Phagocytic communication during apoptotic cell clearance and inflammation

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Abstract

Clearance of apoptotic cells is essential for tissue homeostasis, development and inflammation resolution; it is carried out by professional phagocytes, such as macrophages, and non-professional phagocytes, such as epithelial cells. In most tissues, such as the lung and the brain, professional and non-professional phagocytes reside in close proximity, but it is not known how they co-regulate the cell clearance process. This thesis research has revealed that macrophages redirect the phagocytosis of epithelial cells and this in turn has functional consequences. Data are presented showing that macrophages secrete insulin-like growth factor-1 (IGF-1) after encountering apoptotic cells or the Th2 cytokines IL-4 and IL-13. This IGF-1 from macrophages dampens uptake of the apoptotic cells but enhances the uptake of significantly smaller microparticles by non-professional phagocytes. The IGF-1 mediated redirection of phagocytosis is rapid and reversible. Moreover, macrophages themselves appear resistant to this IGF-1 effect, maintaining normal phagocytic capacity. In a model of allergic airway inflammation, disruption of IGF-1 signaling in airway epithelial cells results in exacerbated disease. Further, productive IGF-1 signaling is most important during allergen sensitization in limiting tissue inflammation. In addition to IGF-1, alveolar macrophages also secrete microparticles with anti-inflammatory properties, and these microparticles inhibit the expression of a variety of inflammatory cytokines produced by airway epithelial cells exposed to HDM allergen. Collectively, these results provide new insights on communication between phagocytes within a tissue, and how this communication can influence tissue inflammation to allergens.

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List of Abbreviations

AB	Apoptotic blebs
ABCA1	ATP-binding cassette transporter-1
ABCA7	ATP-binding cassette transporter-7
ALS	Acid labile subunit
ANOVA	Analysis of variance
Arp2/3	Actin-related proteins 2/3
ARF6	GTP-bound ADP-ribosylation factor 6
ATP	Adenosine triphosphate
ATP11C	Adenosine triphosphatase type 11C
BAI1	Brain angiogenesis inhibitor 1
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CC3	Cleaved caspase-3
CCSP	Club cell secretory protein
Cdc42	Cell division control protein 42 homolog
CDR	Circular dorsal ruffles
CED	Cell death defective
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
ChIP	Chromatin immunoprecipitation

CHO	Chinese hamster ovary cells
CML	Chronic myelogenous leukemia
COPD	Chronic obstructive pulmonary disease
CRKII	Chicken tumor virus no. 10 regulator of kinase
CSF	Colony-stimulating factor
CytoD	Cytochalasin D
DC	Dendritic cell
Dock180	Dedicator of cytokinesis 180
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELMO	Engulfment and cell motility
EM	Electron microscopy
ER	Estrogen receptor
ESCRT	Endosomal sorting complex required for transport
ETC	Electron transport chain
EV	Extracellular vesicle
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FKN	Fractalkine
GAS6	Growth arrest specific 6
GEF	Guanine nucleotide exchange factor

GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GH	Growth hormone
GT	Glucose transporter
GTP	Guanosine triphosphate
GULP	Engulfment adaptor phosphotyrosine binding domain-1
H&E	Hematoxylin and eosin
HDM	House dust mite
HGF	Hepatic growth factor
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSV	Herpes simplex virus
ICAM	Intercellular adhesion molecule
IFIT2	Interferon induced protein with tetratricopeptide repeats 2
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IGF-1R	IGF-1 receptor
IGFBP	IGF binding protein
IL	Interleukin
ILC2	Innate lymphoid cells, type 2
ISX	ImageStreamX
KLF4	Kruppel-like factor 4
LAP	LC3 – associated phagocytosis

LC3	Light chain 3
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LRP1	Low density lipoprotein receptor-related protein 1
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cells
MLCK	Myosin light-chain kinase
MMP	Mitochondrial membrane potential
MMPs	Matrix metalloproteinases
MFG-E8	Milk fat globule EGF factor 8
MP	Microparticle
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OVA	Ovalbumin
PANX1	Pannexin 1
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PI3K	Phosphatidylinositol-4,5-biphosphate 3-kinase
PPAR	Peroxisome-proliferator activated receptor
PROS1	Protein S

PSQ	Penicillin, streptomycin, glutamine
PtdSer	Phosphatidylserine
PTX3	Pentraxin 3
Rac1	Ras-related C3 botulinum toxin substrate 1
RANTES	Regulated on activation, normal T cell expressed and secreted
RhoA	Ras homolog gene family, member A
RhoG	Ras homology Growth-related
ROCK1	Rho-associated coiled-coil-forming kinase I
ROS	Rod outer segments
RPE	Retinal pigment epithelial cells
rtTA	Reverse tetracycline-controlled transactivator
RXR	Retinoid X receptors
S1P	Sphingosine 1-phosphate
S6K	S6-ribosomal protein kinase
SH2	Src homology 2
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
TAM	Tyro3, Axl, Mer
tetO	Tetracycline-responsive promoter element
TGFβ	Transforming growth factor-β
Th	T-helper cells

TIM	T-cell immunoglobulin domain and mucin domain
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRIO	Triple functional domain protein
TSLP	Thymic stromal lymphopoeitin
TULP1	Tubby-like protein-1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UCP2	Uncoupling protein 2
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor
Xkr8	Xk-related protein 8
YFP	Yellow fluorescent protein

Chapter I

Introduction

Billions of cells undergo apoptosis every day as a result of tissue development, pathogenesis, and maintenance of homeostasis[1-3]. These apoptotic cells are recognized and efficiently removed by professional phagocytes, such as macrophages, and non-professional phagocytes, such as epithelial cells[4]. Defects in corpse removal have been linked to the development of inflammation, atherosclerosis and autoimmune disorders[2,5,6]. Within a tissue, such as liver or the lungs, professional and non-professional phagocytes reside in close proximity. As these two different populations are both capable of clearing apoptotic cells, important and unanswered questions arise: (a) Would these different phagocytes communicate and regulate the corpse clearance process, and (b) if there were differential regulation of phagocytosis, how would disruption of this regulation affect tissue integrity.

First, one rationale suggesting that there may indeed be a hierarchy in cell clearance is that professional phagocytes, as sentinels of the immune system, are primed to phagocytose various targets to rapidly respond to any foreign or inflammatory agents. Meanwhile, non-professional phagocytes have primary responsibilities that are essential to maintenance of tissue health. For example, fibroblasts are important for tissue repair as they produce numerous factors involved in extracellular matrix remodeling and growth of new blood vessels. Thus, in the case of a wound or other inflammatory contexts causing tissue damage, it is reasonable to hypothesize that non-

professional phagocytes may be redirected to initiate healing and re-generation as opposed to apoptotic cell clearance. My investigation revealed that macrophages, one type of professional phagocytes, secrete insulin-like growth factor 1 (IGF-1) that influence phagocytic uptake by non-professional phagocytes. Specifically, IGF-1 suppresses apoptotic cell phagocytosis, but enhances uptake of microparticle, a type of extracellular vesicle, by non-professional phagocytes. Interestingly, in a model of allergic airway inflammation, disruption of IGF-1 signaling in airway epithelial cells leads to exacerbated disease. Mechanistically, alveolar macrophage-derived microparticles dampen inflammatory cytokine production by epithelial cells, a type of non-professional phagocyte, upon encountering allergen.

In this chapter, I will provide the necessary background on this investigation; specifically, I will focus the background information on the following topics: apoptotic cell clearance, professional and non-professional phagocytes, insulin-like growth factor-1, extracellular vesicles, and the *in vivo* models of allergic airway inflammation.

1: Apoptotic cell clearance

Apoptotic cell clearance can be divided into distinct events, beginning with the release of “find-me” signals by the dying cell. These factors recruit phagocytes to the vicinity of the apoptotic cell, which is subsequently recognized by phagocyte via the display of “eat-me” signals by the apoptotic cell. Following apoptotic target recognition, several pathways are activated in the phagocyte, resulting in cytoskeleton rearrangement and engulfment of the apoptotic cell. The engulfed apoptotic target, now

in a phagosome, is degraded as the phagosome fuses with lysosomes. In the following sections, these processes will be further discussed in addition to metabolic and immune consequences of apoptotic cell engulfment.

1.1. *Recruitment of phagocytes - "find-me" signals*

Apoptosis, or programmed cell death, is elicited through an extrinsic (ligand-receptor binding mediated) or intrinsic pathway, via toxins or other pathophysiological conditions leading to the activation of caspases. Apoptotic cells then undergo several morphological changes, such as cell shrinkage, chromatin condensation and membrane blebbing[7]. To attract motile phagocytes to their proximity, cells undergoing apoptosis release diffusible "find-me" factors such as lysophosphatidylcholine (LPC); sphingosine 1-phosphate (S1P), fractalkine (FKN)/CX3CL1, and nucleotides ATP and UTP[8-10] (**Figure 1.1**). LPC, initially discovered in the supernatants of apoptotic MCF7 cells, is released upon caspase-3-mediated activation of calcium-independent phospholipase A_2, and can induce the migration of mouse monocytic cells, THP-1[11]. Multiple studies on the role of LPC as a chemotactic factor have shown conflicting reports on whether additional serum factors are necessary for LPC to act as a chemoattractant[12-14]. There is also controversy on the exact receptor that recognizes soluble LPC as initial reports of G-coupled protein receptors, G2A and GPR4, have recently been called into question[14-17]. Additionally, both healthy and autoimmune patients have high serum concentrations of LPC[18], suggesting that the function of LPC as a "find-me" signal may be context and cell-dependent and will require more thorough investigations *in vivo*.

Another lipid mediator, S1P, is a known migratory factor for T- and B- cells[19], and has also been shown to stimulate chemotaxis of THP-1 cells as well as primary monocytes[20]. S1P is thought to be generated during apoptosis via S1P kinase 1[20] and kinase 2, with the latter associated with capase-1 activation[21], and recognized by G coupled protein receptors SIP_1 through $S1P_5$. However, exactly how S1P is released, either extra- and/or intra-cellularly, is currently unknown.

Chemokine CX3CL1, or FKN, is a transmembrane adhesion protein that is cleaved upon apoptosis, releasing a soluble 60kDA fragment, that was shown to stimulate the chemotaxis of human monocyte derived macrophages[22]. In Burkitt lymphoma cells, FKN release was shown to be associated with apoptotic blebs. Mice lacking the FKN receptor, CX3CR1, had fewer macrophages, as compared to control mice, in the germinal centers that typically have high levels of apoptosis due to B-cell turnover[22]. Additionally, both *FKN*$^{-/-}$ mice and *CX3CR1*$^{-/-}$ mice have increased apoptotic debris in the brain following ethanol injection, which induces neuronal apoptosis[23]. Microglia in *CX3CR1*$^{-/-}$ mice did not have a deficiency in the ability to phagocytose apoptotic targets, but had fewer associations with apoptotic debris compared to wild type microglia, suggesting that local movement towards apoptotic debris may be impaired[23].

Recently, nucleotides ATP and UTP have been shown to be released from apoptotic cells and mediate migration through the $P2Y_2$ receptor on monocytes and macrophages[24]. In an *in vivo* model of thymic clearance, co-administration of apyrase, which hydrolyses ATP into AMP and inorganic phosphates, and dexamethasone, a

glucocorticoid that induces apoptosis, led to an increase in apoptotic cell numbers compared to injection with dexamethasone alone[24]. As phagocytes treated with apyrase have normal phagocytic capacity, this result suggested that fewer phagocytes were recruited, due to the breakdown of ATP/UTP, and in turn, the decreased clearance. Additionally, the nucleotide release was shown to be mediated through Pannexin-1, a transmembrane protein that forms a hexameric channel that opens upon cleavage of the C terminal tail via caspase-3 and -7[25]. A question that remains to be investigated regarding all known and yet to be discovered "find-me" signals is the range and stability of these signals. Particularly, whether these signals serve primarily to recruit phagocytes within the microenvironment or whether they serve as long-range homing beacons for phagocytes within the bloodstream or emerging from bone marrow remains to be determined.

1.2. *Detection of apoptotic cells – "eat-me" signals*

Following migration of the phagocytes to the proximity of the dying cell, phagocytes recognize apoptotic cells through the display of "eat-me" signals on their surface[26] (**Figure 1.1**). Changes in glycosylation patterns and charge on the cell surface, expression of intercellular adhesion molecule (ICAM)-3[27], and display of oxidized low-density lipoprotein[28] and calrecticulin[29] are just among a few of the proposed signals. The most extensively characterized "eat-me" signal is the lipid phosphatidylserine (PtdSer). PtdSer is a phospholipid that normally resides on the inner leaflet of the lipid bilayer and upon execution of programmed cell death, relocates to the outer leaflet[30,31].

Recently, adenosine triphosphatase type 11C (ATP11C)[32], a flippase that moves PtdSer from the outer leaflet to the inner leaflet, and a Xk-related protein 8 (Xkr8)[33], a scramblase that nonspecifically moves phospholipids bidirectionally, were shown to be involved in the externalization of PtdSer during apoptosis. Both ATP11C and Xkr8 contain caspase cleavage sites; activation of caspases results in the inhibition of ATP11C and activation of Xkr8[32,33]. Apoptotic cells that overexpressed caspase-resistant ATP11C and Xkr8 could not display PtdSer on the cell surface and were less likely to be engulfed by phagocytes[32-34]. The prominence of PtdSer as an "eat-me" signal is highlighted by the observation that blocking of PtdSer on apoptotic cells, either using Annexin V or competitively with PtdSer-liposomes, is sufficient to decrease their clearance by phagocytes. Additionally, blocking PtdSer recognition by phagocytes *in vivo* leads to production of autoantibodies and immunoglobulin deposition on glomeruli[35]. Lastly, PtdSer exposure leading to phagocytosis is evolutionary conserved in *Drosophila*[36] and the nematode *Caenorhabditis elegans*[37].

PtdSer can be directly recognized by receptors on the phagocyte, such as Brain angiogenesis inhibitor 1 (BAI1)[38], T-cell immunoglobulin domain and mucin domain (TIM) 4[39-41] (and additional family members, TIM3[42,43] and TIM1[44,45]), and Stabilin-2[46]. Certain bridging molecules, such as milk fat globule EGF factor 8 (MFG-E8), growth arrest specific 6 (GAS6), and protein S (PROS1), can also recognize PtdSer and then bind to cognate receptors on the phagocyte. MFG-E8 binds integrin $\alpha_v\beta_3$ on phagocytes[47] and *Mfge8* $^{-/-}$ mice develop autoimmune disease, with production of autoantibodies and glomerulonephritis by 40-weeks of age[48]. Interestingly, the "find-me" signal, FKN, was

found to stimulate macrophage[49] and microglial production[50] of MFG-E8, a way of potentially "priming" phagocytes for cell clearance. GAS6[51] and PROS[52], both found in the serum, serve as bridging molecules for the "TAM" family tyrosine kinase receptors, composed of AXL, TYRO3, and MER, with distinct affinities for each receptor[53,54]. *Mer*$^{-/-}$ mice have higher autoantibodies compared to control mice[55]; further, mice harboring a triple knockout of the TAM receptors have a severe autoimmune phenotype with aberrant proliferation of lymphocytes, among other pathologies[56], suggesting the importance and diverse function of each individual receptor. Three new ligands, galectin-3[57], Tubby and tubby-like protein-1 (TULP1)[58], for the TAM receptors that also mediate apoptotic cell uptake have recently been described, but unlike GAS6 and PROS, they bind apoptotic cells through a PtdSer-independent mechanism, which to this date has not been clearly elucidated.

Receptors for other "eat-me" signals have also been implicated; for example, members of the scavenger receptor family[59-61], including CD36[62] and CD68[63], and lectin-like oxidized LDL receptor-1[64], have been shown to be involved in apoptotic cell clearance. Calrecticulin, expressed on the apoptotic cell surface, can bind to low density lipoprotein receptor-related protein 1 (LRP1)/CD91[29]. As most phagocytes express more than one phagocytic receptor and given the number of phagocytic receptors, one question is the relative contribution of these receptors to clearance *in vivo*. It is possible that some of these receptors serve as tethering molecules[65]. Interestingly, TIM4, a receptor that recognizes PtdSer directly, does not require its cytoplasmic portion to facilitate apoptotic cell uptake[66]. It is also curious whether one phagocytic receptor can

substitute for another receptor, not only during the apoptotic cell uptake but also in downstream physiological consequences. The answer may provide insights on the purpose of individual receptors as well as differences in signaling events.

In addition to “eat-me “signals, “don’t eat-me” signals have also been described (e.g. CD47[67] and CD31[68]). Erythrocytes lacking expression of CD47 are rapidly cleared by splenic red pulp macrophages[67]. The combination of “eat-me” and “don’t eat-me” signals has been suggested to play a key role in limiting phagocytic clearance *in vivo*[29,68].

