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Interleukin-2 Receptor Alpha Nuclear Localization Impacts Vascular Smooth Muscle Cell Function and Phenotype

Kristie Nhi Dinh
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INTERLEUKIN-2 RECEPTOR ALPHA NUCLEAR LOCALIZATION IMPACTS VASCULAR SMOOTH MUSCLE CELL FUNCTION AND PHENOTYPE

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

KRISTIE NHI DINH
B.S., Wright State University, 2019

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ABSTRACT

Dinh, Kristie Nhi. M.S., Department of Biological Sciences, Wright State University, 2021. Interleukin-2 Receptor Alpha Nuclear Localization Impacts Vascular Smooth Muscle Cell Function and Phenotype.

Atherosclerosis is responsible for 50% of all deaths in western society and is the main cause of cardiovascular diseases (Pahwa et al. 2020). Vascular smooth muscle cells (VSMC) play a vital role in the development of atherosclerotic plaques; this is due to their ability to proliferate and migrate in response to inflammation and damage to arteries’ inner linings. To further understand what causes these cells to proliferate and migrate, our lab has previously found that VSMC express all three subunits of the IL-2 receptor (IL-2R). Out of these three subunits, IL-2Rα appeared to change the most depending on the phenotype of the cell. Our studies have shown that IL-2Rα could play a vital role in VSMC switching from a proliferative phenotype to a quiescent phenotype, in response to injury and inflammation.

The present studies found that not only does the nuclear localization of IL-2Rα impact VSMC proliferation, but that commercially available knockout mice are not fully knocked out. In the first study, we utilized immunofluorescent staining, western blotting, and proliferation and migration assays to show that, when IL-2Rα localizes to the nucleus, VSMC proliferation is inhibited, therefore providing a possible means for regulation. In the second study, we used similar methodology in addition to PCR to show that IL-2Rα knock out mice are not completely devoid of IL-2Rα. Because the IL-2Rα
knock out was generated using a neomycin resistance gene insert, we were able to eliminate the majority of IL-2Rα producing cells via treatment with G418, a neomycin analog. Treatment with G418 therefore allowed us to study VSMC nearly depleted of IL-2Rα.

These findings are important in contributing to knowledge of the role of IL2-Rα in VSMC function, which could later aid in developing treatments for intimal hyperplasia and, in turn, atherosclerosis. In addition, knowing that the mice are only partially knocked out is beneficial to future investigators working with these mice.
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Atherosclerosis

Atherosclerosis is the leading cause of death and disability in the Western world (Singh et al. 2002). Over time, our society has seen increases in high-fat diets, sedentary lifestyles, and obesity (Pahwa and Jilal 2020). Because of this, populations have become more vulnerable to developing atherosclerosis. Fatty streaks, which form in the arterial walls, develop into plaques (Herrington et al. 2016). Cardiovascular diseases such as coronary heart disease and stroke, which occur from atherosclerosis, have affected several world populations. Although the mortality rate of these diseases has declined over the years, coronary heart disease still remains the top cause for premature adult mortality worldwide (Herrington et al. 2016). Intimal hyperplasia is a precursor to the development of atherosclerosis.

Intimal hyperplasia occurs when there is a large collection of cells in the tunica intima. The high number of cells stems from proliferation and migration of smooth muscle cells in response to injury or inflammation (Haudenschild 1989). VSMC accumulation through proliferation and migration leads to intimal expansion, therefore causing intimal hyperplasia. Intimal hyperplasia develops as follows. When the endothelial cell layer is damaged, thrombosis occurs (Lemson et al. 2000). VSMC proliferation-inhibiting factors such as heparan sulfate, nitric oxide, and prostacyclin...
decrease. In addition, thrombocyte release of heparinolytic enzymes aide in decreasing proliferation-inhibitor concentrations (Lemson et al. 2000). Meanwhile, growth-stimulating factors such as basic fibroblast growth factor, angiotensin II, and catecholamines accumulate and promote VSMC proliferation (Lemson et al. 2000). Next, VSMC migrate from the media to the intima. This migration is facilitated by the degradation of the extracellular matrix, which contains heparan sulfate proteoglycans, collagen, fibronectin, elastin, and other proteoglycans (Lemson et al. 2000). When damage occurs, tissue-type plasminogen and kinase-type plasminogen activators degrade the extracellular matrix and activate matrix metalloproteases. As the cells continue to proliferate, platelet-derived growth factor (PDGF) aides in their migration to the intima (Lemson et al. 2000).

Intimal hyperplasia leads to atherosclerosis; atherosclerosis is caused by plaque buildup in the arteries, leading to thickening of the blood vessels; it then leads to loss of blood and oxygen flow. The overall process consists of the formation of fatty streaks, formation of atheroma, and then formation of the atherosclerotic plaques (Rafieian-Kopaei et al. 2014). This often results from chronic endothelial injuries that occur through hypertension, smoking, an inactive lifestyle, a high cholesterol diet, and obesity (Rafieian-Kopaei et al. 2014). When fatty streaks form, cholesterol-rich low density lipoproteins (LDL) are trapped; this therefore increases the amount of LDL in the intima, which then leads to spontaneous oxidation (Rafieian-Kopaei et al. 2014). These oxidized particles activate monocytes and T lymphocytes, both of which flow into the vascular
intima through the arterial walls. When VSMC migrate into the lesion site, cytokines and growth factors are released; this leads to the formation of the atherosclerosis plaque, reducing blood flow (Rafieian-Kopaei et al. 2014). Atherosclerosis leads to conditions such as coronary heart disease, chronic kidney disease, and carotid artery disease.

Although millions of patients receive treatment every day for narrowed or occluded arteries, it is essential to find more effective methods. Currently, balloon angioplasties and stent implantations are used; however, these may lead to in-stent restenosis (Kim et al. 2018). To counteract these effects, stents are covered in drugs, such as rapamycin, that inhibit VSMC proliferation. The issue with these nonspecific drugs is that they also suppress re-endothelialization, which may lead to thrombosis (Kim et al. 2018). Studying the proliferation and migration of VSMCs is therefore of significance since these functions lead to intimal hyperplasia in vivo.

