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Expression of the Alpha, Beta, and Gamma Subunits of the Interleukin-2 Receptor by Human Vascular Smooth Muscle Cells

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Expression of the Alpha, Beta, and Gamma Subunits of the Interleukin-2 Receptor by Human Vascular Smooth Muscle Cells

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY SULTAN ALHAYYANI ENTITLED EXPRESSION OF THE ALPHA, BETA, AND GAMMA SUBUNITS OF THE INTERLEUKIN-2 RECEPTOR BY HUMAN VASCULAR SMOOTH MUSCLE CELLS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

Alhayyani, Sultan. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2014. Expression of the Alpha, Beta, and Gamma Subunits of the Interleukin-2 Receptor by Human Vascular Smooth Muscle Cells.

Interleukin 2 (IL-2) is a member of the cytokine family and contributes to the proliferation, survival, and death of lymphocytes [1]. The interleukin-2 receptor (IL-2) is a tripartite receptor commonly expressed on the surfaces of many lymphoid cells and is composed of three non-covalently associated subunits, alpha (α) (CD25), beta (β) (CD122), and gamma (γ) (CD132) [2]. Our laboratory has previously described IL-2 receptor β (IL-2Rβ) expression by vasculature smooth muscle cells (VSMC) in mice and humans [3]. The current work expands our previous observations by assessing the expression of the alpha and gamma subunits of the IL-2R by VSMCs. Analysis of IL-2R expression in human VSMCs revealed no detectable expression of the gamma subunit and low expression of the alpha subunit. Treatment of VSMCs with IL-2 induced VSMC proliferation with a concomitant increase in the expression of the gamma subunit while having no detectable effect on expression of the alpha or beta subunits. Treatment of VSMCs with LPS decreased expression of the beta subunits and had little effect on alpha or gamma expression. Understanding the mechanisms that regulate expression of the IL-2R by VSMCs and how binding of this receptor by IL-2 mediates VSMC proliferation will provide a better understanding of vascular biology and possible mechanisms underlying vascular diseases such as atherosclerosis.

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List of Abbreviations

IL-2 = Interleukin 2

IL-2R = Interleukin 2 Receptor

CD25 = Interleukin 2 Receptor Alpha Subunit

CD122 = Interleukin 2 Receptor Beta Subunit

CD132 = Interleukin 2 Receptor Gamma Subunit

VSMC = Vascular Smooth Muscle Cell

LPS = Lipopolysaccaride

TLR4 = Toll Like Receptor 4

ECM = Extracellular Matrix

α-SMA = Alpha-Smooth Muscle Actin

SM-MHC = Smooth Muscle-Myosin Heavy Chain

CRBP = Cellular Retinol Binding Protein

IL-6 = Interleukin 6

IL-8 = Interleukin 8

IL-11 = Interleukin 11

TNF- α = Tumor Necrosis Factor Alpha

MCPs = Monocyte Chemotactic Peptides

MMIFs = Macrophage Migration Inhibitory Factors

PDGF = Platelet-Derived Growth Factor

c-fos = Proto-Oncogene

c-myc = Proto-oncogene

CDK = Cyclin-Dependent Kinase

INK4 = Tumor Suppressor Protein

KIP/CIP = A Family of Cyclin-Dependent Kinases Inhibitors

P21 = Cyclin-Dependent Kinase Inhibitor 1

P27 = Cyclin-Dependent Kinase Inhibitor 1 B

CD4 Cells = T Helper Cells

CD8 Cells = Cytotoxic T Cells

DCs = Dendritic Cells

NKC = Natural Killer Cells

TCR = T Cell Receptor

CD28 = Co-Stimulatory Receptor Expressed by Naïve T Cells

AP-1 = Activator Protein-1

NF-kB = Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NFAT = Nuclear Factor of Activated T-Cells

T-bet = T-Box Transcription Factor

Rel-A = Transcription Factor

STAT-5 = Signal Transducer and Activator of Transcription 5

Blimp-1 = B lymphocyte induced maturation protein 1

IL-7 = Interleukin 7

IL-9 = Interleukin 9

IL-15 = Interleukin 15

IL-21 = Interleukin 21

Jak-3 = Janus Kinase 3

Shc = Transforming Protein

MAPK = Mitogen-Activated Protein Kinase

PI3K = Phosphatidylinositol 3-Kinase

NKT = Natural Killer T cell

Foxp3 = Fork head Box P3

Treg = Regulatory T cells

TTBS = Tris-Buffered Saline and Tween 20

PBS = Phosphate Buffered Saline

NADPH = Nicotinamide Adenine Dinucleotide Phosphate

NADH = Nicotinamide Adenine Dinucleotide

HVSMC = Human Vascular Smooth Muscle Cell

HEK 293 = Human Embryonic Kidney 293 Cells

ICAM-1 = Intracellular Adhesion Molecule 1

IgG = Immunoglobulin G

HK IL-2 = Humankine IL-2

CS IL-2 = Cell Science IL-2

Acknowledgement

I would like to thank Dr. Lucile Wrenshall for her endless support and guidance, and for being my thesis advisor. Also, I would like to thank Dr. John Miller for being in my committee and for his always contribution to my work. I would like to thank Dr. Barbara Hull, the program director, for her support and suggestions. I would like to thank Dr. Nancy Bigley for her support and suggestions. In addition, I would like to thank Dr. Mohammed Al-Jahdali, the Dean of Science & Art College in Rabigh, King Abdulaziz University, and Dr. Hassan Felemban for their support and guidance. Lastly, many thanks to the Saudi Arabian ministry of high education and King Abdulaziz University, for supporting me financially.

Dedication

I would like to dedicate my thesis to my father (Mohammed Alhayyani), and my mother (Saleha Alhayyani), and my siblings for their endless support and encouragement.

Introduction

Smooth muscle is an involuntary non-striated muscle found within blood vessel walls and many other many tissues. Bundles of myosin and actin filaments within individual smooth muscle cells allow for contraction and relaxation resulting in changing the shape and stiffness of hollow organs. Contraction of vascular smooth muscle cells (VSMC) is responsible for the redistribution of blood to areas where it is needed by altering local blood pressure [4].

