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IL-17 and TNF- α Sustain Neutrophil Recruitment during Inflammation through Synergistic Effects on Endothelial Activation

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IL-17A (IL-17) is the signature cytokine produced by Th17 cells and has been implicated in host defense against infection and the pathophysiology of autoimmunity and cardiovascular disease. Little is known, however, about the influence of IL-17 on endothelial activation and leukocyte influx to sites of inflammation. We hypothesized that IL-17 would induce a distinct pattern of endothelial activation and leukocyte recruitment when compared with the Th1 cytokine IFN- γ . We found that IL-17 alone had minimal activating effects on cultured endothelium, whereas the combination of TNF- α and IL-17 produced a synergistic increase in the expression of both P-selectin and E-selectin. Using intravital microscopy of the mouse cremaster muscle, we found that TNF- α and IL-17 also led to a synergistic increase in E-selectin-dependent leukocyte rolling on microvascular endothelium *in vivo*. In addition, TNF- α and IL-17 enhanced endothelial expression of the neutrophilic chemokines CXCL1, CXCL2, and CXCL5 and led to a functional increase in leukocyte transmigration *in vivo* and CXCR2-dependent neutrophil but not T cell transmigration in a parallel-plate flow chamber system. By contrast, endothelial activation with TNF- α and IFN- γ preferentially induced the expression of the integrin ligands ICAM-1 and VCAM-1, as well as the T cell chemokines CXCL9, CXCL10, and CCL5. These effects were further associated with a functional increase in T cell but not neutrophil transmigration under laminar shear flow. Overall, these data show that IL-17 and TNF- α act in a synergistic manner to induce a distinct pattern of endothelial activation that sustains and enhances neutrophil influx to sites of inflammation. *The Journal of Immunology*, 2012, 188: 6287–6299.

Interleukin-17A (IL-17) is the signature cytokine secreted by Th17 cells, a recently discovered subset of proinflammatory CD4⁺ T cells that were first described in 2005 (1, 2). IL-17 production is characteristic of Th17 cells in the setting of chronic inflammation, but it can also be secreted by a wide variety of cell types associated with innate immune responses, including $\gamma\delta$ T cells, NK cells, and neutrophils (3–5). Given the ubiquitous expression of the IL-17R complex, IL-17 signaling has relevance for a range of cellular targets and organ-specific immune functions (6, 7). For example, IL-17 has an important physiologic role in mucosal immunity and host defense against extracellular bacteria and fungi, as was demonstrated in studies showing increased susceptibility to staphylococcal infection and candidiasis in mice lacking IL-17 (8–10). By contrast, aberrant IL-17 production is

highly associated with inflammatory pathology in the setting of autoimmune disease. Elevated IL-17 expression has been observed in inflamed tissues of patients with rheumatoid arthritis, psoriasis, and multiple sclerosis, and IL-17 deletion attenuates disease severity in mouse models of autoimmunity (11–16). Interestingly, recent studies have also proposed a pathologic role for IL-17 in atherosclerosis, plaque destabilization, and other inflammatory vascular disorders, including cardiac allograft vasculopathy and Kawasaki's disease (17–24). Accordingly, targeted IL-17 depletion has already been adopted as a therapeutic strategy and is currently being tested in clinical trials for several human autoimmune diseases (25, 26).

Despite the heightened interest in IL-17 over recent years, relatively little is known from a mechanistic standpoint as to its unique effects in directing and sustaining leukocyte influx to sites of inflammation, particularly in relation to other T cell-associated cytokines, such as IFN- γ . In addition, although there are many putative cellular targets of IL-17, there is little information on which cell types are most important in mediating its proinflammatory effects. As was demonstrated in early studies (27–30), activation of the vascular endothelium by proinflammatory cytokines, such as TNF- α and IL-1 β , results in the induction of adhesion molecules (e.g., E-selectin) and chemokines (e.g., CXCL8) that play a central role in the now well-known cascade of leukocyte tethering, slow rolling, firm adhesion, and trans-endothelial migration. Differences in the expression of these molecules by vascular endothelium in response to different cytokines can contribute to the unique temporal and spatial patterns of leukocyte subset recruitment to sites of inflammation. For example, the Th1 cytokine IFN- γ has been shown to modulate endothelial activation and Ag-presenting function and can also

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Abbreviations used in this article: IVM, intravital microscopy; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MHEC, mouse heart endothelial cell; qRT-PCR, quantitative RT-PCR; V_{cl} , centerline RBC velocity; WT, wild-type.

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synergize with cytokines such as TNF- α to augment endothelial expression of specific adhesion molecules and chemokines (31, 32). Recent data from our laboratory have demonstrated the inhibitory effect of TGF- β secretion by regulatory T cells on endothelial activation and leukocyte recruitment in response to activation by TNF- α (33). Currently, however, it is unclear whether IL-17 stimulates endothelial activation or modulates the effects of other proinflammatory cytokines. One prior study has suggested that IL-17 alone is capable of potentially activating HUVECs *in vitro*, although this finding contrasts with the experience of our own laboratory, in which IL-17 alone had little effect on the activation of this cell type (34). In addition, studies in nonendothelial cell types have suggested that the predominant effect of IL-17 activity relates to the synergistic induction of genes such as CXCL1 and IL-6 in response to cotreatment with TNF- α (35, 36).

In the current study, we examined the effects of IL-17 on endothelial activation by evaluating its ability to stimulate the expression of selectins, integrin ligands, and chemokines and to potentiate the effects of the classical proinflammatory cytokine TNF- α . In addition, we compared the impact of IL-17 on endothelial activation to that of the signature Th1 cytokine IFN- γ . Furthermore, we tested the functional effects of endothelial activation with IL-17 by using two widely validated models of leukocyte recruitment; namely, an *in vitro* parallel plate flow chamber system, which models leukocyte-endothelial interactions under physiologically relevant levels of laminar shear flow, as well as intravital microscopy (IVM) of leukocyte rolling on microvascular endothelium *in vivo*. Finally, using blocking Abs and transgenic mice, we attempted to elucidate the specific adhesion molecules and chemokine pathways that serve as functional mediators of IL-17 proinflammatory activity. We report that IL-17 and TNF- α promote a distinct pattern of endothelial activation that sustains and enhances neutrophil influx to sites of inflammation, which is primarily mediated through the synergistic induction of endothelial selectins and CXCR2-activating chemokines.

Materials and Methods

Mice

All mice were maintained in a pathogen-free facility at the New Research Building of Harvard Medical School in accordance with the animal research guidelines established by the Committee of Animal Research and the National Institutes of Health. C57BL/6 (wild-type [WT]) mice were purchased from Charles River Laboratories (Wilmington, MA). CXCR2-deficient mice (*Cxcr2*^{-/-}) on the BALB/cJ background and age-matched controls were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were used at 8–12 wk of age.

Primary isolation and culture of mouse heart endothelial cells

Mouse heart endothelial cells (MHEC) were prepared from cell suspensions of cardiac digests by a sequential positive-selection technique using immunomagnetic beads specific for PECAM-1 (CD31) and ICAM-2 (CD102), as previously described (37). Isolated cells were cultured in gelatin-coated tissue culture plates in growth medium containing 20% FBS, 100 mg/ml porcine heparin, and 100 mg/ml endothelial cell mitogen from Biomedical Technologies (Stoughton, MA). Cells were harvested at >80% confluence with trypsin-EDTA and either cultured for experimental assay or frozen in liquid nitrogen for future use.

Flow cytometry for adhesion molecule expression

Confluent MHEC were stimulated for 0.5–16 h with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml). Cells were harvested with trypsin-EDTA and stained with fluorescently labeled Abs (5 μ g/ml) against endothelial adhesion molecules, including P-selectin, E-selectin, ICAM-1, ICAM-2, VCAM-1, mucosal addressin cell adhesion molecule-1 (MAcCAM-1), PECAM-1, and VE-cadherin. Cells were then analyzed by flow cytometry without prior fixation on a BD Biosciences FACSCalibur

cytometer (San Jose, CA) and FlowJo 9.3.1 software (Tree Star, Ashland, OR).

Quantitative RT-PCR analysis

Confluent MHEC were activated with recombinant cytokines for 8 h, and RNA was extracted with an RNeasy kit from Qiagen (Valencia, CA), according to the manufacturer's instructions. RNA was reverse-transcribed using the ThermoScript system by Invitrogen (Grand Island, NY) and amplified by RT-PCR with SYBR Green and a Step-One Detection System from Applied Biosystems (Carlsbad, CA). Primer sequences are available upon request.

