The Immunological and Vascular Effects of IL-6 Signalling in Transplant Arteriosclerosis

by

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Abstract

Transplant arteriosclerosis (TA) is a pathological vascular condition that is a main cause of chronic rejection and eventual failure of cardiac transplants. TA is caused by the activation of T cells towards allogeneic antigens expressed by vascular cells, which results in T cell-mediated injury and dysfunction of allograft arteries. T cells also support the activation and secretion of donor specific antibodies (DSAs) by B cells that contribute to the pathogenesis of TA. Interleukin-6 (IL-6) is secreted within hours of surgical transplantation and plays an important role in activation of allogeneic immune responses and regulation of vascular processes that influences TA. There are two main mechanisms by which IL-6 can signal to cells, classic and trans, that are distinguished by whether it binds to membrane bound or soluble forms of its receptor (IL-6R). In classic signalling IL-6 binds to IL-6R on the surface of cells. In trans-signaling IL-6 binds to soluble IL-6R. In both scenarios, the IL-6/IL-6R complex associates with the signalling subunit gp130 on cell membranes to transduce intracellular signalling events. Interestingly, IL-6 classic and trans-signalling have distinct biological outcomes. I examined the mechanism by which IL-6 classic and trans signalling contributes to immune activation in TA and characterized the biological outcomes of IL-6 signalling in endothelial cells. IL-6 classic and trans signalling were redundant for the activation of peripheral T cells that cause TA. However, eliminating IL-6R expression in T cells significantly reduced the development of DSAs in the serum of graft recipients, suggesting that IL-6 classic signalling in T cells may be required for antibody-mediated pathology in TA. In addition to activating immune cells, IL-6 acts on endothelial cells to induce inflammation and protect the vasculature from injury. IL-6 trans signalling in ECs significantly induced STAT-3, ERK1/2 and Akt activation. This signalling mechanism was needed for ICAM upregulation and the secretion of inflammatory cytokines by IL-6, indicating that IL-6 trans-signaling drives inflammatory activation of ECs. IL-6 classic signalling induced ERK1/2 and Akt activation but not STAT3 and was sufficient to stimulate the secretion of IL-8 and to protect ECs from cell death caused by serum deprivation but did not induce other inflammatory processes in ECs. My results suggest that IL-6 trans and classic signalling differentially affect inflammatory and survival responses in endothelial cells, which may have implications for understanding the vascular effects of IL-6R blockade in patients. Overall, my findings provide new insight into the immune and vascular effects of IL-6 in transplantation.

Dedication

To my mother Karen Bernard,

To my supervisor Dr. Jonathan Choy and to the current and past members of my lab: Umbreen Arshad, Katrina Besler, Catherine Cheneval, Winnie Enns, Naomi Geisbrecht, Martin Lee, Sukh Manku, Ankana Murkahjee, Kevin Rey & Haya Shaalan.

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Acronyms

ADCC	Antibody-dependent cellular cytotoxicity
AICD	Activation-induced cell death
Akt	Protein kinase b
AMR	Antibody-mediated rejection
APC	Antigen presenting cell
DAMP	Danger associated molecular patterns
DC	Dendritic cell
DSA	Donor specific antibody
EC	Endothelial cell
ERK	Extracellular-signal regulated kinase
Gp130	Glycoprotein 130
HLA	Human leukocyte antigen
HUVEC	Human umbilical vein endothelial cells
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon- gamma
IL-6R	Interleukin – 6 receptor
IL-6RTKO	Interleukin-6 Receptor T cell Knock out
JAK	Janus Kinases
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
NO	Nitric oxide
NK	Natural killer cells
P-STAT	Phosphorylated Signal transducer and activator of transcription
PI3K	Phosphoinositide 3-kinases
ROS	Reactive oxygen species
SMC	Smooth muscle cells
SOCS	Suppresor of cytokine signalling
STAT	Signal transducer and activator of transcription
TA	Transplant arteriosclerosis
TNF-α	Tumor necrosis factor alpha
vSMC	Vascular smooth muscle cell
WT	Wildtype

Chapter 1: Introduction

1.1 Heart Transplantation

Over 5000 heart transplants are performed per year worldwide for individuals with end stage heart disease. [1] Advances in immunosuppressive regimens, organ preservation and surgical techniques have greatly reduced acute rejection of cardiac transplants. However, little progress has been made in preventing long term graft loss due to chronic rejection. Consequently, cardiac allografts have a median survival time of 11.1 years. [1] Progressive occlusion and impaired graft function due to transplant arteriosclerosis (TA) is one of the major contributors to chronic rejection of heart transplants.[1–4] Prolonging graft survival and function will depend on understanding the immune mechanisms that cause development of TA.

1.1.1 Types of Transplantation Rejection

There are three major types of rejection: hyperacute, acute and chronic rejection. [5] Hyperacute rejection occurs when the recipient already possesses anti-donor antibodies which, upon transplantation, initiates an immediate cascade of responses that result in antibody-mediated thrombosis and damage.[5] Graft loss due to hyperacute rejection is effectively minimized by human leukocyte antigen (HLA) cross matching assays before surgery. [5] Acute rejection and subsequent necrosis of graft tissue occurs due to immune targeting of the graft by T cells, and manifests normally within the first year after transplantation.[5,6] Modern immunosuppressive regimens have reduced graft loss due to acute rejection with a current survival rate ~90% 1 year post transplant. [1,5,6] Chronic rejection, which occurs months to years after transplantation, results mainly from the occlusion of graft blood vessels, a condition referred to as TA.[3,5] Graft ischemia resulting from TA leads to fibrosis and vascular dysfunction. [5,6]

1.1.2 Innate and Adaptive Immune Response in Transplant Rejection

Both innate and adaptive immune responses play a role in TA. The immune response is triggered by an abundance of foreign antigen in the context of inflammation.[2] Cardiac transplantation is performed between genetically disparate individuals and this genetic disparity provides the source of foreign antigen. The second factor that primes the immune response is inflammation which is facilitated by trauma from the surgical procedure itself as well as ischemia-reperfusion injury inherent in the procedure.[2,5,7,8] Injury to the donor tissue triggers the release of damage associated molecular patterns (DAMPs) and generation of reactive oxygen species (ROS) that activate the innate immune response.[2,5,7] DAMP recognition by their cognate receptors activates antigen presenting cells (APC), such as macrophages and dendritic cells (DC), resulting in maturation and proliferation of these cells that enable them to activate T cells .[5,7] Additionally, APCs secrete cytokines and chemokines that act in an autocrine and paracrine fashion to upregulate adhesion and costimulatory molecules that further propagate the immune response.[5,7] DAMPs also activate complement cascades that result in maintaining inflammation by producing inflammatory fragments and inducing cell death and injury.[5]

DCs are abundant in peripheral tissues and, upon activation by inflammatory cytokines, undergo maturation and migrate to the secondary lymph nodes where they encounter naïve and central memory T cells.[5,7] Activation and proliferation of T cells that recognize foreign peptide-MHC results in their migration into transplant organs where they cause rejection.[3,5]

Allorecognition of donor tissue by the adaptive arm of the recipient immune system following transplantation is the first step in the rejection of vascularized solid organs. Major histocompatibility complex (MHC) molecule and their associated peptides are the main antigens recognized by the host immune system.[5,7] Allorecognition of donor peptides can occur by the direct or indirect pathway of recognition.[9]In the direct

pathway, CD4 and CD8 T cells recognize allo-peptides on donor APCs.[5,7,9] In the indirect pathway, allo-peptides are presented on recipient APC.[5,7,9] Direct recognition by T cells of foreign MHC may seem contradictory to MHC-restriction necessary for T cell mediated immunity; however, the selection process in the thymus that selects for low affinity to self MHC but high affinity for foreign peptide results in a significant population of T cells that are cross reactive for closely related allo-MHC molecules. [7,9] In the indirect pathway, recipient APCs migrate to the graft and pick up antigens for MHC presentation.[7,10]

T cell activation by allo-peptides and co-stimulation occurs in the secondary lymph nodes in the presence of a cytokine milieu which determines their fate.[5,9] CD4 T cells differentiate into T helper cell subsets (Th1, Th2, Th17, Tfh or T-regs) depending on the cytokine environment.[2,5,11] Differentiated effector T cells undergo expansion before returning to the graft via the blood and inducing cell death.[2,5] Th1 cells secrete cytokines, such as IFN-γ and IL-2, that act on surrounding cells and are involved in priming CD8 T cells a well as in activation of macrophages[2,5,8]. Th2 cells produce cytokines IL-4, IL-5 and IL-13 which clear large parasites by activating granulocytes[2,5,8]. Th17 cells secrete IL-17, which is important for neutrophil and macrophage recruitment to sites of inflammation.[2,5] Tfh cells are integral in providing B cell help and the induction of the antibody response[5,12]. T-regs, in contrast, promote tolerance of allo-peptides by secretion of inhibitory cytokines and expression of cell surface molecules that prevent T cell activation [5,7]. CD8 T cells differentiate into cytotoxic T cells (CTL) capable of lysing cells directly by secretion of cytotoxic molecules and upregulation of death receptors .[2,5] CTLs also maintain inflammation by secretion of TNF-α .[2,5]

Many patients develop donor specific antibodies that cause antibody-mediated rejection (AMR) which is associated with poor long term graft survival. [5,7,13] B cells participate in the alloimmune response both by presenting antigen to T cells and by secreting high affinity donor specific antibodies (DSAs). [5,7,13] DSAs recognize MHC class I and II and some non-MHC antigens. [5,13] These antibodies contribute to rejection by inducing complement activation. NK cells recognizing DSAs induce death of graft cells through antibody dependent cytotoxicity (ADCC). [2,5] Complement activation on the

surface of ECs also increases the activation of T cells by this vascular cells type [14] These DSAs develop months to years after transplantation towards donor derived epitopes.[15] Clinically, both DSAs specific to donor HLA and C4d deposition are associated with graft rejection with AMR episodes and often predict poor outcomes. [13,16] Furthermore, AMR may be poorly sensitive to current immunosuppressive regimens. [13,16]

Heart transplant survival rates have not improved for the past thirty years and graft loss due to chronic recognition remains a major hurdle in survival. [1] My project focuses on understanding the etiology of transplant arteriosclerosis, the main contributor to chronic rejection of heart transplants. [1]

1.2 Transplant Arteriosclerosis

Transplant Arteriosclerosis (TA) is a pathological vascular disease that is a leading cause of chronic rejection, preventing the long-term success of heart transplantation. [2,3,17] TA is characterized by intimal hyperplasia in which the innermost endothelial layer of an artery is infiltrated by inflammatory leukocytes and smooth muscle cells causing its concentric expansion. [2,3,8] This arteriosclerotic thickening prevents blood flow to affected grafts. TA pathology is also characterized by vasomotor dysfunction of the arteries that causes vasoconstriction, which further compromises blood flow.[2] Both functional disruption and structural occlusion results in ischemia and eventual tissue death.[2] TA is difficult to diagnose as routine angiographies often fail to detect the damage due to its diffuse nature.[4] Patients afflicted by TA usually are not diagnosed until the disease is in an advanced state.[4]

TA develops in response to the allo-immune targeting of the vasculature.[19] While non-immune mechanisms (donor age, cold ischemia time) contribute to the development of graft vasculopathy TA, immune mediated mechanisms are the main factors at play. In experimental models where syngeneic cardiac transplants are performed there is no development of TA.[2,3,8] Similarly, allogeneic transplants performed into mice that lacks T and B cells are devoid of TA lesions.[2,20] This

demonstrates that the fibroproliferative pathology in TA is driven by an alloimmune response .[2,20] Immunohistochemical staining of coronary arteries obtained from cardiac allograft recipients with transplant arteriosclerosis display an increased infiltration of T cells and macrophages in the extensively thickened intima.[17]

The prevalence of TA is poorly reduced by current immunosuppressive therapies that prevent acute rejection, such as calcineurin inhibitors (Cyclosporine, Tacrolimus) and cell cycle inhibitors (Mycophenolate, Rapamycin, Taxol).[6,10] mTor inhibitors (sirolimus, everolimus) have shown some success in the prevention of TA potentially through the inhibition of smooth muscle cell proliferation.[6,10] Statins (simvastatin), that lower cholesterol and have general anti-inflammatory properties, also show some effect but are sub optimal.[18] This may be a result of the complex interplay of the immune response as well as the contribution of vascular cells in the development of TA. Additionally, the translation of experimental findings to therapeutics as well as the identification of clinically relevant biomarkers remain elusive.

TA specifically affects the arteries of the transplanted heart. The general structure of a healthy artery is displayed in Figure 1.1. The artery is comprised of three layers. The inner most layer, the intima, is one cell layer thick and consists of endothelial cells and underlying basement membrane.[6,14] This is bordered by the internal lamina consisting of a mixture of collagen, proteoglycans and elastic fibers.[6] The second layer is the media, consisting of smooth muscle cells, that is bordered by the internal and external elastic lumina.[6,14] The final layer is the adventitia which consists of connective tissue, fibroblasts, resident leukocytes, progenitor stem cells and nerve endings.[6,14]. This morphology and structure is profoundly altered in TA. The ability of the intima and media of the blood vessel and their component cells to interact in the allo-immune response towards the graft is a feature that must be considered while devising

strategies to target TA. I further explore the immune mechanisms that drive the development of TA in the section below.

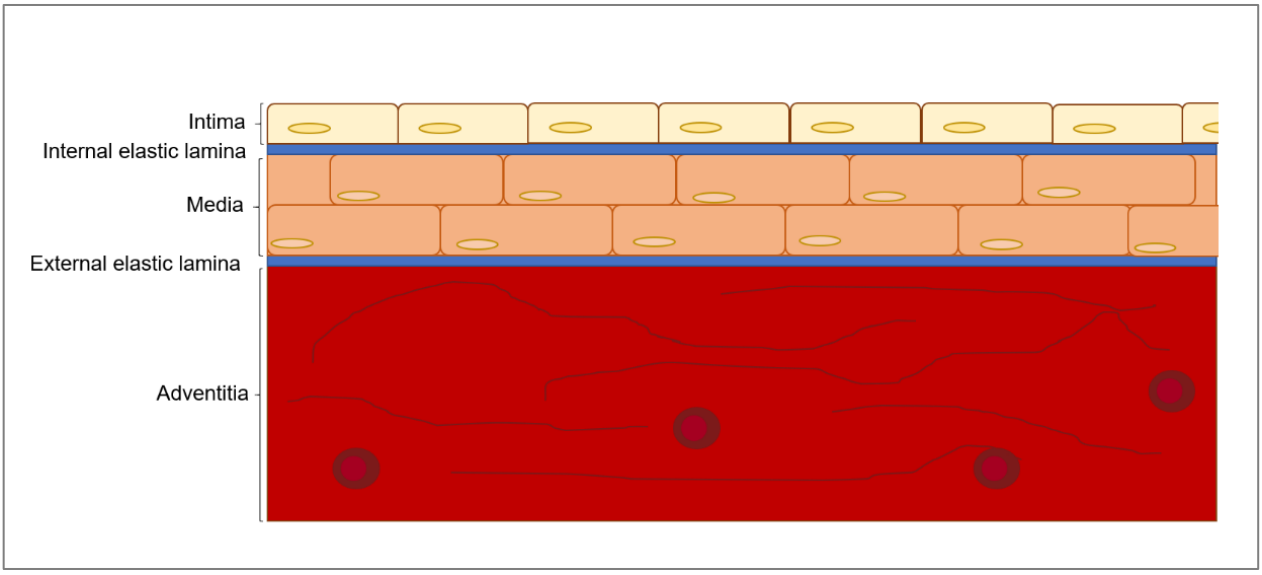


Figure 1.1 Structure of artery. An artery is comprised of three layers: the intima, media and adventitia. The intima is comprised of a single layer of endothelial cells and bordered by the internal elastic lamina. The media is comprised of smooth muscle cells bordered by the outer elastic lamina. The outer layer is the adventitia which is comprised of connective tissue, fibroblasts, nerve endings and progenitor stem cells.