1.3. *Phagocytic signaling – Rac activation*

Phagocytic receptor engagement activates one of two partially redundant signaling pathways resulting in the activation of Rac1, a Rho family GTPase (**Figure 1.1**). Studies in mammalian models and *C. elegans* have identified multiple evolutionarily conserved players that partake in two separate but potentially linked pathways[69]. The first pathway consists of mammalian LRP1/nematode cell death defective-1 (CED-1), an adapter protein, engulfment adaptor phosphotyrosine binding domain-1 (GULP)/CED-6, and an ATP-binding cassette (ABC) transporter (ABCA1 or 7/CED-7) whose precise phagocytic function is unknown[70-73]. In mammals, LRP1 and GULP directly interact, inducing Rac activation and ultimately clearance of apoptotic cells[74]; players that might mediate Rac activation have not yet been identified. In mammalian models, Stabilin-2 has also been shown to signal through GULP[75]. The second signaling pathway consists of CRK (chicken tumor virus no. 10 regulator of

kinase) II/CED-2, Dock180 (dedicator of cytokinesis 180)/CED-5 and ELMO (Engulfment and cell motility)/CED-12[76-80]. The ELMO-Dock180 complex serves as a guanine nucleotide exchange factor (GEF) to activate Rac1/CED-10. The exact role of CRKII in this complex remains something of a mystery; siRNA-mediated knockdown studies show that CRKII, Dock180 and ELMO are all required for efficient clearance of apoptotic cells[81]. However, mutations in the CRKII-binding sites of Dock180 did not seem to dampen apoptotic cell uptake[81]. While Bai1 is directly upstream of ELMO, TRIO (triple functional domain protein), another nucleotide exchange factor for the small GTPase RhoG, and RhoG, were shown to interact with ELMO1[82] to activate Rac1, resulting in cytoskeletal rearrangement and phagocytosis of the apoptotic target. Other Rho family GTPases have also been implicated in apoptotic cell uptake; Cdc42 is important for clearance[83,84], while activation of RhoA suppresses engulfment[85,86].

1.4. *Metabolic and immunological consequences of apoptotic cell engulfment*

Internalized apoptotic targets are degraded in a process known as phagosome maturation in which the phagosome becomes increasingly acidified through fusion with lysosomes[87] (**Figure 1.1**). In recent years, there is increased focus on how the phagocyte processes the engulfed cargo. Catabolic products of lipid and carbohydrates feed into the electron transport chain (ETC) in the mitochondria, creating an electrochemical gradient (termed mitochondrial membrane potential or MMP), which drives ATP production. Phagocytes that have ingested apoptotic cells, but not bacteria or zymosan, have increased MMP, but not enhanced ATP production[88]. Uncoupling

protein 2 (UCP2), which “uncouples” the electrochemical gradient from ATP generation, was also increased following co-incubation of apoptotic targets and phagocytes (**Figure 1.2**). Interestingly, loss of UCP2, which leads to an increase in MMP, negatively affected phagocytic ability[88]. Further, macrophages ingesting apoptotic cells showed greater phosphorylation of S6-ribosomal protein kinase (S6K), a substrate of mammalian target of rapamycin (mTOR)[89]. mTOR, as a major nutrient sensor, controls cellular growth and proliferation[90]. Furthermore, phagocytes ingesting apoptotic targets had greater proliferation and decreased death, suggesting that metabolic derivatives of the engulfed cell may stimulate mTOR activity. However, the relevance of this work to phagocytic clearance *in vivo*, in particular in homeostatic clearance, remains to be seen as mTOR activation only occurred in conditions in which phagocytes were deprived of amino acids[89].

Components of autophagy, a process by which cells can generate energy in nutrient-starved conditions through selective and regulated digestion of internal material, has been linked to apoptotic cell clearance in recent years[91]. Light chain 3 (LC3)-associated phagocytosis (LAP), in which LC3 become associated with the phagosome, can occur after engagement of TIM-4 with apoptotic cells or PtdSer-containing liposomes[92,93] (**Figure 1.2**). LC-3 containing phagosomes mature and degrade the internalized cargo much more rapidly than non-LC-3 containing phagosomes. However, it is unknown whether the contents of LC-3 containing phagosomes are utilized in a similar manner as autophagy.

One area of research that may shed some light on metabolic changes during apoptotic cell clearance is lipid and cholesterol homeostasis. After peroxisome-proliferator activated receptors (PPARs) and liver x-receptors (LXRs) bind fatty acids and oxysterols, they couple with retinoid X receptors (RXRs) to induce transcription of genes involved in lipid and cholesterol metabolism[94]. LXRs and PPARs are activated during apoptotic cell clearance; one consequence is the upregulation of phagocytic receptors such as MER and CD36, as well as opsonins (e.g. GAS6, MFG-E8), by the phagocyte[95-97] (**Figure 1.2**). Mice deficient in PPARs, LXRs, and RXRs, have impaired phagocytic clearance[95,96,98]. Interestingly, MER binding of PtdSer by macrophages elicits cholesterol efflux, mediated through lipid transporter ABCA1[99]. Surprisingly, this efflux did not require the presence of lipids from the apoptotic target, as synthetic targets could mimic the same effect. Recently, it was shown that the increase in transcription and translation of ABCA1 in clearance is mediated through the BAI1-ELMO1-Rac1 pathway and is independent of LXRs[100]. As lipid metabolism and nutrient processing play major roles in pathologies such as atherosclerosis, metabolic syndrome, and tumor progression, further studies on the exact mechanistic relationships may yield therapeutic targets.

One of the unique features of apoptotic cell clearance is that it is immunologically silent, with the production of transforming growth factor-β (TGFβ) and interleukin (IL)-10 accompanying corpse uptake[101-106]. Furthermore, engulfment of apoptotic cells can be immunosuppressive in both professional and non-professional phagocytes that have been treated with toll-like receptor (TLR) agonists[101,102,107-109]. In transplantation studies,

administration of apoptotic cells can induce tolerance by acting on macrophages and/or dendritic cell, allowing the presentation of “self”-antigens and generation of T-regulatory cells[110-113]. However, not all apoptotic cell phagocytosis results in a complete immunosuppressive takeover. Dendritic cell uptake of infected apoptotic cells results in the production of IL-6 and IL-17, which skews naïve T-cells to T_H17[114], suggesting that at times, both a pro-inflammatory and anti-inflammatory program can co-exist. Importantly, proper apoptotic cell clearance is necessary to prevent dying cells from undergoing secondary necrosis. Necrotic cells release their contents, which serve as auto antigens. Once these are phagocytized, breaks in self-tolerance occur through the production of auto-antibodies, deposition of immune complexes, and release of inflammatory cytokines[2]. Multiple mouse knockouts of “find-me” signals, engulfment receptors, and post-phagocytosis processing mediators show the development of a number of pathologies, such as autoimmune disease, atherosclerosis and neuropathy, and diabetes[5].

2: Professional and non-professional phagocytes and their role in disease

Phagocytes in the mammalian system can be generally divided into two categories based on their efficiency of phagocytosis: professional and non-professional. Professional phagocytes are hematopoietic cells of the innate immune system, such as neutrophils, macrophages and immature dendritic cells. Examples of non-professional phagocytes include epithelial cells, fibroblasts, and endothelial cells. In addition to greater proficiency in uptake, professional phagocytes have a more diverse target and

phagocytic receptor repertoire and are capable of producing reactive oxygen and nitrogen species in response to certain ingested targets[115,116]. Studies have shown that inhibition of phagocytosis in either population is detrimental for tissue and organismal health.

2.1. *Professional phagocytes*

Professional phagocytes are a major player in the both clearance and apoptotic processes[117-120]. Macrophages isolated from mice in which α_v integrins are conditionally deleted in myeloid cells have an impaired ability to clear apoptotic cells; these mice develop colitis spontaneously and have high levels of autoantibodies[121]. In a model of herpes simplex virus (HSV)-1 infection, deletion of *Lrp1* in dendritic cells decreased their ability to engulf apoptotic cells, presumably containing the virus, and to cross-present and activate CD8+ T-cells. Mice with deletion of *Lrp1* had greater viral titers and decreased survival compared to control mice[122]. Atherosclerosis is another disease in which failed apoptotic cell clearance, especially by macrophages, can exacerbate pathology. In both mouse models and humans, atherosclerotic lesions contain numerous uncleared apoptotic cells[118,119]. These apoptotic cells undergo secondary necrosis, creating unstable necrotic cores, which can then rupture, triggering acute coronary syndromes, such as myocardial infarction and stroke[123]. As previously mentioned, the BAI1-ELMO1-Rac1 pathway is involved in lipid homeostasis; on an atherosclerosis-prone background, deletion of *Bai1* results in dyslipidemia[100]. Mice with deletion of *Mer*[124] and *Mfge8*[125], also on an atherosclerosis-prone background, have

greater lesion size and accumulation of apoptotic cells as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Furthermore, mice with myeloid-specific deletion of *Lrp1* also have greater atherosclerotic lesion size than control mice due to the inability of knockout macrophages to control lesion apoptosis and accumulation of inflammatory monocytes[126-128].

2.2. *Non-professional phagocytes*

It is increasingly appreciated that non-professional phagocytes, which are often neighbors of current and future apoptotic cells, can play a significant and functionally relevant role in corpse clearance. In fact, in the absence of macrophages, mouse digits are able to form normally in the embryonic stage, albeit slightly delayed, via to the kinetically slower process of phagocytosis by non-professional phagocytes[129]. In the brain, multiple engulfment players, such as LRP1[130], MER[131], and ELMO1[132] have been implicated in synaptic pruning and neurogenesis by astrocytes and other glial cells. During mammary gland involution post-lactation, mammary epithelial cells (MEC) perform the majority of the clearance[133,134]. Profiling of MEC cell lines and primary cells reveal that they express scavenger receptor CD36, LRP1, and integrin $\alpha_v\beta_3$, and produce MFG-E8[133]. Additionally, upon engulfing apoptotic targets, MEC cell lines secrete TGFβ[133]. Mice with a global knockout of MFG-E8 exhibited defective involution, resulting in periductal mastitis, ductal ectasia, and impaired mammary gland development for subsequent litters[135,136]. Gut epithelial cells express BAI1 and can also clear apoptotic gut epithelial cells[137]. Mice with global deletion of *Bai1* have greater

weight loss and more severe disease in a model of dextran sulfate sodium-induced colitis. Specific deletion of *Bai1* in gut epithelial cells resulted in symptoms that mirrored the global knockout, while mice with deletion of *Bai1* in myeloid cells were comparable to wild-type mice, highlighting the importance of non-professional phagocytes mediating clearance in this system. Increased apoptotic cells are found in several pulmonary diseases, such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and asthma[138,139]. While defective clearance by alveolar and lung macrophages is implicated in these diseases, there is evidence that lung epithelial cells are also involved. Epithelial cells lacking the CF transmembrane conductance regulator (CFTR) have decreased corpse uptake due to increased RhoA activity[140]. Mice with conditional deletion of Rac1 in lung epithelial cells that are given house dust mite (HDM) allergen have worse allergic airway inflammation, as measured by immune cell infiltration and cytokine production[141]. Interestingly, these mice have decreased IL-10 levels, presumably due to decreased clearance; exogenous administration of IL-10 during HDM administration was able to significantly reduce disease severity[141].

2.3. *Semi-professional phagocytes*

One main difference between professional and non-professional phagocytes is that for non-professional phagocytes, corpse clearance is secondary to their primary responsibilities. There now exists a subset of phagocytes that function as semi-professional phagocytes wherein the clearance is equally important as non-clearance related duties. Two of the most well studied examples are Sertoli cells and retinal

pigment epithelial (RPE) cells of the eye. First, Sertoli cells are the “nurse cells” of the testis, as the they provide energy and nutrition, such as lactate and amino acids, to developing germ cells during spermatogenesis[142]. Sertoli cells are also primarily responsible for clearing apoptotic germ cells. Both *Elmo1*[143] and *Ucp2*[88] knockout mice have greater numbers of uncleared apoptotic germ cells compared to control mice. *Elmo1*$^{-/-}$ mice have disrupted seminiferous epithelium and decreased sperm output[143]. Additionally, ablation of all three TAM receptors results in infertile male mice, potentially due to their impaired phagocytic activity[144,145]. Another semi-professional phagocyte is the RPE that ingests rod outer segments (ROS) shed by photoreceptors daily. RPE cells lacking *Mfge8* or receptor $\alpha_v\beta_5$ have decreased ROS phagocytosis, however, only mice with *Mfge8* deletion have age-related vision loss[146-149]. Deletion of TAM receptors or just *Mer* alone results in retinitis pigmentosa[144,150-152]. Additionally, deletion of the ligands *ProS* and *Gas6* phenocopies *Mer* deleted mice, although the presence of even a single allele of the genes encoding either PROS or GAS6 rendered the mice completely normal without any defective phenotypes in the eye[153]. Taken together, clearance of apoptotic cells by these different populations of phagocytes is critical to the health status of an organism. While the majority of the studies have been completed using total body knockouts, the relative contributions of each population can be better understood by employment of tissue-specific deletions.

3: Insulin-like growth factor-1 function in health and disease

Our investigations identified a previously unknown biological function for IGF-1 as a modulator of phagocytic uptake in non-professional phagocytes. The current functions predominantly ascribed to IGF-1 are those involved in growth and proliferation, which are summarized below. Comprehension of these downstream physiological effects is necessary for future studies of phagocytic communication in development and tissue homeostasis.

3.1. *IGF-1*

IGF-1 is a 70-amino acid polypeptide with a molecular weight of approximately 7.6kDA[154]. It shares strong amino acid homology with IGF-II and insulin[154]. Due to alternative splicing, several isoforms of IGF-1 exists, with all known transcripts containing exons 3 and 4 that encode the domains of the mature polypeptide[155]. The true biological significance of the distinct isoforms is currently under investigation; there is some evidence that during development, there are kinetic differences in isoform expression patterns[156-158]. The majority of IGF-1 found in extracellular fluid is bound to IGF binding proteins (IGFBPs), of which there are six, with IGFBP3 being the predominant binding partner of IGF-1 and IGF-II[159]. When bound, IGF-1/BP3, and to some extent IGFBP5, also complexes with an additional acid labile subunit (ALS), which is not present in other IGF-1/BP complexes[159]. The primary purpose of IGFBPs is to prolong the half-life of IGF-1 (from 15 minutes to 7-20 hours when in complex with IGFBP3[160,161]) and sequester IGF-1, thereby restricting its bioavailability; however,

IGFBPs may also have secondary effects that can either be inhibitory, potentiate IGF-1 signaling, or independent of IGF-1[162]. Currently, matrix metalloproteinases (MMPs) have been implicated as the proteases that cleave IGFBPs[163-165] and release IGF-1 from the IGF-1/BP complex, with different MMPs having specificities for certain IGFBPs; however, there may be other yet to be defined proteases. The liver serves as one of the major sources of circulating IGF-1. Growth hormone (GH), released by the pituitary gland, signals to hepatocytes to produce IGF-1 and ALS, while hepatic nonparenchymal cells, such as Kupffer cells, have been reported as a source of circulating IGFBP3[166,167] (**Figure 1.3**). Nutrient availability also has an impact on serum-levels of IGF-1. Reduction in protein (25%) and caloric intake (50%) results in significant decreases in detectable IGF-1[168,169]. As IGF-1 is a major factor in cell and tissue growth (detailed below), mice with deletion of IGF-1 in the liver were surprisingly normal compared to their control counterparts, with only minimal retardation in growth despite having much lower levels of serum IGF-1, suggesting that there were numerous peripheral tissues that also produced IGF-1[170] (**Figure 1.3**).

IGF-1 signals through the IGF-1 receptor (IGF-1R), a membrane tyrosine kinase that is composed of two α subunits and two β units, with the transmembrane β units containing the kinase activity. Ligand binding triggers conformational changes that result in ATP binding and autophosphorylation[171] at tyrosine residues 1131, 1135, 1136[172]. IGF-1, IGF-II and insulin bind IGF-1R with different affinities, with IGF-1R preferentially binding IGF-1, followed by II and very weakly, insulin[173]. Recognition of IGF-1 occurs through a high concentration of cysteine residues in the receptor α-subunit[174]. Activation

of IGF-1R recruits adapter proteins containing Src homology 2 (SH2) domains, such as insulin receptor substrate (IRS) 1-4, Shc (in particular p66 isoform), eliciting canonical signaling and activation of the PI3K-Akt-mTOR pathway and the Ras-Raf-MAPK pathway (**Figure 1.4**). The receptor for IGF-II is the cation-independent mannose-6-phosphate receptor, a single membrane-passing protein with a shortened cytoplasmic tail[175,176]. Unlike IGF-1R, IGF-2r does not bind insulin and has much lower affinity for IGF-1. Additionally, binding of IGF-II to IGF-2r elicits very little signaling; instead it appears to act as a sink for IGF-II. Upon binding, IGF-2r is endocytosed and recycled back to the membrane, following release of ligand in the endosome[177,178]. The downstream physiological effects of IGF-1 signaling will be discussed in the following sections.

