Effects of Interleukin-17 on Endothelial Nitric Oxide Synthase and Transplant Arteriosclerosis

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Abstract

Transplant arteriosclerosis (TA), a vascular condition characterized by intimal thickening and vasomotor dysfunction of allograft arteries, is a leading cause of solid organ transplant failure. Properties of the endothelium such as the activity of eNOS control the structural and functional changes that occur in arteries with TA. We have examined the effect of IL-17 on eNOS expression in endothelial cells. Up-regulation of eNOS by IL-17 occurred through a post-translational mechanism because there was no effect of IL-17 on eNOS mRNA levels and inhibition of mRNA translation with cycloheximide did not prevent eNOS induction; but IL-17 treatment of ECs prolonged eNOS protein half-life. To begin examining the role of IL-17 signaling in graft cells in the development of TA, aortic segments from WT and IL-17RA-KO mice were interposed into allogeneic recipients. There were no significant changes in terms of TA development in IL-17RA-KO transplants compared to WT transplants. In summary, these results begin to define the cellular mechanisms by which IL-17 induces eNOS and the relevance of this cytokine to the pathogenesis of TA.

Keywords: Transplantation; Transplant arteriosclerosis; T helper 17; Interleukin-17; endothelial nitric oxide synthase; nitric oxide

Dedication

To my parents, Cliff and Nancy, thank you for all the support. My supervisor, Jonathan Choy, for accepting me into the lab and all the help. My lab partners and friends, Martin, Anna, Nichole, Tallie, Gursev, Anh and Nichole. My dear friend, Cindy, who cheers me on and encourages me to get through the obstacles. Lastly, to everyone else who kindly helped me along the way.

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List of Acronyms

APC	antigen presenting cells
ACT1	NFκB activator 1
AKT	protein kinase B
ANOVA	analysis of variance
BH_4	tetrahydrobiopterin
CHX	cycloheximide
CD4	cluster of differentiation 4
cGMP	cyclic guanine monophosphate
CXCL	chemokine (C-X-C motif) ligand
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
EDTA	ethylenediaminetetraacetic acid
EDHF	endothelial-derived hyperpolarizing factor
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
G-CSF	granulocyte colony-stimulating factor
HSP90	heat shock protein 90
ICAM-1	intracellular adhesion molecule 1
IFNγ	interferon gamma
IL-17	interleukin-17
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein kinase kinase
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
mTORC	mammalian target of rapamycin complex
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells

NO	nitric oxide
NADPH	nicotinamide adenine dinucleotide phosphate
NK cell	natural killer cell
nNOS	neuronal nitric oxide synthase
PI3K	phosphoinositide 3-kinase
PKG	protein kinase G
PRD	positive regulatory domain
RT-qPCR	reverse transcription- quantitative polymerase chain reaction
sGC	soluble guanylyl cyclase
SMC	smooth muscle cell
SP1	specificity protein 1
Th17	T helper 17
TA	transplant arteriosclerosis
T-bet	T-box transcription factor TBX21
TCR	T cell receptor
TGFβ	transforming growth factor beta
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TRAF6	TNF receptor associated factor 6
UTR	untranslated region
vEGF	vascular endothelial growth factor
VE-cad	vascular endothelial cadherin

Chapter 1.

Introduction

1.1. Organ transplantation

Solid organ transplantation remains the only treatment for many diseases that lead to end-stage organ failure. This procedure is therefore life-saving for many people but most grafts eventually fail within 20 years. Graft recipients also need to be maintained on immunosuppressive drugs which have many morbid side effects [1]. As such, the use of organ transplantation as a therapy is currently suboptimal and there is a need for better understanding of the mechanisms by which graft failure occurs in order to specifically prevent it. In general, graft failure occurs because the immune system recognizes genetic differences between the graft and the recipient. The most important genetic difference is that between major histocompatibility complex (MHC) molecules, which present antigens to T cells and activate immune responses that destroy the graft [2]. Through the recognition of foreign peptides presented by either host or donor MHC, host T cells become activated and exert their respective effector functions as cytotoxic or helper T cells resulting in graft destruction.

Rejection of grafts is mediated by the adaptive immune response of the host. T cells from the graft recipient recognize foreign peptide:MHC (pMHC) complexes presented by graft cells. This triggers a response similar to an infection where a specific subpopulation of T cells is activated as a result of T cell receptor (TCR) activation, and these T cells proliferate and lead to the destruction of antigen-bearing cells in the transplanted tissue [3].

Immune-mediated organ damage occurs through multiple mechanisms. Cytotoxic CD8+ effector T cells directly kill graft cells by releasing cytotoxic granules that

contain the death-inducing proteins perforin and granzymes as well as by expressing Fas ligand which activates apoptosis in target cells by engaging Fas [4-6]. CD4+ effector T cells secrete cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) (Th1), which recruit other immune cells as well as boost activation of cytotoxic CD8+ T cells or B cells [7, 8]. These cytokines also induce phenotypic changes in graft cells that lead to tissue remodelling and/or fibrosis [9-11].

Recognition of the foreign graft by the immune system leads to the infiltration of T cells and macrophages as well as the targeting of graft cells by antibodies [12]. Prolongation of graft survival with better MHC matching and immunosuppressants have diminished the incidence of acute rejection but has not changed graft loss due to chronic rejection, which typically occurs after 1 year post-transplantation [13]. Chronic rejection is characterized by fibrosis of the allograft which is caused by a continuous immune-targeted cell death and wound healing of the graft cells [14, 15]. The mechanisms by which chronic rejection develops may be distinct in different organ grafts, but all eventually result in organ dysfunction. In heart transplants, transplant arteriosclerosis is the main reflection of chronic graft failure and it disrupts blood flow inside the allograft causing ischemic organ failure [16].

1.2. Transplant arteriosclerosis (TA)

TA is a vascular condition that affects most solid organ transplants. It is characterized by intimal thickening and vasomotor dysfunction of allograft arteries. Intimal thickening in TA results from the deposition and proliferation of smooth muscle cells and leukocytes underneath the endothelium [16]. There is also the aberrant deposition of extracellular matrix and lipids [17]. As the intima grows, the effective luminal area of affected arteries decreases and this restricts blood flow leading to ischemia of downstream tissues.

Targeting of allogeneic arterial endothelial cells and smooth muscle cells by host T cells causes intimal thickening in TA [18, 19]. Both CD4- and CD8-positive T cells contribute, albeit with different mechanisms. Upon activation, CD4 T cells can develop into three main subsets that migrate to peripheral tissues: Th1, Th2, or Th17. When

activated, Th1 cells secret IFNγ [20-22] which activates endothelial cells to express MHC class I and II [23]. The activated endothelial cells support leukocyte migration into allograft arteries as well as present foreign antigens to T cells [23]. Immune-mediated endothelial damage by cytotoxic T cells and IFNγ causes inflammation in the arterial wall and triggers the migration and proliferation of vascular smooth muscle cells into the intima [24, 25]. This smooth muscle cell migratory process is mediated by cytoskeletal changes in response to growth factors such as platelet-derived growth factor and basic fibroblast growth factor [26-28]. IFNγ also acts directly on vascular smooth muscle cells to promote their proliferation through the mTORC1/S6K1 pathway [18]. All together, the above changes drive the development of intimal thickening by causing the intimal accumulation of smooth muscle cells and leukocytes.

In addition to the described structural changes in TA, luminal occlusion is also driven by functional abnormalities. Arteries are normally capable of expanding to increase blood flow, either in response to acute stimulation or to arterial wall thickening. [29]. During the development of TA, arteries are unable to outwardly expand normally and may actually constrict. This functional abnormality further reduces blood flow [29]. Outward expansion of arteries involves endothelial-dependent production of nitric oxide and immune responses compromise endothelial nitric oxide synthase expression and activity in TA [30](see section 1.4 for nitric oxide and endothelial nitric oxide synthase).

1.3. Blood vessels and endothelial cells

Structurally, arteries are composed of three main layers: intima, media and adventitia. In healthy arteries the intimal layer consists of the space between the luminal endothelial cells and internal elastic lamina. The tunica media is the next inner most layer which consists of elastic fibers and layer(s) of smooth muscle cells responsible for arterial dilation and constriction. Outside the media is the adventitia. It consists of mostly collagenous connective tissue, which anchors the blood vessels next to organs for stability, and of connective tissue cells and leukocytes [31]. Arteries are dynamic tissues that are able to sense and respond to a variety of signals in order to control blood flow through dilation and constriction. Stimuli such as shear stress up-regulate vasodilators including nitric oxide (NO), prostaglandin and endothelial derived

hyperpolarizing factor (EDHF) [32]. EDHF has been classified as an alternate pathway in vasorelaxation from endothelial-derived relaxation factor such as NO, although its chemical identity has not been defined. The constriction of blood vessels is controlled by the production of vasoconstrictors such as endothelin1. The vasodilator NO is crucial to blood vessel physiology and will be the focus of my work.