IVM of leukocyte recruitment during microvascular inflammation *in vivo*

IVM of leukocyte recruitment to postcapillary venules of the mouse cremaster muscle was performed at various time points following intrascrotal injection of IL-17A (1000 ng/mouse) and/or TNF- α (100 ng/mouse). Mice were anesthetized, and surgical exteriorization of the cremaster muscle was performed as previously described (38, 39). In some studies, a cannula was also inserted into the jugular vein to allow for the injection of specific anti-E-selectin (9A9) blocking Abs, as previously described (40). The entire cremaster preparation was typically accomplished in <15 min. Microvessel data were obtained using a specialized Olympus FV 1000 intravital microscope (Olympus, Center Valley, PA) fitted with an Olympus 40 \times water immersion objective (Olympus). Centerline RBC velocity (V_{cl}) in each vessel was measured in real time with a dual photodiode velocimeter (Texas A&M University, College Station, TX). Leukocyte rolling interactions were recorded from five to eight vessels per mouse using an Olympus DP71 CCD video camera and Olympus FluoView 1000 imaging software (Olympus). Systemic leukocyte counts for each mouse were determined from a 50- μ l retro-orbital blood sample and analyzed with a Drew Scientific Hemavet 950FS (Waterbury, CT). Videos were analyzed offline with the National Institutes of Health software package ImageJ (National Institutes of Health, Bethesda, MD). Wall shear rate (W_{sr}) was determined according to the equation $W_{sr} = 2.133 [(8 \times 0.625 \times V_{cl}/D_v)]$, in which D_v is the measured vessel diameter (micrometers). The volumetric blood flow (Q) was calculated from the equation $Q = (V_{cl})(0.625)(A_{cs})$, in which A_{cs} is the cross-sectional area of the cylindrical vessel, and 0.625 is an empirical correction factor. The rolling leukocyte flux was determined by counting the number of cells passing through a plane perpendicular to the vessel axis per minute. The average leukocyte rolling velocity was calculated by measuring the distance traveled and elapsed time for 10 leukocytes in each vessel. The total number of rolling leukocytes per 100- μ m vessel segment was determined by dividing the rolling leukocyte flux (cells/minute) by the average rolling velocity (expressed as micrometers per minute) and multiplying by 100 μ m, as previously described (41). The number of transmigrated leukocytes per vessel was determined by counting the average number of perivascular cells in a $50 \times 100 \mu$ m area adjacent to the vessel wall, as previously described (42). All parameters were also expressed as a ratio normalized for differences in vessel diameter, volume, and systemic leukocyte count.

Isolation and purification of neutrophils from mouse bone marrow

Neutrophils were isolated from bone marrow extracted from the femur and tibia of WT mice using an EasySep kit from Stem Cell Technologies (Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, bones were flushed with isolation media, and the resulting cell suspension was incubated with a negative-selection mixture of biotinylated Abs against mouse CD4, CD5, CD11c, CD45R/B220, CD49b, CD117, TER119, and F4/80, followed by sequential incubations with bispecific anti-biotin/anti-dextran Ab complexes and dextran-coated magnetic particles. Contaminating cells were then removed by magnet separation. Neutrophil purity was typically >90% as determined by flow cytometry for CD11b/Ly6G double-positive cells.

Differentiation of effector CD4⁺ T cells

Effector CD4⁺ T cells were prepared from naive CD4⁺ cells isolated from the spleens of WT mice using anti-CD4 MACS beads from Miltenyi Biotec (Bergisch Gladbach, Germany). Naive CD4⁺ cells were differentiated with plate-bound anti-CD3e (5 μ g/ml) as well as soluble anti-CD28 (2 μ g/ml) and IL-2 (15 U/ml). On day 3, cultures were diluted 1:2 in IL-2 (15 U/ml) containing media and transferred to fresh culture plates without anti-CD3e coating. Media containing IL-2 (15 U/ml) was supplemented again on day 5, and cells were harvested for use on day 6.

Parallel-plate flow chamber assay for leukocyte adhesion and transendothelial migration

Neutrophil and effector CD4⁺ T cell interactions with MHEC monolayers under laminar shear stress were measured with a parallel plate flow chamber, as previously described (37, 43). Briefly, MHEC were cultured on fibronectin-coated 25-mm glass coverslips and incubated for 48 h to allow for monolayer formation. Monolayers were then stimulated for 16 h with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml) before being placed in a flow chamber on the stage of a Nikon Eclipse Ti microscope (Nikon, Melville, NY) controlled by MetaMorph software (Sunnyvale, CA). Neutrophils or effector CD4⁺ T cells were isolated as described above and suspended at $0.5\text{--}1 \times 10^7$ cells/ml in flow buffer. A bolus of $0.5\text{--}1 \times 10^6$ neutrophils or T cells was then perfused across the monolayer at 37°C and allowed to bind at a shear stress of 0.2 dynes/cm². Shear was then increased to 0.8 dynes/cm², and subsequent interactions of perfused leukocytes with the monolayer were recorded for 10 min. In some studies, monolayers were preincubated for 20 min with blocking Abs (20 μ g/ml) against P-selectin (RB40.34), E-selectin (9A9), ICAM-1 (YN1), and ICAM-2 (3C4). Accumulated and transmigrated leukocytes were then quantified offline using ImageJ software (National Institutes of Health). Accumulated cells were defined as the average number of adherent or transmigrated cells in five fields after the 10-min video period. Transmigrated cells were defined as the proportion of initially adherent cells that transmigrated during the 10-min video period.

Statistical analysis

Data are expressed as the mean \pm SEM unless otherwise stated. χ^2 and Student *t* tests were used for two-group comparisons, ANOVA with Bonferroni posttests for multiple group comparisons, and simple linear-regression analysis to control for microvessel shear stress during the IVM studies. All statistical analyses were performed in Prism 5.0 software by GraphPad (La Jolla, CA) and considered statistically significant at $p < 0.05$.

Results

IL-17 and TNF- α promote a synergistic increase in endothelial selectin expression

We tested the ability of IL-17 to promote endothelial selectin expression alone or in combination with TNF- α . Given the distinct temporal dynamics of P-selectin and E-selectin expression during acute inflammation, we first conducted time-course experiments to evaluate the expression of these molecules following cytokine stimulation. MHECs were grown in monolayers, stimulated with rIL-17 and/or TNF- α , and analyzed for surface protein expression of P- and E-selectin by immunofluorescence staining and flow cytometry. In these studies, we found that stimulation of MHEC with IL-17 alone had no statistically significant effect on selectin expression relative to vehicle (Fig. 1A, 1B). However, we found that cotreatment with IL-17 and TNF- α led to a marked, synergistic increase in the surface expression of both P- and E-selectin (Fig. 1A, 1B). Interestingly, although there was no difference in the peak expression of E-selectin at 2 h with IL-17 and TNF- α versus TNF- α alone, combined treatment did lead to a clear increase in the duration of E-selectin surface expression that was sustained up to 16 h (Fig. 1B). Consistent with these findings, we also observed that combination IL-17 and TNF- α produced a synergistic increase in *Psel* and *Esel* mRNA levels after 8 h of activation, as determined by quantitative RT-PCR (qRT-PCR) (Fig. 1C, 1D). By contrast, we found that cotreatment with TNF- α and the signature Th1 cytokine IFN- γ for 16 h led to a reduction in E-selectin expression and had no effect on P-selectin expression relative to TNF- α alone (Fig. 1E, 1F). Finally, we found that costimulation with IL-17 and IL-1 β did not lead to an increase in endothelial selectin expression relative to IL-1 β alone (data not shown).

Endothelial activation with IL-17 and TNF- α synergistically enhances leukocyte rolling in vivo

Given the marked synergistic effect on selectin expression in vitro, we further hypothesized that endothelial activation with IL-17 and

TNF- α would produce a functional increase in leukocyte recruitment in vivo. We chose to test this hypothesis given the established role of selectins as key mediators of leukocyte–endothelial interactions during the initial steps of the leukocyte adhesion cascade (30). Accordingly, we used IVM of the mouse cremaster muscle, which is a well-validated in vivo model of selectin-dependent leukocyte rolling on microvascular endothelium (39, 40, 44). In these studies, intrascrotal injections of IL-17 and/or TNF- α were administered, and then endogenous leukocyte rolling in postcapillary venules of the cremaster muscle was visualized at 2, 6, and 16 h after injection. Baseline hemodynamic parameters, including systemic leukocyte count, vessel diameter (range 20–40 μ m), and intravascular shear stress (range 400–1200 s⁻¹) were similar between treatment groups (Supplemental Table I). A small reduction in shear rate was seen with combination IL-17 and TNF- α , which was statistically significant at early (2 and 6 h) but not late (16 h) time points relative to TNF- α alone. Importantly, these small differences in hemodynamic status did not alter any of the central findings reported below, as linear regression analysis demonstrated that all significant treatment effects were maintained across a wide range of shear rates (Supplemental Fig. 1).

