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Induction of Endothelial Nitric Oxide Synthase Expression by IL-17 in Human Vascular Endothelial Cells: Implications for Vascular Remodeling in Transplant Vasculopathy

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IL-17 is a signature cytokine of Th17 cells, a recently described subset of effector CD4 T cells implicated in the development of several pathologies. We have examined the role of IL-17 in regulating endothelial NO synthase (eNOS) expression in human vascular endothelial cells (ECs) because of the key role of eNOS in determining the pathological outcome of immune-mediated vascular diseases. In cultured ECs, IL-17 increased expression of eNOS, eNOS phosphorylation at Ser1177, and NO production. The induction of eNOS expression by IL-17 was prevented by the pharmacological inhibition of NF-κB, MEK, and JNK, as well as by small interfering RNA-mediated gene silencing of these signaling pathways. The expression of IL-17 was then examined by immunohistochemistry in human arteries affected by transplant vasculopathy (TV), a vascular condition that is a leading reflection of chronic heart transplant rejection. IL-17 was expressed by infiltrating leukocytes in the intima of arteries with TV, and the majority of IL-17–positive cells were T cells. The number of IL-17–positive cells was not correlated with the intima/media ratio, but was negatively correlated with the amount of luminal occlusion. There was also a significant positive correlation between the number of IL-17–positive cells and the density of eNOS-expressing luminal ECs in arteries with TV. Altogether, these findings show that IL-17 induces the expression of eNOS in human ECs and that this may facilitate outward expansion of arteries afflicted with TV. The Journal of Immunology, 2012, 188: 1544–1550.

Vascular endothelial cells (ECs) regulate inflammatory and immune responses, as well as the flow of blood and nutrients into tissues. These aspects of EC biology are regulated by cytokines (1). IL-17 is a signature cytokine produced by Th17 cells, a recently characterized effector subset of CD4 T cells implicated in physiological responses to extracellular bacteria and fungi as well as in the pathogenesis of several immunological conditions (2–4). Despite the established role of IL-17 in pathology, very little is known about how this cytokine affects cells within target tissues during immune responses. IL-17 binds to a heteromeric receptor composed of IL-17RA and IL-17RC subunits, and stimulation of cells with IL-17 leads to activation of the canonical NF-κB, MEK-ERK1/2, PI3K-Akt, JNK, and p38 MAPK pathways (5). Both receptor subunits are expressed on human vascular ECs, and IL-17 triggers phenotypic changes in this cell type that regulate immune responses and angiogenesis (6, 7).

NO is produced by the conversion of L-arginine to L-citrulline and NO by NO synthases (NOSs). There are three isoforms of NOSs: neuronal, inducible (iNOS), and endothelial (eNOS). eNOS is expressed constitutively by the endothelium and stimulates arterial vasodilation (8). eNOS-derived NO also regulates inflammation, immune responses, and angiogenesis (9). Inflammatory cytokines alter the expression and activity of eNOS, and the effects of different inflammatory and immunological conditions on eNOS may be distinct depending on the cytokine milieu (10–12).

Immune targeting of arteries in transplanted organs causes transplant vasculopathy (TV), a leading representation of chronic solid organ transplant failure (13). TV in its full development is characterized by intimal thickening and luminal narrowing of allograft arteries, ultimately leading to acute occlusive events and associated tissue infarction. The pathogenesis of TV involves two main arterial changes: structural changes leading to thickening of the intima and vasomotor dysfunction characterized by defective arterial vasodilation and/or aberrant vasoconstriction (14). Thickening of the arterial intima in TV is caused by immune-mediated damage and dysfunction of the graft endothelium, which leads to leukocyte and smooth muscle cell migration into this layer of the vessel wall (15). Wall thickening due to intimal hyperplasia is opposed by outward remodeling, a process that is believed to involve the acute production of endothelial-derived vasodilators, such as NO, followed by chronic changes that involve extracellular matrix alterations (16). The resultant arterial outward expansion preserves lumen size and blood flow (17). In TV, early endothelial dysfunction results in inadequate vasodilation or aberrant vasoconstriction that prevents arterial expansion, and this contributes to an overall reduction in lumen size termed inward remodeling. This endothelial dysfunction has been suggested to be the main contributor to luminal occlusion in allograft
arteries (18, 19). It is clear that dysregulation of the balance between vasodilatory and vasoconstrictive properties within allograft arteries determines the pathological outcome of TV.