Interleukin 2 (IL-2) is a 15 kD glycoprotein and member of the cytokine family. IL-2 regulates immune cell function by promoting the proliferation, survival, and cell death of lymphocytes expressing the IL-2 receptor (IL-2R) [1]. The IL-2R is a tripartite receptor composed of three non-covalently associated subunits, alpha (α)(CD25), beta (β) (CD122), and gamma (γ) (CD132). The β and α subunits of the receptor bind to IL-2 while the β and γ subunits are responsible for signal transduction [2].

Our laboratory has previously shown that SMCs within blood vessels of IL-2 deficient mice exhibit a loss of smooth muscle cells compared to wild-type controls [3]. Additional work from our lab described expression of the IL-2Rβ by murine and human vascular smooth muscle cells [3]. The current work expands our previous observations by assessing the expression of the α and γ subunits by VSMCs. Analysis of IL-2R expression human VSMCs revealed no detectable expression of the γ subunit and little expression of the α subunit. Exposure of VSMCs to IL-2 increased VSMC proliferation with a concomitant increase in the

expression of the γ subunit while having little effect on expression of the α and the β subunits. In addition, exposure of VSMCs to bacterial lipopolysaccharide (LPS), which has been reported to activate SMCs through interaction with the Toll Like Receptor 4 (TLR4) [5], decreased the expression of β subunit while having little effect on the expression of α and γ. These results suggest that IL-2 and factors that activate SMCs may have a direct effect on expression of the IL-2R subunits by SMCs.

Hypothesis: Human Vascular Smooth Muscle Cells (VSMC) express all three subunits (α, β, γ) of the IL-2R.

Aims of the current study:

- **Determine which subunits of the IL-2 receptor are expressed by smooth muscle cells.**
- **Determine how activation of smooth muscle cells impacts IL-2R expression.**
- **Assess how IL-2 affects smooth muscle cell function.**

Literature Background

Smooth Muscle cells:

Smooth muscle is an involuntary non-striated muscle found within many tissues including blood vessel walls, lymphatic vessels, the gastrointestinal and respiratory tracts, and iris of the eye. Bundles of myosin and actin filaments within individual smooth muscle cells allow for contraction and relaxation resulting in changing the shape and stiffness of hollow organs [4].

Vascular Smooth Muscle Cells (VSMCs) are a major component of blood vessel walls and are responsible for altering local blood pressure thereby providing increased blood flow to areas where it is needed. In addition, VSMCs are important in the remodeling vessel tissue by proliferation, migration, and the synthesis of large amounts of extracellular matrix (ECM), following pregnancy or vascular injury [4]. VSMCs can perform both contractile and synthetic functions, which are characterized by changes in morphology, proliferation, migration rates, and the expression of different marker proteins. Alpha -smooth muscle actin (α-SMA), smooth muscle-myosin heavy chain (SM-MHC), and cellular retinol binding protein (CRBP) are some of the SMC marker proteins involved in SMC contraction as structural components of the contractile apparatus or as a contraction regulator. VSMCs in the vessel wall are diverse. This diversity is reflected by the differential expression of contractile marker proteins, gap of junction proteins, and adhesion molecules. This heterogeneous expression in VSMCs is attributable to variations in gene expression and consequently SMC

function. The diversity of VSMCs within a given length of vessel has been proposed to make the vessel more responsive to various physiological or pathological situations. In addition to their contractile and structural functions within the vessel wall, VSMCs synthesize, secrete, and respond to small soluble signaling molecules [6]. VSMCs synthesize IL-1, IL-6, IL-8, IL-11, TNF- α, monocyte chemotactic peptides (MCPs), and macrophage migration inhibitory factors (MMIFs)-1 α and -1 β [7, 8, 9, 10,11]. These cytokines and growth factors stimulate SMC migration as an early event in vessel remodeling [12,13].

Vascular Smooth Muscle Cells Proliferation:

Under normal conditions VSMCs do not proliferate [14]. However, injuries such as angioplasty, vascular stent implantation, or organ transplantation induce VSMC proliferation [14,15,16]. Growth factors including platelet-derived growth factor (PDGF), basic fibroblast growth factor, and insulin – like growth factor -1 promote VSMC proliferation [17,18]. The actions of these growth factors involve nuclear factors including c-fos and c-myc that become active when the growth factors bind to their respective cell surface tyrosine kinase receptors. These nuclear factors work as transcriptional factors, leading to an increase in the expression of various cell cycle regulatory proteins such as the cyclins and cyclin-dependent kinases (CDK) [18,19]. To keep the proliferative response in check, other factors inhibit VSMC proliferation. Such factors include CDK inhibitors, which are divided into 2 different families based on their structure: the

INK4 family (p14, p15, p16, p18, and p19) and the KIP/CIP family (p21, p27, and p57) [20].

Vascular Smooth Muscle Cell Migration:

In the normal resting condition, VSMCs are non-migratory due, in part, to being surrounded by a highly dense and adhesive matrix and the relative absence of stimulatory factors [21]. Blood flow, sheer stress, cytokines, and peptide growth factors are some of the factors that can affect VSMC migration [21]. The initiation of migration begins with the activation of cell surface receptors such as platelet-derived growth factor (PDGF) receptors which, in turn, activate signal transduction pathways, trigger remodeling of cytoskeleton, decrease the adhesive molecules in the matrix and activate motor proteins [22]. During migration there is a decrease in the expression of cell-cell adhesive molecules and focal contacts in the trailing edge of the cell and an increase adhesion of the cell membrane to the matrix at the leading edge. Regulation of the actin cytoskeleton occurs by several signaling pathways involving molecules such as trimeric G proteins, small G proteins, lipid kinases, Ca^{2+}-dependent kinases, and Rho kinases [23].

There is also a link between migration and proliferation of VSMCs [24]. During VSMC proliferation, migration occurs only when the cell cycle is in the G1 phase but not in the later phases of the cell cycle. When the proliferation of VSMC is inhibited by P21 and p27 proteins, inhibitors of VSMC proliferation, VSMC migration is also inhibited. Overexpression of these inhibitor proteins

leads to a reduction of human vein VSMC migration as well as proliferation [25, 26].