We first analyzed the leukocyte rolling flux through each vessel (rolling cells per minute) as a function of the average leukocyte rolling velocity (Fig. 2A–C). At 2 h of activation, IL-17 alone produced only a marginal increase in leukocyte rolling flux relative to vehicle treatment and did not reduce average rolling velocity to levels seen with TNF- α (Fig. 2A–C). In addition, there was no difference in rolling parameters at 2 h when comparing TNF- α alone to combination IL-17 and TNF- α . After 6 h of activation, however, IL-17/TNF- α cotreatment produced a persistent elevation in the leukocyte rolling flux relative to TNF- α alone (Fig. 2B). Importantly, this effect was not attributable to changes in average rolling velocity (Fig. 2B) or vessel shear stress (Supplemental Fig. 1A). Furthermore, although the leukocyte rolling flux in response to IL-17 and TNF- α had decreased by 16 h, the average rolling velocity remained low, suggesting that the total number of rolling leukocytes in the IL-17/TNF- α -treated vessels was greater than those treated with TNF- α alone. This conclusion is based on the fact that more rolling leukocytes are required to maintain a given leukocyte flux as the average rolling velocity decreases. Accordingly, we quantified the total number of rolling leukocytes per 100- μ m vessel segment for each treatment condition (Fig. 2D) and further expressed this number as a ratio accounting for differences in vessel diameter, volume, and the systemic leukocyte count in each mouse (Fig. 2E). Representative images of total leukocyte rolling in each vessel after 6 h are also included (Fig. 2F–I). Although we did observe a trend toward a positive effect on total leukocyte rolling at 2 h with IL-17 alone, it was statistically significant only in the unadjusted analysis and did not persist at 6 and 16 h of activation (Fig. 2D). However, stimulation with both IL-17 and TNF- α synergistically increased the total number of rolling leukocytes per vessel, which was maximal at 6 h of stimulation but also remained elevated at 16 h relative to TNF- α alone (Fig. 2D, 2E). Representative images of leukocyte–endothelial rolling interactions after 6 h of cytokine activation are shown in Fig. 2F–I, with maximal levels observed after stimulation with IL-17/TNF- α (Fig. 2I) relative to TNF- α alone (Fig. 2H), IL-17 alone (Fig. 2G), or vehicle (Fig. 2F). Importantly, these effects were not attributable to vessel hemodynamic status, as linear regression analysis showed clear differences in total leukocyte rolling with IL-17/TNF- α cotreatment versus TNF- α alone across a wide range of shear rates (Supplemental Fig. 1B).

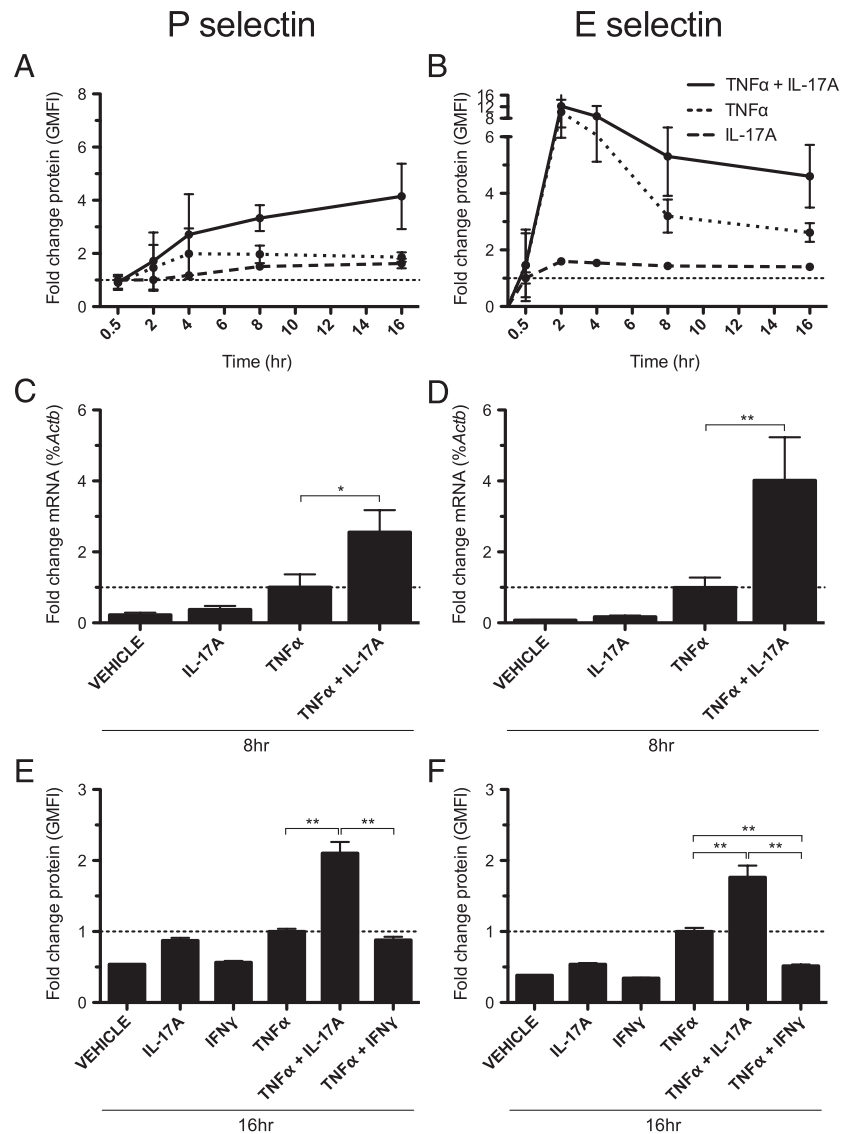


FIGURE 1. IL-17A and TNF- α synergistically increase endothelial selectin expression in vitro. (**A** and **B**) Time course (0.5–16 h) of selectin surface protein expression by MHEC in response to IL-17A (100 ng/ml) and/or TNF- α (50 ng/ml), as determined by immunofluorescent flow cytometry. Data are represented as the fold change in the geometric mean of the fluorescence intensity (GMFI) relative to treatment with vehicle alone. Error bars show the 95% confidence interval for each time point. (**C** and **D**) qRT-PCR of selectin mRNA expression after 8 h of stimulation with IL-17A (100 ng/ml) and/or TNF- α (50 ng/ml). Data are normalized to the level of β -actin (*Actb*) expression and represented as the mean fold change in %*Actb* expression \pm SEM relative to treatment with TNF- α alone. (**E** and **F**) Flow cytometry of selectin expression after 16 h of treatment with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml). Data are represented as the mean fold change in GMFI \pm SEM relative to treatment with TNF- α alone. All data are from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Endothelial activation with IL-17 and TNF- α synergistically enhances the duration of leukocyte slow rolling in vivo

Given the synergistic effects of IL-17 and TNF- α on selectin expression and leukocyte rolling demonstrated above, we anticipated that IL-17 and TNF- α would also potentiate the extent and duration of leukocyte slow rolling. The process of slow rolling represents a key step in the leukocyte adhesion cascade, whereby reduced rolling velocity increases leukocyte exposure to chemokines presented on the apical surface of the endothelium and facilitates leukocyte integrin activation and firm adhesion (45). In these studies, we analyzed the average rolling velocity of leukocytes in each vessel, as well as the proportion of slow-rolling leukocytes with velocities of $<10 \mu\text{m/s}$. First, consistent with the minimal impact of IL-17 on endothelial E-selectin expression in vitro (Fig. 1), we found that treatment with IL-17 alone did not increase the proportion of slow-rolling leukocytes at any time point (Fig. 3A–E). By contrast, we found that treatment with TNF- α alone enhanced the cumulative frequency of slow-rolling leukocytes (Fig. 3A, 3B, 3D) and decreased the average rolling velocity at both 2 and 6 h (Fig. 3E), which is consistent with published literature. At 16 h of stimulation, however, the effect of TNF- α on leukocyte slow rolling had decreased back to levels seen with vehicle treatment alone (Fig. 3C–E). Interestingly,

although slow rolling and average leukocyte rolling velocity were comparable with IL-17 and TNF- α treatment versus TNF- α alone at both 2 and 6 h, coactivation with IL-17 and TNF- α significantly prolonged the duration of slow rolling up to 16 h (Fig. 3C–E). As above, this effect was not attributable to variations in hemodynamic status, as the prolongation in leukocyte slow-rolling velocity in response to IL-17 and TNF- α was maintained across a wide range of shear rates (Supplemental Fig. 1C).

