Intercellular Adhesion Molecule 1 Mediates Migration of Th1 and Th17 Cells Across Human Retinal Vascular Endothelium

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PURPOSE. Autoimmune inflammation of the retina causes vision loss in the majority of affected individuals. Th1 or Th17 cells initiate the disease on trafficking from the circulation into the eye across the retinal vascular endothelium. We investigated the ability of human Th1- and Th17-polarized cells to cross a simulated human retinal endothelium, and examined the role of IgG superfamily members in this process.

METHODS. Th1- and Th17-polarized cell populations were generated from human peripheral blood CD4⁺ T cells, using two Th1- and Th17-polarizing protocols. Transendothelial migration assays were performed over 18 hours in Boyden chambers, after seeding the transwell membrane with human retinal endothelial cells. In some assays intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), or activated leukocyte cell adhesion molecule (ALCAM) blocking antibody, or isotype- and concentration-matched control antibody, was added to the upper chambers.

RESULTS. Th1- and Th17-polarized cells migrated equally efficiently across the human retinal endothelial monolayer. The percentage of IL-17⁺ IFN- γ ⁻ Th17-polarized cells was reduced following migration. Blocking ICAM-1, but not VCAM-1 or ALCAM, significantly reduced migration of Th1- and Th17-polarized cells for a majority of human donors.

CONCLUSIONS. Taken in the context of other literature on transendothelial migration, our results illustrate the importance of investigating the specific tissue and vascular endothelium when considering helper T cell migration in autoimmune inflammation. Our findings further indicate that while generalizations about involvement of specific adhesion molecules in uveitis and other autoimmune disease may be possible, these may not apply to individual patients universally. The observations are relevant to the use of adhesion blockade for therapeutic purposes.

Keywords: uveitis, retina, endothelial cell, Th1 cell, Th17 cell

Many forms of human inflammatory disease that are presumed to have an autoimmune etiology are controlled by helper T cells. Despite differences in their biological activities, either Th1 cells or Th17 cells may direct the effector response, depending on the mode and environment of antigen presentation.^{1–3} In inflammation, circulating T cells access the tissue through a complex molecular interaction with the local vascular endothelium that involves cell adhesion molecules and chemokines.⁴ Immunoglobulin (IgG) superfamily members play a central role in coordinating T cell transendothelial migration. Data generated primarily in mouse systems indicate that extravasation of Th1 versus Th17 subsets may be differentially regulated.^{2,5–8} Human and mouse helper T cell subsets differ in respects that are likely to impact disease, including origin, requirements for differentiation and expansion, and activa-

tion.^{9–12} Little is known of the molecular control of helper T cell trafficking in human autoimmune disease.

Inflammations that involve the tissues within the eye are termed uveitis. Autoimmune uveitis is an important cause of vision loss worldwide¹³; when the disease is based in the retina and/or adjacent intraocular tissues, the prevalence of visual impairment is approximately 50%.¹⁴ Peripheral blood mononuclear cells from individuals with uveitis include leukocyte populations that produce high levels of Th1 and Th17 signature cytokines.^{15–17} Mouse experimental autoimmune uveoretinitis is widely used to study mechanisms of autoimmune uveitis based in the retina.¹⁸ Studies using this clinically relevant model demonstrate that effector T cells migrate across the endothelium of the retinal blood vessels to enter the target tissue.^{19,20} Consistently, histopathological examination of diseased human eyes demonstrates upregulation of IgG superfamily adhesion

molecules on the retinal vascular endothelium,^{21,22} and cultured human retinal endothelial cells upregulate the same molecules following inflammatory stimulation by exposure to tumor necrosis factor- α or lipopolysaccharide.^{23,24}

Apart from the public health significance, there is a practical reason to focus on uveitis for the study of cell trafficking in autoimmune disease. Fresh human retinal tissue may be obtained from eye banks, which harvest corneas from human eyes for transplantation, providing a source of fresh tissue for isolation of human vascular endothelial cells. In this study, we used the Boyden chamber assay to evaluate the migration of Th1- and Th17-polarized cells across a simulated human retinal endothelium, including the role of IgG superfamily adhesion molecules. We show that the polarized T cell subsets migrate equally efficiently across the vascular endothelium, but with some modification in phenotype, and that intercellular adhesion molecule 1 (ICAM-1) is a key molecular mediator of this transendothelial movement.