Systemic blockade of IL-17 activity does not affect intimal thickening of allograft arteries in a complete MHC-mismatched aortic interposition model of TV or in a humanized mouse model of coronary allograft artery rejection, although neutralization of IL-17 has recently been shown to prevent intimal thickening of coronary arteries in hearts transplanted into MHC class II disparate recipients that lack the Th1-inducing transcription factor T-bet (20–22). Yet, the effect of IL-17 on luminal occlusion was not examined in any of these described studies, and the role of IL-17 in regulating the vasoregulatory properties of ECs is not known. Also, the expression of IL-17 in human TV remains uncharacterized. Because of the importance of endothelial expression of eNOS in vascular integrity and vasomotor function, in the current study, we have examined the effect of IL-17 on eNOS expression in human ECs.

We show that IL-17 induces the expression of eNOS and concomitant production of NO in this cell type. The induction of eNOS expression by IL-17 requires NF-κB, MEK1, and JNK signaling pathways. To establish the relevance of our findings in human pathology, the expression of IL-17 was examined in human specimens of TV. IL-17 was expressed in infiltrating T cells, and the abundance of IL-17 was not correlated with the amount of intimal thickening. However, the abundance of IL-17–positive leukocytes was negatively correlated with luminal occlusion and positively correlated with eNOS expression. Together, these findings indicate that IL-17 regulates eNOS expression in the vascular endothelium and that such regulation may impact vascular remodeling in TV.

Materials and Methods

Human cell culture and reagents

Human microvascular ECs (HMVECs) and human coronary artery ECs were purchased from Lonza (Basel, Switzerland) and cultured in EGM-2 MV medium. Cells were used up to the sixth passage as described previously (23). Bovine aortic ECs were cultured in DMEM + 10% FCS and used between passages 5 and 15. Cells were untreated or treated with recombinant human IL-17 (PeproTech, Rocky Hill, NJ) prior to examination of eNOS expression and NO production. Specificity of the EC response to IL-17 was determined by neutralizing IL-17 activity with a neutralizing Ab (R&D Systems, Minneapolis, MN). In some experiments, ECs were incubated with the following inhibitors prior to stimulation with IL-17: pyrrolidine dithiocarbamate (PDTC; 100 μM; Enzo Life Sciences, Farmingdale, NY), rabbit polyclonal Ab to IL-17 (10 μg/ml; R&D Systems), mouse mAb to human CD3 (1/25; DakoCytomation), rabbit polyclonal Ab to eNOS (Abcam, Cambridge, MA), or mouse mAb to CD31 (1/25; DakoCytomation). For single-color staining, primary Ab incubations were followed by incubation of sections with biotin-conjugated secondary Abs followed by HRP-conjugated avidin (DakoCytomation). Staining was visualized using AEC substrate-chromagen (Vector Laboratories, Burlingame, CA), which results in red staining, and sections were counterstained with hematoxylin to visualize cell nuclei. For two-color staining, IL-17 positivity was visualized with AEC substrate chromagen, resulting in red staining. CD3 was detected with an alkaline phosphatase-conjugated secondary Ab followed by visualization with Vector Blue Alkaline Phosphatase substrate (Vector Laboratories), resulting in blue staining.

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS). Identification of differences between two groups was determined using a Student t test and those between multiple groups by two-way ANOVA followed by a least significant difference post hoc analysis. A Pearson’s analysis was performed to determine correlations. In all cases, an α value of ≤0.05 was determined to be significant.