Synergistic effect of IL-17 and TNF- α on leukocyte slow rolling in vivo is E-selectin dependent

We hypothesized that the potentiation of leukocyte slow rolling in response to IL-17 and TNF- α was mediated by an increase in endothelial E-selectin expression relative to treatment with TNF- α alone. This hypothesis was based on the established role of endothelial E-selectin in mediating leukocyte rolling velocities $<10 \mu\text{m/s}$ and on our in vitro findings presented above, which demonstrated a synergistic upregulation of E-selectin in response to combined treatment with IL-17 and TNF- α (40). Accordingly, we conducted intravital studies utilizing a well-validated E-selectin blocking Ab (9A9) to examine the role of E-selectin in mediating the effects of IL-17 and TNF- α on leukocyte slow rolling. In these studies, we found that injection of E-selectin

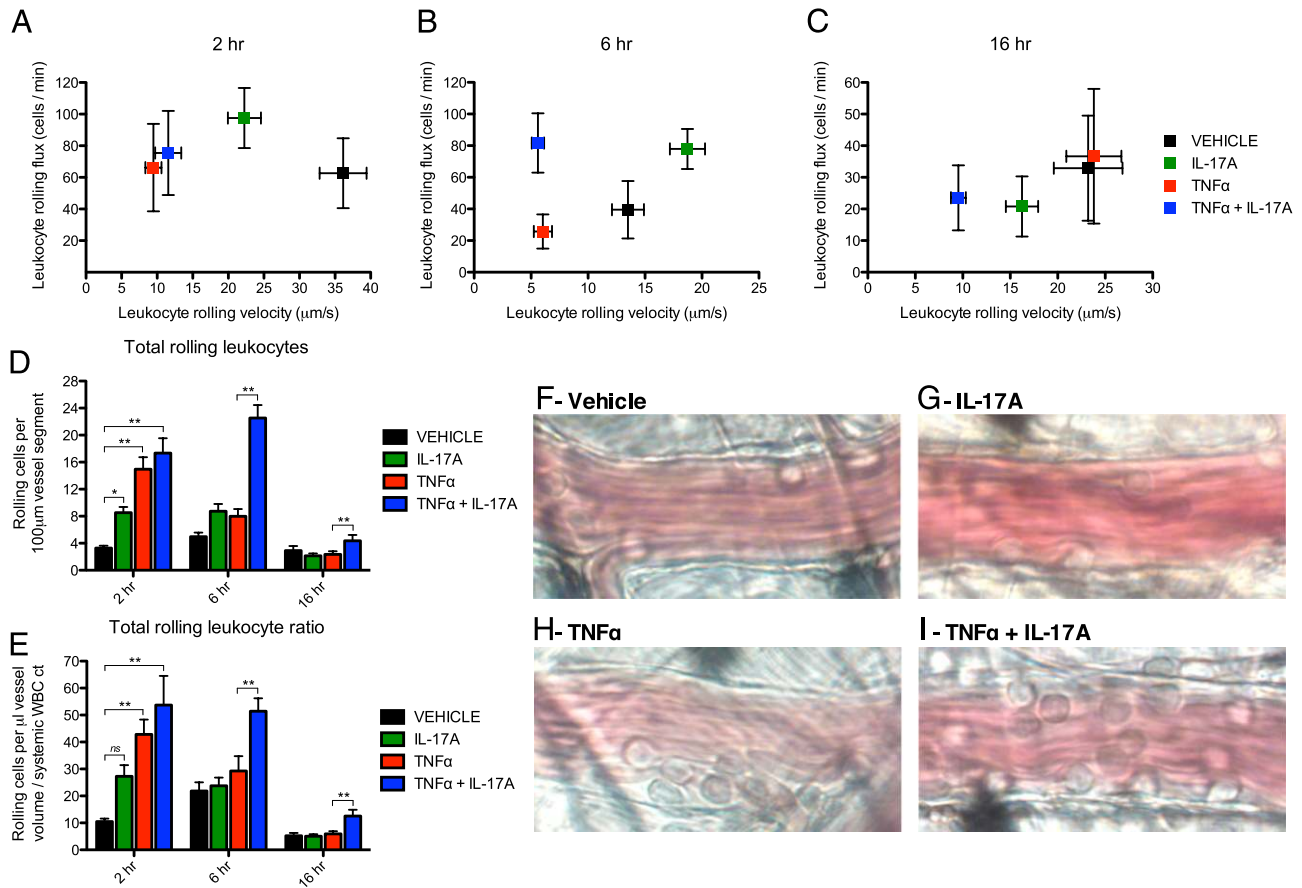


FIGURE 2. IL-17A and TNF- α synergistically enhance rolling interactions of leukocytes with microvascular endothelium in vivo. Leukocyte rolling behavior in the mouse cremaster muscle was quantified by IVM at three time points (2, 6, and 16 h) following intrascrotal injection with IL-17A (1000 ng/mouse) and/or TNF- α (100 ng/mouse). (**A–C**) Leukocyte rolling flux versus average leukocyte rolling velocity for each treatment condition. Data are represented as mean \pm 95% confidence interval. Total number of rolling leukocytes per 100- μ m vessel segment (**D**) and expressed as a ratio (**E**) normalized for differences in vessel diameter, volume, and systemic leukocyte count. Original magnification \times 40. Data are represented as mean \pm SEM. (**F–I**) Representative still images of total rolling leukocytes for each treatment condition after 6 h of stimulation. Sizes of experimental groups and hemodynamic parameters for all data are summarized in Supplemental Table I. * $p < 0.05$, ** $p < 0.01$.

blocking Abs caused a marked reduction in IL-17- and TNF- α -induced leukocyte slow rolling at 16 h and led to a corresponding increase in the average leukocyte rolling velocity (Fig. 3F, 3G). By contrast, injection of anti-E-selectin Abs into mice pretreated with TNF- α alone for 16 h had no significant effect on the proportion of slow-rolling leukocytes (Fig. 3F) or the average rolling velocity (Fig. 3G). These findings suggest that synergistic action between IL-17 and TNF- α extends the duration of E-selectin-dependent leukocyte rolling on microvascular endothelium in vivo.

IL-17 and TNF- α do not enhance endothelial expression of integrin ligands ICAM-1 and VCAM-1

In light of the synergistic effects of IL-17 and TNF- α on endothelial selectin expression and selectin-dependent leukocyte rolling in vivo, we examined whether IL-17 and TNF- α would also support subsequent events in the leukocyte adhesion cascade. Given the central role of endothelial integrin ligands, such as ICAM-1 and VCAM-1, in leukocyte firm adhesion and trans-endothelial migration, we tested the ability of IL-17 and TNF- α to induce the expression of these molecules in cultured endothelial cells (30). Surprisingly, we found that combined treatment with IL-17 and TNF- α for 16 h reduced the expression of VCAM-1 and MAdCAM-1 relative to TNF- α alone (Fig. 4A, 4D) and had no enhancing effect on the expression of ICAM-1, ICAM2,

PECAM-1, or VE-cadherin (Fig. 4B, 4C, 4E, 4F). Similarly, treatment with IL-17 alone had no effect on endothelial expression of any of the integrin ligands studied (Fig. 4). By contrast, we found that cotreatment with TNF- α and the Th1 cytokine IFN- γ increased the expression of VCAM-1 and ICAM-1 relative to TNF- α alone (Fig. 4A, 4B). Combined treatment with IFN- γ and TNF- α also led to a reduction in the expression of MAdCAM-1 and PECAM-1 (Fig. 4D, 4E) and had no enhancing effect on the expression of ICAM-2 or VE-cadherin (Fig. 4C, 4F). Taken together, these data suggest that synergistic action between IL-17 and TNF- α does not enhance endothelial integrin ligand expression, which contrasts sharply to its marked effects on selectin expression (Fig. 1).

Endothelial activation with IL-17 and TNF- α synergistically enhances the expression of neutrophil but not T cell-associated chemokines

Endothelial-derived chemokines are critical regulators of leukocyte integrin activation and firm adhesion, which guide the selective recruitment and chemotaxis of particular leukocyte subsets during inflammation (46). Therefore, we investigated the combined effect of IL-17 and TNF- α on endothelial chemokine expression in vitro. We found that coactivation with IL-17 and TNF- α synergistically increased expression of neutrophilic chemokines agonistic for the CXCR2 receptor, namely CXCL1 (keratinocyte chemoattractant),