MATERIALS AND METHODS

Cytokines and Antibodies

Recombinant human (rh)interleukin (IL)-1 β , rhIL-2, rhIL-6, rhIL-12, and rhIL-23 were purchased from R&D Systems (Minneapolis, MN). Mouse anti-human IL-4 antibody (Ab, clone 3007, isotype IgG1), mouse anti-human interferon (IFN)- γ Ab (clone K3.53, isotype IgG2a), mouse anti-human ICAM-1 (CD54) Ab (clone BBIG-11, isotype IgG1), mouse anti-human vascular cell adhesion molecule 1 (VCAM-1; CD106) Ab (clone BBIG-V1, isotype IgG1), mouse anti-human activated leukocyte cell adhesion molecule (ALCAM; CD166) Ab (clone 105901, isotype IgG1), and mouse IgG1 (clone 11711, isotype IgG1) were also purchased from R&D Systems. Fluorescein isothiocyanate (FITC)-tagged mouse anti-human CD3 Ab (clone UCHT1, isotype IgG1k), Pacific Blue-tagged mouse anti-human CD3 Ab (clone UCHT1, isotype IgG1k), biotin-conjugated mouse anti-human CCR6 Ab (clone 11A9, isotype IgG1k), AlexaFluor 700-tagged mouse anti-human IFN- γ Ab (clone B27, isotype IgG1k), and allophycocyanin (APC)-tagged streptavidin were obtained from BD Biosciences (San Jose, CA). Mouse anti-human CD3 (clone OKT3, isotype IgG2a k), mouse anti-human CD3 (clone UCHT1, isotype IgG1k), mouse anti-human CD28 (clone CD28.2, isotype IgG1k), mouse anti-human IL-4 Ab (clone MP4-25D2, isotype IgG1k), mouse anti-human IFN- γ Ab (clone NIB42, isotype IgG1k), phycoerythrin (PE)-tagged mouse anti-human CD4 Ab (clone OKT4, isotype IgG2b k), eFluor 450-tagged mouse anti-human CD4 Ab (clone OKT4, isotype IgG2b k), PE-tagged mouse anti-human IL-17 Ab (clone eBio64CAP17, isotype IgG1k), peridinin chlorophyll protein-cyanine (PerCP-Cy) 5.5-tagged mouse anti-human IFN- γ Ab (clone 4S.B3, isotype IgG1k), and AlexaFluor 488-conjugated mouse anti-human IL-17 Ab (clone eBio64DEC17, isotype IgG1k) were bought from eBioscience (San Diego, CA). APC-conjugated mouse anti-human CD45RO Ab (clone UCHL1, isotype IgG2a k) was bought from Life Technologies-Molecular Probes (Camarillo, CA).

Cells

Leukocytes were isolated from peripheral blood of healthy adult humans that was collected with consent under a protocol approved by the Oregon Health & Science University Institutional Review Board. Human retinal endothelial cells were isolated from retinas that were dissected from human cadaver eyes within 24 hours of death, as previously

described.²³ To generate sufficient cells for study, the cells were transduced with the mouse recombinant amphotropic retrovirus, LXSNI6E6E7 (gift of Denise A. Galloway, Fred Hutchinson Cancer Institute), as previously reported.²⁴ Cells were cultured in MCDB-131 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Thermo Scientific-Hyclone, Logan, UT) and endothelial growth factors (EGM-2 SingleQuots supplement, omitting FBS, hydrocortisone and gentamicin; Clonetics-Lonza, Walkersville, MD) at 37°C and 5% CO₂.

Generation of Th1- or Th17-Polarized Cells From CD4⁺ CCR6^{-/+} Cells

Human peripheral blood was incubated with a T cell enrichment cocktail (RosetteSep Human CD4⁺ T Cell Enrichment Cocktail; Stem Cell, Vancouver, BC, Canada). Enriched CD4⁺ T cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). The cells were labeled with FITC-tagged anti-CD3 Ab (2 μ g/mL), PE-tagged anti-CD4 Ab (30 ng/mL) and biotin-conjugated anti-CCR6 Ab (5 μ g/mL), and subsequently with APC-tagged streptavidin (700 ng/mL), and sorted on the basis of CD3 and CD4 expression, followed by CCR6 expression on the flow cytometer (BD FACS Vantage; BD Biosciences). CD4⁺ CCR6⁻ or CCR6⁺ T cells were cultured in RPMI-1640 (Life Technologies-Gibco, Grand Island, NY) supplemented with 10% FBS; 2 mM L-glutamine (Life Technologies-Gibco); penicillin-streptomycin (100 U/mL-100 μ g/mL; Life Technologies-Gibco); and 10 mM HEPES (Thermo Fisher Scientific Inc., Fair Lawn, NJ) at 37°C and 5% CO₂, in wells of a 96-well plate previously coated with anti-CD3 Ab (clone UCHT1, isotype IgG1k; 5 μ g/mL) and anti-CD28 Ab (5 μ g/mL). CD4⁺ CCR6⁻ T cells were cultured under Th1-polarizing conditions (rhIL-12 [10 ng/mL] and anti-IL-4 Ab [100 ng/mL]), and CD4⁺ CCR6⁺ T cells were cultured under Th17-polarizing conditions (rhIL-1 β [10 ng/mL]; rhIL-6 [20 ng/mL]; rhIL-23 [10 ng/mL]; anti-IFN- γ Ab [100 ng/mL]; and anti-IL-4 Ab [100 ng/mL]). After 3 days in culture, T cells were transferred to uncoated wells, and 50% of spent medium was replenished with the appropriate polarizing medium and rhIL-2 (50 ng/mL). After 7 days in culture, T cells were returned to anti-CD3 Ab- and anti-CD28 Ab-coated wells, and polarizing medium alone. The cycle of polarization in the absence and presence of rhIL-2 was repeated. Over the 14-day procedure, cultures were moved into wells of progressively wider diameter and medium was replenished as cell growth required.

