International Immunology, Vol. 28, No. 5, pp. 255–261
doi:10.1093/intimm/dxv068
Advance Access publication 27 November 2015

© The Japanese Society for Immunology. 2015. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

SHORT COMMUNICATION

CXCR6 regulates the recruitment of pro-inflammatory IL-17A-producing T cells into atherosclerotic aortas

Matthew J. Butcher*, **Chih-I Wu***, **Tayab Waseem** and **Elena V. Galkina**

Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA 23507-1969, USA

Correspondence to: E. V. Galkina, Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, 700 West Olney Road, Norfolk, VA 23507-1696, USA; E-mail: galkinev@evms.edu

*These authors contributed equally to this study.

Received 30 August 2015, *accepted* 3 November 2015

Abstract

The adaptive immune response is involved in the development and progression of atherosclerosis and IL-17A$^+$ cells play a role in this disease. Although elevated number of CD4$^+$ IL-17A$^+$ (T_{h17}) and IL-17A$^+$TCRγδ$^+$ T cells are found within murine atherosclerotic aortas and human plaques, the mechanisms governing IL-17A$^+$ T-cell migration to atherosclerotic lesions are unclear. The chemokine receptor CXCR6 is expressed on several T-cell subsets and plays a pro-atherogenic role in atherosclerosis. Here, we used CXCR6-deficient (*Cxcr6$^{GFP/GFP}$*) apolipoprotein E-deficient (*Apoe$^{-/-}$*) mice to investigate the involvement of CXCR6 in the recruitment IL-17A$^+$ T cells to atherosclerotic aortas. Flow cytometric analyses revealed reductions in T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells within aged *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* aortas, in comparison with age-matched *Cxcr6$^{GFP/+}$Apoe$^{-/-}$* aortas. Although CXCR6-sufficient IL-17A$^+$ T cells efficiently migrated toward CXCL16, the migration of CXCR6-deficient IL-17A$^+$ T cells was abolished in transwell assays. Importantly, the recruitment of *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* IL-17A$^+$ T cells into the aortas of *Apoe$^{-/-}$* recipients was markedly reduced in short-term adoptive transfer experiments. Altogether these results demonstrate an important role of CXCR6 in the regulation of pathological T_{h17} and IL-17A$^+$TCRγδ$^+$ T-cell recruitment into atherosclerotic lesions.

Key words: atherosclerosis, chemokine receptor, IL-17A, immune system, lymphocytes

Introduction

Atherosclerosis, the major etiological process responsible for 25% of global deaths, is the process through which inflamed arterial plaques form, persist and eventually rupture, resulting in myocardial infarctions and ischemic stroke. The etiology of atherosclerosis critically depends on the immune system, and recent work has demonstrated that many leukocytes are present within arterial lesions. Lesional monocytes, macrophages, dendritic cells and T cells, including T_{h1}, T_{h2}, T_{reg}, T_{h17}, and TCRγδ$^+$ T-cell subsets, have been described (1, 2).

IL-17A, a major cytokine produced by T_{h17} and some TCRγδ$^+$ T cells, is required to efficiently control bacterial and fungal infections at mucosal sites. However, IL-17A also actively participates in major autoimmune diseases (3–5). In the context of atherosclerosis, elevated levels of T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells have been reported within atherosclerotic *Apoe$^{-/-}$* and *Ldlr$^{-/-}$* mice (6–9), coronary artery disease (CAD) and endarterectomy patients (10–12). Mechanistic studies in atherosclerotic mice have yielded at least two unifying hypotheses: that IL-17A plays a pro-atherogenic role by supporting aortic chemokine/cytokine production, myeloid cell recruitment (6, 7, 9, 10, 13–15) and activation; and an atheroprotective role, via the potential regulation of aortic T_{h1} or smooth-muscle-cell collagen deposition (8, 16, 17). Thus, while IL-17A may promote (8, 16, 17), not affect (9, 14, 18), or adversely affect (6, 12, 19) collagen synthesis and plaque stability; to date, the majority of evidence supports a pro-atherogenic role for IL-17A (6, 7, 9, 13–15, 18).

Although multiple T-cell subsets are present within the aortic wall, the mechanisms behind aortic and aortic adventitial T-cell homing are not completely understood. Several adhesion molecules and chemokines/chemokine receptors have been demonstrated to regulate aortic T-cell content. CCL5, CXCL10 and CXCL16 and their respective receptors CCR1, CXCR3 and CXCR6 support the migration of T_{h1} cells, and several studies have implicated CCL19/CCL21, CCL17 and the chemokine receptors CCR7 and CCR4 in the regulation of T_{reg} homing (20, 21). In contrast, the mechanisms through which T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells are recruited to atherosclerotic lesions are unknown; however, several candidates might be involved. The chemokine receptors CCR7

and CXCR5 generally support T-cell migration into secondary lymphoid tissues and the non-lymphoid homing receptors CCR4, CCR5, CCR6 and CXCR6 are expressed by T_{h17} cells (22). Interestingly, while CCR6 plays a central role in T_{h17}-cell recruitment in experimental autoimmune encephalomyelitis (23), rheumatoid arthritis (24), and air pouch inflammation models (25) CCR6 did not affect the recruitment of aortic T_{h17} cells in atherosclerotic mice (26). Thus, the mechanisms through which T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells are recruited to atherosclerotic lesions remains to be addressed.

