January 2013

Statin Inhibition Of Macrophage Integrin-Induced Rac2-Myosin Iia Interaction: An Anti-Inflammatory Effect

Kenneth E. Ike
Yale School of Medicine, macbook3714@gmail.com

Follow this and additional works at: http://elischolar.libraryyale.edu/ymtdl

Recommended Citation
http://elischolar.libraryyale.edu/ymtdl/1803

This Open Access Thesis is brought to you for free and open access by the School of Medicine at EliScholar – A Digital Platform for Scholarly Publishing at Yale. It has been accepted for inclusion in Yale Medicine Thesis Digital Library by an authorized administrator of EliScholar – A Digital Platform for Scholarly Publishing at Yale. For more information, please contact elischolar@yale.edu.
STATIN INHIBITION OF MACROPHAGE INTEGRIN-INDUCED RAC2-MYOSIN IIA INTERACTION: AN ANTI-INFLAMMATORY EFFECT

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Kenneth Ike

2013
ACKNOWLEDGEMENTS

I would like to thank God for strength, guidance and grace.

Franka for being a resilient, loving, single parent.

Bonnie and Chima for their constant love and support.

My loving fiancée Susan for her support and sacrifice during times of great challenge.

The Freeman Family for their love and acceptance

My friends Huston, Charles, and Dean Nancy Angoff who were always there for me no matter the situation.

I would also like to give special thanks to Dr. Jeffrey Bender, Dr. Alan Morrison, and the entire Bender lab for providing the resources and support necessary to complete this project.
ABSTRACT

STATIN INHIBITION OF MACROPHAGE INTEGRIN-INDUCED RAC2-MYOSIN IIA INTERACTION: AN ANTI-INFLAMMATORY EFFECT.
Kenneth E. Ike, Alan Morrison, and Jeffrey R. Bender. Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.

HMG-CoA reductase inhibitors (statins) are pharmaceuticals that are utilized for the treatment of lipid disorders along with the primary and secondary prevention of coronary heart disease. HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis, converting HMG-CoA to mevalonate. The isoprenoid products, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are derived from the mevalonate pathway and serve as substrates in the prenylation of 2% of cellular proteins including the Rho family of low molecular weight G-proteins which mediate multiple cellular signals. Prenylation is an important post-translational modification of proteins that plays a role in the subcellular localization of proteins to certain hydrophobic (membrane) compartments. This localization can affect overall protein function. We demonstrate that manipulation of the prenylation state of Rac2 alters the formation of a signaling complex between Rac2 and Myosin IIA that forms via β2 integrin engagement coupled to chemokine (CCL2, MCP-1) stimulation. This complex appears critical to the translocation of the RNA binding protein, HuR, from the nucleus to the cytosol whereby it functions to exact its effects on mRNA stability. HuR translocation prolongs the half-life of transcripts encoding critical inflammatory, immune and angiogenic cytokines like TNF-α, INF-γ, and VEGF-A. This pathway may represent a pleiotropic effect of statin therapy and may serve as a target for future small molecule therapeutics with anti-inflammatory potential.
# Table of Contents

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>STATEMENT OF PURPOSE AND HYPOTHESIS</td>
<td>18</td>
</tr>
<tr>
<td>METHODOLOGY</td>
<td>19</td>
</tr>
<tr>
<td>RESULTS</td>
<td>26</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>35</td>
</tr>
<tr>
<td>FIGURES</td>
<td>39</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>62</td>
</tr>
</tbody>
</table>
INTRODUCTION

Pleiotropic Effects of Statins

Strictly defined, pleiotropy is the phenomena where a single gene has multiple phenotypic effects. The same process can be and has been applied to pharmacologic agents, like statins. It is well recognized that the targeted effect of statins, or HMG-CoA reductases, is the inhibition of cholesterol synthesis downstream of mevalonate. However, in addition to the well-characterized lipid lowering effects, multiple beneficial off-target effects have been established over the last 25 years of clinical experience. Examples to be reviewed here include actions on endothelial cells, antioxidant effects, effects directly on leukocytes, effects on plaque stability, anti and pro-angiogenic effects, and effects directly on the cardiac myocardium (1).

The effect of statins on endothelial cell function has been linked to the direct effects statins have on endothelial nitric oxide synthase (eNOS) activity, subsequent NO production and inhibition of endothelin-1 (ET-1) synthesis (2). In regards to eNOS activity, there have been numerous studies supporting that statins increase mRNA stability of eNOS providing protection during times of vascular insult which include conditions of hypoxia, increased ox-LDL, and elevated IL-1 and TNF-α levels (3). Statins, particularly atorvastatin, have also been shown to inhibit the production of a protein named caveolin-1 (4). Caveolin-1 is a potent inhibitor of eNOS activity. Statins inhibit the formation of a caveolin-1 complex that inhibits eNOS by modulating its expression.
By both *in vivo* and *in vitro* studies, statins have antioxidant activity by decreasing the amount of reactive oxygen species (ROS). The mechanism involves the inhibition of p21 Rac isoprenylation, which affects its key functional role the NADPH oxidase system of endothelial cells and reduces the production of ROS (5).

There is evidence to support the hypothesis that statins exert a direct inhibition of leukocyte activation. Though the mechanism of this inhibition is not clear, there are several possibilities for how this might occur. First, statins directly reduce the expression of adhesions molecules, particularly intracellular adhesion molecule -1 (ICAM-1) on endothelial cells (6). ICAM-1 is critical for the adhesion, activation, and transendothelial migration of the leukocyte from the vasculature. Further, it is known that integrin adhesion is dependent on small GTPase Rho activation (7). The dependence of Rho activation on isoprenylation provides a potential interventional site for statins. Finally, certain statins, including lovastatin and simvastatin, directly inhibit leukocyte endothelial binding through a specific binding site on expressed adhesion molecules such as LFA-1 (8). The significance of these effects may be a crucial part of the non-lipid lowering contributions to beneficial cardiovascular outcomes. This thesis will attempt to expand on the function of a small G-protein that further elucidates this premise.

Statins have been shown to have multifaceted effects that promote plaque stability. One important mechanism of statins is the ability to decrease scavenger receptor expression and ox-LDL uptake by macrophages (9). A decrease in ox-LDL uptake reduces foam cell formation, which is a key determinant of plaque stability. Another key effect of statins is
that they inhibit the macrophage ability to migrate in plaque and produce matrix metalloproteinases (MMP), which are necessary for the degradation of extracellular matrix (10). The net effect of MMP modulation on plaque stability is still debated but provides another avenue for explanation of the statin contribution to plaque instability.

Statins may be involved in numerous aspects of angiogenesis in a dose dependent manner, although the exact role of statins in angiogenesis is debated (11). Statins have been implicated in the mobilization and maturation of endothelial progenitor cells (EPC’s), which are important in contributing to endothelial regeneration at sites of vascular stress. Risk factors like hypertension, hypercholesterolemia, and diabetes are associated with decreased levels of EPC’s. Statin therapy appears to counteract this effect (12).

The role of vascular endothelial growth factor (VEGF) is interesting and controversial. VEGF is known to have positive effects on vascular remodeling, but prior studies demonstrate that statins decrease the plasma concentration of VEGF (13). This is a seemingly paradoxical effect when considering factors in cardiovascular mortality and morbidity. Studies done in apolipoproteinE/apolipoprotein B-100 mice with different statin types may begin to shed light on this paradox.

Moving from animal models to the clinic, there are numerous trials that have established the vascular and cardio protective effect of statins particularly in regards to acute coronary syndrome (ACS). One such study, the PROVE IT -TIMI 22 trial, randomized
4162 patients to either pravastatin or atorvastatin and found a 28% reduced risk in composite death, MI or rehospitalization for recurrent ACS for subjects in the statin treatment group (14). The Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (or Jupiter) trial examined people with elevated CRP levels, as a marker for inflammatory state, but relatively normal lipid levels, and determined that people who took statins has a 40% relative risk reduction for heart attack, stroke and death after 1 year (15). This study raises further questions about the role inflammation has in cardiac and vascular disease.

**Characterization and Functional Role of Rac2 in Cell Signaling**

Rac2 is part of the Rho subfamily small GTPases (16). There are six subfamilies that include the RhoA-related subfamily (RhoA, RhoB, RhoC), the Rac1-related subfamily (Rac1, Rac2, Rac3, and RhoG), the CDC42 related subfamily (CDC42, g25k, tc10, tcl, chp/wrch-2 and wrch-1) the RnD subfamily (Rnd1, Rnd2, and RhoE/Rnd3), the RhoBTB subfamily and the Miro subfamily (17). Specifically in the Rac1 subfamily, 88% homology of sequence is shared between Rac1, 2, and 3 with major differences lying in their C-terminal residues. Traditionally, this family of proteins is thought to share similar functions as molecular switches with two forms that predominate; a GTP-bound (active) and a GDP-bound (inactive). The transition between GTP to GDP bound states of the GTPase is regulated by two groups of proteins. One group is called the guanosine nucleotide exchange factors or (GEF’s) which serve to bind the GTPases with GTP. This group has a Dbl - homology (DH) domain responsible for catalytic activity and a pleckstrin homology (PH) domain responsible for membrane or lipid localization. The
second groups of regulators are labeled GTPase-activating proteins (GAP’s) and serve to switch GTP for GDP on the GTPases. The active form of the GTPases and including Rac2 are thought to play a role in a variety of cellular processes including cellular proliferation, NADPH oxidase activity, gene expression, cell adhesion, and cell motility (18).