Generation of Th1- or Th17-Polarized Cells From CD4⁺ CD45RO⁺ Cells

A modification of the method described by Kebir et al.²⁵ was used. Mononuclear cells were separated from human peripheral blood by density gradient centrifugation using Ficoll-Paque PLUS. CD14⁺ monocytes and CD4⁺ CD45RO⁺ T cells were isolated from the peripheral mononuclear cells by positive selection using CD14 MicroBeads, followed by negative selection using the Memory CD4⁺ T Cell Isolation Kit (both from Miltenyi Biotec, Auburn, CA). CD4⁺ CD45RO⁺ T cells were cultured for 5 to 6 days in RPMI-1640 supplemented with 10% FBS; 2 mM L-glutamine; penicillin-streptomycin (100 U/mL-100 μ g/mL) and 10 mM HEPES, with CD14⁺ monocytes, anti-CD3 Ab (clone OKT3, isotype IgG2a k; 2.5 μ g/mL); and either a Th1-polarizing cocktail (rhIL-12 [10 ng/mL] and anti-IL-4 Ab [5 μ g/mL]) or a Th17-polarizing cocktail (rhIL-23 [10 ng/mL], anti-IFN- γ Ab [5 μ g/mL], and anti-IL-4 Ab [5 μ g/mL]) at 37°C and 5% CO₂.

FIGURE 1. A majority of Th1-polarized cells produce IFN- γ alone, while Th17-polarized cells include populations that produce IL-17 alone, IFN- γ alone, and both IL-17 and IFN- γ . (A) Th1-polarized cells ($n = 5$ donors) or (B) Th17-polarized cells ($n = 9$ donors) were generated from CD4⁺ CCR6^{-/-} or CD4⁺ CCR6⁺ T cells by exposure to anti-CD3 Ab and anti-CD28 Ab, and either rhIL-12 and α IL-4 Ab, or rhIL-1 β , rhIL-6, rhIL-23, anti-IFN- γ Ab, and anti-IL-4 Ab, respectively. (C) Th1-polarized cells ($n = 11$ donors) and (D) Th17-polarized cells ($n = 10$ donors) were generated from CD4⁺ CD45RO⁺ T cells by incubation with CD14⁺ monocytes and either rhIL-12 and anti-IL-4 Ab, or rhIL-23, anti-IFN- γ Ab, and anti-IL-4 Ab, respectively. Representative flow dot plots showing the intracellular expression of IFN- γ (x-axis) and/or IL-17 (y-axis) by polarized helper T cells. Cells were gated on the lymphocyte population based on size and granularity. Graphs show the percentage of lymphocytes expressing either IL-17 and/or IFN- γ . Bars represent mean and error bars represent standard error of mean.

Detection of Intracellular Cytokine Expression by Polarized T Cells

CD4⁺ CCR6^{-/-} Th1- and CD4⁺ CCR6⁺ Th17-polarized cells were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ g/mL ionomycin calcium salt (both from Sigma-Aldrich) in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin-streptomycin (100 U/mL–100 μ g/mL) and 10 mM HEPES, and concurrently treated with a protein transport inhibitor (BD GolgiStop containing monensin; BD Biosciences) for 5 hours. CD4⁺ CD45RO⁺ Th1- and Th17-polarized T cells were stimulated with 20 ng/mL PMA and 1 μ g/mL ionomycin calcium salt for 3 hours, followed by 2 μ g/mL brefeldin A (Sigma-Aldrich) for an additional 2 hours. Intracellular cytokine production was assessed using a fixation and permeabilization kit (BD Cytofix/Cytoperm Fixation and Permeabilization Solution Kit; BD Biosciences), per the manufacturer's instructions. Briefly, for CD4⁺ CCR6^{-/-} or CD4⁺ CD45RO⁺ T cells, respectively, cells suspended in phosphate buffered saline supplemented with 1% FBS and 0.1% sodium azide were surface-stained with either FITC-tagged anti-CD3 Ab (1 μ g/mL) and eFluor 450-tagged anti-CD4 Ab (30 ng/mL) or Pacific blue-tagged anti-CD3 Ab (8 μ g/mL) and APC-conjugated anti-CD45RO Ab (2 μ g/mL), fixed and permeabilized with a fixation and permeabilization solution (BD Biosciences), and stained for intracellular cytokine expression with either PerCP-Cy5.5-tagged anti-IFN- γ Ab (60 ng/mL) and PE-tagged anti-IL-17 Ab (100 ng/mL) or AlexaFluor 700-tagged anti-IFN- γ Ab (400 ng/mL) and AlexaFluor 488-tagged anti-IL-17 Ab (100 ng/mL). After staining, the T cells were acquired on a flow cytometer (BD LSR II; BD Biosciences). Cells were gated on the lymphocyte population