**VSMC Role in Atherosclerosis: Differentiation into Quiescent and Proliferative Phenotypes**

Vascular smooth muscle cells play a critical role in intimal hyperplasia. Their primary function is to contract, therefore controlling vessel diameter, and regulating blood flow (Gomez and Owens 2012). The artery wall is composed of the adventitia, which is the outermost layer of the wall, the tunica media, and the innermost tunica intima (Davies and Hagen 1994). The adventitia consists of fibroblasts in connective tissue, with collagen and elastin (Davies and Hagen 1994). The medial layer is composed of VSMC, which are densely grouped together in an interstitial matrix; this matrix
includes proteoglycans in addition to collagen and fibronectin (Newby and Zaltsman 2000). The basement membrane separates the media and tunica intima. It consists of collagen, laminin, and heparan sulfate proteoglycans such as perlecan (Newby and Zaltsman 2000). Finally, the tunica intima is composed of a thin layer of endothelial cells, which are on the basement membrane.

VSMC are an important target in developing treatments for atherosclerosis and intimal hyperplasia. They have been shown to be extremely flexible with their ability to quickly respond to injury and inflammation. This response, when smooth muscle cells “switch” from a quiescent to proliferative phenotype, is aptly called phenotype switching. In the quiescent (contractile) phenotype, the cells are spindle-shaped and elongated. They are non-proliferative and non-migratory; however, under inflammatory conditions, quiescent cells can shift into a proliferative phenotype. Quiescent cells also produce less h-caldesmon, collagen and meta-vinculin than proliferating VSMC (Rensen, Doevendans and Van Eys 2007). Cells in the proliferative (synthetic) phenotype are shorter and more rounded. (Rensen, Doevendans and Van Eys 2007). In addition, they produce four to five times more extracellular matrix proteins than quiescent cells, and their proliferation rates are increased by at least 10% (Lemson et al. 2000). In intimal hyperplasia, as discussed before, there is an increased number of cells in the proliferative state compared to the quiescent; with that, there are higher levels of matrix deposition, and more cells found in the intima (Lemson et al. 2000). It is important to note that cells
may contain features of both phenotypes; instead of being set in one phenotype, rather, there is a gradient in phenotype expression.

**Figure 1. Phenotypic switching of VSMCs from proliferative to quiescent states.**

Proliferative cells can be seen to the left of the arrow, with quiescent cells on the right.

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**IL-2 and its Role in Atherosclerosis**

Interleukin-2 (IL-2) is most known to regulate T cell homeostasis; however, our laboratory has previously shown that IL-2 may also play a role in VSMC function (Arumugam et al. 2019). In previous studies, Miller et al. found that IL-2 is retained in the arterial extracellular matrix through heparan sulfate proteoglycans. The authors determined that IL-2 might be of importance to VSMC survival, as IL-2 KO mice lost smooth muscle cells over time (Miller et al. 2012). Recently, our laboratory showed that IL-2 increases expression of the survival-related protein FoxO3a in VSMC. FoxO3a is a forkhead transcription factor regulated by the serine/threonine kinase Akt, which is
responsible for phosphorylation of targets that contribute to cell proliferation, survival, and differentiation (Allard et al. 2008). When akt is silent, VSMCs are quiescent, and FoxO aids in cell survival. We showed that while VSMC growth factors such as PDGF promote akt activation and proliferation, IL-2 shifts the balance towards FoxO and survival (Arumugam et al. 2019). In addition, preliminary data showed that Bcl-xL expression, an anti-apoptotic protein found in intimal VSMCs (Perlman et al. 1997), was found to increase with addition of IL-2.

The IL-2 receptor is a heterotrimeric protein with α (CD25), β (CD122), and γ (CD132) subunits. Arumugam, et al found that VSMC express all three subunits of the IL-2 receptor (IL-2R). In addition, they found that depending on the phenotype, IL-2Rα varied in expression and localization in the cells. This was a novel finding. Proliferating VSMC expressed lower amounts of IL-2Rα in comparison to the quiescent phenotype (Arumugam et al. 2019). IL-2Rα was also observed in the filopodia of proliferative cells, while in quiescent cells, it was more concentrated around the cell body (Arumugam et al. 2019). Little is known about the localization of the IL-2Rα receptor. Data to be presented in this thesis suggest that nuclear localization of IL-2Rα promotes quiescence in VSMC.

Previous studies from our laboratory suggested that IL-2 may play a role in VSMC proliferation and migration. Our laboratory has found that IL-2 binds to heparan sulfate and is present in the extracellular matrix surrounding VSMCs (Wrenshall and Platt 1999). In addition, it associates with endothelial and smooth muscle cells within the arterial wall. Interestingly, IL-2 KO mice were found to lose smooth muscle cells over
time (Miller et al. 2012). More recent data, presented in this thesis, suggests that the IL-2Rα localizes to the nucleus in quiescent, but not proliferating, VSMC. Understanding the role of IL-2Rα in VSMC proliferation and migration, and how its localization impacts these functions, may lead to new approaches for the treatment of intimal hyperplasia.
CHAPTER 2

IL2-Rα Localization to the Nucleus is Dependent on VSMC Phenotype

I. Introduction

Vascular smooth muscle cells are involved in the development of plaques. Intimal hyperplasia, which is an accumulation of smooth muscle cells in the tunica intima, typically occurs in the blood vessels prior to the development of atherosclerosis. When injury or inflammation occurs, VSMC proliferate and migrate from the media to the intima of the arterial wall, causing a narrowing of the vessel lumen. There is a relationship between VSMC and atherosclerosis – as discussed previously, VSMC are capable of phenotype switching in response to injury and inflammation.