In this study, we demonstrate that virtually all T_{h17} cells and IL-17A$^+$TCRγδ$^+$ T cells express high levels of the chemokine receptor CXCR6 in atherosclerotic aortas. In CXCR6-deficient *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* mice, CXCR6$^+$ T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells failed to accumulate within aortic atherosclerotic lesions. We assessed the role of CXCL16/CXCR6-dependent IL-17A$^+$ T-cell chemotaxis in transwell assays and found that T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells from *Apoe$^{-/-}$* mice migrated towards CXCL16 in a dose-dependent manner. Lastly, *in vivo* competitive adoptive transfer experiments demonstrated that IL-17A$^+$ T cells require CXCR6 to home to atherosclerotic lesions. Collectively, our data indicate that the chemokine receptor CXCR6 is required for efficient T_{h17} and IL-17A$^+$TCRγδ$^+$ T-cell recruitment to inflamed atherosclerotic lesions.

Methods

Mice

Cxcr6$^{GFP/+}$ and *Cxcr6$^{GFP/GFP}$* mice (27) (a kind gift of Dr Littman, Howard Hughes Medical Institute, New York University) were crossed with *Apoe$^{-/-}$* mice (Jackson Laboratories, Bar Harbor, MN, USA) to obtain *Cxcr6$^{GFP/+}$Apoe$^{-/-}$* and *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* mice. Mice were bred and maintained under specific pathogen-free conditions in the animal facilities of Eastern Virginia Medical School, Norfolk. Mice of 40–50 weeks old were used for the experiments described, in accordance with the EVMS Institutional Animal Care and Use Committee guidelines.

Flow cytometry

The preparation of aortic cell suspensions and intracellular flow cytometry staining protocols were conducted as previously described (14, 28, 29). Briefly, the mice were anesthetized and their vasculature was perfused with PBS containing 20 U ml^{-1} sodium heparin via cardiac puncture. The aortas were subsequently dissected and digested for 1 h at 37°C with 125 U ml^{-1} Collagenase Type XI, 60 U ml^{-1} Hyaluronidase Type 1-s, 60 U ml^{-1} DNase 1 and 450 U ml^{-1} Collagenase Type I in PBS (Sigma-Aldrich, St Louis, MO, USA). Single-cell suspensions were prepared from the spleens, peri-aortic lymph nodes (PALN) and digested aortas using 70 μm nylon cell strainers. To re-stimulate the cell suspensions for intracellular cytokine staining, the cells were cultured for 5 h at 37°C with complete RPMI1640 (10% FBS, 2% penicillin/streptomycin) supplemented with 10 ng ml^{-1} PMA, 500 ng ml^{-1} Ionomycin C and 600 ng ml^{-1} Brefeldin A (Sigma-Aldrich). To stain the re-stimulated cells, the single-cell suspensions were pre-incubated with anti-mouse CD16/32 antibodies (10 min, room temperature), and stained with the following antibodies: CD45-Pacific Orange (Life Technologies), CXCR3-PerCP Cy5.5, CCR6-APC, CD3ε-APC Cy7, TCRβ-APC, TCRγδ-eF450 (all from eBioscience) or appropriate isotype controls. Intracellular staining for IL-17A-PE or IgG2a-PE (eBioscience) was performed using Fix and Perm kits (Invitrogen, Life Technologies) following the manufacturer's instructions. Following the staining procedure, the samples were acquired using a CyanADP™ (Dako, CO, USA) or an upgraded CytekDevelopment 8DXP-upgraded FACS Calibur (BD Biosciences) and analyzed using FlowJo (Tree Star Inc.). For all flow cytometry experiments, the gates were set based on isotype or saline-recipient *Apoe$^{-/-}$* organs.

Transwell migration experiments

Forty- to fifty-week-old *Apoe$^{-/-}$* or *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* peripheral lymph nodes (cervical, axillary, brachial, inguinal lymph nodes) were sterilely collected and single-cell suspensions were prepared; 0.3–0.4 × 10^6 cells resuspended in 0.1 ml RPMI1640 supplemented with 1% BSA and 10 mM Hepes ('migration media' hereafter) were seeded onto the top well of the transwell. The bottom wells were loaded with 0.6 ml migration media supplemented with the following: media alone (BSA control), 100 ng ml^{-1} recombinant mouse CXCL16 (Peprotech, NJ, USA), 300 ng ml^{-1} CXCL16 or 1000 ng ml^{-1} CCL20 (Peprotech). The loaded cells were allowed to migrate for 3 h at 37°C before being harvested for PMA/Ionomycin-based re-stimulation and intracellular cytokine staining. The migration index indicates the percentage of transmigrated IL-17A$^+$ T cells normalized to the mean of the BSA controls for each genotype (30).

Short-term IL-17A⁺ T-cell homing experiments

Forty- to fifty-week-old *Cxcr6$^{GFP/+}$Apoe$^{-/-}$* and *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* spleens were sterilely collected and the erythrocytes were lysed using ACK lysis buffer (5 min, room temperature, 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and washed with PBS. Following the lysis procedure, *Cxcr6$^{GFP/+}$Apoe$^{-/-}$* and *Cxcr6$^{GFP/GFP}$Apoe$^{-/-}$* splenocytes were labeled with Cell Trace Violet or Cell Trace Far Red (Life Technologies) for 10 min at 37°C and washed twice with 10% FBS/PBS, following the manufacturer's instructions. The labeled cohorts were mixed in a 1:1 ratio and 100 × 10^6 cells (50 × 10^6 cells per donor population) were injected into each 40-week-old *Apoe$^{-/-}$* recipient mouse. As a negative control, some age-matched *Apoe$^{-/-}$* mice were injected with saline as dye-negative controls. At the time of the injection and 72 h post-injection, the donor starting populations and the recipient *Apoe$^{-/-}$* aortas, spleens, blood and PALN were collected for intracellular cytokine staining and assessed for IL-17A$^+$ T cells. All gates were set based on isotype and non-recipient *Apoe$^{-/-}$* controls. To control for differences in the starting percentage of IL-17A$^+$ T cells and the migratory potential of *Cxcr6$^{GFP/+}$* versus *Cxcr6$^{GFP/GFP}$* cells, the fold migration data were normalized to the starting percentage of IL-17A$^+$ T cells and the percentage of donor IL-17A$^+$ *Cxcr6$^{GFP/+}$* T cells of each recipient tissue. Donor CD45$^+$CD3$^+$TCRαβ$^+$GFP$^+$IL-17A$^+$ T cells were considered to be T_{h17} cells, whereas CD45$^+$CD3$^+$TCRαβ$^-$GFP$^+$IL-17A$^+$ T cells were considered to be donor IL-17A$^+$ γδ T cells as 75–80% of CD45$^+$CD3$^+$TCRαβ$^-$IL-17A$^+$ T cells are TCRγδ$^+$.