There are other proteins that may have intimate roles in the functional regulation of these proteins. One such protein is RhoGDI. There are three Rho GDI’s, RhoGDI 1, 2, 3 or alpha, beta, gamma. RhoGDI 2 is LyGDI or D4GDI. These are regulatory proteins known to interact with Rho GTPases and regulate the localization of the Rho GTPases between cytosol and membrane (19). In addition they have domains that regulate the GTP/GDP cycling function carried out by Rho GTPases that is handled in their switch regions. Proteins in the GDI family make a variety of contacts with members of the Rho small GTPase class and may be involved in regulatory processes to varying degrees based on cell type and small GTPase variant.

In addition to the switch regions that regulate GTP-GDP association and the contact points for the GDI proteins, the small GTPases have a prenylation modification that consists of isoprenoid products derived from the mevalonate pathway (20). Prenylation is an important post-translational modification of proteins that often plays a role subcellular localization of proteins to certain hydrophobic compartments (21). The prenylation type is determined by the 4th cysteine residue position from the C terminus (COOH) of the G-protein, containing a CAAX (C-Cysteine, A-Aliphatic, X-serine, methionine, glutamine,
alanine or threonine) motif sequence (22). The sequence will determine whether one of two prenylation groups, farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP), will be added by a farnesylation (by farnesyltransferase) or a geranylgeranylation (by geranylgeranyl transferase) reaction. Rac2 posses a CSLL sequence which signals a geranylgeranylation by the action of geranylgeranyl transferase I (23). This modification allows Rac 2, to remain soluble in the cytosol and associated with the GDI (Guanine dissociation inhibitor) or to move to the plasma membrane to mediate downstream signaling events (18). Multiple crystal structures of various members of the different GTPase families have shown interaction with the GDI molecule (24).

Rac2 and other members of the small GTPase protein family have been linked to numerous effector proteins. A common theme among these interactions is that residues in the switch I region of the proteins are key for guiding reactions with different effector cells. For example, Rac’s interaction with p21 activated kinase (PAK) is dependent on a tyrosine residue at the 40th position, but another residue position determines its interactions with mixed lineage kinase (Mlk 2 and 3). In addition to PAK and Mlk, Rac interactions have been linked to Ser/Thr kinases, PI-4-P5K, POR-1, PLD, PI3K, DAG kinase, PLC-B2, Wave/Scar, POSH, p67 phox, IQGAP and among others that affect a variety of pathways (25). These pathways include interactions with actin, SRF, JNK/p38, NF-kB, NADPH oxidase system, cell-cell contacts and cell cycle progression. The mechanism by which the small GTPases, particularly, Rac function to activate the effector proteins is through disruption of intramolecular auto-inhibitory domains that regulate kinase activity. Upon binding to Rac, the inhibitory domain unfolds and becomes
exposed allowing kinase activity to ensue.

**Macrophage Role in development of Vascular Disease**

Monocytes are a uniquely positioned cell type in the inflammation pathway. Their regulation depends partly on the activity of small GTPases that are expressed intercellularly. In addition, they play a pivotal role in driving the inflammatory response involving plaque deposition in the vasculature. Monocytes interact with adhesion molecules that are expressed on the surface of endothelial cells in response to ox-LDL or other activators. Chemokines like monocyte chemoattractant protein 1 (MCP-1) have been implicated in the migration of the monocyte into the intima layer of the vessel wall and also drive the maturation of monocytes to macrophages (26). The maturation and activation of the monocyte depends partly on those chemokines, and others like macrophage colony stimulating factor (M-CSF) help to drive the expression of scavengers’ receptors on the macrophage and allow for the consumption of ox-LDL molecules by the macrophage. The ox-LDL is believed to bind to CD-36 and lead to further activation of the macrophage within the plaque (27). This activation state leads to the expression of many other inflammatory cytokines that in turn effect endothelial cells, smooth muscle cells, lymphocytes, and other macrophages. Consequently, this further drives the inflammatory environment and the pathology of the plaque. The structural integrity of the plaque is challenged by increased expression of matrix metalloproteinases (MMP’s) and by macrophages that degrade the components of the extracellular scaffolding. This is important because plaque stability plays an important role in rates of spontaneous plaque rupture and downstream consequences.
There is evidence that supports the significance of the different subsets of macrophages in the progression of atherosclerotic plaque. The two subtypes of macrophages include M1 and M2. The M1 subtype is pro-inflammatory and expresses cytokines that include IL-1β, TNF and IL-6. The M2 subtype has a high expression of scavenger receptors and might be expected to phagocytize lipid. The distinction of macrophage phenotype supports the timeline seen in the ApoE knockout mouse model. In the ApoE model of atherosclerotic plaque development, histologic examination of vessel walls demonstrates that early in atherosclerotic plaque progression the M2 subtype macrophage predominates in lesions whereas the M1 subtype dominates in the later phases (28).

Role of Chemokines in development of Atherosclerotic Plaque

Several chemokine/chemokine receptor pairings have numerous studies in mice that show independent and additive effects on their ability to reduce the atherosclerotic burden. Mouse models seem to stratify the influence of the three chemokine/chemokine receptors into 3 major events. The first phase is macrophage recruitment, which has been linked primarily with CCL5, mediated stimulation of CCR5. The second phase is early monocyte adhesion, which has been shown to be mediated by the CCL2/CCR2 interaction. The third phase is chronic monocyte adhesion, which has been shown to be supported by the fractalkine/CX3XR1 interaction (29).

Monocyte chemoattractant protein 1 (MCP-1), also known as CCL2, is a key chemokine that promotes the migration of monocytes/macrophages. This chemokine is part of the CC subgroup of chemokines, is roughly 13 kDa in size, and the gene is located on
chromosome 17q11.2. MCP-1 works by binding to chemokine receptors CCR2 and CCR11. CCR2 is a G-protein coupled receptor located on the surface of inflammatory cells. Binding to CCR2 results in the formation of IP3, activation of PKC, and release of intracellular calcium stores. CCR11 actions are less well described. The intracellular pathways of MCP1 signaling involve numerous downstream effector proteins that culminate in modulating cell activation and trafficking (26).

During times of inflammatory stress, multiple cell types including endothelial cells, smooth muscle cells, and monocytes secrete MCP-1 and create a chemotactic concentration gradient for inflammatory cells to follow. Ox-LDL, but not naïve LDL, has been shown to induce MCP-1 production by endothelial cells and smooth muscle cells, which may represent the start of the recruitment of monocytes and the initiation of atheroma formation.

CX3CL1 (also known as fractalkine) is a member of the CXC subfamily of chemokines. It is also an important chemokine that plays a role in immune cell trafficking. It consists of 397 amino acids with 5 unique domains and is encoded on chromosome 16. Its basal production by endothelial cells and smooth muscle cells is very low, requiring TNF-α for marked increase. CX3CL binds to CX3CR1, which is another G-protein coupled receptor. Pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ which all are implicated in stimulating an NF-κB pathway that induces expression of CX3CL/CX3CR (30).

CCR5 is a unique chemokine receptor known as a participating receptor that allows HIV
infection in macrophages and other CCR5 expressing immune cells. The CCR5 gene is encoded on chromosome 3p21 and responses to ligands CCL3, CCL4, and CCL5 (31).

**Characterization, Activation and Role of β 2 Integrin in Vascular Inflammation**

Integrin’s are defined as the cells’ connection between extracellular and intracellular environments (32). The signaling that occurs in integrin expressing cells is thought to be directional. When integrins bind, signals are sent from the extracellular domain of the integrin to the intracellular domains that are associated with cytoskeletal proteins and other intracellular effectors that act to inform the cell of its present environment. The cell then reacts via intracellular mechanisms to inform the extracellular component of the integrin that a change in affinity is needed to direct adhesion or migration. These signal transduction processes mediated through the integrin molecule are complex. Integrin’s are composed of many domains and have three prominent conformation states. Each integrin molecule contains an alpha and beta subunit. Each subunit contains 3 parts that include an N-terminal extracellular domain, a transmembrane domain and a C-terminal cytoplasmic domain. There are 18 different alpha subunits that can possibly join with 8 beta subunits for different confirmations. There are two major groups of alpha subunits within 18 possible subunits. One group contains an αI domain while the other set does not. This only affects binding with potential ligands by requiring the participation of different beta subunits or not depending on the alpha subunit group. The key-binding site for the integrins is the metal ion dependent adhesion sites (MIDAS) whose conformation is dependent on the conformation of the αI and βI domains. These domains are important because they drive ligand specificity by driving conformational states that accept only
certain inflammatory ligands. The alpha subunit is most important in mediating the binding to particular ligands. In the group of integrins that do not contain the αI domain, ligand binding is primarily determined by the beta subunit. However, the beta subunit has the primary function of communicating with the intracellular domains and transmitting information between components. In terms of the overall conformational states the integrins have a bent closed, extended closed, and extended open state. Each of these states differs by the degree to which the α and β subunit articulate at various extracellular points particularly at the headpiece region and the hybrid region.

Integrins are important components of the immune effector cell. When the cell is recruited to a site of inflammation, it must be able to travel out of circulation and localize itself to specific areas around the precipitating inflammatory event. Part of this process is aided by the ability of the immune cell to express integrins on the cell surface. Integrins such as LFA-1 are able to bind to ligands like ICAM-1 that are expressed on a variety of cell types and help direct the immune response to localized areas. While studying cell-cell adhesions in 1986, Springer was able to discern that LFA-1 interacted with other cell ligands than previously described (33). As part of his investigation of cell-cell adhesions he was able to determine that when LFA-1 deficient lymphocytes were incubated together, cell-cell adhesion would not occur in the presence of phorbol ester. However, when LFA-deficient cells were incubated with normal lymphocytes, adhesion was present indicating that a ligand other that LFA-1 was necessary for cell-cell adhesion to take place. Dustin et al further showed that ICAM-1 expression on dermal fibroblasts was increased three to five times baseline by IL-1 and TNF-alpha (34). More importantly, they showed that ICAM-1 was expressed on a variety of cell types particularly vascular
endothelial cells. Springer et al displayed LFA-1 binding to ICAM suspended in an artificial lipid membrane. These discoveries have led to additional questions regarding whether or not LFA-1 mediated binding influenced the activation state of the immune cell.