based on size and granularity, and data were analyzed using a commercial software program (FCS Express V3; De Novo Software, Los Angeles, CA).

Transendothelial Migration Assay

Human retinal endothelial cells were suspended in modified MCDB-131 medium with 10% FBS, seeded at 30,000 cells on polyethylene terephthalate transwell membranes (0.3 cm² diameter, 3 micron pore size; BD Falcon Labware, Franklin Lakes, NJ) positioned in wells of 24-well plates, and incubated at 37°C and 5% CO₂. The transwell membranes were precoated with bovine type I collagen (50 μ g/mL; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at 37°C. At 4 to 5 days after seeding of the endothelial monolayers, polarized Th1 or Th17 cells, suspended in modified MCDB-131 medium with 10% FBS, were added to upper chambers of transwells at up to 1.2×10^6 T cells/transwell. Lower chambers were filled with medium alone. In some cases, anti-ICAM-1 Ab (10 μ g/mL), anti-VCAM-1 Ab (30 μ g/mL), anti-ALCAM Ab (30 μ g/mL), or mouse IgG1 directed against irrelevant antigen (matched concentration) was added to the upper chamber concurrently with the T cells. Transwells were incubated for 18 hours at 37°C and 5% CO₂, after which time migrated T cells were recovered from the upper and/or lower chambers and counted on a hemacytometer. Collection of cells involved careful aspiration of all medium from each chamber separately. The lower side of the transwell membrane was washed to dislodge lymphocytes that had migrated through the endothelium, but not reached the lower chamber; these lymphocytes were added to those collected directly from the lower chamber. Enzymatic digestion of the endothelium was not performed, to preserve the

FIGURE 2. Th1- and Th17-polarized cells generated by two different protocols migrate equally efficiently across simulated human retinal vascular endothelium. (A) CD4⁺ CCR6⁻ Th1-polarized cells and CD4⁺ CCR6⁺ Th17-polarized cells ($n = 6$ donors), and (B) CD4⁺ CD45RO⁺ Th1- and Th17-polarized cells ($n = 7$ donors) were migrated across a human retinal endothelial cell monolayer in a Boyden chamber for 18 hours (0.5×10^6 T cells/transwell). Phenotypes of polarized cell populations are illustrated in Figure 1. Graphs show the total number of migrated cells for each polarized T cell subset. Bars represent mean and error bars represent standard error of mean. Data were analyzed by two-tailed unpaired Student's *t*-test. ns, no significant difference.

phenotype of the T cells. Conditions were performed in triplicate. For all experiments, the integrity of the endothelial monolayers was confirmed by measuring the diffusive flux of 1 mg/mL Texas Red-conjugated dextran (MWt 70,000; Molecular Probes-Invitrogen, Eugene, OR) across monolayers, per the method of Harhaj et al.²⁶

Statistical Analysis

Data obtained in transendothelial migration assays were presented as mean $\pm$ standard error of mean. Data from two groups were compared by Student's *t*-test, one- or two-tailed, and paired or unpaired, as appropriate, using a commercial scientific graphing and statistics software (GraphPad Prism; GraphPad Software, La Jolla, CA). In all analyses, a significant difference was defined as one yielding a *P* value less than 0.05.

RESULTS

Th1- and Th17-Polarized Cells Migrate in Equal Numbers Across Simulated Human Retinal Endothelium