Previous studies have shown that IL-2 promotes proliferation and migration of VSMC, and as previously discussed, that VSMC expresses all three subunits of IL-2Rα. Our laboratory found that there was lower expression of IL-2Rα in proliferating VSMC in comparison to quiescent VSMC. When we looked closer at the cells that expressed each phenotype, immunofluorescent staining suggested that quiescent VSMC expressed IL-2Rα in their nuclei. However, the antibody we used was weak, as it needed tyramide signal amplification (TSA) for adequate detection. Based on this initial observation, we hypothesized that IL-2Rα localizes to the nucleus in quiescent VSMC, and in this location may impact VSMC proliferation.
II. Results

Due to the weak antibody used, and TSA needed to visualize the staining, we first used four different antibodies (rabbit anti-human polyclonal anti-IL-2Rα antibodies from Bioss Antibodies, BosterBio, MyBioSource, LSBio) to determine whether IL-2Rα was present in the nucleus of vascular smooth muscle cells. As shown in Figure 2, we stained VSMC with 4 distinct anti-IL-2Rα antibodies, specific to various epitopes on the receptor (see Table 1). The nucleus was demarcated using DAPI. When the images merged, the receptor could be seen in the nucleus of VSMC.

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</tr>
<tr>
<td>LS Bio</td>
<td>C-terminus, amino acids 223-272</td>
</tr>
<tr>
<td>My BioSource</td>
<td>IL-2Rα</td>
</tr>
<tr>
<td>Bioss</td>
<td>C-terminus, amino acids 258 - 268</td>
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Table 1. Epitopes of anti-IL-2Rα antibodies from commercially available sources.

To further prove that IL-2Rα was present in the nuclei of quiescent cells, we isolated membrane, cytoplasmic, and nuclear fractions of quiescent VSMC and probed for IL-2Rα using Western blot analysis. Figure 3 shows that the majority of IL-2Rα was localized to the nucleus of VSMC.

In order to show that the antibody tested was specifically recognizing IL-2Rα, we preabsorbed IL-2Rα with the immunizing peptide used to generate the antibody. Presorption of anti-IL-2Rα with the immunizing peptide abrogated staining. In
addition, we decreased IL-2Rα expression in VSMC using siRNA. As seen in Figure 4, knock down of IL-2Rα message with siRNA greatly decreased detection of nuclear IL-2Rα.

VSMC are phenotypically different if grown in SMCM with 2% FBS (proliferative media; see Materials and Methods) compared to serum free DMEM supplemented with insulin, selenium, and transferrin (ITS) (quiescent media) (Kisiday et al. 2005). To determine whether localization of IL-2Ra differed in proliferating vs quiescent VSMC, VSMC were grown in each of the aforementioned media and localization of IL-2Ra was assessed using indirect immunofluorescence. Figure 5a shows that when compared to the cells in SMCM, the cells grown in ITS displayed more IL-2Rα in the nucleus. Figure 5b shows a histogram of 9,000 – 11,000 VSMC cultured under proliferating or quiescent conditions and stained with anti-IL-2Rα. As shown by the black line, the cells grown in ITS had a higher number of nuclei that expressed IL-2Rα. From this, it is suggested that there is more found in the nucleus of cells that are phenotypically quiescent.

The nuclear localization of IL-2Rα in quiescent VSMC led us to ask whether the receptor was indeed absent from proliferating cells or, alternatively, was not recognized by the anti-IL-2Rα antibody due to post-translational modifications, particularly glycosylation, that might occur with proliferation. IL-2Rα is known to be heavily glycosylated, with several N and O glycosylation sites (Greene and Leonard 1986). As a
**Figure 2.** IL-2Ra is expressed in the nuclei of VSMC. VSMC were isolated from human aortas and grown in serum free media; afterwards, they were fixed and stained with 4 distinct anti-IL-2Ra primary antibodies as shown. They were then probed with the appropriate secondary antibodies and imaged using an epifluorescent microscope. The above data are representative of 3 separate experiments.
Figure 3. IL-2Rα is localized to the nucleus of quiescent VSMC. VSMC were cultured in serum free media to induce quiescence. Cells were lysed and fractionated using a commercially available kit (Cell Signaling Technology, MA). Lysates were subjected to Western blot analysis and probed with anti-IL-2Rα antibodies. The purity of the nuclear fraction was assessed using fibrillarin, which localizes to the nucleolus. This experiment is representative of 3 separate experiments.

The epitopes recognized by the antibodies from Boster versus Bioss are quite distinct (see Table 1). The antibody from Bioss recognizes an epitope at the C terminus, whereas the antibody from Boster recognizes an epitope within the N terminus. When probed with anti-IL-2Rα from Bioss, cytoplasmic and membrane localization was detected in...
Figure 4. The IL-2Rα antibody (BosterBio) is specific to detection of the receptor.

(top) VSMC were treated with 25 nM siRNA or vehicle only for 48h, and then later stained with the anti-IL-2Rα antibodies from Boster and the appropriate secondary.

(bottom) Human VSMC were cultured in serum free media supplemented with ITS for 6 days. They were fixed and stained with anti-IL-2Rα (after being pre-absorbed with immunizing peptide), and then probed with an HRP conjugated secondary antibody, finishing with tyramide signal amplification. Nuclei were identified with DAPI. Scale bar = 200 microns. These data are representative of 4 separate experiments.
proliferating VSMC (Figure 6a). However, expression in the nucleus remained low with both antibodies.

Because cytoplasmic vs membrane localization is difficult to distinguish in permeabilized cells, we probed fractionated VSMC lysates with the anti-IL-2Rα from Bioss. Based on this fractionation, the majority of IL-2Rα detected by the antibody from Bioss was in the membrane (Figure 6b). Additional experiments, outside the scope of this thesis, showed that the antibody from BosterBio does not recognize the membrane form of IL-2Rα due to differential glycosylation.

Because we observed nuclear localization of IL-2Rα when VSMC are in the quiescent phenotype, we wanted to see if IL-2Rα would localize to the nucleus if cells were placed under other conditions, such as cellular stress, in which cells do not proliferate. We used tunicamycin to induce stress to the endoplasmic reticulum (ER), bleomycin to induce genotoxic stress, and hydrogen peroxide to cause oxidative stress in VSMC. As seen in Figure 7, conditions of cellular stress increased nuclear localization of IL-2Rα.