Statistics

For comparisons between two groups, unpaired or paired (adoptive transfer experiments) two-tailed Student's *t*-test

were used. For comparisons of >2 conditions with a defined control group (transwell experiments), a one-way ANOVA with Dunnett post hoc tests (PASW Statistics, v18) were used. For all experiments, the means ± SEM are shown.

Results and discussion

Aortic T_{h17} and IL-17A⁺TCRγδ⁺ T cells express high levels of CXCR6 within atherosclerotic plaques

T_{h17} and IL-17A⁺TCRγδ⁺ T cells are present within atherosclerotic *Apoe*⁻/⁻ aortas (7), and several lines of evidence suggest that they play a pro-atherogenic role (6, 7, 9, 14). However, the mechanisms behind their recruitment to the aortic wall are unclear. CXCL16 is one of the most abundant chemokines expressed in murine and human atherosclerotic plaques (21, 31) and its receptor, CXCR6, is known to participate in T-cell migration (32). Prior work has demonstrated a pro-atherogenic role for CXCR6, as CXCR6-GFP knockin *Cxcr6*$^{GFP/GFP}$*Apoe*⁻/⁻ mice, which lack the functional CXCR6 receptor, displayed reductions in atherosclerotic lesions, aortic macrophage and T_{h1} accumulation (32). Thus, we hypothesized that CXCR6 might play a role in the recruitment of T_{h17} and IL-17A⁺TCRγδ⁺