1.2.1 Allo-immune response in TA

Intimal thickening and impaired compliance of arteries is the culmination of a reparative response to vascular injury. The recipient develops an adaptive immune response whereby recipient T cells, recognizing graft derived MHC, become activated in secondary lymphoid tissue and return to the graft via the blood to elicit effector responses. [2] Cytotoxic T cells kill vascular cells.[2,20] Cytokine and growth factor secretion by infiltrating T cells stimulate smooth muscle cells (SMC) to transmigrate from the media into the subendothelial layer of the intima and proliferate.[6] Also contributing to the robust immune response is the secretion of DSA from B cells. [2,3] The vasculature itself contributes to TA pathology and activation of the endothelium results in recruitment of leukocytes via chemokines, cytokines and the upregulation of adhesion molecules.[6] The response of vascular cells to the injurious effector responses results in the morphological and functional changes that characterize TA.

1.2.2 Cell mediated Injury

Vascular rejection is mainly driven by allo-reactive Th1 cells that recognize foreign peptide MHC complexes expressed on the endothelial cells and directly induce vascular injury[2,14] They secrete cytokines such as IFN-γ that augment inflammation and drive morphological changes of graft arteries.[2,8,21] IFN-γ activates vascular ECs by upregulating MHC I and II, adhesion molecules, costimulatory receptors and death receptors that further recruit leukocytes [8,22,23]. Expression of MHC molecules, are also increased in activated ECs and this increases T cell activation and targeting of the allograft blood vessels.[8,22,23] Activated EC and vascular smooth muscle cells (vSMC) secrete various cytokines(IL-6 IL-8, IL-1-a, MCP-1) and chemokines (CXCR3, CXCR5) in response to IFN-γ and Tnf-α, that recruit leukocytes to the graft.. [8,11,13,14,22] Additionally, Th1 cell secretion of IFN-γ promotes SMC infiltration and proliferation in the subendothelial layer of the artery leading to concentric expansion of the intima. [2,24]

CD4 T cell secretion of TNF-α and IFN-γ attenuates nitric oxide (NO) production by ECs which compromises NO-induced vasodilation and consequently arterial function.[2] There is some evidence that Th17 cells contribute to TA by secreting cytokines that contribute to the development of fibrosis and supporting B cell proliferation. Finally follicular helper T cells (Tfh) contribute to the alloimmune response by providing stimulation to B cells that secrete DSAs.[2,25]

Recognition of MHC class I by CD8 T cells leads to cell death of vascular EC through perforin and granzymes which, upon cell entry, activates apoptotic caspase pathways.[2,24] CD8 T cells express death ligands (FasL and TRAIL) which, upon binding their receptors on their target cells, activate apoptotic pathways. [2,24] EC death and subsequent regenerative processes are central to the development of TA. EC injury causes the release of growth factors and bioactive fragments (e.g. perlecan) that promote SMC and fibroblast growth.[2,17,26,27] Injured ECs increase platelet aggregation that also contributes to occlusion of the lumen.[2,14,17,26–29] The presence of endothelial death and repair is evident in cardiac allograft recipient tissue samples and is a diagnostic criteria for TA.[17]

1.2.3 DSA mediated Injury

Tfh differentiation begins with the recognition of the peptide:MHC complex on DCs in the presence of cytokines (IL-6, IL-2) and costimulatory stimulation (ICOS/ICOSL).[12] IL-6 induction of Bcl6 leads to the expression of CXCR5 which homes the Tfh cells to the T/B cell interface in a secondary lymphoid organ.[12] Tfh cells then interact with B cells which act as a source of antigen but also provide costimulation via ICOS/ICOS ligand interactions and help form germinal centres (GC) .[12] In these GCs, B cells differentiate into plasma cells and commence the secretion of high affinity alloantibodies. Tfh cells aid in this task by providing costimulation via CD40/CD40 ligand interactions and cytokine secretion (IL-21). [12,25] Other Tfh produced cytokines also direct antibody class switching. [25]

B cell production of high affinity DSAs contributes to vascular injury of graft arteries in a variety of ways. DSAs and complement maintain inflammation and T cell targeting of ECs by enhancing their recruitment and activation.[13,14] Complement bound to DSAs, in the presence of IFN-γ, activate ECs and enhance their ability to recruit and activate CD4 T cells.[14,30] Complement and DSAs turn on an inflammatory gene profile in ECs that result in the upregulation of adhesion molecules , chemokines, cytokines and cytokine receptors.[13–15] DSAs can also directly crosslink the highly polymorphic MHC molecules present on the surface of EC which induce pathways that promote migration, proliferation, cytoskeletal rearrangement.[13,15,30] EC death is also facilitated by DSAs binding to Fc-gamma receptors (FcγR) on various effector cells such as macrophages and natural killer (NK) cells, monocytes and neutrophils inducing ADCC and secretion of inflammatory cytokines.[2,13,30] This robust allo-immune response by both cells mediated and antibody responses drive the concentric expansion of the intima and vascular dysfunction that characterizes TA.

1.3. Interleukin 6

IL-6 is a pleiotropic cytokine that is critical to a variety of inflammatory and immunomodulatory biological processes. IL-6 is involved in, but not limited to, mediating T and B cell function, inflammation, vascular function and cytoprotection and thus is a key player in allograft rejection. The table below briefly describes the varied and diverse biological effects of IL-6.

Table 1. Biological functions of the cytokine IL-6

Cell type	Action of IL-6
Hepatocytes	Acute phase protein synthesis (SAA, Fibrogen, Albumin)[31,32]
B-cells	IgG switching and development[31,33] B cell maturation[34]
T-cells	Activation (CXC- upregulation and CCL- secretion)[35,36] Differentiation (Th17,Th22 Tfh)[31,34,37] Proliferation[19,34] Survival[31,38] Migration[35]
Macrophages, Neutrophils	Differentiation[39] Survival[39]
Megakarocytes	Platelet production[34]
Epithelial cells	Regeneration[40,41]
Smooth muscle cells	Activation (receptor upregulation)[42] Proliferation[42]
Fibroblasts	Permeability[31] VEGF secretion[39]
Endothelial cells	Activation (cytokine and chemokine secretion, adhesion molecule upregulation, growth factor secretion, MHC molecule upregulation)[2,43] eNOS [2] contractility [44]
Cardiomyocytes	Regeneration, hypertrophy[45]
Osteoclasts	Osteoclast formation and readsoption[31]

1.3.1 IL-6 Signalling

Both stromal and immune cells are capable of synthesizing IL-6 in response to inflammatory stimuli such as cytokines (e.g. Tnf-α , IL-1b) and DAMPs (e.g. (mt), DNA, high mobility group box 1 (HMGB1), and S100 proteins).[46,34,47] IL-6 initiates specific cell response by forming a complex with the IL-6 receptor (IL-6R) and signal transducing protein gp130 (Figure 1.2).[46,34,48] There are two main mechanisms by which IL-6 signals: classic and trans. They are distinguished by whether IL-6 binds to membrane bound (classic) or soluble IL-6R (trans). [46,34,47] The membrane bound form of IL-6R is restricted to leukocytes and hepatocytes, allowing for tight regulation of IL-6 signalling. [46,34,47] The soluble form of IL-6R (sIL-6R) is shed by macrophages and neutrophils in response to inflammatory stimuli, allowing for global IL-6 signalling on most cell types as gp130 is ubiquitously expressed. [46,34,47] sIL-6R can also be cleaved off the surface of these cells by proteases ADAM10 and 17. [46,34,47,49] Minor amounts of sIL-6R result as a product of differential mRNA splicing in humans.[34,47]

IL-6 levels under homeostatic conditions are undetectable but increase 1000 fold under inflammatory conditions.[49] sIL-6R levels are considerably higher (75ng/mL vs 5ng/mL) suggesting that in the context of inflammation most available IL-6 is bound to sIL-6R and signal induction occurs via the trans-signalling pathway.[49]

Signal induction through IL-6 occurs when either IL-6/IL-6R or IL-6/sIL-6R complexes with gp130 on target cell membranes. [46,34,47] The resulting dimerization of signal transducer gp130 leads to the autophosphorylation of constitutively associated janus kinases (JAK1, JAK2 and Tyk2) which go on to recruit and activate three pathways.[34,50] Janus kinases can phosphorylate signal transducer and activator transcription 3 (STAT3) or to a lesser extent STAT 1 and STAT5.[46,47] Alternatively dimerization of gp130 can lead to the phosphorylation of the associated kinase Hck.[48,50] Hck phosphorylation facilitates recruitment of Gab-1 and subsequent activation of mitogen activated protein kinase /extracellular signal-regulated kinase 1/2 (MAPK/ERK1/2) and phosphoinositide-3-kinase-protein kinase B (PI3K/AKT) cascades. [48,50] The MAPK/ERK1/2 pathway can also be activated by Shp2 which recruits the grb2-SOS complex which activates Ras that catalyzes the first step of the MAPK

cascade.[51] IL-6 activation of STAT-3, MAPK/ERK1/2 or PI3K/Akt result in the induction of IL-6 responsive genes. Termination of IL-6 signalling is tightly regulated by suppressor of cytokine synthesis-1 and 3 (SOCS1, SOCS3), which is induced by STAT3 and inhibits Jak1.[46,50]

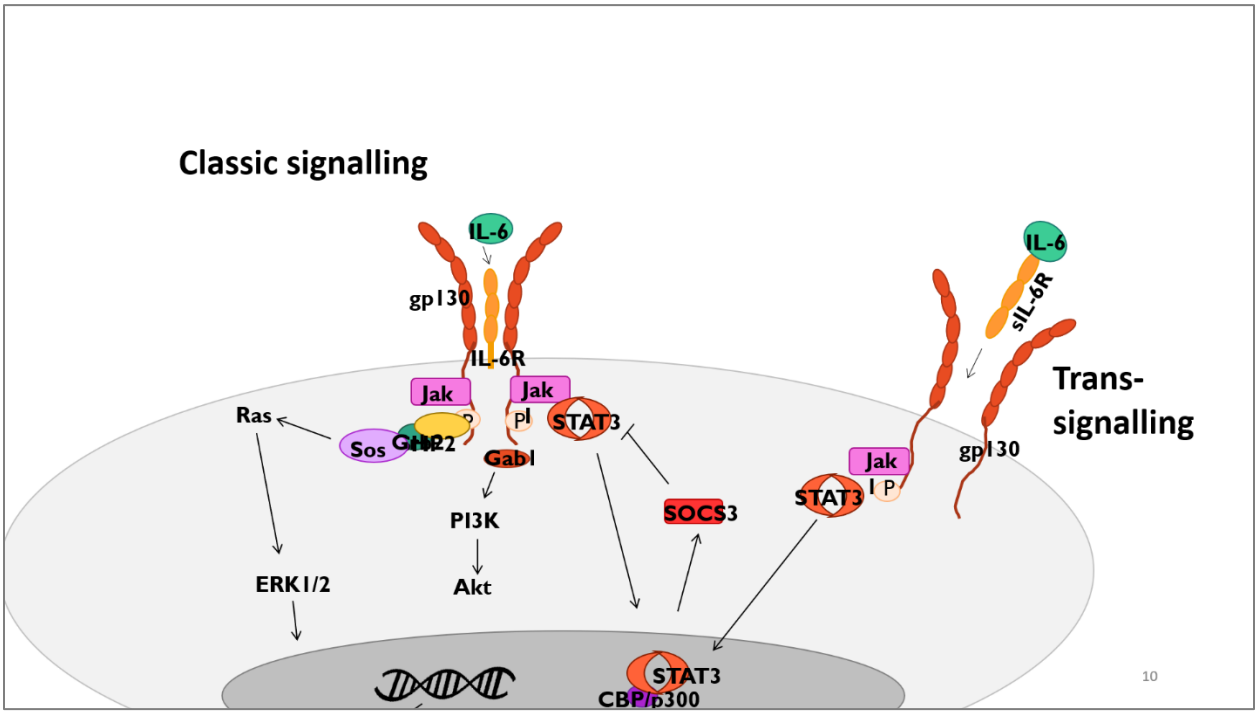


Figure 1.2 IL-6 classic and trans-signalling. In classic signalling IL-6 binds to the membrane bound IL-6 receptor (IL-6R) that is associated with the signalling subunit gp130. In IL-6 trans-signalling, IL-6 binds to soluble IL-6R (sIL-6R) forming an IL-6: sIL-6R complex. The complex then binds to gp130. Dimerization of gp130 leads to phosphorylation of the associated Janus kinases (JAKs) which recruits and activates STAT3. Activation of STAT3 leads to its translocation to the nucleus where it regulates transcription of target genes. IL-6 signalling can also activate MAPK/ERK1/2 and PI3K/Akt pathways

IL-6 classic signalling has been implicated in processes involved in metabolic homeostasis, mitogenesis and the acute phase response while IL-6 trans-signalling has been implicated in inflammatory processes, leukocyte recruitment and apoptotic pathways.[47] While the biological outcomes mediated by IL-6/IL-6R are not neatly categorized, the ability to differentially target IL-6 classic or trans-signalling can ameliorate disease in various experimental systems. Specifically, abrogation of IL-6

trans-signalling is efficacious in preventing several inflammatory diseases such as colitis, acute inflammation, and atherosclerosis.[29,48,52,53] This protective effect may be as a result of inhibiting IL-6 trans-signaling in macrophages. [29,48,52,53] A reduction of adhesion molecules upregulated on the surface EC in the murine model of atherosclerosis was also attributed to inhibiting IL-6 trans-signalling, as was decreased colonic epithelial cells proliferation in the model of colitis.[29,53] Sgp130Fc, a fusion protein comprising of soluble gp130 fused to the Fc portion of IgG, has been shown to inhibit trans-signalling while leaving classic signalling intact.[48] This protein is currently in phase II clinical trials for treatment of inflammatory bowel disease.[48,54] Global inhibition of IL-6 signaling has clinical implications as well. Tocilizumab, an IL-6R neutralizing antibody used to treat several autoimmune diseases, has shown promising results in reducing AMR episodes in kidney transplant recipients with chronic glomerulopathy possibly by inhibiting B cell activation and DSA secretion.[55] These studies highlight the importance of understanding the role of IL-6 classic or trans signaling in disease manifestation.