Results

IL-17 induces eNOS expression and NO production in human ECs

To begin investigating the effects of IL-17 on eNOS expression, HMVECs were treated with IL-17 (10 ng/ml), lysates collected at various time points after stimulation, and eNOS expression examined by immunoblot analysis. IL-17 induced eNOS expression as early as 2 h poststimulation, and this persisted until 8 h poststimulation (Fig. 1A, 1B). eNOS levels were reduced to basal levels after 16 and 24 h (data not shown). In addition to defining the kinetics of IL-17–mediated eNOS induction, HMVECs were also stimulated with various concentrations of IL-17 and eNOS expression examined. IL-17 induced eNOS expression in a dose-dependent manner (Fig. 1C). eNOS expression was significantly increased in response to 10 and 100 ng/ml of IL-17, and a maximal induction of 2.7-fold over unstimulated cells was observed at 10 ng/ml of IL-17 (Fig. 1D). Similar findings were observed in human coronary artery ECs (Fig. 1C). Given the similar effects of IL-17 on HMVECs and human coronary artery ECs, all subsequent experiments were performed using HMVECs. Importantly, the induction of eNOS expression in stimulated ECs was due to

ECs were lysed and immunoblots were performed as described previously (24). Membranes were incubated with Abs to eNOS (BD Biosciences, San Diego, CA), phosphorylated eNOS (Ser1177; BD Biosciences), NF-κB essential modulator (NEMO; BD Biosciences), MEK1 (BD Biosciences), JNK (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich). Quantification of data were performed by normalizing eNOS expression to β-actin levels using ImageJ software (National Institutes of Health), followed by calculating the fold increase as compared with the indicated groups.

Quantification of NO

NO production by ECs was measured by quantifying the accumulation of nitrite in culture supernatants as described previously (24). Briefly, bovine aortic ECs were untreated or treated with IL-17. Culture supernatants were collected after 16 h and nitrite concentrations measured using an NO-specific chemiluminescence analyzer (Sieves, Boulder, CO).

Small interfering RNA-mediated gene silencing

Small interfering RNA (siRNA)-mediated gene silencing was performed as described previously (23). Subconfluent ECs were harvested by trypsinization and 5 × 10^5 cells transfected with 100 pmol control or gene-specific siRNA per reaction using an Amaz sequentially (Lanza). Validated control, NEMO, MEK1, and JNK siRNA was purchased from Qiagen (Valencia, CA). After transfection, cells were rested for 48 h prior to examination of knockdown efficiency and eNOS induction by IL-17.

Human specimens of TV

Eleven formalin-fixed, paraffin-embedded samples of allograft arteries with TV were obtained under ethics-approved protocol from the St. Paul’s Hospital Cardiovascular Registry (Vancouver, BC, Canada). All samples were acquired from explanted cardiac allografts and processed as described elsewhere (25). The mean age of allograft recipients was 55.2 ± 15.8 y, and 10 out of the 11 specimens were from allografts implanted into male recipients.

Morphological analysis

Cross-sections (5 μm) of coronary arteries with TV were stained using an elastic Van Gieson protocol to visualize the elastic laminae. The external and internal elastic laminae, as well as the circumference of the lumen, were highlighted by manual tracing and then the area within each circumference quantified using ImageJ software (National Institutes of Health). The medial, intimal, and luminal areas were calculated from these measurements and the intima/media ratio and percentage of luminal occlusion determined as described previously (26).

Immunohistochemistry

Cross-sections of coronary arteries were stained by immunohistochemistry as described previously (27) using a goat polyclonal Ab to IL-17 (10 μg/ml; R&D Systems), mouse mAb to human CD31 (1/25; DakoCytomation), rabbit polyclonal Ab to eNOS (Abcam, Cambridge, MA), or mouse mAb to CD31 (1/25; DakoCytomation). For single-color staining, primary Ab incubations were followed by incubation of sections with biotin-conjugated secondary Ab followed by HRP-conjugated avidin (DakoCytomation). Staining was visualized using AEC substrate-chromagen (Vector Laboratories, Burlingame, CA), which results in red staining, and sections were counterstained with hematoxylin to visualize cell nuclei. For two-color staining, IL-17 positivity was visualized with AEC substrate chromagen, resulting in red staining. CD3 was detected with an alkaline phosphatase-conjugated secondary Ab followed by visualization with Vector Blue Alkaline Phosphatase substrate (Vector Laboratories), resulting in blue staining.