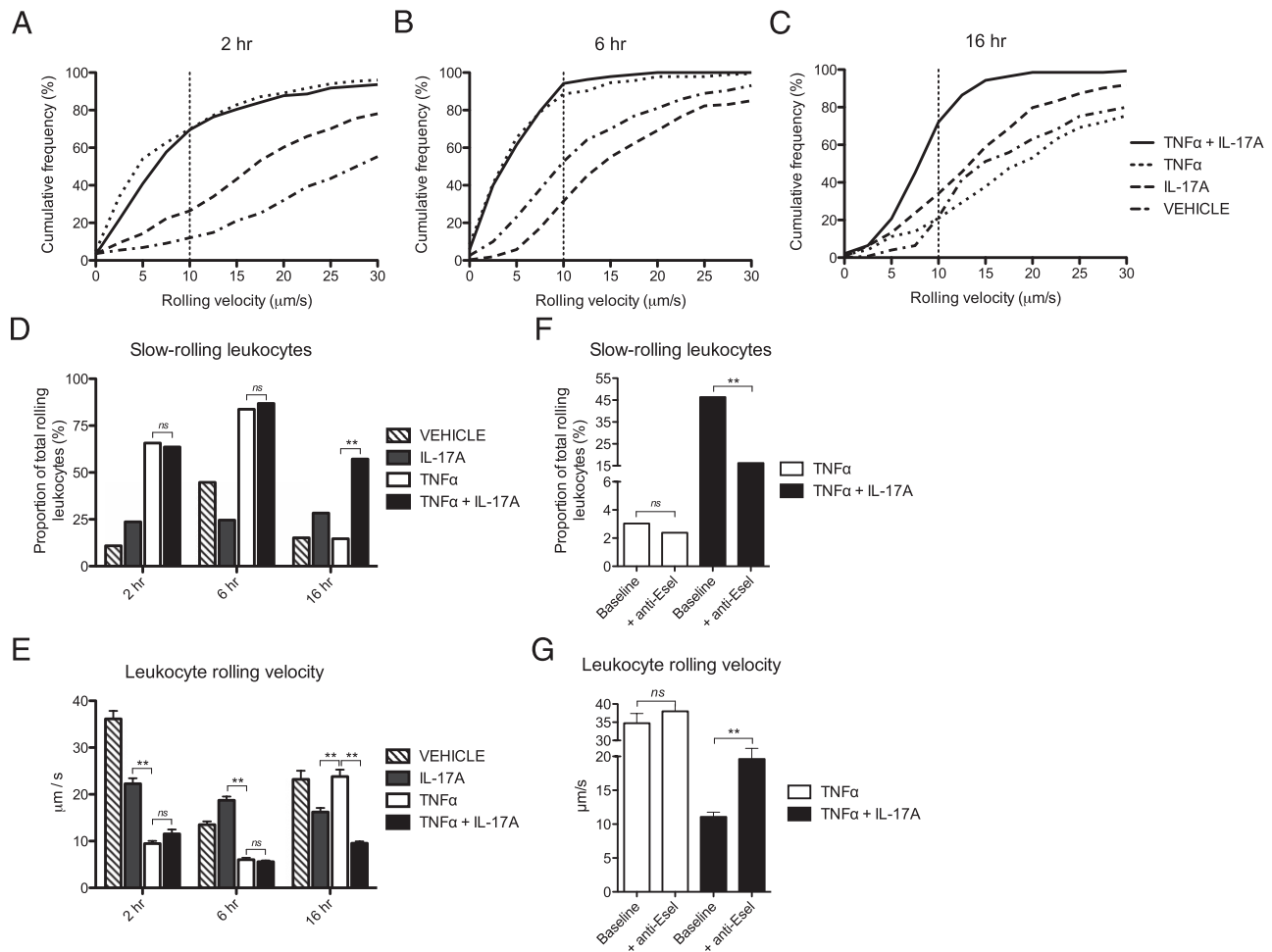


FIGURE 3. IL-17A and TNF- α enhance leukocyte slow rolling on microvascular endothelium in vivo. Leukocyte rolling velocities in the mouse cremaster muscle were quantified by IVM following intrascrotal injection with IL-17A (1000 ng/mouse) and/or TNF- α (100 ng/mouse). (A–C) Cumulative frequency distribution of leukocyte rolling velocity after 2, 6, and 16 h. Proportion of slow-rolling leukocytes (<10 μ m/s) in each vessel (D) and the average leukocyte rolling velocity (E) after 2, 6, and 16 h of cytokine activation. Effects of E-selectin (9A9) blocking Ab injection (50 μ g/mouse) on the proportion of slow-rolling leukocytes (F) and the average leukocyte rolling velocity (G) at 16 h after stimulation with TNF- α alone or in combination with IL-17A. Data (D–G) are represented as mean \pm SEM. Sizes of experimental groups and hemodynamic parameters for all data are summarized in Supplemental Table I. E-selectin blocking studies were performed with $n = 3$ mice/group. ** $p < 0.01$.

CXCL2 (MIP2 α), and CXCL5 (LIX), which are members of the ELR⁺ family of chemokines (Fig. 5A–C). In addition, we found that IL-17 and TNF- α synergistically induced endothelial expression of GM-CSF (Fig. 5D) and G-CSF (Fig. 5E), which have pleiotropic-enhancing effects on granulopoiesis, neutrophil survival, and phagocytic activity (47–49). In contrast to its effects on neutrophilic chemokines, cotreatment with IL-17 and TNF- α had no influence on the expression of the T cell-activating chemokines CCL5 (RANTES), CXCL9 (MIG), and CXCL10 (IP-10) relative to TNF- α alone (Fig. 5G–I). To compare potential differences in Th1- versus Th17-driven inflammation, we also tested the effects of the signature Th1 cytokine IFN- γ on endothelial chemokine expression. Interestingly, we found that IFN- γ alone or in combination with TNF- α had no enhancing effect on the expression of neutrophilic chemokines or levels of GM-CSF and G-CSF (Fig. 5A–E), but did strongly induce the expression of the aforementioned T cell-activating chemokines (Fig. 5G–I). Endothelial expression of the monocyte-activating chemokine MCP-1 was not significantly enhanced with either IL-17/TNF- α or IFN- γ /TNF- α relative to TNF- α alone (Fig. 5F). These data indicate that the signature cytokines produced by Th17 and Th1 cells have distinct synergistic activity with TNF- α in promoting the selective

expression of chemokines by endothelium that are supportive of neutrophil versus T lymphocyte migration, respectively.

IL-17 and TNF- α synergistically enhance leukocyte transendothelial migration in vivo

Having established that IL-17 and TNF- α potentiate endothelial expression of neutrophilic chemokines in vitro, we hypothesized that synergistic action between IL-17 and TNF- α would also have a functional impact in vivo on leukocyte transendothelial migration, which represents the physiologically critical endpoint of leukocyte recruitment to sites of infection or tissue damage. Using IVM, we quantified the number of transmigrated leukocytes in the perivascular space at 2, 6, and 16 h after cytokine activation, as previously described (42). Transmigration data are presented as the absolute number of perivascular cells per 100- μ m vessel segment (Fig. 6A) and as a ratio of absolute cells normalized for differences in vessel diameter, volume, and the systemic leukocyte count in each mouse (Fig. 6B). Representative images showing transmigrated cells in the perivascular space at 16 h for each treatment condition are also shown (Fig. 6C–F). First, treatment with IL-17 alone did not enhance the number of transmigrated cells above levels observed with vehicle treatment at any of the

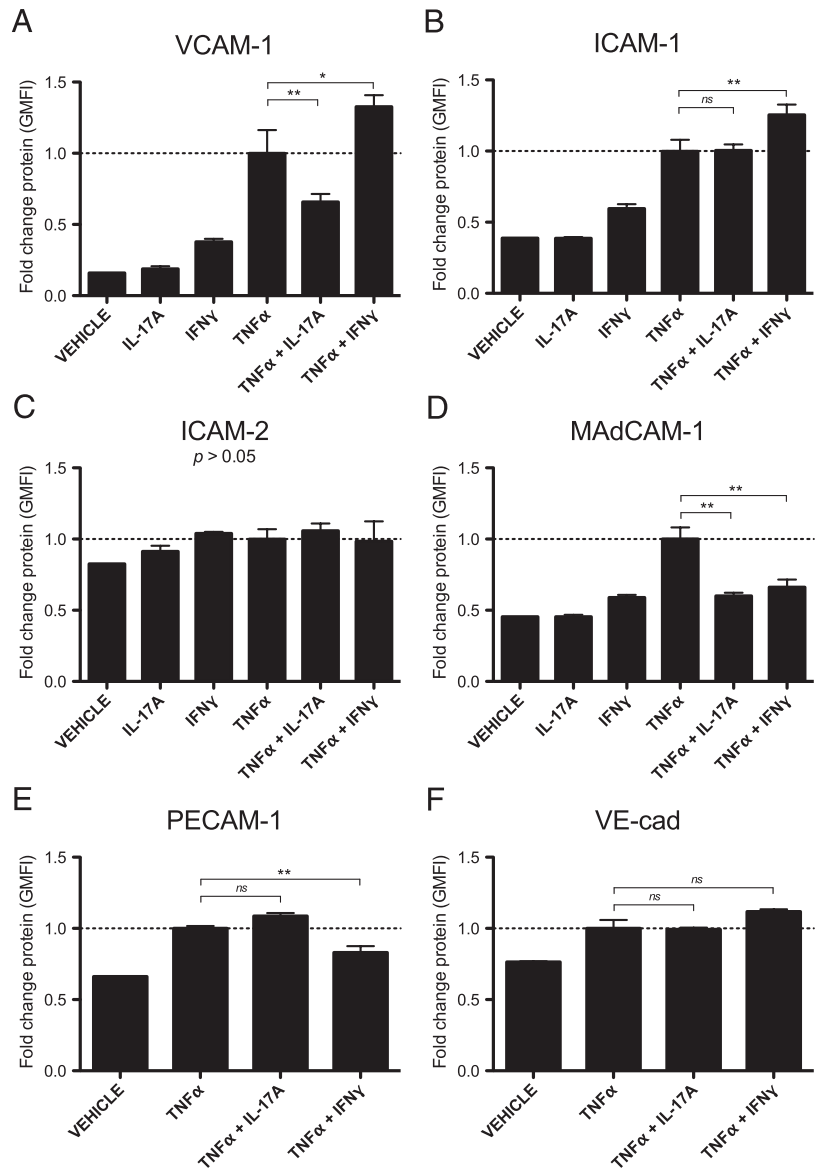


FIGURE 4. IL-17A does not enhance endothelial expression of integrin ligands in vitro. (A–F) Immunofluorescent flow cytometry of integrin ligand expression after 16 h of treatment with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml). Data are represented as the mean fold change in the geometric mean of the fluorescence intensity (GMFI) \pm SEM relative to treatment with TNF- α alone. All data are from at least three independent experiments. * p < 0.05, ** p < 0.01.

time points studied (Fig. 6A, 6B, 6D). In addition, using a sub-maximal dose of TNF- α (100 ng/mouse), we observed a trend toward an increase in leukocyte transmigration at all time points, although this effect did not achieve statistical significance (Fig. 6A, 6B, 6E). Interestingly, however, coadministration of IL-17 and TNF- α produced a clear, synergistic induction in the number of transmigrated perivascular leukocytes at both the 6- and 16-h time points (Fig. 6A, 6B, 6F). These data demonstrate that IL-17 and TNF- α are capable of synergistically enhancing and sustaining leukocyte transmigration in vivo.