3.2. *IGF-1 in growth and development*

IGF-1, as its moniker suggests, is a major player in the development and growth of an organism. In humans, IGF-1 levels peak during pubertal maturation and then decline with age. Disruption of IGF-1 (and GH) signaling is a major area of research in the treatment of dwarfism and acromegaly. A series of total body knockout mice on the IGF-1 family members and receptors revealed the importance of these factors in development. *Igf1*$^{-/-}$ mice are born 60% of normal birth weight and the majority die as neonates, which may be dependent on the background[179-181]. Of the ones that survive to adulthood, the mice are infertile, have impaired bone, brain, and muscle development, and continue to lose weight until they reach a steady-state weight of approximately 30%

of their wild type counterparts[179,181,182]. *Igf2*$^{-/-}$ mice are also born smaller than control mice (60% of normal birth weight) and show a slight delay in ossification, similar to the *Igf1*$^{-/-}$ mice[180,183]. Interestingly, a heterozygous loss of *Igf2* is sufficient for decreased embryonic growth[183], while heterozygous deficiency of *Igf1* results in only a 10 to 20% reduction in birth weight in mice compared to control mice[179], suggesting that IGF-II is more important than IGF-1 for intrauterine growth. Importantly, *Igf2*$^{-/-}$ mice are fertile, survive to adulthood, and gain weight comparable to control mice[183,184], unlike *Igf1*$^{-/-}$.

A more severe phenotype is seen in *Igf1r*$^{-/-}$ mice, which are born at only 45% of normal birth weight, but die quickly after birth from respiratory failure, possibly due to muscle hypoplasia[180]. *Igf1r*$^{-/-}$ mice also have defects in bone and neuronal development and skin abnormalities. Double knockouts further revealed receptor preferences for each ligand. *Igf1*$^{-/-}$ *Igf1r*$^{-/-}$ knockouts phenocopy *Igf1r*$^{-/-}$ mice, while *Igf2*$^{-/-}$ *Igf1r*$^{-/-}$ are born weighing only 30% of normal weight and rapidly die from respiratory failure[180]. The additive effect of deleting *Igf2* and *Igf1r* suggests that IGF-II must signal through a second receptor, which later was identified as the insulin receptor[185,186]. Interestingly, ablation of *Igf2r*$^{-/-}$ results in pups that are much larger (135% of normal birth weight)[187]. These pups have organomegaly, are polydactyl, exhibit heart abnormalities, and die perinatally. Crossing *Igf2r*$^{-/-}$ onto either an *Igf1r* or *Igf2* null background rescued the mice, most likely because the excess IGF-II is prevented from signaling and stimulating overgrowth[187-189].

While a full transcriptome analysis has not been performed on all tissues affected by IGF-1, the results of the knock out mice and the knowledge of the activated signaling

pathways stimulated by IGF-1 binding suggest that IGF-1 modifies the gene expression of transcripts associated with cellular growth and proliferation. Additional studies have shown that IGF-1 is a major anti-apoptotic factor and mediates cell differentiation. Lungs from *Igf1*$^{-/-}$ E18.5 embryos show hypoplasia, a reduction in extracellular matrix, altered microvasculature, and increased inflammatory cells[190]. Microarray analysis show that, in the absence of IGF-1 signaling, genes involved in the pathways of MAPK, Wnt, cell adhesion signaling, tight junctions, leukocyte transendothelial migration, among others, can be either upregulated or repressed. For example, genes involved in lung organogenesis (e.g. *Klf2, Fgf18, Aqp5*) and vasculogenesis (e.g. *Ctgf, Vegfa, Xlkd1*) are all down-regulated[190]. cDNA microarrays of Chinese hamster ovary (CHO) cells stimulated with an IGF-1 analogue revealed significant increases in genes associated with cell division and proliferation, such as *Ska2, Mns1*, *Mrpl22*[191]. Since IGF-1 has such pleiotropic effects, selected areas of interest will be further detailed below. [192]

3.3. *IGF-1 in cancer*

The IGF-1 receptor is overexpressed in numerous cancers and neoplastic cell lines[193-196]. IGF-1 treatment promotes the proliferation and survival of many cell types[197-199] and is important for oncogenesis[200]. Decreasing IGF-1 levels in mouse models of colon cancer[201] and breast cancer[202] via a deletion of liver specific IGF-1 reduced cellular metaplasia, tumor size, and metastasis. Mice undergoing caloric restriction had decreased mammary tumor growth, in part due to decreased IGF-1 signaling and downstream activation of epithelial-to-mesenchymal transition process[203,204]. Microarray

profiling of MCF-7 breast cancer cells treated with IGF-1 showed a distinct change in gene expression of over 800 genes. Importantly, when this profile was compared to estrogen receptor (ER)-negative human breast cancer tumors, it positively correlated with poor prognostic markers and poorer survival and metastatic outcomes[205]. Several large population studies have shown a positive correlation between IGF-1 serum levels and increased risk of colorectal cancer[206,207], breast cancer[208-210], and prostate cancer[211-213]. Cancer development and growth is not only impacted by serum IGF-1, as cancer cells can also release IGF-1 that then acts in an autocrine or paracrine manner[197,214,215]. In chronic myelogenous leukemia (CML), histological staining of biopsies of patients in blast crisis showed high expression of IGF-1, which was later determined to be induced by the oncogene Bcl-Abl[216]. Treating CML blast crisis cell lines with an anti-IGF-1R antibody reduced cell proliferation and increased apoptosis[216]. Currently, several small molecule inhibitors of IGF-1/IGF1-r pathway and monoclonal antibodies to IGF-1R have been identified and tested in Phase 1-3 clinical trials[198,217]. Some of these inhibitors also target IGF-II and the insulin pathway, and are used in combination with other therapies against cancer, such as chemotherapy, anti-estrogen therapy, mTOR inhibitors, etc. While some of these treatments have been shown to be active and effective in *in vitro* and *in vivo* studies, their efficacy in clinical studies have had mixed results[217,218].

3.4. *IGF-1 in the immunity branch*

IGF-1 has also been shown to modulate immune responses and affect certain immune cells, particularly related to the area of survival and proliferation. In a model of allogeneic bone marrow transplantation, subcutaneous administration of IGF-1 improved reconstitution of T- and B-cells and splenic myeloid populations without aggravating graft versus host disease[219]. IGF-1R is expressed on immature and mature thymocytes, with the double negative $CD4^-$ $CD8^-$ population expressing much higher surface levels as compared to single positive thymocytes[220]. In insulin-deficient diabetes, thymic atrophy typically occurs, resulting in lymphopenia and possible predisposition of the body to infection. Diabetic rats given IGF-1, at levels in which hypoglycemia still occurred, had significant repopulation of the thymus[221]. Similar to T-cells, IGF-1 also stimulates proliferation of B-cells and B-cell progenitors[222-224]. Additionally, IGF-1 promotes class switching and antibody production in plasma cells[224].

In the myeloid lineage, human macrophages, granulocytes, and activated macrophages all express the IGF-1R[225]. In THP-1 cells, IGF-1 was shown to promote migration in THP-1 monocytes[226] and decrease either spontaneous[225] or Fas-induced[227] apoptosis in neutrophils. Macrophages and monocytes have also been shown to be producers of IGF-1 transcript and protein through treatment with a variety of factors, including prostaglandin E_2[228], tumor necrosis factor α[228], colony-stimulating factors (CSF)[229,230], or calcium ionophores[231].

Because of the well-documented role of IGF-1 in immune cell activation, proliferation and survival, increased or aberrant IGF-1 signaling has been implicated in

the pathogenesis of a variety of immune-related diseases. For example, Graves' disease is an autoimmune disorder causing hyperthyroidism and connective tissue disease, particularly in the eye orbit. IGF-1R is highly expressed on T- and B-cells and fibroblasts from these patients compared to cells isolated from non-diseased individuals[232]. IGF-1 signaling in fibroblasts promotes the production of IL-16 and RANTES (regulated on activation, normal T cell expressed and secreted), which recruit inflammatory T-cells that then expand. Additionally, B-cells encountering IGF-1 produce auto-antibodies involved in the exacerbation of tissue and muscle swelling of the eye[232]. IGF-1 signaling or increased IGF-1 serum levels have also been linked to rheumatoid arthritis, inflammatory bowel disease, and experimental autoimmune encephalomyelitis (an animal model of inflammatory demyelination of the central nervous system), with the latter potentially benefiting from IGF-1 treatment[233]. However, because IGF-1 has such pleiotropic effects, deciphering the exact direct role and mechanism of IGF-1 signaling in these pathologies as well as whether IGF-1 is beneficial or harmful during disease development, progression, or resolution remains a challenge.

3.5. *IGF-1 promotes wound healing*

As a stimulator of mitogenesis, IGF-1 is beneficial for wound healing. Fluid derived from wounds show elevated levels of IGF-1[234-236]. Cells of the dermis and epidermis upregulate IGF-1 expression within 1-3 days following injury[236-238]. Mechanistically, IGF-1 promotes collagen synthesis, keratinocyte migration and differentiation of myofibroblasts[239-241]. A topical application of IGF-1 onto skin lesions

showed improved re-epithelialization, without affecting granulocytes and monocytes/macrophages[240]. Skeletal muscle growth, maintenance and repair is also facilitated by IGF-1; satellite cells produce IGF-1 that in turn promotes the proliferation of and differentiation of the satellite cells into myoblasts[242]. Because of these effects, IGF-1 is prohibited by the World Anti-Doping Agency for all sporting events. Overproduction of IGF-1 can adversely lead to scar formation and fibrosis, with the latter being of particular importance in idiopathic pulmonary fibrosis (IPF). In IPF, IGF-1 serves as an anti-apoptotic signal to fibroblasts, but not lung epithelial cells, and promotes fibroblast differentiation into myofibroblasts [243,244].

Other physiological effects of IGF-1 that are not discussed here include aging, glucose metabolism and diabetes, neuropathy, stroke and Alzheimer's Disease.

4: Extracellular vesicles

Our studies showed that while IGF-1 dampened apoptotic cell phagocytosis by non-professional phagocytes, IGF-1 treatment enhanced uptake of small liposomes. Physiologically, these small liposomes are similar to microparticles (MPs). We found that macrophages release MPs basally and these MPs could influence the immune response of epithelial cells to allergens. MPs belong to the extracellular vesicle (EV) family that recently have been shown to be important for cell-cell communication and will be further discussed in this section.

4.1. *Overview of extracellular vesicles*

In 1967, Peter Wolf discovered small lipid-rich particles, separated from platelets by ultracentrifugation, which he termed “platelet dust[245].” Electron microscopy showed that these particles may be derived from platelets and can be induced to emerge[245]. Since then, the “platelet dust” and other such released particles have been identified and renamed under the broad category of “extracellular vesicles (EVs).” EVs are lipid bilayer containing vesicles and can be further divided into subcategories based on their biogenesis and size: exosomes, MPs, and apoptotic blebs/bodies (**Figure 1.5**). Apoptotic blebs (AB) are the largest of EVs, as their reported size range is from 1μm to 5μm[246]. During apoptosis, caspase-3 activation of Rho-associated coiled-coil-forming kinase I (ROCK1) initiates actomyosin contraction, stimulating bleb formation[247,248]. Additionally, it was recently shown that Pannexin 1 (PANX1) is also involved in the regulation of apoptotic body release in T-cells[249]. MPs are approximately 100nm to 1000nm in diameter and are formed by outward blebbing of the plasma membrane[250]. The actual molecular mechanism behind MP formation and release is still unknown, but there is evidence that an increase in intracellular calcium is necessary[251-253]. In tumor cells, actomyosin contraction needed for MP shedding appears to be mediated by GTP-bound ADP-ribosylation factor 6 (ARF6) signaling to myosin light-chain kinase (MLCK)[254]. Lastly, exosomes are the smallest EVs, at about 30 to 100nm[255], and the most well studied of all EVs. Unlike ABs and MPs, exosomes are generated within multivesicular bodies, which then fuse with the plasma membrane, releasing these small vesicles into the extracellular space. Exosome generation and secretion is thought

to be mediated by the endosomal sorting complex required for transport (ESCRT) machinery[256] and Rab GTPases[257,258]. As this thesis involves the study of MPs, the remainder of this section will be mostly focused on the role of MPs in physiology.

4.2 *Microparticles*

MPs may be the most heterogeneous subtype of all EVs, with a dynamic size range, cargo content, and array of cell surface markers. MPs retain markers of the cells from which they are derived[250], but also may share classic exosome markers, such as CD63 and CD9, albeit expressed at a lower frequency compared to exosomes[259,260]. As their content is derived from the parental cell, their cargo may contain protein, DNA, RNA, miRNA, etc. MPs are released constitutively at a very low rate, although higher levels of MPs are routinely generated from tumor cells upon induction[261]. Hence, the microenvironment of the parental cell can greatly influence the MP cargo. One key characteristic of MPs is the externalization of PtdSer through the activity of phospholipid scramblases[262]; however, there is emerging evidence that there may be MPs that are PtdSer negative[263]. Because of the PtdSer exposure, MPs have been reported to be internalized by scavenger receptors or integrin[264,265].

Atherosclerosis, rheumatoid arthritis, and cancer are a few examples of pathologies with which MPs have been associated. MPs can serve as biomarkers of disease in addition to having significant effects on cellular functions. While healthy individuals all have circulating MPs, the MP profiles change with disease. For example, hypertensive individuals have greater numbers of MPs released from endothelial cells

and platelets[266]. Elevated levels of leukocyte, endothelial cell, and erythrocyte-derived MPs also positively correlate with graft versus host disease[267], rheumatoid arthritis[268] and inflammatory bowel disease[269], and favorable disease treatment appears to decrease MP numbers. Functionally, MPs have a coagulant property due to the exposed PtdSer, and thus, can contribute to thrombosis and exacerbate the clinical complications of atherosclerosis[270]. MPs released from ovarian cancer cells contain MMPs, which can then degrade the extracellular matrix and promote metastasis[271,272]. Leukocyte-derived MPs can stimulate release of pro-inflammatory cytokines from endothelial cells *in vitro*[273,274], but in other cases, dampen TNFα and IL-8 production by macrophages treated with zymosan or LPS (lipopolysaccharide)[275]. The diverse array of consequences initiated through MP contact is most likely to due to the heterogeneous nature of MPs.

There are several challenges to the field of EV biology that revolve around the standardization of isolation and characterization of EVs. Some of the studies involving MPs were completed using MPs from cells induced to undergo apoptosis. Whether or not these are actually MPs and not undescribed apoptotic blebs is unknown. Currently, differential centrifugation and “sizing” are some of the primary ways to separate and define each subgroup. For example, flow cytometry is used as a way to size MPs; however, most conventional flow cytometers have a threshold of 500nm (300nm for newer models)[276]. Polystyrene beads are often used as sizing guides on flow cytometers; however, due to the nature of the interaction between the beads and the

laser, the bead sizes are often misrepresented. Hence, multiple methods of validation are necessary to ensure the proper study of EVs.

5: Allergic airway inflammation

To investigate the physiological relevance of IGF-1 mediated phagocytic modulation, we used a mouse model of allergic asthma. Over 300 million people are affected by asthma, a chronic inflammatory disease of the airways. It is characterized by airway remodeling and narrowing, overproduction of mucus, and hyper-reactivity of smooth muscle cells to stimuli such as allergen, smoke, cold, or exercise[277]. Asthma typically manifests clinically as coughing, chest tightness, wheezing, and shortness of breath. Current treatments for asthma include inhaled corticosteroids to dampen inflammation, and β_2-agonists to dilate the bronchi and bronchioles. Asthma sufferers can be divided into two categories: allergic and non-allergic.

Allergic asthmatics are typically younger; most children and approximately 50% of adults who have asthma have allergic asthma[278]. Generally, children become sensitized to an allergen (as measured by serum IgE levels or a positive skin prick test) and develop a variety of atopic manifestations, such as atopic dermatitis[279]. Pathophysiologically, allergic asthma is well characterized by elevated levels of Th2 T-cells, eosinophils, dendritic cells, mast cells, and Th2-associated cytokines, such as IL-4, -13, and -5. In contrast, non-allergic asthma is typically adult onset with co-morbidities including nasal polyposis and gastroesophageal reflux disease[280]. By definition, non-allergic asthmatics have a negative skin prick test and serum IgE levels

are either normal or low, despite having local IgE production in the airways[281]. Non-allergic asthma tends to be more severe than allergic asthma and generally refractory to current therapies.