Interleukin-2:

Interleukin 2 (IL-2) is a 15 kD glycoprotein, a member of the cytokine family, and is produced predominately by activated $CD4^+$ and $CD8^+$ T cells [1]. IL-2 regulates immune cell function by promoting proliferation, survival, and cell death of lymphocytes expressing the IL-2R [27]. IL-2 also acts as a down-regulator of T cells leading to apoptosis. This feedback mechanism targets re-activated T cells in order to restrict the expansion of antigen-specific immune responses [28, 29]. Other cells such as activated dendritic cells (DCs) and natural killer (NK) cells produce IL-2, but the biological relevance of this production is unclear [30, 31, 32]. IL-2 is produced following binding of the T cell receptor (TCR) by costimulatory molecules such as CD28 on naïve T cells. Transcriptional induction of the IL-2 gene is stringent. The promoter/enhancer region for the IL-2 gene is approximately 500 base pairs upstream of the transcription initiation site [33]. After TCR/costimulatory molecules bind, the TCR signaling induces an increase in AP-1 and NF-kB levels, which in turn cause the dephosphorylation of NFAT, leading to its translocation from the cytoplasm to nucleus [34, 35]. These transcription factors bind to specific sites within the first 300 base pairs of the minimal promoter, leading to IL-2 gene transcription. T-box transcription factor (T-bet) has been recently found to repress IL-2 production through its interaction with Rel-A within the proximal IL-2 promoter [36]. IL-2

inhibits its own production through a classical auto-regulatory feedback loop, this loop depends on activation of signal transducer and activator of transcription-5 (STAT-5), and the IL-2-dependent induction of the transcriptional repressor B-lymphocytes maturation protein-1 (Blimp-1) [37]. Thus, when naïve T cells become activated, IL-2 is produced and binds to the IL-2 receptor (IL-2R), which, in turn, leads to activation of STAT-5 and induction of Blimp-1, thence IL-2 gene repression.

Interleukin-2 Receptor:

The IL-2R is a tripartite receptor composed of three non-covalently associated subunits: alpha (α; CD25), beta (β; CD122), and the gamma (γ; CD132). The γ subunit is common to the receptor complexes for at least six different interleukin receptors: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The β and the γ chains are related to the class I cytokine receptor superfamily, and exhibit primarily folded β-sheet structures [2]. These three subunits are required for high affinity IL-2 binding. IL-2 initially binds only the α subunit, which then promotes association with the β and γ subunits. This IL-2/IL-2R quaternary complex induces signaling pathways through the cytoplasmic β and γ tails [38, 39]. The proteins Jak-3 via γ and Jak-1 via β phosphorylate key tyrosine residues, which leads to binding between the adaptor Shc and either Stat-3 or Stat-5. Shc activates two pathways: the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). These two pathways are important for cell survival and growth. After Stat is further phosphorylated, it dimerizes and

translocates into the nucleus to regulate genes important in growth and function of T cells [39]. IL-2/IL-2R binding on the cell surface is short-lived. After 10 to 20 minutes, the IL-2/IL-2R complex is internalized into the cytoplasm. IL-2, CD122, and CD132 are degraded by lysosomes, whereas CD25 recycles to the cell surface [40, 41, 42] [43]. CD132 is a shared subunit not only for IL-2R, but also for IL-4, IL-7 IL-9, IL-15 and IL-21, and is expressed on all hematopoietic cells [2, 44]. Lymphoid progenitors, NK cells, NKT cells, memory phenotypic CD8 T cells, and $Foxp3^+$ Treg cells express CD122 subunit [45]. Most lymphoid cells, except subsets of developing pre-T and pre-B cells, do not express CD25. Activated effector cells, and natural $CD4^+$ $Foxp3^+$ Treg cells express CD25 [46].

Materials & Methods

Human Vascular Smooth Muscle Cell Culture:

Human aortae, obtained from organ donors, were washed with PBS and the adventitia was removed by gently scraping the outer surface with a scalpel blade. A 2 cm^2 piece was placed in a Petri dish and cut into1 mm^2 pieces. The pieces were placed lumen side down onto the surface of a TC-75 culture flask. The flask was placed upright in an incubator at 37°C to allow the tissue to adhere to the surface of the flask. After 4-6 hours, 10 ml of smooth muscle cell medium was carefully poured into the flask so as not to dislodge the tissue. The aortic pieces were cultured for 1-2 weeks before removal, and remaining cells were passaged 4 times before use in assays.

In-cell Western*:*

VSMCs were cultured in black 96-well plates at an initial density of 5,000 cells per ml. After incubation under conditions indicated in the figure legends, cells were fixed in ice-cold methanol with 2% acetic acid v/v. After washing 3 times with Tris-buffered saline/Tween-20 (TTBS), the cells were blocked for 2 hours with Odyssey blocking buffer (Licor) containing 2% mouse serum. The anti-CD25, anti-CD122, and anti-CD132 antibodies were added at 1μg/well in TTBS and incubated with the fixed, blocked cells in triplicate overnight at room temperature. Isotype control antibodies were used at the same concentration as the primary antibodies. The next day the cultures were washed 3X with TTBS,

and probed for 1.5 h with streptavidin-680 (SA-680, Invitrogen) used at 1μg/well in TTBS. After a final wash, the plate was allowed to air dry in the dark and read at 680 nm using a Synergy plate reader. (For additional details see appendix A)

IL-2 Binding Studies:

VSMC were cultured in 96-well plates at an initial density of 5,000 cells per well. Cells were probed with one of two different IL-2 preparations previously conjugated to a small molecular weight infra-red dye. Briefly, human IL-2 from a bacterial source (unglycosylated) or mammalian cell line (glycosylated) was labeled with an infrared dye (680LT, LI-COR Biosciences, Lincoln, NE) per the manufacturer's instructions. The activated infra-red dye was added to the IL-2 at a molar ratio of 1:1. Following a 2 hour incubation at 20°C, unconjugated dye was removed from the preparation using a Zebra Spin de-salting column.