Endothelial activation with IL-17 and TNF- α promotes a synergistic increase in neutrophil but not effector CD4⁺ T cell transmigration under laminar shear flow in vitro

Given the enhancing effect of IL-17 and TNF- α on leukocyte transmigration in vivo, as well as the differential activity of IL-17 and IFN- γ on endothelial expression of neutrophil and T cell-activating chemokines, respectively, we hypothesized that endothelial activation with IL-17 and TNF- α would also lead to a selective increase in neutrophil but not T cell transmigration. To circumvent the limitation of IVM in accurately differentiating

endogenous leukocyte subsets, we chose to test this hypothesis using an in vitro parallel-plate flow chamber system, which allows for the controlled comparison of leukocyte subset recruitment under physiologically relevant levels of laminar shear flow (37, 43, 50). In these studies, neutrophils or effector CD4⁺ T cells were perfused across MHEC monolayers that had been preactivated with cytokines for 16 h, with the specific goal of determining if endothelial activation with IL-17 and TNF- α would selectively enhance the transmigration of either subset relative to treatment with IFN- γ and TNF- α . The number of accumulated and transmigrated leukocytes was then recorded over a 10-min period by video microscopy. In this model, in which perfused leukocytes are initially allowed to adhere at subphysiologic levels of shear stress (<0.2 dynes/cm²) before increasing flow to experimental levels (0.8 dynes/cm²), there was no statistically significant effect on neutrophil or T cell accumulation observed across treatment groups after 16 h of activation (Fig. 7A, 7G). Furthermore, we found that IL-17 alone did not significantly increase the transmigration of neutrophils or effector CD4⁺ T cells above levels observed with vehicle treatment alone (Fig. 7B, 7H). However, consistent with the chemokine expression data presented above

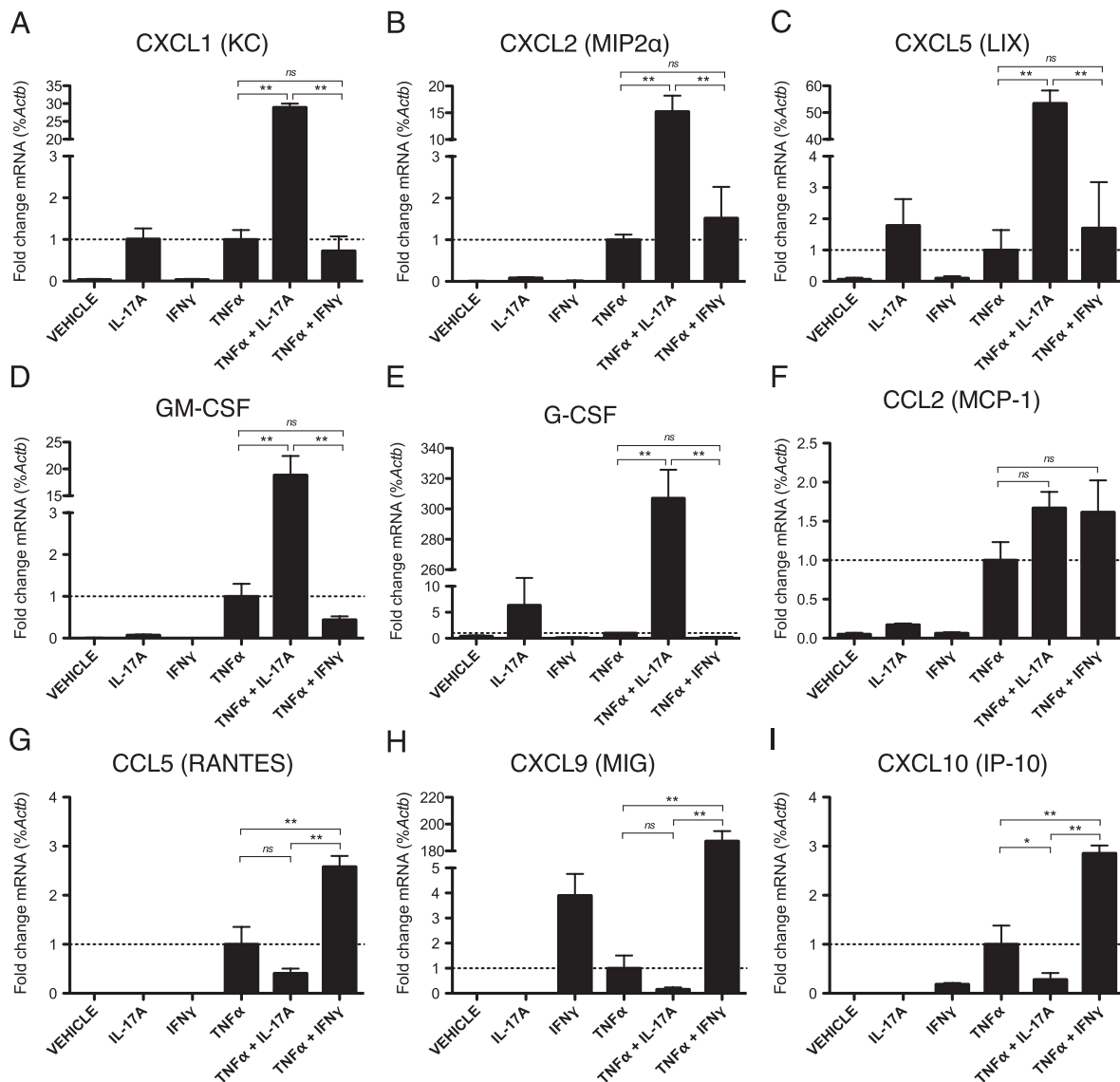


FIGURE 5. IL-17A and TNF- α synergistically increase endothelial chemokine expression in vitro. (A–I) qRT-PCR of chemokine mRNA expression after 8 h of stimulation with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml). Data are normalized to the level of β -actin (*Actb*) gene expression and represented as the mean fold change in %*Actb* expression \pm SEM relative to treatment with TNF- α alone. All data are from at least three independent experiments. * p < 0.05, ** p < 0.01.

(Fig. 5), we found that endothelial activation with IL-17 and TNF- α for 16 h led to a marked, synergistic increase in the level of neutrophil but not CD4⁺ T cell transmigration (Fig. 7B, 7H). By contrast, endothelial activation with IFN- γ and TNF- α selectively increased the transmigration of effector CD4⁺ T cells (Fig. 7H) but did not significantly alter neutrophil transmigration (Fig. 7B).

Synergistic effect of IL-17 and TNF- α on leukocyte transendothelial migration is dependent on neutrophil CXCR2 as well as endothelial selectins and ICAMs

To further examine the role of specific leukocyte–endothelial interactions in mediating the synergistic effects of IL-17 and TNF- α on neutrophil transmigration, we used blocking Abs against endothelial P-selectin, E-selectin, ICAM-1, and ICAM-2. We chose these targets in light of their respective roles in promoting neutrophil integrin activation, firm adhesion, and transmigration (30). In these studies, MHEC monolayers that had been preactivated with IL-17 and TNF- α for 16 h were incubated with blocking Abs just prior to neutrophil perfusion through the flow chamber. The total

number of accumulated and transmigrated cells was then determined as described above. We found that pretreatment with blocking Abs against P- and E-selectin led to a reduction in neutrophil accumulation, as well as a small but statistically significant reduction in the rate of neutrophil transmigration (Fig. 7C, 7D). Importantly, although treatment with blocking Abs against endothelial ICAM-1 and ICAM-2 did not affect neutrophil accumulation, it did lead to a significant reduction in neutrophil transmigration (Fig. 7C, 7D). These findings suggest that although endothelial selectins may contribute to neutrophil transmigration in response to IL-17 and TNF- α , there is perhaps a larger role for selectin-independent activation of neutrophil ICAM ligands, such as LFA-1 and Mac-1. In light of our findings that IL-17 and TNF- α synergistically increase endothelial expression of the CXCR2-activating chemokines CXCL1, CXCL2, and CXCL5 (Fig. 5A–C), we further hypothesized that activation of neutrophil CXCR2 by these molecules represented the central upstream event, leading to the increase in ICAM-dependent neutrophil transmigration. Accordingly, using neutrophils derived from *Cxcr2*^{-/-}

