 7 weeks were sectioned and aortic sinus lesions were analyzed. Strikingly, mice treated with IL-2/JES6-1 had significantly reduced lesion size (85608±12744 μm²) compared with either control IgG–treated mice (194121±19015 μm²) or anti–CD90.2-treated mice (225220±24237 μm²; Figure 4A). Lesional area positive for macrophages (Mac-3+ area) was also reduced in IL-2 complex-treated mice (Figure 4B; P<0.05). Although lesion size was reduced by IL-2/JES6-1, the percentage of macrophages, collagen, or neutral lipids (Oil Red O) did not differ between groups (Figure VA–VF in the online-only Data Supplement). There was no significant effect on lesion size or composition after anti-CD90.2 treatment compared with control IgG–treated mice.

IL-2/Anti-IL-2 Complex Treatment Reduces Serum VLDL Levels and It Is Associated With Reduced Hepatic Apolipoprotein B Expression

We did not observe weight changes (Figure 4C) as a consequence of either ILC depletion or expansion. However, injection of IL-2/JES6-1 caused a change in lipid profile. Levels of total cholesterol (Figure 4D) were decreased, whereas triglycerides (Figure 4E) were increased in IL-2/JES6-1–treated mice.
High-performance liquid chromatography analysis revealed that the reduction in cholesterol was located in the chylomicron and VLDL fraction, whereas LDL and high-density lipoprotein levels were similar between groups (Figure 4F).

The increased levels of triglycerides were mainly located in the VLDL compartment (Figure 4G). To test if expansion of CD25+ ILCs affected lipoprotein production, we measured genes associated with lipid metabolism. Transcription of apolipoprotein B (apoB) was markedly reduced in the livers from IL-2/JES6-1-treated mice (Figure 4H), whereas hepatic lipase (lipc), apolipoprotein C-III (apoC3), and the triglyceride-synthesizing enzyme diglyceride acetyltransferase (dgat) were expressed at similar levels between groups (Figure VIA–VIC in the online-only Data Supplement).

Previous studies have demonstrated that type 2 ILCs may promote hepatic fibrosis. To address this, we fed mice HFD...
for 7 weeks and treated with phosphate-buffered saline or IL-2/JES6-1 for the past 4 weeks. Liver was sectioned and stained with hematoxylin and eosin as well as Masson trichrome to detect collagen deposition. Strikingly, we found that mice treated with IL-2/JES6-1 displayed marked hepatic eosinophilic and lymphoid inflammation (Figure 4I). Leukocyte accumulation was mainly found in portal areas (Figure VID in the online-only Data Supplement). Moreover, we observed moderate fibrosis in areas with intense leukocyte accumulation (Figure VID in the online-only Data Supplement) in IL-2/JES6-1-treated mice. The amount of ILC2s in liver digests was increased after IL-2 complex treatment (Figure VIE in the online-only Data Supplement). To assess liver function, we measured soluble markers of liver damage in serum. Levels of aspartate aminotransferase were similar between groups but alanine aminotransferase was mainly found in portal areas (Figure VID in the online-only Data Supplement). Strikingly, we found that mice treated with IL-2/JES6-1 (Figure VIIF in the online-only Data Supplement) after HFD feeding for 7 weeks.}

**Figure 3.** Interleukin (IL)-2-anti-IL-2 complex (IL-2/JES6-1) treatment expands innate lymphoid cell (ILC) 2s in the aorta of rag1<sup>−/−</sup>ldlr<sup>−/−</sup> mice. A, Percentages of aortic lin<sup>−</sup>CD25<sup>−</sup>CD127<sup>−</sup>CD25<sup>+</sup> cells and (B) CD90<sup>−</sup>CD127<sup>−</sup>CD25<sup>+</sup> cells compared between groups (3–4 mice/pool). C, Comparison of ILC2 (lin<sup>−</sup>CD90<sup>−</sup>CD25<sup>−</sup>ST2<sup>+</sup>) cells from mice treated with or without IL-2/JES6-1 in aortas with or without perivascular adipose tissue (PVAT). **P<0.01, ***P<0.001.