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the specific actions of IL-17 because blockade of IL-17 activity with a neutralizing Ab eliminated eNOS induction (Fig. 1E). Some reports have suggested that ECs can express iNOS, although this is controversial (28, 29). In our experiments, iNOS was not expressed in unstimulated ECs and was not induced by IL-17 (data not shown).

Phosphorylation of human eNOS on Ser\(^{1177}\) leads to activation of this enzyme (30). Consistent with our observation that IL-17 increases eNOS expression, stimulation of ECs with IL-17 also increased the levels of Ser\(^{1177}\)-phosphorylated eNOS (Fig. 2A). In addition, IL-17 increased significantly the production of NO from ECs (Fig. 2B). Taken together, the above data show that IL-17 induces eNOS expression and concomitant NO production in ECs.

**Induction of eNOS expression by IL-17 requires NF-κB, MEK1, and JNK signaling pathways**

IL-17 activates NF-κB, PI3K-Akt, MEK-ERK1/2, JNK, and p38 signaling pathways (31). To begin to determine the signaling pathways necessary for the induction of eNOS expression in response to IL-17, ECs were treated with pharmacological inhibitors to NF-κB (PDTC), PI3K (LY294002), MEK (PD98059), JNK (SP600125), and p38 (SB203580) prior to stimulation with IL-17. Treatment of ECs with these inhibitors did not affect basal levels of eNOS (data not shown). However, pharmacological inhibition of NF-κB, MEK1, and JNK prevented IL-17-mediated induction of eNOS, whereas inhibition of PI3K and p38 had no effect (Fig. 3A, Supplemental Fig. 1).

The role of NF-κB, MEK1, and JNK pathways in the induction of eNOS by IL-17 was then examined by siRNA-mediated gene silencing of signaling molecules required for activation of each pathway. Activation of the canonical NF-κB pathway requires IκB degradation that is triggered by the activation of IκB kinase (IKK)-β and, in certain instances, IKKα (32, 33). This pathway is entirely dependent on NEMO (IKKγ), which complexes with IKKβ (and IKKα in certain circumstances) and facilitates their activation. As such, we inhibited expression of NEMO using siRNA gene silencing and examined the effects on eNOS expression. Transfection of ECs with NEMO siRNA markedly inhibited expression of this signaling molecule (Fig. 3B). IL-17 induced the expression of eNOS in ECs transfected with control RNA, but did not induce eNOS expression in ECs transfected with NEMO siRNA (Fig. 3B). In addition to the NF-κB pathway, we inhibited expression of MEK1 and JNK. There was marked re-
duction of MEK1 and JNK protein levels after transfection of ECs with the respective siRNA sequences (Fig. 3C, 3D). When eNOS expression was examined, inhibition of either MEK1 or JNK completely prevented the induction of eNOS expression by IL-17 (Fig. 3C, 3D).

Characterization of IL-17 expression and accumulation in TV
To determine the potential contribution of IL-17 to vascular changes and to the regulation of eNOS expression in TV, we examined expression of this cytokine in formalin-fixed, paraffin-embedded specimens of human allograft arteries by immunohistochemistry. IL-17 was expressed by cells in the intima of arteries with TV (Fig. 4A). The staining was specific for IL-17 because adsorption of the primary Ab with an excess concentration of rIL-17 prior to its addition to the tissue specimens eliminated the staining (Supplemental Fig. 2). Morphologically, IL-17–positive cells resembled infiltrating leukocytes, and two-color immunohistochemistry for IL-17 and CD3 indicated that the majority of IL-17–positive cells were T cells (Fig. 4B, 4C). IL-17 positivity in our examination could reflect IL-17 expression by leukocytes and/or binding of IL-17 to its receptor on the surface of these cells.