1.3.2 Role of IL-6 in transplant rejection and TA

IL-6 is secreted within hours of surgical transplantation and is a key regulator of both the initial innate immune response and subsequent adaptive immune response that causes rejection. Analysis of tissue from cardiac transplant recipients with multiple rejection episodes show increased gene expression of IL-6.[11,56,57] Initial IL-6 production is antigen independent, secreted by graft cells, but is sustained by infiltrating leukocytes. Immune responses mediated by IL-6 drive TA by enhancing T and B cell responses, maintaining inflammation and mediating vascular activation.

There are a multitude of studies that demonstrate that IL-6 is integral to supporting T cell responses in transplant rejection and TA. Experimental murine models of cardiac transplantation have identified a role for IL-6 in supporting Th1 effector function. Specifically, allogeneic cardiac transplant recipients in which IL-6 was neutralized displayed impaired Th1 secretion of IFN-γ.[56,58]. Our lab and others have demonstrated that IL-6 also supports T cell expansion and infiltration by directly increasing T cell proliferation and cytokine production and thus vascular

injury.[19,46,56,57,59] Non-tolerance of the donor graft is in part due to the ability of IL-6 to skew the balance of the Th1/Treg ratio such that the allogeneic Th1 cells predominate.[11] IL-6 does this by supressing the induction of Foxp3 expression which inhibits transforming growth factor beta (TGF-β) signalling via upregulation of SMAD7.[60] TGF-β and IL-6 work in conjunction to promote the differentiation CD4 T cells into Th17 cells tipping the balance from T-reg differentiation.[61–63] In models of transplantation where IL-6 has been neutralized, T-reg expansion is enhanced.[58] Th17 and Tfh differentiation is also supported by IL-6 signaling as evidenced by studies where inhibition of IL-6 signalling impaired Th17 secretion of IL-21 as well as Tfh differentiation in skin transplant recipients.[58] Finally, IL-6 acts to increases T cell survival through prevention of apoptosis by a variety of mechanisms.[34,57,64] One such mechanism is by reducing the susceptibility of T-cells to activation induced cell death (AICD) by increasing c-FLIP expression.[19] C-FLIP prevents procaspase8 cleavage and activation of the pro-apoptotic pathway.[19] Another mechanism is by induction of anti-apoptotic factors such as Bcl-2.[19,57,62]

B cell maturation and DSA generation is supported by this inflammatory cytokine as well. IL-6, in addition to IL-21, promotes Bcl-6 expression and Tfh differentiation which recruit B cells to germinal centres.[46,34,62,63,33,65] (Tfh cells secrete cytokines and provide costimulation that facilitates the maturation of naïve B cells, antibody class switching and high-affinity antibody production. [46,34,62,63,33,65]

IL-6 also acts on the cells comprising the vascular tissue by supporting both EC and vSMCs activation and proliferation and the production of potent chemoattractants such as MCP-1/CCl-2 and IL-8/CXCL8.[35,66,42]. ICAM , VCAM and VLA-4, which are instrumental for leukocyte infiltration into the graft, are upregulated in ECs in response to IL-6.[35,66,67] Upregulation of the coreceptor gp130 occurs after activation and increases vSMCs their responsiveness to IL-6 trans signalling.[42] In addition, IL-6 secretion impairs the vasomotor function of arteries by inducing EC production of endothelium derived constriction factors (e.g. ET-1) which can bind to receptors on vSMCs activating a cascade that culminates in calcium release from the sarcoplasmic reticulum resulting in smooth muscle contraction.[68,44] Dysfunction in EC occurs by IL-6

induced ROS generation which, if overproduced, can damage cell membranes and disrupt proteins and DNA.[50] Fibrosis in cardiac allografts has been correlated with the induction of connective tissue growth factor (CTGF) production by IL-6.[69]

In contrast to immune activation, IL-6 also mediates a variety of immunomodulatory and regenerative biological processes. Particularly relevant in the context of transplantation, IL-6 has been shown to be involved in regeneration of some cell types including epithelial cells and cardiomyocytes.[70] IL-6 also promotes EC and cardiomyocyte survival and IL-6R expression is upregulated on these cells in the context of stress, injury and inflammation via the MAPK and PI3K pathways.[45] In cardiomyocytes, survival and cytoprotection is mediated via the classic signalling pathway by the induction of NO production and iNOS expression.[45]

1.4 Thesis objective

My project examines the mechanisms by which IL-6 classic and trans signalling contributes to immune activation in TA and characterizes the biological outcomes of IL-6 signalling in endothelial cells.

The Aims are:
1. To examine how IL-6 classic and trans-signalling affects immune responses that cause TA
2. To characterize the biological effects of IL-6 in endothelial cells

Chapter 2: Immunological Effects of IL-6 in a murine model of TA

2.1 Introduction

Interleukin -6 (IL-6) plays an important role in the activation of allogeneic immune responses in TA and supports a multitude of both inflammatory and immunoregulatory processes. Various studies have provided insight into the effect of IL-6 on peripheral T cell activation and its role immune mediated injury. However, these studies have not separated the contribution of IL-6 classic signalling and trans-signalling to the development of vascular rejection. Zhao et al. found that eliminating total IL-6 signalling prevented T cell differentiation in secondary lymphoid organs, decreased T cell proliferation and impaired T cell effector functions.[57] However elimination of IL-6 signalling was not enough to prevent graft loss in this model.[57] Whether the effects on T cell development or effector function relied on IL-6 classic or trans signalling was not explored. Our lab has shown that the source of IL-6 is important in the development of TA.[19] Specifically donor derived IL-6 is necessary for peripheral CD4 and CD8 proliferation and survival that contributes to development of TA.[19] As such, we sought to examine the signalling mechanism by which IL-6 classic signalling is immune activating in TA. IL-6 classic signalling may be sufficient for T cell proliferation and survival, although others have shown that IL-6 trans signaling is important in this regard.[47] It is therefore of interest to determine whether IL-6 classic signalling is necessary or redundant for peripheral T cell activation in TA. Of similar importance is the effect of IL-6 signalling in T cells and its capacity to contribute to B cell production of DSAs. To examine the role of IL-6 classic signalling in T cells, allogeneic transplants into mice that lack IL-6R specifically in T cells (IL-6R-TKO) were performed. IL-6 trans-signalling can be selectively inhibited with the fusion protein sgp130Fc. To explore how IL-6 trans-signaling contributes to TA pathogenesis, we performed allogeneic transplants into transgenic mice with high circulating levels of sgp130Fc.

My results show that the activation of peripheral T cell responses, as indicated by CD4 and CD8 T cell accumulation in transplanted artery segments, was not affected in IL-6R-TKO or sgp130Fc graft recipients. The accumulation of macrophages in transplanted artery segments was also not affected in IL-6R-TKO or sgp130Fc mice. However, eliminating IL-6R in T cells markedly and significantly reduced the production of donor-specific antibodies toward allogeneic antigens. These findings suggest that IL-6R expression on T cells and IL-6 trans-signaling are redundant for the activation of peripheral T cell responses but IL-6R expression in T cells is needed for the development of antibody responses in transplantation.

2.2 Methods and Materials

2.2.1 Animals

C57Bl/6(H-2^b), Balb/c(H-2^d) were obtained from Jackson Laboratories. IL-6RTKO mice were generated by crossing C57Bl/6(CD4 Cre) and C57Bl/6 – *IL-6Ra$^{fl/fl}$* to create mice lacking IL-6R on T cells. Genotype was confirmed by analysis of CD4Cre and IL-6Rfl Gene expression by RT-PCR. DNA was extracted from ear punches using Tissue PCR Kit (Sigma). Transgenic mice expressing fusion protein sgp130Fc were kindly provided by Dr. Stefan Rose-John and the Christian-Albrechts University of Kiel. Mice were bred in house and used for experimentation at 8 to 12 weeks of age. All protocols used in this study were reviewed and approved by the Simon Fraser University Animal Care Committee following the guidelines set out by the Canadian Council on Animal Care.

2.2.2 Murine model of transplant arteriosclerosis

Murine aortic interposition transplantation was performed by Winnie Enns [71] Mice were anesthetized and a longitudinal incision made in abdomen. A short segment of abdominal aorta ~1 cm in length from Balb/c(H-2^d) donor mice was grafted into the resected infra-renal aorta of C57Bl/$6^{IL\text{-}6RTKO}$ and C57Bl/$6^{sgp130Fc}$ recipient mice as well as their WT littermates. Arteries were harvested at day 30 when rejection of the artery segment involves alloimmune reactions and results in arterial changes that resemble vascular rejection.

2.2.3 Air pouch model of inflammation

To assess immune cell infiltration in the context of acute inflammation, C57Bl/6 WT and C57Bl/6 ^{sgp130Fc} were anesthetized and an air pouch created in dorsal cervical thoracic by injecting 6 mL of sterile air. After 3 days, the pouches were reinjected with 4 mL of air. On day 6, 1 mL of 2% non-gelling λ-carrageenan (Sigma) in sterile PBS (Sigma) were injected into the pouches. Controls received sterile PBS. To examine the exudates, the mice were sacrificed 72 hours after administration of carrageenan and the

pouches were washed with 9% saline solution supplemented with 54 mM EDTA . The lavage fluid was immediately cooled on ice and centrifuged at 5000 rpm for 10 minutes at 4°C to collect infiltrating immune cells.

2.2.4 Histology, immunohistochemistry and morphological analysis

Arteries were harvested 30 days post transplant and fixed with 4% (v/v) paraformaldehyde, and flash frozen in OCT Medium (Electron Microscopy Sciences). Sections of 0µm were prepared. Luminal narrowing, intimal and medial area were quantified following staining with hematoxylin-eosin. Luminal narrowing was calculated using the following formula:

$$\%\text{Luminal Narrowing} = \frac{\text{Intimal area}}{(\text{Intimal area} + \text{Lumen area})}$$

Area was measured using ImageJ (NIH).

Arterial sections were stained with polyclonal anti-CD31 antibody (Abcam) to assess endothelial integrity and anti-Mac-3 (clone M3/84) (BD Pharmingen), polyclonal CD4 (BD Pharmingen), polyclonal CD8 (BD Pharmingen) to quantify macrophage, CD4 T cell, CD8 T cell infiltration respectively. Sections were incubated with above primary antibody, followed by a biotin conjugated secondary antibody. Arterial sections were then incubated with by streptavidin – HRP. Staining was visualized using AEC substrate-chromagen kit (Vector) and imaged using Olympus CX31 Microscope (Olympus Life Science).

Endothelial integrity was quantified by measuring the length of EC staining divided by total lumen length. The accumulation of macrophages, CD4 and CD8 T cells was quantified by manual counting of positive cells divided by intimal area. Quantification was done using ImageJ (NIH)

2.2.5 Cell Isolation and Flow Cytometry

Splenocytes were harvested from C57Bl/6$^{IL\text{-}6RTKO}$ and C57Bl/6^{sgp130Fc} recipient mice as well as their WT littermates were isolated. CD4 and CD8 T cells were purified from splenocytes by negative selection using CD4 and CD8 EasySep T cell Enrichment kits (StemCell Technologies) according to the manufacturer's instructions. The isolated CD4 and CD8 T cells were resuspended and cultured in RPMI + 10% FBS (Invitrogen, Carlsbad, CA).

Total splenocytes or isolated T cells were incubated with fluorescently conjugated antibodies to Pe anti mouse IL-6R (UV4) (Biolegend), APC anti mouse CD4 (clone RM4-5) (BD Biosciences), APC anti mouse CD8 (clone 53-6.7) (BD Biosciences), APC anti mouse CD11b (clone M1/70) (eBioscience) for 30 mins at 4ºC to assess IL-6R expression on T cells,

For P-STAT-3 expression analysis, T cells isolated by negative selection using EasySep Mouse T cell Isolation Kit (Stem Cell Technologies). Cells were reconstituted in RPMI (VWR) supplemented with 10% FBS. Cells were treated with 50ng/mL mouse IL-6 (R&D Systems) for 30 mins then stained with APC anti-mouse P-STAT3 (Thermofisher) and analyzed by flow cytometry.

To examine exudates from air pouch, cells were incubated with APC anti mouse CD11b (clone M1/70) (BD Biosciences), FITC anti mouse F4/80 (clone BM8) (BD Biosciences), FITC anti mouse Ly6G (clone 1A8) (BD Biosciences) for 30 mins to assess monocytes, macrophages and neutrophils respectively

Data was acquired on BDFACs Jazz and analysed using FlowJo (Treestar Inc). Negative and positive gates were set using appropriate isotype controls.

2.2.6 Donor Specific antibody assay

BALB/C splenocytes were incubated with serum obtained from graft recipients. Donor-specific antibody binding was detected using a PE conjugated polyclonal goat anti-mouse antibody (eBioscience). Splenocytes were costained for anti-CD3 (clone OKT3)

(BD Biosciences) and the mean fluorescence intensity of donor-specific antibody staining of CD3+ cells quantified. Data were acquired on a BD FACS Jazz and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR).