Members of the Bender lab have contributed significantly to the understanding of the integrin-mediated processes particularly in T-cells. In 2006, Wang et al. was able to demonstrate in human peripheral T-cells that engagement of LFA-1 leads to the prolongation of TNF-a, GM-CSF, IL-3 mRNA half-life and also had an effect on the mRNA encoding for a chimeric B-globin mRNA reporter construct containing a destabilizing class II AU-rich element from the GM-CSF mRNA 3’untranslated region (35). In 2010, Rangolam et al. found in T-cells that LFA-1-induced stabilization of ARE-containing mRNA’s occurs in T-cells and was dependent on HuR. This involves a pathway linking Vav-1, Rac1 and 2, MKK3, and p38MAPK proteins (36). In 2012, Zhang et al. demonstrated that the ICAM – LFA-1 - HuR axis is key for stabilization of VEGF and MMP-9 labile transcripts in a macrophage model of angiogenesis (37). It is the role of Rac’s in the integrin induced, HuR-mediated signaling cascade and the importance of HuR in the macrophage inducible factor production that has led to the work described in this thesis.

Characterization and Functional Role of Myosin IIA in Cytoskeletal Reorganization

Myosin IIA is classified as a nonmuscle or atypical motor protein that associates with actin. This association plays a key role in cell migration and adhesion. In terms of
structure, Myosin IIA has three separate structural domains: globular head, neck region and rod (38). The first part is a globular head domain that contains regions responsible for actin binding. This domain also contains an enzymatic Mg$^{++}$ ATPase motor domain. The neck region contains an essential light chain (ELC) and a regulatory light chain (RLC). The ELC is responsible for stabilization of the heavy chain structure. The RLC is responsible for overall structure conformation, which is pivotal for actin association. The coiled coil rod domain allows for dimerization.

On phosphorylation of the RLC, Myosin IIA transforms from a closed 10S assembly to an open 6S assembly that has an exposed rod domain. This rod domain is then able to dimerize with the rod domain of other heavy chain assemblies with concomitant actin interaction with the globular head domain. Studies have shown that there are two residues on the RLC that are important in terms of regulation. The Ser19 and Thr18 have been identified as two sites that are integral in promoting Myosin IIA association with actin through altered baseline conformational changes. Several kinases have been implicated in RLC phosphorylation including myosin light chain kinase (MLCK), rho associated coiled coil-containing kinase (ROCK), and CDC42 binding kinase CDC42BP. ROCK also acts to inhibit protein phosphatase 1 (PP1) which is a key dephosphorylator of Myosin IIA (39).

Myosin IIA is a key regulator of slow retrograde flow in the lamellum, which is part of the leading edge of motile cells (39). When Myosin IIA is knocked down or inhibited with blebbistatin, actin bundles disappear from the lamellum but not the lamellipodium.
This finding is key to understanding the overall role for myosin in cell motility. Myosin IIA also has a role in insuring proper cellular adhesion via its interaction with a variety of other proteins linked to integrins. In the traditional model, integrins are associated with actin/myosin clusters via actin linkage proteins that include α-actinin, talin and viniculin. Interactions between these proteins are important for the activation of a variety of kinases (focal adhesion kinase, Src, paxillin) that transmit downstream signals through small GTPase mediated pathways.

In vitro studies involving Rac 1 address this point. Leeuwen et al showed that in the presence of bradykinin stimulation, Rac1 was activated and regulated the calcium dependent phosphorylation of MSII in PC12 and N1E-115 cells (40). This phosphorylation led to increased cell spreading through the reorganization of the cytoskeletal elements. Notably, this change in phenotype was in contrast to Rho mediated cell rounding suggesting a possible inhibition of Rho mediated cytoskeletal reorganization. In terms of releasing contacts made by cell adhesion molecules, the role of Myosin IIA has also been explored. Morin et al. showed in lymphocytes that disrupting the interaction of LFA-1 with Myosin IIA resulted in an inability of lymphocytes to disengage interactions with ICAM-1 at the uropod (41). After inhibition with blebbistatin or siRNA knockdown, the lymphocytes displayed a more elongated phenotype and a decreased inability to migrate on ICAM1/CXCL-12 slides compared to cells in DMSO.
As mentioned above, blebbistatin, which is a highly specific inhibitor of Myosin IIA was used in many assays. Blebbistatin is a small molecule, uncompetitive, inhibitor that preferentially binds to the ADP+P bound form of Myosin IIA at the head region. It does not interfere with actin myosin binding or ATP induced actin myosin dissociation (42).

**Leukocyte HuR Regulation in Integrin induced RNA Stabilization**

Human antigen R or HuR is an RNA binding protein that is a member of the embryonic lethal abnormal vision (ELAV) family of proteins (43). It is expressed in a variety of cell types and is detected in both the nucleus and cytoplasm. It is involved in insuring the stabilization of many short-lived mRNA’s that contain AU (adenylate and uridylate) rich elements (AREs). This process is key in the important steps involved in gene expression. Post-transcriptional modification of mRNA can occur on many levels that involve the processes of translocation, translation, editing, and degradation of the mRNA molecule. This is particularly important in the context of the mRNA for a large number of inflammatory cytokines including TNF-a, IL-6 and IL1β..

Many potential pathways have been explored to elucidate HuR shuttling. Studies examining the role of AMPK in decreased HuR shuttling or increased HuR nuclear levels show that AMPK activation serves as a unifying pathway in many potential inflammatory or noxious cell stimuli including oxidative stress and hypoxia. However, studies of various potential signaling pathways have yielded no direct regulatory answers. There has been an important observation of the actions of importin-α, which is a key cytoplasmic associated protein of HuR important in the importation of the protein into the nucleus. Studies have shown that specific targeted activation of AMPK leads to modifications of
importin-α, which increases its cytoplasmic levels and thus nuclear HuR levels (44). Other observations have helped to shed light on potential pathways involved in the regulation of HuR shuttling and its ability to stabilize mRNA (45).

**Characterization of ApoE KO mouse model in study of Atherosclerosis**

The ApoE deficient mouse model was developed to study the process of atherosclerosis (46,47). The Maeda group provided the original mouse strain, which today is distributed by Jackson Laboratory. Murine models have a number of caveats given the animal’s relative differences in cholesterol physiology. At baseline, mice have high density lipoprotein (HDL) levels as high as 85mg/dl (48). These mice do not develop atherosclerotic plaques on regular chow diet and often require long intervals on a western diet before the development of atherosclerotic lesions. This is possible because of the expression of ApoE, which allows the mice to clear lipids efficiently. The ApoE glycoprotein is synthesized in the liver, brain, monocytes and macrophages. With the knockout of the ApoE gene, the mice develop atherosclerotic lesions on normal chow diet and develop more significant lesions in shorter time frames on high fat diets. This allows for study of atherosclerotic plaque development and other factors driving inflammation in a more practical timeframe.

Despite the relative value of the ApoE deficient murine model there are several shortcomings that raise the question as to whether or not findings in the model can be completely extrapolated to humans (49). The first is the question of gender. Several studies have shown that male and female murine models respond differentially to high fat
diet. Tangirala et al have shown that male models develop atherosclerotic lesions in the aorta more frequently than models of the female gender at baseline (50). In addition, their ApoE\(^{-}\) model also displayed a trend towards higher incidence of atherosclerotic plaque lesions in male mice compared to female mice but the difference was not significant. After the study released by Tangirala there have been multiple studies that have shown differences in incidence and size of atherosclerotic lesions in murine aorta based on the timing in which the high fat diet is started and based on the gender of the model. The gender differences have prompted questions into whether or not estrogen plays a role in influencing the development of atherosclerotic plaque in the model.

Several studies performed in the ApoE\(^{-}\/-\) model have shown that manipulation of estrogen levels leads to the same protective effects in the model. Elhage et al and Bourassa et al have demonstrated that estrogen supplementation in ApoE \(-/-\) mice leads to inhibited fatty streak development and reduced atherosclerotic lesion development respectively (49). However, several studies have also shown that estrogen manipulation leads to non-protective effects in the ApoE \(-/-\) model. A study utilizing tamoxifen, an estrogen receptor agonist/antagonist based on tissue type, showed a regression of atherosclerotic plaque in the ApoE \(-/-\) model (49).
STATEMENT OF PURPOSE AND HYPOTHESIS

Cardiovascular disease is the leading cause of death in the United States (51). A majority of cardiovascular disease stems from atherosclerotic coronary disease and HMG-CoA reductase inhibitors have remained a standard of care in primary and secondary prevention for the past quarter century. Our laboratory is interested in identifying novel signaling pathways influenced by statins to develop new classes of therapeutics that target inflammation and the immune system.

The hypothesis of this thesis is: Isoprenylation is crucial to a signaling complex between Rac2 and Myosin IIA that promotes the translocation of HuR from the nucleus to the cytosol.

The purpose of this thesis is to:

1. Demonstrate the importance of Rac2 and Myosin IIA in the translocation of HuR

2. Demonstrate the role of β2 integrin adhesion in the formation of a signaling complex between Rac2 and Myosin IIA in the setting of chemokine signaling.

3. Demonstrate the disruption of both the translocation of HuR and the complex between Rac2 and Myosin IIA in the setting of atorvastatin treatment and reversal with isoprenyl specific-rescue.