We used two alternative protocols to generate human Th1- and Th17-polarized cells for the transendothelial migration studies. Polarized T cell populations were characterized by flow cytometry on intracellular expression of the Th1 and Th17 prototype cytokines, IFN- γ , and IL-17. Since cells expressing IFN- γ or IL-17 average approximately 11% and 0.5% of the CD4⁺ T cell population in a healthy human, respectively,²⁷ polarization procedures are necessary to generate cell populations for comparative studies. Although the T cells are derived from healthy adult humans, polarization yields populations that are relevant for the study of human autoimmune disease.²⁵ By the first method, human CD4⁺ CCR6⁻ or CCR6⁺ T cells were exposed to rhIL-12 and anti-IL-4 Ab, or rhIL-1 β , rhIL-6, rhIL-23, anti-IFN- γ Ab, and anti-IL-4 Ab, in the presence of anti-CD3 Ab and anti-CD28 Ab, to polarize to a Th1 or Th17 phenotype, respectively. Cells were also exposed intermittently to rhIL-2 to stimulate proliferation. Purity of CD4⁺ CCR6⁻ T cells was $92.55 \pm 1.12\%$ ($n = 4$ donors) and purity of CD4⁺ CCR6⁺ T cells was $94.70 \pm 1.65\%$ ($n = 8$ donors). Immunophenotyping demonstrated $28.36 \pm 6.20\%$ IFN- γ ⁺/IL-17⁻ cells, $0.16 \pm 0.04\%$ IFN- γ ⁺/IL-17⁺ cells, and 0.74

$\pm 0.50\%$ IFN- γ ⁻/IL-17⁺ cells in the Th1-polarized preparations (Fig. 1A), and $17.77 \pm 1.47\%$ IL-17⁺/IFN- γ ⁻ cells, $9.78 \pm 2.24\%$ IL-17⁺/IFN- γ ⁺ cells and $27.81 \pm 2.99\%$ IL-17⁻/IFN- γ ⁺ cells in the Th17-polarized preparations (Fig. 1B). By the second method, which was adapted from the protocol published by Kebir et al.,²⁵ human CD4⁺ CD45RO⁺ T cells were incubated with rhIL-12 and anti-IL-4 Ab or rhIL-23, anti-IFN- γ Ab and anti-IL-4 Ab, along with CD14⁺ monocytes, to generate Th1- and Th17-polarized populations, respectively. Purity of CD4⁺ CD45RO⁺ T cells was $94.68\% \pm 0.28\%$ ($n = 5$ donors). Immunophenotyping demonstrated $23.31 \pm 2.67\%$ IFN- γ ⁺/IL-17⁻ cells, $2.34\% \pm 0.46\%$ IFN- γ ⁺/IL-17⁺ cells and $6.73\% \pm 1.18\%$ IFN- γ ⁻/IL-17⁺ cells in the Th1-polarized preparations (Fig. 1C), and $13.39\% \pm 2.12\%$ IL-17⁺/IFN- γ ⁻ cells, $5.03\% \pm 1.23\%$ IL-17⁺/IFN- γ ⁺ cells and $20.56 \pm 3.36\%$ IL-17⁻/IFN- γ ⁺ cells in the Th17-polarized preparations (Fig. 1D).

The Boyden chamber assay provides an *in vitro* system for studying leukocyte diapedesis across a biological barrier. To compare the ability of human Th1- and Th17-polarized cell populations to migrate across simulated retinal vascular endothelium, transwell membranes were coated with collagen I and seeded with human retinal endothelial cells. After 4 to 5 days, when the endothelial cells had formed an intact monolayer, as verified by limited diffusion of high molecular weight dextran, T cell preparations were migrated through the transwells for a period of 18 hours. There was no significant difference in the absolute number of cells that migrated across the retinal endothelial monolayer between Th1- and Th17-polarized populations generated from CD4⁺ CCR6^{-/+} cells (Fig. 2A). The same populations generated from CD4⁺ CD45RO⁺ cells also did not differ significantly in terms of number of migrated cells (Fig. 2B). These data imply that Th1- and Th17-polarized cells migrate equally efficiently across simulated human retinal vascular endothelium. Vital dye staining of migrated cells, performed using trypan blue in selected experiments, indicated that few cells were nonviable at the end of the migration interval (data not shown).

Migration Across Simulated Human Retinal Endothelium Alters the Phenotype of Th17-Polarized Cells

Although we observed no difference in absolute numbers of human Th1- versus Th17-polarized cells migrating across simulated retinal endothelium, we sought to determine whether the cell phenotype was impacted by the act of migration. Immunophenotyping of cells at the time of loading into the Boyden chamber ("premigrated") and of cells recovered from the lower chamber of the transwell ("migrated") revealed no significant difference in the percentage of cells expressing IFN- γ and/or IL-17 for the CD4⁺ CCR6⁻ Th1-polarized population (Fig. 3A). However, there was a significant reduction in the percentage of CD4⁺ CCR6⁺ Th17-polarized IL-17⁺/IFN- γ ⁻ cells between premigrated and migrated subsets (Fig. 3B). We observed the same result for migration experiments using Th1- and Th17-polarized cells generated from CD4⁺ CD45RO⁺ cells; the percentage of IL-17⁺/IFN- γ ⁻ cells was significantly decreased in migrated versus premigrated subsets for Th17-polarized (Fig. 3E), but not Th1-polarized (Fig. 3D) cells. To determine the cause of decreased IL-17⁺ expression by Th17-polarized IL-17⁺/IFN- γ ⁻ cells, in selected experiments we also immunophenotyped the cells that remained in the upper chamber of transwell at the end of the experiment ("nonmigrated"). CD4⁺ CCR6⁺ Th17-polarized cells showed significant reduction in the percentage of IL-17⁺/IFN- γ ⁻ between premigrated and nonmigrated subsets and between migrated and nonmigrated subsets (Fig. 3C). CD4⁺