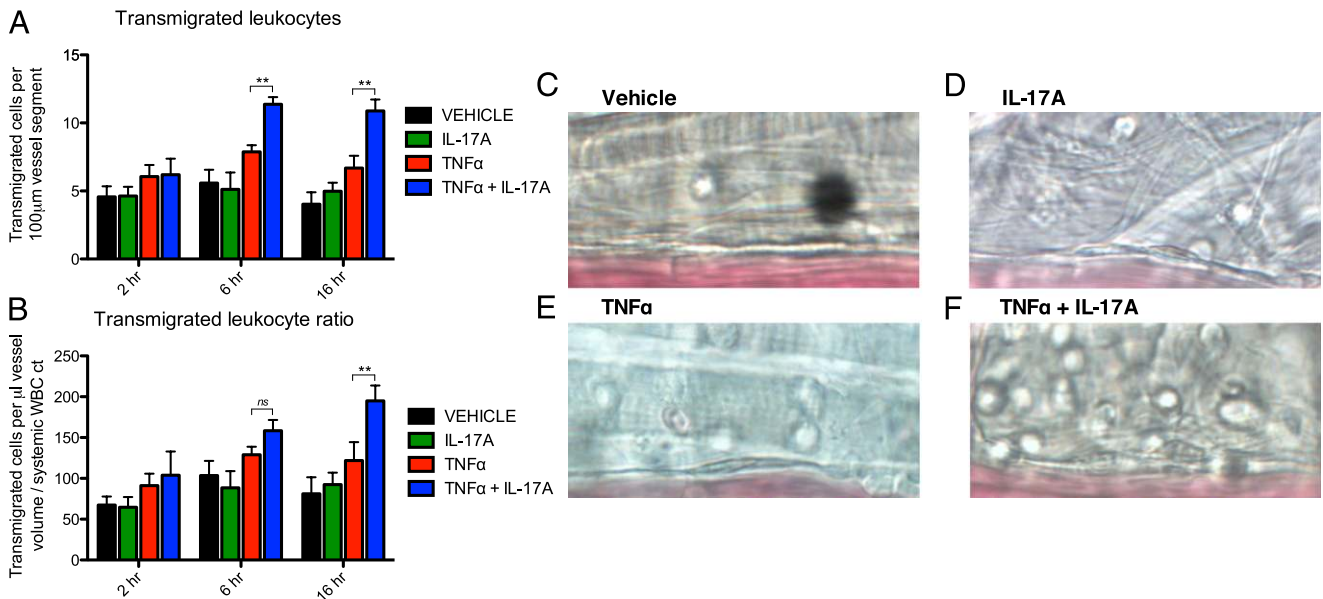


FIGURE 6. IL-17A and TNF- α synergistically increase leukocyte transendothelial migration in vivo. (A–F) Transmigrated leukocytes in the perivascular space ($50 \times 100 \mu\text{m}$ area) were quantified at three time points (2, 6, and 16 h) following intrascrotal injection with IL-17A (1000 ng/mouse) and/or TNF- α (100 ng/mouse). Data are represented as the mean \pm SEM of the total number of transmigrated cells per field of view (A) and as a ratio normalized for differences in microvessel volume and systemic leukocyte count (B). (C–F) Representative still images of transmigrated leukocytes for each treatment condition after 16 h of stimulation. Original magnification $\times 40$. Sizes of experimental groups and hemodynamic parameters for all data are summarized in Supplemental Table I. $**p < 0.01$.

mice, we demonstrated that CXCR2 deficiency had no effect on neutrophil accumulation (Fig. 7E) but completely abrogated the enhancing effect of IL-17/TNF- α on neutrophil transmigration (Fig. 7F). This finding indicates that the synergistic increase in neutrophil transmigration in response to IL-17 and TNF- α primarily reflects enhanced endothelial expression of CXCR2 ligands.

Discussion

Recent interest in the function of IL-17 stems from its defining role in mediating Th17 effector functions and a growing awareness of its importance for innate immunity. IL-17 has established roles in host defense against infection and the pathophysiology of autoimmune disease and may also contribute to atherosclerosis, plaque instability, and other forms of vascular inflammation. Given the beneficial effects of IL-17 gene deletion or Ab neutralization in several experimental models of inflammation, clinical trials have begun to test the therapeutic impact of IL-17 blockade in human autoimmune disease. Relatively little is known from a mechanistic perspective, however, regarding the functional importance of specific cell types or inflammatory molecules in mediating IL-17 activity. This has been particularly true for research into the role of IL-17 in endothelial activation, which plays a central part in regulating leukocyte influx to sites of inflammation. In particular, it is unclear if IL-17 is capable of directly inducing endothelial activation and leukocyte adhesion during inflammation or if its primary function is to modulate the effects of other proinflammatory cytokines. Although one prior study has suggested a potent role for IL-17 alone in endothelial activation, this finding contrasts with the experience of our own laboratory and highlights the conflicting nature of published literature on the question (34). For example, several studies in nonendothelial cell types have suggested that the predominant effect of IL-17 activity during inflammation derives from its ability to synergistically upregulate proinflammatory gene expression when combined with TNF- α or other innate cytokines

such as IL-1 β (35, 36). Furthermore, although Th1- and Th2-driven adaptive immune responses are typically neutrophil poor, observational data have noted that Th17 cells and neutrophils are often associated at sites of chronic inflammation. In this regard, there has been limited investigation into how the effects of IL-17 on endothelial activation might differ from other signature T cell cytokines, such as IFN- γ , and thus contribute to functional differences in leukocyte subset recruitment during Th17- versus Th1-dominant inflammation.

We sought to better define how IL-17 contributes to inflammation by examining its role in modulating endothelial activation and leukocyte subset recruitment in vitro and in vivo. We also explored the differential roles of IL-17 and IFN- γ in these processes to compare the potential influence of Th17- versus Th1-dominant immune responses. Our data demonstrate that IL-17 treatment alone, even at high concentrations, was less potent than TNF- α alone in promoting endothelial activation and leukocyte recruitment. Furthermore, we show that combined treatment with IL-17 and TNF- α promotes the synergistic activation of endothelial cells to express adhesion molecules and chemokines that specifically enhance and sustain neutrophil recruitment during inflammation. Specifically, we demonstrate that IL-17 and TNF- α synergistically enhance endothelial expression of P- and E-selectin in vitro and promote a synergistic increase in E-selectin-dependent leukocyte rolling in vivo. Furthermore, we show that endothelial activation with IL-17 and TNF- α synergistically enhances leukocyte transmigration in vivo and leads to an increase in the expression of neutrophil but not T cell-associated chemokines in vitro and a functional increase in neutrophil but not effector CD4 $^+$ T cell transmigration under laminar shear flow. Finally, we show that the enhancement of neutrophil transmigration in response to endothelial activation with IL-17 and TNF- α is chiefly mediated by neutrophil CXCR2 and endothelial ICAM expression, suggesting an important role for the CXCR2 ligands CXCL1, CXCL2, and CXCL5. Importantly, we also show that the effects of synergistic action between TNF- α and IL-17 versus