5.1. *Allergic asthma mouse model*

Allergic asthma is studied in the laboratory setting by sensitizing and challenging mice with allergens, such as HDM, cockroach, ragweed, or ovalbumin (OVA)[282]. In our laboratory, Dr. Ignacio Juncadella, a former post-doctoral associate, established an HDM experimental allergy model in which HDM is given intranasally in both the sensitizing and challenge phases[141]. This model improves upon the commonly used intraperitoneal/intranasal allergen delivery system by mimicking the natural route by which humans encounter allergen.

House dust mite contains several allergens that can initiate the immune response. For example, Der p 1 has cysteine protease activity that can disrupt epithelial tight junctions, allowing allergen passage through the epithelial cell barrier and increasing contact with antigen presenting cells[283,284]. Another allergen, Der p 2 can bind LPS with nanomolar affinity and promote TLR-4 signaling[285]. In a simplified model of experimental allergy, airway epithelial cells, upon encountering allergen, produce a variety of chemokines and cytokines that attract immature dendritic cells (DCs) to their vicinity and assist in DC maturity. DCs that have captured allergen then migrate to the mediastinal lymph nodes, presenting to T-cells and driving Th2 differentiation and production of classic Th2 cytokines. These cytokines then assist in B-cell class switching to produce

allergen specific IgE antibodies, which can diffuse back into the tissue and bind to receptors on mast cells and basophils. Repeated exposure to allergen causes local restimulation of resident and/or recruited Th2 cells as well as activation of mast cells, causing release of chemokines, cytokines and other mediators. Th2 cells and type 2 innate lymphoid cells (ILC2s) produce IL-5, which recruits eosinophils into the airways. Sustained inflammation results in physiological changes that include, globlet cell hyperplasia and increased mucus production, extracellular matrix deposition, and smooth muscle cell proliferation[278,286,287] (**Figure 1.6**).

5.2. *Epithelium response in allergic airway inflammation*

A key cell type in the pathogenesis of allergic asthma is the airway epithelial cell, which, as described above, can function as a non-professional phagocyte. In addition to being a physical barrier, airway epithelial cells produce a number of important cytokines that are essential for the pathogenesis of allergic airway inflammation. For example, thymic stromal lymphopoeitin (TSLP) not only primes DCs for promoting a Th2 response, but also plays a role in CD4 T-cell homeostasis[288] and can directly promote *Il4* transcription in combination with T-cell receptor stimulation[289]. The overexpression of *Tslp* in lungs alone is able to induce spontaneous development of inflammatory responses akin to symptoms of allergic airway inflammation. Mice lacking the TSLP receptor that have been given allergen have greatly attenuated disease[290,291]. Additionally, there are several *TSLP* polymorphisms associated with asthma and airway hyper-responsiveness[292]. Airway epithelial cells also produce IL-25 and IL-33, which are

key cytokines involved in the recruitment and activation of ILC2s. As early responders, ILC2s produce IL-5 and IL-13, resulting in eosinophil recruitment and mucus production, respectively[293]. With respect to apoptotic cell clearance, deletion of *Rac1* specifically in airway epithelial cells reduces their ability to engulf apoptotic cells, resulting in exacerbated airway inflammation[141].

5.3. *Macrophage response in allergic airway inflammation*

With respect to professional phagocytes, resident alveolar macrophages appear to play an anti-inflammatory role in the pathogenesis of airway inflammation. Depletion of alveolar macrophages (by administration of clodronate liposomes) in mice given OVA or HDM results in elevated levels of inflammatory cytokines and eosinophils[294]. Additionally, transfer of naïve alveolar macrophages into the airways of mice whose sensitized alveolar macrophages had been depleted resulted in a decrease in inflammatory cytokines[295]. Allergen-sensitized alveolar macrophages appear to have decreased phagocytic capacity compared to naïve alveolar macrophages, and subsequently, may produce less anti-inflammatory cytokines[296]. In contrast, lung macrophages, which could be derived from peripheral blood monocytes, were shown to promote airway hyperreactivity through inducing histamine release from basophils and mast cells[297] or inhibiting β-adrenergic function via production of oxygen radicals[298]. Given that asthma is a multifactorial disease, it is not surprising that “one” cell type can have opposing roles. Furthermore, cells can engage in communication that further adds another layer of complexity to disease pathogenesis.

6: Focus of this dissertation

The primary hypothesis tested in this proposal is that, under conditions of inflammation where both professional phagocytes and non-professional phagocytes are in proximity, there will be communication between phagocytes. We further hypothesize that such communication would influence the engulfment of one or both of the phagocytes, and when such communication is disrupted, this would affect the severity of inflammation. I have tested this hypothesis and provide evidence that IGF-1 secreted by macrophages (professional phagocytes) influences the responses of airway epithelial cells (non-professional phagocytes), and this in turn modulates airway inflammation to the house dust mite allergen.

7: Brief summary of the following chapters

In Chapter II, I will detail the experimental procedures used for the remainder of this thesis. Chapter III details the major findings of my thesis; how macrophages influence phagocytosis by non-professional phagocytes and tissue inflammation. Specifically, in chapter III, I describe how IGF-1 suppresses apoptotic cell uptake by non-professional phagocytes, but enhances the uptake of small particles. Further, macrophages, the source of IGF-1, appear to be resistant to the effect of IGF-1. I will also discuss the *in vivo* results of disrupting IGF-1 signaling in airway epithelial cells in a model of allergic airway inflammation as well as the potential role that macrophage-derived MPs play in possibly dampening inflammation. I will summarize my work in

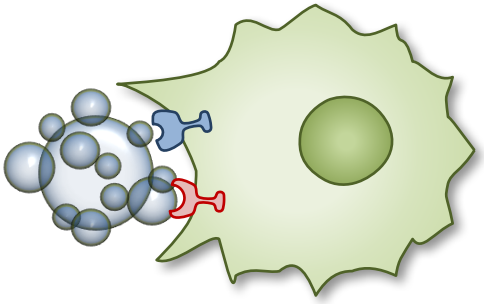
Chapter IV and detail potential future avenues of investigation to expand this current work.

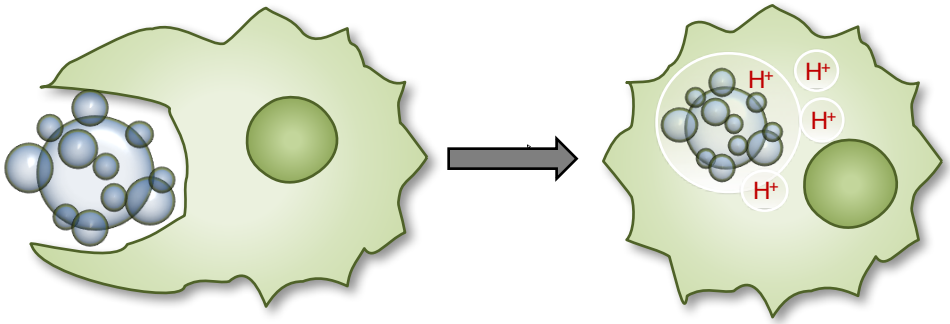
Figure 1.1 – Phagocytic clearance process.

When cells undergo apoptosis, they release soluble mediators, termed “find-me” signals, which recruit phagocytes to their vicinity (1). Following phagocyte migration, recognition of the apoptotic cell occurs via the display of “eat-me” signals (2). Binding of “eat-me” signals, such as phosphatidylserine, to receptors on the phagocytes elicits cytoskeleton rearrangement and engulfment of the apoptotic target (3). The engulfed target, now contained in a phagosome, is degraded as the phagosome becomes more acidic as it fuses with lysosomes.

1. Release of ”find-me” signals and phagocyte recruitment

2. Phagocyte recognition of “eat-me” signals displayed by apoptotic cells





3. Cytoskeleton rearrangement, phagocytosis, and degradation of ingested apoptotic target through increased phagosome fusion with lysosomes (phagosome maturation)

Figure 1.2 – Metabolism and apoptotic cell clearance.

Following apoptotic cell recognition and phagocytosis, a variety of metabolic consequences can occur. For example, recognition of apoptotic cells by Bai1 leads to cholesterol efflux through an LXR independent mechanism. Additionally, phagocytes, via PPAR, can upregulate phagocytic receptors, such as Mer. Apoptotic cell uptake also increases expression of UCP2, a mitochondrial uncoupling protein. Changes in mitochondrial membrane potential, in turn, influence phagocytic capacity. This figure is published in "Han, C.Z. and Ravichandran, K.S. Metabolic connections during apoptotic cell engulfment. *Cell* (2011)[299]."

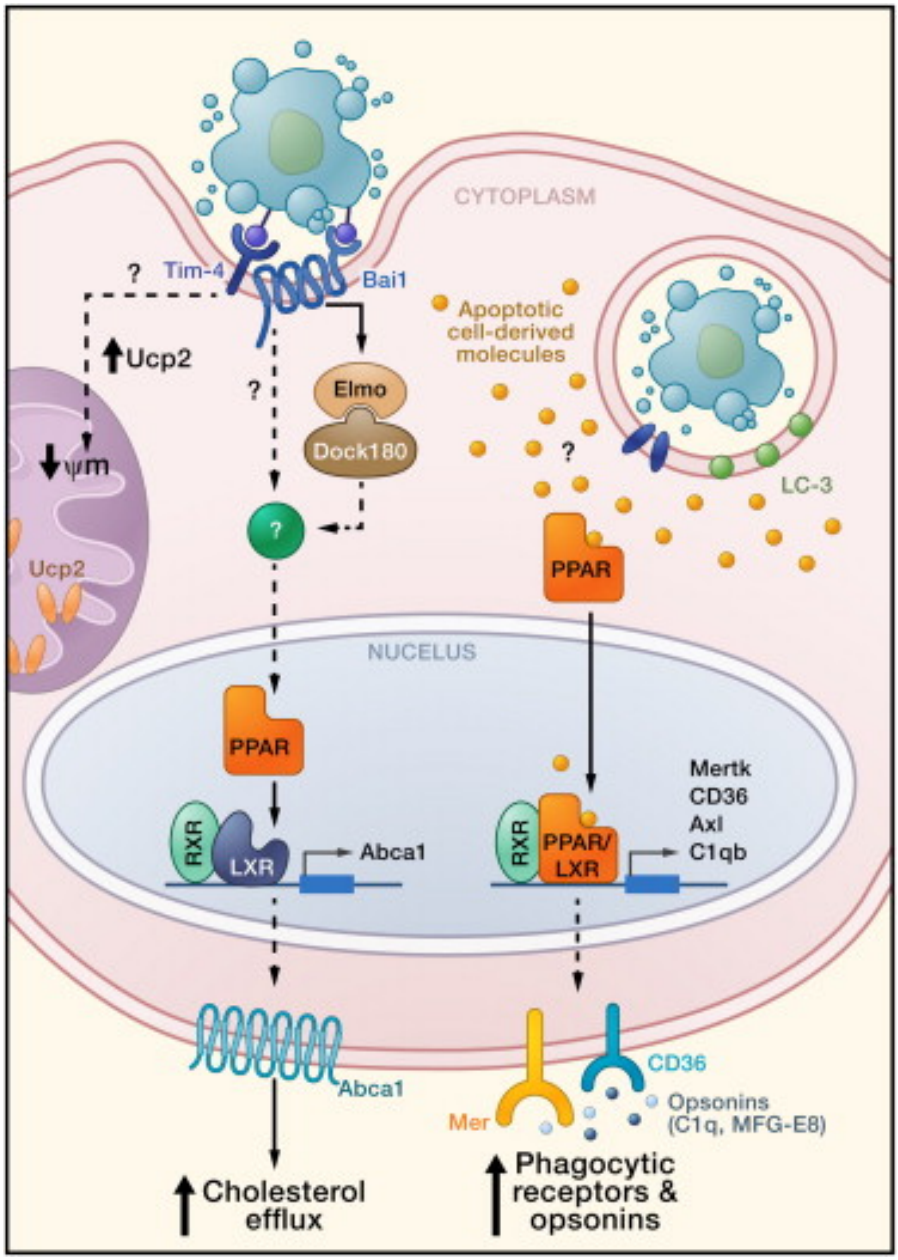

CYTOPLASM
Tim-4
Bai1
?
Ucp2
Elmo
Dock180
?
Apoptotic cell-derived molecules
?
LC-3
ψm
Ucp2
?
PPAR
NUCELUS
PPAR
RXR
LXR
Abca1
Mertk
CD36
Axl
C1qb
RXR
PPAR/ LXR
Abca1
CD36
Mer
Opsonins (C1q, MFG-E8)
Cholesterol efflux
Phagocytic receptors & opsonins

Figure 1.3 – Sources of IGF-1 *in vivo*. The liver is one predominant source of circulating IGF-1. The pituitary gland releases growth hormone, which signals to the liver. In turn, the liver produces IGF-1 and IGFBPs, which bind IGF-1 and prolong the half-life of IGF-1 as well as offer selectivity in IGF-1 binding to IGF-1R. A variety of peripheral tissues also produce IGF-1, which then acts in a paracrine or autocrine manner. This figure is adapted from Pollak (2008)[198].

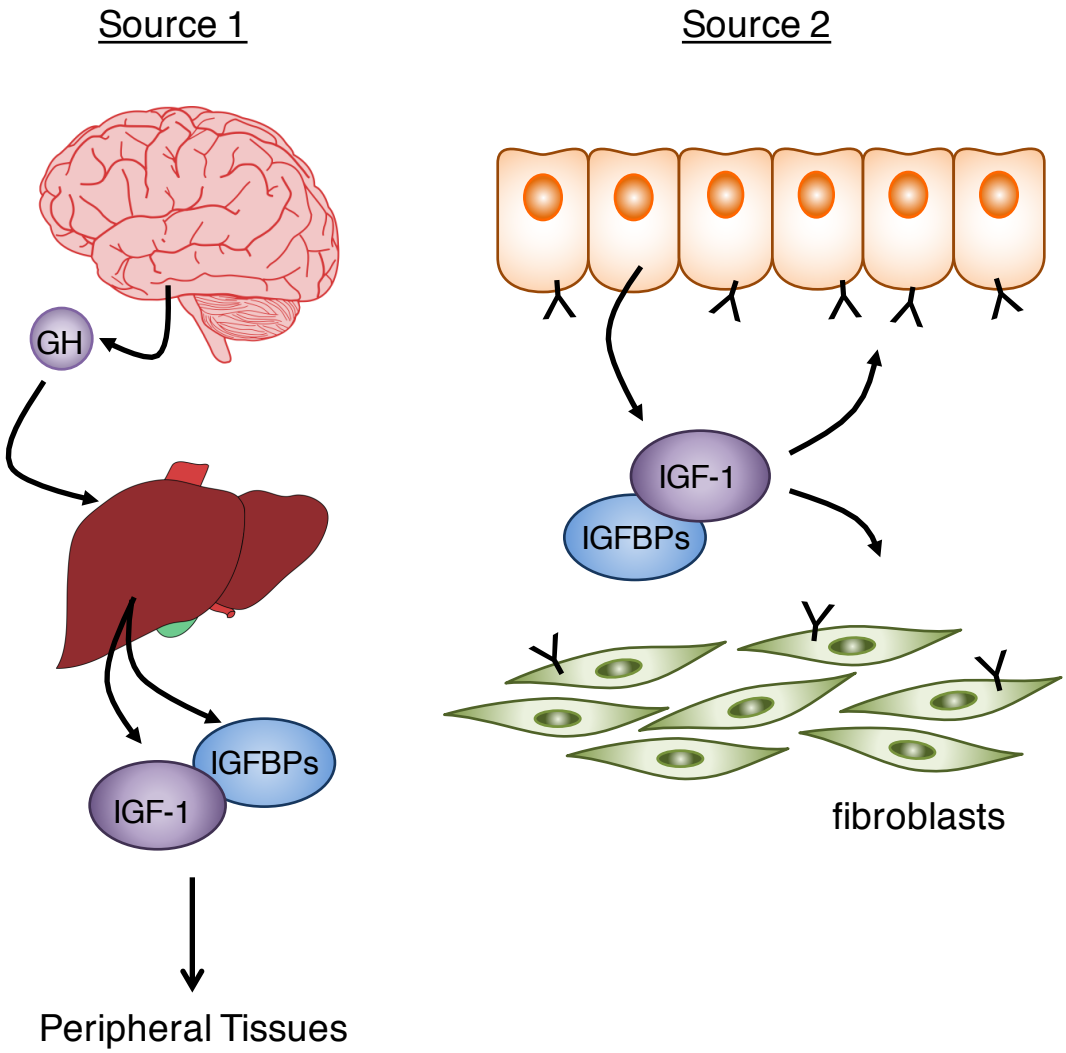

Source 1
Source 2
GH
IGF-1
IGFBPs
IGF-1
IGFBPs
fibroblasts
Peripheral Tissues

Figure 1.4 – Generalized IGF-1R downstream signaling.