**High Serum Levels of IL-5 and Eosinophilia in Mice Treated With IL-2/JES6-1**

We measured the serum levels of cytokines to determine if either IL-2/JES6-1 or anti-CD90.2 treatment had systemic inflammatory effects. Serum levels of IFN-γ, IL-17, TNFα (not detected), MCP-1 (monocyte chemotactic proteins), IL-4, or IL-13 were not affected by depletion or expansion of CD25<sup>+</sup> ILCs (Figure VIIA–VIIE in the online-only Data Supplement), although we observed a slight increase in IL-6 in mice treated with IL-2/JES6-1 (Figure VIIF in the online-only Data Supplement). Strikingly, treatment with IL-2/JES6-1 resulted in high levels of IL-5 in serum (Figure 5A) and increased levels of eosinophils in blood (Figure 5B). We further investigated whether IL-2/anti-IL-2 complex treatment led to accumulation of eosinophils in tissues. Leukocyte fractions from tissues were isolated and analyzed by flow cytometry. Eosinophil accumulation to visceral adipose tissue and liver (Figure 5C) was dramatically enhanced by IL-2/anti-IL-2 treatment. Furthermore, eosinophil accumulation in the aorta was increased by 75-fold IL-2/JES6-1 treatment (Figure 5C) and 99% of the eosinophils were located in PVAT (182±59 eosinophils in aorta without PVAT and 1906±11400 eosinophils in aorta with PVAT).}

**IL-5 Blockade Inhibits IL-2 Complex-Induced Eosinophilia But Not Affect Atherosclerosis**

We hypothesized that the high levels of IL-5 induced after IL-2 complex injections affected atherosclerosis. To investigate this, mice (ldlr<sup>−/−</sup>rag1<sup>−/−</sup>) were fed HFD for 7 weeks and treated for the past 4 weeks with IL-2 complexes supplemented with anti-IL-5 (clone: TRFK5) or an isotype control (n=7/group). Anti-IL-5 efficiently blocked eosinophilia in IL-2/anti–IL-2-treated mice (>90% reduction; Figure 6A) as well blocked eosinophil accumulation in the atherosclerotic aorta (>90% reduction; Figure 6B). Moreover, accumulation of eosinophils was found to be reduced in the liver or the epididymal adipose tissue (Figure VIIIA and VIIIB in the online-only Data Supplement). Anti-IL-5 treatment did not affect levels of aortic ILC2s (Figure 6C). Aortic sinus lesions were comparable between mice treated with IL-2/anti-IL-2 complexes plus either control IgG (141 594±25 343 μm<sup>2</sup>) or anti-IL-5 (100 582±25 470 μm<sup>2</sup>; Figure 6D, P=0.2) and cholesterol levels were similar (Figure 6E). The serum levels of triglycerides were higher in the anti-L5-treated group, but the difference did not reach statistical significance (Figure 6F). No difference in body weight or epididymal visceral adipose tissue was observed (Figure VIIIC and VIIID in the online-only Data Supplement).
Figure 4. Interleukin (IL)-2-anti-IL-2 complex (IL-2/JES6-1) treatment reduces atherosclerosis and affects serum cholesterol and triglyceride levels. Mice were fed high-fat diet (HFD) for 7 weeks and treated with control IgG, anti-CD90.2, or IL-2/JES-1 for the past 5 weeks (n=12–13). A, Lesion area and (B) macrophage area of lesions in aortic sinuses were quantified. C, Weight, (D) total cholesterol, and (E) triglycerides were measured in serum. High-performance liquid chromatography of serum for (F) cholesterol and (G) triglycerides (n=3/treatment). H, Apolipoprotein B (apoB) mRNA expression in liver (n=7–11). In a follow-up experiment, mice were fed HFD for 7 weeks and treated with phosphate-buffered saline (PBS) or IL-2/JES6-1 for the past 4 weeks (n=10–11). I, Hematoxylin and eosin staining of liver sections (×40). CM indicates chylomicron; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low-density lipoprotein. *P<0.05, **P<0.01, ***P<0.001.
Notably, although eosinophils were dramatically reduced in the liver, levels of alanine aminotransferase (Figure 6G), and aspartate aminotransferase (Figure VIII in the online-only Data Supplement) and hepatic inflammation assessed by histology (Figure 6H; Figure VIIIIF in the online-only Data Supplement) were similar between the 2 treatment groups.