Cells were probed with one of the two fluor-conjugated monomeric IL-2 preparations at the concentrations indicated in the figure legend for 20 mins at 4°C. To determine if antibodies against the β subunit of the IL-2R would block binding of the fluor-IL2, some cells were pre-incubated with 40ug/ml of anti-CD122 for 30 minutes. Cells were then fixed with ice-cold methanol with 5% acetic acid for 15 minutes at 4°C. The fixed cultures were washed 2 times with PBS and read at 680 nm using a Synergy plate reader. (For additional details see appendix B).

SMC Proliferation Assay:

VSMCs were cultured in clear 96-well plates at an initial density of 5,000 cells per ml. Some cells were treated with IL-2 as indicated in the figure legend, and incubated for 2, 4, or 6 days. Twenty µl of CellTiter 96 Aqueous One solution reagent (Promega) was added to each well of the 96-well plate containing the samples in 100µl of culture medium, incubated at 37°C for 1-4 hours, then read at 490nm using a Synergy plate reader. The CellTiter 96 Aqueous One Solution reagent contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium, which is reduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion occurs by NADPH or NADH produced by dehydrogenase enzymes only in cells that are metabolically active. (For additional details see appendix C)

Results

Human Vascular Smooth Muscle Cell can express the Alpha, Beta, and Gamma Subunits of the IL-2 Receptor.

To determine if human vascular smooth muscle cells (HVSMC) express the Alpha, Beta, or Gamma subunits of IL-2R, we examined their expression using an In-Cell Western analysis, in which cell surface expression of receptors is assayed in fixed, cultured cells using fluorescently-labeled antibodies. As shown in Figure 1, HVSMCs express Beta subunit, and to a lesser degree the Alpha subunits. Little to no expression of the Gamma subunit was detected.

Dye-labeled glycosylated IL-2 binds vascular smooth muscle cells.

In light of the above results, we next asked whether IL-2 binds the IL-2R expressed by HVSMCs. Cultured cells were incubated with glycosylated human IL-2 labeled with a fluorescent dye (680LT, LI-COR Biosciences, Lincoln, NE). The presence of carbohydrates mimics the *in vivo* situation wherein HVSMC's would be exposed to a glycosylated IL-2. To confirm that retention of the fluor-IL-2 was mediated by the IL-2R, some cells were pre-incubated with an anti-CD-122 antibody. As seen in figure 2, fluor-monomer IL-2 bound to HVSMCs. Pre-incubation with anti-CD-122 decreased the binding of fluor-IL-2 three fold.

Dye labeled, non-glycosylated IL-2 binds vascular smooth muscle cells.

To assess the involvement of carbohydrates on IL-2 in mediating binding to the IL-2R expressed by HVSMCs, binding of glycosylated fluor-IL-2 (above)

was compared to binding of a non- glycosylated form of recombinant IL-2. Using the same procedure as detailed above, HVSMC's were found to bind non-glycosylated fluor-IL-2, and this binding was also inhibited by an anti-CD122 antibody. An approximately 2 fold decrease in the binding of non-glycosylated fluor-IL-2 was detected in wells pre-incubated with Anti-CD122 antibody compared to wells probed with dye labeled non-glycosylated IL-2 alone (Figure 3).

IL-2Rβ expression decreases in vascular smooth muscle cells after exposure to lipopolysaccharide (LPS).

Having confirmed that HVSMCs express the Beta and Alpha subunits of the IL-2R, we asked whether treatment of HVSMCs with LPS influenced IL-2R subunit expression. To do so, VSMC were cultured with LPS for 48 hours, and IL-2R expression was assessed. No appreciable change in the expression of the Alpha or Gamma subunits was observed in response to exposure to LPS (data not shown). However, Beta expression was observed to decrease (Figure 4).

Exposure of Vascular Smooth Muscle Cells to IL-2 increases expression of the IL-2R gamma subunit (γ).

Based on the above results, we asked how IL-2 affects IL-2R subunit expression. Cultured VSMCs were cultured with IL-2 for 24 hours and IL-2R subunit expression was assessed. Interestingly, Gamma subunit expression increased in

cells exposed to IL-2 compared to media only (Figure 5). No appreciable change occurred in Alpha and Beta expression (data not shown).

Exposure of Vascular Smooth Muscle Cells to IL-2 increases SMC Proliferation.

To determine if IL-2 affects VSMC proliferation, HVSMC's were treated with monomeric IL-2 and the proliferative response was assessed. Treatment of HVSMCs with IL-2 resulted in an approximately 25 fold-increase in the number of viable cells compared to wells receiving no IL-2.

Discussion

Interleukin 2 is a 15-kD cytokine produced mainly by T cells. It plays a critical role in regulating T cell proliferation, survival and death [1]. IL-2 binds with high affinity to a tripartite receptor composed of alpha (CD25) (α), beta (CD122) (β), and gamma (CD132) (γ) subunits that are expressed mainly on the surfaces of lymphoid progenitors, NK cells, NKT cells, memory T cells, and T-reg cells [45]. However, more recent studies from our lab and others have described expression of the IL-2R on non-lymphoid cells including vascular smooth muscle cells [47, 3].

Our laboratory recently described expression of the β subunit of the IL-2R by murine and human VSMC's [3]. Our current work reveals that VSMCs express both the β and α subunits, and can be induced to increase expression of the of γ subunit. To examine if the glycosylation state of IL-2 affects binding to the IL-2R expressed on VSMC's, we compared the binding of a glycosylated form of IL-2 with a non-glycosylated form. No appreciable difference was detected between the glycosylated and non-glycosylated forms, suggesting that the IL-2R expressed on VSMC's does not bind IL-2 in a carbohydrate-dependent manner. Since IL-2's main function is regulating T cell proliferation, we asked if treatment of VSMC's with IL-2 induces a proliferative response. Interestingly, IL-2 increases VSMC proliferation, suggesting that in vivo exposure of VSMC's to IL-2 may affect the development of new blood vessels and/or the regeneration of vasculature following injury.