Binding of IGF-1 to its receptor, IGF-1R, elicits activation of two canonical receptor tyrosine kinase pathways, resulting in changes in expression of a variety of genes and proteins associated with proliferation, survival and differentiation. Additionally, IGF-1 signaling results in translocation of glucose transporters (GTs) to the membrane surface.

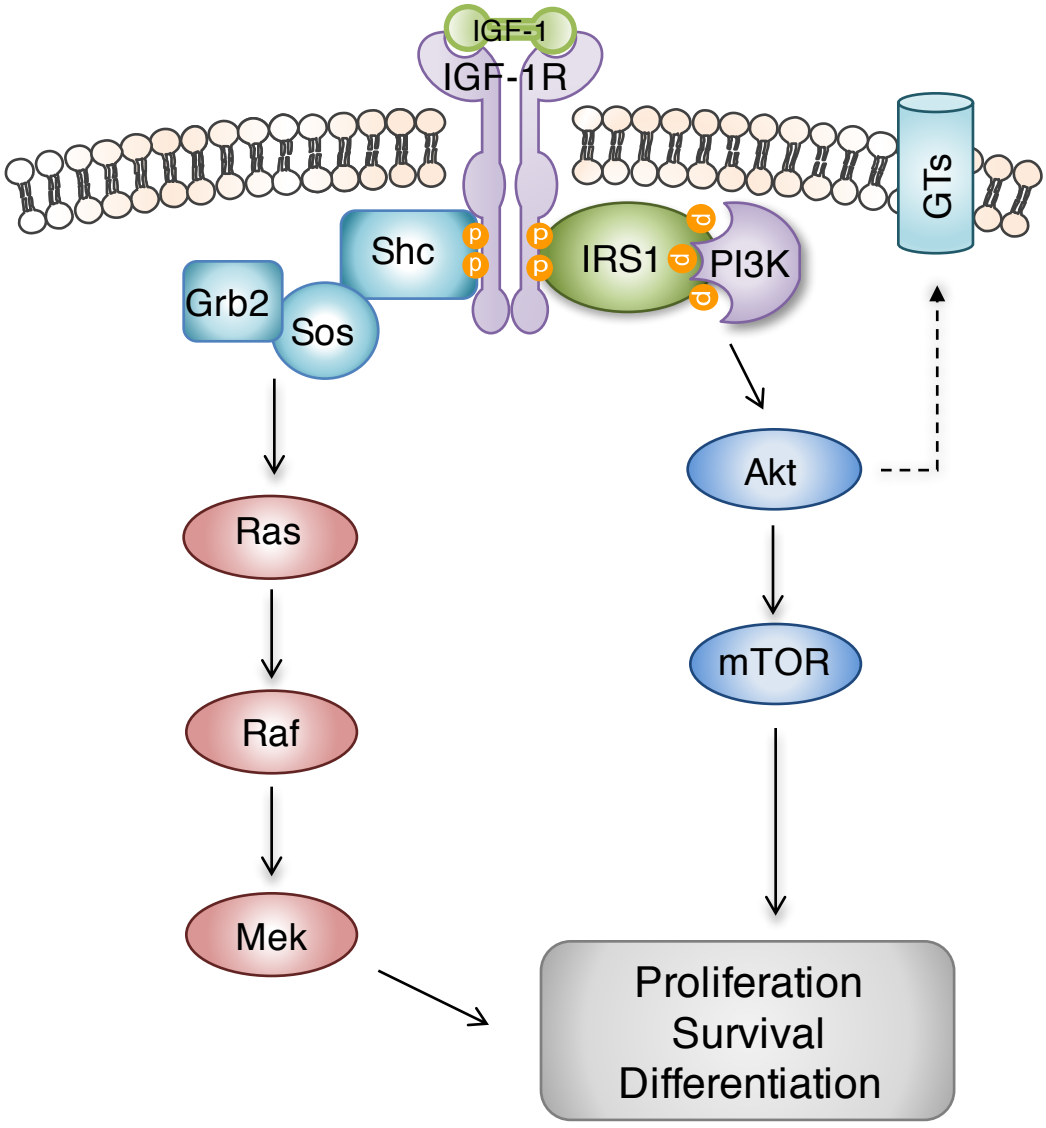
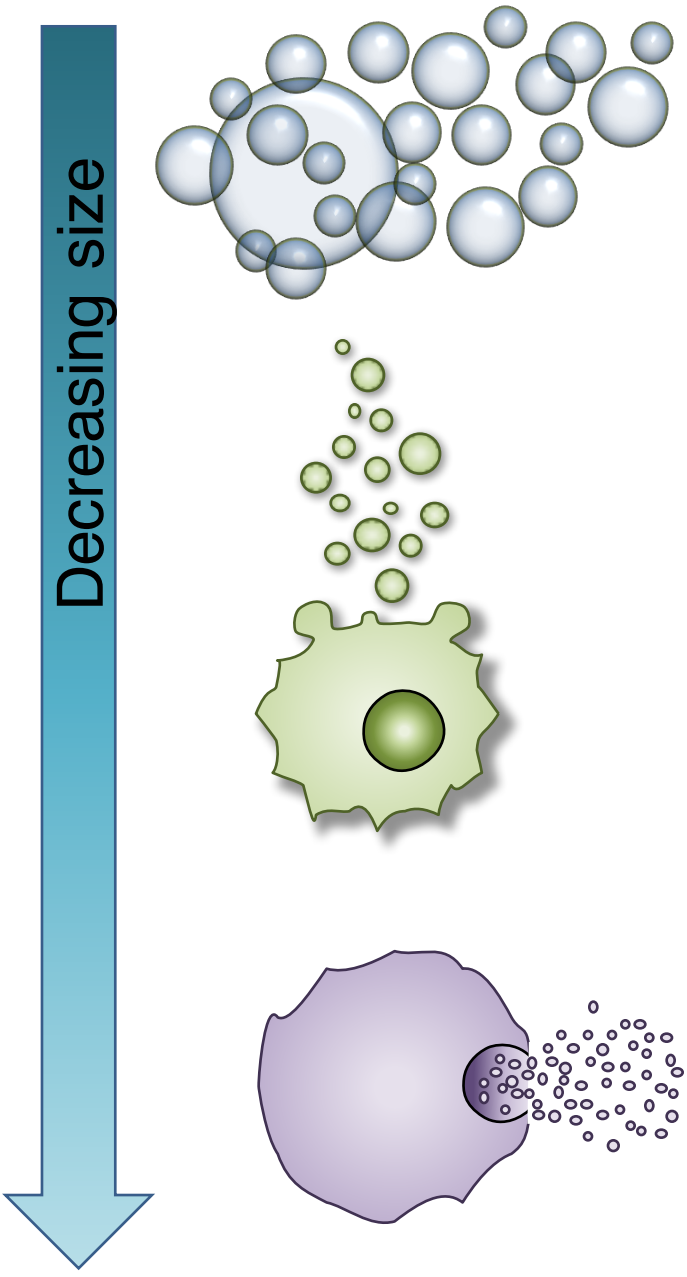

IGF-1
IGF-1R
GTs
Shc
IRS1
PI3K
Grb2
Sos
Akt
Ras
mTOR
Raf
Mek
Proliferation
Survival
Differentiation

Figure 1.5 – Types of extracellular vesicles.

Extracellular vesicles can be categorized into three categories: apoptotic blebs, released during apoptosis; MPs, which bud from the plasma membrane; and exosomes, which are formed in multivesicular endosomes and secreted by the cell.



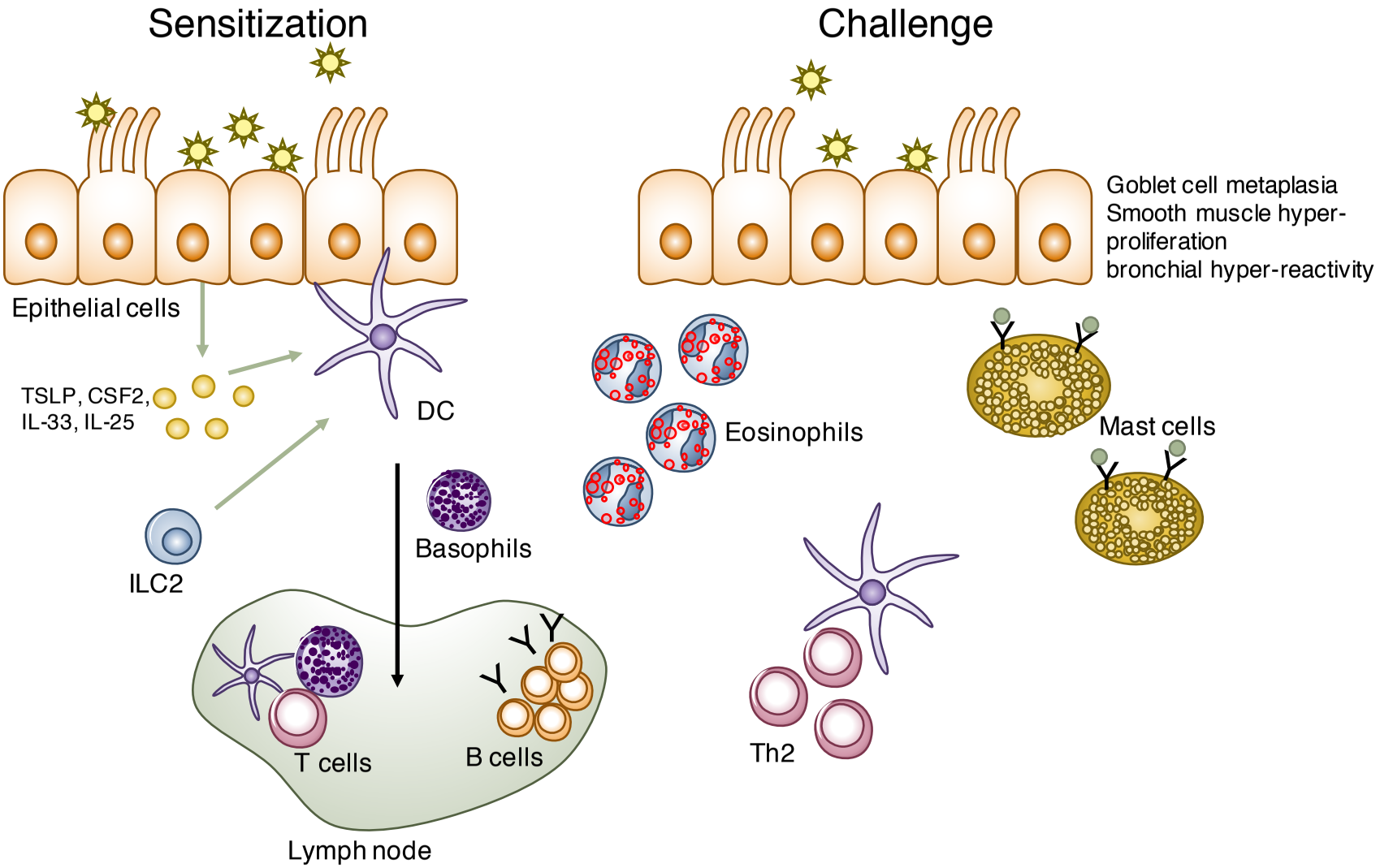
Apoptotic blebs
(1-5µm)

Microparticles
(100nm – 1µm)

Exosomes
<100nm

Figure 1.6 – Immunological effects during allergic airway inflammation.

Epithelial encounter with allergens leads to production of a variety of inflammatory cytokines that aid in recruitment and maturation of conventional dendritic cells and type 2 innate lymphoid cells. DCs can also be activated directly via sampling of airway space for allergens. DCs then migrate to draining lymph nodes and skew CD4 T-cells to Th2 cells. B cells also begin class switching. In the challenge phase, recruited and local T cells are re-activated by DCs and produce Th2 cytokines. The summation of these changes results in airway remodeling and bronchial hyper-reactivity. Figure is adapted from Lambrecht and Hammad (2015)[278].


Sensitization
Challenge
Epithelial cells
TSLP, CSF2,
IL-33, IL-25
DC
ILC2
Basophils
T cells
B cells
Lymph node
Eosinophils
Th2
Mast cells
Goblet cell metaplasia
Smooth muscle hyper-
proliferation
bronchial hyper-reactivity

Chapter II

Materials and Methods

1: Mice

C57BL/6J, *Igf1r*$^{fl/fl}$, and *Igf1*$^{fl/fl}$ mice were obtained from Jackson Laboratories. CCSP-$_{rtTA/tetO}$-Cre were kindly provided by Dr. Jeffrey Whitsett at Cincinnati Children's Hospital[300]. To generate IGF-1R deletion in Club cells, we crossed CCSP-$_{rtTA/tetO}$-Cre mice to *Igf1r*$^{fl/fl}$ [301]. To achieve deletion, mice were given doxycycline (1mg/mL) in drinking water containing 0.4% sucrose for at least 7 days prior to beginning allergen administration, unless otherwise noted. We also crossed *Igf1*$^{fl/fl}$ mice[181,182] to LysM-Cre mice to conditionally delete *Igf1* in the myeloid lineage. We have previously reported on the generation of CCSP-Cre/YFP mice[141]. For all *in vivo* experiments, except generation of apoptotic thymocytes, mice between the ages of 8 and 12 weeks were used. No blinding was performed for any *in* vivo experiments. Mice were allocated to experimental groups based on genotype and age-matching. All animal procedures were performed according to the protocols provided by the Institutional Animal Care and Use Committee (IACUC) of the University of Virginia.

2: Induction of airway inflammation

Mice were given drinking water containing doxycycline (1mg/mL) seven days prior to first HDM administration. Mice were primed intranasally with 10μg of low endotoxin house dust mite extraction (Indoor Biotechnologies) on days 0, 2, 4 and then

challenged intranasally on days 10, 12, and 14. On day 16, mice were euthanized and analyzed for eosinophilic airway inflammation. Alternatively, mice were given three doses of low endotoxin HDM on days 0, 2, and 4 and analyzed on day 6 ("sensitization phase"). For the "challenge phase," mice that had not received any doxycycline were given three doses of low endotoxin HDM on days 0, 2 and 4, then given doxycycline (via drinking water) from day 4 until the mice were analyzed. These mice were also challenged intranasally with three doses of low endotoxin HDM on days 10, 12, and 14 and analyzed on day 16. For airway inflammation experiments, sample size was selected to be above 3 mice per group per experiment.

3: Collection of BAL fluid, lymph nodes, and lung

For airway inflammation experiments, 0.8mL of PBS was delivered intratracheally through a cannula. Recovered BAL fluid was centrifuged and the supernatant was frozen at -80^{o}C for subsequent Luminex analysis. Collected cells were stained for surface markers to distinguish macrophages, neutrophils, T-cells, and eosinophils. For lung harvests, mice were perfused through the right ventricle with PBS and the lungs were carefully excised and placed in type 2 collagenase (Worthington Biochemical Corporation) dissolved in HBSS containing Ca^{2+} and Mg^{2+}. Lungs were minced and then incubated at 37^{o}C for one hour, with vigorous pipetting to separate the tissue every 15 minutes. The lung homogenate was then passed through a 70μm nylon strainer, spun down and treated with red blood cell lysis buffer (Sigma-Aldrich) for 5 minutes. The cells were then washed and resuspended in PBS containing 0.1%BSA. Draining lymph

nodes were carefully extracted, and a single cell suspension was made by passage through a 70μm nylon strainer using the flat end of a syringe. Cells were washed and then resuspended in PBS containing 0.1%BSA.

4: Cell staining and total cell numbers

The collected cells were stained for macrophages, neutrophils, T cells, and eosinophils using the following markers: CD11c (eBioscience, cl. N418), Siglec F (BD Biosciences, cl. E50-2440), Ly6G (eBioscience, clone 1A8), CD11b (eBioscience, cl. M1/70), F4/80 (eBioscience, cl. BM8), CD3 (eBioscience, cl. 145-2C11), CD4 (eBioscience, cl. RM4-5), CD44 (eBioscience, cl. IM7), CD69 (eBioscience, cl. H1.2F3). Absolute cell numbers were determined using AccuCount Particles (Spherotech). Flow cytometry data were collected on FACS Canto I (Becton Dickinson) and analyzed with FlowJo (Treestar, Inc).

5: Microscopy and histology

For hematoxylin and eosin and Periodic acid Schiff staining of lung sections, mice were perfused with PBS and a cannula inserted into the trachea. The lungs were gently inflated with 10% formalin at a constant fluid pressure at 25 cm. The trachea was tied off and the entire heart and lung were removed and placed in 10% formalin and then transferred to 70% ethanol. Lungs were paraffin embedded, sectioned and stained by HistoTox Labs (Boulder, CO) for hematoxylin and eosin and Periodic acid Schiff Staining. Additional lung sections were embedded and sectioned by Research Histology

Core at University of Virginia. The immunohistochemical staining for IGF-1R and cleaved caspase 3 was performed by the University of Virginia Biorepository and Tissue Research Facility. Approximately 6-10 images were taken per mouse, with a total of 3-4 mice per group, and blindly scored by two independent scorers for inflammation, PAS staining, and cleaved caspase 3 positive cells. Immunofluorescence staining of lung sections was performed by Melissa Bevard of the University of Virginia Cardiovascular Research Center Histological Services.