The following data is based on VSMC in vitro. To determine whether IL-2Rα localized to the nucleus in situ, we examined normal and diseased human arteries for expression of IL-2Rα by Western blot. We acknowledge that not all cells in the arterial wall are VSMC, but they will comprise the majority. As seen in Figure 6B, the anti-IL-2Rα antibody from Bioss recognized a 50kD form of IL-2Rα in the membrane fraction and a 45kD form in the nuclear fraction. The anti-IL-2Rα from Boster strongly
Figure 5. Nuclear localization of IL-2Rα is increased in quiescent vs proliferating VSMC. (A) VSMC were grown in SMCM and ITS for 3 days, fixed, and stained with IL-2Rα (BosterBio), followed by the appropriate Cy5 conjugated secondary antibody. (B) The histogram represents the summation of 9000 (ITS) and 11,000 (SMCM) VSMC. Cells were imaged using a Cytation (Biotek) imaging plate reader and data was processed using FCS Express 7 flow cytometry software. These data are representative of 3 separate experiments.
Figure 6. Differential detection of IL-2Rα using antibodies recognizing distinct epitopes. (A) VSMC were cultured in SMCM for 72 hours, fixed with methanol, and stained with anti-IL-2Rα from BosterBio and Bioss. (B) The Western blot displays the presence of IL-2Rα in the membrane, detected using the anti-IL-2Rα antibody from Bioss. Smooth muscle cell lysates were utilized. Blot was imaged using the ChemiDoc and Image Lab. Control Western blot uses Na-K-ATPase antibody from Proteintech. These data are representative of over 5 experiments.
Figure 7. IL-2Rα localizes to VSMC nuclei under conditions of stress. Cells were grown in DMEM for 72 hours and treated with tunicamycin for endoplasmic reticulum (ER) stress, bleomycin for genotoxic stress, and hydrogen peroxide for oxidative stress. IL-2Ra was detected as previously described. These data are representative of 4 separate experiments.

recognized a 45kD form in the nucleus but did not recognize the 50kD form in the membrane fraction (Figure 3). Based on these data, we used the latter size differences to identify membrane vs nuclear IL-2Rα in lysates of diseased and normal human arteries. In comparing tissue from an abdominal aortic aneurysm (AAA) to a normal artery, the membrane form predominated in the aneurysmal tissue and the nuclear form predominated in the normal tissue. In a second specimen from a popliteal aneurysm, the
membrane form predominated at the site of the aneurysm. However, more “normal” tissue distal to the aneurysm weakly expressed the nuclear form. In the adjacent popliteal vein, where inflammation would be markedly decreased, the nuclear form predominated. We chose aneurysmal tissues given their high level of inflammation, however atherosclerotic tissue without aneurysmal changes revealed similar findings (not shown).

In summary, these data suggest that quiescent VSMC, cultured without serum in vitro or present in non-inflamed arteries in vivo, express IL-2Rα mainly in the nucleus. In contrast, VSMC exposed to proliferative or inflammatory stimuli in vitro or in vivo express IL-2Rα mainly in the cell membrane.

![Figure 8](image.jpg)

**Figure 8.** The “nuclear” form of IL-2Rα predominate in normal arteries and the membrane form predominates in inflamed arteries. Tissue lysates were prepared from diseased or normal human arteries and probed with anti-IL-2Rα antibodies as previously described. These data are representative of over 5 experiments.
Based on our findings that IL-2Rα localizes to the nucleus of quiescent cells, we next asked, using VSMC isolated from IL-2Ra KO mice, how the absence of IL-2Rα might impact proliferation and migration of VSMC. Proliferation and migration are characteristics of synthetic/proliferative VSMC, and these functions are implicated in the development of intimal hyperplasia. We first measured proliferation of cells using a click chemistry-based proliferation assay, in which dividing cells incorporate the non-radioactive nucleoside analog 5-ethynyl-2’-deoxyuridine (EdU) (Wong et al. 2019). Click chemistry is termed from when alkyne and azide groups “click” together when exposed to catalytic copper (Hein et al. 2008). The alkyne that attaches to the nucleoside analog is detected by the azide, which is bound to a fluorescent marker (Hein et al. 2008).

KO VSMC displayed increased baseline and stimulated proliferation when compared with WT VSMC. These data suggest that nuclear localization of IL-2Rα promotes quiescence; if there is no IL-2Rα to localize into the nucleus, however, then the cells will assume a proliferative phenotype. The data in Figure 9 may suggest that IL-2Rα could play a role in inhibiting VSMC proliferation.

We then tested the impact of IL-2Rα on migration by testing migration of WT and IL-2Rα KO VSMC. To measure migration, we used a Boyden chamber assay in which VSMC are cultured on a filter and stimulated to migrate through it using a chemotactic agent in the media. Figure 10 shows that, in comparison to the WT cells, the cells without the receptor present had higher numbers of migrating cells both at baseline and when
Figure 9. IL-2Rα deficient VSMC exhibit increased proliferation when stimulated with FBS. were incubated for 72h with increasing concentrations of FBS. EdU was added for the last 24h and its incorporation was detected using click chemistry. Total cells were counted using the nuclear stain DAPI. Cells were counted using an imaging plate reader. These data are representative of over 5 experiments.

stimulated by PDGF. This result suggests that the receptor plays a role in regulating cell proliferation and migration.

We next wanted to see if we could alter nuclear localization of IL-2Rα using a peptide. We chose to use a peptide because it is specific to the receptor and should have
minimal off target effects. To produce a peptide mimetic that might alter nuclear localization of IL-2Ra, we first needed to determine which sequences might be causing nuclear localization of the receptor. To do so, we used *in silico* analyses to find DNA binding and nuclear localization sequences. The results showed that 12 amino acids in the C terminus were predicted to bind DNA. A second analysis showed that 6 of these amino acids were predicted to be a nuclear localizing sequence. From these data, we created a peptide that consisted of the DNA binding sequence (C12AA); we hoped that

![Figure 10](image.png)

**Figure 10.** IL-2Ra deficient VSMC exhibit increased migration in response to PDGF. WT and KO-G VSMC were grown in DMEM and plated. 18-24 hours after being plated, 50 ng/ml PDGF was added to the bottom chamber to stimulate migration. VSMC nuclei were stained with Sytox, imaged using an epifluorescence microscope, and then quantified using ImageJ. These data are representative of over 5 experiments.
we hoped that this peptide would compete with the receptor in binding to the DNA or nuclear localization sites. Because of this, we hypothesized that the peptide mimetic would lower nuclear localization of IL-2Rα.