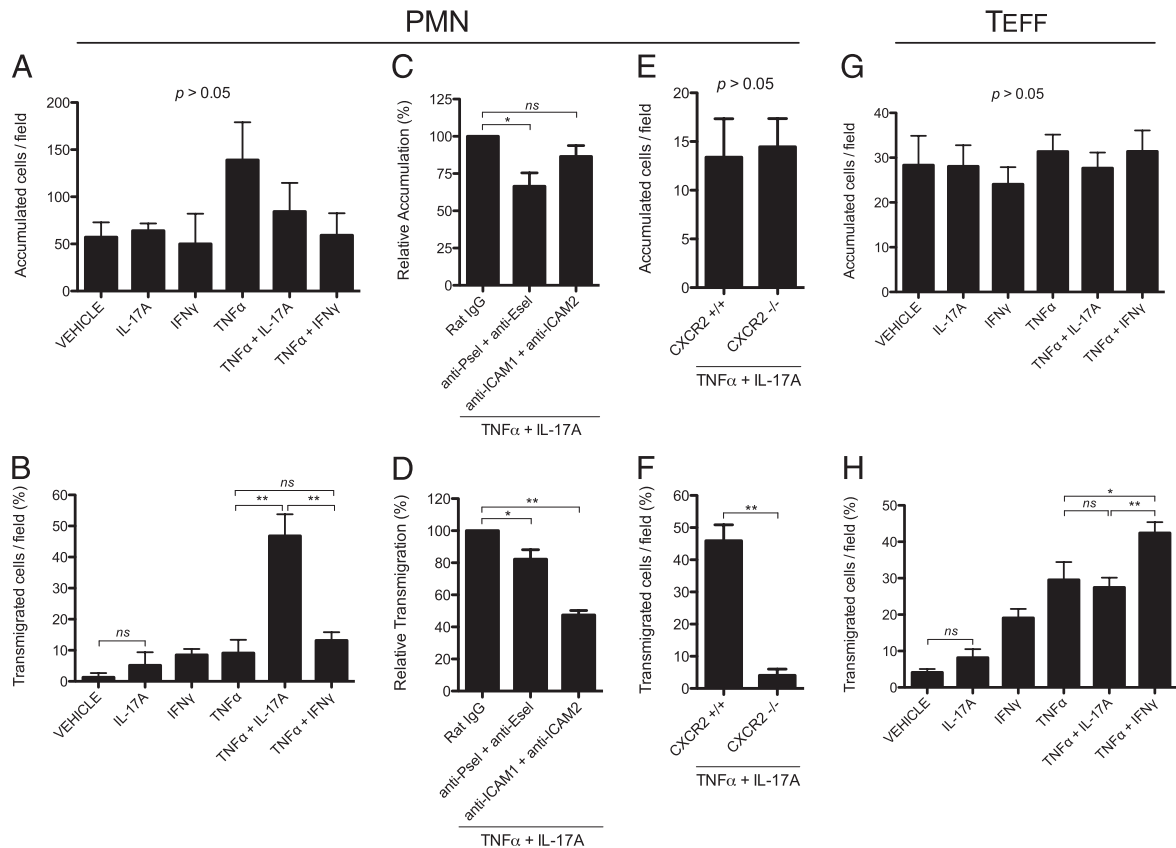


FIGURE 7. IL-17A and TNF- α synergistically increase neutrophil but not effector CD4⁺ T cell transmigration through endothelium under laminar shear flow. **(A and B)** Bone-marrow-derived neutrophils (PMN) were perfused at a defined shear stress (0.8 dynes/cm²) across MHEC monolayers that had been preactivated for 16 h with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml). Accumulated (A) and transmigrated (B) cells were then quantified by video microscopy. **(C and D)** MHEC were preactivated for 16 h with both IL-17A (100 ng/ml) and TNF- α (50 ng/ml) and incubated with blocking Abs (20 μ g/ml) against E-selectin (9A9), P-selectin (RB40.34), ICAM1 (YN1), and ICAM2 (3C4). Neutrophils were then perfused, and accumulated (C) and transmigrated (D) cells were quantified. Neutrophils from *Cxcr2*^{-/-} and *Cxcr2*^{+/+} mice were perfused over MHEC that had been activated for 16 h with both IL-17A (100 ng/ml) and TNF- α (50 ng/ml), and accumulated (E) and transmigrated (F) cells were quantified. **(G and H)** Effector CD4⁺ T cells (TEFF) were differentiated in vitro from naive CD4⁺ cells isolated from the spleens of WT mice. Cells were then perfused over MHEC that had been activated for 16 h with IL-17A (100 ng/ml), IFN- γ (100 U/ml), and/or TNF- α (50 ng/ml), and accumulated (G) and transmigrated (H) cells were quantified. All data are expressed as mean \pm SEM. All data are from at least three independent experiments, except for (E) and (F), which were from two independent experiments. * $p < 0.05$, ** $p < 0.01$.

TNF- α and IFN- γ are clearly distinct and associated with unique patterns of adhesion molecule and chemokine expression, as well as functional differences in the recruitment of neutrophils versus T lymphocytes, respectively.

In the current study, we identify a novel role for IL-17 in potentiating classical TNF- α -mediated inflammatory responses in endothelial cells. Although synergistic activity between IL-17 and TNF- α has been previously reported in other cell types with regard to chemokine expression (35, 36), the current study represents the first demonstration, to our knowledge, of the functional relevancy of this mechanism for endothelial activation and leukocyte recruitment during inflammation in vivo. In particular, synergistic and protracted upregulation of P- and E-selectin expression in response to IL-17 and TNF- α has not been previously reported, and this finding represents a clear alteration in the well-characterized temporal dynamics of E-selectin expression in response to TNF- α , which classically involves an early peak after 2–4 h of activation followed by a gradual regression toward baseline levels by 16–24 h (28, 51). As was previously demonstrated in several in vivo models, the robust induction of E-selectin at early time points during acute inflammation has a central role in driving peak levels of neutrophil influx, likely by promoting neutrophil slow rolling and integrin activation (40, 52–54). In-

terestingly, E-selectin has also been shown to enhance neutrophil integrin activation and recruitment in vivo in a cooperative manner with CXCR2 activation by chemokines such as CXCL1 (55), which has particular relevance to the current study in light of our data demonstrating a synergistic increase in endothelial expression of CXCL1, CXCL2, and CXCL5 in response to IL-17 and TNF- α . Synergistic regulation of neutrophil recruitment through enhanced E-selectin-dependent rolling and increased production of CXCR2-activating chemokines may, therefore, represent critical features of IL-17 and TNF- α proinflammatory activity on endothelium.

Although beyond the scope of the current study, elucidation of the signaling pathways that drive the synergistic induction of E-selectin and other genes in response to IL-17 and TNF- α represents an important avenue of future investigation. Interestingly, we have found that endothelial activation with IL-17 and TNF- α does not appear to enhance nuclear translocation of the proinflammatory transcription factor NF- κ B relative to TNF- α alone (data not shown), which is consistent with prior data in other cell types and suggests that synergy between IL-17 and TNF- α at the molecular level may be driven by a noncanonical mechanism of action (35, 36). Several prior studies in nonendothelial cell types have instead proposed a role for MAPK-dependent effects on the stability of specific mRNA transcripts (e.g., IL-8), although this has not yet been validated in

endothelial cells and may not be relevant to all genes that are synergistically regulated by IL-17 and TNF- α (35, 36, 56–59).

We believe these observations also contribute to our broader understanding of IL-17 activity during both innate and adaptive immunity. For example, in the setting of acute inflammation, recent studies have demonstrated that $\gamma\delta$ T cells are capable of secreting high levels of IL-17 in response to pathogen-associated activation of TLR1, TLR2, and Dectin 1 (60, 61). Importantly, this activation occurs well prior to the development of Th17-driven adaptive immunity and takes place independently of Ag-specific TCR engagement. Neutrophils, likewise, have also been proposed as an important innate source of IL-17, as suggested by a recent study of ischemia-reperfusion injury in the kidney (4). That IL-17 is produced at high levels by innate immune cells in response to pathogens and ischemia suggests that synergistic action between IL-17 and TNF- α could represent a highly conserved mechanism of host defense against infection and tissue injury. Similarly, in the setting of adaptive immunity, in which Th17 cells likely represent a predominant source of IL-17, synergistic action between IL-17 and TNF- α could sustain neutrophilic influx to sites of chronic inflammation. In this regard, it is interesting to consider the possibility of a positive-feedback loop related to Th17 and IL-17 activity, which could contribute to the indolent course of many forms of autoimmunity or the pathophysiology of acute exacerbations. For example, a recent study from our laboratory has demonstrated that Th17 cells have an enhanced ability to bind E-selectin relative to Th1 cells, which could preferentially enhance their recruitment to sites of inflammation following IL-17/TNF- α -mediated upregulation of E-selectin (62). Newly recruited Th17 cells could then further amplify local production of innate cytokines once present at the site of inflammation. For example, we have observed that Th17 cells are capable of directly producing high levels of TNF- α (G.K. Griffin, unpublished observations) in conjunction with IL-17, which in turn could stimulate further production of TNF- α and IL-1 β by macrophages (63). Although the activating effects of these cytokines would undoubtedly reinforce local chemokine expression and leukocyte influx, one prior study has made the important observation that Th17 cells and neutrophils are also capable of direct chemotactic cross talk involving the secretion of the neutrophil chemokine CXCL8 and the Th17 chemokine CCL20, respectively, which could further support their mutual recruitment to sites of inflammation (64).

Finally, the current study has clear implications for the therapeutic targeting of autoimmunity and other IL-17-associated diseases. The human relevance of our *in vitro* and *in vivo* findings using the murine system is supported by the fact that we have also found synergistic regulation of E-selectin expression in human saphenous vein endothelial cells (data not shown) and by a recent report published during the preparation of this manuscript showing that TNF- α and IL-17 synergistically enhance the prothrombotic phenotype of HUVECs (65). Although clinical trials are currently ongoing testing the efficacy of anti-IL-17 therapy in human autoimmune disease, in light of the present findings, it is interesting to consider the potential impact of dual anti-IL-17 and anti-TNF- α therapy, either in patients not responding to TNF- α blockade alone or as an adjunct during episodes of acute exacerbation. Likewise, as more is learned about the signaling pathways relevant to IL-17 and TNF- α proinflammatory activity, targeted approaches may become possible whereby synergistic action is selectively inhibited without interfering in the unique functions of these cytokines during host defense. Furthermore, although studies in animal models testing the role of IL-17 in atherogenesis have shown variable effects on total plaque burden, IL-17 may still have the potential to enhance atherothrombotic risk by stimulating neutrophil recruitment and

inflammation within established plaques (17–20, 24, 66). This notion may be particularly relevant to patients with rheumatoid arthritis or to recipients of cardiac allografts, in whom IL-17-associated inflammatory disease is superimposed on an elevated baseline cardiovascular risk (67–70). In light of ongoing clinical trials to test the efficacy of anti-IL-17 therapy for human autoimmune disease, our data support the promise of these approaches for a variety of IL-17-associated vascular inflammatory disorders in which endothelial inflammation is critically involved in the initiation and progression of disease.

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Disclosures

The authors have no financial conflicts of interest.

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