2.2.7 ELISA

Serum was collected from C57Bl/6 and C57Bl/6^{sgp130Fc} was collected and sgp130Fc levels quantified using an sp130 DuoSet ELISA kit as per the manufacturer's instructions (eBioscience)

2.2.8 Statistical analysis

Comparisons between two groups was analyzed with a students t-test and between more than two groups using two-way ANOVA with Sidak's and Tukey's multiple comparisons tests in GraphPad Prism

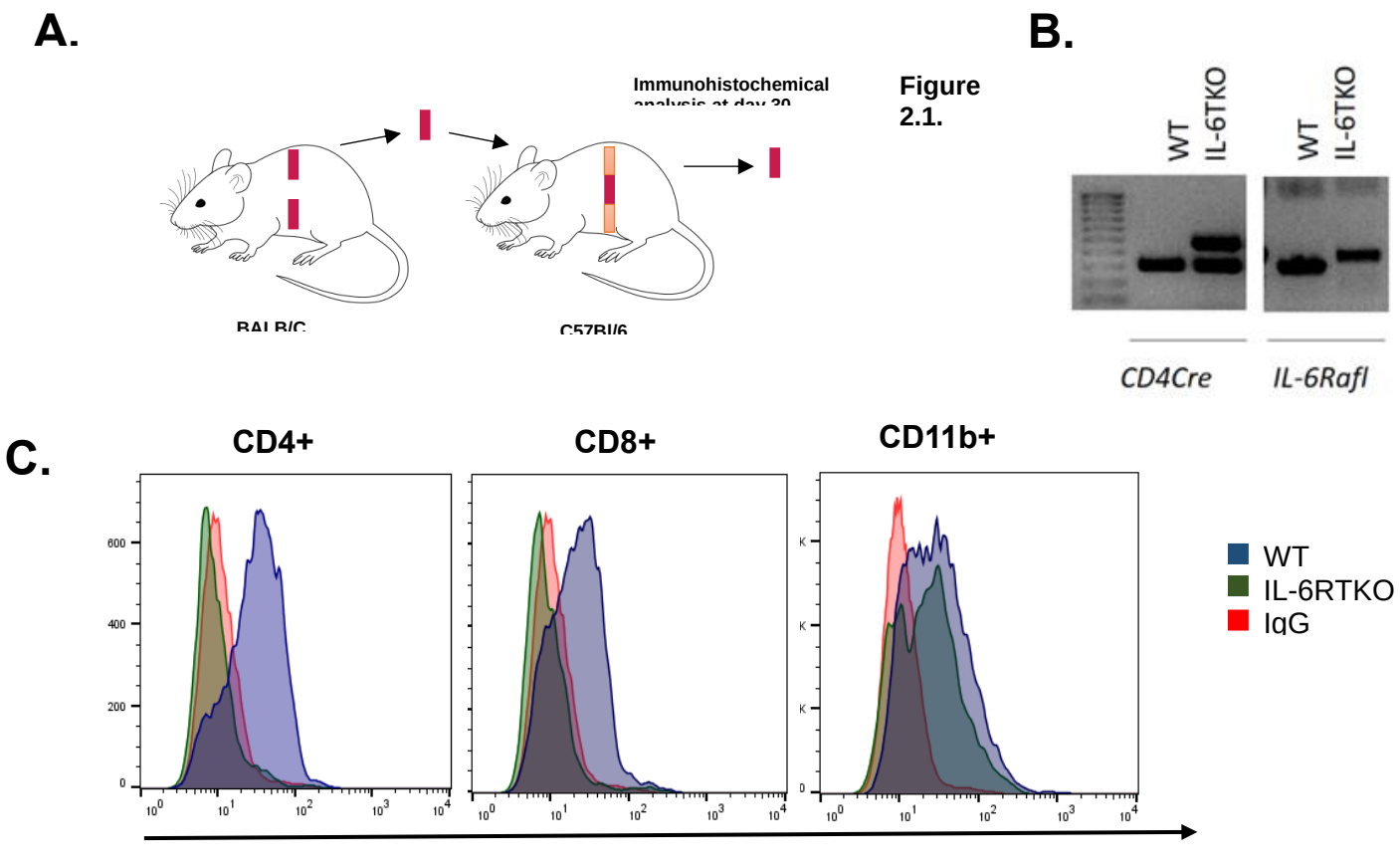
2.3 Results

2.3.1 Generation and confirmation of IL-6RTKO and sgp130Fc mice

The role of IL-6 classic signalling in peripheral T cell activation was examined using transgenic mice that lack IL-6R specifically in T cells (Figure 2.1A). These mice were created by crossing C57Bl/6 mice containing the IL- 6R gene flanked by *loxp* sites with C57bl/6 mice expressing the *Cre* recombinase gene under a CD4 promoter (Figure 2.1B). The C57Bl/6$^{IL\text{-}6RTKO}$ offspring do not express IL-6R on CD4 and CD8 T cells but maintain expression of this receptor in other cells (Figure 2.1C). Also, T cells do not respond to IL-6 alone in IL-6RTKO mice (Figure 2.1D)

The role of trans signalling was examined using transgenic mice expressing the fusion protein sgp130Fc. Levels of sgp130Fc were assessed by ELISA (Figure 2.2A). To confirm the sgp130Fc mice were generating levels of sgp130Fc sufficient to block systemic trans signalling, we examined monocyte infiltration using an air pouch model of inflammation (Figure 2.2B).[52] Rabe et al. have demonstrated that IL-6 trans-signaling is necessary for infiltration of monocytes in this model of inflammation. Consistent with this study, macrophage infiltration into the air pouch after challenge with carrageenan was reduced compared to WT littermates and neutrophil infiltration was maintained in both sgp130Fc and WT mice (Figure 2.2 C and D).

Figure 2.1.



Generation and confirmation of IL-6RTKO mice. A. Schematic of murine aortic interposition model. Segments of abdominal aorta from Balb/c donor mice were interposed into the infrarenal aorta of WT or IL-6RTKO recipient mice. The grafts were harvested after 30 days. B. Representative agarose gel resulting from PCR genotyping of genomic DNA from ear punches of WT and IL-6TKO mice. Bands indicate floxed IL-6R mutant (671 bp) and wild-type (530 bp), CD4 Cre (500 bp and 324 bp) and wild-type (324 bp). C. Splenocytes were harvested from WT and IL-6RTKO mice and stained for CD4, CD8, CD11b and IL-6R to determine IL-6R expression and on CD4 T cells, CD8 T cells, and myeloid cells respectively. D. T cells were isolated from WT and IL-6RTKO mice, stimulated with 50ng/mL IL-6 and stained for CD4 and p-STAT3 to determine activation via flow cytometry. Representative histograms are shown.

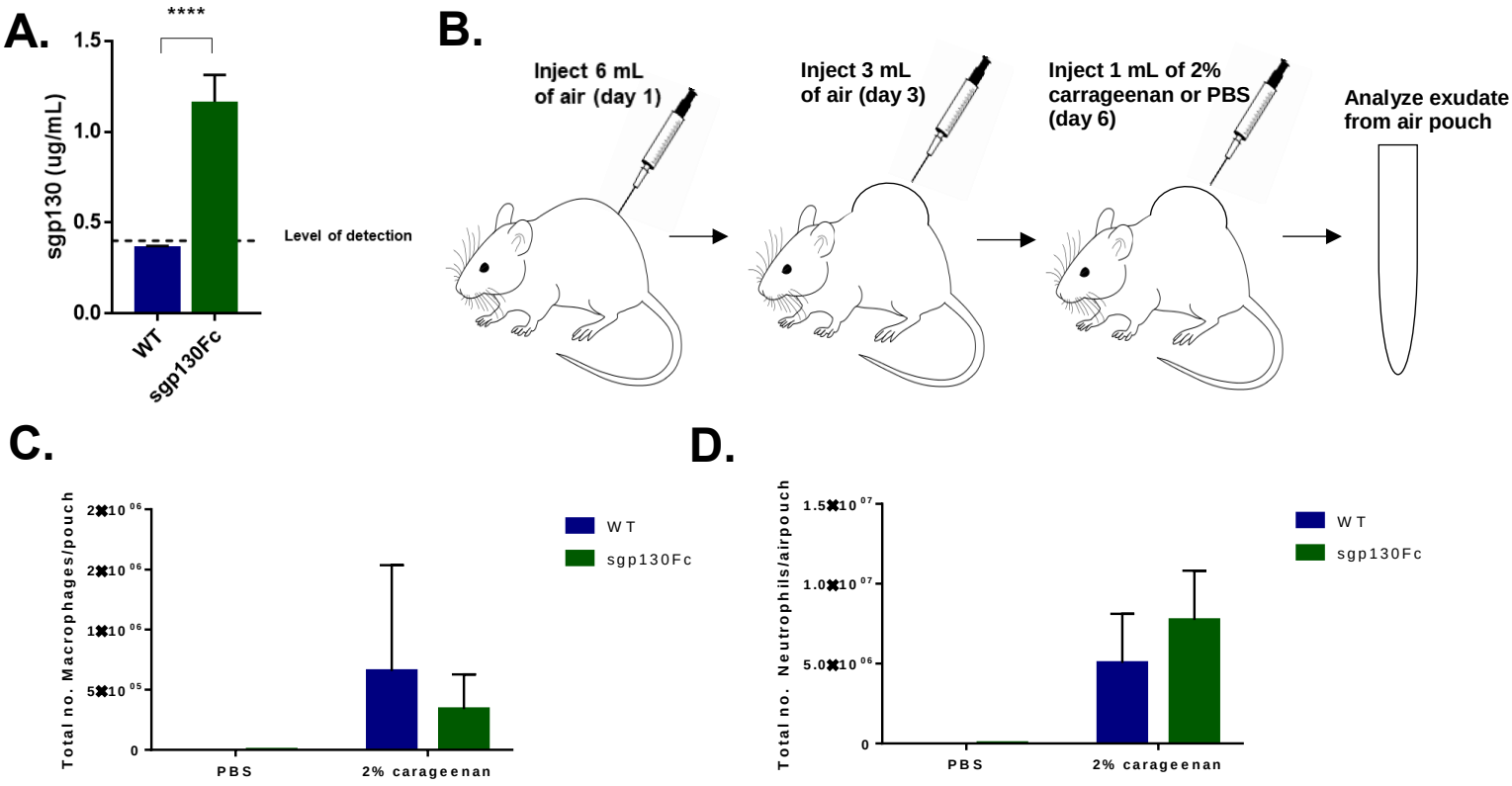


Figure 2.2. Generation and confirmation of sgp130Fc mice. A. sgp130Fc levels determined by ELISA for WT and sgp130Fc mice. **B.** Scheme of air pouch model of inflammation. Exudate from air pouch of WT and sgp130Fc was stained with **C.** Cd11b and F4/80 for Macrophages (WT (n=3) and sgp130Fc (n=3)) or **D.** Cd11b and GR-1 for neutrophils)WT (n=3) and sgp130Fc (n=3))infiltrating the air pouch 72 hours after injection of 2% carrageenan or phosphate-buffered saline as measured by flow cytometry. Data depict the mean ± SD of the indicated values ****=P<0.0001 determined using unpaired t-test.

2.3.2 Eliminating IL-6R expression on T cells does not affect leukocyte infiltration and intimal thickening

T cell mediated injury of vascular cells causes intimal thickening in the aortic interposition model of TA that I am using. Eliminating IL-6R expression in T cells, and by extension classic signalling, did not affect intimal thickening in IL-6RTKO recipients (Figure 2.3A). Also, the accumulation of CD4 T cells or CD8 T cells into the graft was assessed via immunohistochemical staining and there was no difference between WT and IL-6RTKO recipients (Figure 2.3B and 2.3C). There is also no reduction in the infiltration of macrophages into the intima (Figure 2.3D). Splenocytes were harvested at day 7 which corresponds to T cell activation in this model . My data suggests that IL-6

classic signalling is redundant for activation of peripheral in CD4 and CD8 T cells that migrate to allograft arteries to cause TA (supplementary Figure 1)

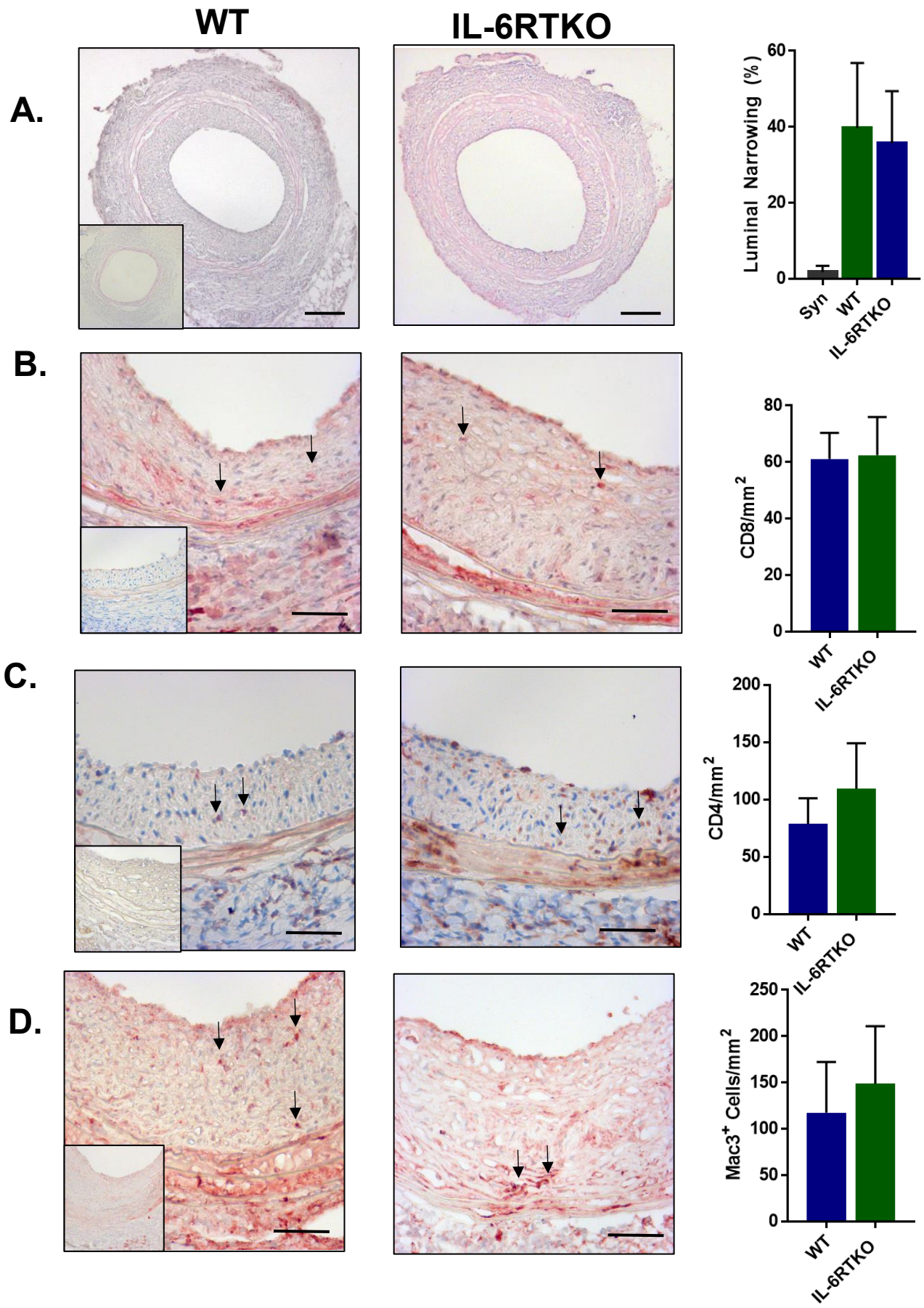


Figure 2.3 Elimination of IL-6R expression on T cells has no effect on luminal narrowing or leukocyte accumulation. Segments of abdominal aorta from Balb/c donor mice were interposed into the infrarenal aorta of WT (n=5) or IL-6RTKO (n=6) recipient mice. The grafts were harvested after 30 days and cross-sections stained **A.** with H&E to visualize intimal thickening (scale bar 0.1 mm). Inset is syngeneic control **B.** CD4 to visualize CD4 T cells (scale bar 0.1 μm)**C.** CD8 to visualize CD8 T cells (scale bar 0.1 μm)**D.** Mac-3 to visualize macrophages (scale bar 0.1 μm) (Magnification: 40×). Data n.s. comparing WT to IL-6RTKO. Data depict the mean ± SD of the indicated values.