Figure 5. Interleukin (IL)-2-anti-IL-2 complex (IL-2/JES6-1) treatment causes high levels of IL-5 and recruitment of eosinophils to perivascular adipose tissue (PVAT) and liver. A, Levels of IL-5 (pg/mL) in serum and (B) eosinophils in blood of mice treated with control IgG, anti-CD90.2, or IL-2/JES6-1. C, Eosinophils were quantified in visceral adipose tissue (VAT), liver, and aorta±PVAT. Numbers indicate percentages of eosinophils (lin-Siglec-F+) of live CD45+ leukocytes. **P<0.01, ***P<0.001. PBS indicates phosphate-buffered saline.
Discussion

ILCs have been shown to play important roles in inflammatory diseases and in maintaining barrier homeostasis. Previous work has demonstrated that several cytokines influence atherosclerotic disease in different ways. Many of these cytokines, previously considered to be Th cell derived have recently been shown to be produced by ILCs. Our study demonstrates that ILCs, in particular CD25+ ILC2s, can influence atherosclerosis. A few recent studies have indirectly suggested a role for ILCs in atherosclerosis. The ILC2-activating cytokines IL-25 and IL-33 have been shown to reduce atherosclerosis. Also, the transcription factor Id3 was shown to regulate IL-5 production of lin−Sca1+CD127+CD25−ST2+. However, this is the first study to directly examine the effects of ILC expansion or depletion on atherosclerosis.

We demonstrate that IL-5-producing CD25+ ILCs are present in atherosclerotic mouse aortas. These cells are expanded
by treatment of mice with IL-2/anti-IL-2 complexes, inhibiting atherosclerotic lesion formation. IL-5 has been shown to be atheroprotective by promoting the secretion of natural antibodies by B-1 cells. As ldlr−/−rag1−/− mice lack B-1 cells, our model allows us to study the B-1 cell–independent effects of IL-5. One such effect is the role for IL-5 in inducing mobilization and proliferation of eosinophils. We found that IL-2/JES6-1 treatment generated a marked increase in circulating and tissue eosinophils. However, blockade of IL-5 in mice given IL-2 complexes did not significantly affect atherosclerosis. This intriguing finding suggests that CD25+ ILCs may act in an IL-5–independent manner to limit atherosclerotic burden. The atheroprotective potential of ILC2s in our study probably relates, in part, to alterations in lipid metabolism. Expansion of CD25-expressing ILCs resulted in reduced VLDL cholesterol levels. This atheroprotective phenotype was associated with reduced expression of the Apob gene in the liver. However, mRNA levels of Lipe, Dgat2, or Apoc3 were not affected by IL-2/JES6-1 treatment, showing that other aspects of liver function were intact. As IL-5 blockade did not affect cholesterol levels, the cholesterol-lowering effect of IL-2 complexes may possibly be mediated by IL-13, other ILC2-related soluble mediators or by cell–cell dependent mechanisms. Arguing against a potential role of IL-13 in this study is the fact that IL-2 complex treatment did not increase levels of IL-13 in serum, although it is difficult to estimate how well local production of IL-13 in the tissue is reflected in the circulation. Also, we cannot completely exclude the role of other CD25-expressing cells in affecting cholesterol. Surprisingly, the IL-2/JES6-1-treated mice exhibited elevated triglyceride levels. Others have shown that deficiency in IL-5 production results in increased adiposity. Conversely, we observed a decrease in epididymal visceral adipose tissue in mice treated with IL-2/anti-IL-2 complexes, which have high levels of circulating IL-5. Because anti-IL-5 appeared to block the rise in triglycerides in HFD-fed ldlr−/−rag1−/− treated with IL-2/anti-IL-2 complexes, thus the elevated numbers of eosinophils may not be responsible for the IL-2/anti-IL-2 effect on serum triglycerides. Possibly, ILC2 activation compromises storage of triglycerides in adipose tissue, leading to high levels of triglycerides in the serum, through an IL-5–independent mechanism. Altogether our data suggests that ILC2s may affect atherosclerosis primarily by influencing metabolism rather than acting directly in the lesion. However, further investigation is required to determine if there are direct atheroprotective effects of ILC2s within lesions, such as by influencing macrophage phenotype. We found that HFD did not influence numbers of ILCs in the aorta or percent ILCs in the spleen. However, the proportion of CD25+ ILCs to total leukocytes was reduced in the atherosclerotic artery, indicating that relative levels of anti-atherogenic CD25+ ILCs drop as atherosclerosis progresses.

