Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Sialomucin CD43 regulates T helper type 17 cell intercellular adhesion molecule 1 dependent adhesion, apical migration and transendothelial migration

Francisco E. Velázquez,[1] Marina Anastasiou,[1,2] Francisco J. Carrillo-Salinas,[1] Njabulo Ngwenyama,[1] Ane M. Salvador,[1] Tania Nevers[1] and Pilar Alcaide[1]

[1]*Department of Immunology, Tufts University School of Medicine, Boston, MA, USA and* [2]*Laboratory of Autoimmunity and Inflammation, University of Crete Medical School, Crete, Greece*

doi:10.1111/imm.13047
Received 10 September 2018; revised 7 December 2018; accepted 15 January 2019.
Correspondence: Dr Pilar Alcaide, Tufts University School of Medicine, 136 Harrison Avenue, M&V 701, Boston, MA 0211, USA.
Email: pilar.alcaide@tufts.edu
Senior author: Pilar Alcaide

Summary

T helper type 17 lymphocytes (Th17 cells) infiltrate the central nervous system (CNS), induce inflammation and demyelination and play a pivotal role in the pathogenesis of multiple sclerosis. Sialomucin CD43 is highly expressed in Th17 cells and mediates adhesion to endothelial selectin (E-selectin), an initiating step in Th17 cell recruitment to sites of inflammation. CD43$^{-/-}$ mice have impaired Th17 cell recruitment to the CNS and are protected from experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis. However, E-selectin is dispensable for the development of EAE, in contrast to intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1). We report that CD43$^{-/-}$ mice have decreased demyelination and T-cell infiltration, but similar up-regulation of ICAM-1 and VCAM-1 in the spinal cord, compared with wild-type (WT) mice, at the initiation of EAE. CD43$^{-/-}$ Th17 cells have impaired adhesion to ICAM-1 under flow conditions *in vitro*, despite having similar expression of LFA-1, the main T-cell ligand for ICAM-1, as WT Th17 cells. Regardless of the route of integrin activation, CD43$^{-/-}$ Th17 cell firm arrest on ICAM-1 was comparable to that of WT Th17 cells, but CD43$^{-/-}$ Th17 cells failed to optimally apically migrate on immobilized ICAM-1-coated coverslips and endothelial cells, and to transmigrate under shear flow conditions in an ICAM-1-dependent manner. Collectively, these findings unveil novel roles for CD43, facilitating adhesion of Th17 cells to ICAM-1 and modulating apical and transendothelial migration, as mechanisms potentially responsible for Th17 cell recruitment to sites of inflammation such as the CNS.

Keywords: autoimmunity; CD43; intercellular adhesion molecule 1; leukocyte recruitment; T helper type 17 cells.

Introduction

T helper type 17 lymphocytes (Th17 cells) are dominant pro-inflammatory cells in initiating and sustaining autoimmune reactions in a variety of tissues and organs. These include devastating diseases such as multiple sclerosis, a demyelinating disease of the central nervous system (CNS), which is mediated in part by autoreactive Th17 cells that are recruited to the CNS.[1–3] The selective recruitment of T-cell subsets is a highly regulated process that requires sequential interactions of T-cell-expressed selectin ligands with endothelial selectins, followed by chemoattractant-induced T-cell integrin activation that leads to firm adhesion and T-cell apical migration (also

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function associated antigen 1; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PMA, phorbol myristate acetate; PSGL-1, P-selectin glycoprotein ligand 1; TEM, transendothelial migration; Th17 cells, T helper type 17 lymphocytes; VCAM-1, vascular cell adhesion molecule 1

known as locomotion) to sites of transendothelial migration (TEM). β_1 and β_2 integrins are components of VLA-4 ($\alpha_4\beta_1$) and LFA-1 ($\alpha_L\beta_2$), the main ligands of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), respectively, and are responsible for mediating T-cell arrest, apical and transendothelial migration.[4] Using the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis, numerous studies have demonstrated that the initial adhesion of T cells to inflamed vessels is predominantly mediated by VLA-4–VCAM-1 interactions, whereas LFA-1–ICAM-1 becomes engaged in the latter steps of apical and transendothelial migration.[5] Selectin–selectin ligand interactions, on the other hand, are dispensable for the development of EAE.[6–8] Numerous findings support the view that Th17 cells play an essential role in triggering the initial phases of autoimmune CNS inflammation, whereas Th1 cells likely contribute to pathogenesis later in disease progression.[1,9–11] Although different T-cell subsets use a similar repertoire of ligands to traffic to sites of inflammation, their expression patterns can differ, resulting in selective recruitment.[4,12] Hence, it is critical to identify the adhesion molecules that mediate such selective recruitment.

Sialomucin CD43 is a transmembrane protein highly expressed in Th17 cells, which functions as an E-selectin ligand *in vitro* and *in vivo* and selectively regulates Th17 cell recruitment to sites of inflammation including the CNS during EAE, as we previously reported.[13,14] CD43 is also expressed to a lower extent in other leukocytes that include Th1 cells and skin-resident T cells, in which it mediates E-selectin adhesion but only in cooperation with other selectin ligands such as P-selectin glycoprotein ligand 1 (PSGL-1).[13,15,16] In addition, CD43 has been reported to participate in a variety of cellular processes that include cell differentiation, proliferation, adhesion, anti-adhesion and T-cell co-stimulation,[17,18] but its absence does not influence other processes such as Th17 or Th1 cell differentiation *in vitro*, or in response to myelin oligodendrocyte glycoprotein (MOG) immunization *in vivo*.[14] These multiple functions of CD43 can be attributed to the variety of ligands reported to bind CD43 in different cells. Among these, ICAM-1 stands out as one that was shown to directly bind CD43 in an *in vitro* model system that involved CD43 expressing human immortalized T cells and immobilized ICAM-1 under static conditions.[19] However, whether this interaction is functional and/or involved in Th17 cell interactions with vascular endothelial cells under physiological flow conditions as a mechanism that mediates Th17 cell recruitment and inflammation *in vivo* has not been studied to date. Genetic deficiency of CD43 results in protection from EAE resulting from decreased Th17 cell infiltration into the CNS.[14,20] Given that E-selectin–selectin ligand interactions are dispensable for T-cell recruitment to the CNS in EAE,[6] we reasoned that CD43 regulates Th17 cell adhesion to endothelial ICAM-1 and modulates Th17 cell infiltration to sites of inflammation that do not require selectin interactions such as the CNS in EAE.

Here, we report the novel finding that CD43 facilitates adhesion of Th17 cells to ICAM-1 under flow conditions *in vitro* independently of LFA-1 expression. Our results also demonstrate that CD43 does not interfere with LFA-1-mediated firm arrest of Th17 cells to ICAM-1, but modulates its ability to mediate chemokine-induced apical and transendothelial migration. These results position CD43 as an adhesion molecule that modulates Th17 cell recruitment in an inflammatory context that is independent of selectin interactions, such as in EAE, through modulating adhesive interactions with endothelial ICAM-1.

Materials and methods

Reagents

Recombinant mouse IL-23, E-selectin, and P-selectin Fc-chimeras were from R&D Systems (Minneapolis, MN). Recombinant mouse IL-12, IL-2, IL-6, tumor necrosis factor-α, recombinant human transforming growth factor-β, and the following antibodies to mouse cytokines and adhesion molecules: IL-4 (clone 11B11), interferon-γ (clone XMG 1.2), IL-2 (clone JES6-1A12), CD4 (clone GK 1.5), CD3 (clone 145-2C11), CD28 (clone 37.51), IL-17A (clone 2C11-18H10.1), CD43 activation-associated glycoform (clone 1B11), CD44 (clone IM7), anti-LFA-1 (clone M17/4) and the corresponding isotype controls are all from BioLegend (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA) and complete freund's adjuvant (CFA) were obtained from Sigma (St. Louis, MO), and carrier-free CCL20 and stromal cell-derived factor-1α were from Peprotech (Rocky Hill, NJ). Ionomycin was from Sigma and Vibrant CFSE, Alexa 680 and Phalloidin Alexa Fluor 546 were from Life Technologies (Carlsbad, CA). MOG was purchased from Anaspec (Fremont, CA) and pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Avidin and biotin were purchased from Vector Laboratories (Burlingame, CA), and fibronectin was purchased from Gibco (Carlsbad, CA).

Mice

All mice were bred in the pathogen-free facility at Tufts University School of Medicine, in accordance with the guidelines of the institutional animal care and use committee at Tufts University School of Medicine and the NIH Animal research guidelines. C57BL/6 (wild-type; WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME) or used as littermates from CD43 heterozygous crosses. CD43$^{-/-}$ were generated in our laboratory

from intercrosses of PSGL-$1^{-/-}$ $CD43^{-/-}$ (provided by Dr. McEver, Oklahoma Medical Research Foundation, OK) with C57BL/6 (WT) mice as described previously.[14] ICAM-$1^{-/-}$ mice, lacking all ICAM-1 isoforms, also named ICAM-1^{null} as described elsewhere,[21,22] were obtained from Daniel Bullard (University of Alabama Birmingham, AL). Mice were killed at 7–12 weeks of age for harvest of naive $CD4^+$ T cells and at 1–2 weeks of age for the generation of primary mouse heart endothelial cells. The genotypes were determined by polymerase chain reaction (PCR), and null mutations were also confirmed by FACS analysis of spleen cells.[14]

EAE induction and immunohistochemistry

Eight- to twelve-week-old and aged-matched WT and $CD43^{-/-}$ female mice were immunized using 100 μg of MOG emulsified 1 : 1 in CFA. Mice were also given 200 ng of pertussis toxin to permeabilize the blood–brain barrier. Mice were clinically scored in a blinded fashion using the following criteria: 0, no disease; 1, flaccid tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness. The spinal cords were harvested at days 14 or 16 post-injection, and used for immunohistochemistry staining or RNA isolation. Spinal cord sections (5-μm thick) were dehydrated in increasing concentrations of ethanol (70–90%), and incubated in luxol fast blue solution (Sigma) overnight at 56°, rinsed with 95% ethanol, incubated with lithium carbonate solution for 30 seconds, and with xylene. Where indicated, spinal cord sections were frozen in OCT (Sakura Finetek, Torrance, CA), sectioned, fixed in acetone and stained with anti-ICAM-1 (YN1.1) or anti-CD4 (GK1.5) antibodies (BioLegend) using the ABC solution (Vectastain Elite ABC-HRP kit; Vector Laboratories) as previously described for other tissues.[23]

Preparation of effector T cells

$CD4^+$ cells were isolated from spleen and lymph node cell suspensions of WT or genetically deficient mice using positive selection by immunomagnetic MACS beads (Invitrogen, Carlsbad, CA). Th1 cells were derived from naive T cells by anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) stimulation in the presence of IL-12 (10 ng/ml), anti-IL-4 (500 ng/ml), and IL-2 (25U/ml), as previously described.[24,25] To achieve Th17 differentiation, naive T cells were stimulated in the presence of transforming growth factor-β (3 ng/ml), IL-6 (30 ng/ml), IL-23 (20 ng/ml), plus anti-interferon-γ (10 μg/ml), anti-IL-4 (10 μg ml), and anti-IL-2 (10 μg/ml) monoclonal antibody. On day 3, Th1 and Th17 cultures were diluted 1 : 1 with fresh medium containing IL-2 (25 U/ml) and IL-23 (20 ng/ml), respectively. Cells were harvested on day 4 and immediately used in experiments. In the studies involving adhesion to ICAM-1, Th17 cells and Th1 cells were treated for 5 min with 50 ng/ml of PMA to induce integrin activation, before being perfused in the flow chamber apparatus. Note that studies of T-cell subsets in many laboratories use populations that do not uniformly produce the detectable defining cytokines by flow cytometric assays, and usually the signature cytokine-producing cells are < 40% of the population,[24–28] reflecting the nature of the assays not inducing synchronized cytokine expression in all cells rather than the indicated per cent-positive undergoing appropriate differentiation. No significant differences were observed in Th17 cell differentiation between WT and $CD43^{-/-}$ mice, as we and others have previously described.[14,28]