2.3.3 Eliminating IL-6 trans-signalling does not affect luminal narrowing and leukocyte infiltration

Aortic interposition grafts were placed into WT or sgp130Fc recipients to examine the role of IL-6 trans-signaling. When intimal thickening was examined, inhibition of systemic IL-6 trans-signalling by sgp130Fc did not affect intimal thickening in sgp130Fc graft recipients (Figure 2.4A). Additionally, CD4 and CD8 T cell infiltration into the graft at day 30 was not affected by inhibiting IL-6 trans-signalling (Figure2.4B and C). Blockade of trans-signaling also did not decrease macrophage infiltration into the graft (Figure 2.44D). These findings indicate that IL-6 trans- signaling may not be required for activation of peripheral T cell response that cause TA.

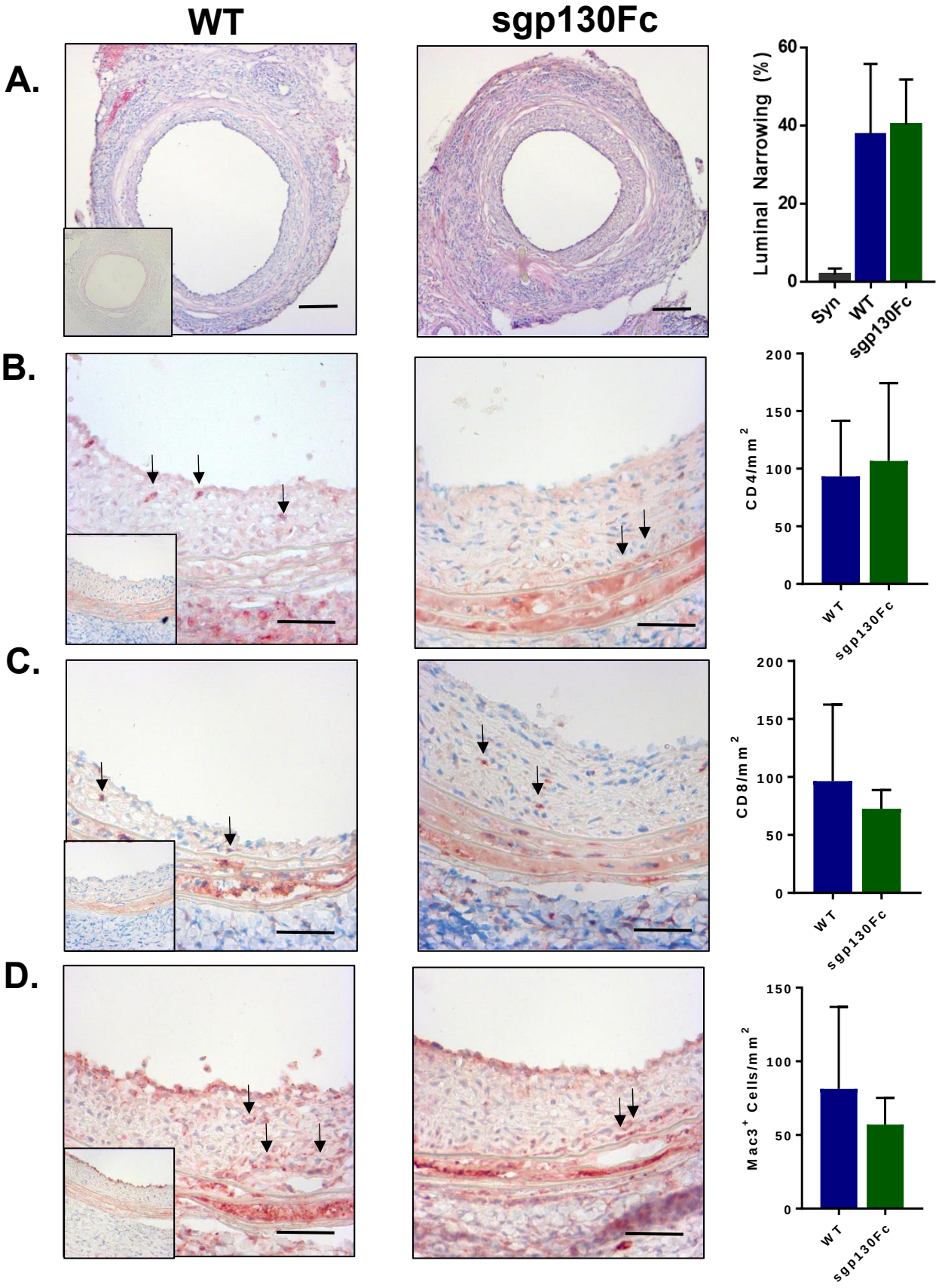


Figure 2.4. Elimination of IL-6 trans signalling has no effect on intimal thickening or accumulation of leukocytes. Segments of abdominal aorta from Balb/c donor mice were interposed into the infrarenal aorta of WT(n=5) or sgp130Fc (n=6) recipient mice. The grafts were harvested after 30 days and cross-sections stained with **A.** H&E to assess intimal thickening (scale bar 0.1mm) **B.** CD4 to visualize CD4 T cells (scale bar 0.1 µm) **C.** CD8 to visualize CD8 T cells (scale bar 0.1 µm) D. Mac-3 to visualize macrophages (scale bar 0.1 µm). (Magnification: 40×). Data n.s. comparing WT to IL-6RTKO. Data depict the mean ± SD of the indicated values.

2.3.4 Eliminating IL-6R expression on T cells reduces the development of donor specific antibodies

Tfh cells that reside in lymphoid organs support the activation and secretion of donor specific antibodies by B cells which is important to the pathogenesis of TA.[13,25] DSAs are not abundant in our model until 3 weeks post-transplant (Figure 2.5A). As such, these antibodies minimally contribute to intimal thickening that is assessed at day 30 post transplant. Our model can be used to examine the development of DSAs, but additional models are needed to examine antibody-mediated rejection. To assess the presence of DSA, sera from transplant recipients was collected and incubated with donor splenocytes. Eliminating IL-6R in T cells significantly reduced the development of DSA in the serum of graft recipients but inhibiting trans-signalling did not (Figure 2.5 B and C). My findings thus far suggest that IL-6 classic signalling in T cells is non-redundantly required for the development of DSA in TA.

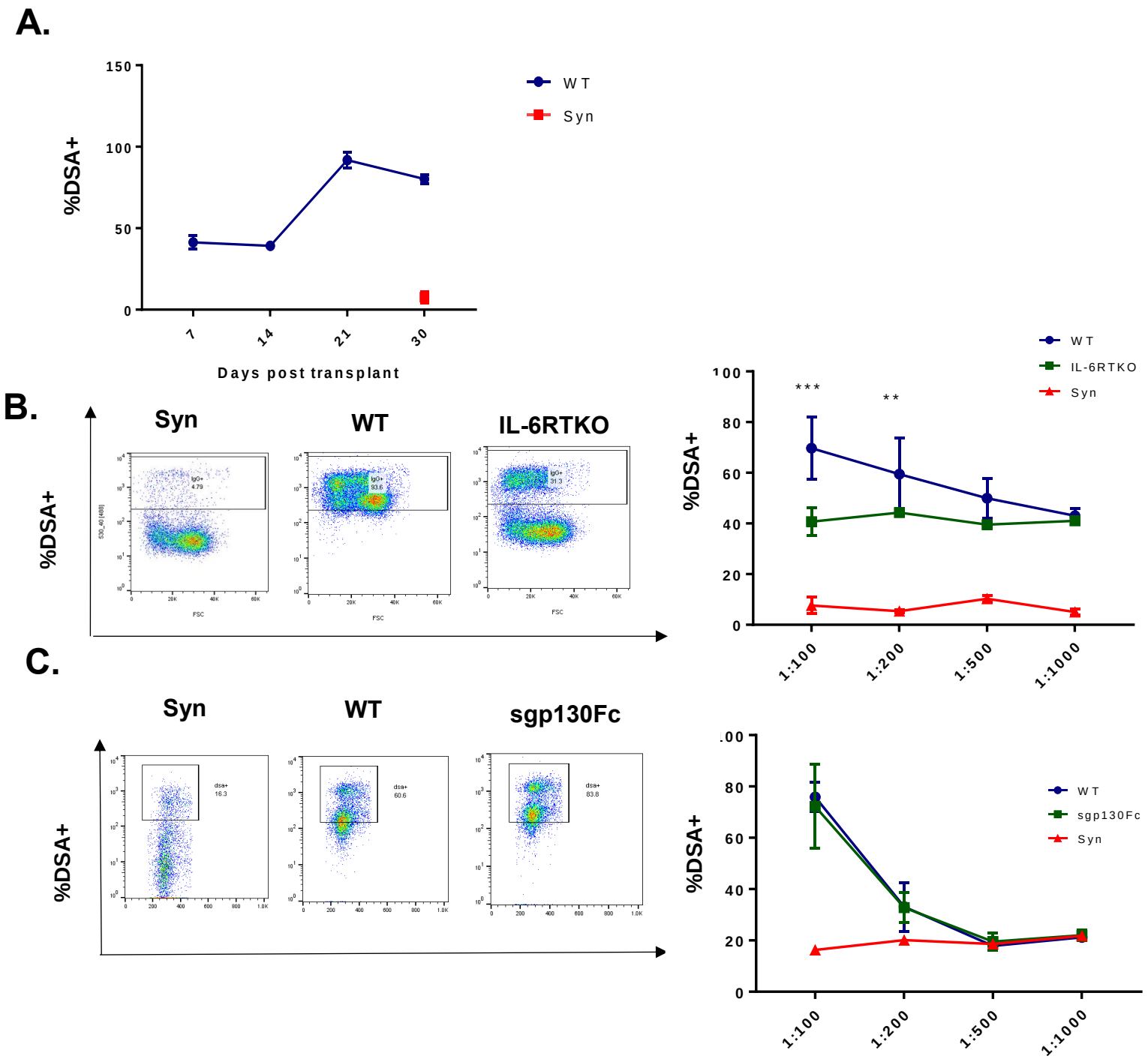


Figure 2.5 Elimination of IL-6R expression on T cells causes not elimination of IL-6 trans-signaling causes a reduction in donor specific antibodies. A. Sera at day 7,14, 21 and 30 from allogeneic WT (n=3)recipients incubated with donor Balb/c splenocytes for 30 mins and flow cytometric analysis performed for CD3 and anti-mouse IgG **B.** Sera at day 30 from mice that received syngeneic (syn) transplants (n=3) or from WT(n=5) or IL-6R-TKO(n=6) that received allogeneic transplants OR **C.** Sera at day 30 from mice that received syngeneic (syn) transplants or from WT or sgp130Fc that received allograft artery segments were analyzed and flow cytometric analysis performed for CD3 and anti-mouse IgG. Graph depicts the mean ± SD of the frequency of CD3+ cells bound with donor specific antibody. ***P < 0.001, **P<.01 comparing WT vs IL-6RTKO.

2.4 Discussion

IL-6 signalling is critical to multiple pathophysiological processes that culminate in the development of TA. My studies build on previous research implicating IL-6 in T cell activation and trafficking into the graft. Our lab previously demonstrated the role of graft derived IL-6 in supporting the activation, proliferation and survival of T cells and the resultant cell mediated damage of donor arteries.[19] This chapter aimed to delineate the role of IL-6 classic and trans-signalling in the development of the allo-immune response in TA.

We first examined the contribution of IL-6 classic signalling in T cells to immune activation by performing allogeneic aortic transplants into recipients that lacked IL-6R on T cells. Luminal narrowing, an indicator of vascular changes induced by cell mediated injury, was not affected in IL-6RTKO transplant recipients. Accumulation of CD4 T cell, CD8 T cells and macrophages was not affected indicating that peripheral T cell responses are not dependent on IL-6R expression in T cells. Strikingly, IL-6R expression was needed for development of DSAs. Our studies show that IL-6 classic signalling is dispensable for T cell activation and resultant cell mediated injury but is required for development of DSAs and possibly AMR.

T cells lacking IL-6R have been demonstrated to still be responsive to IL-6 signalling upon addition of sIL-6R in vitro.[29] Jones et al. demonstrate that attenuated Th17 differentiation in IL-6R$^{-/-}$ mice can be rescued upon addition of hyper IL-6 (a synthetic cytokine where IL-6 and sIL-6R are fused via a linker protein) .[29] Therefore, it is possible IL-6 trans-signalling can compensate for lack of IL-6 signalling in this system. The role of IL-6 trans-signalling was then investigated by performing allogeneic aortic transplants into recipients that express high levels the fusion protein sgp130Fc. Sgp130 binds to the IL-6: sIL-6R complex and prevents IL-6 trans-signaling while retaining classic signalling. Further, histological assessments of graft arteries displayed no difference in intimal thickening between WT and sgp130Fc recipients. Inhibiting trans-signalling also did not affect accumulation of CD4 T cells, CD8 T cells or macrophages. This was not due to insufficient levels of circulating sgp130 as we confirmed abrogation

of trans-signalling using the air pouch model of inflammation where trans-signalling is necessary for macrophage infiltration. Future studies could investigate the effect of elimination of trans-signalling on T cell activation separately from trans-signalling in the vasculature.

IL-6 contributes to the production of high affinity donor specific antibodies by contributing to Tfh differentiation and subsequent B cell help as well as acting on B cell directly to induce their maturation and antibody class switching. A novel finding of this study is that elimination of IL-6 classic signalling in T cells is needed for the production of DSAs suggesting that IL-6 is needed for the actions of Tfh cells. In showing this key role of IL-6 classic signalling in DSA production, we have characterized the effect of IL-6 on one aspect of the humoral response. DSA contributes to the manifestation of TA in many ways. DSA recognize MHC and non MHC antigens on donor tissue and facilitate cell lysis.[13] Furthermore DSA can activate and promote the proliferation of endothelial cells. De novo DSAs develop months to years after transplantation and clinicians are recognizing the significant contribution to chronic rejection of solid organs. Diagnostic assessment of antibody mediated rejection has recently been standardized and so assessment of AMR contribution to chronic rejection is of renewed interest.[16] Therefore our novel finding that IL-6 classic signalling is necessary for DSA generation has far reaching implications. Indeed, Tocilizumab, an IL-6R neutralizing antibody, has shown promising results in reducing AMR episodes in kidney transplant recipients. Further investigative studies looking at how eliminating classic signalling in T cells affect Tfh differentiation and effector function could be performed by harvesting splenocytes at day 7 and quantifying expression of CXCR5, PD1, Bcl6 and IL-21.