The role of eosinophils in atherosclerosis has not yet been addressed in experimental models. Human carotid plaques were found to have expression of eotaxin and CCR3 but no significant levels of eosinophils were detected. We found that most eosinophils were localized to the PVAT and not to actual lesion, indicating that eosinophils do not influence lesion development through actions within the plaque. Further studies using eosinophil-deficient mice models are needed to further understand the role of eosinophils in atherosclerosis development. Treatment with IL-2/JES6-1 immune complexes has been shown to reduce atherosclerosis in ldlr−/− mice, which has been attributed to the expansion of regulatory T cells. The ldlr−/− rag1−/− mouse lacks regulatory T cells, and the only known CD25+ cells that will respond to IL-2/JES6-1 are ILCs. Thus, our findings suggest that part of the effect of IL-2/JES6-1 treatment observed in these studies may be because of expansion of CD25-expressing ILCs. It should be noted that expansion of ILCs is probably more pronounced in ldlr−/−rag1−/− than in immunocompetent mice because of reduced competition for available IL-2 complexes in the absence of regulatory T cells.

Given the atheroprotective effect of IL-2/JES6-1 treatment, it may be considered surprising that depletion of ILCs by anti-CD90.2 did not affect lesion size or composition. There are several possible explanations for this finding. First, depletion of ILCs by anti-CD90.2 injections removes several different classes of ILCs with potentially opposite effects on atherosclerosis. It is conceivable, given knowledge from cytokine knockout mice, that ILC1s, ILC3s, and CD90-expressing natural killer cells may promote atherosclerosis, whereas ILC2s may be atheroprotective. The role of these subsets needs to be addressed in future studies. Second, treatment of mice with CD90 effectively depleted all splenic cells capable of secreting these cytokines, but failed to remove all ILC2s from the aortic wall. This is consistent with previous reports of failure of anti-CD90.2 treatment to deplete dermal ILC2s. It is possible that aortic ILCs, rather than lymphoid organ-resident ILCs, determine atherosclerotic progression in ldlr−/−rag1−/− mice. Third, to avoid infections, our ldlr−/−rag1−/− mice were given antibiotics from birth and for the duration of the experiment. Finally, our study does not address the potentially important interaction of ILCs with T and B cells.

In summary, we demonstrate that expansion of CD25-expressing ILCs, including ILC2s, results in atheroprotection in ldlr−/−rag1−/− mice. Our study highlights the potential of ILC modulation as a pharmacological target for the treatment of cardiovascular disease and demonstrates the interaction between innate type 2 immunity and metabolism.

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Disclosures
None.