We believe that this pleotropic effect of statin may help to highlight this signaling pathway as a potential target in the development of novel pharmacotherapeutics.
METHODOLOGY

THP1 Cell Line
The human leukemic cell line (THP-1) was established using blood from a 1-year-old boy with acute monocytic leukemia. These cells display several characteristics that define its monocytic phenotype. Tsuchiya et al displayed that these cells phagocytized sheep erythrocytes and 0.8 mm diameter latex particles (52). These cells also displayed the ability to activate T-lymphocytes in the presence of Con A. In addition, these cells display lysozyme production. Furthermore, these cells express a-naphthyl butyrate esterase that is inhibited by NaF, which is an established distinguishing characteristic of monocytic cells. These cells differentiate into a more macrophage like phenotype when exposed to PMA. THP1 cells were cultured in RPMI supplemented media with FBS, Pen/Strep/Glut with cell media replaced every 48-72 hours.

C57BL/6 & Rac2KO Bone Marrow Derived Macrophages
Bone marrow derived macrophages were isolated from the femurs and tibias of C57BL/6 and Rac2KO mice and placed in 10ml of fully supplemented RPMI media in a 50ml conical tube. The fully supplemented RPMI media is 500ml RPMI, 2 µl 2-µME, 5ml HEPES, 5ml Pen/Strep, 5ml L glutamine, 5ml sodium pyruvate, 50ml FBS. Remove media and wash bones with 70% EtOH (3X). Remove EtOH and wash with 1X CMF-PBS (3X). Grind bones in 10ml RPMI using mortar/pestle. Filter bone homogenate through a 40µm strainer into a new 50ml conical tube. Add 10ml RPMI to mortar/pestle and repeat homogenization/filter. Wash mortar/pestle with 10ml RPMI and filter this into the same tube. Centrifuge at 1500 RPM for 5 minute at 4°C. Aspirate supernatant and re-suspend pellet in 2ml lysis buffer. Add 3ml ACK lysis buffer and pipette up and down with a glass Pasteur pipette (better yield). Incubate for 5 minute at RT. Add 10ml RPMI to halt lysis. Centrifuge at 1500 RPM for 5 min at 4°C. Re-suspend pellet in 5ml 1X CMF-PBS. Centrifuge at 1500 RPM for 5 min at 4°C. Obtain L cell media (-20°C). Re-suspend pellet in 10ml L cell media + fully supplemented RPMI media and filter through a 70µm strainer. Do cell counts. After cell counts, centrifuge media at 1500 RPM for 5 minute at 4°C. Re-suspend pellet L cell media + fully supplemented RPMI and plate 4~5 x10⁶ cells in 7ml media in an Optilux petri dish (100x20mm). Cell counts and plate 4x10⁶ cells in 7ml media in an Optilux petri dish (100x20mm). Add 4ml fresh, pre-warmed macrophage media (L Cell) to each plate. Macrophages may be used on days 7-9, after rinsing with 1xCMF-PBS, wash plates vigorously with 1x CMF-PBS containing 5mM EDTA

Peripheral Blood Monocyte Extraction Assay
Peripheral Blood Monocytes were isolated from healthy human volunteers (53). Draw blood from subject using heparinized collection tubes. Place blood into 50 ml conical tube and dilute 50:50 with 1xPBS. Pellet leukocyte/RBC fraction by centrifugation of
cells for 15 minutes at 200g at room temperature. Using sterile pipet add room temperature 1xPBS to final volume of 40ml. Slowly layer Ficoll-Hypaque solution underneath the leukocyte/RBC/PBS mixture by placing tip at bottom of tube (up to 10ml or 3ml/10ml sample). Centrifuge 20-30 minutes in GH-3.7 rotor at 900g at 18°C no brake. Using sterile pipet remove upper layer that contains plasma and most of cell platelet function. Using another pipet transfer mononuclear lymphocyte cell layer to another tube “white cloudy band”. Add HBSS and centrifuge 10 minutes at 450g. Remove supernatant and re-suspend in HBSS. Re-suspend cells in complete RPMI-1640 and use for assays.

**Subcellular Fractionation Assay**

**Goal:**
To biochemically determine Rac2 localization under non-stimulatory and stimulatory events.

**Reagents:**
Thermo Scientific sub-cellular fractionation kit, 1xPBS, Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB), Nuclear Extraction Buffer (NED), Chromatin Extraction Buffer (NED), Cytoskeletal Extraction Buffer (PEB), Tabletop micro-centrifuge

**Method:**
Prepare Buffer reagents according to recommendation of Thermo scientific subcellular fractionation kit (78840). (500:500:250:250:250) ratio for CEB, MEB, NED, MEB, PEB Buffers respectively. Our samples vary from 5x10^6 to 8x10^6 cells per sample so approximate packed cell volume is 50µl. Add halt protease inhibitor cocktail in 1:100 ratio to each volume of buffer prior to use. Harvest cells. If suspension, spin cells down in original media 1500rpm, 5 min, 25°C, remove media, then wash 1x with 1X PBS (1500rpm, 5 min, 25°C). Remove PBS and add 500µl CEB Buffer. If adherent, remove media with glass pipet. Wash with 10ml 1x PBS by swirling around plate. Remove PBS and add 500-800µl CEB buffer directly to plate sample. Use cell scraper to dislodge adherent cells, swirling buffer around plate in between successive cell scrapings. Place sample on ice and use pipet to transfer buffer from plate to micro-centrifuge tube. Place sample with CEB buffer in 4°C for 10 minutes with gentle rock. Remove sample from 4°C and micro-centrifuge for 5 minutes, 500g, 4°C. After centrifuge, transfer supernatant to clean pre-chilled micro-centrifuge tube. Re-suspend pellet gently in 300µl of CEB buffer and place in 4°C for 5 minutes with gentle rock (wash step). Centrifuge pellet for 5 minutes, 500g, 4°C and discard supernatant. Add 500 µl of MEB buffer to pellet and re-suspend. Vortex on highest settling for 5 seconds. Place in 4°C with gentle rock for 10 minutes. Centrifuge sample 5 minutes, 3000g, 4°C. Remove supernatant and add to clean, pre-chilled micro-centrifuge tube. Re-suspend pellet in 300µl of MEB buffer, vortex on
highest setting for 3 seconds and place in 4°C for 5 minutes with gentle rock (wash step). Centrifuge sample 5 minutes, 3000g, 4°C and discard supernatant. Re-suspend pellet in 250µl NEB buffer. Vortex on highest setting for 15 seconds. Place in 4°C and rock for 30 minutes. Centrifuge for 5 minutes, 5000g, 4°C. Transfer supernatant to clean pre-chilled micro-centrifuge tube. Re-suspend pellet in 250µl NEB buffer with (5µl 100mM CaCl₂ and 3µl Micrococcal Nuclease per 100µl of NEB). Vortex on highest setting for 15 seconds. Place in 37°C water bath for 5 minutes. Vortex on highest setting for 15 seconds. Centrifuge at 5 minutes, 16000g, room temperature. Transfer supernatant to clean micro-centrifuge tube. Re-suspend pellet in 100% l of NEB buffer with (5% l 100mM CaCl₂ and 3% l Micrococcal Nuclease per 100% l of NEB). Vortex on highest setting for 15 seconds. Discard supernatant. Add room temperature PEB Buffer and vortex on highest setting for 15 seconds. Incubate at room temperature for 10 minutes. Centrifuge for 5 min, 16000g and transfer to new centrifuge tube. Save fraction on ice for same day use. For extended storage freeze with liquid nitrogen and store in -80°C.

Immunofluorescence Assay

Goal:
Characterize association between Rac2, RhoGDI and LyGDI and localization of Rac2 under specific stimulatory conditions by immunofluorescence (54).

Reagents:
Rac2 antibody - (Santa Cruz) (polyclonal) (rabbit), RhoGDI antibody - (Santa Cruz) (rabbit), LyGDI antibody - (Santa Cruz) (mouse and rabbit), Goat Serum IgG, Normal IgG - rabbit (Santa Cruz), Normal IgG - mouse (Santa Cruz), 1x PBS .3% Triton, 1x PBS, 4% Paraformaldehyde, 100% Methanol, 70% EtOH, 6 well glass tissue culture treated plate, Glass coverslips (fisherbrand 22x22-1), Microscope slides (poly-lysine treated), Styrofoam container with wells (leftover cuvette holder suffices), .01% w/v poly-l-lysine (sigma)

Method:
Sterilize glass coverslips in 6 well plates with 1 of two methods. 1)Add EtOH to wells and let sit for 15 minutes, then remove excess and let dry at room temp or overnight or 2) First coat slides with poly-l-lysine, then UV irradiate overnight. Coat glass coverslips with .01-w/v poly-l-lysine for 1 hour then remove excess. Allow glass slides to dry at room temp or in 37 degree. Place cells in appropriate media and experimental condition in each of the 6 well at 250,000 cell/ml density / 2ml total. Remove media from each of the wells. Wash 1x with 1x PBS Sterile. Fix cells by adding 1ml 1xPBS 4%. Paraformaldehyde and let sit for 15 minutes. Wash cells 3x with 1x PBS (5 min/wash). Permeabilize cells with 100% Methanol (10 minutes @ -20°C). Wash cells 3x with 1x
PBS (5min/wash). Block w/goat serum 5% by volume (50ul in 950 1xPBS, .3% triton) 1 hour at 4°C. Incubate in primary antibody (1 to 500 antibody to 1xPBS, .3% triton) overnight. Wash cells 3x with 1x PBS (5 minutes/wash). Incubate cells with secondary antibody (alexa-flour 488/568) for 2 hours. Wash cells 3x with 1x PBS (5 minutes/wash). Stain cell nucleus with DAPI for 15-20 seconds. Wash cells 3x with 1x PBS. Mount cells with Tris-Cl mounting solution and store in dark overnight. Visualize cells.