Evaluation of T-cell interactions with adhesion molecules and apical and transendothelial migration under defined flow conditions in vitro

T-cell interactions with immobilized E-selectin and ICAM-1 (20 μg/ml) under defined laminar flow conditions were recorded using real-time video microscopy (20× objective) and using a parallel plate apparatus and the Nikon Elements NIS software. For adhesion studies, T cells were perfused at a concentration of $0{\cdot}5 \times 10^6$ cells/ml on immobilized adhesion molecules, and T-cell accumulation was measured in eight different fields after the initial minute of each flow rate (shear stress 1 dyne/cm^2), as previously described.[14] Where indicated, Th17 cells were incubated with function blocking antibodies to LFA-1 (M17/4), or their respective IgG isotype controls at 20 μg/ml during 20 min before the Th17 cells (unstimulated, stimulated 5 min with 50 ng of PMA, or perfused across ICAM-1-coated coverslips pre-incubated with 1 μg/ml CCL20 for 15 min) were perfused in the flow chamber. For detachment assays, a bolus of 3×10^6 Th17 cells (PMA-treated or unstimulated) was perfused across ICAM-1-coated coverslips at 1 dyne/cm^2, paused for 30 seconds, then flow was re-established and fluid shear stress was increased stepwise every 15 seconds. Th17 cells remaining adherent after each step in shear flow were determined. For apical migration assays, a bolus of 1×10^6 Th17 cells was perfused at 1 dyne/cm^2 over ICAM-1-coated coverslips plus 1 μg/ml of CCL20, paused for 30 seconds, then imaged during 20 min, taking frames every 15 seconds, at 1 dyne/cm^2 of shear stress to track Th17 cell motility on ICAM-1. Quantification of distance and velocity was performed using ImageJ software, tracking each individual cell per frame for the duration of the video. The distance covered was calculated as the sum of distances each cell covered during any relocation of the cell during the 20-min duration of the videos. In experiments involving primary endothelial cells, mouse heart endothelial cells were generated from WT and ICAM-$1^{-/-}$ 1- to 2-week-old mice and grown to

confluence as we have previously described as a useful model to evaluate T-cell TEM under flow conditions *in vitro*.[26,29] Monolayers were treated with tumor necrosis factor-α (100 ng/ml) for 4 hr and with CCL20 (200 ng/ml) or stromal cell-derived factor-1α (250 ng/ml) for 15 min, before being inserted in the flow chamber and 2×10^6 Th17 or Th1 cells were perfused across. Per cent TEM is represented as (TEM cells/total accumulated + TEM cells) × 100. Apical migration on endothelial cells was quantified by tracking individual cells on ImageJ for 10–20 frames post adhesion. All adherent cells present in all frames for the duration of the video, cells that although not present at time 0, apically migrate into the field of view, and cells that are adhered during several time-points but occasionally apically migrate outside the field of view are all incorporated in the data analysis for both WT and $CD43^{-/-}$ Th17 cells.

Transwell assays

Th17 cells were stained with CFSE (Life Technologies) and added to ICAM-1 (20 μg/ml) coated or non-coated 3-μm pore diameter transwells (Costar, Sigma), in the presence or absence of CCL20 (200 ng/ml) in the bottom chamber, and allowed to transmigrate for 3 hr. Fluorescence in the bottom compartment was determined in relative fluorescence units (RFU) quantified in a fluorescence reader, Spectramax M5 (Molecular Devices, San Jose, CA).

Flow cytometry

Flow cytometry was performed to evaluate the expression of Th17 cell surface LFA-1 as described previously.[26] The data were acquired on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Treestar, Ashland, OR).

Real-time quantitative PCR

Total RNA was extracted from mouse spinal cords harvested from MOG-immunized WT and $CD43^{-/-}$ mice at the indicated time-points or from control phosphate-buffered saline-injected mice. Spinal cords were processed directly using Trizol (Invitrogen) and RNA was extracted using an RNeasy lipid tissue minikit (Qiagen, Germantown, MD). RNA was then reverse transcribed using the ThermoScript RT-PCR system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and amplified by real-time PCR with SYBR green PCR mix (Applied Biosystems, Foster City, CA). Samples were quantified in triplicate using 40 cycles performed at 94° for 30 seconds, 60° for 45 seconds, 72° for 45 seconds using an ABI Prism® 7900 Sequence Detection System. The following primers were used: *Esel* F 5′-TGA CCA CTG CAG GAT GCA T-3′; R 5′-ATC CAA CGA ACC AAA GAC TCG-3′; *Icam1* F 5′- GCT GTG CTT TGA GAA CTG TG-3′; R 5′-GTG AGG TCC TTG CCT ACT TG-3′; *Vcam1* F 5′-ACT CCT TAG TCC TCG GCC A-3′; R 5′-TGG TTT CTT GTG ACC CTG AGC-3′; *bactin* F 5′-TCC TTC GTT GCC GGT CCA-3′; R 5′-ACC AGC GCA GCG ATA TCG TC-3′.

Immunofluorescence

Wild-type and $CD43^{-/-}$ Th17 cells were added to ICAM-1- and CCL20-coated coverslips for 30 min at 37° under static conditions, or perfused under flow conditions at 1 dyne/cm^2 for 15 min across ICAM-1- and CCL20-coated or fibronectin (5 μg/ml) -coated coverslips. Coverslips with adhered cells were fixed with formaldehyde and acetone and stained with fluorescent Alexa fluor 546 phalloidin antibody and DAPI, and imaged using Nikon Elements NIS software at 40×. Fluorescence intensity was analyzed using ImageJ software in four or five cells per field of three different image replicates. Three representative fields of view were imaged per each cell type and condition.

Statistical analysis

Data are expressed as the mean ± SD. Statistical analyses were performed using Student's *t*-test or non-parametric Mann–Whitney *U*-test, or by analysis of variance and Newmal–Keuls post test when comparing multiple groups, using GraphPad Prism software (GraphPad Software, San Diego, CA). When the unpaired *t*-test was used, the outcome was confirmed to be normally distributed. Differences were considered statistically significant at $P \leq 0{\cdot}05$.

Results

ICAM-1 is similarly expressed in the spinal cord of WT and $CD43^{-/-}$ mice in response to MOG immunization at the initiation of EAE

We and others have previously reported that $CD43^{-/-}$ mice are protected from EAE and have decreased $CD4^+$ T cells, specifically Th17 cells, infiltrating the spinal cord.[14,20] As expected, $CD43^{-/-}$ mice had decreased $CD4^+$ T-cell infiltration in the spinal cords compared with WT mice 16 days after MOG immunization, which is the peak of T-cell infiltration that initiates demyelination and spinal cord damage in EAE (Fig. 1a,b). This correlated with decreased areas of demyelination in the white matter, as shown in the red-highlighted portions of the spinal cord stained with luxol fast blue (Fig. 1c), as well as with decreased clinical score compared with WT mice at the same time-point after MOG immunization

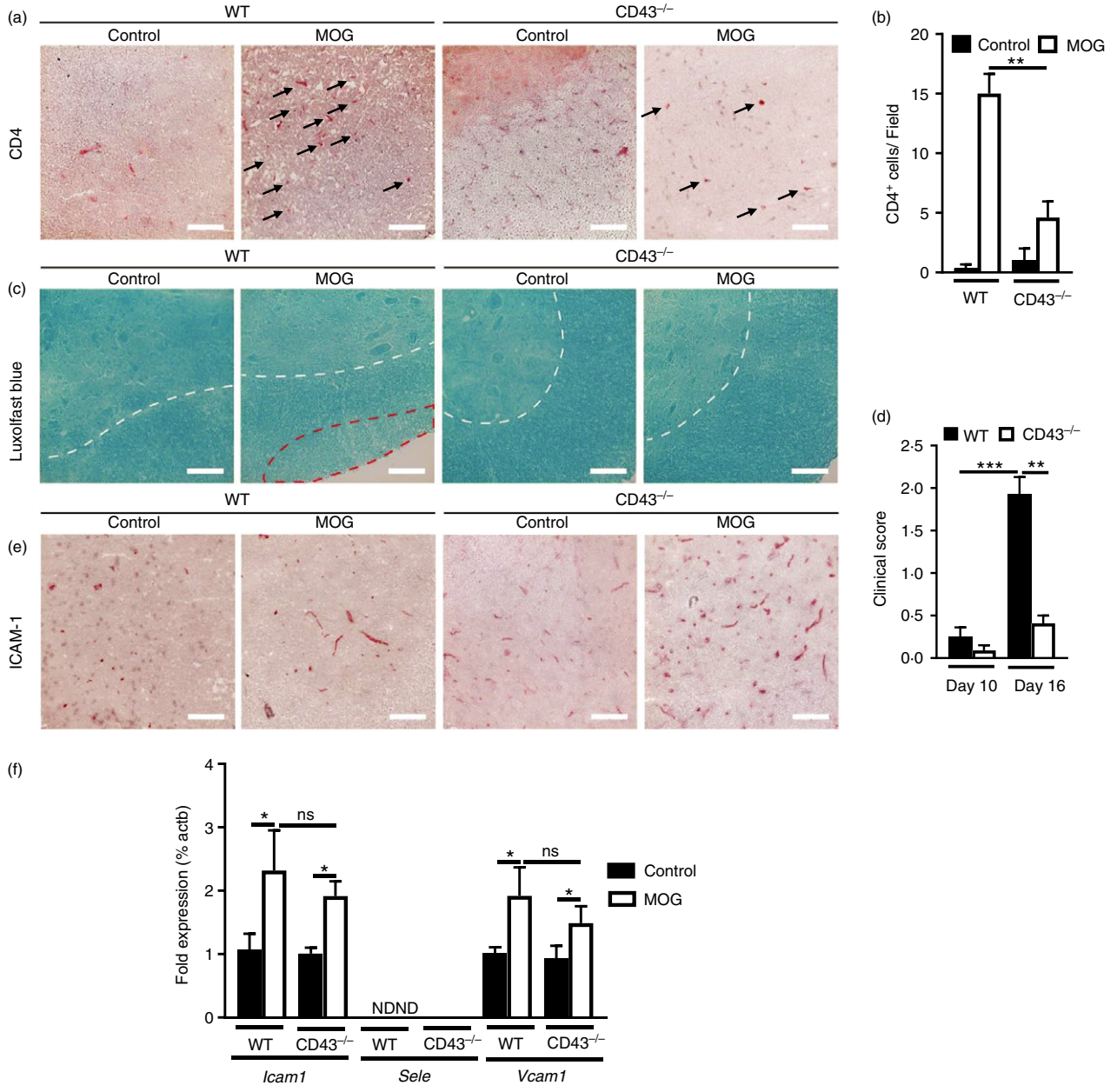

Figure 1. Myelin oligodendrocyte glycoprotein (MOG) immunization does not induce spinal cord T-cell infiltration, demyelination and experimental autoimmune encephalomyelitis (EAE) in CD43$^{-/-}$ mice, and results in comparable intercellular adhesion molecule 1 (ICAM-1) expression in the spinal cord in wild-type (WT) and CD43$^{-/-}$ mice. (a–c) Representative photomicrographs and quantification of WT and CD43$^{-/-}$ spinal cord sections from mice immunized with MOG or phosphate-buffered saline (PBS) (control) (16 days), stained for CD4^{+} T-cell infiltration (a, b), and luxol fast blue for changes in demyelination (red dotted line indicates area of demyelination in the white matter) (c). (d) Clinical score at the indicated time-points post immunization: $n = 8$ WT MOG and $n = 11$ CD43$^{-/-}$ MOG, $n = 3$ PBS controls. (e, f) ICAM-1 protein (e) and gene expression quantification by quantitative RT-PCR (f) 14 days post immunization: $n = 3$ PBS controls, $n = 4$ WT and $n = 6$ CD43$^{-/-}$. Scale bar = 50 μm. Representative images and quantification of $n = 4$ WT MOG, $n = 6$ CD43$^{-/-}$ MOG, and $n = 3$ PBS controls, three sections/mouse. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

(Fig. 1d). We next determined the protein and mRNA levels of ICAM-1, known to regulate cell recruitment to the spinal cord,[30] in unimmunized and MOG-immunized WT and CD43$^{-/-}$ mice at the peak of T-cell infiltration. ICAM-1 protein was constitutively expressed in the endothelium of both WT and CD43$^{-/-}$ mice and similarly expressed in the spinal cord vessels of WT and CD43$^{-/-}$ mice upon MOG immunization (Fig. 1e). Quantitative PCR studies reflected a two-fold induction in *Icam-1* and *Vcam-1* gene expression in both WT and

$CD43^{-/-}$ immunized mice compared with their phosphate-buffered saline-treated controls (Fig. 1f). In contrast, E-selectin was undetectable in all groups, in agreement with previous reports.[6,8] Taken together, our results indicate that, in contrast to WT mice, $CD43^{-/-}$ mice have decreased T-cell infiltration in the spinal cord, intact myelin and do not present symptoms of EAE, but have similar induction of ICAM-1 expression in the spinal cord in response to MOG immunization.