FIGURE 3. Migration across simulated human retinal endothelium reduces the percentage of IL-17⁺/IFN- γ ⁻ cells in the Th17-polarized population. (A) CD4⁺ CCR6⁺ Th1-polarized cells ($n = 5$ donors), (B) CD4⁺ CCR6⁺ Th17-polarized cells ($n = 9$ donors), (D) CD4⁺ CD45RO⁺ Th1-polarized cells ($n = 11$ donors), and (E) CD4⁺ CD45RO⁺ Th17-polarized cells ($n = 12$ donors) were phenotyped according to intracellular expression of IFN- γ and/or IL-17 ahead of the migration assay ("pre-migrated," square) and following migration across a human retinal endothelial cell monolayer in a Boyden chamber ("migrated," circle). For (C) CD4⁺ CCR6⁺ Th17-polarized cells ($n = 4$ donors) and (F) CD4⁺ CD45RO⁺ Th17-polarized cells ($n = 7$ donors), cells that did not move through the Boyden chamber were phenotyped at the end of the migration assay ("nonmigrated," diamond). Data were analyzed by two-tailed paired Student's *t*-test. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$ and **** $P < 0.0001$.

CD45RO⁺ cells Th17-polarized cells showed significant reduction in the percentage of IL-17⁺/IFN- γ ⁻ cells between migrated and nonmigrated subsets (Fig. 3F). The reduction in IL-17⁺/IFN- γ ⁻ cells between pre-migrated and nonmigrated cells, which was observed for CD4⁺ CCR6⁺ Th17-polarized cells, is consistent with loss of IL-17 expression over time. However, reduction in expression of IL-17 in migrated cells in comparison with nonmigrated cells, which was observed for cells generated by both Th17-polarization protocols, suggests phenotypic modification is effected by the act of transendothelial migration.

Migration of Th1- and Th17-Polarized Cells Across Simulated Human Retinal Endothelium Is Reduced by Antibody-Mediated Blockade of ICAM-1

We investigated the involvement of three IgG superfamily adhesion molecules in the migration of Th1- and Th17-polarized cells across simulated human retinal endothelium by incorporating Ab blockade in the Boyden chamber assay. Antibody that targeted human ICAM-1, or VCAM-1, or ALCAM, or isotype-matched Ab directed against irrelevant antigen in the same concentration, was added to the upper chambers of transwells 1 hour prior to the addition of T cells. For CD4⁺ CCR6⁺ T cells polarized to a Th1 phenotype, a significant reduction in cell migration was observed in the presence of Ab-mediated blockade for ICAM-1 in 4 of 5 donors (Fig. 4A), for VCAM-1 in 0 of 5 donors (Fig. 4B), and for ALCAM in 1 of 5 donors (Fig. 4C). For CD4⁺ CCR6⁺ T cells polarized toward a Th17 phenotype, a significant reduction in T cell migration was observed in the presence of Ab-mediated blockade for ICAM-1 in 8 of 10 donors (Fig. 4D), for VCAM-1 in 1 of 9 donors

(Fig. 4E), and for ALCAM in 1 of 6 donors (Fig. 4F). Similar results were obtained when T cell subsets generated from CD4⁺ CD45RO⁺ cells were migrated. Th1-polarized CD4⁺ CD45RO⁺ cells from 3 of 7 donors showed significantly reduced migration when ICAM-1 was blocked (Fig. 5A), 0 of 7 donors when VCAM-1 was blocked (Fig. 5B), and 1 of 7 donors when ALCAM (Fig. 5C). Th17-polarized CD4⁺ CD45RO⁺ cells from 6 of 7 donors showed significantly reduced migration when ICAM-1 was blocked (Fig. 5D), 0 of 7 donors when VCAM-1 was blocked (Fig. 5E), and 2 of 7 donors when ALCAM was blocked (Fig. 5F). These results, generated with Th1 and Th17 cells polarized according to two protocols, are consistent with a role for ICAM-1 in the transendothelial migration of Th1- and Th17- polarized cells in a majority of persons. Although VCAM-1 and ALCAM may participate in this process, the data indicate that this is in a minority of individuals. For cells exposed to Th1 or Th17 polarization by either protocol, there was no significant difference in the percentage of migrated IFN- γ ⁺/IL-17⁻ cells, IFN- γ ⁻/IL-17⁺ cells and IFN- γ ⁻/IL-17⁺ cells between blocking Ab and isotype-matched control Ab for ICAM-1, VCAM-1 and ALCAM (Supplementary Figs. S1, S2). This result suggests that any blockade effects that occur in the Boyden chamber assay do not preferentially involve one of the defined cell populations.