**Rac GTPase Activation Assay**

**Goal:** To determine activation state of Rac2 in biochemical assay across multiple experimental conditions (55).

**Reagents:**
Rac2 antibody – Proteintech monoclonal, or C terminal class. THP1 cells 10x10^6 per plate. PBD-Pak beads. Lysis Buffer: 1ml HEPES, 7.5ml NACL (1M), 500ul triton X100, 5ml Glycerol, 400 µl EDTA, 400 µl EGTA, 35 ml h2o, protease inhibitor (1 tab per 10ml per aliquot), GTPγS (non-hydrolyzable GTP), RPMI Media with FBS and Pen/Strep/Glut

**Method:**
Spin down at 1500rpm for 5 minutes at 25°C. Take off excess media. Lyse Cells in lysis buffer 500µl (as per above). Allow cells to sit in lysis buffer on ice 5 minutes. Spin down at 1000g 5minutes at 4°C. Take Supernatant do protein concentration. Take 50ug per sample for TCL, re-suspend 4x buffer, boil, save -20°C. Take equal amount protein per sample and add GTPγS (100µM – 5µl in 500µl solution) for 15 minutes at 37°C. During this time re-suspend beads by flicking and place on ice. Add 12ul of beads to TCL from GTPγS treatment to beads and rock at 4°C for 40 minutes. Spin down beads 400g 2 minutes, take off supernatant. Perform 2 washes of beads at 400g 2 minutes in lysis buffer. Take beads and add to 40µl of 2x sample buffer (4x dissolved with H2O 2x volume). Boil, run gel, transfer, Western blot for Rac2 and Myosin IIA

**Immunoprecipitation Assay**

**Goal:** Characterize association between any two desired proteins across multiple experimental conditions (56).

**Reagents:**
Rac2 antibody - Santa Cruz (polyclonal) (rabbit), Rac2 antibody - Millipore (polyclonal) (rabbit), Rho-GDI antibody - Santa Cruz (rabbit), Ly-GDI antibody - Santa Cruz (mouse and rabbit), IP Buffer: 25mm Tris-Cl pH 7.4, 150mm NaCl, .1% Triton X-100, 2.5mm EDTA, 1mm DTT, Protein A/G Beads

**Method:**
Add 4ug of IP antibodies to 40 µl of bead slurry in 500µl of PBS in 1.5 ml centrifuge tube and rock in 4°C overnight. Lyse 10x10^6 cells in 1 ml IP buffer
Spin down samples at 9300g at 4°C for 10 minutes in microcentrifuge. Take supernatant. Do protein concentration. Add 40µl of A/G bead slurry to each sample and rock in 4°C
for 30 minutes (pre-clear). Spin down beads (6000 rpm, 6 minutes, 4°C) and take lysate. After overnight A/G antibody incubation spin down beads (6000 rpm, 6 minutes, 4°C). Take off PBS supernatant. Add 500µg lysate to respective antibody samples. Rock sample overnight at 4°C. Wash samples 2x with IP Buffer (6000 rpm, 6 minutes, 4°C). Bring up in 40µl of sample buffer. Boil 4 minutes. Spin down beads at 10,000 rpm for 10 minutes and take sample buffer. Run 20µl per lane in small gel (4-15% gradient).

Western: Rac2, Rho-GDI, or Ly-GDI depending on experimental combination.

ICAM Localization Assay

Goal:
To utilize ICAM binding as signal for Rac2 activation across many different experimental conditions.

Reagents:
Petri Dishes, 10µg/ml Goat Anti Human Igg-Fc in 50mM Tris-Cl pH 9.5 (100µl Anti Human Igg for 10ml Tris-Cl), IgG, Tris-CL pH 9.5 *make 1M stock Tris base and pH with HCL then filter sterilize, PBS-CMF, PBS + 2% dialyzed FBS 10ml in sterile 490 ml PBS, rH ICAM 100ng/ml (-20°C ), rH Fc (-20°C), Assay Buffer: 20mM HEPES pH 7.4, 135 mM NaCl, 5mM KCl, 2mM Glucose, 1mM MgCl2, .6mM CaCl2, sterile filter. 4x Lysis Buffer: 20mM HEPES pH 7.4, 150mM NaCl, 4% TritonX100, 40% Glycerol, 16mM EDTA, 16mM EGTA, 4 non EDTA protease inhibitor tablets

Method:
Coat plate with 5 µl of Igg Fc. Incubate at room temperature for 1 hour. Wash 2x with PBS-CMF + 2% dialyzed FBS (simple swish and suction). Block by adding 5 ml PBS + 2% dialyzed FBS and let sit room temp for 1 hour. Take off PBS. Add 5 ml of rh ICAM (100ng/ml in 2% dialyzed FBS / or 1 µl rh ICAM in 10ml dialyzed FBS) Swish around to ensure even coating and put in 4°C overnight. If Fc plate use rh Fc (100ng/ml). Remove media from rh ICAM or rh Fc coated plates. Take 10 million cells and separate out into plates +/- PMA and incubate for 30 minutes. Prepare Assay buffer with PMA. 4ml assay buffer. Dilute PMA 1 to 10 in DMSO and take 2 µl add to 4ml assay buffer). After 30 minute incubation wash cells 2x with PBS. Bring up in 1.2ml of assay buffer with PMA per sample. Transfer to labeled ICAM plates and place in incubator for 60 minutes. Gently remove assay buffer and add CEB buffer to each plate. Scrape plates and continue with subcellular fractionation protocol

Cytochalasin D and Blebbistatin Inhibition Assay

Goal:
To determine relationship of Rac2, actin and Myosin interaction.

**Reagents:**
Rac2 ab – Protein monoclonal, Myosin Antibody, THPI cells 10x10^6 per plate
Lysis Buffer: 1ml HEPES, 7.5ml NACL (1M), 500ul triton X100, 5ml Glycerol, 400 μl EDTA, 400 μl EGTA, 35 ml h2o, protease inhibitor (1 tab per 10ml per aliquot), MCP-1 (100ng/ml final concentration) (stock is 100ng/%l), Cytochalasin D (.01μm, .1μm, 1μm and 10μm formulations) (5mg in 1ml stock), Blebbistatin (2μM, 10μM, 50μM formulations), GTPγS, 10x10^6 cells per plate, RPMI Media with FBS and Pen/Strep/Glut

**Method:**
Method adapted from DuoLink In Situ manual. Plate slides on cover slip and permeabilize. Add blocking solution to each sample. Incubate the slides in pre-heated chamber for 30 min at 37°C. Remove blocking solution from each sample. Add primary antibody to each sample. Incubate antibody in humidity chamber (variable timing). Dilute two PLA probes in 1:5 antibody diluent. Remove primary antibody from slides. Wash slides in 1X buffer x2 5 minute each time. Add PLA probe solution. Incubate slides in pre-heated humidity chamber for 1 hour at 37°C. Dilute ligation stock 1:5 in high purity water and mix. Remove PLA probe from slides. Wash slides in 1x wash buffer A 2x at 5 minute with gentle agitation. Prepare ligase-ligation solution. Add ligase-ligation solution to each sample. Incubate slides in preheated humidity chamber for 30 minutes at 37°C. Dilute amplification stock 1:5 in water and mix. Remove polymerase from freezer and prepare...
amplification-polymerase solution. Add amplification-polymerase solution to each sample. Incubate slides in pre-heated humidity chamber for 100 minutes at 37°C. Remove amplification-polymerase solution. Wash in 1x wash buffer B 2x for 10 minutes. Wash in .01x wash buffer B fro 1 min. Let slides dry in dark at room temperature. Mount slides
RESULTS

Baseline Localization of Rac2

Prior studies have established a relationship between small GTPases like Rac2 and RhoGDI (24). In figure 1, we utilize two protocols differing in wash conditions that minimize contamination of protein fractions to accurately determine Rac2 localization under non-stimulatory conditions. Figure 1, vertical lane 2 shows a Rac2 signal in the cytoplasmic fraction. Figure 1, vertical lane 4 shows no signal in the membrane fraction. At baseline, Rac2 is localized to the cytosol. Figure 1, vertical lanes 2 and 4 also show a signal for RhoGDI confirming its cytosolic predominance at baseline. We utilize immunofluorescence to establish co-localization between Rac2 and RhoGDI in untreated THP1 cells (Figure 2A). Consistent with earlier biochemical findings, the untreated THP1 cells show an overlap pattern that has a high correlation coefficient of .916 (Figure 2B). The limitations to these findings include elevated nonspecific binding of IgG rabbit antibody in control slides for immunofluorescence microscopy (Figure 3A, 3C). The IgG rabbit is the same species as the Santa Cruz polyclonal Rac2 antibody utilized in the assay. Interpretation of these results may be compromised by species pre-determined non-specific binding and antibody quality. At the time of the experiment, the Rac2 rabbit polyclonal was the best antibody available and was considered the standard for Rac2 detection. More data and exploration of the Rac2 RhoGDI association will need to be done to fully understand the relationship between these proteins.