**Figure 11. VSMC treated with a peptide mimetic of the C terminus of IL-2Rα express IL-2Rα in the nucleus.** (top) VSMC were treated with 100 uM C12AA for up to 72h as noted above. IL-2Rα was detected using anti-IL-2Rα antibodies from Boster. (bottom) VSMC were treated with TAMRA labeled C12AA (red) and imaged using confocal microscopy. Nuclei were detected using Sytox (green). These data are representative of over 5 experiments.
Figure 11 shows the results of our treating VSMC with increasing durations of C12AA. The upper panel of Figure 11 shows that the peptide mimetic increases (rather than decreases) nuclear localization of IL-2Rα. The bottom panel demonstrates that a fluorophore-labeled peptide enters the cell and appears to enter the nucleus. Although we do not yet understand why C12AA increases IL-2Rα localization to the nucleus, we continued to use this peptide to see how increased nuclear localization impacts VSMC function and phenotype. Once we found that C12AA increased nuclear localization of IL-

![Graph 1](image1.png)

**Figure 12.** Treatment of VSMC with C12AA, which increases nuclear localization of IL-2Rα, inhibits migration and proliferation of VSMC. Cells were grown in DMEM and treated with peptide for 72h (proliferation) or 18h (migration). Proliferation and migration were assessed as previously described. This experiment is representative of over 5 experiments.
2Rα, we asked how this peptide affected VSMC migration and proliferation. As shown in Figure 12, the number of unstimulated cells remained relatively stable. The number of proliferating and migrating cells, however, decreased with increasing concentrations of peptide used. This suggests that increased nuclear localization of IL-2Rα inhibits VSMC proliferation and migration.

III. Summary and Discussion

In this study, we have shown that IL-2Rα localizes to the nucleus in quiescent VSMC. We also demonstrated that IL-2Rα is localized to the nucleus when VSMC are exposed to various types of cellular stress. We have generated evidence from westerns, immunofluorescent staining, and exposing the VSMC to stressful conditions in order to support our claim. We have shown that IL-2Rα could play a part in VSMC proliferation and migration.

There are several theories as to how the receptor could be acting in the nucleus, to play a role in VSMC proliferation and migration. The receptor could possibly be a chromatin remodeling factor or a transcription factor. The mechanism of how nuclear IL-2Rα inhibits proliferation will be the subject of future studies.

How localization of IL-2Rα is controlled is also an important area of investigation. Our laboratory has preliminary data (not shown) suggesting that IL-2Rα could be moving in and out of the nucleus by way of phosphorylation. We have seen that phosphorylation increases nuclear localization; this could be similar to FOXO3A, which moves in and out of the nucleus based on phosphorylation.
FOXO3A interacts with several different kinases, which causes it to translocate to the cytoplasm. On the other hand, interaction with other kinases cause it to undergo phosphorylation at specific residues, which induce nuclear localization and transcriptional activity (Wang et al. 2017). Acetylation also occurs, which aids in transcriptional activity and expression of stress resistance genes. It has been found that phosphorylation and acetylation happen at the same time and could possibly have an effect on each other (Wang et al. 2017). This information is relevant because FOXO3A and other proteins that shuttle between the nucleus and cytoplasm serve as models for mechanisms that may alter localization of IL-2Rα.

Data in this thesis shows that the anti-IL-2Rα antibody from Boster is present in the nucleus of quiescent VSMC, while the antibody from Bioss is detectable in the membrane of proliferating VSMC. There are several possibilities as to why the anti-IL-2Rα antibody from Boster does not detect membrane IL-2Rα; however, our laboratory has found, in preliminary studies, that glycosylation could be playing a role. It is possible that the membrane form of IL-2Rα is more heavily glycosylated and that this glycosylation could be blocking the N terminal epitope recognized by the anti-IL2Rα from Boster. In turn, the nuclear form of the receptor may be less glycosylated. This hypothesis is supported by our finding that the membrane form of IL-2Rα is larger than the nuclear form (50 vs 45 kD). Glycosylation of the membrane form of IL-2Rα may serve as a means to promote membrane localization and block nuclear localization.
IL-2R is a tripartite receptor; however, our studies are focused on the alpha subunit. When the alpha subunit binds to the beta and gamma subunits, affinity for IL-2 increases. However, the findings presented here are independent of IL-2. Although preliminary data suggests that IL-2 increases nuclear localization of IL-2Rα, many other stimuli also increase nuclear localization (Figure 7). These findings are similar to a study conducted by Deho et al., who found that the alpha subunit controls mast cell production independent of IL-2.

If we can determine the mechanism by which IL-2Rα localizes to the nucleus and inhibits proliferation, then perhaps therapies can be generated that prevent plaques from forming as quickly. If cells can be manipulated to remain in the quiescent phenotype, proliferation and migration would decrease, therefore slowing the formation of intimal hyperplasia.

IV. Materials and Methods

Immunofluorescence

VSMC cells were seeded in 8-well chamber slides, then cultured in 10% DMEM (proliferative media) or ITS-supplemented DMEM (quiescent media) for 72h at 37°C. The cells were fixed in ice-cold methanol and stained. Slides were stained with a primary antibody and then an appropriate secondary antibody. All antibodies were validated by Western blot. VSMCs were imaged using the EVOS FL epifluorescence microscope (Thermo Fisher Scientific).
**Protein extraction**

Cells were grown to approximately 70-80% confluence in SMCM or ITS media. The media was aspirated, and the cells were washed with PBS. Once PBS was removed, RIPA lysis buffer + protease inhibitors were added to each plate, and a cell scraper was used to scrape the cells into a conical tube. The lysates were incubated on ice. They were then sonicated, incubated on ice again, and centrifuged at 4°C. The supernatant, containing soluble protein, were placed in separate tubes and stored at -80°C. Lysates were measured for protein concentration by BCA prior to use.