IL-2 is used as an immunotherapy due to its ability to increase T-cell proliferation and differentiation. Although this treatment can cause regression of tumor metastases, it is associated with multiple side effects including vascular leak syndrome, left ventricular dysfunction, increased cardiac output, and decreased systemic vascular resistance [48]. The most significant dose-limiting side effect is vascular leak syndrome, which entails increased vascular permeability causing a leakage of fluid from the circulatory system into the interstitial space resulting in ascites, hydrothorax, pulmonary edema, and hypotension.

Previous studies have shown that IL-2 increases the expression of adhesion molecules such as ICAM-1 in endothelial cells and beta-2 integrin in neutrophils. Increased expression of these adhesion molecules has been proposed to be part of the mechanism underlying vascular leak syndrome [49, 50]. Whether IL-2 binding to its receptor in SMCs increases expression of these adhesion molecules is under investigation. However, given the responsivity of VSMC's to IL-2 described in the current work, it seems likely that some aspect of vascular leak syndrome could be caused by a direct effect of high dose IL-2 on VSMC's.

Abnormal VSMC proliferation is thought to play a critical role in atherosclerosis and restenosis. In response to injury of the blood vessel endothelial cell layer, cholesterol, and cellular waste products accumulate in the damaged area, inducing endothelial cells to produce pro-inflammatory cytokines. As a response to this inflammation, leukocytes adhere to the endothelial layer

[51]. This may cause the production of cytokines including IL-2. Based on our finding that IL-2 increases SMC proliferation *in vitro*, exposure of VSMC's to IL-2 during an inflammatory event would cause them to proliferate, causing fibromuscular lesions, which are characterized as abnormal growth within the vessel wall. As a result of this process, the artery wall thickens, leading to reduced blood flow, which may contribute to atherosclerosis or restenosis. Our lab has previously described the binding of IL-2 to heparan sulfate (HS) carbohydrates within the blood vessel wall [3]. Cleavage of the HS carbohydrates by heparanase was shown to release biologically active IL-2. Liberation of HS-bound IL-2 by heparanase released by T-cells during extravasation through the vessel wall could, in turn, induce the proliferation of VSMCs [3].

Previous work has demonstrated that SMC's produce multiple immunoregulatory factors including IL-6, IL-8, and TNF- α [52, 53, 54, 55]. Given these previous studies, future work assessing the ability of VSMC's to produce IL-2, which could work in an autocrine fashion as it does with T cells is reasonable. The current work demonstrates the involvement of the Beta subunit of the IL-2R in binding IL-2. Expanding these studies to assess involvement of the alpha and possibly the gamma subunits would better define the interaction of IL-2 with the IL-2R expressed on VSMC's. One approach would be to assess how small interfering RNA (siRNA) specific for the alpha, beta, or gamma subunits affect the ability of SMC's to proliferate in response to exposure to IL-2 in vitro. In addition, determining if IL-2 induces the proliferation of vessel endothelial cells would provide a broader vision of the influence of IL-2 on non-

lymphoid cells of the vasculature. The aortas of IL-2 deficient mice are characterized by an abnormal cytoarchitecture of the SMC layer [3] suggesting that IL-2 may have an effect on the development or maintenance of a normal vessel wall. In addition, smooth muscle cell α-actin (SMC α-actin) is a protein known to play a critical role in the differentiation of VSMC. Determining if administration of IL-2 to IL-2 knock-out mice results in a more normal VSMC layer and the expression of SMC α-actin would allow an assessment of the role played by IL-2 during blood vessel development. Understanding the mechanisms that regulate expression of the IL-2R by VSMCs and how binding of this receptor by IL-2 effects VSMC biology will provide a better understanding of the mechanisms underlying vascular development and diseases of the vasculature such as atherosclerosis, and restenosis.