$CD43^{-/-}$ Th17 cells have reduced adhesion to ICAM-1 compared with WT Th17 cells

We had previously demonstrated that Th17 cells require CD43 to roll on E-selectin *in vitro* and *in vivo*, and that recruitment of Th17 cells to the spinal cord was significantly impaired in $CD43^{-/-}$ mice.[14] However, E-selectin is dispensable for T-cell-mediated inflammation in EAE,[6] and our data (Fig. 1) indicate that E-selectin mRNA is undetectable in the spinal cord at the peak of T-cell infiltration in EAE. In contrast, ICAM-1, which has previously been reported to associate with immobilized CD43 in *in vitro* assays using immortalized cells,[19] is expressed in the spinal cord after MOG immunization in WT and $CD43^{-/-}$ mice[4,21] (Fig. 1). To determine whether CD43 regulates Th17 adhesion to ICAM-1 as a mechanism potentially mediating Th17 cell recruitment in EAE, we next evaluated the adhesion of WT and $CD43^{-/-}$ Th17 cells to ICAM-1 under defined shear flow conditions *in vitro*. Accumulation of T cells on ICAM-1 under flow conditions requires activation of the integrin LFA-1, and this can be induced by a variety of agents including physiological stimulation by chemokines or with PMA, an approach which bypasses chemokine receptor signaling.[31,32] Th17 cells were treated with PMA or perfused across ICAM-1 coverslips coated with chemokine CCL20, a chemokine previously reported to induce mouse Th17 cell arrest to ICAM-1 and to human and mouse Th17 cell arrest on endothelial cells.[26,33] These conditions promote rapid integrin activation and a shift from the low-affinity to the high-affinity conformation that allows adhesion to ICAM-1.[34–36] Both PMA and CCL20 induced adhesion of WT and $CD43^{-/-}$ Th17 cells to ICAM-1, compared with unstimulated Th17 cells, which adhered to ICAM-1, but to a lesser extent. However, under these conditions, $CD43^{-/-}$ Th17 cell adhesion to ICAM-1 was significantly impaired compared with WT Th17 cells (Fig. 2a,b). In contrast, $CD43^{-/-}$ Th1 cells, which we previously reported as infiltrating the spinal cord in a similar way to WT Th1 cells,[14] adhered to ICAM-1 in similar numbers to WT Th1 cells, indicating that CD43 modulates specifically Th17 cell adhesion to ICAM-1 (Fig. 2c,d). To better understand the role of CD43 in Th17 cell adhesion to ICAM-1, we next determined whether the absence of CD43 altered LFA-1 expression, the main ligand of ICAM-1 in T cells, as a mechanism of the observed decreased adhesion. We found that WT and $CD43^{-/-}$ Th17 cells, unstimulated or stimulated with PMA or with CCL20 for 5 min and 15 min, respectively (the time required to induce integrin activation), had similar surface expression to LFA-1 (Fig. 2e,f). Our results demonstrate that the lack of CD43 significantly impairs Th17 cell adhesion to ICAM-1 under flow conditions, and that this effect is Th17-cell-specific and is independent of CD43 modulating LFA-1 expression.

Function blocking of LFA-1 abolishes adhesion to ICAM-1 of $CD43^{-/-}$ Th17 cells but not WT Th17 cells

To functionally determine if CD43 was regulating adhesion of Th17 cells to ICAM-1 independently of LFA-1 expression, we performed similar adhesion studies under shear flow conditions using Th17 cells treated with anti-LFA-1 function blocking antibody or an isotype IgG control. PMA was used to trigger integrin activation in these function blocking studies. Anti-LFA-1 treatment of WT Th17 cells resulted in significantly decreased adhesion to ICAM-1 compared with IgG-treated Th17 cells, confirming that LFA-1 is the major ligand for ICAM-1. However, WT Th17 cell adhesion to ICAM-1 was not completely abolished, in contrast to $CD43^{-/-}$ Th17 cell adhesion to ICAM-1, which was decreased to the level of non-PMA-treated (unactivated) Th17 cells and significantly decreased compared with WT anti-LFA-1-treated cells (Fig. 3a). This effect was also Th17 cell subset-specific, as it was not observed in Th1 cells (Fig. 3b). To ensure that blocking LFA-1 on Th17 cells was not altering the functionality of CD43, we performed similar studies on E-selectin. As expected, $CD43^{-/-}$ Th17 cells adhered to E-selectin in lower numbers than WT Th17 cells (Fig 3c), in contrast with Th1 cells, in which lack of CD43 did not impair adhesion to E-selectin (Fig. 3d), as we have previously reported.[14] Moreover, no differences in adhesion to E-selectin were observed between IgG and anti-LFA-1-treated $CD43^{-/-}$ Th17 cells (Fig. 3c) or $CD43^{-/-}$ Th1 cells (Fig. 3d), confirming the specificity of anti-LFA-1 in functionally blocking T-cell subset adhesion to ICAM-1. Taken together, these data demonstrate that LFA-1 is the major functional ligand for ICAM-1 expressed on Th17 cells, and that anti-LFA-1 function blocking antibody inhibits the adhesion of WT Th17 cells to ICAM-1 more efficiently than the adhesion of $CD43^{-/-}$ Th17 cells to ICAM-1.

$CD43^{-/-}$ and WT Th17 cells show comparable firm arrest on ICAM-1 and shear-stress-induced detachment from ICAM-1

Our findings, thus far, indicated that the absence of CD43 resulted in decreased adhesion of Th17 cells to

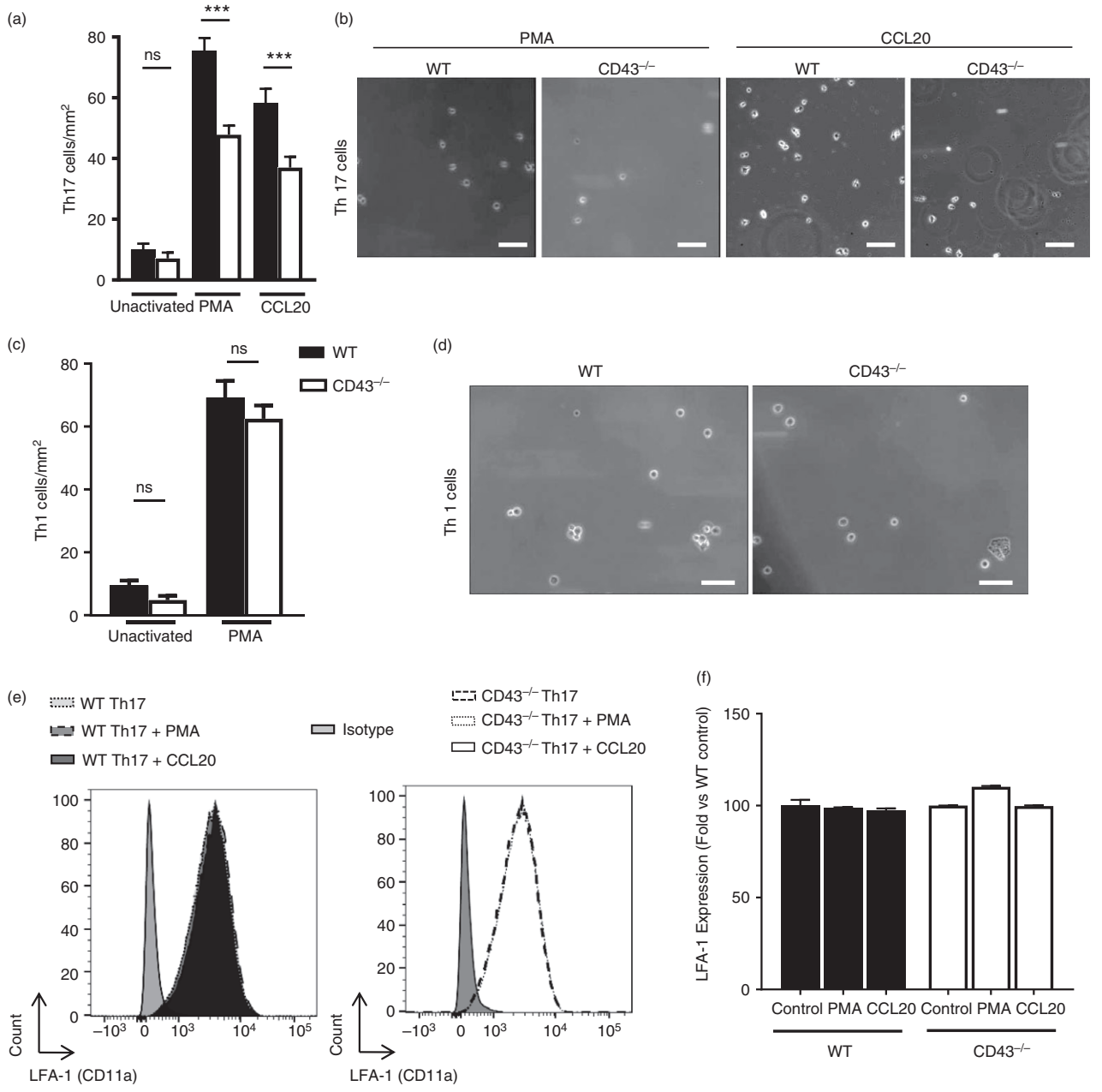

Figure 2. CD43$^{-/-}$ T helper type 17 (Th17) cells have impaired adhesion to intercellular adhesion molecule 1 (ICAM-1) compared with wild-type (WT) Th17 cells. (a–d) WT and CD43$^{-/-}$ Th17 cells (a, b) and Th1 cells (c, d) were treated for 5 min with phorbol 12-myristate 13-acetate (PMA) or left untreated, and perfused at a concentration of 0·5 × 10^6 cells/ml over immobilized ICAM-1 (20 μg/ml) or ICAM-1 and CCL20 (1 μg/ml) -coated coverslips, at a shear stress of 1 dyne/cm^2. Videos were recorded for 1 min and adhesion was quantified in six different fields of view. Representative images of PMA and CCL20 WT and CD43$^{-/-}$ Th17 cells conditions (b) and PMA-treated Th1 cells (d) adhesion to ICAM-1. Scale bar is 50 μm; $n \geq 3$ independent experiments with duplicate conditions per experiment. (e, f) Flow cytometry staining (e) and quantification (f) of surface LFA-1 expression in WT and CD43$^{-/-}$ Th17 cells that were unstimulated, treated for 5 min with PMA or treated for 15 min with CCL20. LFA-1 expression is represented as MFI WT or MFI CD43$^{-/-}$ relative to the MFI isotype control, and normalized to WT control cells. Data represent $n = 3$ independent experiments for PMA- and CCL20-treated cells, and $n = 6$ independent experiments for control unstimulated cells, and show the mean ± SD values. ***$P < 0{\cdot}001$.