**Western blotting**

Protein amounts of cell samples were determined by BCA to ensure equal amounts per lane, prior to being treated by SDS-PAGE. After being run on a gel, proteins were transferred to a PVDF membrane and incubated with 5% nonfat milk in TBST [0.5% Tween 20, 10 mM Tris (pH 8.0), 150 mM NaCl] for one hour, rocking at room temperature. The membrane was then rocked overnight at 4°C in blocking buffer with primary antibodies (Boster, 1:1000) against IL-2Rα. It was washed three times for ten minutes each using TBST, and then incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit) at dilution 1:10,000 for one hour, rocking at room temperature. The membrane was washed three more times for ten minutes each, and then developed for five minutes, rocking with Luminata Crescendo Western HRP substrate (Millipore Sigma).
VSMC proliferation

Proliferation was measured by incorporation of EdU, which was then assessed using click chemistry. Once cells were cultured at 37°C in SMCM or ITS media, they were transferred to a 96 well plate and stimulated to proliferate for 72h with either PDGF or FBS. VSMC were treated with EdU for the last 24 hours of the incubation period. The cells were fixed in paraformaldehyde and EdU was detected using the following labeling solution (THPTA, CuSO₄, Cy3 picolyl azide, Na ascorbate). Incorporation of EdU were measured using a fluorescence plate reader (Cytation, Biotek).

VSMC migration

Migration was assessed using the Boyden chamber assay (Arumugam et al. 2019). VSMCs were plated in the Boyden chambers, which are filter inserts placed in 24-well plates. VSMC were plated into the upper well formed by the insert. VSMC were allowed to settle for 12-18h and stimulated to migrate through the filter by adding a chemotaxis agent (PDGF) to the lower chamber. VSMC were allowed to migrate for 6 hours, and then the cells on the filter inserts were fixed with methanol at -20°C. The cells on the top side of the filter were removed using a cotton swab, while cells migrating through to the underside of the filter were washed with PBS, and then stained with Sytox. The cells were imaged using the EVOS FL epifluorescence microscope (Thermo Fisher Scientific), and then counted using Image J (Arumugam et al. 2019).
CHAPTER 3

Incomplete knock out of IL-2Rα knock out mice

I. Introduction

VSMC are found to play a role in atherosclerosis; as discussed in Chapters 1 and 2, IL-2R may also contribute to VSMC phenotype switching, which in turn could be a point of treatment against plaque development. The source of VSMC in these studies stems primarily from mice, in addition to human tissue donations. Mice are important model organisms due to their genetic similarity to humans, in addition to their anatomical and physiological likeness (Bryda 2013). Like humans, mice have about 30,000 genes, 95% of which are shared between both species. It is advantageous to use mice, due to their quick reproduction and high numbers of offspring (Bryda 2013).

Data from Chapter 1 indicated that IL-2Rα is expressed in the nucleus of VSMC. To help define the function of nuclear IL-2Rα, we isolated VSMC from IL-2Rα WT and KO mice. Surprisingly, we found that IL-2Rα protein was expressed in KO VSMC. Our findings outlined below show that commercially available IL-2Rα KO mice are not complete knock outs, raising a cautionary note for investigators using these mice.
II. Results

We acquired IL-2Rα KO mice to determine how IL-2Rα affects the function of VSMCs. WT and KO mice were identified by genotyping using the protocol provided by Jax mice. As a first step following isolation and culture of VSMC from each of these mice, we examined expression of IL-2Rα protein. Figure 13 displays the use of 4 commercially available anti-IL-2Rα antibodies from Bioss Antibodies, BosterBio, and LSBio to assess IL-2Rα protein in WT and KO VSMC. As shown in Figure 13, IL-2Rα was detected in both WT and KO VSMC using each antibody.

To further support that there was receptor in the KO cells, we assessed IL-2Rα protein expression by Western blot analysis. As shown in Figure 14, expression of IL-2Rα protein in WT and KO cells is comparable. This supports the fact that the KO VSMC are not fully knocked-out, with presence of the receptor in both cell immunofluorescence staining and western blotting. It is also of note that all the bands are the same size; this is important because IL-2Rα has multiple splice variants. By comparing the WT and KO VSMC to human VSMC and T cells, we found that the KO cells express full-length IL-2Rα.

To confirm that the anti-IL-2Rα antibody used was specific for its epitope and not exhibiting any non-specific binding, we pre-absorbed the antibody with immunizing peptide prior to staining. This preabsorption abrogated staining, demonstrating that detection of protein by this anti-IL-2Rα antibody is specific to the IL-2Rα epitope (Figure 15).
Figure 13. IL-2Rα is detected in WT and KO VSMC with use of antibodies. VSMC were isolated from WT and KO mice, cultured, and then stained with three different anti-IL-2Rα antibodies as noted. These data are representative of 3 separate experiments.
Figure 14. Full length IL-2Rα is being expressed in KO VSMC isolated from mice.

Lysates were prepared from Jurkat T cells, WT VSMC, and IL-2Rα KO VSMC. Isolated proteins were separated by SDS-PAGE and IL-2Rα was detected by Western blot analysis. All bands fall between 37 and 50 kD, indicating protein being detected is full-length IL-2Rα. This experiment is representative of over 5 separate experiments.

Given our results suggesting that VSMC isolated from IL-2Rα KO mice are producing IL-2Rα protein, we asked whether we could detect the WT IL2RA gene in genomic DNA isolated from KO VSMC. To increase the sensitivity of detection, we used WT and KO primers both individually and together. While both primers used simultaneously did not detect a WT product, use of the WT primer alone detected a band of predicted WT size consistent with our WT controls (Figure 16). Use of the KO primer
Figure 15. Preabsorption of anti-IL-2Rα with immunizing peptide abrogates detection of nuclear IL-2Rα in VSMC. WT VSMC were stained with anti-IL-2Rα with/without pre-absorption of the antibody with the immunizing peptide. These data are representative of over 5 experiments.

alone detected the predicted IL2RA mutant gene isolated from KO but not WT VSMC. Sequencing of the WT band from IL-2Rα KO VSMC confirmed the presence of IL-2Rα. These results demonstrate that VSMC isolated from KO mice express the WT IL-2Rα gene.