1.4. Nitric oxide and endothelial nitric oxide synthase

NO is a short-lived bioactive gas that serves as a potent signaling molecule in vasodilation and neurotransmission as well as a bactericidal agent produced by macrophages. NO was identified as a main mediator of arterial vasodilation in seminal studies by Palmer et al. in which it was determined that this gas completely mimicked the effects of endothelial-derived relaxation factor from endothelial cells [33]. Subsequent work showing that mice lacking endothelial nitric oxide synthase (eNOS), the enzyme that produces NO in endothelial cells, are hypertensive firmly established the importance of this bioactive gas in regulating arterial vasodilation [34]. NO readily diffuses into cells making it effective as a signaling molecule. NO signals through the activation of soluble guanylyl cyclase (sGC) in smooth muscle cells to cause vasodilation. Activated sGC converts GTP into cyclic guanine monophosphate (cGMP)[35]. In smooth muscle cells, elevated cGMP levels activate nucleotide-dependent protein kinase G (PKG) which increases myosin light chain phosphatase activity and potassium-driven hyperpolarization of the smooth muscle cell, thereby leading to relaxation of smooth muscle cells and vasodilation.

NO is synthesized by a family of nitric oxide synthases that includes eNOS, neuronal NOS (nNOS) and inducible NOS (iNOS). All NOSs convert L-arginine to L-citrulline and NO with the aid of 5 cofactors: nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), and heme [33, 36, 37]. Production of NO from each NOS isoform has specific biological effects. In blood vessels, eNOS-derived NO stimulates arterial vasodilation, regulates inflammation, and suppresses smooth muscle cell proliferation and platelet aggregation [20]. In TA, production of NO by eNOS is compromised as the endothelium becomes injured by immunological processes and this

contributes to pathological luminal occlusion by preventing compensatory vasodilation [38]. Given the importance of eNOS expression and activity in defining the outcome of TA, it is important to understand the cellular mechanisms by which this enzyme is regulated.

eNOS is expressed constitutively by vascular endothelial cells. Its expression and activity, however, is tightly regulated at transcriptional, post-transcriptional and post-translational levels. During steady state of eNOS expression, two positive regulatory domains (PRD) are found in the eNOS core promoter region that binds to Sp1. This is essential for basal eNOS promoter activity. Growth factors such as vEGF have been shown to up-regulate eNOS mRNA transcription through activation of the ERK1/2-MAPK pathway [39].

At the post-transcriptional level, eNOS mRNA translation and stability are largely regulated by *cis*-acting RNA elements in the 5' and 3' untranslated regions (UTR) of the eNOS mRNA. These *cis*-acting RNA elements can bind to regulatory proteins that directly stabilize or destabilize the mRNA transcript. Searles et al identified 51- and 75-kDa proteins in endothelial cell extracts that bind to the 3'-UTR of eNOS [40]. The 51-kDa protein was found to be more abundant in post-confluent endothelial cells than proliferating cells suggesting a role in destabilization of the eNOS mRNA [40]. Many extracellular factors such as lipopolysaccharides (LPS) and TNFα can also induce RNA-protein interaction to the eNOS UTRs and affect eNOS mRNA stability [41, 42]. MicroRNAs also regulate eNOS levels by interfering with transcript stability of proteins that contribute to eNOS levels [43-45]. In addition, sONE, an antisense eNOS mRNA transcript, was identified to negatively regulate eNOS mRNA levels [46]. In a subsequent study using electrophoretic mobility shift assays, Ho et al showed that ribonucleoprotein binding to eNOS 3' UTR forms a complex which stabilizes eNOS mRNA from inhibitory elements such as sONE, siRNA and miRNA [47]. Altogether these reports show the intricacy of eNOS regulation at the post-transcriptional level.

At the post-translational level, eNOS activity is regulated by phosphorylation at many sites, with that at serine-1177 by AKT being particularly important [48]. In addition, myristoylation and palmitoylation of eNOS in the cytosol directs protein localization to the

plasma membrane [49]. Binding partners such as caveolin-1, calmodulin, and heat shock protein 90 (HSP90) also regulate eNOS localization as well as the activity of this enzyme [50]. Caveolin-1 sequesters eNOS at the plasma membrane caveolae and decreases its activity. On the contrary, HSP90 increases eNOS activity by changing eNOS conformation to increase binding affinity for activating the co-factor calmodulin. A necessary cofactor in NO synthesis, BH_4, is needed for eNOS activity [33, 37, 51]. When BH_4 levels are insufficient, eNOS electron transfer becomes uncoupled which leads to the production of superoxide [52-54]. eNOS protein stability is also regulated through modifications that control its binding to accessory proteins. Association of eNOS with HSP90 and caveolin-1 has been shown to protect eNOS against degradation with HSP90 masking eNOS from proteases and preventing caveolin-1 from sequestering eNOS to the plasma membrane rendering it inactive [55-57].

1.5. Th-17 cells, IL-17, and TA

Upon activation, CD4 T cells undergo differentiation into distinct effector lineages that produce unique cytokines. As mentioned, CD4+ T cells can be activated to differentiate into three main effector lineages in the periphery [7]. Th1 cells secret IFNγ and are responsible for clearing intracellular bacteria and viruses. IFNγ activates macrophages to phagocytize and digest foreign pathogens and CD8+ cytotoxic T cells to kill target cells [22]. IFNγ also alters a broad range of cellular transcriptional activity that can cause tissue remodelling [21, 58]. Th2 cells secrete IL-4, IL-5, and IL-13 and are responsible for clearing helminthes from the body by activating B cell proliferation and IgE class switching [59]. Lastly, Th17 cells secrete IL-17, IL-21 and IL-22 and are responsible for clearing extracellular bacterial and fungal infections at the epithelial/mucosal barrier [60]. The induction of Th17 cell differentiation is initiated by TGF-β, IL-1β, IL-6, and is amplified by IL-21 and IL-23 [61].

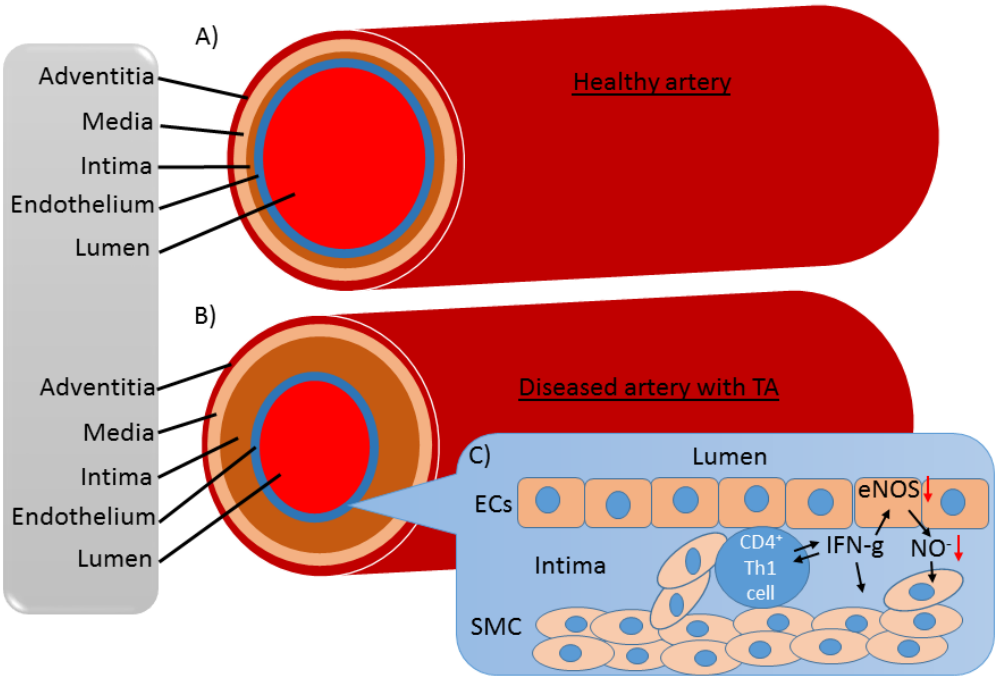


Figure 1. Structure of healthy and diseased arteries with TA.
A) A healthy artery is composed of 3 major layers, tunica intima, tunica media, and tunica adventitia. B) A diseased donor artery with transplant arteriosclerosis shows infiltration of several cell types such as T cells, macrophages, and smooth muscle cells (SMC) into their non-native locations. C) These infiltrating cells are attracted by the cytokines secreted as a result of immunological response against the donor tissue, in this case, specifically against the endothelium. IFNγ secreted by CD4+ Th1 cells play a major role here as it down-regulates eNOS mRNA causing dilatory dysfunction as well as activating the ECs to up-regulate inflammation-associated markers such as ICAM-1 and MHC class II that facilitate the clearance of antigens in the event of viral infections [30]

IL-17 is produced by Th17 cells and innate immune cell types, such as macrophages, NK cells, NKT cells and γδ T cells [62-65]. IL-17 binds to a heterodimeric receptor comprised of IL-17RA and IL-17RC. Binding of IL-17 to its receptor activates the signaling pathways ERK, JNK, NFκB, p38, and AKT-PI3K signaling pathways [66, 67]. Upon receptor engagement, a signaling cascade involving ubiquitinylation and phosphorylation is initiated. Protein sequence alignment has shown IL-17 shares sequence homology with receptors in the Toll-like receptor/IL-1 signaling pathways [68]. NFκB activator 1 (ACT1), an E3 ubiquitin ligase, acts as a bridge between the cytoplasmic tail of IL-17RA and TRAF6, a second E3 ubiquitin ligase indispensable in IL-17 activation of NFκB and JNK pathways [69, 70]. Following ACT1-mediated lysine-63 ubiquitinylation, TRAF6 acts as a signaling transducer in the NFκB and JNK pathways.