References
Innate Lymphoid Cell Expansion and Atherosclerosis

Significance

Atherosclerosis is a chronic inflammatory disease of arteries and a major cause of myocardial infarction and stroke. Leukocytes are important in regulating the disease and immune pathways are considered potential targets of next-generation pharmaceuticals for the treatment of cardiovascular disease. In this study, we investigated the role of a newly discovered leukocyte subset termed innate lymphoid cells (ILCs) in a mouse model of atherosclerosis. We either depleted or expanded ILCs to study the effect on atherosclerosis. Although ILC depletion did not significantly affect atherosclerosis, expansion of CD25-expressing ILCs led to a dramatic reduction of atherosclerosis, and it was associated with reduced very low-density lipoprotein cholesterol levels and increased levels of the type 2 cytokine interleukin-5. However, the role of ILCs in affecting atherosclerosis was, in our experimental setting, not contingent on interleukin-5. We propose that expansion of type 2 ILCs may have beneficial effects on atherosclerosis through various mechanisms, including lipid metabolism.
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Supplemental Figure I. High-fat diet feeding does not influence levels of ILCs in the aorta

(A) Gating strategy of CD45^+ lineage (lin) CD90^+CD127^+CD25^+ ILCs in digested aorta. Isotype control anti-CD25.

(B) Number of CD45^+ leukocytes, (C) lin^-CD90^+CD127^+ ILCs or (D) lin^-CD90^+CD127^+CD25^+ ILCs per aorta. n=5 mice/pool.
Supplemental Figure II. Gating strategy for aortic CD90⁺CD127⁺CD25⁺ ILCs from rag1⁻/⁻/ldlr⁻/⁻ mice.

(A) Gating strategy for identification of aortic ILCs.

(B) Aortic lin⁺CD90⁺CD127⁻ (non-ILCs), CD25⁻ ILCs and CD25⁺ ILCs sorted by FACS and stimulated with PMA/ionomycin. Levels of IFNγ and IL-17 in supernatants from stimulated cells was measured in two separate experiments. (C) Quantification of splenic ILCs in rag1⁻/⁻/ldlr⁻/⁻ mice fed chow or high-fat diet (n=7-9).
Supplemental Figure III. Gating strategy for identification of splenic lineage-CD90^+CD127^+ ILCs.
Representative gating of splenic lin^CD90^+CD127^+ ILCs from a rag1^-/-ldlr^-/- mouse fed high-fat diet and treated with control IgG.
Supplemental Figure IV. Aortic ILCs from anti-CD90.2 injected mice produce type 2 cytokines.
(A) Expression of CD90 on aortic Lin^CD127^CD25^+ cells from anti-CD90.2 injected rag1^-/-ldlr^-/- mice. ILCs (CD25^+ or CD25^-) or non-ILCs were sorted from anti-CD90.2 treated mice and stimulated with PMA/ionomycin for 24h. (B)IL-5, (C) IL-4, (D) IFNγ and (E) IL-17 was measured in the supernatant.
Supplemental Figure V. No effect of ILC depletion or expansion on lesion composition
(A) Collagen (Van Gieson), (B) macrophage (Mac-3) and (C) Oil Red-O staining of aortic sinus. Quantification of (D) Collagen, (E) macrophages and (F) Oil Red O comparing treatment groups (n=8-10).
Supplemental Figure VI. Effects of CD25+ ILC expansion on liver and adipose tissue.

Hepatic RNA was isolated from mice treated with control IgG, anti-CD90.2 or IL-2/JES6-1. (A) mRNA expression of apolipoprotein C-III (Apoc3), (B) diglyceride acetyltransferase (Dgat), and (C) hepatic lipase (Lipc) was measured by qRT-PCR (n=7-11. Mean ± SEM). In a follow-up experiment, mice were fed HFD for seven weeks and treated with PBS or IL-2/JES6-1 for the last four weeks. (D) Representative liver sections from rag1^-/-ldlr^-/- mice stained with H&E or Masson’s Trichrome stain (magnification 10x and 40x).