IL-2Rα KO mice were generated by replacing exons 2 and 3 of IL-2Rα with a neomycin resistance gene insert as a selectable marker (Willerford, et al, 1995). Given this information, we asked whether treatment of KO VSMC with G418 (a neomycin analog) would decrease or eliminate IL-2Rα protein expression. We predicted that KO cells would be resistant to G418 (assuming the Neo gene is transcribed) and WT cells would be sensitive and therefore eliminated. To this end, we cultured WT and KO VSMC
Figure 16. **IL-2Rα KO VSMC express WT IL-2Rα DNA.** DNA was isolated from murine VSMC and tails. DNA was amplified using IL-2Rα primers per Jax protocol. The WT primers amplify a 146 bp product, and the mutant a 280 bp product. Primer sets were added together, or singly, as indicated. PCR was performed using these primers and amplified products were separated electrophoretically in a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. PCR products were visualized using UV light and the image was captured using ChemiDoc MP imaging. These data are representative of over 5 experiments.
Figure 17. G418-treated KO VSMC display less IL-2Rα than WT VSMC. WT and KO VSMC were grown in DMEM for 96 hours, with 2 mg/ml G418. Cells were stained with IL-2Rα primary (BosterBio) and the appropriate secondary before imaging. These data are representative of over 5 experiments.
with increasing concentrations of G418. G418 nearly eliminated WT cells at a concentration of 2 mg/ml (Figure 17). However, 85% of VSMC isolated from KO mice survived G418 treatment. IL-2Rα production by these treated cells was significantly diminished compared to WT VSMC as shown by immunofluorescence (Figure 17).

These data demonstrate that the Neo resistance gene is expressed in IL-2Rα KO VSMC, and that treatment of KO VSMC with G418 killed susceptible WT cells and significantly decreased IL-2Rα protein production.

While it is shown in Figure 17 that treatment of IL-2Rα KO VSMC with G418 nearly eliminated IL-2Rα producing cells, we wanted to confirm our results using a different approach, specifically Western blot analysis. Figure 18 shows that lysates of VSMC treated with G418 have less IL-2Rα in comparison to cells that have not been G418-treated.

We found that VSMC isolated from KO mice expressed the wildtype IL2RA gene, but the level of IL2RA gene seemed qualitatively low compared to the amount of protein expressed. This incongruity prompted us to ask if KO VSMC were picking up receptor from an exogenous source. Several studies have shown the presence of soluble CD25 in conditions such as non-Hodgkin’s lymphoma, hepatocellular carcinoma, multiple sclerosis, and coronary artery disease (Jo et al. 2010, Cabrera et al. 2010). Given these reports, we wanted to see if FBS possibly contained IL-2Rα. We added FBS to serum free media in concentrations of 0, 0.5, and 2% to see if KO VSMC cultured in FBS
express more IL-2Ra protein. As shown in Figure 19, IL-2Ra KO VSMC cultured in FBS express increased IL-2Ra when compared to those in serum free media.

**Figure 18.** G418 decreases IL-2Ra protein expression in VSMC isolated from IL-2Ra KO mice. VSMC, isolated from IL-2Ra WT and KO mice, were cultured under serum free conditions in the presence/absence of G418 at 2 mg/ml. VSMC were subsequently lysed and extracted proteins were separated by SDS-PAGE and analyzed by Western blot for expression of IL-2Ra. Histone H3 was used to normalize the amount of protein run for both samples. Relative intensity of the bands was generated using a BioRad imager, by calculating the intensity of the histone H3 bands, and normalizing the IL-2Ra to the baselines. This experiment is representative of 4 experiments.
Figure 19. WT and KO VSMC display higher proliferation rates and expression of IL-2Rα with higher concentrations of FBS. Cells were grown in DMEM for 3 days and treated with varying concentrations of FBS. They were then imaged after being fixed and stained with the according primary and secondary antibodies (BosterBio). These data are representative of over 5 experiments.
Based on the number of cells per field, the latter data suggested that KO cells proliferated more than WT when exposed to FBS, consistent with our prior observations (Figure 9). In addition, these cells were noticeably smaller in morphology (Figures 19, 20). KO VSMC were not only much smaller than WT cells, but they also took up less DAPI (Figure 20). This observation is consistent with the finding that DNA content is directly proportional to cell size (Amodeo & Skothiem 2016). In comparing nuclear area and DNA content of WT and KO VSMC, however, we noted that the decrease in DNA content was out of proportion to the decrease in nuclear area. KO nuclei were approximately half the size of WT, but only had 1/6 the amount of DNA. This finding suggests that KO VSMC are hypodiploid, which implies a severe dysregulation of cell proliferation and/or division. Hypodiploid cells are mainly associated with an aggressive form of acute lymphoblastic leukemia (Comeau & Mullighan 2017).

Because our data suggested that VSMC were taking up IL-2Rα from serum, we assessed the presence of IL-2Rα in FBS by Western blot. For comparison, we included purified IL-2Rα from a commercially available source that is comprised of amino acids 1-213. Soluble IL-2Rα, released from the cell surface by enzymatic cleavage, is cleaved between amino acids 182 – 192 and is therefore smaller than the commercially available protein. IL-2Rα was detected in FBS, however its molecular weight was approximately 45kD, consistent with full length IL-2Rα from VSMC lysates (Figure 21). Commercially
Figure 20. IL-2Rα deficient VSMC are smaller and contain less DNA compared to WT cells. (top) Phase and IL-2Rα overlay images from WT and KO VSMC, treated and probed for IL-2Rα as in Figure 19. Scale bar = 100 μM. (bottom) Nuclei of VSMC from Figure 19 were imaged for area and DAPI intensity using a Cytation imaging plate reader (Biotek). Data accrued from these images were then processed using flow cytometry software. These data are representative of over 5 experiments.
Figure 21. IL-2Rα is found in FBS. Purified IL-2Rα protein and FBS were separated by SDS-PAGE and analyzed by Western blot for expression of IL-2Rα. The purified protein was produced in HEK-293 cells and is truncated at amino acid 213. These data are representative of 4 experiments.

available IL-2Rα was approximately 37 kD. These results suggest that IL-2Rα in serum is full length IL-2Rα and not the soluble, truncated form.