In conclusion, our studies posit a redundant role of IL-6 classic and trans-signalling in activation of peripheral T cell responses in TA. IL-6 signalling via either pathway is sufficient for peripheral T-cell activation as well accumulation of leukocytes in the graft. We identified a novel role of IL-6 classic signalling in T cells in DSA production. This may be of clinical relevance as antibody mediated organ rejection is not responsive to conventional therapies and understanding the immunological mechanisms involved may inform treatment strategies.

Chapter 3 Biological effects of IL-6 signalling in endothelial cells

3.1 Introduction

Endothelial cell injury and activation are key events that triggers a cascade of biological processes that influence the formation of TA lesions. The allo-immune response induces injury by cytotoxic molecules, ligation of death receptors, complement mediated lysis and ADCC.[11,13,19] The release of proteins, growth factors and cytokines act on adjacent vSMC to trigger their intimal migration and proliferation.[2,8,17] Activation of ECs also promotes TA development by leukocyte infiltration into allograft arteries.[2,23,26] Our lab has shown that systemic abrogation IL-6 signalling (inhibiting both classic and trans-signalling) increases endothelial injury indicating that IL-6 has protective effects.[19] Little is known about the contribution of IL-6 classic and trans-signalling to vascular protection or injury in TA and the signalling mechanisms by which this is regulated.

Vascular protection by EC could be due to IL-6 promotion of EC survival and or promotion of EC proliferation. There is evidence of IL-6 signalling enhancing EC proliferation.[29,74]Classic signalling has been shown to promote the formation of vascular endothelial cells into tube-like structures while IL-6 trans-signalling inhibits the morphological differentiation. .[29,74] Not much is known about IL-6 in EC survival.

The aim of this chapter is to understand the biological effects of IL-6 signalling in EC in order to target inflammatory processes that promote TA development and preserve protective processes. My findings show that IL-6 trans-signalling in ECs significantly induces STAT-3 activation, ICAM upregulation as well as the secretion of inflammatory cytokines MCP-1. IL-6 classic signalling is cytoprotective for EC and sufficient for IL-8 secretion. Neither IL-6 classic nor trans-signalling have a role in EC proliferation. My results suggest that IL-6 trans- signalling induces some inflammatory responses in endothelial cells and targeting this pathway specifically may have implications for inhibiting vascular injury.

3.2 Materials and Methods

3.2.1. Cell culture

Initial experiments on the activation of signalling cascades were conducted in human umbilical vein endothelial cells (HUVEC) (Lonza) cultured in EBM media (Lonza) supplemented with hydrocortisone, GA-1000, hEGF, BBE, FBS, and ascorbic acid .This media was discontinued so HUVEC were cultured in Medium 200 (Thermofisher) and supplemented with Large Vessel Endothelial Supplement (fetal bovine serum, hydrocortisone, hEGF, bFGF, heparin, and ascorbic acid) (Thermofisher) Cells were kept at 37 °C with 5% CO2 and used at passages 3 through 6.

3.2.2 Immunohistochemistry

Human TA samples were kindly provided by Dr. Bruce McManus at St. Paul's Hospital, Vancouver, BC. Formalin fixed, paraffin embedded sections were deparaffinized and rehydrated with xylene and ethanol washes. Antigen retrieval was done by heating sections to 121^{o}C in Citrate buffer at pH 6.0 Sections were stained with primary anti-IL-6R antibody (LS-C733461) (Abcam) then incubated with biotin conjugated secondary antibody followed by streptavidin- HRP. Staining was visualized using AEC substrate-chromagen kit (Vector Labs). Slides were imaged using Olympus CX31 Microscope (Olympus Life Science).

3.2.3 Western Blot

To assess IL-6 classic and trans activation of STAT-3, MAPK and Akt pathways, HUVEC in serum-low media or complete media were treated with 50ng/mL IL-6 (Prepotech) or 50 ng/mL IL-6 and 50 ng/mL sIL-6R (R&D systems) for 5, 15, 30 and 60 mins. Cells were then lysed with cold RIPA buffer containing 0.05 M NaF, 0.05 M β-glycerophosphate, 1 μM PMSF, and 2 μM sodium orthovanadate, then incubated 30 mins on ice. Lysates were boiled for 15 minutes in Laemmeli buffer (Sigma) with 5% β-mercapetoethanol before running on SDS-PAGE gels. Membranes were then incubated

in either anti-STAT3 (124H6) (New England Biolab), polyclonalanti-phospho STAT3 (New England Biolab), anti- p44/42 MAPK (Tyr705) (ERK1/2) (137F5) (Cell Signalling), anti-phospho- p44/42 MAPK (ERK1/2) (D13.14.4E) (Cell Signalling), anti-Akt (C73H10) (New England Biolab), anti-phospho-Akt (C31E5E) (New England Biolab), and loading control anti-β-actin (AC-15) (Sigma) overnight. Membranes were then incubator with HRP-conjugated anti mouse or anti rabbit secondary antibodies (Jackson ImmunoResearch) for 1 hour before development with Clarity ECL Western Substrate (BioRad). Membranes were imaged using Fujifilm LAS-4000(GE Healthcare Life Sciences) and quantified using ImageJ (NIH).

To assess IL-6R expression, HUVEC and A549 Human lung carcinoma epithelial cells were grown to confluency and lysed as above. Membranes were incubated with anti-IL-6R antibody (17506) (Abcam) or anti-β-actin (AC-15)(Sigma) overnight.

3.2.4 Flow Cytometry

To assess ICAM-1 and IL-6R protein expression on the cell surface of HUVEC, flow cytometric analysis was performed 24 hours after IL-6 stimulation. HUVEC were lifted with 0.25% trypsin EDTA and stained with a fluorescently conjugated antibody to PE anti human ICAM (clone HA58) (BD Biosciences) and APC anti human IL-6R (clone UV4) (Biolegend).

To assess HUVEC survival, both floating and attached cells were harvested after IL-6 stimulation. Cells were then washed with PBS and stained with Propidium Iodide (Abcam) in the dark for 30 min.

Data was acquired on BDFACS Jazz and analysed using FlowJo (Treestar Inc). Negative and Positive gates were set using appropriate isotype controls.

3.2.5 ELISA

Supernatants were collected from HUVEC treated with either 50ng/mL IL-6 (Preprotech) or 50ng/mL IL-6 and 50ng/mL IL-6R (R&D systems) for 24, 48 and 72 hours and MCP-1

and IL-8 levels quantified using an DuoSet ELISA kit as per the manufacturer's instructions (eBioscience)

3.2.6 MTS Cell Proliferation Assay

HUVEC were plated at a subconfluent concentration and expansion of the cellular population over 48 h measured using an MTS assay (Abcam) as per the manufacturer's instructions. Reduction of MTS tetrazolium compound by metabolically active cells cause a formazan product. The color change is measured by microplate reader at 500 nm.

3.3 Results

3.3.1 Expression of IL-6R on EC

The expression of IL-6R is tightly regulated and the responsiveness of ECs to IL-6 classic signaling is controversial. We examined the in-situ expression of IL-6R in coronary arteries from healthy individuals and individuals diagnosed with TA. IL-6R expression is minimal in the endothelium of healthy coronary arteries but apparent on the endothelium of arteries with TA, indicating that its expression may be induced by inflammatory stimuli (Figure 3.1A and B). When IL-6R expression was examined in HUVECs, it was detected by both Western blot and flow cytometry (Figure 3.1 C and D). HUVECs also express abundant levels of gp130 (Figure 3.1E). As such, HUVECs are an appropriate model to examine IL-6 responsiveness in ECs.

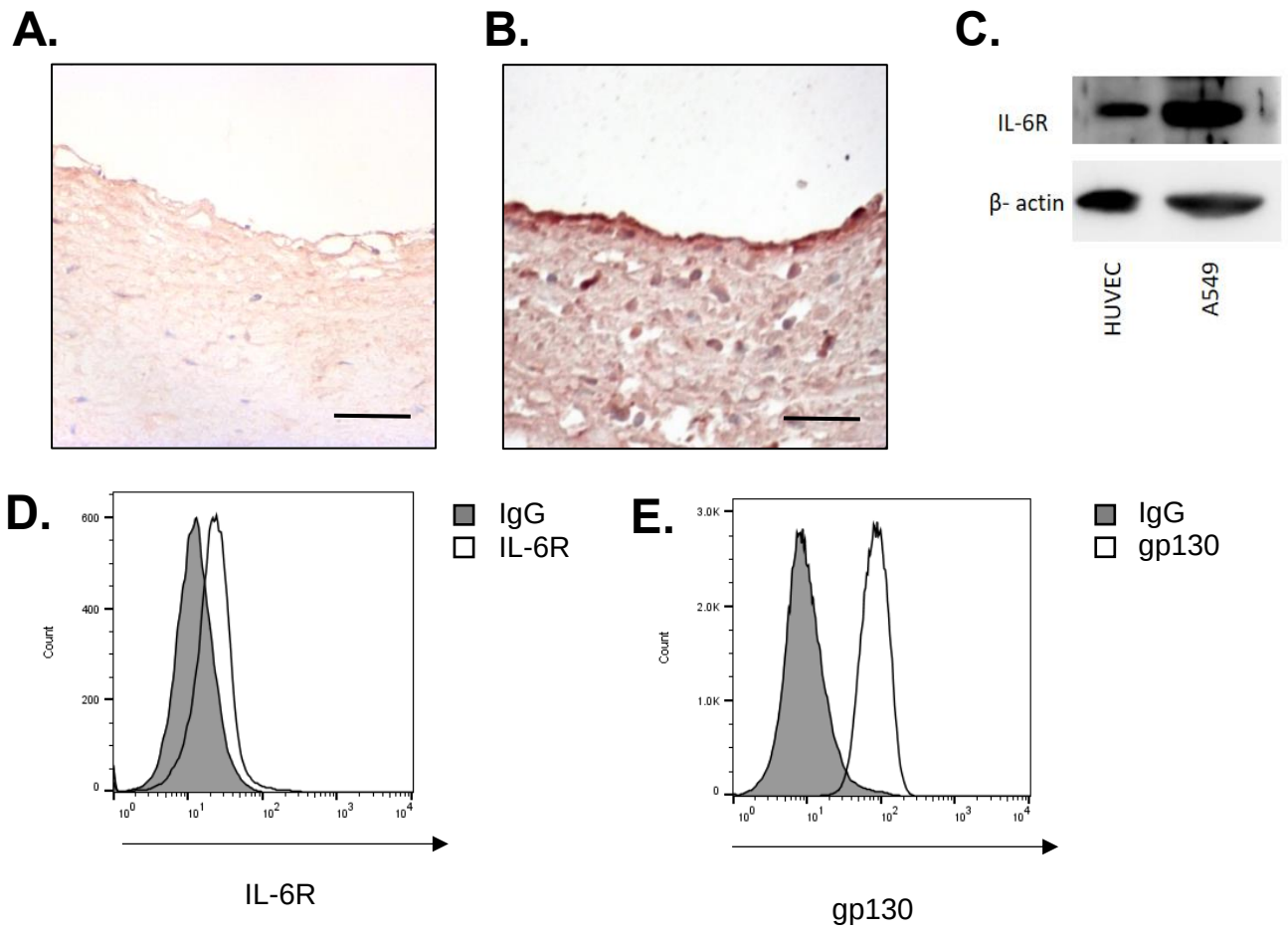


Figure 3.1. Expression of IL-6R on endothelial cells. Immunohistochemical analysis of IL-6R expression on **A.** Healthy human coronary arteries and **B.** human arteries afflicted with transplant arteriosclerosis (scale bar = 1µm) **C.** Whole cell lysates of HUVEC and A549 Human lung carcinoma epithelial cells (positive control) were analyzed for IL-6R via western blot. Representative histogram for **D.** HUVEC stained with anti- IL-6R for surface expression of IL-6R and anti IgG for control, **E.** HUVEC stained with anti- gp130 for surface expression of gp130 and anti IgG for control.

3.3.2 IL-6 classic and trans-signalling differentially induces STAT-3 phosphorylation in HUVEC but not ERK1/2 and Akt

The responsiveness of human endothelial cells to IL-6 classic and trans-signalling was then examined. Endothelial cells were treated with IL-6 alone (classic) or IL-6 + soluble IL-6R (trans) for 5, 10, 30 and 60 minutes, then lysed and activation of cell signalling pathways analysed via Western blot. There was minimal induction of Stat-3 phosphorylation induced by IL-6 classic signalling and its levels were not significantly

above basal levels. IL-6 trans-signalling significantly induced Stat-3 phosphorylation at 15, 30 and 60 mins. (Figure 3.2A). ERK1/2 phosphorylation and Akt phosphorylation were examined in HUVEC cultured for 16 hours in media containing only 0.5% fetal bovine serum (serum-low condition). Complete media was not used as it contains growth factors capable of activating the MAPK/ERK 1/2 and P13K/Akt pathways. There was significant ERK 1/2 phosphorylation induced by both IL-6 classic and trans-signalling at 60 mins that was comparable in magnitude (Figure 3.2B). Also, both classic and trans-signalling significantly induced comparable levels of Akt phosphorylation at 60 mins (Figure 3.2C). As such, IL-6 classic and trans-signalling differentially induce Stat-3 activation in endothelial cells but similarly induce ERK1/2 and Akt activation