ICAM-1 under shear flow conditions independently of LFA-1 expression. However, the main ligand mediating ICAM-1 adhesion was confirmed to be LFA-1, as many CD43$^{-/-}$ Th17 cells still adhered to ICAM-1. We reasoned that such a decrease in adhesion of CD43$^{-/-}$ Th17 cells to ICAM-1 may not fully explain the protection from EAE and decreased Th17 cell infiltration in the spinal cord observed in CD43$^{-/-}$ mice, and hypothesized

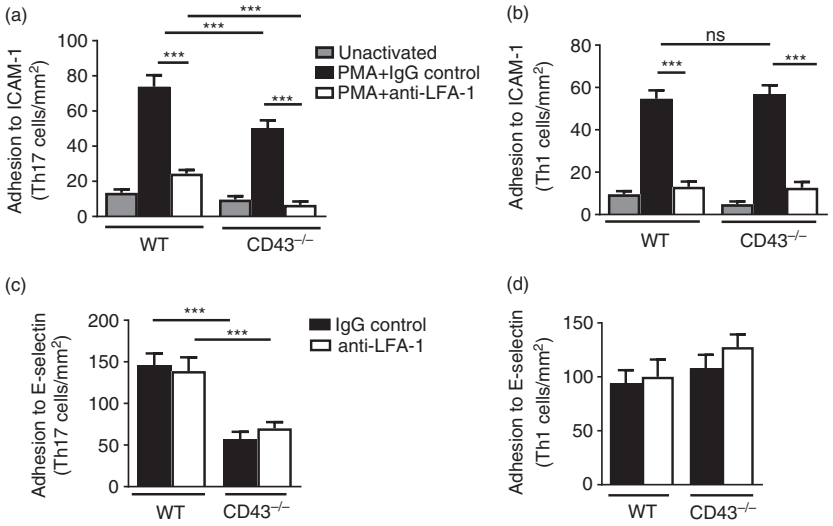

Figure 3. Wild-type (WT), but not CD43$^{-/-}$ T helper type 17 (Th17) cells, adhere to intercellular adhesion molecule 1 (ICAM-1) in the presence of anti-LFA-1 function blocking antibody under physiological flow conditions. (a–d) phorbol 12-myristate 13-acetate (PMA)-activated Th17 cells (a–c) and Th1 cells (b–d) from WT and CD43$^{-/-}$ mice were perfused at a concentration of 0·5 × 10^6 cells/ml over ICAM-1- (20 μg/ml) (a, b) or E-selectin- (20 μg/ml) (c, d) coated coverslips at 1 dyne/cm^2, and treated with either IgG isotype control antibody or function blocking anti-LFA-1 (40 μg/ml) during 20 min before perfusion. T-cell adhesion was visualized during 1 min and quantified in six different fields of view. Data are representative of $n \geq 3$ independent experiments and are shown as mean ± SD. ***$P < 0{\cdot}001$.

that additional adhesive mechanisms regulated by CD43 in an LFA-1/ICAM-1-dependent manner may be influencing other steps of the Th17 cell recruitment cascade. We next sought to address whether CD43, in addition to modulating Th17 cell adhesion to ICAM-1, also regulates other functions of LFA-1, such as Th17 cell spreading and firm arrest upon engagement of endothelial cell surface ICAM-1.[36–38] To test this, we perfused a bolus of integrin-activated Th17 cells with PMA or CCL20 across ICAM-1 under shear flow conditions, allowed them to firmly arrest, and using live video microscopy, quantified the formation of pseudopods, cell structures formed upon ICAM-1 engagement that reflect firm arrest and precede apical and transendothelial migration. As expected, fewer CD43$^{-/-}$ Th17 cells adhered to ICAM-1 compared with WT Th17 cells, regardless of using PMA or CCL20 as the integrin activation stimulus (Fig. 4a,b). CCL20 induced pseudopod formation and spreading in more cells than PMA; however, those PMA- or CCL20-activated CD43$^{-/-}$ Th17 cells that adhered to ICAM-1 were able to form pseudopods and spread to the same extent as WT Th17 cells (Fig. 4c). To further investigate this phenomenon, we next evaluated the adhesion strength of WT and CD43$^{-/-}$ Th17 cells that were firmly arrested on ICAM-1 using detachment assays. In these studies, we added three-fold more Th17 cells than in the adhesion and apical migration assays to allow many cells to firmly arrest on ICAM-1, and studied whether they detached or remained arrested. Live cell imaging was performed at increasing laminar shear stresses, and quantification of Th17 cells that initially adhered to ICAM-1; those that remained arrested on ICAM-1 as the shear stress increases served as a way to determine the maximum adhesion strength. We found that integrin activation with PMA induced firm arrest of both WT and CD43$^{-/-}$ Th17 cells, compared with non-PMA-treated (integrin inactivated) controls. Consistent with our previous findings (Fig. 2), WT Th17 cells adhered in greater numbers to ICAM-1 than CD43$^{-/-}$ Th17 cells (Fig. 4d). Moreover, a small fraction of both groups (~20% of the initially adhered) detached as the shear stress increased from 0 to 2 dynes/cm^2, whereas the majority of the WT and CD43$^{-/-}$ Th17 cells (≥ 80% of the initially adhered) remained arrested and did not detach as the shear increased to 10 dynes/cm^2 (Fig. 4e). Taken together, these data indicate that CD43 does not regulate firm arrest of Th17 cells on ICAM-1.

CD43 regulates Th17 cell integrin-mediated apical migration on ICAM-1

We next focused on further evaluating the role of CD43 in regulating LFA-1 integrin-mediated apical migration of Th17 cells on ICAM-1, a step occurring upon T-cell firm adhesion that controls T-cell migration on the apical side of the endothelium in response to chemokine signals that precede TEM.[4,38–40] We perfused WT and CD43$^{-/-}$ Th17 cells on coverslips coated with ICAM-1 and CCL20, and monitored T-cell apical migration under shear stress. Specific parameters such as the distance covered by apically migrating cells and the velocity at which they migrate on ICAM-1 were quantified in both Th17 cell

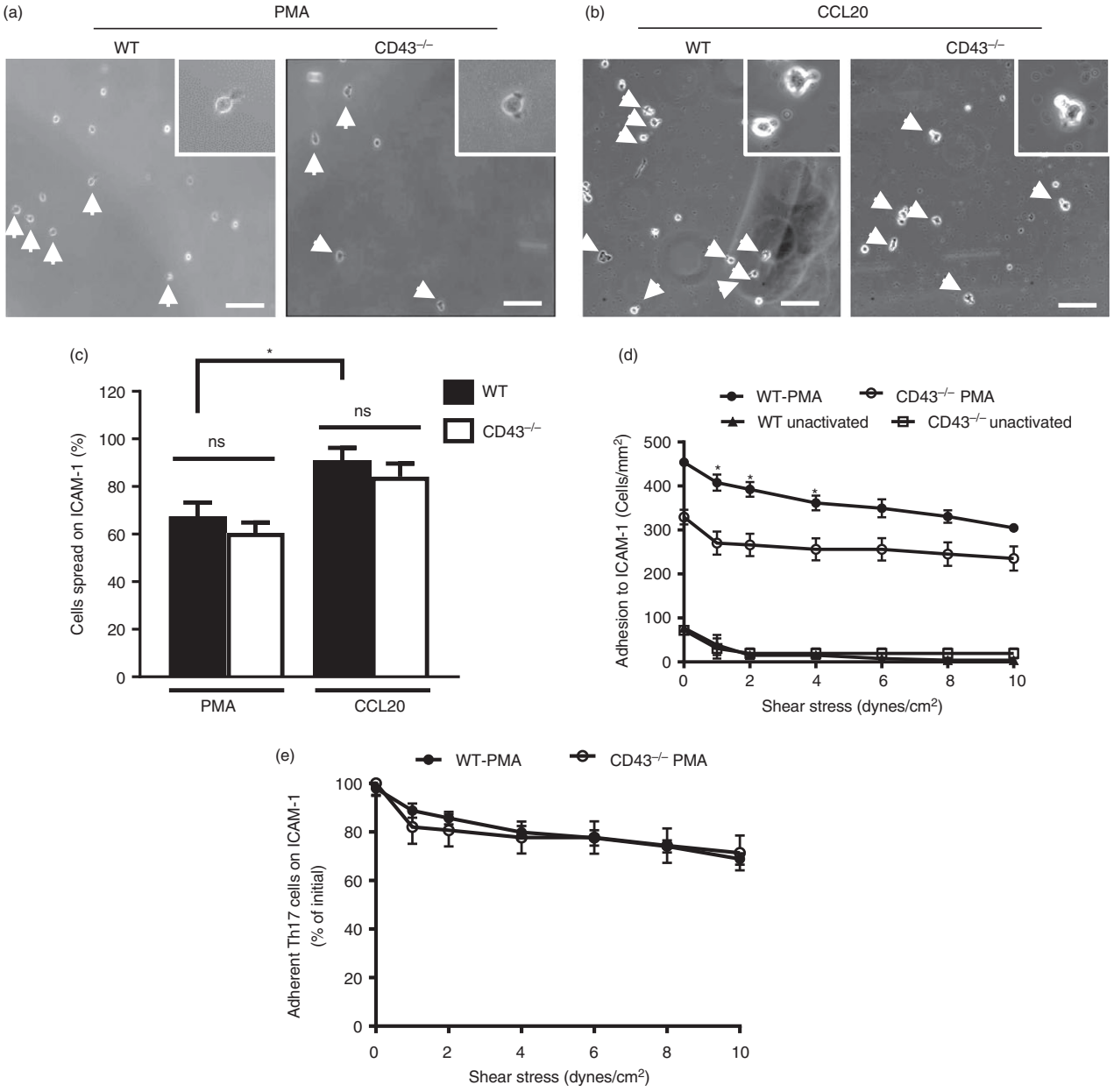

Figure 4. CD43$^{-/-}$ T helper type 17 (Th17) cells that firmly adhere to intercellular adhesion molecule 1 (ICAM-1) form pseudopods and adhere to ICAM-1 with similar strength as found in wild-type (WT) Th17 cells. (a–c) A bolus of 1 × 10^6 CD43$^{-/-}$ and WT Th17 cells, phorbol 12-myristate 13-acetate (PMA)-treated, was perfused across ICAM-1-coated coverslips, or left untreated and perfused across ICAM-1- and CCL20-coated coverslips, at shear stress 1 dyne/cm^2 and recorded during 1 min. Representative images of WT and CD43$^{-/-}$ adhesion with white arrowheads pointing to Th17 cells forming pseudopods, and zoomed view of a single spread cell forming pseudopods (a, b), and quantification of cells with pseudopods ([no. of cells with pseudopods/no. of total accumulated cells] × 100 (c). (d, e) A bolus of 3 × 10^6 WT and CD43$^{-/-}$ Th17 cells was perfused over ICAM-1- (20 μg/ml) coated coverslips, paused for 30 seconds, and shear stress was increased every 30 seconds up to a maximum of 10 dynes/cm^2. Th17 cells that remained adhered to ICAM-1 were quantified on triplicate coverslips for each independent experiment and represented as total adhered cells (d) and as the percentage of cells of each genotype that remain adhered as shear stress increased (e). Scale bar is 50 μm Data show the mean ± SD values. *P < 0·05, PMA WT Th17 versus PMA CD43$^{-/-}$ Th17 cells at each indicated shear stress, and is representative of three or more independent experiments.

types. All WT cells were actively apically migrating, but 7·94% of CD43$^{-/-}$ Th17 cells remained static on ICAM-1 (Fig. 5a). Fewer CD43$^{-/-}$ Th17 cells adhered to ICAM-1, and the frequency of apical migration of those adhered was slightly impaired. Remarkably, the CD43$^{-/-}$ Th17 cells that were motile, showed a decrease in the distance covered on ICAM-1, calculated as the sum of distances each cell covered during any relocation of the cell for the