(E) Levels of aspartate aminotransferase and (F) alanine aminotransferase were measured in serum. (G) Epididymal visceral adipose tissue and (H) liver resident ILC2s (lin^CD90^-CD25^-ST2^-) after seven weeks of high-fat diet and IL-2/JES6-1 treatment. (n=10-11)
Supplemental Figure VII. Levels of serum cytokines in ILC depleted or IL-2/JES6-1 treated mice

Serum levels of (A) IFNy, (B) IL-17, (C) MCP-1, (D) IL-13, (E) IL-4, (F) IL-6 comparing control IgG, anti-CD90.2 and IL-2/JES6-1 treated rag1-/ldlr-/- mice. (n= 7-10).
Supplemental Figure VIII. Liver inflammation and fibrosis by IL-2 complex treatment is independent of IL-5

(A) Quantification of eosinophil accumulation to (A) liver and (B) epididymal VAT of mice treated with IL-2 complex combined with either anti-IL-5 or control IgG (ctrl IgG). (C) Body weight, (D) epididymal VAT and serum levels of (E) aspartate aminotransferase. (F) Liver sections stained with H&E or Masson’s trichrome (10x and 40x magnification). n=7/group.
MATERIALS AND METHODS

Mice
All mice used were bred in the pathogen-free facility at the New Research Building (Harvard Medical School, Boston, MA), in accordance with the guidelines of the Committee of Animal Research at the Harvard Medical School and the National Institutes of Health Animal Research Guidelines. Sulfatrim (Sulfamethoxazole/Trimethoprim) was administered to mice starting at birth and continuing for the duration of the experiment.

The \textit{ldlr}^{-/} \textit{rag1}^{-/-} mice were generated by breeding \textit{ldlr}^{-/-} and \textit{rag1}^{-/-} C57BL/6 mice, both purchased from Jackson Laboratories (Bar Harbor, ME). Age matched groups of female \textit{ldlr}^{-/-} and \textit{ldlr}^{-/-} C57BL/6 mice were fed high-fat diet (HFD) containing 1.25\% cholesterol (Cat. No. D12108C, Research Diets Inc.) \footnote{1} for seven weeks starting at eight weeks of age. In the main cohort, mice were treated with isotype control IgG, anti-CD90.2 or IL-2 complexes for the last five weeks of the experiment. In a follow up study mice were fed HFD for eight weeks and injected with PBS or IL-2 complexes for the last four weeks of the experiment. Mice were randomly assigned to groups.

Innate lymphoid cell depletion and expansion
Mice were injected intraperitoneally twice a week with 0.25 mg of cell depleting rat anti-mouse anti-CD90.2 mAb (clone 30H12; BioXCell) or an IgG2b isotype control antibody (clone LTF2; BioXCell). In order to expand CD25$^{+}$ ILCs, mice were injected i.p. twice a week with 6 \mu g IL-2/JES6-1-complexes that were prepared by mixing IL-2 (R&D) with anti-IL-2 (clone:JES6-1A12, BioXCell) \footnote{2}.

IL-5 blockade
Mice were fed HFD for seven weeks and injected with IL-2/JES6-1 co-administered with either anti-IL-5 (15 mg/injection; clone: TRFK5, BioXCell) or an isotype control (15 mg/injection; clone: HRPN) for the last four weeks before sacrifice.

Tissue digestion and cell sorting
Atherosclerotic artery from the ascending aorta to iliac bifurcation was to enzymatic digestion. In some experiments, perivascular adipose tissue (PVAT) was removed from aortas before digestion. Aortas were minced and subsequently digested with 450 U/ml collagenase I-S (C1639, Sigma), 125 U/ml collagenase XI (C7657, Sigma), 60 U/ml hyaluronidase (H3506, Sigma) and 60 U/ml DNase1 (D5025, Sigma) for 1 hour. Cells were washed with PBS twice and then stained with fluorochrome-conjugated antibodies and viability stain. Cells sorted by FACS (FACS Aria, BD Biosciences) were resuspended in RPMI containing 10\% fetal bovine serum with PMA (1ug/ml) and ionomycin (20 ng/ml) and cultured for 24h at 37 °C before collection of supernatant.