As such, Zhong and colleagues have shown that deubiquitinating enzymes are crucial in regulating IL-17 signaling. Overexpression of the deubiquitinase USP25 significantly inhibited IL-17- but not TNFα-mediated NFκB activation [71]. Activation of the described signaling pathways by IL-17 leads to the induction of chemokines and pro-inflammatory cytokines such as G-CSF, CXCL-1, CXCL-2, CXCL-5, and CXCL-8 that participate in clearing pathogens by recruiting neutrophils and inducting the production of anti-microbial peptides [62, 72-74].

Th17 cells have been shown to play a role in the pathogenesis of several autoimmune diseases but their contribution to organ transplant rejection and TA remains unclear. Th17 cells are capable of mediating cardiac graft rejection and TA in a model in which graft recipients are unable to mount Th1 responses due to T-bet deficiency. In these experiments, elimination of IL-17 by neutralizing antibody resulted in a significant reduction in acute rejection and TA [75]. Also, in a corneal transplant model, IL-17-deficient mice experienced delayed graft rejection compared to control mice [76]. However, it has also been suggested that the role of Th17 cells may be redundant in the corneal transplant model, as overall graft survival time was unchanged between the two groups and IL-17-deficiency only delayed development of allogeneic rejection [76]. In another study where cardiac graft rejection was augmented by TLR9 activation and graft recipients were immunosuppressed with co-stimulatory blockade, elimination of IL-6 and IL-17 prevented the rejection augmented by TLR9 signaling [77].

The role of IL-17 in vascular rejection and TA has been directly examined in a handful of studies. Systemic blockade of IL-17 in a rat aortic interposition model reduced acute inflammation but not intimal thickening that is reminiscent of TA [78-80]. In work by Fogal et al, neutralization of IL-6 resulted in the expansion of T regulatory cells (Tregs) that can secrete IL-10 to suppress inflammatory responses [81], decreased Th17 markers and reduced intimal expansion possibly due to an increase in Tregs [82].

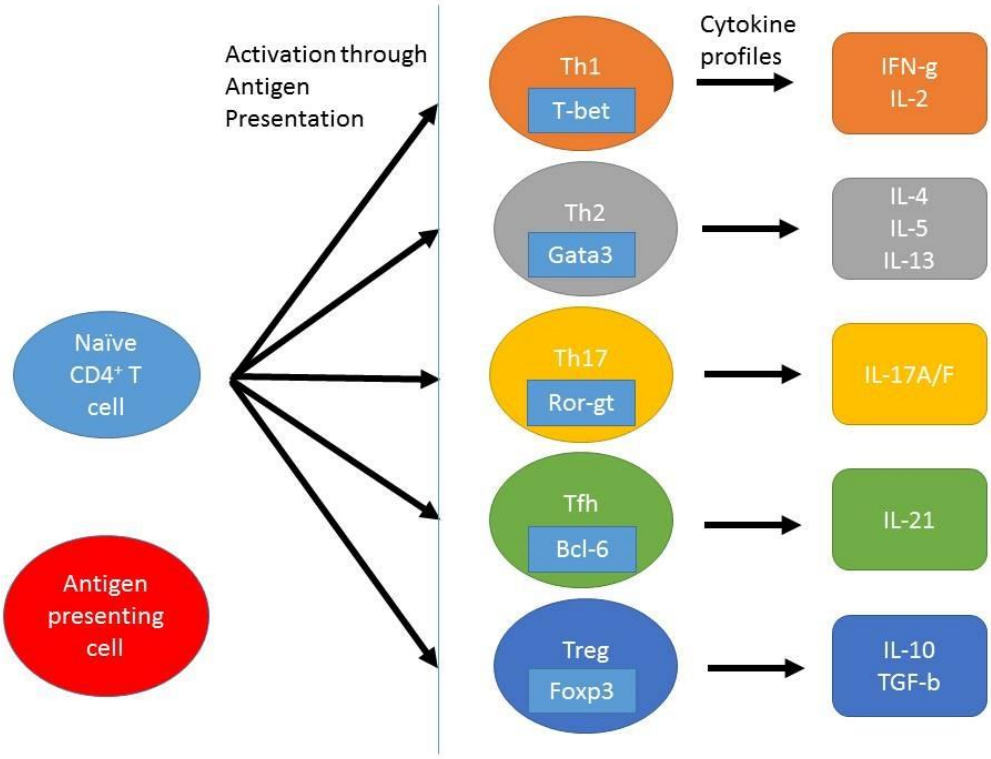


Figure 2. Differentiation of CD4+ T cells.
Naïve CD4+ T cells have many lineages of differentiation after antigen recognition and activation. Each subtype of activated CD4+ T cells are driven by a principal transcription factor and have a distinct cytokine secretion profile.

The effect of IL-17 on endothelial cells has been studied mainly as it pertains to immune activation of this cell type. Endothelial cell activation involves the up-regulation of cell adhesion molecules and chemokines that support leukocyte migration [23, 83]. IL-17 by itself was shown to minimally up-regulate cell adhesion molecules and chemokine secretion in endothelial cells but synergized with TNFα to up-regulate these inflammatory molecules [8]. It has also been reported that IL-17 can cause endothelial damage by promoting endothelial apoptosis by activating caspase-3 and caspase-9 [84] although the mechanism is unclear. In the same study, it was shown that IL-17 can increase the secretion of von Willebrand factor, an endothelial specific protein that induces platelet aggregation, as a result of endothelium damage [84, 85].

1.6. Preliminary studies

Because of the importance of NO in vascular biology, in previous work I studied the effect of IL-17 on eNOS expression in ECs. I showed that IL-17 up-regulates eNOS protein levels and NO production through the coordinated activation of the MEK1-ERK, JNK and NFκB signaling pathways. In addition, the abundance of IL-17 was significantly correlated with increased eNOS expression and increased luminal area in human specimens of TA. These findings suggest that IL-17 may affect the development of TA by increasing eNOS expression in the endothelium (Appendix A) [86].

Chapter 2.

Aims, Rationale and Hypothesis

My previous data established that IL-17 up-regulates eNOS but the mechanism remains largely unknown. Also, the role of IL-17 in vascular changes that occur in TA remains poorly defined. As such, the overall goal of my project is to obtain insight into the mechanism by which IL-17 up-regulates eNOS expression and to examine its role in TA using an aortic interposition model of this condition. We hypothesize that IL-17 signaling induces an increase in eNOS protein levels through increasing eNOS gene transcription, increasing eNOS mRNA stability and/or translation, or direct eNOS protein stabilization. This increase in eNOS protein levels may affect the outcome of TA. to determine this, my thesis had two aims:

Aim 1. Obtain insight into the mechanism by which IL-17 up-regulates eNOS.

To determine whether the induction of eNOS protein levels by IL-17 occurs through transcriptional, post-transcriptional, or post-translational mechanisms.

Aim 2. Determine the role of IL-17 in transplant arteriosclerosis.

To study the role of IL-17 in TA using an established model of TA involving aortic interposition grafting between minor histocompatibility antigen mismatched strains of mice. Aortic segments from wild-type (WT) and IL-17 receptor-A-knockout donor mice (both on a C57Bl/6 background) will be interposed into the infrarenal aorta of WT 129J recipient mice. Graft recipients will be sacrificed at 60 days post-transplantation and intimal thickening and luminal occlusion will be examined by histological analysis of arteries.

Chapter 3.

Mechanisms of eNOS up-regulation by IL-17

3.1. Rationale

As I have previously shown, IL-17 up-regulates eNOS protein levels in a dose- and time-dependent manner. Induction of eNOS peaks at 6 h post-stimulation and is optimal at a dose of 10 ng/mL. We also determined that IL-17 increases eNOS levels by acting through the coordinated activation of MEK1/ERK, JNK, NFκB pathways [86]. These findings led us to investigate how IL-17 is up-regulating eNOS in ECs. Determining whether the up-regulation of eNOS by IL-17 occurs through gene transcriptional, post-transcriptional or post-translational mechanisms will unveil the contribution of IL-17 toward vascular tone balance in an immune-targeted environment such as arteries with TA.

3.2. Materials and Methods

3.2.1. Human primary cell culture and reagents

Human microvascular ECs (HMVECs) and human coronary artery ECs (HCAECs) were purchased from Lonza (#CC-2543, #CC-2585 respectively) and cultured in EGM-2 MV supplemented medium (#CC-3102, 5% FBS, VEGF, R3-IGF-1, hFGF-B, hEGF, hydrocortisone, ascorbic acid, gentamicin, amphotericin-B). Cells were used up to the sixth passage. Cell passaging was done every 2-3 days or when sub-confluent using 0.25% trypsin in 5% EDTA. Cells were growth factor starved for 24 h prior to experiments to ensure basal eNOS expression. Cells were untreated or treated with recombinant human IL-17 (#200-17, PeproTech) prior to examination of eNOS

expression and NO production. In some experiments, cells were incubated for 30 min with the following inhibitors (prior to IL-17 stimulation): CHX (which inhibits translation, #239763-1GM, ethanol; Calbiochem), and MG132 (which inhibits proteasomal degradation, #80053-194, ethanol, VWR).