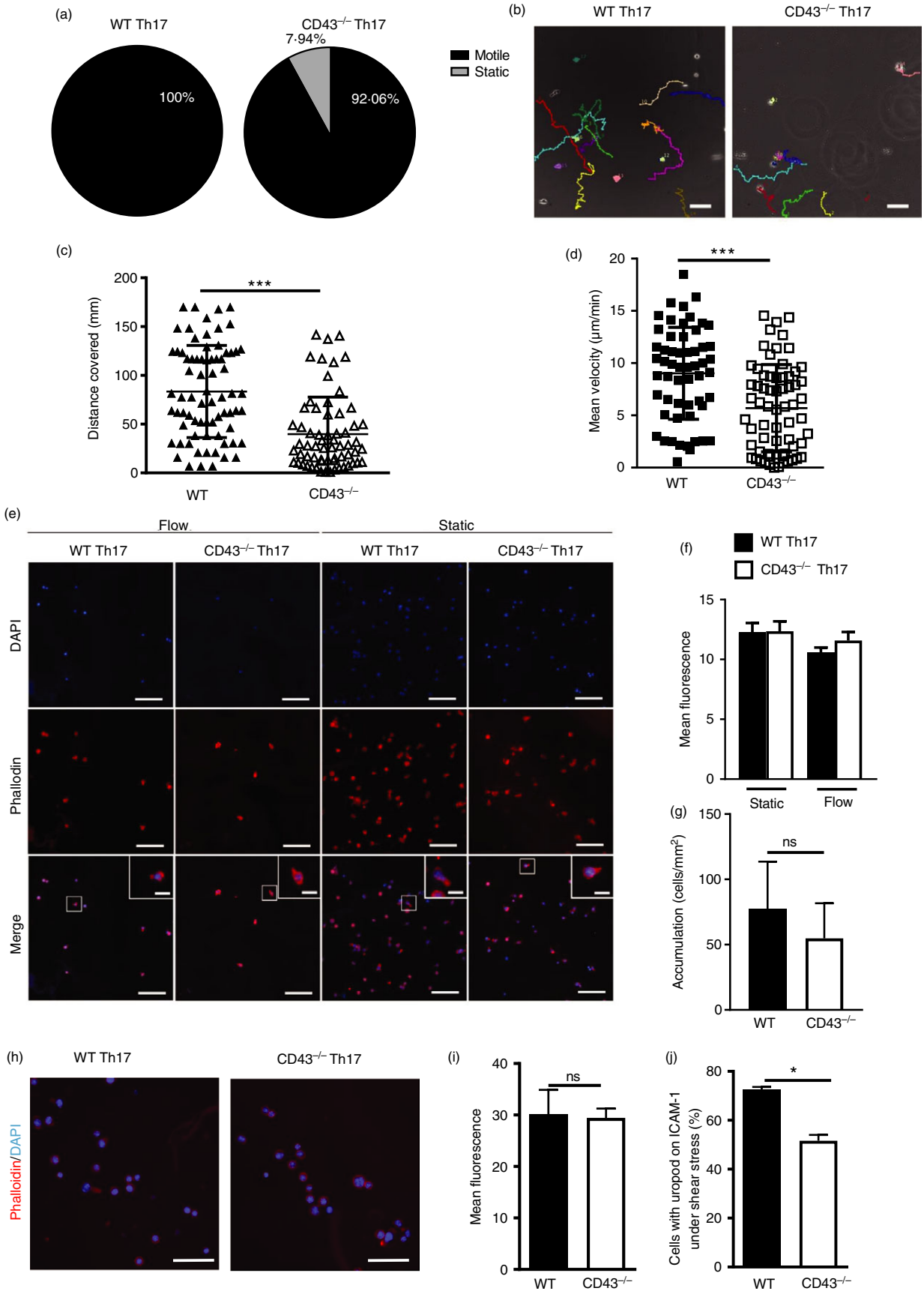
(a)
WT Th17
CD43−/− Th17
7·94%
100%
92·06%
(b)
WT Th17
CD43−/− Th17
Motile
Static
(c)
Distance covered (mm)
200
150
100
50
0
***
WT
CD43−/−
(d)
Mean velocity (μm/min)
20
15
10
5
0
***
WT
CD43−/−
(e)
Flow
Static
WT Th17
CD43−/− Th17
WT Th17
CD43−/− Th17
DAPI
Phallodin
Merge
(f)
WT Th17
CD43−/− Th17
Mean fluorescence
15
10
5
0
Static
Flow
(g)
Accumulation (cells/mm2)
150
100
50
0
ns
WT
CD43−/−
(h)
WT Th17
CD43−/− Th17
Phalloidin/DAPI
(i)
Mean fluorescence
40
30
20
10
0
ns
WT
CD43−/−
(j)
Cells with uropod on ICAM-1 under shear stress (%)
80
60
40
20
0
*
WT
CD43−/−

Figure 5. CD43$^{-/-}$ T helper type 17 (Th17) apical migration on intercellular adhesion molecule 1 (ICAM-1) is impaired compared with wild-type (WT) Th17 cells in an actin cytoskeletal polymerization independent manner. 1×10^6 WT Th17 and CD43$^{-/-}$ Th17 cells were perfused over ICAM-1- (20 μg/ml) and CCL20- (1 μg/ml) coated coverslips and time lapses were recorded for 20 min to quantify apical migration (a–f, j), or over fibronectin- (5 μg/ml) coated coverslips for 10 min under shear flow conditions (1 dyne/cm^2) (g–i). (a) Percentage of WT and CD43$^{-/-}$ Th17 cells that apically migrate on ICAM-1. (b) Representative pictures from the last frame of videos indicating tracks (colored lines) of WT and CD43$^{-/-}$ Th17 cells apically migrating for the duration of the video. Scale bars: 50 μm. (c, d) Distance (c) and velocity (d) covered by Th17 cells apically migrating on ICAM-1 and CCL20-coated coverslips. (e, f) Representative immunofluorescence images of phalloidin staining in Th17 cells on ICAM-1- and CCL20-coated coverslips at 40× magnification (scale bar 50 μm), zoomed view of a representative phalloidin-positive cell in the upper right corner (scale bar 10 μm) (e) and quantification of corrected total fluorescence (f). (g–i) Th17 cells were perfused across fibronectin-coated coverslips under shear flow conditions (1 dyne/cm^2) and adhesion was quantified in several fields of view (g), previous to fixation and staining with phalloidin, as indicated in the representative immunofluorescence images (h) and quantification of total mean fluorescence (i). (j) Quantification of Th17 cells adhered to ICAM-1 presenting uropods under shear flow conditions (1 dyne/cm^2). Data show the mean ± SD values and are representative of three or more independent experiments for the ICAM-1 studies, and $n = 3$ independent experiments for the fibronectin studies. *$P < 0{\cdot}05$; ***$P < 0{\cdot}001$.

duration of the video (Fig. 5b,c), and decreased velocity compared with motile WT Th17 cells (Fig. 5d), demonstrating a role for CD43 in apical migration on ICAM-1 (see Supplementary material, Videos S1 and S2). We next sought to determine whether CD43 modulates F-actin polymerization, a process required for the actin-cytoskeletal rearrangement involved in T-cell arrest and apical migration.[41] More Th17 cells adhered to ICAM-1 in response to CCL20 under static conditions compared with under flow conditions, and consistent with our previous results, more WT Th17 cells adhered compared with CD43$^{-/-}$ Th17 cells, whether flow was present or not. WT and CD43$^{-/-}$ Th17 cells arrested on ICAM-1 in response to CCL20 signaling and similarly expressed phalloidin, a maker of F-actin polymerization (Fig. 5e,f). We next evaluated whether CD43 regulates Th17 cell adhesion to fibronectin, the ligand of integrin β_3 recently reported to be expressed in Th17 cells and to contribute to Th17 cell migration to the CNS during EAE,[42] and stained F-actin with phalloidin. WT and CD43$^{-/-}$ Th17 cells adhered to fibronectin in similar numbers under flow conditions, and no differences were observed in the intensity of F-actin on WT and CD43$^{-/-}$ Th17 cells adhered to fibronectin (Fig. 5 g,h). Apical migration not only requires actin polymerization, which can be determined by phalloidin staining, but also active actin dynamics that change the shape of T cells to promote directionality to the sites of TEM. Such directionality is controlled by the uropod, the posterior protrusion in migrating lymphocytes that contains several proteins, including CD43.[43] We found that WT and CD43$^{-/-}$ Th17 cells formed uropods when interacting with ICAM-1 under shear flow conditions in response to CCL20 (Figs 4b and 5e). However, the frequency of CD43$^{-/-}$ Th17 cells with uropods in these conditions was significantly decreased compared with WT Th17 cells (Fig. 5j). Taken together, these data indicate that CD43 modulates chemokine triggered Th17 cell apical migration on ICAM-1, but not adhesion to fibronectin or the expression of F-actin in either ICAM-1 or fibronectin under flow conditions, and suggest that defective uropod formation in the absence of CD43 may be responsible for the decreased apical migration observed on ICAM-1.

CD43$^{-/-}$ Th17 cells have impaired ICAM-1-dependent migration towards CCL20 under static conditions, and decreased adhesion, apical migration on endothelial cells and transendothelial migration under flow conditions

Given that CD43 contributes to Th17 cell apical migration on ICAM-1, and that this step precedes TEM, we next evaluated whether CD43 played a role in Th17 cell migration across ICAM-1-coated trans wells in response to CCL20. Fluorescently labeled Th17 cells were added to the upper chamber of the ICAM-1-coated and non-coated transwells, and transmigration through the pores was quantified in the lower chamber using a fluorescence read out. Minimal transmigration occurred in the absence of ICAM-1, and we found that ICAM-1-mediated migration in response to CCL20 was significantly impaired in CD43$^{-/-}$ compared with WT Th17 cells (Fig. 6a). Our data to this point supported a role of CD43 as a regulator of Th17 cell adhesion and apical migration on ICAM-1 under flow conditions, and in ICAM-1-mediated transmigration under static conditions. To further investigate whether this CD43–ICAM-1 axis was functional in Th17 cell interactions with the vascular endothelium, we next evaluated the adhesion, apical migration and TEM steps of the Th17 cell recruitment cascade under flow conditions *in vitro*, in a system involving WT and CD43$^{-/-}$ Th17 cells, WT and ICAM-1$^{-/-}$ primary endothelial cells, and CCL20. As expected, high numbers of WT Th17 cells accumulated on WT endothelial cells in response to CCL20, in contrast to the lower numbers that accumulated in ICAM-1$^{-/-}$ endothelial cells. Notably, CD43$^{-/-}$ Th17 cell accumulation on WT endothelial cells was decreased compared with WT Th17 cells. CD43$^{-/-}$ Th17