Effect of Atorvastatin on Rac2 localization
Isoprenylation is an important post-translational modification that effects small GTPase localization. To better characterize the effect of statin induced de-isoprenylation on Rac 2 localization, we treat THP1 cells with 10μM atorvastatin. Figure 4, vertical lane 3, row 1 shows that Rac2 localizes predominantly to the cytosol after 10μM atorvastatin treatment. Vertical lane 6, row 1 shows that a small fraction of Rac2 can become membrane associated after atorvastatin treatment. This is most likely explained by generalized dysregulation due to loss of isoprenylation. The deregulated state allows a small but detectable fraction of Rac2 to associate with the membrane. The western blot in figure 4 also shows a signal intensity difference between the untreated, PMA, PMA+atorvastatin cytosolic lanes. As we discover later, this is due to the mechanism of antibody binding for the directed class of antibody (Figure 5A, 6). At the time of the experiment, the Santa Cruz antibody was considered the standard for Rac2 detection. The antibody is reacting to the non-isoprenylated C-terminus of Rac2 and displays a variation in prenylation level. The samples contain equal amounts of protein. The diminished signal of HSP90 is due to statin effects on HSP90 expression. Samples are treated with GTPγS, which is a nonhydrolyzable form of GTP that allows complete loading of Rac2 to promote the activated state, before and after separation to determine protein fractions that could be activated. In terms of the Rac2 relationship with Rho GDI after 10μM Atorvastatin treatment, immunofluorescence microscopy shows a drop in the correlation coefficient to .875 (Figure 2B). This is most likely representative of some disruption to the Rac2 RhoGDI interactions due to loss of isoprenylation. The same limitations to immunofluorescence analysis apply as stated above and more work is needed to characterize the statin effect.
Effect of β2 integrin engagement and PMA on Rac2 activation

Rac2 activation is important to its cellular functions. Our experiments show that β2 integrin engagement with PMA co-stimulation causes Rac2 activation to levels detectable by the activation assay (Figure 7). Figure 7 vertical lane 4 shows a detectable Rac2 signal by pulldown with PBD-pak domain. Notably, in vertical lane 1 & 2, the Fc portion of recombinant ICAM or PMA alone does not activate Rac2 to detectable levels. In vertical lane 3, ICAM alone shows a very small level of Rac2 activation. Findings in Figure 7 suggest that integrin engagement requires stimulation to the high affinity states (with PMA or other physiologic products) and thus the presence of integrin ligand alone is not sufficient to load Rac2 to detectable levels for the Rac GTPase activation assay.

Mass spectrometry findings and Complex development

During the course of performing many of our pulldown assays we observed non-specific antibody binding forming bands at roughly 40kDa and 200kDa. We provide a Coomassie Blue stain that displays the pattern of these bands in relation to a PMA time-course experiment (Figure 8). After mass spectrometry analysis, these bands were discovered to be actin (coverage 78.7%) and non-muscle myosin IIA (coverage 61.5%) (Figure 9). This result suggests that Rac2 activation in the setting of PMA could involve specific cytoskeletal complexes necessary for creating an apparatus conducive for downstream signaling.

Significance of isoprenylation in complex development
THP1 cells with 0.1, 1, 10 and 100μM atorvastatin display concentration dependent loss of complex formation in the setting of PMA and GTPγS (Figure 10). We observe loss of actin and myosin IIA signal at atorvastatin concentrations ≥ 10μM. This observation correlates with an increase in signal intensity for Rac2 as shown in the pulldown row, suggesting loss of prenylation as measured by Rac2 c-terminal class antibody (see below for further detail). In addition, the Myosin IIA signal is lost at 10μM atorvastatin. These results show that complex formation under the conditions of PMA + GTPγS is an isoprenylation dependent process.

**Complex formation is geranylgeranyl pyrophosphate dependent**

We sought to recover complex formation by providing prenylation substrates geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) after co-incubation of THP1 cells with 10μM Atorvastatin over 24 hours (Figure 11). In lane 1 of figure 11, actin signal is at baseline while MyosinIIA signal is absent. In lane 2, complex formation is demonstrated with increased actin and Myosin IIA signal in the context of PMA stimulation. In lane 3, atorvastatin inhibits PMA induced complex formation as there is a drop in signal for both actin and Myosin IIA. Complex recovery is suboptimal in lane 4 with 5μM FPP, but near positive control levels with 5μM GGPP in lane 5 and complete with 5μM GGPP+5μM FPP in lane 6. In addition, the isoprenylation state of Rac2 is shown by western blot. Lanes 2 and 3 show loss of isoprenylation. Lanes 4 and 5 show recovery of isoprenylation with GGPP alone or GGPP+FPP. These results support that the complex is dependent on isoprenylation substrate GGPP and that Rac2 isoprenylation is recovered with GGPP substrate.
Complex formation is actin dependent

We demonstrate that complex formation is dependent on actin polymerization (Figure 12). THP1 cells were co-incubated with CCL2 and cytochalasin D. We inhibit actin polymerization with .1, 1, and 10µM cytochalasin D and observe a dose dependent loss of actin and Myosin IIA signal by western blot. This experimental result supports the notion that formation of the complex is dependent on actin polymerization. In addition, we utilize CCL2 in place of PMA to provide a more physiologic stimulatory signal.

Complex formation requires Myosin IIA

Further characterization of our complex was achieved with dose dependent inhibition of Myosin IIA using Blebbistatin (Figure 13). Despite decreases in Myosin IIA signal by western blot, actin levels remained stable. This shows that actin polymerization and recruitment is independent of Myosin IIA recruitment to the signaling complex.

Demonstration of Complex existence in BMDM and human WBC

Complex formation is demonstrated in BMDMs under conditions of CCL2 coupled β2 integrin engagement (Figure 14). The significance of this figure is that the CCL2 coupled β2 integrin engagement signal does not stimulate Rac1 or CDC42 association with Myosin IIA suggesting specificity for the Rac2-Myosin IIA interaction. Further, complex formation is inhibited in Rac2KO BMDM’s. To further demonstrate the existence of the complex in human peripheral white blood cells, a screening assay was performed. Figure 14 shows formation of the complex under experimental conditions of PMA and GTPγS loading in subjects not currently taking statins. However, for subjects taking statins formation of the complex does not occur to the same extent. This screening figure gives
some insight into the regulation of the complex in human cells and allows a first molecular insight into pharmacologic statin therapy influencing this signaling complex. More controls and further experiments are needed to expand on this concept.

**Significance of Rac 2 Isoprenylation in Complex Formation**

The significance of Rac2 isoprenylation is an important consideration in the formation of the complex. A novel aspect to antibody binding was discovered when a new monoclonal antibody became available for Rac2 (Figure 5A). The original Santa Cruz and Abcam antibodies for Rac2 were polyclonal and recognized Rac2 by binding to the C-terminus of the small GTPase. In states when Rac2 was isoprenylated by the GTases, these antibodies appear to have more difficulty binding to the C-terminus of the small GTPase and display a quantifiable decrease in signal. When Rac2 was non-isoprenylated (in the case of statin treatment), these antibodies bind readily and reproducibly to the C-terminus. Around 2010-2011 antibodies were introduced to the market that recognized full recombinant Rac2 (Proteintech) and bound to the protein regardless of prenylation state. The differential binding provides a useful tool to access prenylation levels while using full protein antibodies as loading controls as shown in (Figure 5B and 6).

Given this unique tool we were able to quantify the amount of time it takes for Rac2 loss of isoprenylation to occur. As shown in figure 16A, we observe peak effect at 24 hours with 10μM atorvastatin. By utilizing the Rac2 activation assay, we determined that non-isoprenylated Rac2 is able to achieve activation secondary to dysregulation of the GDI’s ability to inhibit GTP loading. Our localization experiment hinted at the potential Rac2 activation when Rac2 localized to the membrane in that assay. In figure 16B, it is clear
that non-isoprenylated Rac2 can become activated at higher concentrations than isoprenylated Rac2 as shown by the activation assay. This raises questions as to whether or not Rac2 fits in the general regulation model proposed for small GTPases.

HuR translocation is dependent on CCL2 coupled β2 integrin engagement and inhibited by loss of prenylation

Prior to use of physiologic stimuli CCL2, we utilized PMA to activate cells. In prior experiments, we have shown the ability of PMA coupled β integrin engagement to induce HuR translocation to the cytoplasm and its dependence on isoprenylation (Figure 17). Figure 17 row 1 shows no HuR translocation in untreated THP1 cells plated on poly-l-lysine. In overlay in lane 1, DAPI nuclear stain and HuR stain overlap. Row 2 shows that PMA is insufficient to produce HuR translocation. Row 3 shows HuR translocation in THP1 cells treated with PMA and plated on ICAM. Row 4 shows inhibition of HuR translocation in THP1 cells treated with 10μM Atorvastatin, PMA and plated on ICAM.

With the use of physiologic stimuli, we demonstrate biochemically that CCL2 coupled β integrin engagement and GGPP substrate are necessary for Rac2-Myosin IIA interaction by pulldown assay in the context of 10μM atorvastatin treatment (Figure 18A). In row 1, lane 1 and 2 there is no detectable pulldown of Myosin IIA in the presence of squalene or no precursor. In row 1, lane 3 there is a minor detectable amount of MyosinIIA in pulldown with FPP as substrate. In row 1, lane 4, GGPP allows for a detectable pulldown of Myosin IIA. In addition, there are no detectable amounts of isoprenylated Rac2 with GGPP rescue. We also demonstrate by confocal analysis that HuR translocation is dependent on CCL2 coupled β integrin engagement and isoprenylation
With provision of prenylation substrate GGPP and GGPP+FPP, we are able to rescue HuR translocation in the presence of atorvastatin.

**Interactions between Rac2 and Myosin IIA**

The β2integrin induced Rac2-myosin IIA interaction is unique to the literature and was explored further in our experiments. Comparison of the activating signals of CCL2, PMA+ICAM, ICAM and ICAM+CCL2 show that the Rac2-myosin IIA interaction is achieved when dual signals from either CCL2 coupled β2 engagement or PMA coupled β2 engagement is present (Figure 19). This further reinforces a multistep process as being required for activation of Rac2. In order to discern if this interaction was even more specific, experiments were conducted in knockout mice. Figure 14 shows the specificity of this interaction in Rac2KO mice versus C57BL/6.