Figures

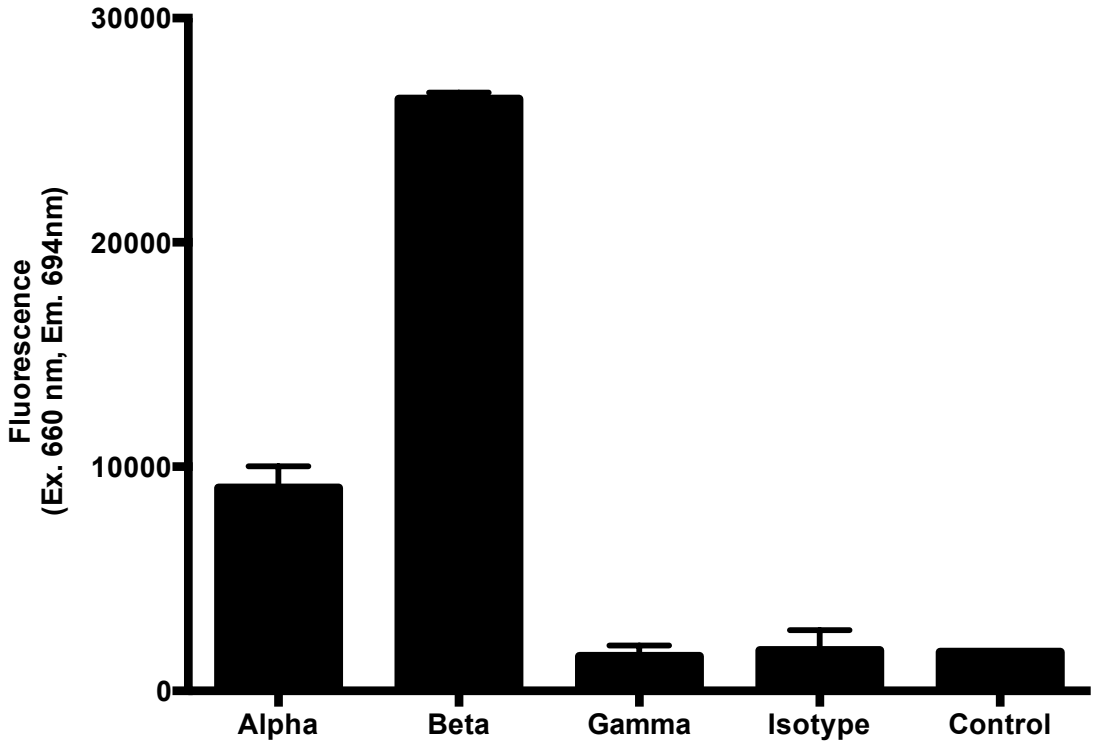

Figure 1. Human Vascular Smooth Muscle Cells Express the Alpha (α), Beta (β) but not the Gamma (γ) subunit of IL-2R. Using in-cell Western analysis, cultured VSMCs were probed with antibodies directed against α, β, and γ subunits of the IL-2R. Isotype indicates biotinylated mouse IgG, and control indicates cells alone with no antibodies. Error bars represent the means ± SD of triplicate wells, and representative of 3 separate experiments. Analysis of variance (ANOVA): $P < 0.05$

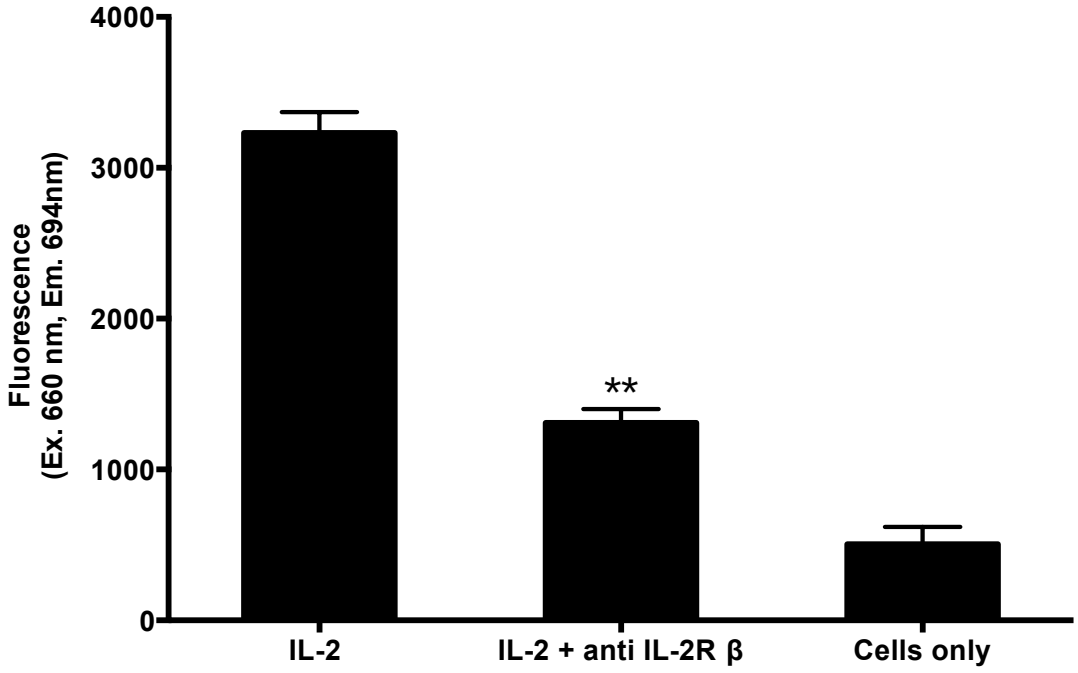

Figure 2. VSMCs bind dye-labeled glycosylated IL-2 via IL-2R β.

Cultured VSMCs were probed with dye labeled glycosylated IL-2 after pre-incubation with or without anti- IL-2R β antibodies. Error bars represent the means ± SD of triplicate wells, and representative of 3 separate experiments. Analysis of t test: **($P < 0.05$).

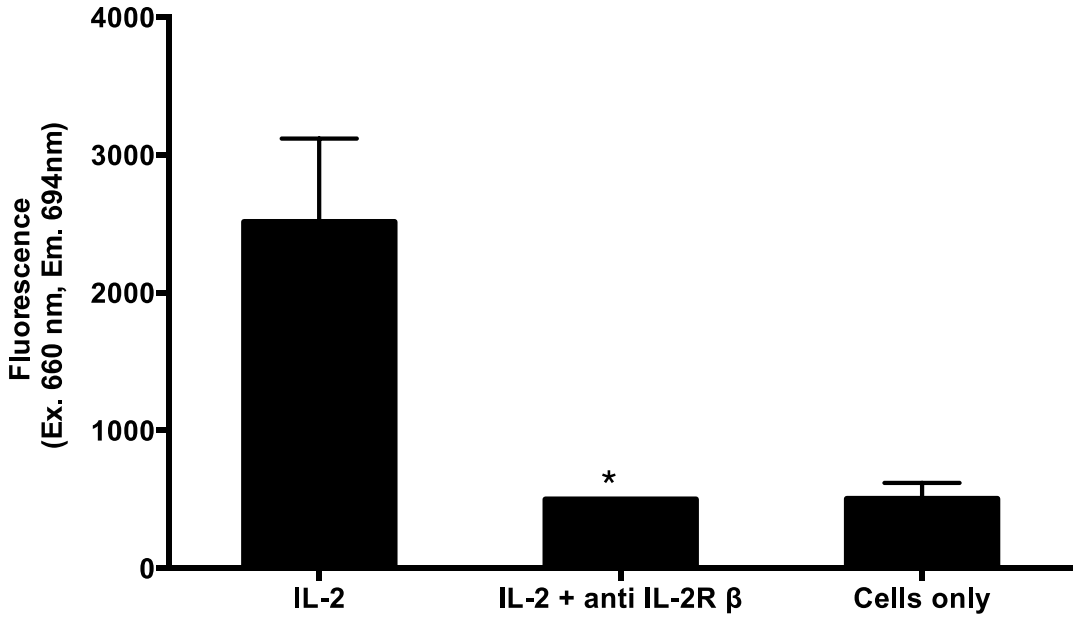

Figure 3. VSMCs bind dye-labeled non-glycosylated IL-2 via IL-2R β. Cultured VSMCs were probed with dye labeled non-glycosylated IL-2 after pre-incubation with or without anti- IL-2R β antibodies. Error bars represent the means ± SD of triplicate wells, and representative of 3 separate experiments. Analysis of t test: *($P < 0.05$).