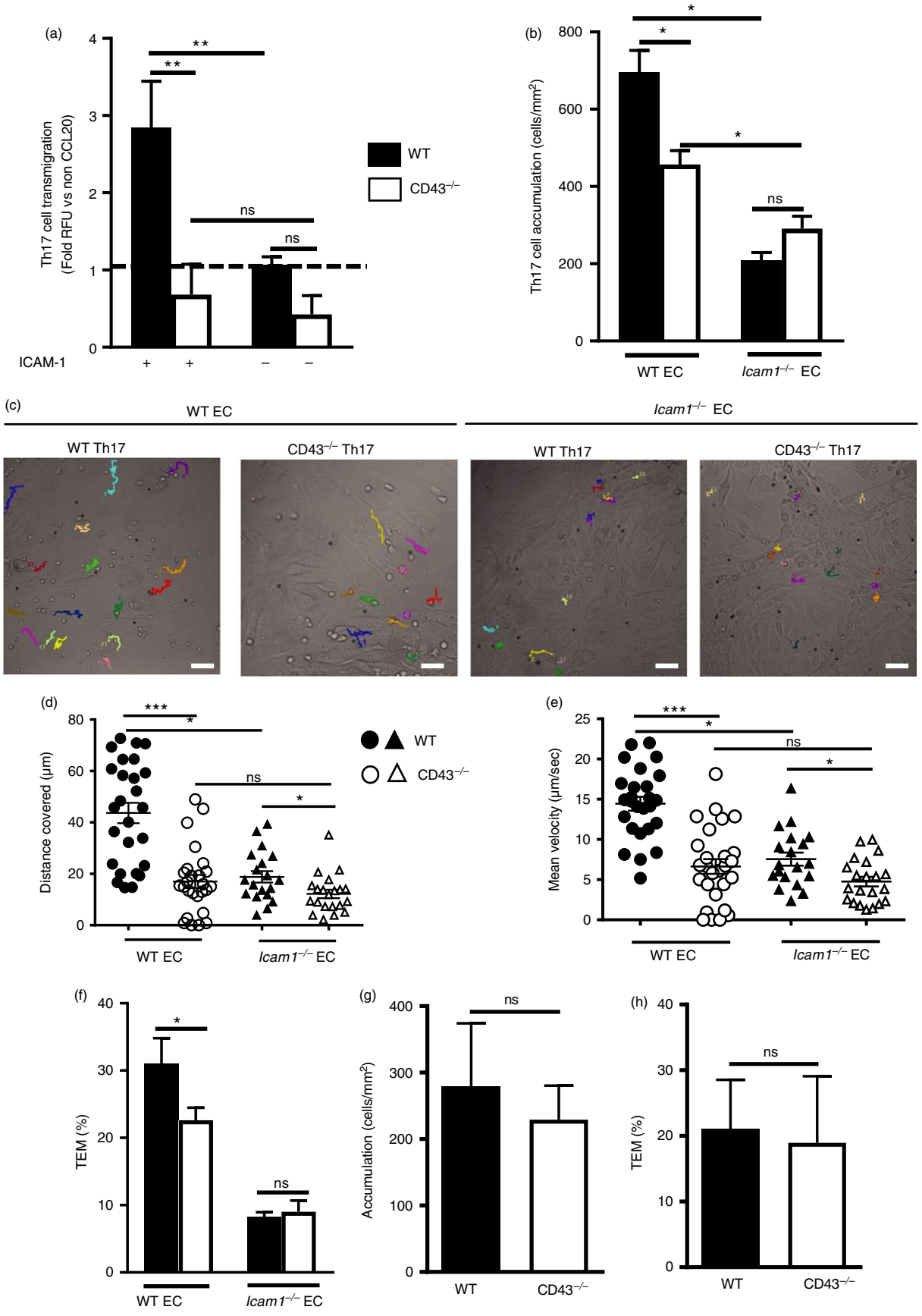
(a)
Th17 cell transmigration (Fold RFU vs non CCL20)
WT
CD43−/−
ICAM-1
+ + − −
(b)
Th17 cell accumulation (cells/mm2)
WT EC
Icam1−/− EC
(c)
WT EC
Icam1−/− EC
WT Th17
CD43−/− Th17
WT Th17
CD43−/− Th17
(d)
Distance covered (µm)
WT
CD43−/−
WT EC
Icam1−/− EC
(e)
Mean velocity (µm/sec)
WT EC
Icam1−/− EC
(f)
TEM (%)
WT EC
Icam1−/− EC
(g)
Accumulation (cells/mm2)
WT
CD43−/−
(h)
TEM (%)
WT
CD43−/−

Figure 6. CD43$^{-/-}$ T helper type 17 (Th17) cells have significantly impaired intercellular adhesion molecule 1 (ICAM-1) -dependent apical migration and transendothelial migration (TEM) compared with wild-type (WT) Th17 cells. (a) WT and CD43$^{-/-}$ Th17 cells were fluorescently labeled and added to the upper chamber of ICAM-1-coated or uncoated transwells, in the presence or absence of CCL20 in the bottom chamber of the transwells. Th17 cell transmigration was quantified by determining the fluorescence in the bottom chamber after 3 hr. Transmigration is represented as relative fluorescence units (RFU), relative to non-CCL20 conditions (marked by the dotted line). Data are representative of $n \geq 3$ independent experiments. (b–f) 2×10^6 Th17 cells were perfused across tumor necrosis factor-α (TNF-α) activated primary mouse heart endothelial cells in the presence of CCL20 (200 ng/ml) and recorded for 8 min. Adhesion (b), apical migration, including distance covered and velocity of migration (c–e), and TEM (f) were quantified using NIKON ELEMENTS and IMAGEJ software. Representative images of the distance covered by Th17 cells apically migrating on endothelial cells are shown. Scale bar is 50 μm (d). (g, h) 2×10^6 Th1 cells were perfused across TNF-α activated primary mouse heart endothelial cells in the presence of stromal cell-derived factor-1α (SDF-1α; 250 ng/ml), recorded for 8 min, and adhesion (g) and TEM (h) were quantified. $n = 3$ independent experiments with duplicate conditions. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

cells also showed decreased accumulation on ICAM-1$^{-/-}$ endothelial cells compared with accumulation on WT endothelial cells, and no significant differences were found between WT and CD43$^{-/-}$ Th17 cell adhesion to ICAM-1$^{-/-}$ endothelial cells (Fig. 6b). These data are in support of ICAM-1 contributing to Th17 cell adhesion to endothelial cells in addition to other endothelial adhesion molecules. We next determined the role of CD43 in apical migration and TEM, and whether this was dependent on endothelial ICAM-1. We found that apical migration on WT endothelial cells was impaired in CD43$^{-/-}$ Th17 cells, which migrated a shorter distance and at a slower velocity compared with WT Th17 cells (Fig. 6c). Although WT Th17 cells had defective apical migration on ICAM-1$^{-/-}$ endothelial cells compared with their motility on WT endothelial cells, both parameters were comparable between CD43$^{-/-}$ Th17 cells on WT and on ICAM-1$^{-/-}$ endothelial cells (Fig. 6c–e). Moreover, WT Th17 cell TEM was impaired in ICAM-1$^{-/-}$ endothelial cells compared with WT endothelial cells, indicating a critical role for ICAM-1 on Th17 cell TEM. CD43$^{-/-}$ Th17 cell TEM across WT endothelial cells was decreased compared with WT Th17 cells, but similar TEM was observed between WT and CD43$^{-/-}$ Th17 cells across ICAM-1$^{-/-}$ endothelial cells (Fig. 6f). In contrast to the decreased adhesion and TEM observed in CD43$^{-/-}$ Th17 cells compared with WT Th17 cells, CD43$^{-/-}$ Th1 cell adhesion (Fig. 6 g) and TEM (Fig. 6h) across primary endothelial cells was comparable with WT Th1 cells. To further evaluate if the observed effects on Th17 cell adhesion, apical migration and TEM were mediated by CD43 regulation of LFA-1 functions, we performed Th17 cell adhesion and TEM studies on primary endothelial cells in the presence of an anti-LFA-1 function blocking antibody. Treatment of WT and CD43$^{-/-}$ Th17 cells with anti-LFA-1 resulted in a significant decrease in adhesion to primary endothelial cells, and no significant differences were observed between WT and CD43$^{-/-}$ Th17 cells (Fig. 7a,c). Moreover, of the very few cells that adhered to endothelial cells, none of them were able to transmigrate (Fig. 7b). Taken together, our data support that CD43 facilitates Th17 cell TEM in response to CCL20 in an LFA-1/ICAM-1-dependent manner, in part by regulating apical migration on endothelial ICAM-1 that precedes TEM. These data also demonstrate that CD43 is essential for effective ICAM-1-dependent transmigration of Th17 cells, but not Th1 cells, and that Th17 cell chemotaxis towards CCL20 in the absence of ICAM-1 is not regulated by CD43. Hence, Th17 cell adhesion, apical migration and TEM are mediated by LFA-1, which cooperates with CD43 in these functions required for optimal Th17 cell recruitment.

Discussion

In this study, we have comprehensively investigated the contribution of CD43 to Th17 cell interactions with endothelial ICAM-1 using *in vitro* model systems to study the different steps of the T-cell recruitment cascade in which ICAM-1 is involved: adhesion, firm arrest, apical migration and TEM. We report the novel findings that Th17 cells use CD43 as a pro-adhesive molecule that facilitates adhesion to ICAM-1, as well as LFA-1/ICAM-1-mediated apical migration and TEM. These results, combined with our data using the *in vivo* model of EAE to evaluate the initial step of Th17 cell recruitment to the CNS, suggest that the CD43 regulation of Th17 cell interactions with ICAM-1 is a mechanism responsible for the observed decreased demyelination and EAE clinical score in CD43$^{-/-}$ mice, and could potentially regulate Th17 cell trafficking to other sites of inflammation.

The physiological role of CD43 is complex and yet does not yield a coherent picture in T cells, with studies showing different roles in T-cell activation in mice and human.[44,45] More recently, it was reported that different CD43 epitope targeting with antibodies in human T cells results in distinct signaling, leading to different T-cell differentiation pathways.[45] We and others reported that CD43$^{-/-}$ mice are protected from EAE by mechanisms that include decreased CD4$^+$ T-cell recruitment, and more specifically Th17 cell recruitment to the CNS, but not to Th17 cell differentiation *in vivo* and *in vitro*.[14,20] Hence, the results reported here indicate that the phenotypic difference between WT and CD43$^{-/-}$ Th17 cells in

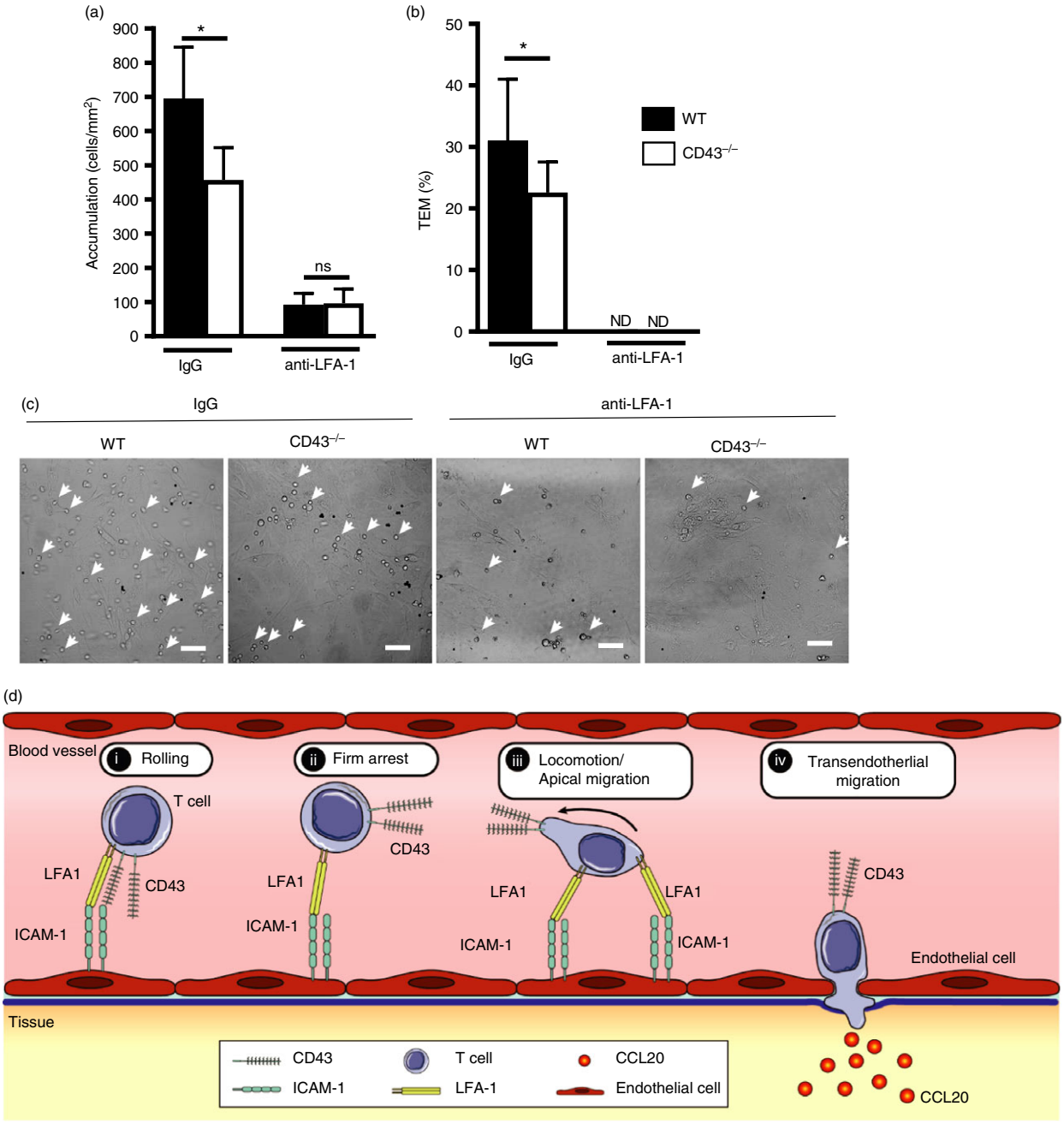

Figure 7. Blockade of LFA-1 results in decreased adhesion and complete inhibition of transendothelial migration (TEM) of both wild-type (WT) and CD43$^{-/-}$ T helper type 17 (Th17) cells. Th17 cells were treated with IgG isotype control antibody or function blocking anti-LFA-1 (40 μg/ml) during the 20 min before perfusion across tumor necrosis factor-α (TNF-α) activated primary mouse heart endothelial cells in the presence of CCL20 (200 ng/ml) at 1 dyne/cm^2. Adhesion (a) and TEM (b) were quantified. (c) Representative pictures are shown from the frames taken after 8 min of adhesion and TEM. (d) Schematic representation of the role of CD43 in modulating Th17 cell interactions with endothelial intercellular adhesion molecule 1 (ICAM-1) in the absence of selectins in the vasculature. (i) Th17 cells use LFA-1 to adhere to ICAM-1 on the vascular endothelium in the absence of E-selectin and this is facilitated by CD43. (ii) LFA-1 mediates firm arrest of Th17 cells to ICAM-1. (iii) CD43 facilitates LFA-1-mediated apical migration on endothelial ICAM-1, and (iv) TEM. Our data suggest that the reorientation of CD43 to the uropod of the Th17 cell upon adhesion to ICAM-1 (indicated with black arrow) is necessary for Th17 cells to successfully apically migrate (iii) and transmigrate to sites of inflammation such as the spinal cord in experimental autoimmune encephalomyelitis (iv). *$P < 0{\cdot}05$.