IL-17 is negatively correlated with luminal narrowing and positively correlated with eNOS expression in TV
To examine the potential role of IL-17 in the vascular changes that occur in TV, the number of IL-17–positive cells in the intima of allograft arteries was counted and normalized to the total number
of intimal T cells. This measure was then examined in relation to intimal thickening, as determined by the intima/media ratio, and to the extent of luminal occlusion. The intima/media ratio provides a measure of intimal thickening, and the extent of luminal occlusion reflects the net effect of intimal thickening and vascular remodeling (either outward or inward). There was no significant correlation between the number of IL-17–positive cells and the intima/media ratio of allograft arteries (Fig. 4D). However, there was a significant negative correlation between the number of IL-17–positive cells and percentage of luminal occlusion of allograft arteries with TV (Fig. 4E). Moreover, there was no correlation between the number of IL-17–positive cells and time posttransplantation at which the arteries were obtained, suggesting that our observations are not confounded by temporal differences in the allogeneic immune response (Supplemental Fig. 3). Because the intima/media ratio reflects thickening of the intima, whereas the percentage of luminal occlusion reflects the net effect of intimal thickening and remodeling, these data indicate that IL-17 may be involved in supporting outward remodeling in TV.

The production of NO by eNOS is involved in outward remodeling (34, 35). Because we observed the induction of eNOS by IL-17 in cultured ECs and our examination of clinical samples of TV suggests that IL-17 may be involved in outward remodeling, we examined the expression of eNOS in allograft arteries with TV. eNOS was observed in cells lining the lumen in all arteries. Staining of adjacent sections for CD31 confirmed the expression of eNOS in luminal ECs (Fig. 5A). The number of eNOS-positive cells lining the lumen of allograft arteries was then quantified and normalized to the total length of CD31 positivity lining the lumen of each artery. This measure, which provides a quantitative determination of the density of eNOS-positive cells in the luminal endothelium of allograft arteries, was then examined in relation to the number of IL-17–positive cells. There was a significant positive correlation between the number of IL-17–positive cells and the number of eNOS-positive cells in allograft arteries with TV (Fig. 5B). These findings support our in vitro data and suggest that IL-17 is involved in the induction of eNOS expression in TV.

**Discussion**

Modulation of eNOS expression and activity affects the outcome of inflammatory vascular diseases (36). Despite the clear contribution of immune responses to these pathologies, the effect of IL-17 on eNOS expression has been poorly characterized. We show that IL-17 induces eNOS expression in human ECs and that this effect requires the coordinated activation of NF-κB, MEK1, and JNK signaling pathways. Further, through examination of human specimens of TV, we provide evidence consistent with the notion that IL-17 induces eNOS expression in the endothelium of allograft arteries and that this induction offsets luminal occlusion by supporting outward vascular expansion.

The cytokine regulation of eNOS expression and activity is complex. IL-17 has been reported to induce eNOS mRNA expression in bone marrow-derived cells, but protein expression and the signaling pathways regulating eNOS induction were not examined (37). We show that, in ECs, IL-17 induces eNOS protein expression through activation of NF-κB, MEK1, and JNK pathways. This mechanism is similar to the induction of eNOS expression by shear stress, which involves MEK-mediated activation of NF-κB (38). The human eNOS promoter contains binding sites for NF-κB and AP-1 (39), and the induction of eNOS promoter activity by shear stress requires NF-κB, but not AP-1, binding to cis-acting promoter elements (38). MEK1 is also able to induce eNOS expression in response to mechanical strain in bone marrow-derived stromal cells (40). In addition to MEK1 and NF-κB, we find that JNK is also required for the induction of eNOS expression by IL-17. JNK is known to induce eNOS expression in response to lysophosphatidylcholine in human umbilical vein ECs, but the downstream mechanisms by which this occurs remain undefined (41).