6: Airway hyper-reactivity

Mice were anesthetized and given a tracheotomy tube that delivered increasing concentrations of aerosolized methacholine. The tracheotomy tube in turn was connected to the inspiratory and expiratory ports of a volume-cycled ventilator (flexiVent; SCIREQ Scientific). Airway resistance was measured at baseline and after each dose of methacholine.

7: Macrophage isolation

To obtain bone marrow-derived macrophages, femurs were removed from 8 week old mice and flushed with 5mL of sterile PBS containing 5% fetal bovine serum (FBS). The cell suspension was centrifuged, treated with red blood cell lysis buffer, washed, and then plated onto sterile petri dishes in DMEM containing 10% L929 media, 10% FBS and 1% penicillin/ streptomycin/glutamine (PSQ). Media was replenished every 2 to 3 days and differentiated cells were used at day 6 post-harvest. Resident

peritoneal macrophages were obtained by flushing the peritoneal cavity of mice with 10mL of cold PBS containing 5% FBS. Collected cells were spun down, resuspended in X-VIVO 10 (Lonza) and plated at a concentration of $3x10^5$ cells per well in a 24 well plate for IGF-1 secretion assays, and $5x10^5$ per well in a 24 well plate for engulfment assays. Floating cells were removed the next day and the remaining peritoneal macrophages were used 2 days after isolation. Alveolar macrophages were isolated by flushing the lungs with 1mL of cold PBS instilled intratracheally (five flushes). Collected cells were centrifuged, resuspended in F12K media containing 10% FBS and 1%PSQ, and seeded at $1x10^5$ cells per well in a 48-well plate. All floating cells were removed the next day and the remaining cells were used in assays 2 days after isolation.

8: *In vivo* cytokine or apoptotic cell administration

Mice were treated intranasally with 1μg of recombinant mouse IL-4, IL-5, or IL-13 (eBiosciences), $1x10^6$ apoptotic Jurkat cells, or PBS as control, for two consecutive days. On the third day, BAL fluid was recovered and centrifuged; the supernatant was stored at -80^{o}C for subsequent cytokine analysis. For cytokine or apoptotic cell administration, sample size was selected to be 2 or more mice per group per experiment.

9: Cytokine and IGF-1 analysis

IL-4, IL-5, IL-6, and CCL-11 in the BAL fluid of mice that were sensitized with HDM (sensitization phase only) were quantified by a multiplex Luminex performed by

the University of Virginia Flow Cytometry Core Facility. Secretion of IGF-1 from J774 cells, peritoneal macrophages and collected BAL fluids, as well as TSLP from BAL fluids, were measured by ELISA (R&D Systems).

10: *In vitro* cell systems

LR73 (hamster fibroblasts), SVEC-40 (mouse endothelial cells), BEAS-2B (human bronchial epithelial cells, ATCC #CRL-9609), 16HBE14o- cells (human bronchial epithelial cells), MH-S (mouse alveolar macrophage cells ATCC #CRL-2019), and Jurkat (human T-cells) were either available in the laboratory or obtained from ATCC.

11: *In vitro* engulfment assay

LR73, SVEC-40, BEAS-2B, and 16HBE14o- cells were seeded in a 24 well plate. Thymocytes were isolated from 4-6 week old mice and induced to undergo apoptosis with dexamethasone and then stained with either TAMRA (Invitrogen, C-1171) or CypHer5E (GE Healthcare, PA15401). LR73 and SVEC-40 cells were incubated with apoptotic thymocytes at a 1:10 phagocyte-to-target ratio, BEAS-2B at a 1:5 phagocyte-to-target ratio, and 16HBE14o- at a 1:20 phagocyte-to-target ratio for 2 hours. Mouse IGF-1 (Sigma-Aldrich), human IGF-1 (Sigma-Aldrich), or human IGF-II (Sigma-Aldrich) were added to the phagocytes at the same time as addition of apoptotic targets. For IGFBP3 studies, IGFBP3 (Sigma-Aldrich) was added to media or supernatant from J774 cells for one hour to allow IGFBP3 to bind to any available IGF-1; then the mix was

added to the phagocytes along with apoptotic targets. Targets were then removed by washing three times with PBS, the cells were dissociated from the plate with trypsin, and the engulfment assessed by flow cytometry.

12: *In vivo* engulfment assay

CCSP-Cre/YFP mice were administered PBS or 1μg of IGF-1 intranasally. One hour later, 100 million CypHer5E-labelled apoptotic thymocytes or PtdSer liposomes with or without IGF-1 were injected intranasally, and the mice were euthanized 3.5 hours later. The BAL fluid was harvested and the lungs excised, minced, and digested into a single cell suspension. The cells were then stained with appropriate markers: CD11c and Siglec F for alveolar macrophages and EpCam (along with YFP expression) for airway epithelial cells, and analyzed by flow cytometry to assess apoptotic cell or liposome uptake by the airway epithelial cells and the alveolar macrophages.

13: Liposome construction

Liposomes were prepared by dissolving the lipids (phosphatidylserine, dioleoyl phosphatidylcholine, cholesterol and the lipid DiD dye) in chloroform, evaporating chloroform under flow of argon gas in a glass vial, then subjecting the lipid layer to overnight lyophilization to remove traces of organic solvent. Normal saline was then added for hydration, and intense vortexing was preformed to prepare multilamellar vesicles. Liposomes were repeatedly filtered through a 0.2um Nuclepore polycarbonate

filter to prepare smaller particles. Particle size was verified by dynamic light scattering using Nicomp 370.

14: Microparticle isolation

MH-S cells were seeding at $4x10^6$ cells per 10cm tissue culture-treated dish. Primary mouse alveolar macrophages were seeded at $2x10^6$ cells per 60mm tissue culture-treated dish. After adherence, the media was replaced with .22μm filtered media to remove any contaminating MPs. After an overnight incubation, the supernatant was harvested and spun at 5000xg for 6 minutes to remove cell debris and apoptotic bodies. The pellet was discarded and the resulting supernatant was filtered through a .8μm filter and spun again at 17,000xg for 10 minutes at 20^{o}C. The pelleted MPs were then washed with HBSS and then spun again at 17,000xg for 10 minutes at 20^{o}C. For engulfment assays, MPs were stained with TAMRA for 20 minutes and then added to BEAS-2B cells for 90 minutes. For flow cytometry, purified particles were stained with CD11c, Siglec F, and Annexin V (BD Biosciences, Cat. No. 550475) and processed on an ImagestreamX™ imaging flow cytometer (Amnis). MP size distribution and concentration was characterized using qNano (IZON Science) with a NP400 membrane. At least 500 particles were counted and measured at two different pressures. MPs were prepared for cryo-electron microscopy using standard methods[302].

15: Immunoblotting

LR73, J774 or BEAS-2B cells were seeded in a 60mm dish at a concentration of $5x10^5$. Cells were serum-starved for 6 hours and then stimulated with 100ng/mL of IGF-1 for various time points. Cells were lysed in RIPA buffer and used in Western blots. The blots were probed for phospho-Erk1/2 (Cell Signaling Technology, #4370), phospho-Akt (Cell Signaling Technology, #4060), phospho-IGF-1R (Cell Signaling Technology, #3024), total Erk2 (Santa Cruz Biotechnology, #sc-154-G), total Akt (Cell Signaling Technology, #4691), total IGF-1R (Cell Signaling Technology, #9750), and anti-B-actin-HRP (Sigma-Aldrich, #A3854) followed by chemiluminescence detection.

16: RNA-Seq

BEAS-2B cells were treated with HDM and alveolar macrophage-derived MPs for 3 hours. Total RNA was extracted and an mRNA library was prepared using Illumina TruSeq platform and followed by transcriptome sequencing using an Illumina NextSeq 500 cartridge. R v3.2.3 was used for graphical and statistical analysis and the R package DESeq2 (v1.10.3) was used for differential gene expression analysis of RNA-Seq data. Justin Perry assisted with RNA-Seq analysis and heat map generation.

17: Quantitative RT-PCR

Total RNA was extracted from cells using Quick-RNA Miniprep Kit (Zymo Research) or RNeasy Mini Kit (Qiagen) and cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturers' instructions.

Quantitative gene expression for mouse *Igf1* (Mm00439560_m1), human *TSLP* (Hs00263639_m1), *CSF2* (Hs00929873_m1), *IL6* (Hs00985639_m1), *IL8* (Hs00174103_m1), *FGF2* (Hs00266645_m1), *IFIT2* (Hs01922738_s1), *PTX3* (Hs00173615_m1), *KLF4* (Hs00358836_m1) or housekeeping human *HPRT* (4326321E) or mouse *Hprt* (Mm01545399_m1) was performed using Taqman probes specific for each gene (Applied Biosystems) run on a StepOnePlus Real Time PCR System (Applied Biosystems).

18: Statistical analysis

Statistical significance was determined using GraphPad Prism 5 or 6. Unpaired Student's two-tailed *t*-test, one-sample *t*-test, or a one-way or two-way ANOVA were performed according to the test requirements. Grubbs' Outlier Test was used to determine outliers, which were excluded from the final analyses. *p*-values of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks) were considered significant.

Chapter III

Macrophages redirect phagocytosis by non-professional phagocytes and influence tissue inflammation

1: Abstract

Both professional phagocytes (such as macrophages) and non-professional phagocytes (such as epithelial cells) clear billions of apoptotic cells and other particles on a daily basis *in vivo*. Since professional and non-professional phagocytes reside in proximity in essentially all tissues, a fascinating unanswered question is whether there is cross-communication between phagocytes. We hypothesized that there is likely crosstalk between phagocytes in mediating cell clearance, and that this might impact inflammation and tissue function. Here, we show that macrophages release a soluble growth factor and MPs that influence the nature of engulfment by nearby non-professional phagocytes and their response. During apoptotic cell engulfment or in response to inflammation-associated cytokines, macrophages released insulin-like growth factor-1 (IGF-1). The binding of IGF-1 to IGF-1 receptor, on non-professional phagocytes, redirected their phagocytosis such that the engulfment of larger apoptotic cells was dampened while the uptake of MPs was enhanced. In contrast, macrophages were refractory to this IGF-1 mediated engulfment modulation. Macrophages also released MPs, whose uptake (enhanced via IGF-1 signaling) by epithelial cells dampened their inflammatory cytokine production. Consistent with these observations, deletion of the IGF-1 receptor specifically in airway epithelial cells *in vivo* resulted in

exacerbated lung inflammation after exposure to an airway allergen. These genetic and functional studies reveal a novel mode of communication, via IGF-1 and MPs, between macrophages and epithelial cells that can critically influence the magnitude of tissue inflammation *in vivo*.

2: Introduction

In the human body, approximately a million cells are turned over every second, as part of normal healthy living[1]. Such turnover of cells, which occurs predominantly via apoptosis, takes place in all tissues at varying levels[2,3]. Phagocytes recognize these apoptotic cells via the display of 'eat-me' signals on the dying cells, such as phosphatidylserine exposure on the plasma membrane, changes in surface charge, and modifications to certain cell surface proteins[8,31]. After engagement of eat-me signals by receptors on phagocytes, the apoptotic cells are quickly cleared such that even in tissues with a high rate of turnover, very few apoptotic cells are seen in the steady state. Defects in proper clearance of apoptotic cells results in their secondary necrosis, predisposing the body to inflammation and autoimmunity or chronic inflammatory diseases[2,5,6].

In early studies of cell clearance, significant focus was placed on the role of professional phagocytes, such as tissue resident macrophages or recruited motile phagocytes, due to their ability to engulf large number of corpses. However, it is increasingly appreciated that non-professional phagocytes, which are often neighboring cells, can play a significant and functionally relevant role in corpse clearance[4]. For example, retinal pigment epithelial cells are known to ingest spent rod outer segments from the previous day[148,149,153,303]; mammary epithelial cells are known to perform the majority of the clearance during mammary gland involution post-lactation[134]; fibroblasts are known to clear dying cells during embryogenesis[129]; and epithelial cells of the airways and gut can mediate clearance of dying neighbors[137-141,304]. However, in most

tissues, professional and non-professional phagocytes reside in close proximity; yet the intriguing and important question of how these phagocytes divide the labor of clearance has not been addressed. Moreover, when tissues become damaged or inflamed (such as by infections, allergens, etc.), the recovery process requires both efficient cell clearance and tissue repair, with the repair often being a key role for non-professional phagocytes. A fascinating unanswered question is whether there is regulation among professional and non-professional phagocytes in mediating cell clearance, and how the distribution of clearance duties impacts inflammation and organ/tissue function. Our studies presented here identify a novel modality of communication, wherein macrophages engulfing apoptotic cells release a soluble mediator, insulin-like growth factor-1 (IGF-1); this soluble IGF-1, in turn, suppresses the phagocytic ability of nearby non-professional phagocytes. This IGF-1 mediated communication between macrophages and non-professional phagocytes also impacts the magnitude of inflammation *in vivo*.

3: Results

IGF-1 dampens engulfment by non-professional phagocytes

To address the potential cross-regulation among phagocytes, we took a candidate screening approach and initially tested a panel of eleven soluble mediators that have previously been linked to tissue repair, inflammation dampening, and tissue morphogenesis[305]. We used LR73 fibroblasts, a non-professional phagocyte cell line, under assay conditions that can monitor increased or decreased apoptotic cell

engulfment. Ten of the factors tested did not affect the ability of LR73 cells to ingest apoptotic targets; however, addition of insulin-like growth factor-1 (IGF-1) significantly dampened apoptotic cell uptake (**Figure 3.1A**). This engulfment attenuation via IGF-1 was seen at physiological IGF-1 concentrations reported in mouse and human serum (<100 to 600 ng/mL)[306-309] (**Figure 3.1B**). When we tested some of the factors that did not affect phagocytosis, such as EGF, FGF2, VEGF, PDGF-AA and PDGF-BB in a dose titration, we did not detect a significant alteration in the engulfment of apoptotic cells (**Figure 3.2A**). The lack of an effect on phagocytosis by these factors was not due to their inability to bind to their cognate receptors, as they induced early downstream signaling events (**Figure 3.2B, C, D and E**) as indicated by phosphorylation of Erk1/2. We also extended our observations to other non-professional cell types as the engulfment dampening effect of IGF-1 was also seen with airway epithelial cell lines BEAS-2B (**Figure 3.1C**) and 16HBE14o- (**Figure 3.3A**), as well as the endothelial cell line SVEC-40 (**Figure 3.3B**).

One possible explanation for the inhibition of engulfment seen with IGF-1 could be due to its effect on apoptotic cells, such as the masking of the eat-me signal phosphatidylserine (PtdSer) on the apoptotic cells. However, this was not the case, as Annexin V, which binds to PtdSer[31-33,310], was still able to efficiently stain apoptotic cells treated with a range of IGF-1 concentrations (**Figure 3.4A**). Moreover, the effect of IGF-1 on phagocytes was rapid, as adding IGF-1 simultaneously with apoptotic cells inhibited engulfment comparably to pre-treatment of phagocytes before adding apoptotic cells (data not shown). Additionally, IGF-1 mediated suppression of

phagocytic uptake was reversible as washing phagocytes pre-treated with IGF-1 resulted in near complete restoration of engulfment capacity (**Figure 3.4B**). Collectively, these data suggest that the ability of IGF-1 to modulate engulfment by non-professional phagocytes is rapid and reversible.

IGF-1 mediated engulfment suppression occurs via the IGF-1 receptor

We then assessed whether the effect of IGF-1 on LR73 cells was mediated by the IGF-1 receptor. We initially confirmed that LR73 cells express the IGF-1 receptor (IGF-1R) and that phosphorylation of Akt, a signaling molecule downstream of the IGF-1R, occurs after treatment with IGF-1 (**Figure 3.1B**). In blood, IGF-1 is typically bound to IGF binding proteins (IGFBPs); the IGFBPs, along with an acid-labile subunit, stabilize and sequester IGF-1 from IGF-1R[311]. Addition of IGFBP3 to media containing IGF-1 restored the phagocytic capability of LR73 cells (**Figure 3.4C**), suggesting that binding of active IGF-1 to its receptor was necessary. Also when we treated human BEAS-2B epithelial cells with a neutralizing antibody against the human IGF-1R, the engulfment-dampening effect of IGF-1 was reversed (**Figure 3.4D**). We then tested OSI-906, a small molecule inhibitor of IGF-1R that blocks receptor auto-phosphorylation that is currently being tested in Phase II and III clinical trials[312]. Increasing concentrations of OSI-906 rescued the engulfment ability of LR73 cells exposed to IGF-1 in a dose-dependent manner, correlating with the decreased tyrosine phosphorylation of IGF-1R and downstream Erk phosphorylation (**Figure 3.4E**). Addition of NVP-AEW541, another small molecular inhibitor of IGF-1R, also reversed the engulfment

suppression via IGF-1 (**Figure 3.5**)[313]. These data suggested that productive signaling through the IGF-1R is necessary for modulation of apoptotic cell engulfment by IGF-1. We also tested IGF-II and insulin, which share structural similarity with IGF-1 and can also bind IGF-1R[309,314]. IGF-II and insulin could cause reduction in apoptotic cell uptake (**Figure 3.6**), but their effective concentrations to achieve suppression were higher compared to IGF-1, likely due to their reported lower binding affinities for IGF-1R[315].