DISCUSSION

In this study, we used the Boyden chamber assay to investigate the migration of human Th1 and Th17 cells across a simulated human retinal vascular endothelium. This model system is directly relevant to the initiation of autoimmune inflammation within the retina, which may be effected by either helper T cell

FIGURE 4. Blockade of ICAM-1 reduces human retinal endothelial transmigration of Th1- and Th17-polarized cells generated from $CD4^+$ $CCR6^-$ T cells and $CD4^+$ $CCR6^+$ T cells, respectively. (A–C) Th1-polarized cells and (D–F) Th17-polarized cells were migrated across a human retinal endothelial cell monolayer in a Boyden chamber for 18 hours (0.5×10^6 T cells/transwell) in the presence of blocking Ab directed against (A, D) ICAM-1; (B, E) VCAM-1; and (C, F) ALCAM (closed bars) or isotype-matched control IgG1 (open bars). Graphs show the total number of migrated T cells for each donor. Bars represent mean and error bars represent standard error of mean. $n = 3$ transwells/condition. Data were analyzed by one-tailed unpaired Student's *t*-test. * $P < 0.05$ and ** $P < 0.01$.

subset, depending on the specific circumstances of antigen presentation. To strengthen the conclusions of our work, all experiments were performed using Th1 and Th17 cells generated by two different protocols: (1) $CD4^+$ $CD45RO^+$ T cells exposed to rhIL-12 and anti-IL-4 Ab (Th1-polarized); or rhIL-23, anti-IFN- γ Ab and anti-IL-4 Ab (Th17-polarized) in the presence of $CD14^+$ monocytes; and (2) $CD4^+$ T cells lacking CCR6, exposed to rhIL-12 and α IL-4Ab (Th1-polarized); or $CD4^+$ T cells expressing CCR6, exposed to rhIL-1 β , rhIL-6, rhIL-23, anti-IFN- γ Ab, and anti-IL-4 Ab (Th17-polarized), in the presence of anti-CD3 and anti-CD28 Abs. Both Th17-polarizing protocols generated populations of IFN- γ^+ /IL-17 $^-$ cells, IFN- γ^+ /IL-17 $^+$ cells, and IFN- γ^- /IL-17 $^+$ cells; Th17-polarized IFN- γ^+ /IL-17 $^-$ cells are functionally distinct from Th1-polarized IFN- γ^+ /IL-17 $^-$ cells.²⁸ For Th1, the majority of phenotyped cells were IFN- γ^+ /IL-17 $^-$; and for $CD4^+$ $CCR6^-$ Th1-polarized cells in particular, IL-17-expressing cells were effectively eliminated. We observed no difference in the total number of Th1 and Th17 cells migrating across the simulated human retinal endothelium. On the other hand, the IFN- γ^- /IL-17 $^+$ subset was relatively depleted in Th17-polarized cell populations recovered from lower chambers of the transwells. By incorporating Ab blockade of IgG superfamily adhesion

molecules into the assay, we demonstrated the common requirement for ICAM-1, but not VCAM-1 and ALCAM, in the migration of both Th1- and Th17- polarized cells.

Th1 cells and Th17 cells may act individually or in concert to initiate autoimmune inflammation,^{1,2,5} and transendothelial emigration from the bloodstream is the first event at the target organ.²⁹ On this basis, one might expect no difference in the ability of human Th1-polarized and Th17-polarized cells to navigate simulated human vascular endothelium. This is what we observed in migrating cells polarized by two alternative protocols across human retinal endothelium. However, using a similar Boyden chamber assay, Kebir et al.²⁵ demonstrated that human $CD4^+$ $CD45RO^+$ Th17-polarized cells moved across a human brain endothelial monolayer in greater numbers than $CD4^+$ Th1-polarized cells. While variations in the polarization procedures may result in functional differences,³⁰ in the setting of leukocyte transendothelial migration, the most likely explanation for such disparate observations relates to the vascular endothelium. Multiple groups, most notably Chi et al.³¹ who studied 53 human endothelial cultures, have used molecular profiling technology to demonstrate molecular phenotypic differences between endothelial cells located in different vascular beds. Retinal and brain endothelial cells