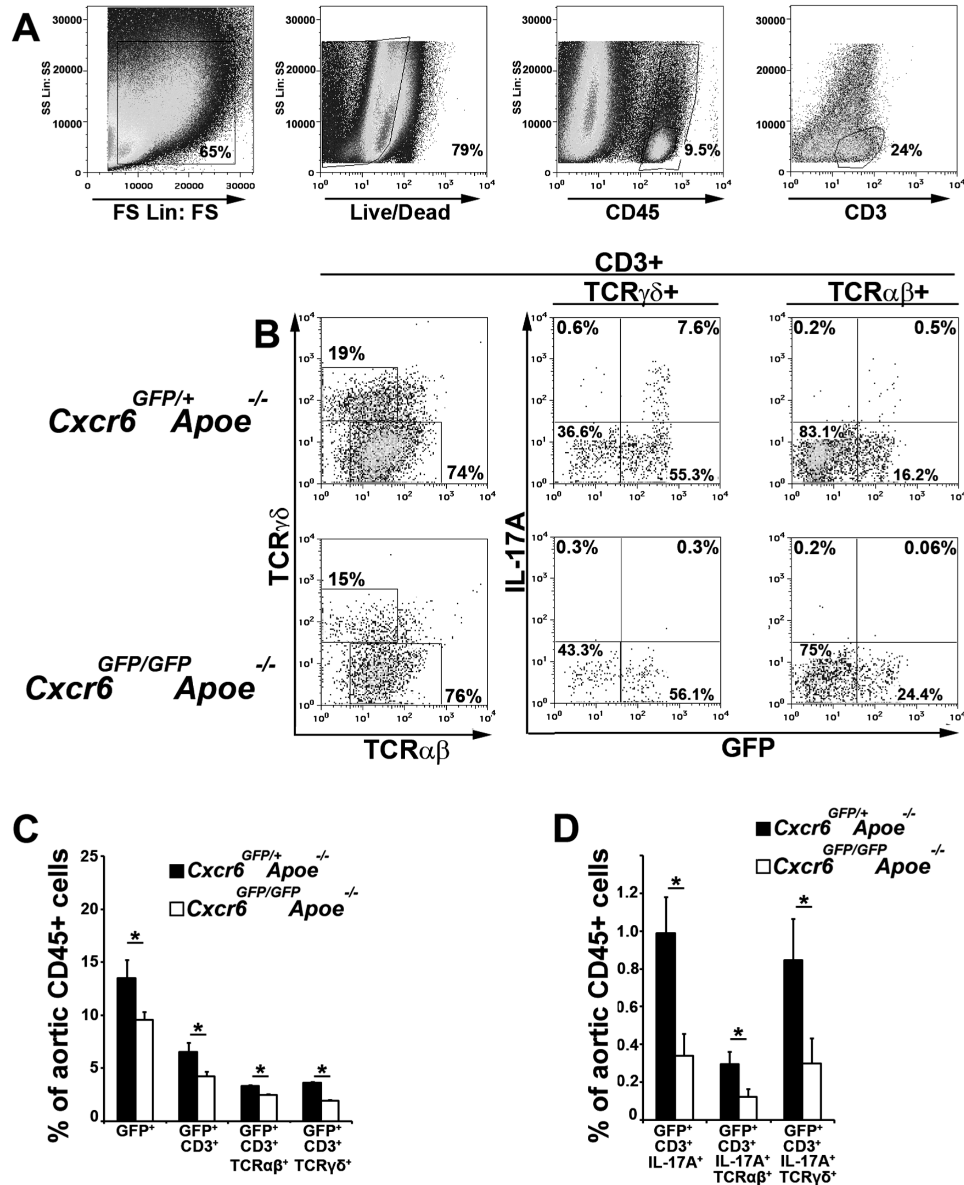

Fig. 1. Aortic T_{h17} and IL-17A⁺TCRγδ⁺ T cells express high levels of CXCR6 in atherosclerosis. Forty- to fifty-week-old *Cxcr6*$^{GFP/+}$*Apoe*⁻/⁻ and *Cxcr6*$^{GFP/GFP}$*Apoe*⁻/⁻ aortas were assessed for CD3ε, IL-17A, TCRαβ, TCRγδ and CXCR6-GFP expression by intracellular cytokine staining. (A) Representative flow cytometry gating scheme. For the analysis of CXCR6^{GFP} and IL-17A expression by T cells, the aortic samples were gated on Forward/Side Scatter, Live events (Live/Dead Aqua low-negative), CD45⁺ cells and CD3⁺ T cells. (B) Representative TCRαβ and TCRγδ staining amongst aortic T cells, and CXCR6^{GFP} expression and IL-17A staining within the CD3⁺TCRγδ⁺ and CD3⁺TCRαβ⁺ T-cell gates. (C) The average percentages of various CXCR6/GFP-expressing cells and (D) IL-17A⁺GFP⁺ populations among CD45⁺ aortic leukocytes. The mean ± SEM are shown. n = 13 *Cxcr6*$^{GFP/+}$*Apoe*⁻/⁻ mice, n = 7 *Cxcr6*$^{GFP/GFP}$*Apoe*⁻/⁻ mice, three to six independent experiments. *$P < 0.05$.

T cells to atherosclerotic plaques. To test this hypothesis, we examined 40- to 50-week-old atherosclerotic *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ mice for aortic CXCR6-expressing T_{h17} and TCRγδ$^+$ T cells (Fig. 1). CXCR6-GFP expression levels correlate with CXCR6 protein expression in *Cxcr6*$^{GFP/+}$ mice (33). As we previously reported, the percentage of CD45$^+$GFP$^+$ leukocytes was significantly decreased in *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ versus *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ mice and approximately 25–35% of CD3$^+$ cells expressed GFP within the aortas of *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ mice [adapted from (32)]. In addition, we observed significantly less CXCR6-GFP-expressing CD3$^+$ T cells, CD3$^+$TCRαβ$^+$ T cells and CD3$^+$TCRγδ$^+$ T cells in *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ aortas in comparison with *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ controls (Fig. 1C). To determine if CXCR6 might be required for the accumulation of T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells, we examined aged *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ aortas for IL-17A-expressing T cells. As previously described (7), we observed populations of aortic CD3$^+$TCRαβ$^+$ and CD3$^+$TCRγδ$^+$ T cells that expressed IL-17A (Fig. 1B). Interestingly, all aortic IL-17A$^+$ T cells expressed high levels of CXCR6-GFP in *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ mice, and CXCR6^{GFP}-expressing IL-17A$^+$ T cells included both T_{h17} and TCRγδ$^+$ T cells (Fig. 1B and D). Under steady-state conditions, CXCR6^{GFP}-expressing T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells were virtually absent in *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ aortas (Fig. 1B and D). Together, these results suggest that although there are many leukocytes that express CXCR6 within atherosclerotic plaques, aortic T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells are defined by high expression of IL-17A and CXCR6.

T_{h17} cells rely on CXCL16/CXCR6-dependent chemotaxis to migrate to atherosclerotic plaques

As all aortic IL-17A$^+$ T cells express high levels of CXCR6, CXCR6 has been reported to be involved in the recruitment of IL-17A$^+$TCRγδ$^+$ T cells to the dermis (34) and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ mice displayed a 60% reduction in aortic IL-17A$^+$ T-cell content, we hypothesized that aortic T_{h17} and IL-17A$^+$TCRγδ$^+$ T-cell recruitment would partially rely on CXCL16/CXCR6-dependent chemotaxis. To test this hypothesis, we first examined the migratory potential of splenic *Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ T_{h17} cells *in vitro* (Fig. 2). In transwell experiments, *Apoe*$^{-/-}$ splenic T_{h17} cells (Fig. 2A) migrated in a dose-dependent manner toward CXCL16, whereas CXCR6-deficient T_{h17} cells were unresponsive (Fig. 2B). In addition to CXCR6 and IL-17A, T_{h17} cells also express the chemokine receptor CCR6 (23, 24). Thus, as a positive control, we also determined the chemotactic potential of WT and CXCR6-deficient T_{h17} cells toward the CCR6 ligand, CCL20 (Fig. 2). As expected, *Apoe*$^{-/-}$ T_{h17} cells chemotactically migrated toward CCL20 *in vitro*. Thus, as CXCR6-dependent T_{h17} migration has not been demonstrated in atherosclerosis, we sought to formally test whether the CXCL16/CXCR6 axis is required for the recruitment of aortic T_{h17} cells *in vivo*. To test this, we adoptively transferred fluorescently labeled Cell Trace Violet$^+$ *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and Far Red$^+$ *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ splenocytes to 40-week-old atherosclerotic *Apoe*$^{-/-}$ recipients and tracked T_{h17} and IL-17A$^+$TCRγδ$^+$ T cell recruitment to the aorta and PALN 72 h post-transfer (Fig. 3). As we demonstrated previously