3.2.2. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

ECs were collected after treatment with 10ng/mL of IL-17 for up to 6 h and trypsinized by incubation with 300 µL of 0.25% trypsin in 5% EDTA. The cell suspensions were pelleted and supernatant aspirated. Cellular total RNA was extracted using the RNeasy Mini kit purchased from Qiagen (#74104). RNA was double eluted in 50 µL of double distilled water (ddH2O). Total RNA was measured using a spectrophotometric Nano-drop 1000. All sample RNA concentrations were normalized to the lowest concentration. Reverse transcriptase reaction was performed to generate total cDNA. RNA was heated at 65 ^{o}C for 10 min and chilled on ice to allow for primer annealing. dNTPs, RNase inhibitor, reverse transcriptase, random hexamers and RT buffer were then added to the mixture and held at 42 ^{o}C for 1 h to generate cDNA. The mixture was then heated to 65 ^{o}C to inactivate the RT enzyme. Total cDNA concentrations were measured using Nano-drop for normalization in the qPCR reaction. eNOS gene expression array primers with Taqman flurogenic probes were purchased from Applied Biosystems (Hs01574659_m1). 20 ng of cDNA was combined with Taqman qPCR master mix and loaded onto a 96 well plate for a total of 20 µL per well. The plate was centrifuged at 1500 rpm for 5 min to dispel air bubbles in the wells. The plate was subjected to 2 min at 50 ^{o}C, 10 min at 95 ^{o}C, then for 30 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 min. Quantitative PCR was performed in the Applied Biosystems 7900HT Real-Time PCR system and each sample normalized to the housekeeping gene GAPDH to account for loading errors. Three individual repeat experiments were performed and results compiled using Excel.

3.2.3. Western blot analysis

ECs were lysed in RIPA buffer (50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 1% Triton-X100, 0.1% SDS, 10% glycerol, 0.02% sodium azide and 1% sodium deoxycholate). Lysates were diluted with 6X Lameli SDS loading buffer (60mM Tris pH 6.8, 12% SDS, 47% glycerol, 600mM DTT, 0.06% bromophenol blue) and heated to 95^{o}C for 10 min before loading onto 10% polyacrylamide gel and run at 100V for 2 h. Size differentiated proteins were then transferred for 1 h to nitrocellulose membranes at 100V for 1 h. Membranes were incubated with primary antibodies against eNOS (#610296, BD Biosciences), HSP-90 (#4874, Cell Signaling Technology), ubiquitin (#8017, Santa Cruz Biotechnology), and β-actin (#A1978, Sigma-Aldrich) for 2 h in 5% bovine serum albumin. Four 10-min washes with PBS-tween 0.05% on shaker were followed by incubation with HRP-conjugated secondary antibodies specific to the origins of the primary antibodies for 1 h. Secondary antibody-bound proteins were then visualized using Clarity Western ECL substrate (#170-5060, Bio-Rad) and detected with Fujifilm LAS-4000 imager. 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.

3.2.4. Immunoprecipitation

ECs were lysed with 300 µL of RIPA buffer or plasma membrane lysis buffer (10mM Tris-HCl, 50mM NaCl, 1.25mM EDTA, 1% Triton-X100) after treatment with IL-17. The lysates were kept on ice for 30 min then centrifuged at 10000 g for 10 min to collect cellular debris. Nano-drop was used to determine protein concentration of lysates. The supernatants were then pre-cleared with 1 µg of mouse control IgG (#sc-2025, Santa Cruz Biotechnology) and 20 µL of protein-G PLUS-Agarose beads (#sc-2002, Santa Cruz Biotechnology) for 1 h on rocker. The beads were then pelleted at 5000 g for 5 min and supernatant transferred to new tubes. 1 µg of eNOS antibody was added to the lysates per 300 µg of cellular protein and incubated on rocker at 4 ^{o}C overnight. 20 µL of protein-G beads were added the next morning and incubated for 4 h on rocker at 4 ^{o}C. The beads were then collected by centrifugation at 5000 g for 5 min

and washed 2 times with 1 mL of PBS. Samples were diluted with 6X SDS loading buffer in PBS and heated to 95 ^{o}C for 10 min before using for western blot.

3.2.5. Cellular fractionation

Cells were harvested using 0.25% trypsin in 5% EDTA and pelleted at 1500 RPM for 5 min. Cytosolic, plasma membrane and nuclear fractions were separated using Abcam Cell Fractionation Kit (#ab109719, Abcam) according to Abcam Cell Fraction Standard Protocol v5. Antibodies against GAPDH (#2118S, New England Biolabs) and VE-cadherin (#2500S, New England Biolabs) were used as cytosolic and plasma membrane fraction control markers respectively. Fractions were analyzed by western blot.

3.2.6. Statistical analysis

Identification of differences between two groups was determined using a Student's t test and those between multiple groups by two-way ANOVA followed by a least significant difference post hoc analysis. In all cases, an α value of ≤ 0.05 was determined to be significant.

3.3. Results

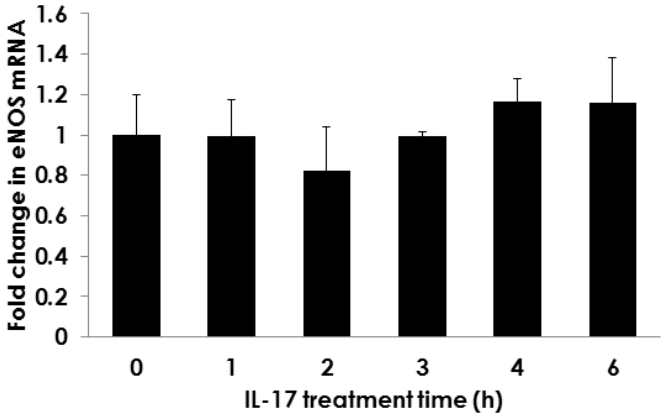
3.3.1. IL-17 does not affect eNOS mRNA levels

In order to determine if IL-17 is up-regulating eNOS protein expression through increasing the transcription of eNOS mRNA, endothelial cells were treated with 10 ng/mL of IL-17 for up to 6 h. Total cellular RNA was extracted reverse transcribed into cDNA and qPCR for eNOS mRNA performed. eNOS mRNA levels were calculated as relative mRNA copies to the housekeeping gene GAPDH and then expressed as a fold change compared to untreated cells.

eNOS mRNA levels were not affected by treatment of IL-17 during the course of 6 h whereas eNOS protein levels were increased as compared to untreated at time zero

(Fig. 3A, 3B). This indicates that IL-17 does not up-regulate eNOS protein expression by inducing eNOS gene transcription.

A)



B)

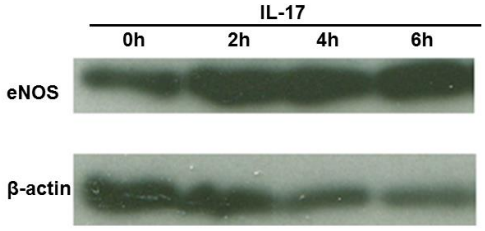


Figure 3. Effect of IL-17 on eNOS gene expression and mRNA stability.
A) eNOS mRNA levels were measured by RT-qPCR and normalized to GAPDH levels. The graph is the mean ± SE of the fold induction of eNOS mRNA levels over three individual experiments. B) Representative western blot of IL-17-induced eNOS protein up-regulation over the course of 6 h.

3.3.2. Inhibition of mRNA translation does not block IL-17 up-regulation of eNOS

Because IL-17 did not affect eNOS mRNA levels, we next examined whether IL-17 increased eNOS protein levels potentially through the amplification of mRNA translation. ECs were treated with a broad spectrum eukaryotic translation inhibitor, CHX, prior to being stimulated with IL-17. eNOS protein levels were determined after 6 h. If IL-17 increases eNOS protein levels by increasing eNOS mRNA translation, then the addition of CHX should abolish this effect.

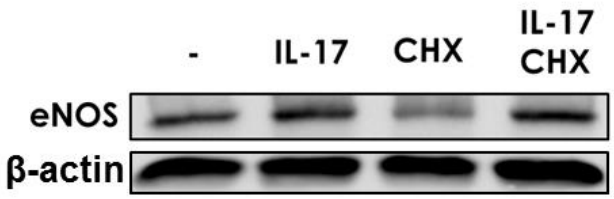
As seen in figure 4, IL-17 induced an up-regulation of eNOS protein expression by approximately 1.5-fold of basal levels. CHX alone did not affect significantly the levels of basal eNOS expression. When the ECs were treated with both IL-17 and CHX, the induction of eNOS protein levels was comparable to the levels observed in cells treated with IL-17 alone. Because blocking mRNA translation did not prevent the induction of eNOS by IL-17, these findings suggest that IL-17 up-regulates eNOS through a mechanism independent of mRNA translation.