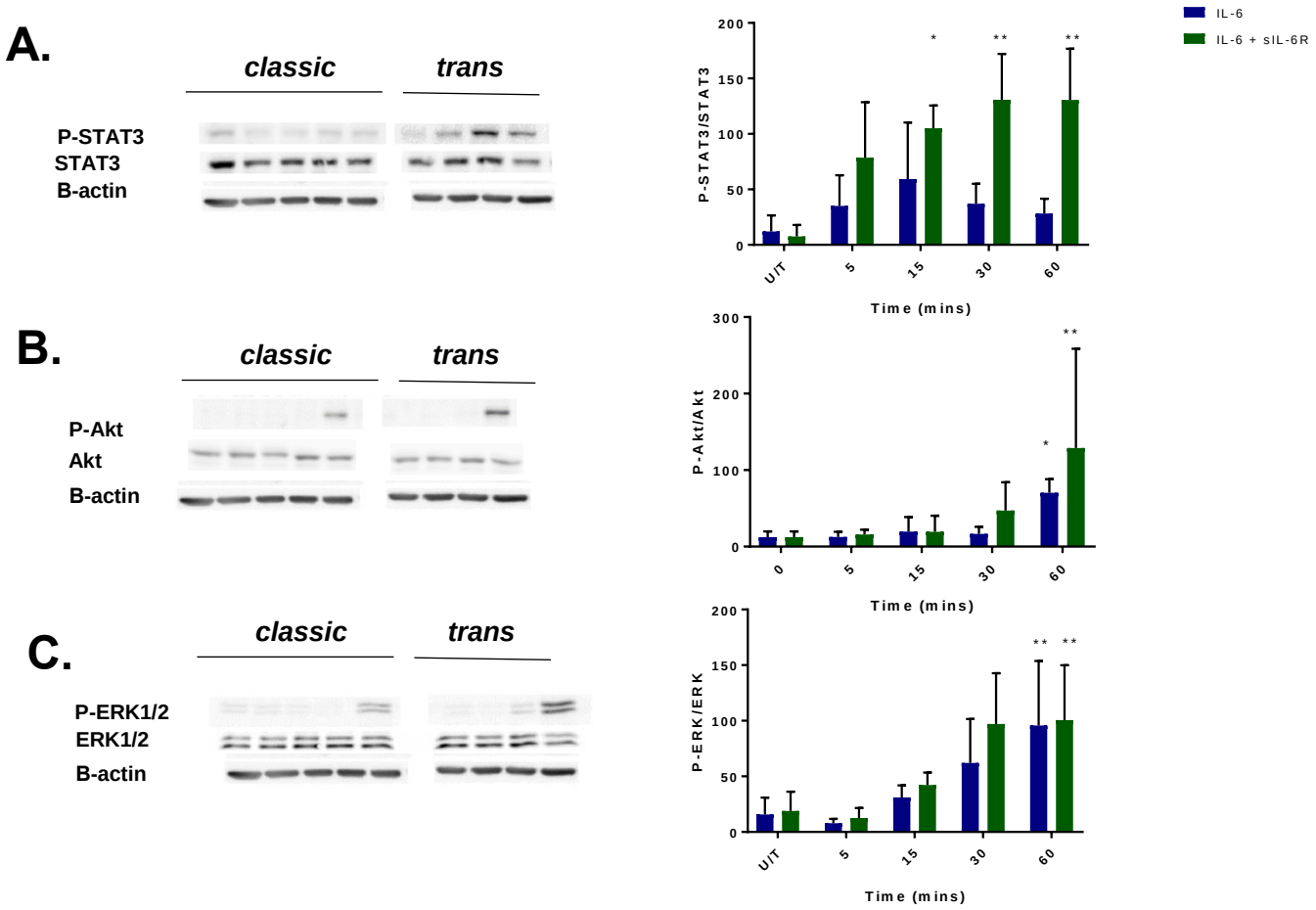


Figure 3.2. IL-6 classic and trans-signalling differentially induces STAT-3 phosphorylation in HUVEC but not ERK1/2 and Akt. HUVEC were stimulated with 50 ng/mL IL-6 (classic) or 50 ng/mL IL-6 and 50ng/mL sIL-6R (trans) 0, 5, 15, 30 and 60 minutes. **A.** STAT3, P-STAT3 **B.** Akt, P-Akt **C.** Erk1/2, P-Erk1/2 levels were analyzed by Western blot. * = P <0.05 and ** = P <0.01 comparing classic vs trans and determined using unpaired t-test.

3.3.3 IL-6 trans-signalling is necessary for ICAM upregulation

The effect of IL-6 signalling on the vasculature is complex and has important implications for the development of TA. IL-6 has inflammatory effects on endothelial cells but the signalling mechanisms involved remain poorly examined. ICAM is an adhesion molecule that is up-regulated by IL-6 and is required for leukocyte migration into sites of inflammation.[2] IL-6 trans but not classic signalling significantly upregulated ICAM expression on the surface of endothelial cells (Figure 3.3A). In order to ascertain the mechanism by which this occurs, cells were treated with an Akt inhibitor LY294002

and an MAPK inhibitor PD98059 for 1 hour before IL-6 treatment. Inhibition of the PI3K/Akt pathway prevented the induction of ICAM, indicating the involvement of this pathway in this inflammatory response (Figure 3.3B). MAPK inhibition did not affect ICAM upregulation (Figure 3.3C). STAT3 inhibition could not be assessed because its inhibition with pharmacological inhibitors had an adverse effect on HUVEC viability. Currently we are investigating methods to inhibit STAT3 by overexpression of a dominant negative (DN) mutant of STAT3.

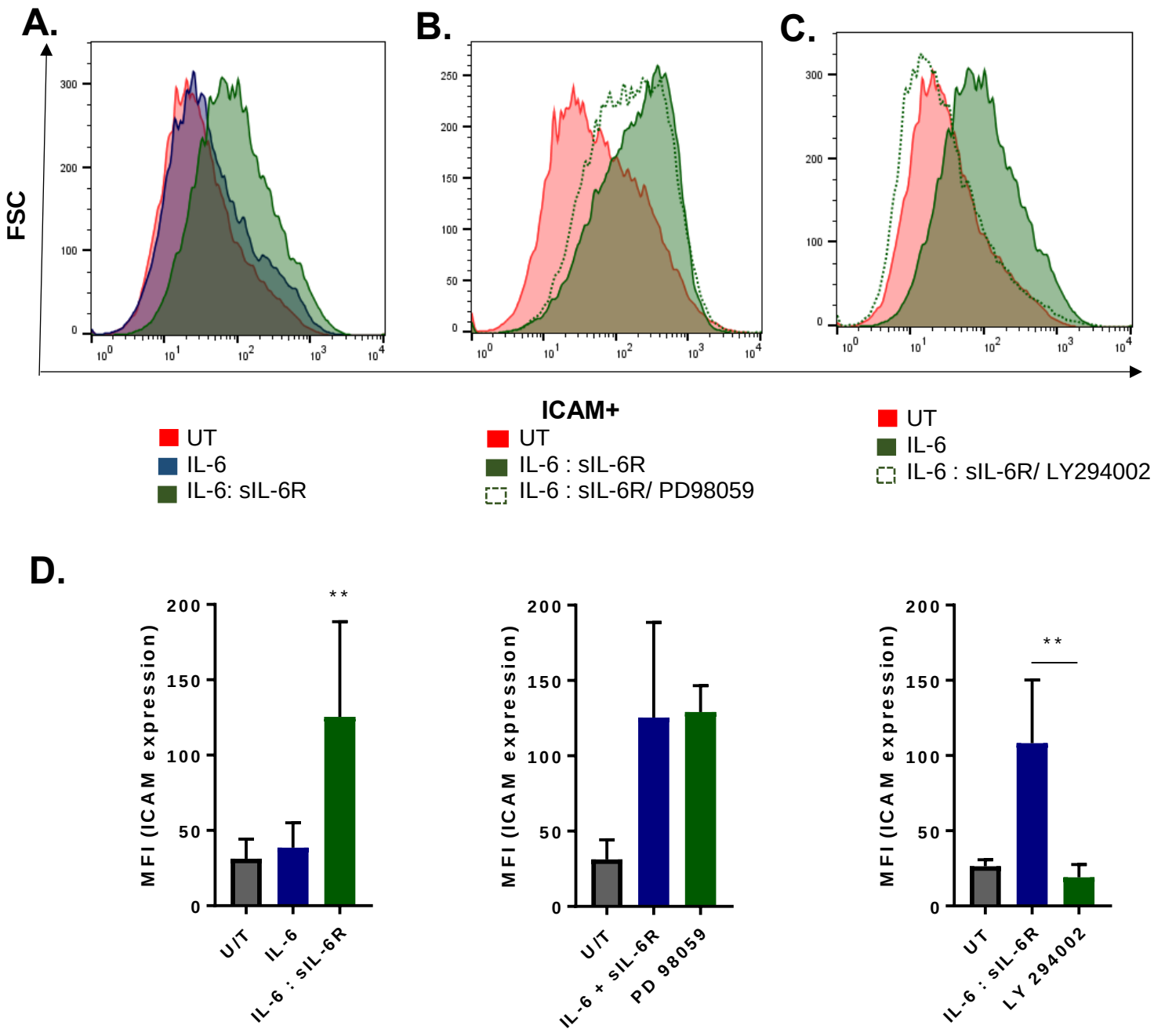


Figure 3.3. IL-6 trans-signalling induces for ICAM expression. **A.** HUVEC were treated with 50 ng/mL IL-6 (classic signaling) or 50 ng/mL IL-6 and 50ng/mL sIL-6R (trans-signaling) for 24hrs, stained for ICAM and analyzed by flow cytometry. **B.** HUVEC were treated with PD98059 (MAPK/ERK 1/2 Inhibitor) for 60 mins before treatment described in A. **C.** HUVEC were treated with LY294002 (PI3K/Akt inhibitor) for 60 mins before treatment described in A. **D.** Quantification of corrected MFI was plotted for 3 independent experiments. * = P <0.05 and ** = P <0.01 compared to UT group unless otherwise indicated and determined using unpaired t-test.

3.3.4. IL-6 trans-signalling is necessary for MCP-1 secretion but both IL-6 classic and trans-signalling induce IL-8 secretion

Next, we examined whether IL-6 classic or trans-signalling differentially regulates inflammatory chemokine secretion by endothelial cells. MCP-1 is a potent chemoattractant for monocytes and is secreted by endothelial cells in response to IL-6.[32] Endothelial cells were treated with IL-6 or IL-6 + sIL-6R overnight and supernatants analysed by ELISA for the presence of MCP-1. IL-6 trans-signalling significantly induced MCP-1 secretion at 24 and 48 hours whereas IL-6 classic signalling did not (Figure 3.4A). We then examined the effect of IL-6 classic and trans-signalling on IL-8 secretion. IL-8 is important for recruitment of neutrophils.[35,66,42] Both IL-6 classic and trans-signalling significantly induced IL-8 secretion at 48 and 72hrs (Figure 3.4B). Thus, IL-6 trans-signalling is needed for MCP-1 secretion that recruits monocytes but IL-6 classic signalling is sufficient to induce IL-8 that recruits neutrophils

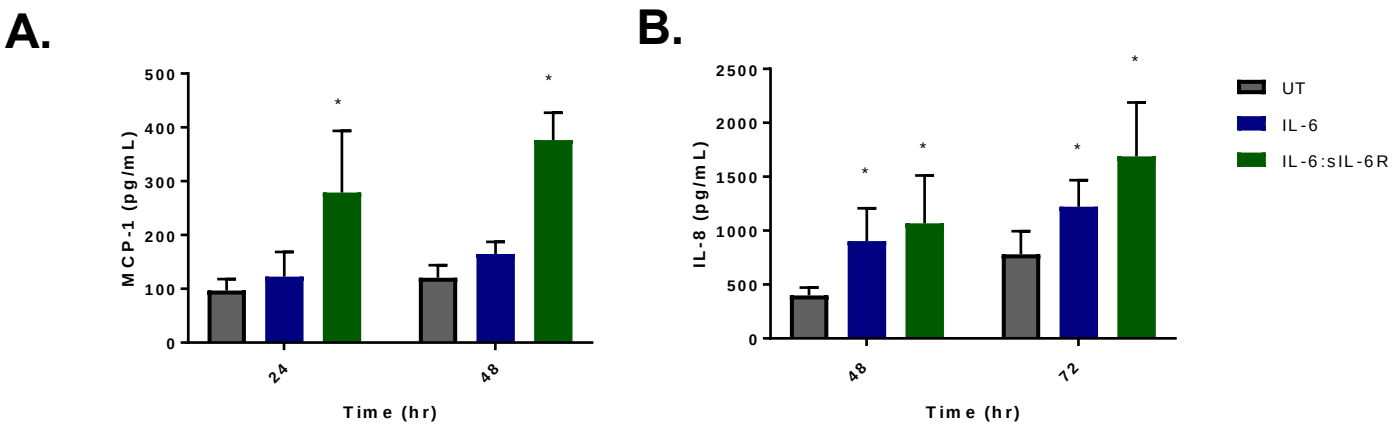


Figure 3.4. IL-6 trans-signalling is necessary for MCP-1 secretion but both IL-6 classic and trans-signalling is needed to induce IL-8 secretion. HUVEC were treated with 50 ng/mL IL-6 (classic signaling) or 50 ng/mL IL-6 and 50ng/mL sIL-6R (trans-signaling) for 24, 48 and 72 hrs and supernatant analyzed via ELISA assay for **A.** MCP-1 and **B.** IL-8. Data from 3 independent experiments. * = P <0.05 compared to UT and determined using unpaired t-test.

3.3.5 IL-6 does not affect EC proliferation but IL-6 classic signalling is cytoprotective

The vascular reparative and protective effects of IL-6 were then assessed. To examine whether IL-6 classic or trans-signalling influenced endothelial cell proliferation, HUVEC were plated at a subconfluent concentration and expansion of the cellular population over 48 h measured using an MTS assay. Neither IL-6 classic nor trans-signalling influenced proliferation. (Figure 3.5A). We then investigated whether IL-6 classic or trans-signalling protected endothelial cells from cell death from serum starvation. HUVEC were maintained in M-200 supplemented with LVES and treated with IL-6 or IL-6 + sIL-6R for 24 hours before media was changed to M-200 supplemented with 0.1% FBS for 16hrs (serum deprived condition). IL-6 classic signalling was cytoprotective for endothelial cells but IL-6 trans-signaling did not have a significant effect (Figure 3.6B)

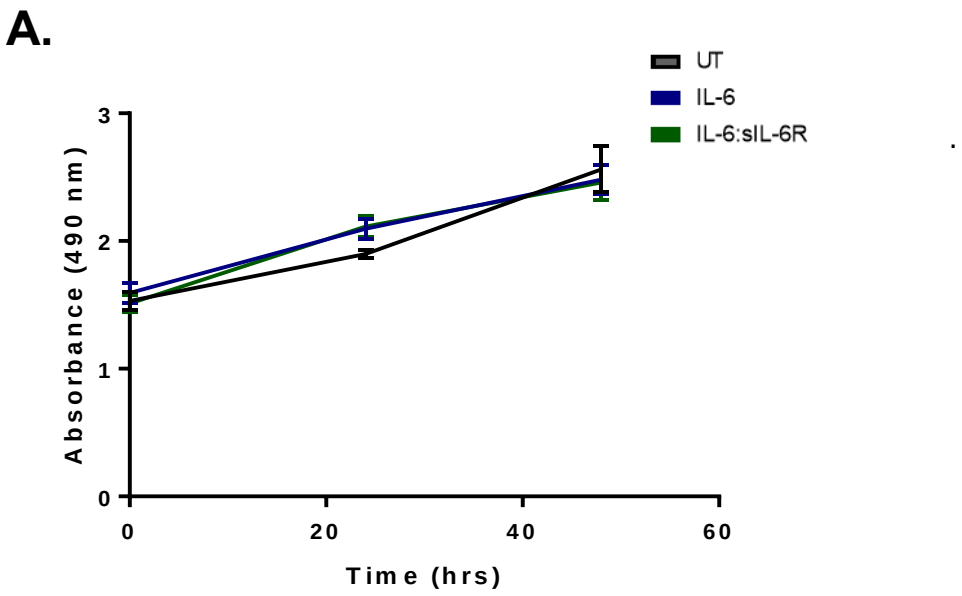


Figure 3.5. IL-6 classic or trans-signalling does not affect endothelial cell proliferation. **A.** MTS Assay showing the effect 50 ng/mL IL-6 (classic signaling) or 50 ng/mL IL-6 and 50ng/mL sIL-6R (trans-signalling) on the expansion of endothelial cells over at 48 hours. Data quantified from 3 independent and n.s when compared to UT.