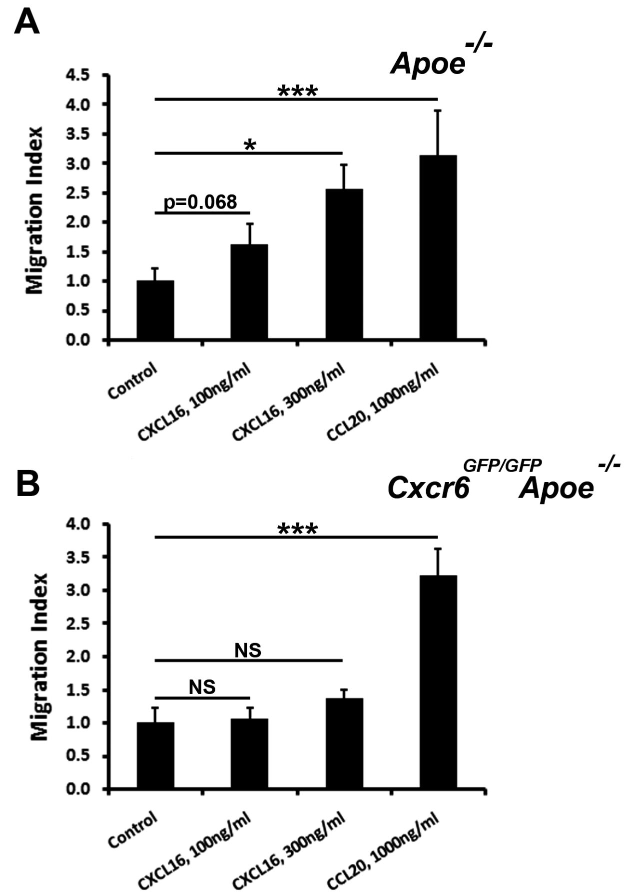

Fig. 2. T_{h17} cells recovered from atherosclerotic mouse spleens chemotactically migrate toward CXCL16. Forty- to fifty-week-old *Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ peripheral lymph nodes were collected and $0.3–0.4 \times 10^6$ cells were seeded into transwells containing complete RPMI1640 supplemented with BSA, 100 ng ml^{-1} CXCL16, 300 ng ml^{-1} CXCL16 or 1000 ng ml^{-1} CCL20 for cell migration experiments. The cells were allowed to migrate for 3 h before the cells in the bottom well were collected for re-stimulation and intracellular cytokine staining. The results depict IL-17A$^+$ T cell migration normalized to the mean of the BSA controls (30). (A) Fold *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ IL-17A$^+$ T-cell transwell migration. (B) Fold *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ IL-17A$^+$ T-cell transwell migration. The results are shown as the mean ± SEM and are representative of three technical replicates from three to four independent experiments. The data are expressed as the mean ± SD of triplicate wells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NS, not significant.

that *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice display elevated atherogenesis (13.5 ± 1.7% and 11.3 ± 1.9% of aortic lesion area, respectively) in comparison with *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ mice (5.0 ± 0.5% of lesion area) (32), *Cxcr6*$^{GFP/GFP}$ and *Cxcr6*$^{GFP/+}$ splenocytes were used to specifically track the migration of CXCR6-GFP$^+$ IL-17A$^+$ T cells. In these experiments, *Cxcr6*$^{GFP/+}$ and *Cxcr6*$^{GFP/GFP}$ donor T cells and IL-17A$^+$ T cells accumulated to a similar extent in peripheral blood and lymphoid organs, including the PALN (Fig. 3A and B), spleens, peripheral lymph nodes (data not shown). In contrast, CXCR6-deficient T cells (1 ± 0.2-fold *Cxcr6*$^{GFP/+}$ versus 0.26 ± 0.15-fold *Cxcr6*$^{GFP/GFP}$ T cells, P = 0.016), IL-17A$^+$ T cells, T_{h17} cells and IL-17A$^+$TCRγδ$^+$ T cells failed to accumulate within atherosclerotic lesions, in comparison with