3.3.3. IL-17 increases eNOS protein stability

In order to determine whether IL-17 increases eNOS protein levels through post-translational effects that could involve altered proteasomal degradation, we initially used a proteasome inhibitor, MG132, to block protein degradation within cells. The rationale was that if IL-17 increases eNOS protein levels through reducing proteasomal degradation there would be an equal amount of eNOS protein in cells treated with IL-17 in the absence or presence of MG-132. If IL-17 was not affecting protein degradation, eNOS protein levels would be higher in cells treated with IL-17 and MG132 as compared to those treated with MG132 only. However, these experiments led to highly variable results that were not interpretable (data not shown). There was minimal and variable induction of eNOS in response to IL-17, potentially because proteasomal degradation of IκB is needed for activation of the NF-κB pathway, which is involved in IL-17-mediated up-regulation of eNOS [87].

Because we were not able to use MG132 to evaluate the effects of the proteasome on IL-17 induction of eNOS, we measured the life-span of eNOS protein in endothelial cells to determine whether it was being affected by IL-17. Cells were treated with CHX for 30 min. and then left untreated or treated with IL-17. Cell lysates were prepared at 0, 3, 6, 9, and 12 h post-treatment with IL-17 and eNOS protein levels examined by western blot. As seen in figure 5A, during the course of 12 h, treatment of ECs with CHX reduced eNOS protein levels to approximately 50% of basal levels. However, in response to treatment with IL-17, eNOS protein levels did not decrease and were significantly higher than in cells not treated with IL-17. These findings suggest that IL-17 up-regulates eNOS protein levels by prolonging its half-life.

A)



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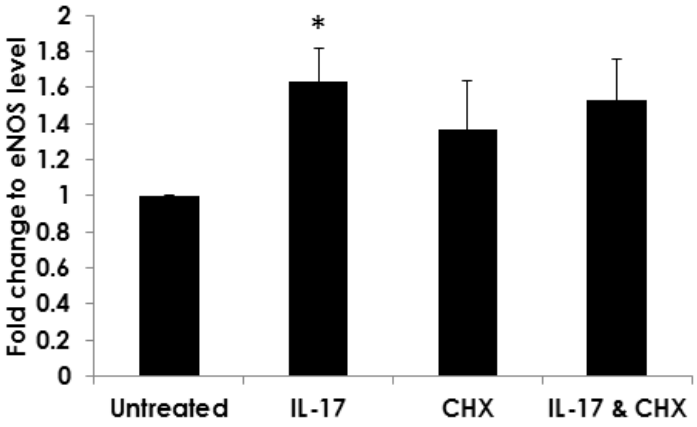


Figure 4. Effect of translation inhibition on the induction of eNOS by IL-17.
A) CHX was added to ECs 30 min prior to stimulation of ECs by IL-17 for 6 h. eNOS expression was determined by western blot analysis. Representative blot of four individual experiments. B) Quantification of effect of IL-17 on eNOS induction after the inhibition of translation by CHX. Data are the mean±SE of eNOS protein levels relative to untreated ECs

Under complete inhibition of translation, there should not be any neonascent protein synthesis. To investigate whether the slight increase in eNOS expression in response to IL-17 that occurred in the presence of CHX was due to incomplete inhibition of mRNA translation higher doses of CHX were used. Higher doses of CHX (20 µg/mL) eliminated the increase in eNOS levels induced by IL-17. However, eNOS levels remained lower in untreated cells compared to IL-17 treated cells (Figure 5C). This substantiates our conclusion that IL-17 increases eNOS protein stability in ECs. Importantly, CHX did not reduce cell viability in any of our experiments (data not shown).

3.3.4. Effect of IL-17 on eNOS protein localization

One potential mechanism by which IL-17 increases eNOS protein stability in cells could be related to its intracellular localization. It has been shown that eNOS is extensively modified post-translationally through several mechanisms such as myristoylation, palmitoylation, and phosphorylation that can affect its localization. Myristoylation of eNOS increases the association of the protein with the plasma membrane lipid bilayer and palmitoylation of eNOS increases plasma membrane localization by increasing its binding to the caveolae protein caveolin-1 (CAV-1) [55]. Phosphorylation primarily controls the activation of eNOS via alteration of its binding affinity to other binding partners such as calmodulin (CaM) [88]. Furthermore, binding of eNOS to its partners such as HSP90 likely takes place mainly in the cytosol and this may increase eNOS protein stability.

In order to examine whether eNOS localization is affected when ECs are treated with IL-17, a cell fractionation procedure was used to separate the plasma membrane, cytosol, and nuclear compartments of treated or untreated ECs. eNOS levels were determined in each fraction after stimulation with IL-17. The presence of GAPDH and VE-cadherin were used to assess the purity of cytosolic and membrane fractions, respectively.

Challenging ECs with IL-17 increased total cytosolic eNOS but had no effect on membrane associated forms of eNOS. This indicates that IL-17 triggers a process that is associated with the accumulation of eNOS mainly in the cytosol.

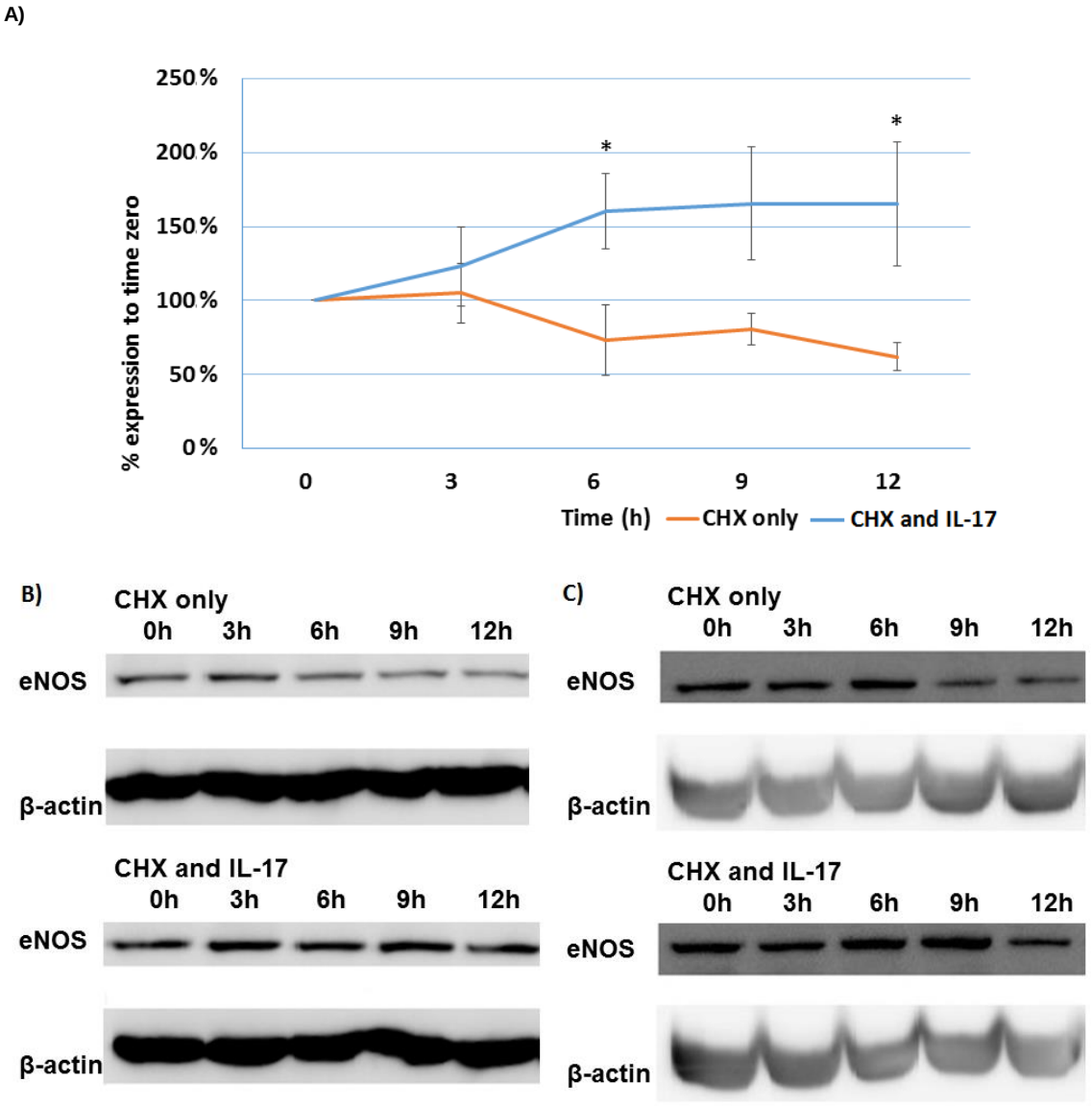
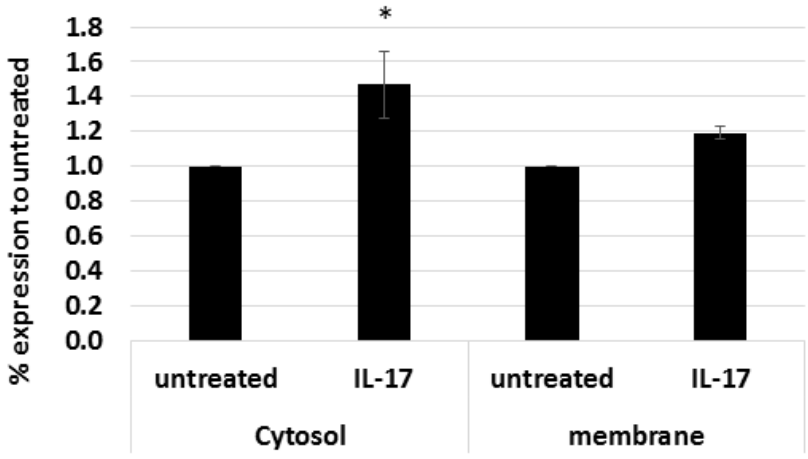


Figure 5. Effect of IL-17 on eNOS half-life.
A) Treatment with CHX reduced eNOS levels over the course of 12 h. However, treatment of IL-17 in addition to CHX prevented the degradation of eNOS and prolonged its half-life. Quantification of five individual experiments. B) Representative western blot of data summarized in A treated with 1 µg/mL CHX and also with or without IL-17. C) Representative western blot of ECs treated with 20 µg/mL CHX and also with or without IL-17.