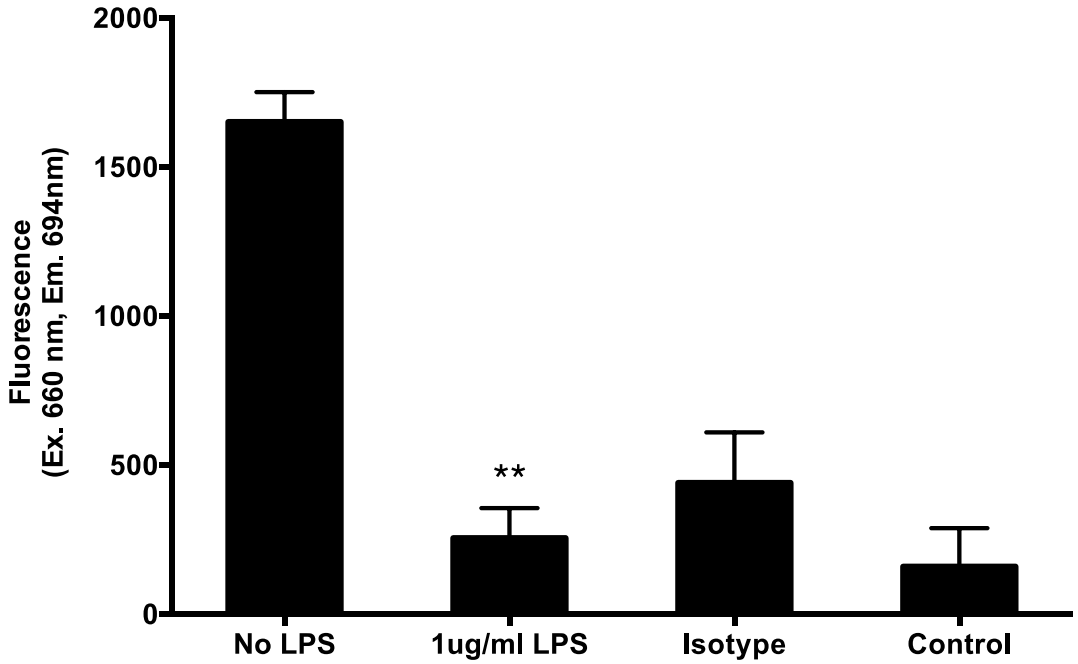

Figure 4. **Exposure of VSMCs to lipopolysaccharide decreases IL-2Rβ expression.** Using in-cell Western analysis, cultured vascular smooth muscle cells were probed with an antibody directed against the β subunit of the IL-2R after 48 hours incubation with lipopolysaccharide (LPS; 1 ug/ml). Isotype indicates biotinylated mouse IgG, and control indicates cells alone with no antibodies. Error bars represent the means ± SD of triplicate wells, and representative of 3 separate experiments. Analysis of t test: **($P < 0.05$)

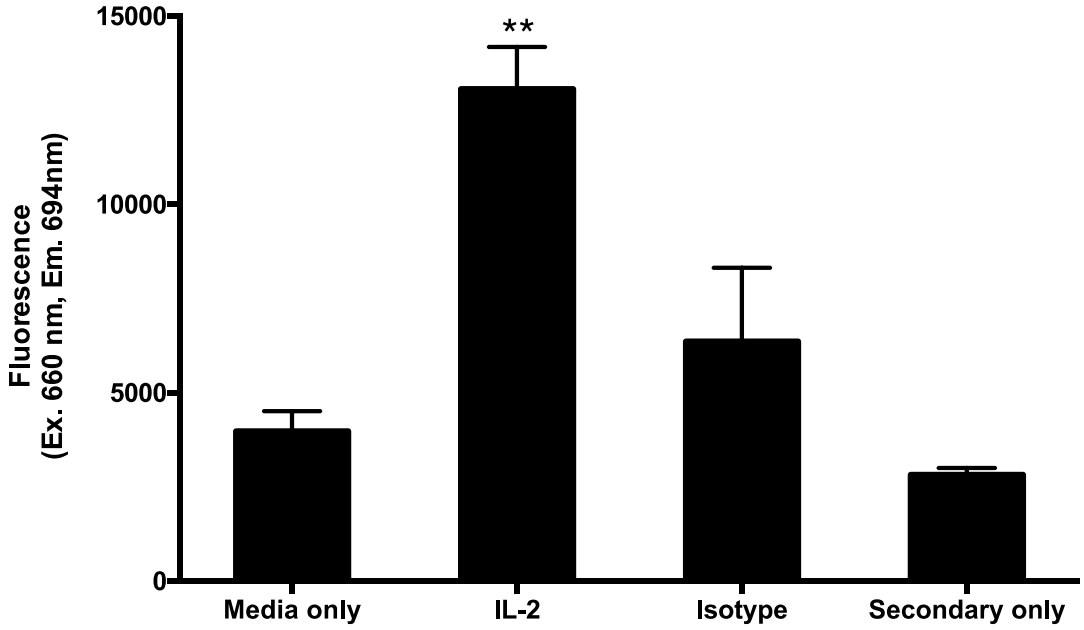

Figure 5. Treatment of Vascular Smooth Muscle Cells with IL-2 increases expression of the IL-2R gamma subunit . Using in-cell Western analysis, cultures were probed with an antibody directed against the gamma subunit of the IL-2R following 24 hours incubation with IL-2 (4 ng/ml). Isotype indicates biotinylated mouse IgG, and control indicates cells alone with no antibodies. Error bars represent the means ± SD of triplicate wells, and representative of 3 separate experiments. Analysis of t test: **($P < 0.05$)