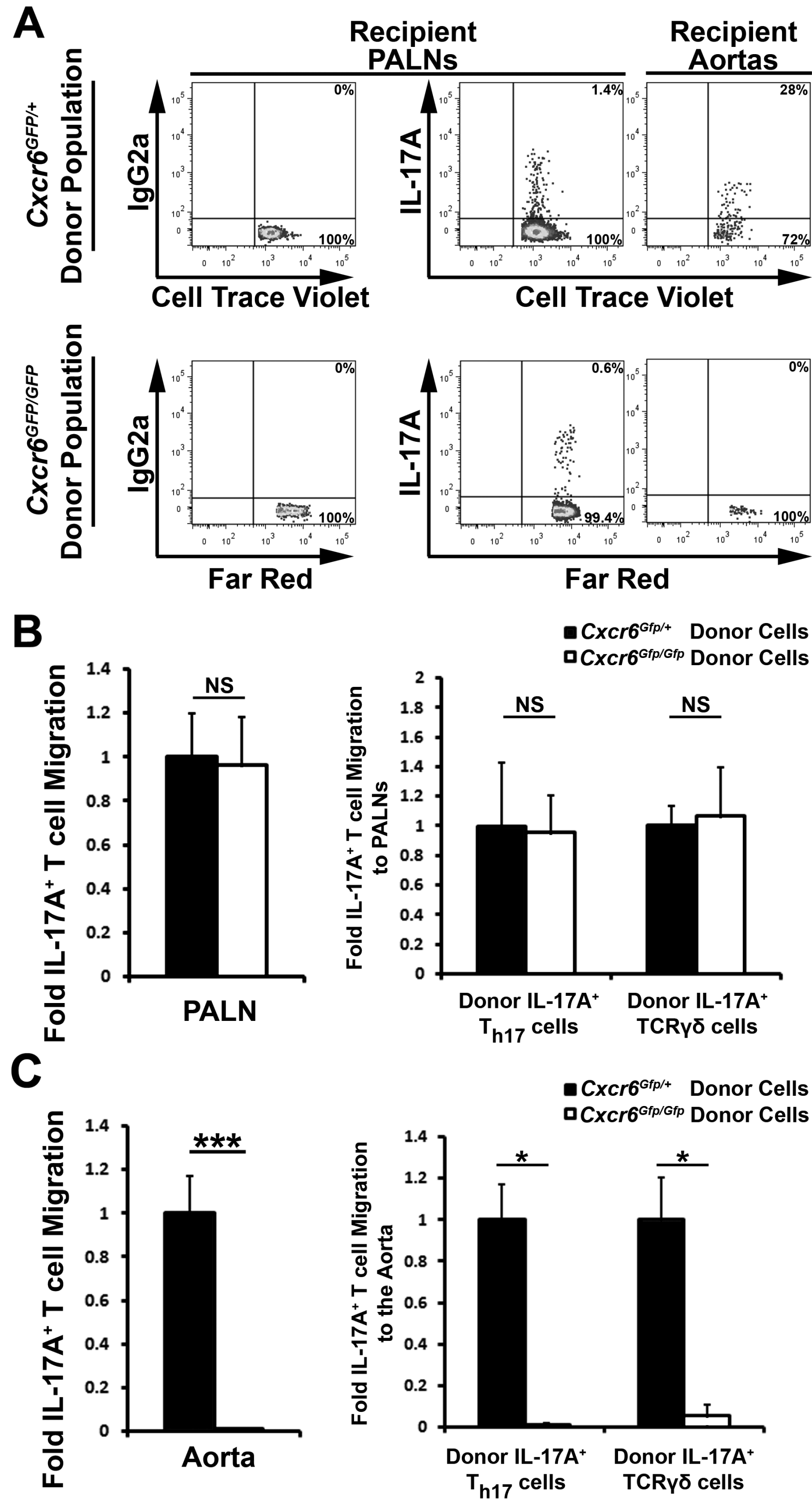

Fig. 3. CXCR6 is required for short-term IL-17A⁺ T-cell homing to atherosclerotic aortas; 40-week-old *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ splenocytes were labeled with Cell Trace Violet or Far Red fluorescent dyes, mixed in a 1:1 ratio, and adoptively transferred to 40-week-old atherosclerotic *Apoe*$^{-/-}$ recipients. Seventy-two hours post-transfer, the recipient spleens, PALN, and aortas were assessed for donor IL-17A⁺ T cells. (A) Representative flow cytometry plots from two pooled recipient *Apoe*$^{-/-}$ mice. All flow cytometry plots are gated on CD45⁺ dye⁺ donor leukocytes. Recipient *Apoe*$^{-/-}$ PALN isotype control staining (left), and IL-17A staining in the PALNs (middle) and aortas (right) within the gated *Cxcr6*$^{GFP/+}$ and *Cxcr6*$^{GFP/GFP}$ donor populations. (B and C) Quantification of the IL-17A⁺ T-cell homing results. The percentage of IL-17A⁺ *Cxcr6*$^{GFP/+}$ and IL-17A⁺ *Cxcr6*$^{GFP/GFP}$ T cells that successfully migrated to the recipient *Apoe*$^{-/-}$ PALNs, and aortas, normalized to the starting percentage of IL-17A⁺ T cells and IL-17A⁺ *Cxcr6*$^{GFP/+}$ T cells within the recipient organs. The mean ± SEM are shown. *n* = 9 independent transfer experiments. **P* < 0.05, ****P* < 0.001. NS, not significant.

CXCR6-sufficient T cells (Fig. 3A and C). Thus, CXCR6 is required for the efficient recruitment of IL-17A$^+$ T cells to atherosclerotic lesions.

Interestingly, peri-aortic and splenic IL-17A$^+$ T cells, T_{h17}, and IL-17A$^+$TCRγδ$^+$ T cells, express high levels of CCR6 (data not shown). Within the aorta, T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells are similarly 98.96 ± 0.84% and 95.1 ± 2.62% positive for CCR6, respectively. However, as the deficiency of CXCR6 almost completely abolished the accumulation of IL-17A$^+$ T cells into aorta, CCR6 may not compensate for the defective homing of CXCR6-deficient IL-17A$^+$T cells to the aorta. This observation is in line with recent studies that demonstrated that CCR6 is dispensable for the recruitment of aortic T_{h17} cells in *Ccr6*$^{-/-}$*Apoe*$^{-/-}$ and *Ccr6*$^{-/-}$*Ldlr*$^{-/-}$ mice (26, 35). Thus, despite high expression of the chemokine receptor CCR6, T_{h17} cells might instead rely majorly on CXCR6-depedent chemotaxis to migrate toward atherosclerotic lesions.