A.

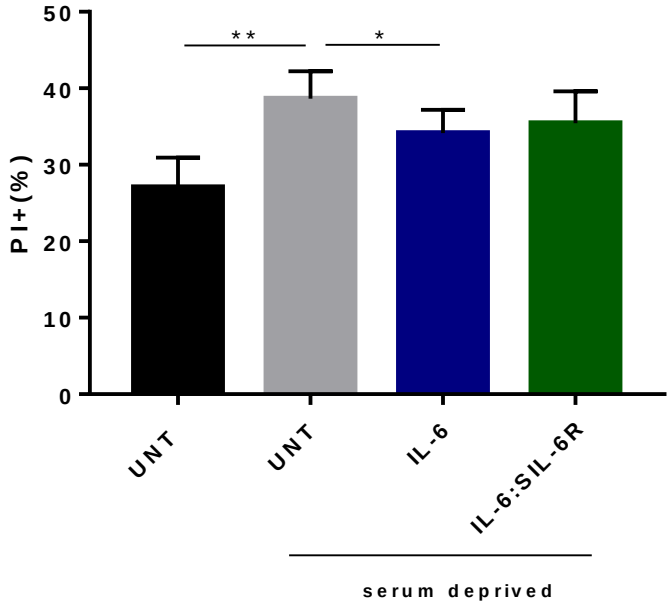


Figure 3.5. IL-6 classic signalling is protective for endothelial cells A. HUVEC were treated with 50 ng/mL IL-6 (classic signaling) or 50 ng/mL IL-6 and 50ng/mL sIL-6R (trans-signalling) or for 24 hours before media was changed to serum deprived media for 16hrs. Data quantified from 3 independent experiments. * = P <0.05, ** = P <0.01 determined using unpaired t-test.

3.4 Discussion

IL-6 has inflammatory and protective effects on endothelial cells but the mechanisms by which this cytokine exerts these effects are poorly understood. In particular, very little is known about the contribution of IL-6 classic and trans-signalling to the various outcomes induced by this cytokine in ECs. Also expression of IL-6R and responsiveness of ECs to IL-6 classic signaling is controversial We know from studies and clinical trials that in some diseases systemic abrogation of IL-6 signalling is counter productive because either there is no attenuation of pathological processes or total elimination has other adverse effects such as reduced ability to protect from infections.[19,75] It is important to examine the nuanced role of IL-6 so that new approaches can be developed that preserve protective processes and target the pathophysiological effects of IL-6.

Earlier studies suggest that IL-6R expression is restricted to leukocytes and hepatocytes and whether EC express IL-6R is contested.[49] My studies show that IL-6R is expressed on the surface of endothelial cells and that the receptor is upregulated under inflammatory conditions. Endothelial cells also express gp130. IL-6R must associate with coreceptor gp130 to initiate intracellular signalling pathways because the short intracellular domain of IL-6R lacks signalling domains .[49] Cultured endothelial cells are responsive to IL-6 classic signalling by activating both the ERK1/2 and Akt pathways . However, IL-6 alone does not induce STAT3 activation in ECs. The activation of the MAPK and PI3K cascade activates a multitude of downstream targets that control cell survival, growth and inflammation. Therefore, regulation of IL-6R expression allows for distinct biological effects under conditions of inflammation and homeostasis. While our experiments do not explore whether this differential activation is due to a difference in magnitude of signal strength, by understanding the signalling pathways involved in immune activation or cytoprotection, it may be possible to target pathways involved in inflammatory processes while retaining the protective effects. Future experiments where IL-6R expression is increased by lentiviral transduction can allow us to look at the role of signal threshold in differential activation of signaling pathways by IL-6 classic and trans pathways.

Our experiments also show that IL-6 classic signalling has a cytoprotective role for endothelial cells in vitro. IL-6 classic signalling rescues HUVEC death from serum deprivation. Cytoprotection in other cell types has been linked to IL-6 induction of the PI3K/Akt pathway and inhibition of caspase 9.[76] Potential studies using chemical inhibitors and or gene silencing techniques could examine the signaling pathways and regulatory proteins responsible for this protective effect.

An interesting biological effect mediated by IL-6 classic signalling is its ability to induce IL-8 secretion. IL-8 is the main chemoattractant for neutrophils, which may set the stage for resulting T cell-mediated damage of donor tissue. IL-6 classic signalling may be sufficient to stimulate the release of IL-8 that recruits neutrophils, which could then shed IL-6R creating a positive feedback loop that propagates the inflammatory immune response. Future studies can look at the signalling pathways involved with IL-8 secretion by chemical inhibition studies. Exploring the intracellular signalling basis for this effect allows us to determine whether inhibiting a specific pathway with chemical inhibitors is a viable strategy in TA.

We next looked at the biological effects mediated by IL-6 trans-signalling. My results suggest that IL-6 trans-signalling induces inflammatory responses in endothelial cells. Our in vitro studies support that endothelial cells in an inflammatory environment are responsive to trans-signalling. We show that IL-6 trans signalling is sufficient for ERK1/2 and PI3K/Akt activation and necessary for STAT-3 activation. Phosphorylation of STAT-3^{Tyr705} results in association with p300/CBP and transcription of IL-6 responsive genes.[29,50] IL-6 trans-signalling is necessary for the upregulation of ICAM on endothelial cells and this is in part mediated by the PI3K/Akt pathway indicating a novel role for IL-6 induction of this cascade in non-mitogenic responses. We also attempted to assess the effects of STAT-3 inhibition however the chemical inhibition of STAT-3 had a negative effect on cell viability (data not shown). We are now in the process of transfecting HUVEC with a DN form of STAT-3 to examine if the STAT3 pathway cross-talks with the PI3K pathway to regulate the expression of ICAM on endothelial cells.

In summary, ECs are responsive to IL-6 classic and trans-signalling and induce distinct outcomes. IL-6 classic signalling activates both the MAPK and PI3K/Akt

pathway and is involved in mediating endothelial cell survival. It also induces IL-8 secretion, which may play a role in initiating the innate immune response in inflammatory conditions. My studies also show that IL-6 trans-signalling activates both the MAPK and PI3K/Akt pathways and is necessary for STAT-3 activation. IL-6 trans-signalling promotes activation of endothelial cells by upregulating ICAM in part via the PI3K/Akt pathways. EC also secrete inflammatory cytokines MCP-1 and IL-8 in response to IL-6 trans-signalling. The experiments detailed in this chapter begin to answer the nuanced role of IL-6 classic and trans-signalling in both the cytoprotective and inflammatory effects on vascular endothelial cells and how these processes are regulated by different intracellular signalling cascades

Chapter 4: General discussion and conclusion

Transplant arteriosclerosis (TA) is a major hindrance to long term survival of cardiac allografts. TA occurs months to years after transplantation and by current methods is difficult to diagnose. Re-transplantation, which accounts for 3% of all transplantation procedures in North America, is the sole treatment option for individuals diagnosed with TA.[1] Advances in the management and treatment of TA require understanding the immune mechanisms involved in the pathophysiology of this vascular disease.

The first chapter examined the cellular signalling mechanisms of IL-6 and its contribution to immune activation in a murine model of TA. There is no consensus in the field as to how IL-6 classic or trans signalling regulates T cell responses. Our lab showed IL-6 classic signalling was sufficient for expansion and survival of T cells in vitro.[19] Nish et al found that IL-6 classic signalling was not integral to T cell survival in vivo while Stefan Rose et al identified IL-6 trans-signalling as being integral to this process.[63,77] The role of T cell activation is also unclear with some studies providing evidence that IL-6 classic signalling drives T cell activation while IL-6 trans-signalling drives immune responses at the site of inflammation. [60,78] In contrast, others identify a necessary role for IL-6 trans- signalling in T cell activation and differentiation.[60,78] My studies show a redundant role for IL-6 classic and trans-signalling in peripheral T cell activation. This is supported by evidence that IL-6 trans-signalling can compensate for lack of IL-6R expression on T cells allowing for normal effector function.[29]

My studies also clearly show that IL-6 classic signalling is needed for donor specific antibody generation. Examination of sera from IL-6RTKO yielded significantly less DSA compared to WT counterparts. This suggests that IL-6 classic signalling plays a non-redundant role in Tfh cells and resulting B-cell help. IL-6 activation of STAT1 is necessary for the induction of Bcl6, the regulator needed for Tfh differentiation.[79] It could be that Tfh cells depend on STAT1 induced Bcl-6 expression for differentiation and that this requires IL-6 classic signalling. This has implications for chronic DSA mediated injury and reparative responses that culminate in TA lesions.

The third chapter of my thesis characterized IL-6 signalling in endothelial cells. These cells are exposed to a cytokine milieu post transplantation and are active participants in the allo-immune response. The activation and injury of endothelial cells contribute to the initiation of processes that lead to the development of TA lesions. Previous studies have implicated IL-6 in both a protective and deleterious capacity. IL-6 promotes endothelial cell dysfunction by inhibiting NO production, inducing vasoconstrictive factors and upregulating inflammatory cytokines and adhesion molecules.[2] Paradoxically, IL-6 has been demonstrated to be cytoprotective for endothelial cells. Very little is known about the cellular signalling mechanisms that mediate these effects and the conditions under which we observe these effects.

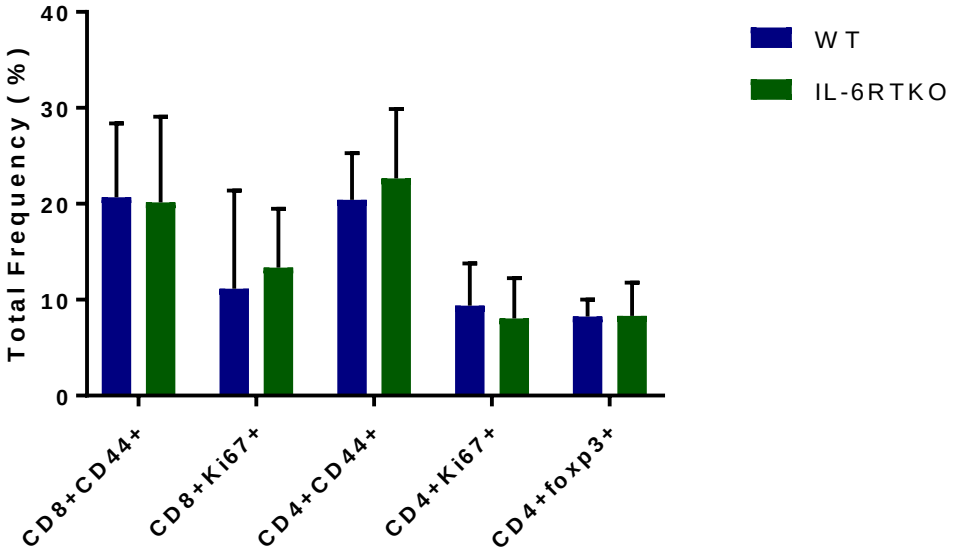
I showed that endothelial cells are responsive to both IL-6 classic and trans-signalling. They express low levels of IL-6R under basal conditions and upregulate the receptor in conditions of inflammation. In these cells, IL-6 classic signalling is sufficient to activate MAPK/ERK1/2 and PI3K/Akt pathways while trans-signalling activates STAT3 in addition to MAPK/ERK1/2 and PI3K/Akt pathways. We examined whether IL-6 signalling was involved in cytoprotection and found that IL-6 classic signalling protected HUVEC from death due to serum deprivation. This cytoprotection was not related to any effect on proliferative capacity. Both the MAPK and Akt pathways are involved in mitogenic processes and are possible mediators of this biological outcome.[32]

We then examined the signalling mechanisms by which IL-6 is inflammatory. IL-6 trans- signalling activates STAT3, MAPK and Akt pathways in human vascular endothelial cells. The balance between STAT3 activation vs MAPK and PI3K depends on the phosphorylation of gp130.[51] Both SOCS3 (which curtails STAT3 activation) and Shp2 (which recruits and activates MAPK) bind to the same motif and the balance tips towards STAT3 when gp130 is phosphorylated.[51] Studies also show that inflammatory stimuli also increase gp130 expression and downregulate mIL-6R expression, rendering endothelial cells more responsive to trans-signalling.[80] This allows for IL-6 trans-signalling to mediate distinct biological effects compared to IL-6 classic signalling.

My studies reveal multiple inflammatory effects mediated by IL-6 trans-signalling. ICAM expression on the surface of endothelial cells was mediated by IL-6 trans-signalling and inhibition of the PI3K/Akt pathway resulted in a reduction in ICAM upregulation. ICAM is one of the molecules that facilitates enhanced recruitment of leukocytes to sites of inflammation.[2] STAT3 activation has also been implicated in ICAM upregulation where it binds to IFN-γ response element (IRE) in the promoter region of the ICAM gene.[27] Ongoing studies are investigating the participation of this pathway in vascular endothelial cells. Additionally IL-6 trans-signalling significantly induces both IL-8 and MCP-1 gene expression and, as I show, protein secretion.[80] Studies in other cell types such as fibroblasts implicate the MAPK pathway in IL-8 and MCP-1 secretion induced by IL-6.[51,80,81] Further studies using pharmalogical inhibitors can elucidate the signalling mechanism by which this is controlled in EC. Trans-signalling mediates pro inflammatory responses in endothelial cells and facilitates leukocyte/EC interactions and contributes to the pathogenesis of TA

Overall my studies have elucidated the redundant role of IL-6 classic and trans-signalling in peripheral T cell activation but an obligate role of IL-6 classic signalling in DSA generation. Finally, I characterized inflammatory and cytoprotective processes mediated by IL-6 classic and trans-signalling in human vascular endothelial cells. These findings could be exploited when devising strategies to precisely target pathological effects of IL-6 in TA.

Supplementary Figures



Supplementary Figure 1. T cell activation in WT and IL-6RTKO mice. Splenocytes were harvested from WT and IL-6RTKO recipients at day 7 post-transplantation and flow cytometric analysis performed for CD4 or CD8 and CD44, Ki67 and Foxp3 to quantify the number of effector, proliferating T cells. Graphs depict the mean ± SEM of the frequency of CD4 and CD8 T cells that were positive.

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