A)



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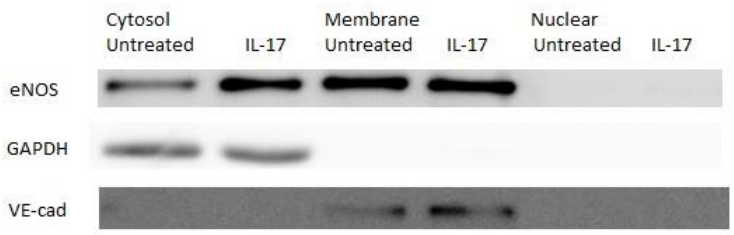

Figure 6. Effect of IL-17 on eNOS localization within cells.
A) IL-17 induces eNOS protein levels in the cytosol. No difference in eNOS protein levels were found associated in the membrane fraction after IL-17 treatment. GAPDH was used as a cytosolic fraction control. VE-cad was used as a membrane fraction control. *$p < 0.02$. n=4. B) Representative western blot of endothelial cell fractionated and blotted against indicated antibodies.

3.4. Discussion

We have determined that IL-17 does not increase eNOS mRNA levels, indicating that it does not increase protein levels through the up-regulation of eNOS gene transcription or mRNA stability (Fig 3). The next step we took was to examine eNOS mRNA translation using CHX to block translation. This showed that the IL-17-induced up-regulation of eNOS was not a result of increased mRNA translation. We

subsequently used a CHX-based approach to examine eNOS protein half-life in the presence or absence of IL-17. This experiment yielded crucial information suggesting that IL-17 prolongs eNOS protein half-life, a process that is related to increased eNOS localization in the cytosol.

The mechanism by which IL-17 increases eNOS protein stability is unknown. When eNOS is synthesized, it is myristoylated to increase its affinity for lipid bilayers [89]. When cellular conditions change, eNOS could be further palmitoylated and associated with CAV-1 at the plasma membrane, which renders eNOS inactive and away from possible degradation [49, 55, 56]. When an activating stimulus, such as a calcium influx, is encountered, a calcium-binding messenger protein, calmodulin, binds eNOS in the cytosol to activate it [88]. Another eNOS binding partner, HSP90, is also involved in eNOS activity and stability as it has been shown to bind eNOS in the cytosol and shield eNOS from calpain-mediated degradation [57]. We began to examine the mechanism by which IL-17 might increase eNOS protein stability by fractionating untreated and IL-17-treated ECs into cytosol and plasma membrane fractions. The amount of eNOS in the cytosolic fraction was significantly more in the IL-17-treated group compared with control. This result suggests that eNOS may become associated with cytosolic binding partners such as CaM or HSP90. We have attempted to examine the effect of IL-17 on the association of eNOS with HSP90 by co-immunoprecipition experiments. Although we were able to immunoprecipitate eNOS and detect its up-regulation in IL-17-treated groups, we were not able to detect HSP90 in our experiments. It has been suggested that the HSP90:eNOS complex is very fragile and a slight increase in ionic strength of the lysis buffer could dissociate such a complex [90]. However, after attempting several preparations of weakly ionic buffers, we still could not detect HSP90 in our pull-down fraction. Future experiments are needed to clarify the precise mechanism by which eNOS protein stability is increased by IL-17.

Our observation that IL-17 is up-regulating eNOS by prolonging its half-life is interesting because we determined previously that a concerted effect of MEK1-ERK, JNK and NFκB signaling is required for the increase in eNOS protein levels. Putting the two results together, it may be that eNOS is post-translationally modified by a common kinase or ubiquitin ligase shared amongst these three pathways. It has been suggested

that IKKi, a crucial kinase required for IL-17 signaling at a very early stage of receptor engagement, is necessary for downstream NFκB and MAP kinase activation [91]. Another kinase, IKKβ, required for NFκB activation has also been shown to phosphorylate targets that are not involved in NFkB activation [92]. In addition, phosphorylation and ubiquitinylation are essential protein modifications involved in IL-17 signaling. Ramet et al have also shown that HDL and apolipoprotein A-I enhance eNOS protein half-life through the activation of ERK1/2 and AKT/PI3K pathways [93]. It is plausible that eNOS is targeted at multiple phosphorylation sites after IL-17 stimulation and this increases its protein stability. Changes in eNOS ubiquitinylation could also be controlled by IL-17. NFκB activator 1 (Act1) is an E3 ubiquitin ligase that binds to the cytoplasmic domain of IL-17RA and mediates Lys63 ubiquitinylation of TRAF6, another E3 ligase signaling intermediate involved in IL-17 signaling [54, 70]. The involvement of Act1 implicates potential deubiquitinylating regulatory mechanisms (which may contribute to a decrease in eNOS ubiquitinylation leading to a slowed protein turnover) [71]. We attempted to examine eNOS ubiquitinylation but were not able to detect this protein modification on immuno-precipitated eNOS from human vascular endothelial cells. This could be due to a very small fraction of eNOS that is ubiquitinylated at any given time in a cell and the ubiquitinylation state of eNOS may vary [94]. To address this, we have begun to overexpress eNOS protein by exogenous plasmid transfection to increase the amount of total eNOS and amplify ubiquitinylated eNOS. Examining these protein modifications are the focus of future studies.

Although we did not detect an effect of IL-17 on eNOS mRNA levels, this cytokine may still have effects on gene transcription and mRNA stability if the two processes offset each other. It has been shown that eNOS level can be regulated at the level of gene transcription and that IL-17 activates downstream signaling pathways that affect eNOS promoter activity, such as NFκB [39, 66, 67]. To definitively examine whether gene transcription of eNOS is affected, cells can be cultured with radiolabeled uracil during IL-17 treatment. The neonascent mRNA will contain the radiolabeled uracil and the difference in signal will be caused by IL-17 treatment.

In summary, our *in-vitro* findings indicate that IL-17 affects eNOS protein stability positively by increasing protein half-life. The effect by IL-17 could increase the

production of nitric oxide in blood vessels to control vascular function. Future studies on the exact mechanisms by which IL-17 can directly increase eNOS protein half-life are currently underway.

Chapter 4.

The Effect of IL-17 Signaling in Graft Cells on the Development of TA

4.1. Rationale

I showed previously that IL-17 levels in clinical samples of TA were correlated with increased eNOS levels and luminal expansion [86]. However, experimental evidence for a role of IL-17 in modifying TA has not been obtained. We have begun to examine this using an aortic interposition model of TA in which aortic segments from WT or IL-17 receptor A (IL-17RA)-deficient mice on a C57Bl/6 background (H-2^b) were interposed into the infrarenal aorta of recipient 129J mice (H-2^b). This is a minor histocompatibility antigen mismatched model that results in moderate vascular damage that reflects the changes that occur in the human condition.

4.2. Materials and Methods

4.2.1. Aortic interposition grafting

Breeding pairs of IL-17RA knockout mice on a C57Bl/6 background were provided by Dr. Alison Budelsky at Amgen Inc. [95]. Gender matched female or male mice from 8 to 12 weeks of age were used as artery donors into minor histocompatibility antigen mismatched 129J mice. Wild type C57b/6 donor mice were used as controls. Segments of infrarenal aorta from donor mice were interposed into the infrarenal aorta of recipient mice as established in the Choy lab [96]. All the transplants were performed by Ms. Winnie Enns. Mice receiving transplants were sacrificed at 60 days and transplanted artery segments extracted and frozen in Tissue-Tek O.C.T. freezing media

(#25608-930, VWR) with 5-methylpentane and liquid nitrogen. The blocks were stored at -80 °C and sectioned using a cryostat at -20 °C. All procedures were approved by the SFU animal ethics review committee.