Next, we sought to probe the potential signaling pathways downstream of IGF-1R that might be involved in the engulfment dampening effect of IGF-1. Using a series of small molecule inhibitors, we found that common downstream components of growth factor and receptor tyrosine kinase signaling such as Akt, mTOR, Erk or PI3-Kinase[316] were not essential for this IGF-1 effect on non-professional phagocytes (**Figure 3.7**). Since other growth factors in the panel we tested (such as PDGF and EGF) elicit similar signaling cascades, but did not decrease phagocytic capacity, this suggested IGF-1 might induce a distinct type of signaling. IGF-1 has been shown to lead to the activation of the small GTPase RhoA[317]; this was of particular interest as activation of RhoA and subsequent activation of Rho kinase (ROCK) has been previously shown to dampen apoptotic cell engulfment[85,86]. Initially, it appeared treatment of LR73 cells with Y27632, a ROCK inhibitor, was able to partially rescue IGF-1 induced engulfment suppression (**Figure 3.8**). However, as ROCK inhibition basally increases phagocytosis of apoptotic cells (consistent with what has been previously reported), we normalized the change in phagocytosis for each inhibitor concentration to the control (**Figure 3.8**, *right panel*). After normalizing, we observed that ROCK inhibition did not increase corpse uptake in

LR73 cells in the presence of IGF-1 more than the increase observed basally due to ROCK inhibition. We confirmed our observations with a second ROCK inhibitor, GSK269962 (**Figure 3.8**). Collectively, these results suggest that inhibition of ROCK does not appear to specifically rescue IGF-1 induced engulfment. However, activated Rac1 (which has been positively correlated with promoting apoptotic cell engulfment[76,83]) could bypass the attenuation of apoptotic cell uptake by IGF-1 (**Figure 3.9**) suggesting that IGF-1R acts at or before a step that can be bypassed by active Rac1.

Macrophages are insensitive to engulfment suppression by IGF-1

As non-professional and professional phagocytes are both capable of clearing apoptotic cells, we then tested whether IGF-1 also impairs apoptotic cell engulfment by professional phagocytes, such as macrophages. When IGF-1 was added to the macrophage cell line J774, these cells continued to engulf apoptotic cells at levels comparable to control (**Figure 3.10A**). Kinetic analysis showed that J774 cells engulfed apoptotic cells at levels comparable to controls at all time points analyzed (**Figure 3.11A**). Importantly, IGF-1 mediated signaling in J774 cells was initiated, as confirmed by increased phosphorylation of Akt (**Figure 3.10A**) or IGF-1R (**Figure 3.11A**). Another macrophage cell line, IC-21 (**Figure 3.12**), and primary macrophages (either bone marrow-derived or resident peritoneal macrophages) were also unaffected in their ability to engulf apoptotic corpses, even after addition of supra-physiological concentrations of IGF-1 (**Figure 3.10B, C**). It is noteworthy that the surface expression of IGF-1R was confirmed in all macrophage cell types analyzed (**Figure 3.11C**). Together, these data

suggest that IGF-1 mediated modulation of phagocytosis does not extend to macrophages despite eliciting canonical early IGF-1 signaling.

IGF-1 enhances small particle uptake by non-professional phagocytes

Non-professional phagocytes can engulf apoptotic cells and particles ranging in different sizes[143]. We next assessed whether IGF-1 mediated suppression of non-professional phagocyte engulfment also extends to smaller particles, such as PtdSer containing liposomes. Surprisingly, contrary to the effect on apoptotic cells, IGF-1 treatment enhanced the uptake of liposomes that were ~150 to 200nm in size (**Figure 3.10D**). This effect was again specific to IGF-1, as LR73 cells treated with EGF or VEGF had no significant difference in liposome uptake (**Figure 3.10D**). This enhancement in liposome uptake was also reversible and dependent on IGF-1R signaling, as the inhibitor OSI-906 reduced the liposome uptake (**Figure 3.10E**). IGF-1 did not affect macrophage uptake of smaller particles, as peritoneal macrophages treated with increasing concentrations of IGF-1 had comparable levels of liposome uptake as control-treatment conditions (**Figure 3.10F**). This suggested that that IGF-1 is not a generic inhibitor of apoptotic cell uptake, but rather it modifies phagocytosis by non-professional phagocytes in a target-dependent manner.

Rac1 activation results in changes in the actin cytoskeleton and engulfment of apoptotic cells requires both the polymerization and de-polymerization of actin. As these factors complicated any studies on the contribution of actin dynamics to the molecular mechanism of IGF-1 mediated redirection of phagocytosis, we used the IGF-1 induced

enhancement of liposome uptake to examine how the actin cytoskeleton is modified following IGF-1 treatment. First, we treated LR73 cells with Cytochalasin D (CytoD), which binds to the barbed end of actin filaments and prevents further addition of monomeric actin, thereby promoting actin depolymerization[318], and found that it was able to potently suppress the IGF-1 mediated enhancement of liposome uptake (**Figure 3.13A**). Next, we confirmed this finding by treating LR73 cells with Latrunculin A, which also promotes actin depolymerization, albeit via a different mechanism[319] (**Figure 3.13B**). Interestingly, treatment with Jasplakinolide, a peptide that induces actin polymerization[320], was not able to reverse the effect of IGF-1 on liposome uptake in non-professional phagocytes (**Figure 3.13C**). These pharmacological studies suggested that IGF-1 may dampen phagocytosis of apoptotic cells by modulating the steps involved in actin polymerization. Previous studies have shown that cross-linked actin networks are important for many cellular migration and other phagocytic events. Arp2/3 complex plays a key role in formation of such branched networks through binding to existing actin filaments, nucleation and subsequent elongation/growth of new filaments, resulting in the dendritic array of actin filaments. To test the possible targeting of the Arp2/3 complex by IGF-1 signaling in our phagocytic context, we used CK666, a small molecule inhibitor of Arp2/3[321]. Treatment of LR73 cells with CK666 had minimal effect on the IGF-1 mediated enhancement of liposome uptake (**Figure 3.13D**). Collectively, these data suggest that the IGF-1 mediated modulation of phagocytosis involves changing of G-actin dynamics, but not Arp2/3 mediated functions.

Macrophages release IGF-1 during apoptotic cell engulfment

High levels of IGF-1 are found the in the serum, largely attributed to production in the liver in response to growth hormone signaling. Intriguingly, mice globally lacking IGF-1 are born with a dwarfism phenotype and some are perinatally lethal[180]; however, a liver-specific knockout of IGF-1 showed no obvious growth or developmental defect, suggesting that there are other peripheral sources of IGF-1[181]. Macrophages have previously been shown to produce IGF-1 in response to IL-4[322,323]. This raised the possibility that macrophages within a tissue, via IGF-1 release, could redirect phagocytosis by nearby non-professional phagocytes. We first confirmed that peritoneal macrophages treated with IL-4 produce IGF-1 (**Figure 3.14A**). When we asked whether macrophages might produce IGF-1 during apoptotic cell recognition, resident peritoneal macrophages exposed to apoptotic Jurkat cells (but not live cells) produced IGF-1 (**Figure 3.14A**). We ruled out that this IGF-1 production was derived from the Jurkat cells themselves (**Figure 3.14A**). IGF-1 protein induction appears to be from newly transcribed *Igf1* message, as we could also detect an increase in the *Igf1* mRNA in a time-course analysis (**Figure 3.15**). Macrophage-produced IGF-1 could also suppress apoptotic cell uptake by non-professional phagocytes, and this effect was again reversed by addition of exogenous IGFBP3 with the supernatants (**Figure 3.14B**). Taken together, these data suggest that IGF-1 released from macrophages can have the capacity to dampen engulfment by neighboring non-professional phagocytes.

Macrophages and IGF-1 influence engulfment by airway epithelial cells in vivo

We next asked whether macrophage-mediated modulation of apoptotic cell uptake by non-professional phagocytes could be recapitulated *in vivo*. We chose the lung as a tissue suitable to address this question, as both alveolar macrophages and airway epithelial cells are known to engulf apoptotic cells. Moreover, alveolar macrophages, similar to the resident peritoneal macrophages, are derived from fetal monocytes[324-336], and are readily isolated via their $CD11c^+$ Siglec F^+ expression. The epithelial cells of the airways can also be isolated and tracked via available genetic tools[337]. When we assessed the expression of the IGF-1 receptor in the airways by immunofluorescence, IGF-1R expression was prominent in the epithelial cell layer (**Figure 3.16**) but was also detectable at lower levels in alveolar macrophages. To examine whether IGF-1 would affect engulfment by airway epithelial cells *in vivo*, we used mice in which the airway epithelial cells are specifically marked by YFP expression. This was achieved by crossing YFP-reporter mice with *CCSP*-Cre mice that express Cre under the Club cell secretory protein promoter (CCSP), resulting in Cre expression specifically in airway epithelial cells of the trachea, bronchi and bronchioles[300,338]. When apoptotic cells or liposomes were administered intranasally along with IGF-1 (**Figure 3.14C**), YFP-positive airway epithelial cells had decreased uptake of apoptotic cells, but increased uptake of liposomes, compared to control mice receiving apoptotic cells or liposomes without IGF-1 (**Figure 3.14D, E**). Importantly, the lung macrophages isolated from the same IGF-1 treated mice were unaffected in their ability to engulf these targets

(**Figure 3.14D, E**), indicating that IGF-1 can differentially affect engulfment by professional and non-professional phagocytes closely residing in the same tissue.

To expand upon our *in vitro* findings regarding IGF-1 release by macrophages, we then asked whether alveolar macrophages produce IGF-1 *in vivo* after exposure to IL-4 or apoptotic cells (**Figure 3.14F**). Analysis of bronchoalveolar lavage (BAL) fluid demonstrated that intranasal administration of either IL-4 or apoptotic Jurkat cells caused a reproducible increase in IGF-1 (**Figure 3.14G**). We also tested whether IL-13 or IL-5 could also induce IGF-1[339]. Interestingly, IL-13 (whose receptor shares a common subunit with the IL-4 receptor[340]) could also induce IGF-1, but administration of IL-5 failed to enhance IGF-1 levels in the BAL fluid (**Figure 3.14G**). Although macrophages are one of the primary producers of IGF-1, the source of IGF-1 is difficult to distinguish in these experiments. To better address this, we crossed mice carrying floxed *Igf1* loci (*Igf1*$^{fl/fl}$ mice)[181,182] to the LysM-Cre mice, where the Cre is expressed under the lysozyme M promoter specific for the myeloid lineage. Cre-mediated deletion was confirmed by the absence of detectable *Igf1* mRNA in alveolar macrophages from LysM-Cre/*Igf1*$^{fl/fl}$ mice (**Figure 3.17**). After intranasal administration of IL-4 or IL-13 or apoptotic Jurkat cells in LysM-Cre/*Igf1*$^{fl/fl}$ mice, IGF-1 was not detectable in the BAL fluid (**Figure 3.14H**). This suggested that the macrophage/myeloid population represents the predominant source of soluble IGF-1 after exposure to IL-4, IL-13 or apoptotic cells in the lung. Further, IL-4 and IL-13 are classic Th2 cytokines involved in the pathophysiology of allergic asthma, suggesting that an airway model of allergic

inflammation, could provide a system to assess the relevance of the IGF-1/IGF-1R communication between these cell types *in vivo.*

IGF-1R in airway epithelial cells is critical for limiting inflammation

Failure to clear apoptotic cells is known to lead to secondary necrosis and exacerbate inflammatory conditions. As IGF-1/IGF-1R signaling alters apoptotic cell engulfment by non-professional phagocytes, we next assessed whether IGF-1 mediated communication between macrophages and airway epithelial cells impacts inflammation in response to allergens. We had previously established a model of airway inflammation induced by the allergen house dust mite (HDM), where apoptotic cell recognition by airway epithelial cells influenced inflammation[141,341]. In this model, HDM is intranasally administered in both the allergen sensitization and challenge stage, thereby modeling the natural route of allergen encounter (**Figure 3.18A**). Initially, we tested the response of LysM-Cre/*Igf1*$^{fl/fl}$ mice to HDM. However, these mice turned out to be unsuitable for this analysis, as we continued to detect variable levels of IGF-1 in the BAL fluid of these mice after HDM administration; this was likely due to the leakage of serum-derived IGF-1 into the lungs due to the inflammation around the vasculature. Therefore, we addressed the relevance of the IGF-1/IGF-1R axis by targeting the IGF-1 receptor on the epithelial cells.

We obtained mice with a floxed *Igf1r* locus (*Igf1r*$^{fl/fl}$)[301] and crossed them to the CCSP-$_{rtTA/tetO}$-Cre transgenic mice[300,337]. The latter strain drives Cre expression under the CCSP promoter in the epithelial cells of the trachea, bronchi and bronchioles[300,337].

This strain also provides temporal control of the *Igf1r* deletion such that Cre expression is induced only after exposure to doxycycline in the drinking water. Given the well-studied role of IGF-1 in development and growth, this system permits airway epithelial cells to develop normally and the deletion of *Igf1r* only when desired (at 8 weeks of age), prior to allergen exposure. When we administered doxycycline, we observed near complete loss of IGF-1R expression in the epithelial cells of CCSP-Cre/*Igf1r*$^{fl/fl}$ mice (**Figure 3.18B**). This suggested that inducible and efficient deletion of IGF-1R in the airway epithelial cells of CCSP-Cre/*Igf1r*$^{fl/fl}$ mice is achievable.

We next tested the CCSP-Cre/*Igf1r*$^{fl/fl}$ mice in our HDM-model of allergic airway inflammation. After sensitization and challenge with low-endotoxin HDM (**Figure 3.18A**), the CCSP-Cre/*Igf1r*$^{fl/fl}$ mice had significantly greater airway inflammation based on several parameters: cellular infiltration and analysis of BAL fluid, lung histology, and pulmonary function tests. First, there was marked increase in the number of eosinophils and CD4 T-cells in the BAL fluid (**Figure 3.18C**) and a trending increase in inflammatory cells in the lungs (**Figure 3.19A**) compared to controls. Second, the lung draining lymph nodes from CCSP-Cre/*Igf1r*$^{fl/fl}$ mice were significantly larger in size and contained more CD4 T-cells relative to control mice (**Figure 3.18D**). Third, histological analysis of lung sections showed that CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given HDM had increased peribronchial and perivascular cellular infiltration (**Figure 3.18E**) as well as greater mucus accumulation, another indicator of lung inflammation (**Figure 3.18F**). Fourth, CCSP-Cre/*Igf1r*$^{fl/fl}$ mice treated with HDM displayed greater airway reactivity in response to methacholine challenge, a measure of the bronchial hyper-responsiveness seen in

allergic airway inflammation[342,343], compared to control mice (**Figure 3.18G**). We also saw a slight trend towards greater number of apoptotic cells, albeit not significant, (detected via cleaved caspase 3 staining of lung sections), likely due to increased inflammation in CCSP-Cre/*Igf1r*$^{fl/fl}$ compared to control mice (**Figure 3.19B**). Together, these data suggested a requirement for IGF-1R in the airway epithelial cells in minimizing inflammation in this airway inflammation model. This was initially surprising, as we were expecting the loss of IGF-1R on the airway epithelial cells (and the likely improved apoptotic cell clearance) to attenuate, rather than worsen, the allergen-induced inflammation. This prompted us to determine the window in which IGF-1/IGF-1R signaling was most responsive to dampening inflammation caused by allergen exposure.