FIGURE 5. Blockade of ICAM-1 reduces human retinal endothelial transmigration of Th1- and Th17-polarized cells generated from $CD4^+ CD45RO^+$ T cells. (A–C) Th1-polarized cells and (D–F) Th17-polarized cells were migrated across a human retinal endothelial cell monolayer in a Boyden chamber for 18 hours (0.5×10^6 T cells/transwell) in the presence of blocking Ab directed against (A, D) ICAM-1; (B, E) VCAM-1 and (C, F) ALCAM (closed bars) or isotype-matched control IgG1 (open bars). Graphs show the total number of migrated T cells for each donor. Bars represent mean and error bars represent standard error of mean. $n = 3$ transwells/condition. Data were analyzed by one-tailed unpaired Student's *t*-test. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.0001$.

share anatomical features and physiological properties that create similar biological barriers,^{32,33} but work directly comparing the two populations has revealed multiple molecular and functional differences, including differences in expression of molecules involved in leukocyte extravasation.^{34–37} Our findings emphasize the importance of understanding the pathobiology of the local vascular endothelium when studying any autoimmune disease, as results obtained with one endothelial cell subset may not be relevant to another.

Phenotypic comparison of migrated Th17-polarized cells with premigrated cells revealed a reduction in the percentage of $IL-17^+/IFN-\gamma^-$ cells. This outcome was not observed for the $CD4^+ CD45RO^+$ cells Th1-polarized cells, which contained a small percentage of this cell subset. While the capacity of human or mouse Th17 cells for absolute lineage conversion has come under question recently,³⁸ multiple publications have described a reduction of IL-17 production by Th17 cells, in contrast to Th1 cells, under various in vivo and in vitro conditions; this change, which may be accompanied by an increase in IFN- γ production, has been most convincingly

demonstrated using fluorescent reporter mice to track T cells that have activated IL-17.³⁹ Our results suggest that, in the context of transendothelial migration, there is potential for effect on IL-17 production by human Th17 cells. For both polarization protocols, there was reduction in the percentage of $IFN-\gamma^-/IL-17^+$ T cells in the course of transit from upper to lower chambers of the transwell. For $CD4^+ CCR6^+$ Th17-polarized cells, additional reduction of $IFN-\gamma^-/IL-17^+$ T cells also occurred within the upper well over the course of the assay. Influence of the retinal vascular endothelium is a logical explanation for this observation, based on reports that, depending on local cytokine milieu and endothelial cell subpopulation, coculture with endothelial cells may either induce or suppress IL-17 production by Th17 cells.^{40–42} The absence of Th17-polarizing conditions during the assay may also have contributed to loss of $IFN-\gamma^-/IL-17^+$ $CD4^+ CCR6^+$ T cells in the upper chamber, as previously described.⁴³

Electron microscopic analyses of mouse eyes after induction of experimental autoimmune uveoretinitis show lymphocytes move transcellularly across the retinal vascular endothelium.^{44,45} As originally described, the endothelial

transmigratory cup that coordinates trans- and paracellular movement is enriched in ICAM-1 and VCAM-1.⁴⁶ However, migration assays performed in Boyden chambers seeded with human brain endothelial cells identify ICAM-1 and ALCAM as the critical IgG superfamily players in diapedesis of helper T cells across the blood-brain barrier.^{47–49} For a majority of donors, we found ICAM-1, but not VCAM-1 or ALCAM, mediated migration of both Th1- and Th17-polarized cells across simulated human retinal endothelium. Studies in mouse experimental autoimmune uveoretinitis also support an important role for ICAM-1 in T cell extravasation; upregulation of ICAM-1 immediately precedes this event in retinal venules,⁵⁰ and severity of inflammation is reduced by blockade of ICAM-1 or its ligand, leukocyte function-associated antigen 1 (LFA-1).^{51–53} While it is most likely that ICAM-1 blockade was effective at the level of the endothelium, blocking may also have been effected on T cells, which express ICAM-1.⁵⁴ In contrast, VCAM-1 is induced on endothelium remote from extravasation and after the onset of inflammation.⁵⁰ ALCAM has not been previously studied with regard to autoimmune uveitis, although it is known to be expressed by human retinal endothelium.⁵⁵ By analyzing Ab blockade experiments by donor, some interindividual differences are apparent, ICAM-1 does not mediate migration in all individuals and conversely, VCAM-1 or ALCAM mediates migration in exceptional individuals.

In conclusion, our work shows that human Th1- and Th17-polarized cells migrate equally efficiently across human retinal vascular endothelium, and that ICAM-1 is involved in coordinating the movement of both cell subsets. Our results suggest the possibility of using ICAM-1 blockade therapeutically to treat different forms of autoimmune uveitis, mediated by either Th1 cells or Th17 cells. However, since ICAM-1 usage was not universal across cell isolates generated from all human donors, variation in therapeutic effectiveness could be expected. Our observations reveal some differences in helper T cell migratory behaviors from those published in relation to the vascular endothelium in other tissues, and highlight the importance of an individualized approach to autoimmune diseases involving different tissues.

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