Liver was dissected and passed through a 70 \mu m cell strainer. Non-parenchymal cells from the liver were separated from parenchymal cells by centrifugation at 50 RCF. The non-parenchymal cells in the supernatant were isolated and put on a Lympholyte gradient (Cedarlane, Ontario, Canada). Cells were centrifuged at 1250 RCF and the lymphocyte fraction was isolated. Cells were washed and resuspended in PBS.

Epididymal visceral adipose tissue was minced and incubated at 37 °C for 40 minutes with 4 mg/ml collagenase type II (Worthington) supplemented with 0.5\% bovine serum albumin (BSA). Digested tissue was passed through 70 \mu m cell strainer and washed with PBS (+0.5\% BSA) and centrifuged at 500 RCF where after cells were resuspended in PBS.
Flow cytometric analyses and cell sorting
Splenocytes, mesenteric lymph nodes and aortic digests were stained using the following antibodies (from Biolegend unless indicated): CD90.2 (53-2.1), CD127 (A7R34), Siglec-F (E50-2440, BD Biosciences), CD25 (PC61), CD11b (M1/70), Gr-1 (RB6-8C5), B220 (RA3-6B2), NKp46 (29A1.4), NK1.1 (PK136) CD45.2 (104), ST2 (DiH9). Live/Dead Viability staining (Life Technologies) or 7-AAD (BD Pharmingen) was used for exclusion of non-viable cells. Samples were run on a DXP12 flow cytometer (Cytek) and analyzed using FlowJo software (Treestar).

Multiplexed Cytokine assays
Serum cytokines were analyzed using a Luminex bead-based multiplex assays specific for IL-2, 4, 5, 6, 10, 12p40, 12p70, IL17a, IFNγ, TNFα, and MCP-1. Recombinant cytokine standards (Bio-Rad, Hercules, CA) were used to calculate cytokine concentrations and data were analyzed using StarStation 2.3 software (Applied Cytometry, Sheffield UK). Cytokines in supernatants from PMA/ionomycin-treated aortic cells were measured by multiplex analysis (Eve Biotechnology, Calgary, Alberta, Canada).

Serum lipid analysis
Mouse blood cholesterol and triglycerides were quantified on the c501 module of the Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN), and lipoprotein profiles were further analyzed by high-performance liquid chromatography (HPLC; Liposearch, Tokyo, Japan).

Immunohistochemical staining of aortic lesions and liver
Frozen sections (8 µm) were stained with antibodies specific for Mac-3 (M3/84, BD Pharmingen) to detect macrophages or with Van Gieson staining kit (Sigma) for collagen assessment. Sections were stained with Oil Red O to measure neutral lipids. Lesions area was presented as average± SEM. Liver sections (formalin fixed and paraffin-embedded) were stained with H&E and Masson’s trichrome.

Quantitative RT-PCR (qRT-PCR) analysis
Total RNA was extracted from cultured cells by Rneasy kit (QIAGEN Inc., Valencia, California, USA) and reverse-transcribed using the ThermoScript RT-PCR system and random hexamer primers according to the manufacturer’s instructions (Invitrogen). cDNA was amplified by real-time PCR with SYBR Green PCR mix (Applied Biosystems) and Step-One Detection System (Applied Biosystem) according the manufacturer’s instructions. Levels of specific gene expression in the samples were normalized to expression of hprt and values were expressed as relative to control IgG treated mice.

Serum markers of liver function
Alanine aminotransferase, aspartate aminotransferase, and total bilirubin were measured in serum using the ALTL, ASTL and BILTS assays (all Roche Diagnostics) and the c501 analyzer.

Statistical analysis
All statistical analyses were performed using Prism software. Differences between two groups of mice were analyzed by Student’s t test or by the Mann-Whitney U tests (for nonparametric data) and expressed as mean ± SEM. One-way ANOVA with Tukey’s Multiple Comparison post test or Kruskal-Wallis with Dunn’s multiple comparison tests for three or more group experiments was used depending on normal distribution. A value of p<0.05 was considered to be significant. For experiments with n≤3 no statistical test was performed.
References