4.2.2. Histology and morphological analysis

Cross-sections (8 μm) of aortic interposition grafts were stained using an elastic van Gieson protocol to visualize the external and internal elastic laminae. The circumference of each elastic laminae as well as the lumen was measured by manual tracing and then the area within each circumference quantified using ImageJ software (National Institutes of Health). The program was calibrated with a micrometer to measure the absolute values. From these measurements, the area of medial, intimal, and luminal regions of the artery were calculated. Relative intimal thickening was determined by calculating the intima to media ratio as well as the percent luminal occlusion [19].

4.3. Results

4.3.1. Effect of graft expression IL-17RA in mediating arterial changes in TA

To investigate whether or not IL-17 signaling in graft cells plays a role in TA, we performed aortic interposition grafting using artery segments from IL-17RA KO or wild type mice. The transplants were harvested at 60 days and morphological characteristics of TA were quantified. This included intima to media ratio (which provides a relative measure of intimal thickening), percent luminal narrowing, absolute intimal area, and absolute medial area.

Artery grafts from both experimental groups showed extensive arteriosclerotic thickening characterized by the development of a cellular intima. The elastic laminae of the arteries remained intact. No pathological changes were observed in syngeneic grafts.

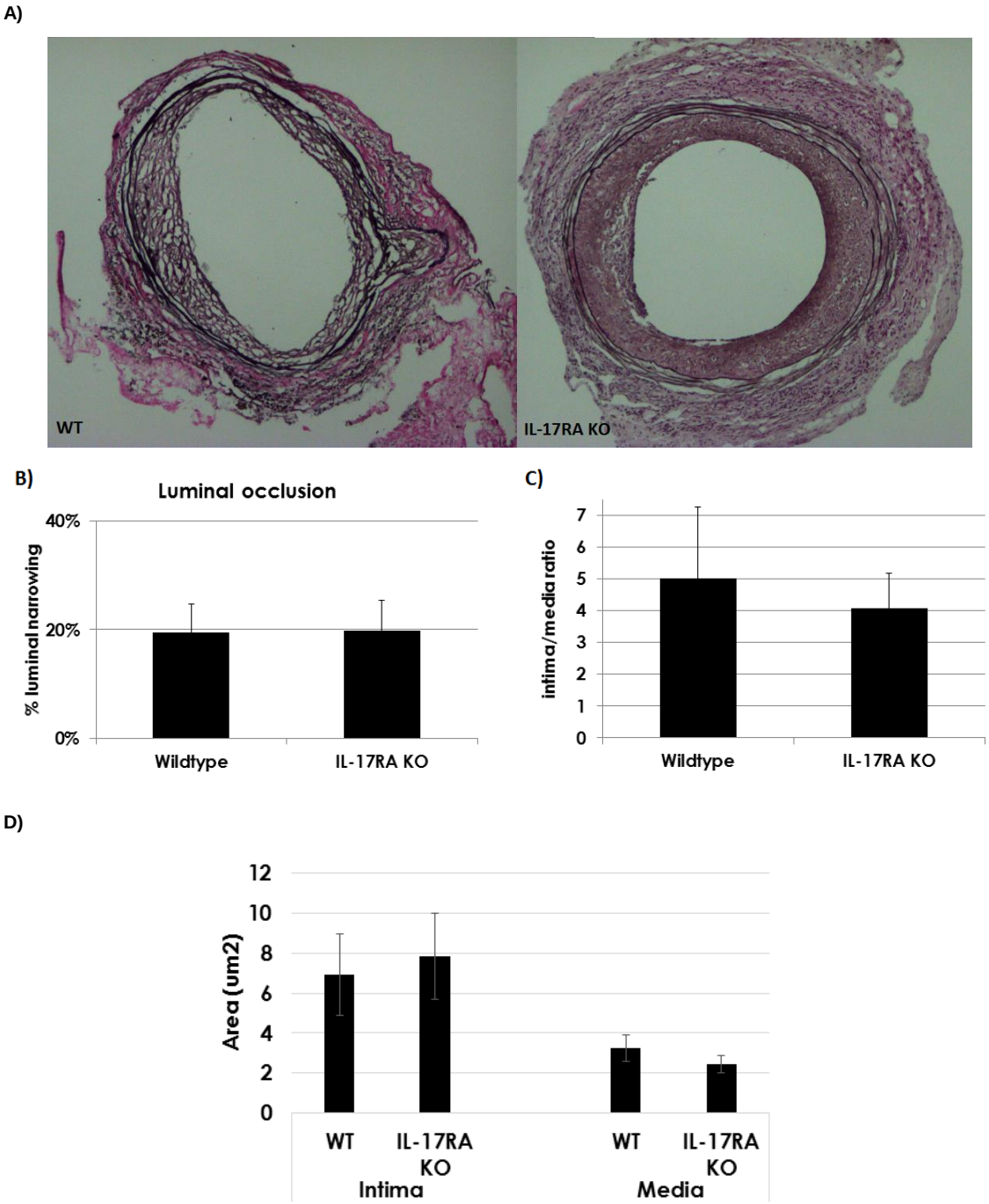

Figure 7. Effect of IL-17 signaling on morphological changes that occur in a minor-MHC mismatched murine model of TA.

Aortic segments from wildtype (n=8) and IL-17RA KO (n=8) donors were interposed into the infrarenal aorta of allogeneic recipient mice. Grafts were harvested at day 60. Arterial cross-sections were cut and stained using a standard van Gieson protocol. A) Representative artery cross-section of WT and IL-17RA KO allografts respectively. n=8. B) Luminal occlusion of the arteries as measured by % luminal narrowing. C) Intimal thickening of the arteries as measured by intima to media ratio. D) Absolute intimal and medial areas of the transplanted arteries

As seen in figure 7, no significant difference was observed in the intima to media ratio of IL-17RA KO arteries compared to wild type arteries. This suggests that deficiency in IL-17 signaling does not affect the amount of intimal thickening while taking into account possible artery size differences. Also, no significant difference was observed in luminal narrowing between WT and IL-17RA KO grafts. Finally, no significant difference was observed in the absolute intimal and medial areas of arteries between the groups. Altogether, our findings suggest that there is no obvious effect on vessel remodelling in IL-17RA KO grafts in the model of TA that we have used.

4.4. Discussion

We examined the effect of IL-17 signaling in graft cells on the development of TA using an aortic interposition model. We determined that a lack of IL-17 signaling in vascular cells did not affect intimal thickening, luminal narrowing or medial thickness. These findings indicate that IL-17 signaling in graft cells likely does not affect obvious structural changes that occur in allograft arteries in our model.

The involvement of IL-17 in transplant rejection has been studied previously although no reports have examined the role of IL-17 signaling in graft or vascular cells specifically. As mentioned earlier, it has been shown that systemic blockade of IL-17 does not affect intimal thickening in a complete MHC mismatched aortic interposition model but reduces inflammation associated with acute allograft rejection [78, 80]. Our data support this finding in a minor histocompatibility mismatch setting, although we do not know whether elimination of IL-17 signaling in recipient cells affects TA. This is the focus of current studies. Also, Th17 responses were shown to contribute to the development of TA in a MHC II mismatch model in which graft recipients were not able to mount a Th1 response (i.e. were T-bet deficient) [75]. The main difference between this latter report and our findings is that deficiency in IL-17 signaling in the donor artery does not obviously affect the development of TA. This could be due to a difference in models. The study by Yuan et al use graft recipients that were unable to mount a Th1 response. In our model, a Th1 response can still be established. The immune response in clinical TA samples is predominated by Th1 cells. This is important because preventing the development of Th1 responses in graft recipients artificially alters the

pathogenesis of TA. Indeed, IFNγ is a major inducer of smooth muscle cell migration and proliferation and this aspect of vascular remodelling no longer contributes in T-bet KO recipients.

As seen from our *in-vitro* data that IL-17 can directly up-regulate eNOS, we hypothesized and confirmed that this accumulation of eNOS protein can produce NO which is beneficial in outward remodelling of obstructed arteries in TA [86]. However, we did not test whether or not superoxide, a toxic radical produced by uncoupled eNOS, accumulated as well in the supernatant [52]. Superoxide rapidly react with NO to form a toxic compound peroxynitrite [97]. Peroxynitrite can react directly with transition metals needed for proper enzymatic reactions. The outcome of peroxynitrite modification with enzyme transition metal centre can affect enzymatic activity, cytoskeletal organization as well as cell signal transduction and eventually lead to cell death [98]. Therefore, although we observed an increase in NO production as measured through nitrite accumulation, the possibility of peroxynitrite formation due to superoxide production remains unclear in experiments.

In our examination of clinical samples of TA, we observed that IL-17 correlated with eNOS levels and with increased luminal area suggesting that this cytokine might increase arterial expansion in TA. One possible mechanism by which luminal area can expand without effects of arteriosclerotic thickening is through alterations in vascular dilation and constriction. We attempted to examine this by directly measuring artery function using wire myography. Unfortunately vasodilation and constriction could not be reliably measured in our model due to overwhelming vascular damage caused by the rejection process. Furthermore, another complication is that our model may not completely reflect the clinical conditions where all patients receive immunosuppression. In order to better evaluate the impact of IL-17 on arterial functional changes in TA, we may have to optimize a model that incorporates less immunological damage and/or immunosuppression.

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Appendix A.