the recruitment steps cannot be attributed to a role of CD43 in Th17 cell differentiation. However, how exactly CD43 regulates Th17 cell recruitment to the CNS is unclear, as, on the one hand, CD43 regulates Th17 cell trafficking to sites of inflammation through E-selectin-mediated adhesion,[14,26] but on the other hand, E-selectin

is dispensable for the development of EAE.[6] Our results shed light on this complexity, demonstrating that E-selectin is not expressed in the CNS at the initial time of Th17 cell recruitment in EAE, hence Th17 adhesion to E-selectin is not involved in early T-cell recruitment in EAE. Moreover, CD43$^{-/-}$ mice have similar expression of ICAM-1 and VCAM-1, both being critical in T-cell recruitment to the CNS,[4,27] and ICAM-1, being previously reported as one of the multiple CD43 ligands in an *in vitro* system involving immortalized cells and immobilized CD43.[19]

Our *in vitro* data unveil new mechanisms that contribute to CD43 regulation of Th17 cell interactions with endothelial ICAM-1. We report for the first time, to our knowledge, that CD43$^{-/-}$ Th17 cells have impaired adhesion to ICAM-1, and that this interaction is independent of CD43 altering LFA-1 expression or modifying LFA-1-mediated firm adhesion to ICAM-1. These findings are supported by the similar expression of LFA-1 in unstimulated, PMA-treated or CCL20-treated WT and CD43$^{-/-}$ Th17 cells. The residual adhesion of WT Th17 cells, but not CD43$^{-/-}$ Th17 cells, to ICAM-1, observed in the presence of function blocking antibody to LFA-1 could either mean that CD43 and ICAM-1 interact in the absence of LFA-1, in line with a previously reported interaction between ICAM-1 and CD43 using an immortalized cell line model system,[19] or more accessibility of anti-LFA-1 in Th17 cells lacking CD43. The WT Th1 cells adhere to ICAM-1 in similar numbers to the CD43$^{-/-}$ Th1 cells; hence, CD43 regulation of adhesion to ICAM-1 is Th17-cell-specific, which is in line with previous work suggesting that the composition and function of CD43 differs among these two T-cell subsets.[14] Our data demonstrate that just like in other activated T cells, LFA-1 is the major ligand for ICAM-1 in Th17 cells, and that CD43 regulates its adhesive function. The results obtained in the detachment assays indicating that the percentage of WT and CD43$^{-/-}$ that remain adhered to ICAM-1 after the initial increase of shear stress from 0 to 2 dynes/cm^2 is comparable among the two cell types support the idea that CD43 does not impair the firm arrest function of LFA-1.

Our results indicating that Th17 cell apical migration on ICAM-1 is impaired in CD43$^{-/-}$ Th17 cells point to CD43 playing an additional role in the Th17 cell recruitment steps following firm arrest, which may explain the drastic reduction of Th17 cell infiltration in the CNS and protection in EAE.[14,20] Not only did fewer Th17 cells adhere and apically migrate on ICAM-1, but those that did were significantly impaired in the distance they covered and the speed of migration. We report for the first time these parameters in Th17 cell adhesion to ICAM-1 and to primary endothelial cells, and the Th17 cells mean velocity is in line with what has previously been reported for mouse LPS-activated T cells and Th1 cells and human T cells in response to chemokines *in vitro*, or slightly faster.[38,46,47] Interestingly, function blocking of LFA-1 abrogated most of the adhesion and completely inhibited TEM of WT Th17 cells, supporting the idea that CD43 is not directly involved in adhesion and TEM, but regulates such functions of LFA-1. Because very few anti-LFA-1-treated cells adhered per field, we were unable to accurately quantify apical migration, but as this step precedes TEM and TEM was completely inhibited, it is likely that LFA-1 blockade also impairs such a step. Cytoskeletal rearrangement is typically involved in apical migration upon LFA-1 engagement.[36,38,39] CD43 has been reported to interact with the ezrin/rodesin/moesin (ERM) cytoskeletal protein complex that provides a link between the plasma membrane and the cytoskeleton. It is through this interaction that CD43 is pulled by the ERM complex to different parts of the cell surface in a way that allows adequate conformational changes required for cellular functions, such as T-cell migration and antigen presentation during the immune synapse.[48] Our data indicate that F-actin is similarly expressed in WT and CD43$^{-/-}$ Th17 cells apically migrating on ICAM-1. This phenotype is observed under static and under flow conditions of 1 dyne/cm^2, although there is the possibility that higher shear stress is required to observe significant cytoskeletal changes that allow apical migration resisting the flow. However, cytoskeletal rearrangement during apical migration not only requires actin polymerization, but also active complex dynamics that result in uropod formation, a protrusion in the rear part of the T cell that contains proteins such as CD43 and promotes directionality to the sites of TEM.[43] Our results indicate that a smaller fraction of CD43$^{-/-}$ Th17 cells form uropods under flow conditions compared with WT Th17 cells, supporting a role for CD43 in establishing Th17 cell directional apical migration mediated by LFA-1, as previously suggested for lymphocytes.[44] There is also the possibility that the presence of CD43 on the Th17 cell surface facilitates some undefined inside out signaling mechanisms to allow LFA-1 to establish apical migration on ICAM-1.[38,49] Our findings are also in line with a previously described role of CD43 on integrin-mediated adhesion in a process in which myosin regulates CD43 movement to the leukocyte uropod to facilitate integrin enrichment in the leukocyte contact area with the endothelium.[50] Whether this is the case during Th17 cell apical migration mediated by LFA-1 on endothelial ICAM-1, and the exact actin dynamics and cellular distribution of CD43 and LFA-1 as Th17 cells apically migrate on ICAM-1 and on the vascular endothelium require further investigation.

LFA-1 integrin-mediated apical migration precedes TEM, and this usually occurs in response to chemokine signaling. Our transwell studies using CCL20, the main chemoattractant for mouse and human Th17 cells,[14,25,33,51] indicate that CD43 also plays a role in

ICAM-1-mediated Th17 cell transmigration. Because these studies were performed in the absence of flow, when minimal apical migration is required for transmigration, our results suggest that this is a direct effect of CD43 on ICAM-1-dependent transmigration, rather than a result of the impaired apical migration step. Moreover, our mechanistic data evaluating adhesion, apical migration and TEM across WT and ICAM-$1^{-/-}$ primary endothelial cells under flow conditions, corroborate that Th17 cell interactions with ICAM-1 are critical for Th17 cell adhesion, apical migration, and TEM. Although the decreased adhesion to the endothelium cannot be exclusively attributed to endothelial ICAM-1 because CD43 is a major ligand for E-selectin, which is also expressed on activated endothelial cells, the limited apical migration and TEM confirm a major role for CD43 in regulating such functions of LFA-1. It is worthwhile noting that although our data confirm a major role for CD43 in Th17 cell apical endothelial migration and TEM, molecules such as E-selectin, also known to bind CD43 on Th17 cells and mediate rolling, the previous step to firm adhesion, can additionally contribute to Th17 interactions with the endothelium in addition to the later steps mediated by ICAM-1.[14] Our adhesion and TEM studies in the presence of an anti-LFA-1 function blocking antibody also support the idea that most adhesion of Th17 cells to endothelial cells is mediated by LFA-1/ICAM-1-mediated interactions, with additional adhesion molecule contribution. In agreement with our data and that from others demonstrating a role for CCL20 in Th17 cell arrest to ICAM-1 and to human endothelial cells,[26,33] our results presented here demonstrate that this is modulated by CD43 and impacts the outcome of TEM. We suggest that CD43 localization to the opposite side of where the Th17 cell is interacting with a ligand, the uropod, might provide the spatial organization and polarization of the cell that is needed for apical migration, in a similar way to how T-cell adhesion to antigen-presenting cells in the immune synapse[48] (Fig. 7d).

Preventing Th17 cell infiltration in the CNS has significant therapeutic interest, as Th17 cells are the first T-cell subset being recruited and orchestrating CNS damage.[1,11,14,52] Attempts to do so have not been successful in humans, with serious side effects that include enhanced opportunistic viral infections resulting in leukoencephalopathy.[53,54] In mice, ICAM-1 has been implied to be important for the development of EAE, with one of its isoforms being sufficient for spontaneous development of EAE.[5,30,55] However, blocking LFA-1, the main ligand of ICAM-1 in T cells, has not proved to be effective in preventing disease.[56] Recently, Th17 cells have been reported to use integrin β_3 to migrate to the CNS during EAE;[42] hence, the protection observed in CD43$^{-/-}$ mice could also be attributed to impaired interactions of CD43$^{-/-}$ Th17 cells with integrin β_3 ligands such as fibronectin. However, our *in vitro* data indicate that WT and CD43$^{-/-}$ Th17 cells adhered to fibronectin in similar numbers, suggesting that the protection of CD43$^{-/-}$ mice from EAE may be independent of integrin β_3-mediated Th17 cell recruitment, although this would require *in vivo* confirmation. Our data suggest the intriguing possibility that targeting some functions of LFA-1 in an indirect manner through modulating CD43 may be more effective in preventing Th17 cell recruitment to the CNS specifically, and potentially diminishing side effects that involve inhibiting the recruitment of functions of other T-cell subsets. In our mechanistic studies *in vitro*, however, we used primary mouse heart endothelial cells as a well-established model system to study the Th17 cell recruitment cascade, in lieu of primary brain endothelial cells. Our findings suggest that these new aspects of CD43 regulation of Th17 cell interactions with the vascular endothelium, could take place in similar inflammatory settings, in addition to inflammation in the CNS.

In summary, we report a comprehensive characterization of the role that CD43 plays in Th17 cell interactions with vascular endothelial cells and suggest it as a mechanism involved in Th17 cell recruitment to the CNS through ICAM-1-mediated adhesion. Our data contribute to a better understanding of Th17 cell trafficking to sites of inflammation, and shed new light into the complex roles for sialomucin CD43 in T-cell biology.

Acknowledgements

FEV and PA designed all studies and wrote the manuscript. FEV also performed the majority of the experiments. MA performed TEM studies under flow conditions, and apical migration studies on ICAM-1 and endothelial cells, and adhesion studies on fibronectin. FJC performed EAE studies, immunofluorescence, statistical analysis and figure panels. AMS, NN and TN helped with the generation of T cells and performance of flow chamber studies.

These studies were supported by NIH grants, HL097406 and HL123658 (PA), HL123658-01S1 (FV), and T32AI007077-34 (NN).