Importantly, the recruitment of *Cxcr6*$^{GFP/+}$ IL-17A$^+$ T cells to the aorta was disproportionately lower (1 ± 0.2-fold *Cxcr6*$^{GFP/+}$ versus 0.052 ± 0.03-fold *Cxcr6*$^{GFP/GFP}$ T-cell recruitment, P = 0.039) than the proportion of recruited total *Cxcr6*$^{GFP/+}$ T cells (1 ± 0.19-fold *Cxcr6*$^{GFP/+}$ versus 0.26 ± 0.15 *Cxcr6*$^{GFP/GFP}$ T-cell recruitment, P = 0.016). We therefore determined whether *Cxcr6*$^{GFP/+}$ IL-17A$^-$ T cells might express other chemokine receptors in addition to CXCR6. Aortic *Cxcr6*$^{GFP/+}$IL-17A$^-$TCRαβ$^+$ T cells, including IFNγ$^+$ T_{h1} cells, majorly express the T_{h1}-related chemokine receptor CXCR3 (80 ± 2.5% of *Cxcr6*$^{GFP/+}$ IL-17A$^-$IFNγ$^+$ T_{h1} cells), whereas *Cxcr6*$^{GFP/+}$ T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells are mostly negative (18 ± 4% and 20 ± 1.6%, respectively). Thus, *Cxcr6*$^{GFP/+}$ IL-17A$^-$ T cells likely compensate for CXCR6-deficient conditions via CXCR3, whereas CXCR6 is required for the recruitment of T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells to atherosclerotic lesions.

Altogether, these data demonstrate that IL-17A$^+$ T cells, including T_{h17} and IL-17A$^+$ TCRγδ$^+$ T cells, require CXCR6 in order to efficiently migrate to atherosclerotic lesions in *Apoe*$^{-/-}$ mice. These observations are important as it is currently unclear which chemokine receptors are required for the migration of IL-17A-producing cells in atherosclerosis, and aortic T-cell homing experiments are technically challenging. Prior work on the functions of T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells have demonstrated that both subsets accumulate in atherosclerotic lesions and likely promote atherogenesis by supporting further monocyte recruitment and macrophage maturation (6, 7, 9, 14, 15, 18, 19, 36). T_{h17} cells are known to express the chemokine receptors CCR6 (23, 24) and CXCR6 (22) but the precise combination of chemokine receptors required for T_{h17} migration to atherosclerotic lesions was unclear. Several studies have reported the presence of IL-17A-producing T cells in sites of chronic inflammation (23–25) and have implicated the chemokine receptor CCR6 in their recruitment. However, the potential roles of other complementary T_{h17} chemokine receptors were not assessed. Prior work involving CCR6-deficient *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice demonstrated a decidedly pro-atherogenic role for CCR6; however, IL-17A expression was unaltered between CCR6-deficient and CCR6-sufficient mice (26, 35). Here, we expand on these observations by reporting that CCR6 does not compensate for a genetic CXCR6-deficiency in short-term aortic IL-17A$^+$ T-cell recruitment experiments, within atherosclerotic *Apoe*$^{-/-}$ mice.

Additionally, work from our group investigated the effects of CXCR6 on atherosclerosis using *Cxcr6*$^{GFP/+}$*Apoe*$^{-/-}$ and *Cxcr6*$^{GFP/GFP}$*Apoe*$^{-/-}$ mice (32). In that study, atherosclerotic lesions—particularly in the aortic arch, macrophage accumulation, T-cell homing and IFNγ production—were significantly decreased in CXCR6-deficient mice. Thus, we hypothesized in the present study that CXCR6 might affect T_{h17} and IL-17A$^+$TCRγδ$^+$ T-cell recruitment to the aorta. Our results here demonstrated that all IL-17A$^+$ T cells within the aorta express CXCR6 and that CXCR6-deficient T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells were unable to effectively migrate in transwell and short-term homing experiments. CXCL16 is expressed by endothelial and smooth muscle cells, macrophages and dendritic cells (37). CXCR6 is the only known chemokine receptor for CXCL16, which can serve as a chemoattractant or mediate cell adhesion for T cells (37). It is possible that this dual function of CXCL16 provides a unique combination that successfully supports IL-17A$^+$ T-cell migration into atherosclerotic lesions. Additionally, CXCL16-expressing cells are detected within human and murine atherosclerotic vessels, and circulating CXCL16 levels are elevated in patients with acute coronary syndromes (21). CXCL16 levels also positively correlate with the clinical outcomes of stroke and CAD. Thus, elevated CXCL16 production might accelerate the recruitment of T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells in these conditions, thereby supporting further inflammation and atherogenesis. In summary, our results demonstrate that T_{h17} and IL-17A$^+$TCRγδ$^+$ T cells use CXCL16/CXCR6 in order to efficiently migrate toward atherosclerotic lesions.

Funding

National Heart, Lung, and Blood Institute (HL107522 to E.V.G.).

Acknowledgements

We thank T. Phillips for her technical assistance.

Conflict of interest statement: The authors declared no conflict of interests.

References

1 Galkina, E. and Ley, K. 2009. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu. Rev. Immunol.* 27:165.
2 Libby, P., Lichtman, A. H. and Hansson, G. K. 2013. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* 38:1092.
3 Gaffen, S. L. 2009. Structure and signalling in the IL-17 receptor family. *Nat. Rev. Immunol.* 9:556.
4 Kolls, J. K. and Lindén, A. 2004. Interleukin-17 family members and inflammation. *Immunity* 21:467.
5 Reynolds, J. M., Angkasekwinai, P. and Dong, C. 2010. IL-17 family member cytokines: regulation and function in innate immunity. *Cytokine Growth Factor Rev.* 21:413.
6 Erbel, C., Chen, L., Bea, F. *et al.* 2009. Inhibition of IL-17A attenuates atherosclerotic lesion development in apoE-deficient mice. *J. Immunol.* 183:8167.
7 Smith, E., Prasad, K. M., Butcher, M., Dobrian, A., Kolls, J. K., Ley, K. and Galkina, E. 2010. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 121:1746.
8 Taleb, S., Romain, M., Ramkhelawon, B. *et al.* 2009. Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J. Exp. Med.* 206:2067.