To further characterize the interactions between Rac2 and myosin IIA, a proximity ligation assay (PLA) was utilized. Figure 20 shows the common stimulatory conditions, Fc alone, CCL2+Fc, ICAM alone and CCL2+ICAM used and the resultant interactions between Rac2 and MyosinIIA as measured by PLA. The signal is quantified by the red emissions. In Figure 20 row 4 lane 2, we demonstrate that β2 integrin engagement and CCL2 exposure promotes an interaction at a distance of 40nm between Rac2 and myosin IIA as indicated by the strength of signal compared to other conditions.

In order to link chemokine coupled integrin engagement with HuR translocation in a single display, quantify results and show potential clinical significance we utilized our adhesion assay in a study of peripheral derived human monocytes (Figure 21). We plated
the monocytes under stimulatory conditions as shown in (Figure 21). All exhibited some spreading but there was a robust HuR response to CCL2 coupled β2 integrin engagement. The translocation factors show appreciable differences and a large percentage of cells under ICAM+CCL2 underwent HuR translocation compared to individual stimulators.

To further characterize the importance of Rac2 and myosin IIA in the precipitation of this phenomenon we utilized Rac2KO and Myosin IIA KO BMDM. In figure 22, CCL2 coupled β2 integrin induced translocation of HuR is seen in the WT C57BL/6 mice but not in the Rac2KO. Translocation factors are shown with percent cells with HuR translocation. In our myosin knockout experiment, we utilize heterozygous myosin IIA and myosin IIA KO mice to demonstrate that CCL2 coupled β2 integrin translocation is not observed in the knockout. Quantification of HuR translocation factors and percentage support these findings in figure 23.
DISCUSSION

The dysregulation of small GTPases is an important consequence of statin therapy. It is one of many pleiotropic effects that statins demonstrate in vitro and in vivo. Many studies have been completed in an attempt to explain the clinical significance of small GTPase dysregulation given the importance of these molecules in the function of macrophages. The small GTPases hold a central position in the outside in signaling of β2 integrins. These signaling cascades have been linked to monocyte/macrophage activation. Given the centrality of macrophages in the inflammatory response to plaque formation and progression, regulation of the small GTPase class of proteins takes on clinical significance. Our aim was to explore the importance of Rac2 isoprenylation and the significance the modification had on its localization and function.

Our results show that Rac2 is a dynamic protein whose regulation is dependent on isoprenylation. Traditional models in the literature state that Rac2 is isoprenylated and bound to GDI while in the cytosol (19). These models predict that an activation signal causes members of the GTPase class to be dissociated from GDI and localize to the membrane. Once at the membrane, the small GTPases can interact with exchange factors that load it with GTP and allow for interaction with downstream effectors. While this thesis does not readdress the Rac2 RhoGDI association in the literature, we do provide immunofluorescent and western blot evidence that show Rac2 and RhoGDI in similar subcellular fractions. Using a subcellular fractionation protocol we demonstrate that Rac2 can be localized to the membrane when provided the two signals consisting of PMA and GTPγS. Further, our results confirm that Rac2 activation is a two-part phenomenon.
requiring integrin engagement and either PMA or CCL2 exposure. Using immunofluorescent staining, we show that statin pretreatment decreases the association of RhoGDI with Rac2, again supporting this known relationship.

When Rac2 becomes activated through a coupled CCL2-β2 integrin activation signal, a complex of proteins including Rac2, actin and myosin IIA are assembled. This complex is conserved in THP1 monocytes, BMDM, and in an initial screening of human peripheral blood monocytes. We demonstrate that formation of the complex is isoprenylation dependent. This is a key finding that underlies the purpose of studying this modification and this class of proteins. The dependency on isoprenylation is demonstrated by specific substrate supplementation with GGPP and FPP, which allows recovery of complex assembly after 10μM atorvastatin therapy. We are further able to characterize the complex as dependent on actin polymerization and myosin IIA activation because both cytochalasin D and blebbistatin inhibit complex formation.

The formation of the complex reorganizes the cytoskeleton and creates an environment conducive for downstream signaling. We observe HuR translocation to the cytoplasm as a significant event tied to CCL2 coupled β2 integrin activation of Rac2. HuR translocation is significant because of its effects on stabilization of inflammatory and angiogenic mRNA transcripts. As mentioned above, the Bender lab has shown prolongation of message half-lives for transcripts that include TNF-α, IL-3, VEGF and MMP-9 in an HuR dependent manner (35, 36, 37). Our experiments show consistent linkage of CCL2 coupled β2-integrin Rac2 activation with HuR translocation through a
variety of modalities. We also show the entire process to be isoprenylation dependent, which directly links the effect of statin not only to complex formation but also the propagation of inflammatory signals to HuR-mediated gene expression.

To further characterize the determinants of HuR translocation, cells from wild type, Rac2KO, and Myosin IIA KO’s were utilized to discern the relative contributions of each protein. Knockout experiments demonstrate both Rac2 and Myosin are critical to HuR translocation. In fact, bone marrow-derived macrophages from their respective knock out mice demonstrate phenotypic similarities in their diminished response to integrin signaling. Given the novelty of the Rac2-myosin IIA interaction, we conducted experiments to more closely study their relationship under CCL2 coupled β2-integrin activation. Utilizing the Proximity Ligation Assay (PLA), we were able to quantify the close relationship between Rac2 and Myosin IIA.

There remains more to explore in the mechanisms related to regulation of Rac2 prenylation. Our assays did not assess a time frame for cell dependent recovery of either isoprenylation or protein turnover. Future experiments are needed to elucidate this story.

While our studies focused on a CCL2 coupled β2 integrin stimulation, more studies should be carried out with other important chemokines such as CCL5 and CX3CL, which are important in macrophage recruitment and activation in vitro and in vivo. Also, experiments involving oxLDL assays should be completed to better characterize the microenvironment of the plaque and study Rac2 specific signals.
As more basic and clinical studies are completed, the relative contribution of the HuR activation event and integrin mediated signaling pathway in the progression of atherosclerotic plaques will be uncovered. HuR serves as a critical molecular switch in gene expression with respect to the activation and differentiation of immune cells. The above-described integrin-Rac-myosin axis appears to play a critical role in HuR activation and serves as a pleiotropic target of statin therapy. Fully understanding the mechanisms behind this pleiotropic effect of statins has the potential to yield Rac2 and myosin as potential targets for novel molecular therapeutics that modulate the immune system.
Figure 1: **Baseline Rac2 and RhoGDI subcellular localization.** THP1 cells were lysed with separation of cytoplasmic and membrane fractions utilizing thermo scientific subcellular fractionation buffers. Two separate subcellular fractionation protocols were utilized for comparison. Western blot analysis performed with anti-Rac2 (C-terminus) and anti-RhoGDI antibodies.
Figure 2: Effect of atorvastatin on Rac2 RhoGDI interaction. A) THP1 cells were adhered to poly-l-lysine coverslips, permeabilized, and immunofluorescent costaining was performed with RhoGDI, Rac2, and DAPI for nuclear definition in untreated and treated samples. Treated samples were incubated on poly-l-lysine coverslips in 10μM atorvastatin for 24hrs at 37°C prior to permeabilization. Untreated samples were also incubated overnight at 37°C B) Overlap coefficients from raw images were calculated using ImageJ software version 1.43.
Figure 3: **Immunofluorescence characterization of controls.** A) THP1 cells were adhered to poly-l-lysine coverslips, permeabilized, and immunofluorescent costaining was performed with IgG mouse and DAPI in row 1 and IgG rabbit and DAPI in row 2. Images were overlaid and merges are noted. B) Using the same process applied in A, THP1 cells underwent immunofluorescent costaining with anti-Na+/K+ ATPase and DAPI for nuclear definition. C) C57BL/6 and Rac2KO BMDMs were adhered to poly-l-lysine coverslips, permeabilized and immunofluorescent costaining was performed with anti-Rac2
Figure 4: **Effect of Atorvastatin on Rac2 localization.** THP1 cells subjected to the indicated treatment conditions. PMA incubation lasts for 30 minutes and was performed prior to cell lysis. Atorvastatin incubation lasted for 24 hours prior to lysis. Lysis was performed with separation of cytoplasmic and membrane fractions using thermo scientific subcellular fractionation buffers. Samples were incubated with 100μM GTPγS for 15 minutes at 37°C before or after subcellular separation as indicated. Anti-Rac2 C-terminus antibodies were used for detection. Localization markers are provided for cytoplasmic fraction (HSP90) and membrane fraction (Na/K ATPase).
Figure 5: Characterization of C-terminus and full recombinant Rac2 antibody classes. Antibodies directed at C-terminus display isoprenylation dependent binding compared to monoclonal antibodies. A) This is a graphical representation of the differences between the two major antibodies used in our assays. As shown, the Abcam and Santa Cruz antibody are targeted towards the C-terminus of the Rac2 protein which is the same location that the isoprenylation modification targets. The Millipore antibody was designed to recognize a recombinant form of the Rac2 protein. B) THP1, C57BL/6 and Rac2KO cells were incubated with 10μM atorvastatin for 24 hours. Samples were then lysed, treated with 100μM GTPγS, and subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. Anti-Rac2 antibodies to the C-terminus (Abcam) and full recombinant (Proteintech) Rac2 protein were used for detection by western blot.
Figure 6: **Effect of Rac2 localization in BMDM.** BMDM where isolated from femurs and tibia of C57BL/6 mice. Cells where incubated in L-cell media + fully supplemented RPMI for 7 days after which they were exposed to 10uM atorvastatin for 24hrs, lysed utilized subcellular fraction protocol, and loaded into gel. Western blots were stained with anti-Rac2 Abcam (c-terminus) and Millipore (full recombinant) antibodies.
Figure 7: Effect of β 2 integrin engagement and PMA on Rac2 activation. THP1 cells were either incubated on Fc coated or ICAM coated plates for 60 minutes with PMA co-incubation as indicated. GTPγS sample is provided as positive control. Cellular lysis and pulldown with PBD-pak beads were performed according to Rac GTPase activation assay. Aliquots were taken for total cell lysate (TCL). Anti-Rac2 C-terminus antibody or anti-Rac1 antibody was used for protein detection by western blot in pulldown material and total cell lysates as indicated.
Figure 8: Effect of time dependent PMA incubation on complex formation. THP1 cells were incubated with PMA for 0, 1.5, 5 and 15 minutes as indicated. Cellular lysis and pulldown were performed according to Rac GTPase activation assay. Samples were run through 4-15% polyacrylamide gel at 200mV for 30minutes. Gel was then stained with Coomassie brilliant blue for 1 hour and then destained with destaining solution. Indicated bands on polyacrylamide labeled at roughly 40kDa and 200kDa were extracted and sent for mass spectrometry analysis. Image developed on Licor system.
**Actin – 41.7 kDa**