Supplemental background data

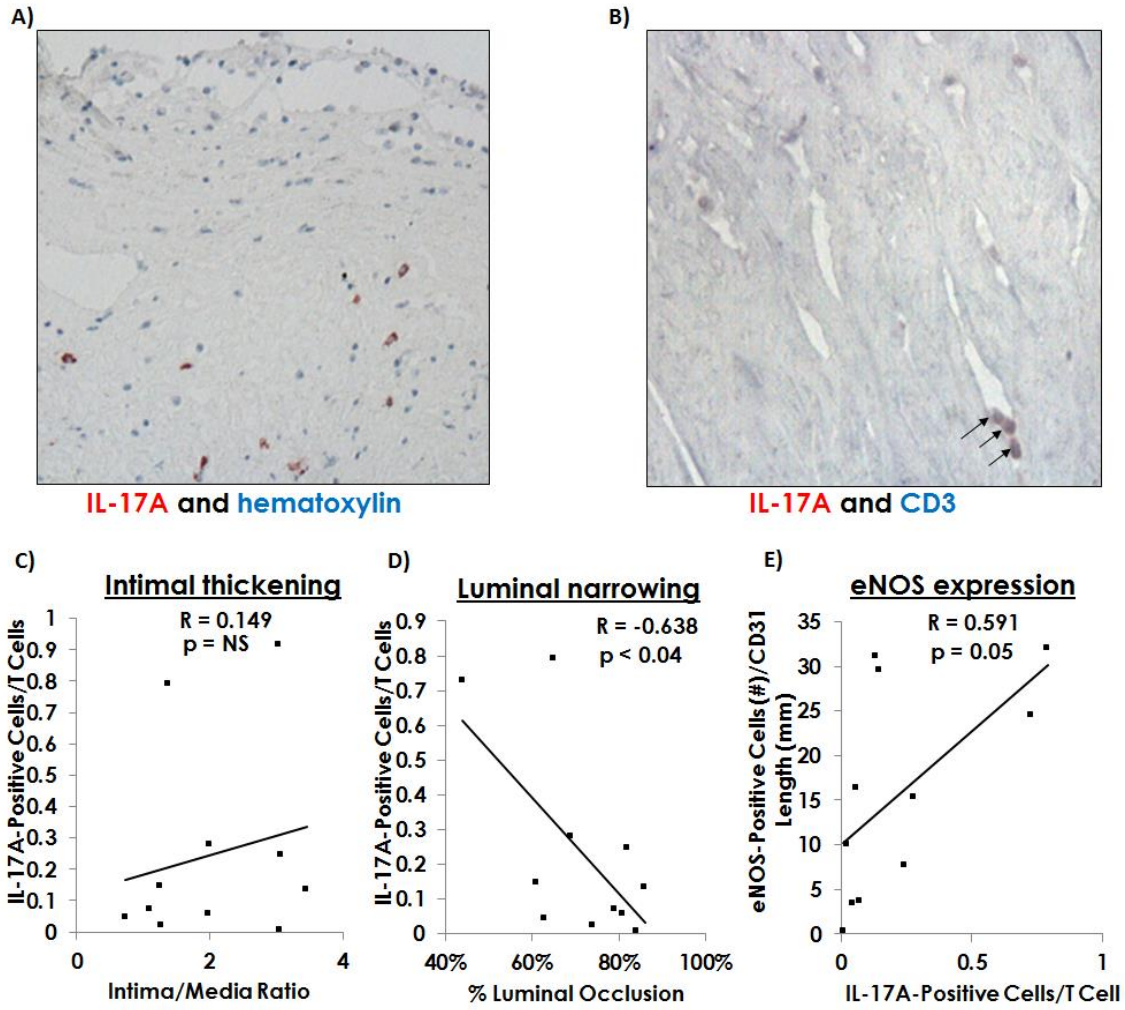

Figure A1. Previous findings on human TA specimen.

A) Intimal region of human TA specimen was immunohistologically stained for IL-17A and hematoxylin, showing presence of IL-17A-expressing cells in the intima. Representative of 11 samples from separate individuals. Mag=x200. B) Intimal region of human TA specimen immunohistologically stained for IL-17A and CD3, showing presence of IL-17A-expressing T cells in the intima. Representative of 11 samples from separate individuals. Mag=x400. C) Quantification of intimal thickening as measured by intima/media ratio in the human TA specimen. No correlation was found between intima/media ratio and the amount of IL-17A-positive T cells. D) Quantification of luminal narrowing as measured by percent luminal occlusion in the TA specimen. A negative correlation was found between percent luminal occlusion and IL-17A-positive T cells in the intima of samples. E) Quantification of eNOS expression in the intima of human TA specimen. A positive correlation was found between the amounts of IL-17A-positive T cells in the intima and eNOS-positive cells lining the artery. [86]

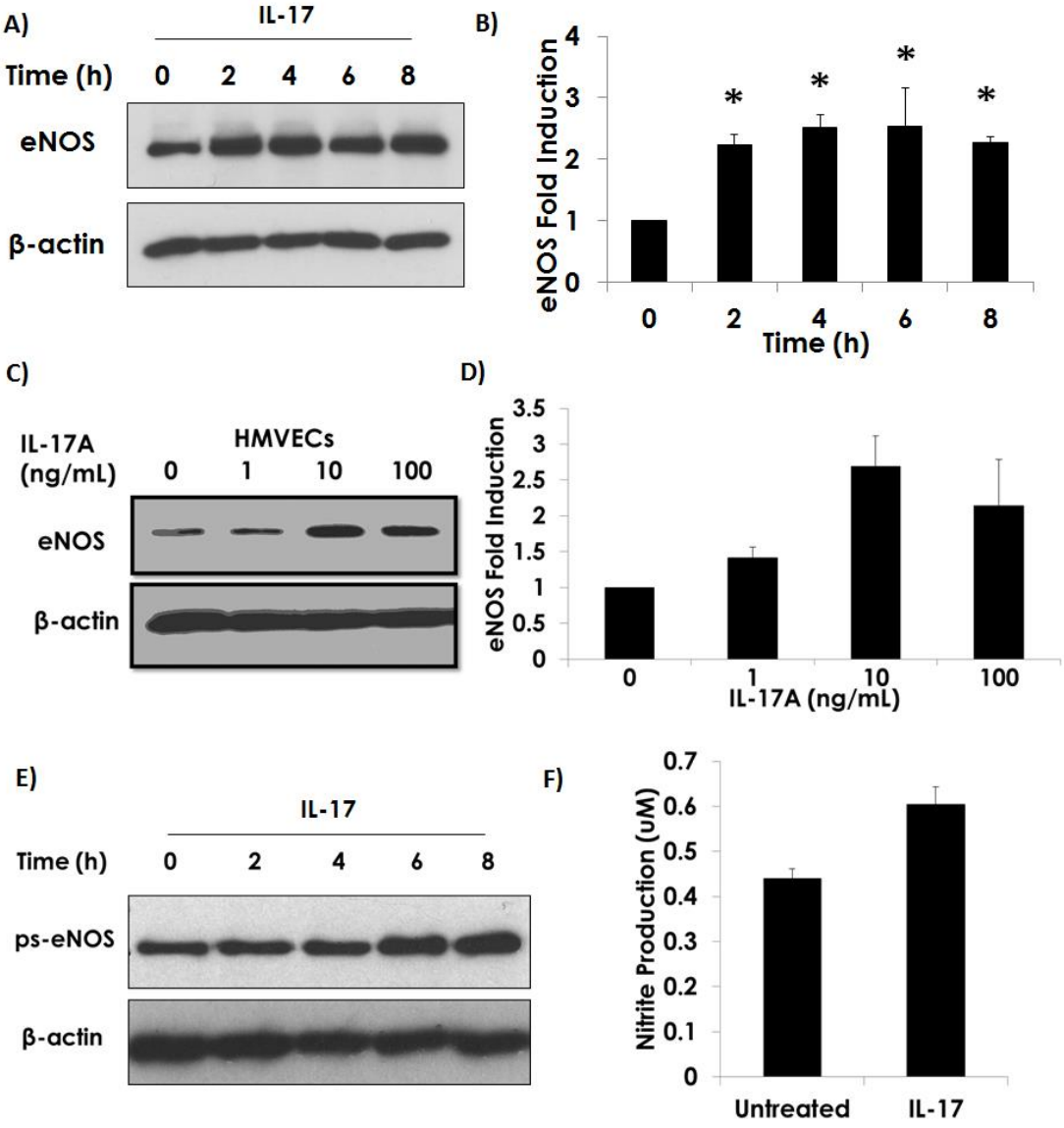

Figure A2. Preliminary findings of eNOS protein level up-regulation by IL-17.

A) IL-17 up-regulated eNOS protein levels time-dependently over the course of 6 h. d B) Quantification of EC time-course treatment with IL-17. C) IL-17 up-regulated eNOS protein levels dose-dependently, peaking induction at 10 ng/mL. D) Quantification of IL-17 treatment at different doses. E) IL-17 induced serine-1177 phosphorylation of eNOS activating enzymatic function to produce NO. F) Nitrite, stable byproduct of NO degradation, accumulation after IL-17 treatment was significantly increased compared with untreated. [86]