In contrast to the role of NF-κB in the induction of eNOS expression by IL-17, this signaling pathway has been reported to inhibit eNOS expression in ECs in response to TNF-α (42). As such, the regulation of eNOS expression by NF-κB is context specific and could depend on variations in the strength of activation of this signaling pathway caused by different cytokines, on differences in the activation of parallel signaling pathways, and/or on the differential activation of downstream DNA-binding proteins. In support of the first possibility, IL-17 is a weaker activator of NF-κB than TNF-α (43). Interestingly, attenuation of eNOS expression by TNF-α is somewhat offset by increased eNOS enzymatic activity caused by this cytokine in human umbilical vein ECs (12). Increased eNOS enzymatic activity in this setting is due to a TNF-α–mediated increase in the production of the eNOS cofactor tetrahydrobiopterin. We have observed that stimulation of ECs with IL-17 increases both eNOS expression and NO production.

In allograft arteries, agonist-induced vasodilation is severely compromised by IFN-γ–mediated inhibition of eNOS expression (19, 44). Thus, biological processes that support vasodilation through the induction of NO production in ECs have the potential to preserve lumen diameter and maintain blood flow through allograft arteries. Preservation of lumen diameter by outward remodeling of arteries is also required for the maintenance of proper blood flow to downstream tissues in the presence of intimal hyperplasia. It is known that flow-mediated outward vascular remodeling is defective in eNOS-deficient mice (34), although eNOS is also required for inward remodeling (45). Altogether, our in vitro experiments and examination of clinical samples of TV indicate that IL-17 may contribute to outward remodeling by inducing eNOS expression in luminal ECs. Rao et al. (21) examined

**FIGURE 5.** eNOS expression in luminal ECs is positively correlated with IL-17 expression in human TV. A. Adjacent sections of formalin-fixed, paraffin-embedded samples of human arteries with TV were immunohistochemically stained for eNOS and CD31 (red) and also counterstained with hematoxylin (blue). Arrows depict eNOS expression in the luminal ECs. Original magnification ×400. B. The number of eNOS-positive cells that were lining the lumen and that overlapped with CD31-positivity was normalized to the length of CD31-positivity (eNOS-positive cells [#]/CD31 length [mm]). This was correlated with the number of IL-17–positive cells in the intima of allograft arteries.
the effects of IL-17 on vascular changes in a humanized mouse model of TV in which immunodeficient mice housing human coronary arteries are adoptively transferred with allogeneic human T cells. In this model, IL-17 blockade did not significantly affect intimal thickening or the total vessel area. Luminal occlusion was not directly examined (21). Consistent with our findings, Madhur et al. (46) showed recently that IL-17 does not affect intimal thickening but supports outward remodeling in a mouse model of atherosclerosis. Finally, an alternative explanation for the negative correlation between IL-17–positive cells and luminal occlusion that we observe could be that IL-17 responses predominate early in allogeneic responses. However, there was no correlation between the number of IL-17–positive cells and the time post-transplantation at which allograft artery specimens were obtained.

In summary, the role of IL-17 in regulating intimal thickening has been examined in experimental models of vascular diseases such as TV, atherosclerosis, and arteritis. However, the involvement of this cytokine in processes that regulate vasomotor function and in arterial remodeling has remained unclear. Our identification of a role for IL-17 in the induction of eNOS in human ECs and in the potential preservation of lumen diameter in human arteries with TV provides important insights into the pathogenesis of TV specifically and vascular disease in general.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Inhibition of p38 MAPK does not affect the induction of eNOS expression by IL-17. ECs were treated with DMSO or the p38 inhibitor SB203580 (SB) prior to being left unstimulated or stimulated with IL-17. Cell lysates were prepared 6 h later, and eNOS and β-actin expression determined by immunoblot analysis.
Supplemental Figure 2. Positive staining for IL-17 is prevented by adsorption of the primary antibody with IL-17. Serial sections of human allograft arteries with TV were incubated with isotype control, anti-IL-17, or anti-IL-17 that had been pre-incubated with an excess concentration of recombinant IL-17 for 30 min. Arrows denote IL-17-positive cells. Mag = 200X.
Supplemental Figure 3. The number of IL-17-positive cells in arteries with TV is not correlated with time post-transplant. The number of IL-17-positive cells (normalized to total number of T cells) in the intima of human specimens of TV was examined in relationship to the time post-transplantation at which the arteries were obtained.