III. Summary and Discussion

In this study, we have provided evidence from immunofluorescence staining, western blotting, and PCR showing that commercially available IL-2Rα KO mice are not fully knocked out. Although other studies have used these mice to study the IL-2/IL-2R system, our results do not necessarily negate those findings. In fact, our data suggest that the phenotype of a complete IL-2Rα KO mouse would be more severe than that of mice currently used. However, for future studies, researchers should be careful if they are considering using these mice for experiments.
Although our data shows that the IL-2Rα mice are incompletely knocked out, the mechanism behind the incomplete KO is not known. Knockout mice are usually generated by replacing a portion of the coding region of the gene by a drug resistance marker. For correct insertion of the drug resistance marker and deletion of the targeted coding region, homologous recombination must occur at both the 5’ and 3’ ends of the DNA to be inserted. If homologous recombination only occurs on the 5’end, then the segment of DNA is inserted but the coding region is not deleted (LePage, et al, 2006). In addition, the neomycin resistance coding sequence and its PGK1 promoter can have unintended consequences on the targeted gene and on adjacent genes if they are left in the gene. Given these issues, some recommend using a Neo insert that is flanked via cre-specific recombinase, so that the insert may be deleted (LePage, et al, 2006). However, this technology had not yet been invented at the time the IL-2Rα KO mice were generated.

The ability to clear IL-2Rα protein with G418 revealed that VSMC take up IL-2Rα from serum. Given the low level of IL-2Rα detected in IL-2Rα KO cells cultured in FBS, however, the majority of IL-2Rα seen in KO cells appears to be coming from murine IL-2Rα production. The molecular weight of IL-2Rα in bovine serum indicates that the cells are taking up full length, rather than soluble, IL-2Rα. This is a novel finding. The presence of full-length IL-2Rα in FBS suggests that IL-2Rα may be coming from exosomes or microvesicles rather than enzymatic cleavage of a soluble, truncated form. Soluble IL-2Rα is generated when IL-2Rα is cleaved at the surface of the cell by a
membrane metalloproteinase (Durda et al. 2015). This data is interesting in that cells may be giving IL-2Rα (whether cleaved or uncleaved) to each other; cells could therefore influence the behavior of other cells.

In a previous study, it was found that human T cell leukemia cells spontaneously released high amounts of the receptor, which was detected in culture supernatants (Rubin et al. 1985). While the full-length receptor was found associated with cell lysates, a smaller, soluble version was also found. In addition, both T and B cell lines were found to produce soluble IL-2R in vitro. Rubin et al. hypothesized that the soluble IL-2R may play a role in regulating the inflammatory response, by binding soluble IL-2. We have not identified any studies, however, that demonstrate or postulate uptake of soluble IL-2Rα by other cells.

In summary, we have determined that commercially available knockout mice are not fully knocked out. We found that treating VSMC with G418 decreases the expression of IL-2Rα. We also found that there is IL-2Rα present in serum, suggesting that IL-2Rα production from one cell or cell type could be influencing other cells.

IV. Materials and Methods

The methods below only include those not already described in Chapter 2.

DNA extraction

Mice were obtained from Jackson Laboratories. Tail clips were enzymatically treated overnight in a 55°C water bath using tail extraction buffer [50 mM Tris-HCl (pH 7.4), 100 mM EDTA, 100 mM NaCl], 20% SDS, and 10 mg/ml Proteinase K (Alfa
Aesar, Lot#T08G006). DNA was extracted using 5M NaCl, ethanol, and TE buffer [1M Tris base pH 10-11 (Fisher BioReagents, Lot#198843), 0.5M EDTA (Sigma, Lot#SLBP6617V), H$_2$O]. It was then suspended in water and treated with 10 ug/ml RNase A (Sigma, Lot#027K1108) at 37°C for 30 minutes. The DNA was precipitated using 3M NaAC and ethanol. Purity values were obtained using the Synergy H1 Hybrid Reader (BioTek).

**PCR**

Mice were genotyped using the protocol provided by Jackson Laboratories. EconoTaq PLUS GREEN 2X Master Mix (Lucigen) and IL-2Rα WT (CD2KO-0461, CD2KO-0462) and KO (CD2KO-6916, CD2KO-6917) primers (Integrated DNA Technologies) were used for DNA amplification. The DNA was amplified using the 3Prime thermocycler (Techne) with the following touchdown protocol (developed using Korbie, Mattick 2008).

**DNA electrophoresis**

1.5% agarose (Fisher Scientific, Lot#124315) and 10% TBE buffer [Tris base (Fisher BioReagents, Lot#198843), Boric acid (Fisher Scientific, Lot#140681), EDTA pH 8.3 (Sigma, Lot#SLBP6617V)] were mixed in a flask and microwaved, stirred, and microwaved once more. Once settled to warm temperature, ethidium bromide (Fisher BioReagents, Lot#116199) was added, the solution swirled and then poured into the
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**Table 2. Touchdown amplification protocol.** The Touchdown amplification protocol begins with an annealing temperature above the optimal melting temperature of the primers, and progresses lower through cycles. The second part of the run consists of a standard amplification stage (Korbie, Mattick 2008).

casting tray to solidify. Once solidified, the gel buffer was poured over top of the gel, and used to wash out the wells. The DNA ladder and samples were loaded and run for 1 hour at 100 V. The gel was read using a ChemiDoc Imaging System (Bio-Rad).
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