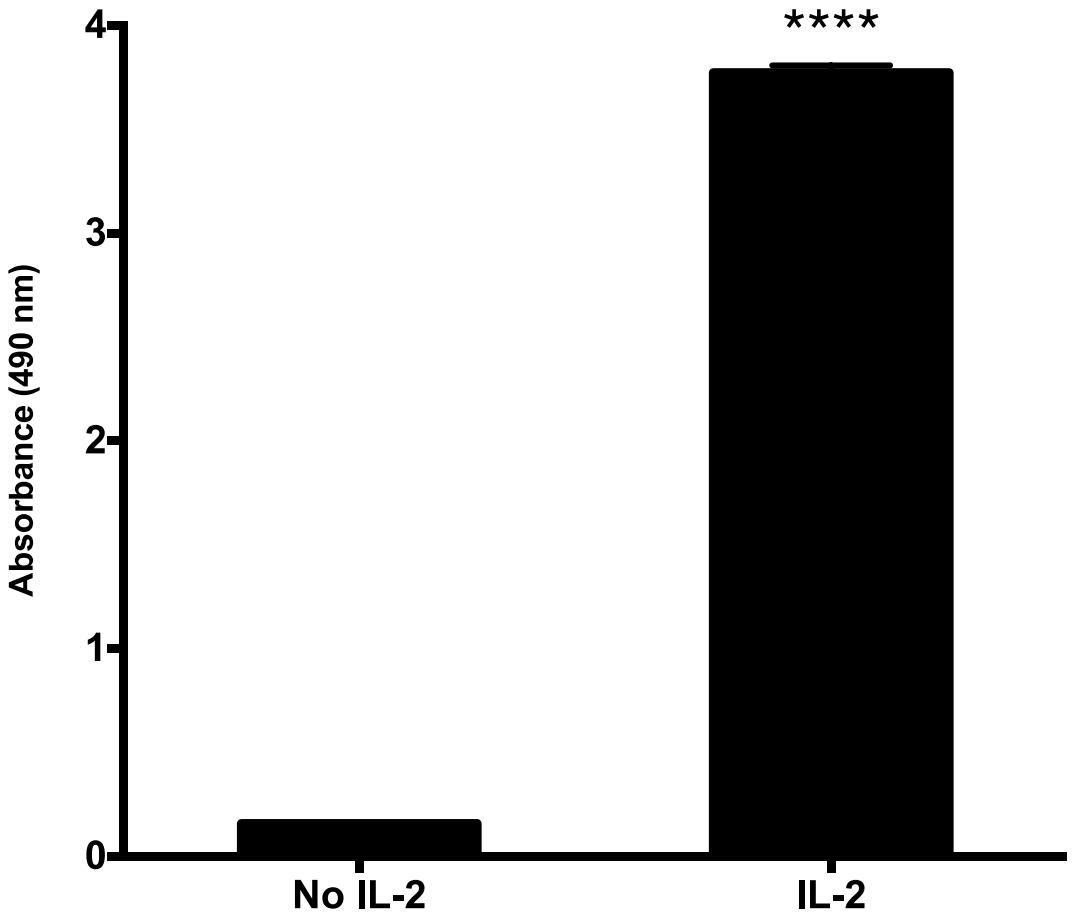

Figure 6: Exposure of Vascular Smooth Muscle Cells to IL-2 increases SMC Proliferation.

VSMCs incubated for 48 hours with 25ng/ml IL-2 or media alone. Proliferation of VSMCs was assessed by MTS-based assay" CellTiter 96 One Aqueous Solution Cell Proliferation Assay". Error bars represent the means ± SD of triplicate wells, and representative of 4 separate experiments. Analysis of t test: ****($P < 0.05$)

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Appendix A

In-Cell Western Protocol

Materials required:

Fixative: Ice-cold Methanol with 2% v/v Acetic Acid.

Assay Buffer: Tris-baffered Saline + 0.1% Tween (TTBS)

1) Gently wash plate 1X with PBS (100 μL/well)

2) Fix for 5 minutes in ice-cold Methanol with 2% v/v Acetic Acid (100 μL/well). After fixation, put the plate in the fridge for 5 mins.

3) Gently wash 3X with PBS (100 μL/well).

4) Block for 2 hours at room temperature with Odyssey block solution with 1% v/v Mouse serum (150 μL/well).

5) Wash 3X with TTBS (100uL/well).

6) Incubate wells with primary antibodies suspended in TTBS overnight at room temperature.

Antibody	Concentration/ml
Anti-alpha	10μg/ml
Anti-Beta	10μg/ml
Anti-Gamma	10μg/ml

Next Day

7) Wash wells 3X with TTBS (100 μL/well).

8) To detect biotinylated antibodies, probe wells with Avidinylated secondary used at 10μg/ml. Incubate for 1.5 hours at room temperature.

9) Wash the plate 3X with TTBS.

10) Read the plate immediately.

11) Place in dry dark place (closed drawer) and allow to dry overnight for 16 hours.

11) Re-read dry plate the next morning.

Appendix B

IL-2 Binding Studies

Materials Required:

Fixative: Ice-cold Methanol with 2% v/v Acetic Acid.

Assay Buffer: PBS only

Protocol:

1) Wash wells 2X with PBS.

3) Pre-incubate 500ng/ml fluor IL-2 for 20 minutes at 4C.

4) Wash wells 3X with PBS.

5) Place 100 μL of Fix solution in each well and incubate at 4C for 15 minutes.

6) Wash wells 2X with PBS.

7) Read on Synergy (wet).

8) Allow wells to air dry in the dark and re-read (dry).

Appendix C

SMC Proliferation Studies

1- Maintain a plate of SMCs in SMC medium supplemented with 25ng/ml human recombinant IL-2 for 48 hours.

2- Thaw the CellTiter 96® AQueous One Solution Reagent. It should take approximately 90 minutes at room temperature, or 10 minutes in a water bath at 37°C, for completely thawing.

3- Pipet 20µl of CellTiter 96® AQueous One Solution Reagent into each well of the 96-well assay plate containing the SMC cells in 100µl of SMC medium.

4- Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO2 atmosphere.

5- Record the absorbance at 490nm using a 96-well Synergy plate reader.