Disclosures

The authors have no financial conflict of interests.

References

1 Hofstetter H, Gold R, Hartung HP. Th17 cells in MS and experimental autoimmune encephalomyelitis. *Int MS J* 2009; **16**:12–8.

2 Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol* 2009; **27**:485–517.

3 Lee Y, Awasthi A, Yosef N, Quintana FJ, Xiao S, Peters A *et al.* Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol* 2012; **13**:991–9.

4 Schnoor M, Alcaide P, Voisin MB, van Buul JD. Crossing the vascular wall: common and unique mechanisms exploited by different leukocyte subsets during extravasation. *Mediators Inflamm* 2015; **2015**:946509.

5 Laschinger M, Vajkoczy P, Engelhardt B. Encephalitogenic T cells use LFA-1 for transendothelial migration but not during capture and initial adhesion strengthening in healthy spinal cord microvessels *in vivo*. *Eur J Immunol* 2002; **32**:3598–606.

6 Doring A, Wild M, Vestweber D, Deutsch U, Engelhardt B. E- and P-selectin are not required for the development of experimental autoimmune encephalomyelitis in C57BL/6 and SJL mice. *J Immunol* 2007; **179**:8470–9.

7 Engelhardt B, Kempe B, Merfeld-Clauss S, Laschinger M, Furie B, Wild MK *et al.* P-selectin glycoprotein ligand 1 is not required for the development of experimental autoimmune encephalomyelitis in SJL and C57BL/6 mice. *J Immunol* 2005; **175**:1267–75.

8 Eppihimer MJ, Wolitzky B, Anderson DC, Labow MA, Granger DN. Heterogeneity of expression of E- and P-selectins *in vivo*. *Circ Res* 1996; **79**:560–9.

9 Angiari S, Donnarumma T, Rossi B, Dusi S, Pietronigro E, Zenaro E *et al.* TIM-1 glycoprotein binds the adhesion receptor P-selectin and mediates T cell trafficking during inflammation and autoimmunity. *Immunity* 2014; **40**:542–53.

10 Arbelaez CA, Glatigny S, Duhen R, Eberl G, Oukka M, Bettelli E. IL-7/IL-7 receptor signaling differentially affects effector $CD4^+$ T cell subsets involved in experimental autoimmune encephalomyelitis. *J Immunol* 2015; **195**:1974–83.

11 Rothhammer V, Heink S, Petermann F, Srivastava R, Claussen MC, Hemmer B *et al.* Th17 lymphocytes traffic to the central nervous system independently of α4 integrin expression during EAE. *J Exp Med* 2011; **208**:2465–76.

12 Zarbock A, Ley K, McEver RP, Hidalgo A. Leukocyte ligands for endothelial selectins: specialized glycoconjugates that mediate rolling and signaling under flow. *Blood* 2011; **118**:6743–51.

13 Alcaide P, King SL, Dimitroff CJ, Lim YC, Fuhlbrigge RC, Luscinskas FW. The 130-kDa glycoform of CD43 functions as an E-selectin ligand for activated Th1 cells *in vitro* and in delayed-type hypersensitivity reactions *in vivo*. *J Invest Dermatol* 2007; **127**:1964–72.

14 Velazquez F, Grodecki-Pena A, Knapp A, Salvador AM, Nevers T, Croce K *et al.* CD43 Functions as an E-Selectin Ligand for Th17 cells *in vitro* and is required for rolling on the vascular endothelium and Th17 cell recruitment during inflammation *in vivo*. *J Immunol* 2016; **196**:1305–16.

15 Fuhlbrigge RC, King SL, Sackstein R, Kupper TS. CD43 is a ligand for E-selectin on CLA^+ human T cells. *Blood* 2006; **107**:1421–6.

16 Hirata T, Merrill-Skoloff G, Aab M, Yang J, Furie BC, Furie B. P-Selectin glycoprotein ligand 1 (PSGL-1) is a physiological ligand for E-selectin in mediating T helper 1 lymphocyte migration. *J Exp Med* 2000; **192**:1669–76.

17 Pedraza-Alva G, Rosenstein Y. CD43 – One molecule, many tales to recount. *Signal Transduction* 2007; **7**:372–85.

18 Rosenstein Y, Santana A, Pedraza-Alva G. CD43, a molecule with multiple functions. *Immunol Res* 1999; **20**:89–99.

19 Rosenstein Y, Park JK, Hahn WC, Rosen FS, Bierer BE, Burakoff SJ. CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. *Nature* 1991; **354**:233–5.

20 Ford ML, Onami TM, Sperling AI, Ahmed R, Evavold BD. CD43 modulates severity and onset of experimental autoimmune encephalomyelitis. *J Immunol* 2003; **171**:6527–33.

21 Bullard DC, Hu X, Schoeb TR, Collins RG, Beaudet AL, Barnum SR. Intercellular adhesion molecule-1 expression is required on multiple cell types for the development of experimental autoimmune encephalomyelitis. *J Immunol* 2007; **178**:851–7.

22 Dunne JL, Collins RG, Beaudet AL, Ballantyne CM, Ley K. Mac-1, but not LFA-1, uses intercellular adhesion molecule-1 to mediate slow leukocyte rolling in TNF-α-induced inflammation. *J Immunol* 2003; **171**:6105–11.

23 Nevers T, Salvador AM, Grodecki-Pena A, Knapp A, Velazquez F, Aronovitz M *et al.* Left ventricular T-cell recruitment contributes to the pathogenesis of heart failure. *Circ Heart Fail* 2015; **8**:776–87.

24 Flynn KM, Michaud M, Madri JA. CD44 deficiency contributes to enhanced experimental autoimmune encephalomyelitis: a role in immune cells and vascular cells of the blood–brain barrier. *Am J Pathol* 2013; **182**:1322–36.

25 Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B *et al.* CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol* 2008; **181**:8391–401.

26 Alcaide P, Maganto-Garcia E, Newton G, Travers R, Croce KJ, Bu DX *et al.* Difference in Th1 and Th17 lymphocyte adhesion to endothelium. *J Immunol* 2012; **188**:1421–30.

27 Ellies LG, Jones AT, Williams MJ, Ziltener HJ. Differential regulation of CD43 glycoforms on $CD4^+$ and $CD8^+$ T lymphocytes in graft-versus-host disease. *Glycobiology* 1994; **4**:885–93.

28 Nacher M, Blazquez AB, Shao B, Matesanz A, Prophete C, Berin MC *et al.* Physiological contribution of CD44 as a ligand for E-Selectin during inflammatory T-cell recruitment. *Am J Pathol* 2011; **178**:2437–46.

29 Lim YC, Garcia-Cardena G, Allport JR, Zervoglos M, Connolly AJ, Gimbrone MA Jr *et al.* Heterogeneity of endothelial cells from different organ sites in T-cell subset recruitment. *Am J Pathol* 2003; **162**:1591–601.

30 Steffen BJ, Butcher EC, Engelhardt B. Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse. *Am J Pathol* 1994; **145**:189–201.

31 Dransfield I, Cabanas C, Craig A, Hogg N. Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J Cell Biol* 1992; **116**:219–26.

32 Dustin ML, Springer TA. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 1989; **341**:619–24.

33 Ghannam S, Dejou C, Pedretti N, Giot JP, Dorgham K, Boukhaddaoui H *et al.* CCL20 and β-defensin-2 induce arrest of human Th17 cells on inflamed endothelium *in vitro* under flow conditions. *J Immunol* 2011; **186**:1411–20.

34 Laudanna C, Kim JY, Constantin G, Butcher E. Rapid leukocyte integrin activation by chemokines. *Immunol Rev* 2002; **186**:37–46.

35 Askari JA, Tynan CJ, Webb SE, Martin-Fernandez ML, Ballestrem C, Humphries MJ. Focal adhesions are sites of integrin extension. *J Cell Biol* 2010; **188**:891–903.

36 Shamri R, Grabovsky V, Gauguet JM, Feigelson S, Manevich E, Kolanus W *et al.* Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat Immunol* 2005; **6**:497–506.

37 Marlin SD, Springer TA. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* 1987; **51**:813–9.

38 Smith A, Bracke M, Leitinger B, Porter JC, Hogg N. LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. *J Cell Sci* 2003; **116**(Pt 15):3123–33.

39 del Pozo MA, Sanchez-Mateos P, Nieto M, Sanchez-Madrid F. Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. *J Cell Biol* 1995; **131**:495–508.

40 Schenkel AR, Mamdouh Z, Muller WA. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat Immunol* 2004; **5**:393–400.

41 Serrador JM, Nieto M, Sanchez-Madrid F. Cytoskeletal rearrangement during migration and activation of T lymphocytes. *Trends Cell Biol* 1999; **9**:228–33.

42 Du F, Garg AV, Kosar K, Majumder S, Kugler DG, Mir GH *et al.* Inflammatory Th17 cells express integrin αvβ3 for pathogenic function. *Cell Rep* 2016; **16**:1339–51.

43 Sanchez-Madrid F, Serrador JM. Bringing up the rear: defining the roles of the uropod. *Nat Rev Mol Cell Biol* 2009; **10**:353–9.

44 Cannon JL, Collins A, Mody PD, Balachandran D, Henriksen KJ, Smith CE *et al.* CD43 regulates Th2 differentiation and inflammation. *J Immunol* 2008; **180**:7385–93.

45 Modak M, Majdic O, Cejka P, Jutz S, Puck A, Gerwien JG *et al.* Engagement of distinct epitopes on CD43 induces different co-stimulatory pathways in human T cells. *Immunology* 2016; **149**:280–96.

46 Knowlden SA, Capece T, Popovic M, Chapman TJ, Rezaee F, Kim M *et al.* Regulation of T cell motility *in vitro* and *in vivo* by LPA and LPA2. *PLoS ONE* 2014; **9**:e101655.

47 Steiner O, Coisne C, Cecchelli R, Boscacci R, Deutsch U, Engelhardt B *et al.* Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood–brain barrier endothelium. *J Immunol* 2010; **185**:4846–55.

48 Allenspach EJ, Cullinan P, Tong J, Tang Q, Tesciuba AG, Cannon JL *et al.* ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. *Immunity* 2001; **15**:739–50.

49 Ramsay AG, Evans R, Kiaii S, Svensson L, Hogg N, Gribben JG. Chronic lymphocytic leukemia cells induce defective LFA-1-directed T-cell motility by altering Rho GTPase signaling that is reversible with lenalidomide. *Blood* 2013; **121**:2704–14.

50 Sanchez-Mateos P, Campanero MR, del Pozo MA, Sanchez-Madrid F. Regulatory role of CD43 leukosialin on integrin-mediated T-cell adhesion to endothelial and extracellular matrix ligands and its polar redistribution to a cellular uropod. *Blood* 1995; **86**:2228–39.

51 Wang C, Kang SG, Lee J, Sun Z, Kim CH. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunol* 2009; **2**:173–83.

52 Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T_H1 and T_H17 cells. *Nat Med* 2008; **14**:337–42.

53 Ransohoff RM. Natalizumab and PML. *Nat Neurosci* 2005; **8**:1275.

54 Theien BE, Vanderlugt CL, Nickerson-Nutter C, Cornebise M, Scott DM, Perper SJ *et al.* Differential effects of treatment with a small-molecule VLA-4 antagonist before and after onset of relapsing EAE. *Blood* 2003; **102**:4464–71.

55 Hu X, Barnum SR, Wohler JE, Schoeb TR, Bullard DC. Differential ICAM-1 isoform expression regulates the development and progression of experimental autoimmune encephalomyelitis. *Mol Immunol* 2010; **47**:1692–700.

56 Hu X, Wohler JE, Dugger KJ, Barnum SR. β2-integrins in demyelinating disease: not adhering to the paradigm. *J Leukoc Biol* 2010; **87**:397–403.

Supporting Information

Additional Supporting Information may be found in online in the Supporting Information section at the end of the article:

Video S1. Wild-type Th17 cells apically migrating on ICAM-1-coated coverslips.

Video S2. CD43$^{-/-}$ Th17 cells apically migrating on ICAM-1-coated coverslips.