78.7% Coverage

**Myosin-9 (nonmuscle myosin IIA) – 226 kDa**

61.5% Coverage

Figure 9: Mass spectrometry results demonstrate actin and Myosin IIA. A) 41.7 kDa band has 78.7% homology with actin. B) 226 kDa band has 61.5% coverage of nonmuscle Myosin IIA. These proteins are identified from Coomassie bands in (Figure 8).
Figure 10: **Effect of atorvastatin complex formation.** THP1 cells were incubated with 0.1, 1, 10 and 100μM of atorvastatin over 24 hour period. Samples were treated with 100μM GTPγS for 15mins at 37°C, lysed and subjected to pulldown with PBD-pak beads. Aliquots were set aside for total cell lysates (TCL). Two polyacrylamide gels were run at 200mV for 30 mins. Gel 1 was stained with Coomassie brilliant blue for 1 hour and then destained with destaining solution. Image developed on Licor System. Gel 2 was transferred to western and stained with anti-Rac2 C-terminus antibody and anti-actin as indicated.
Figure 11: **Effect of GGPP and FPP supplementation on Atorvastatin mediated complex inhibition.** THP1 cells were incubated with 10μM atorvastatin for 24 hours and provided no substrate, 5μM FPP, 5μM GGPP or 5μM FPP + 5μM GGPP as indicated. Negative control without statin therapy or PMA is provided. Positive control with PMA is provided. Samples were lysed and separated aliquots for pulldown and total cell lysates. Pulldown samples were treated with 100μM GTPγS for 15 mins at 37°C, and subjected to PBD-pak beads. Aliquots were set aside for total cell lysates (TCL). Two polyacrylamide gels were run at 200mV for 30 mins. Gel 1 was stained with Coomassie brilliant blue for 1 hour and then destained with destaining solution. Image of gel developed on Licor system. Gel 2 was transferred to western and stained with anti-Rac2 and anti-actin as indicated.
Figure 12: Effect of Cytochalasin D on complex formation. THP1 cells were incubated with 0.1, 1, and 10μM cytochalasin D and 100ng/mL of CCL2 as indicated. Negative control without CCL2 or Cytochalasin D is provided. Positive control with CCL2 is provided. Samples were lysed, treated with 100μM GTPγS for 15mins at 37°C, and subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. Anti-Rac2 antibodies (full recombinant), anti-actin, and anti-Myosin IIA antibodies were used for detection by western blot.
Figure 13: **Effect of Blebbistatin on complex formation.** THP1 cells were incubated with 2, 10, and 50μM blebbistatin as indicated. Negative control is provided with no PMA. Positive control is provided with PMA. Samples were lysed, treated with 100μM GTPγS for 15mins at 37°C performed according to Rac GTPase activation assay. Samples were run through 4-15% polyacrylamide gel at 200mV for 30mins. Gel was then stained with Coomassie brilliant blue for 1 hour and then destained with destaining solution. Image was developed on Licor System.
Figure 14: Knockdown of Rac2 Myosin IIA interaction in Rac2KO BMDMs. BMDM from C56BL/6 and Rac2KO are exposed to CCL2. Samples were lysed, treated with 100uM GTPγS for 15mins at 37°C, and subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. Anti-Rac2 (full recombinant), anti-Rac1, and anti-CDC42 antibodies were used to detect respective proteins by western blot. Total cell lysates (TCL) are provided.
Figure 15: **Effect of PMA and history of current statin use on complex formation in human derived peripheral WBC’s.** Human monocytes were isolated from whole blood in heparinized tubes from 4 volunteers who varied by gender and statin usage. Whole blood was fractionated by centrifugation for 30 minutes at 900g. Mononuclear lymphocyte cell layer was isolated, added to HBSS solution and re-suspended in RPMI-1640. Cells were then stimulated with PMA for 30 minutes, lysed, treated with 100μM GTPγS for 15 minutes at 37°C, denatured in sample buffer and run through 4-15% polyacrylamide gel at 200mV for 30 minutes. Gel was then stained with Coomassie brilliant blue for 1 hour and then destained with destaining solution. Image was developed on Licor System.
Figure 16: Characterization of time to loss of isoprenylation with Atorvastatin. A) THP1 cells were incubated with 10μM Atorvastatin for 0, 1.5, 3, 6, 12, 24 and 48 hours. The first sample group was lysed, subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. The second sample group was lysed, treated with 100μM GTPγS for 15mins at 37°C, and subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. Anti-Rac2 antibodies to the c-terminal (Abcam) and full (Millipore) recombinant Rac2 protein were used for detection by western blot as indicated. B) Quantification of ratio of pulldown +/- GTPγS to loading control is provided.
Figure 17: **Effect of Atorvastatin on $\beta$ 2 integrin engagement and PMA induced HuR translocation.** THP1 cells were adhered to either poly-l-lysine or ICAM coated coverslips and incubated in PMA or Atorvastatin 10$\mu$M for 24 hours as indicated. These cells were then permeabilized, and immunofluorescent costaining was performed with anti-HuR and DAPI for nuclear definition.
Figure 18: **Effect of Rac2 isoprenylation state on Rac2-Myosin IIA interaction and subsequent HuR translocation.** A) THP1 cells were incubated with 10μM Atorvastatin for 24 hours and were terminally incubated with CCL2 for 15 minutes. Samples were provided no precursor, squalene, FPP, or GGPP over the 24 hours prior to lysis as indicated. Samples were lysed, treated with 100μM GTPγS for 15 minutes at 37°C and subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. B) Samples with same treatment from conditions if figure A were fixed and stained for confocal analysis. Anti-HuR, anti-actin and DAPI staining for nuclear definition are utilized.
Figure 19: Effect of β2 integrin engagement and CCL2 on Rac2 interaction with Myosin IIA. Human monocyte cells are incubated with PMA and 100mg/mL of MCP1 for 15 minutes. Samples were lysed and subjected to pulldown with PBD-pak beads according to Rac GTPase activation assay. Anti-Rac2 (full recombinant) and anti-Myosin IIA antibodies were used to detect Rac2 and Myosin IIA by western blot.
Figure 20: **Characterization of CCL2 coupled B2 integrin engagement on Rac2 Myosin IIA interaction as determined by proximity ligation assay.** Samples are subjected to CCL2 alone, ICAM alone and CCL2+ICAM as indicated. Cells were fixed to coverslips, permeabilized, and stained with anti-Rac2 (full recombinant) and anti-Myosin IIA. A PLA probe was then utilized in conjunction with a ligation-ligase solution to hybridize the two PLA probes in close proximity. An amplification solution containing fluorescently labeled oligonucleotides was provided with polymerase allowing the creation of a rolling circle amplification product signal detected by microscopy. Individual images were overlaid and merges displayed as indicated.
Figure 21: Effect of CCL2 coupled β 2 Integrin engagement on HuR translocation. A) Human peripheral monocytes were subjected to ICAM alone, ICAM+Mg/Mn, Fc+Mg/Mn, or ICAM+CCL2 or Fc as a negative control for the ICAM-Fc fusion protein, and stimulated with either divalent cation or CCL2. B) Analysis of the translocation is provided as measurements of percent translocation and translocation factor determination.
Figure 22: Significance of Rac2 in Chemokine-coupled β2 Integrin-induced HuR Translocation. A) BMDM isolated from C57BL/6 and RAC2KO are subjected to CCL2+ICAM. B) Quantitative analysis of HuR translocation by translocation factor and translocation percentage are provided.
Figure 23: Significance of Myosin IIA in Chemokine-coupled β2 Integrin-induced HuR Translocation. A) BMDM from myosin knockout and heterozygous mice are subjected to ICAM+CCL2 or CCL2 alone stimulation. Samples are stained with DAPI, HuR, and actin and subfigures are merged. B) Quantitative analysis of HuR translocation by translocation factor and translocation percentage for each experimental condition are provided.
REFERENCES

14) Murphy, Sabina A., Christopher P. Cannon, Stephen D. Wiviott, Carolyn H. McCabe, and Eugene Braunwald. "Reduction in Recurrent Cardiovascular Events With Intensive Lipid-Lowering Statin Therapy Compared With Moderate Lipid-


28) Khallou-Laschet, Jamila, Aditi Varthaman, Giulia Fornasa, Caroline Compain, Anh-Thu Gaston, Marc Clement, Michaël Dussiot, Olivier Levillain, Stéphanie