9 van Es, T., van Puijvelde, G. H., Ramos, O. H. *et al.* 2009. Attenuated atherosclerosis upon IL-17R signaling disruption in LDLr deficient mice. *Biochem. Biophys. Res. Commun.* 388:261.
10 Butcher, M. and Galkina, E. 2011. Current views on the functions of interleukin-17A-producing cells in atherosclerosis. *Thromb. Haemost.* 106:787.
11 Eid, R. E., Rao, D. A., Zhou, J. *et al.* 2009. Interleukin-17 and interferon-gamma are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells. *Circulation* 119:1424.
12 Erbel, C., Dengler, T. J., Wangler, S. *et al.* 2011. Expression of IL-17A in human atherosclerotic lesions is associated with increased inflammation and plaque vulnerability. *Basic Res. Cardiol.* 106:125.
13 Gao, Q., Jiang, Y., Ma, T. *et al.* 2010. A critical function of Th17 proinflammatory cells in the development of atherosclerotic plaque in mice. *J. Immunol.* 185:5820.
14 Butcher, M. J., Gjurich, B. N., Phillips, T. and Galkina, E. V. 2012. The IL-17A/IL-17RA axis plays a proatherogenic role via the regulation of aortic myeloid cell recruitment. *Circ. Res.* 110:675.
15 Usui, F., Kimura, H., Ohshiro, T. *et al.* 2012. Interleukin-17 deficiency reduced vascular inflammation and development of atherosclerosis in Western diet-induced apoE-deficient mice. *Biochem. Biophys. Res. Commun.* 420:72.
16 Danzaki, K., Matsui, Y., Ikesue, M. *et al.* 2012. Interleukin-17A deficiency accelerates unstable atherosclerotic plaque formation in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 32:273.
17 Gistera, A., Robertson, A. K., Andersson, J. *et al.* 2013. Transforming growth factor-b signaling in T cells promotes stabilization of atherosclerotic plaques through an interleukin-17-dependent pathway. *Sci. Transl. Med.* 5:196ra100.
18 Madhur, M. S., Funt, S. A., Vinh, A. *et al.* 2011. Role of interleukin 17 in inflammation, atherosclerosis, and vascular function in apolipoprotein e-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 31:1565.
19 Erbel, C., Akhavanpoor, M., Okuyucu, D. *et al.* 2014. IL-17A influences essential funcitons of the monocyte/macrophage lineage and is involved in advanced murine and human atherosclerosis. *J. Immunol.* 193:4344.
20 Galkina, E. and Ley, K. 2007. Leukocyte influx in atherosclerosis. *Curr. Drug Targets* 8:1239.
21 Zernecke, A. and Weber, C. 2014. Chemokines in atherosclerosis: proceedings resumed. *Arterioscler. Thromb. Vasc. Biol.* 34:742.
22 Lim, H. W., Lee, J., Hillsamer, P. and Kim, C. H. 2008. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. *J. Immunol.* 180:122.
23 Yamazaki, T., Yang, X. O., Chung, Y. *et al.* 2008. CCR6 Regulates the Migration of Inflammatory and Regulatory T Cells. *J. Immunol.* 181:8391.
24 Hirota, K., Yoshitomi, H., Hashimoto, M. *et al.* 2007. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J. Exp. Med.* 204:2803.
25 Alcaide, P., Maganto-Garcia, E., Newton, G. *et al.* 2012. Difference in Th1 and Th17 lymphocyte adhesion to endothelium. *J. Immunol.* 188:1421.
26 Manthey, H. D., Cochain, C., Barnsteiner, S. *et al.* 2013. CCR6 selectively promotes monocyte mediated inflammation and atherogenesis in mice. *Thromb. Haemost.* 110:1267.
27 Unutmaz, D., Xiang, W., Sunshine, M. J., Campbell, J., Butcher, E. and Littman, D. R. 2000. The primate lentiviral receptor Bonzo/STRL33 is coordinately regulated with CCR5 and its expression pattern is conserved between human and mouse. *J. Immunol.* 165:3284.
28 Butcher, M. J., Herre, M., Ley, K., and Galkina, E. 2011. Flow cytometry analysis of immune cells within murine aortas. *J. Vis. Exp.* 53. pii: 2848. doi:10.3791/2848.
29 Galkina, E., Kadl, A., Sanders, J., Varughese, D., Sarembock, I. J. and Ley, K. 2006. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J. Exp. Med.* 203:1273.
30 Valcu, M. and Valcu, C. M. 2011. Data transformation practices in biomedical sciences. *Nat. Methods* 8:104.
31 Aslanian, A. M. and Charo, I. F. 2006. Targeted disruption of the scavenger receptor and chemokine CXCL16 accelerates atherosclerosis. *Circulation* 114:583.
32 Galkina, E., Harry, B. L., Ludwig, A. *et al.* 2007. CXCR6 promotes atherosclerosis by supporting T-cell homing, interferon-gamma production, and macrophage accumulation in the aortic wall. *Circulation* 116:1801.
33 Geissmann, F., Cameron, T. O., Sidobre, S. *et al.* 2005. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol.* 3:e113.
34 Gray, E. E., Suzuki, K. and Cyster, J. G. 2011. Cutting edge: Identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J. Immunol.* 186:6091.
35 Wan, W., Lim, J. K., Lionakis, M. S. *et al.* 2011. Genetic deletion of chemokine receptor Ccr6 decreases atherogenesis in ApoE-deficient mice. *Circ. Res.* 109:374.
36 Lim, H., Kim, Y. U., Sun, H. *et al.* 2014. Proatherogenic conditions promote autoimmune T helper 17 cell responses in vivo. *Immunity* 40:153.
37 Matloubian, M., David, A., Engel, S., Ryan, J. E. and Cyster, J. G. 2000. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat. Immunol.* 1:298.