In the above experiments, the analyses were performed after the full course of the disease, where the mice had been sensitized and re-challenged with the HDM allergen. The requirement of the IGF-1/IGF-1R signaling in dampening inflammation could have been at the sensitization phase or the challenge phase. To distinguish between these possibilities, we selectively deleted IGF-1R within airway epithelial cells in two temporal stages (by inducing *Cre* via doxycycline administration at different times): IGF-1R was deleted either *before* allergen sensitization (**Figure 3.20A**), or *after* the initial allergen sensitization phase but before the allergen challenge phase (schematic in **Figure 3.21**). Additionally, IGF-1R has also been implicated in fibrosis development in the lung, particularly studied in idiopathic pulmonary fibrosis. Hence, we also examined whether IGF-1 signaling through IGF-1R functions at the recovery phase

of an inflammatory disease by allowing mice that underwent the full sensitization and challenge administration to recover for five days after the last HDM (**Figure 3.21**). Mice with IGF-1R deletion prior to the sensitization phase showed a significant increase in inflammatory parameters (**Figure 3.20B, C**), while deleting IGF-1R after the sensitization phase had minimal effect on the extent of inflammation (**Figure 3.22**). Furthermore, after mice were allowed to recover after HDM administration, the residual inflammation did not significantly differ between control and CCSP-Cre/Igf1r$^{fl/fl}$ mice (**Figure 3.23**).

Airway epithelial cells encountering allergen are known to produce a variety of cytokines, such as thymic stromal lymphopoietin (TSLP) and colony stimulating factor 2 (CSF2), for dendritic cell maturation, as well as IL-33 and IL-25 that drives type 2 innate lymphoid cells (ILC2) to proliferate and produce IL-13[278]. Additionally, primary bronchial epithelial cells isolated from asthmatics have been shown to produce elevated levels of IL-6, IL-8, and CSF2 in comparison to control patients[344]. One potential mechanism for exacerbated disease in HDM-treated CCSP-Cre/*Igf1r*$^{fl/fl}$ mice is increased expression of epithelial-derived pro-inflammatory cytokines. Importantly, in the HDM-sensitized CCSP-Cre/*Igf1r*$^{fl/fl}$ mice, there were higher levels of TSLP and IL-6 compared to control mice (**Figure 3.20D, E**). This is of relevance, as studies in humans and mice have shown that TSLP from airway epithelial cells can exacerbate airway inflammation[290,345-347], and elevated IL-6 has been seen in the sputum of mild to moderate asthmatic subjects[348]. These data suggested that IGF-1R signaling in airway epithelial cells limits airway inflammation during the initial antigen exposure/ allergen sensitization phase. In

terms of the potential trigger(s) for IGF-1 release from the alveolar macrophages during the allergen sensitization phase, while IL-4 is often considered a classic Th2 cytokine produced during the adaptive immune response[338,349], both IL-4[350-353] and IL-13[278] can be produced during the allergen sensitization phase by resident mast cells and type 2 innate lymphoid cells, respectively. In fact, IL-4 was enhanced (along with the cytokines IL-5 and CCL-11/eotaxin-1) in the BAL fluid of CCSP-Cre/*Igf1r*$^{fl/fl}$ mice sensitized with HDM (**Figure 3.20C**). Thus, IL-4 and/or apoptotic cells present during the initial allergen exposure could trigger IGF-1 production from alveolar macrophages.

Alveolar macrophage-derived microparticles suppress inflammatory cytokine transcription in airway epithelial cells

Recently, it has been reported that alveolar macrophages can release MPs that may contain anti-inflammatory mediators in the context of a smoking-induced lung injury[354]. As IGF-1 enhances uptake of liposomes by non-professional phagocytes, we next asked whether these small particles and their uptake by epithelial cells might be of relevance in dampening allergen-induced inflammation. As our initial engulfment studies used artificial liposomes, we first isolated MPs from alveolar macrophages to explore their possible effect on airway epithelial cells in the context of house dust mite-induced inflammation. Using differential centrifugation and filtration, we isolated MPs from alveolar macrophages and characterized them in several ways (**Figure 3.20F**). First, cryo-electron microscopy revealed membrane bound spherical structures about 100nm in diameter or larger (**Figure 3.20G, H**). Second, ImageStreamX™ analysis revealed

that these particles, isolated from either an alveolar macrophage cell line or primary mouse alveolar macrophages, carry markers indicative of lung macrophage origin, either single-positive for CD11c or SiglecF, and have varying levels of Annexin V exposure (**Figure 3.20I**). Third, assessing the size of the particles via tunable resistive pulse sensing analysis showed that the mean particle size was about 357±148.5nm (**Figure 3.20J**), well within the range of described MP size (between 100 and 1000nm)[262]. IGF-1 treatment could also increase the uptake of these macrophage-derived MPs by BEAS-2B airway epithelial cells, paralleling our data with the liposome uptake (**Figure 3.20K**). Importantly, while BEAS-2B cells treated with HDM showed increased mRNA expression of *TSLP, CSF2, IL6, and IL8*, concurrent addition of MPs isolated from alveolar macrophages led to significantly lower expression of *TSLP, CSF2, IL6,* and *IL8* (**Figure 3.20L**). These results suggest that MPs released from alveolar macrophages have the potential to dampen inflammatory responses in epithelial cells. Next, to take an unbiased approach to better understand gene expression changes in the lung epithelium during allergic airway inflammation, we performed RNA-Seq on human bronchial epithelial cells. BEAS-2B cells were stimulated with HDM allergen, in the presence or absence of alveolar macrophage-derived MPs for three hours. Total RNA was extracted and used to create mRNA libraries for RNA-Seq. RNA-Seq results show that human bronchial epithelial cells treated with HDM display a large increase in several genes well associated with asthma in humans, such as fibroblast growth factor 2 (FGF2)[355] (**Figure 3.24A**). We validated four of these genes (FGF2, Kruppel-like factor 4 (KLF4), Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), and

Pentraxin 3 (PTX3)) and demonstrate that alveolar-derived MPs were able to suppress transcription of these HDM-induced genes (**Figure 3.24B**). Collectively, these data suggest that the IGF-1 released by macrophages can influence inflammation by promoting MP uptake by airway epithelial cells and by inhibiting inflammatory cytokine release.

4: Discussion

Our studies presented here identify several new concepts in apoptotic cell clearance and tissue inflammation. First, it has long been known that professional and non-professional phagocytes reside in proximity and that they both have the ability to engulf dying cells; whether one regulates engulfment by the other was not known. Our work identifies a rapid, transient, and reversible mode of regulation, wherein the release of soluble IGF-1 by the macrophages redirects the type of particle uptake by non-professional phagocytes via IGF-1/IGF-1R signaling. Specifically, IGF-1 suppresses phagocytosis of apoptotic cells, but enhances the uptake of MPs, by non-professional phagocytes. This transient effect might allow the macrophages to temporarily 'redirect' the non-professional phagocytes toward other function(s). Such a hierarchy in cell clearance between professional and non-professional phagocytes could provide both the temporal and spatial cross-communication between different phagocytes within a given tissue.

Second, our data identify a two-part regulation of epithelial cells by macrophages in the context of airway inflammation. Macrophages release IGF-1 in response to IL-4, IL-13, and apoptotic cells, the latter being a novel inducer discovered through this work. In airway epithelial cells, HDM has been implicated in inducing apoptosis via endoplasmic reticulum stress[356] or DNA double stranded breaks[357]. In addition to mast cells and ILC2s, basophils have been shown to be an early innate source of IL-4, preceding secretion by Th2 T-cells, in helminth models, ovalbumin and papain (a protease structurally related to Der p1, a component of HDM) administration[358-361].

Macrophages also release MPs with anti-inflammatory properties; the released IGF-1 then redirects the epithelial cells to take up more of these smaller particles, resulting in the dampening of the production of inflammatory cytokines by epithelial cells in response to the house dust mite allergen. How this decrease in inflammatory cytokine production in epithelial cells is achieved is currently unknown as it can be mediated by the cargo content of MPs or by recognition of specific surface proteins displayed on MPs. Nevertheless, the immunosuppressive effects of MPs suggest that macrophages can impact nearby non-professional phagocytes in multiple ways, and that IGF-1/IGF-1R axis can be a key modulator in the airway hyper-responsiveness to allergens (model figure in **Figure 3.25**).

Further investigation is needed to connect IGF-1 signaling or MPs to human asthma. Gene profile studies of severely asthmatic children, comparing those in convalescence with those having an acute exacerbation, found that *IGF1R* expression was decreased in the peripheral blood mononuclear cells of those having an acute exacerbation[362]. This is obviously encouraging and supportive of our data. However, another microarray study of bronchial biopsies from 4 asthmatic and 4 control patients showed no significant differences in IGF-1R[363]. Therefore, it is difficult to definitively conclude from these data whether IGF-1R signaling in asthmatics is perturbed. Our study finds a role for IGF-1 signaling in the sensitization phase of allergic airway inflammation, a time in which the innate immune response dominates. Given the pleiotropic activities of IGF-1, it is highly likely that IGF-1 signaling may be beneficial in specific time periods during allergen encounter, and may even be detrimental in others.

With respect to MPs, currently there are no clear studies on the role of macrophage-derived MPs in human asthma. However, several studies, albeit conflicting, examine serum-MP levels in human asthma as biomarkers of disease[364,365]. Importantly, the majority of the MPs detected in these studies were derived from platelets. Our unbiased approach of using RNA-Seq to examine gene expression in bronchial epithelial cells exposed to HDM and/or MPs showed changes in expression of a variety of genes known to be associated with allergic airway inflammation. Further examination into the interplay between these genes and the MPs, as well as the linkage to phagocyte crosstalk, will provide insight into how these factors regulate allergic airway inflammation.

Lastly, the current functions ascribed to IGF-1 are predominantly those involved in growth, cellular proliferation, and aging[366-369]. The data presented here identifies a previously unappreciated biological function for IGF-1 that could be relevant during development as well as tissue homeostasis.

5: Acknowledgement of co-author contributions

While I performed nearly all of the work presented in this chapter, I received help from others in completing these studies. Dr. Ignacio Juncadella taught me the initial *in vivo* airway engulfment assays; Dr. Jason Kinchen helped with cloning and advice on the cytoskeletal studies; Dr. Monica Buckley assisted on some of the *in vivo* assays; Dr. Alexander Klibanov provided the liposomes; Dr. Kelly Dryden performed the electron microscopy of the MPs; Dr. Uta Erdbrugger provided guidance for the IZON qNano

measurements and advice on studies related to extracellular vesicles; Dr. Suna Onengut-Gumuscu performed the RNA-Seq runs on my samples; Dr. Yun M. Shim helped assess lung function of the mice via the methacholine challenge; and Dr. Kenneth Tung helped with scoring the lung histology.

Figure 3.1 – IGF-1 dampens apoptotic cell engulfment by non-professional phagocytes.

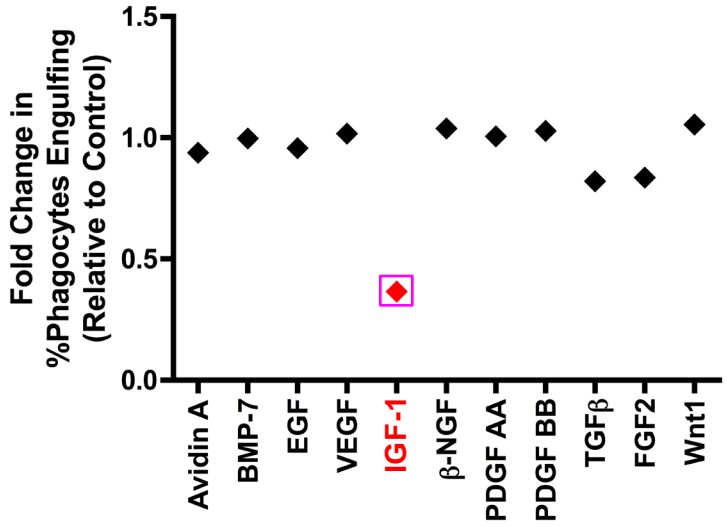
(A) Schematic summarizing the results of testing 11 growth factors on modulating the ability of LR73 cells to engulf apoptotic cells. The engulfment in the presence of each factor was normalized to the vehicle control in every experiment. For 10 of these factors, the normalized value is close to 1, as they did not modulate uptake, while IGF-1 resulted in an inhibition of engulfment.

(B) (Left panel) Representative engulfment assay with LR73 cells treated with increasing concentrations of IGF-1 and apoptotic thymocytes as targets (n=3). (Right panel) Representative Western blot of IGF-1R expression and phosphorylation of Akt in LR73 cells stimulated with IGF-1 for the indicated times.

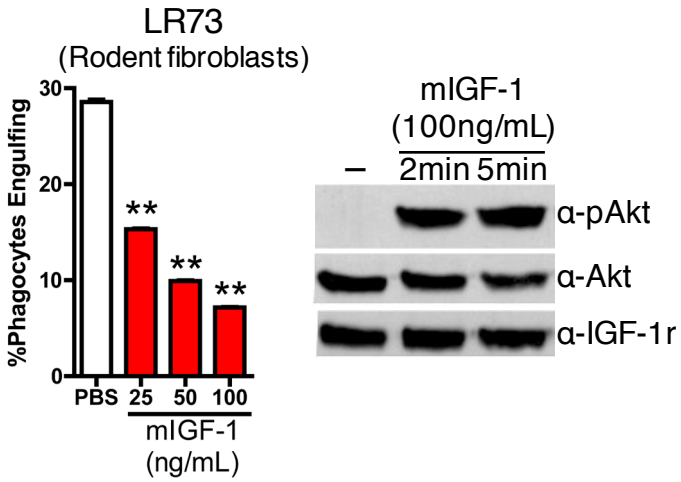
(C) Engulfment of BEAS-2B cells treated with various concentrations of human IGF-1 using apoptotic thymocytes as targets (n=3)

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

A.


Fold Change in %Phagocytes Engulfing (Relative to Control)
1.5
1.0
0.5
0.0
Avidin A
BMP-7
EGF
VEGF
IGF-1
β-NGF
PDGF AA
PDGF BB
TGFβ
FGF2
Wnt1

B.


LR73
(Rodent fibroblasts)
%Phagocytes Engulfing
30
20
10
0
**
**
**
PBS
25
50
100
mIGF-1
(ng/mL)
mIGF-1
(100ng/mL)
–
2min
5min
α-pAkt
α-Akt
α-IGF-1r

C.

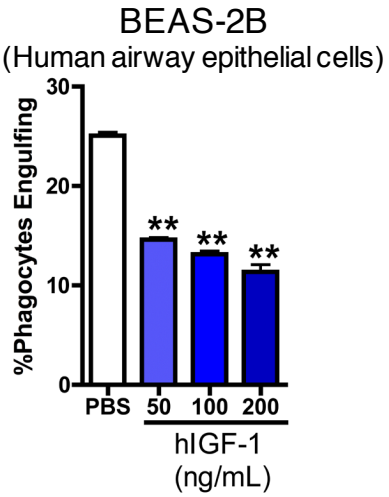
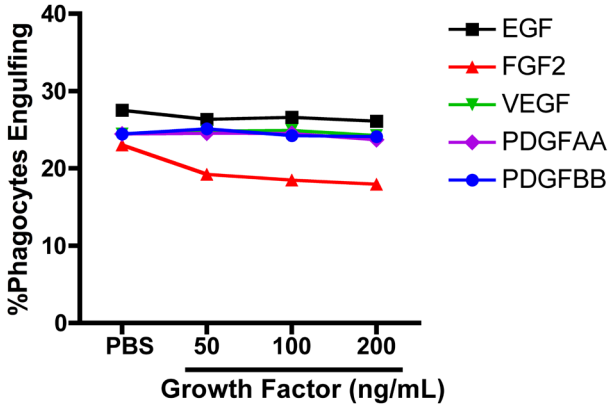

BEAS-2B
(Human airway epithelial cells)
%Phagocytes Engulfing
30
20
10
0
**
**
**
PBS
50
100
200
hIGF-1
(ng/mL)

Figure 3.2 – Erk1/2 is phosphorylated in LR73 cells stimulated with EGF, VEGF, PDGF AA/BB.

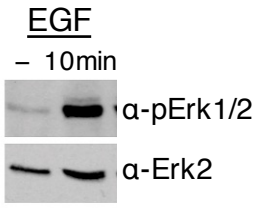
(A) Representative engulfment assay in which LR73 cells were treated with the indicated growth factors at increasing concentrations and assessed for engulfment of apoptotic thymocytes (n=3).

(B-D) Serum-starved LR73 cells were stimulated with 100ng/mL of the indicated growth factors for 10 minutes and the phosphorylation of Erk1/2 was assessed by immunoblotting.

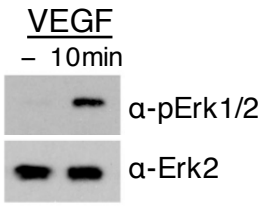
A.


%Phagocytes Engulfing
40
30
20
10
0
PBS
50
100
200
Growth Factor (ng/mL)
EGF
FGF2
VEGF
PDGFAA
PDGFBB

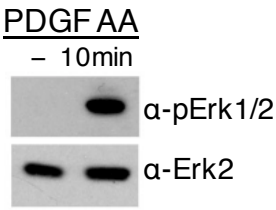
B.


EGF
– 10min
α-pErk1/2
α-Erk2

C.


VEGF
– 10min
α-pErk1/2
α-Erk2

D.


PDGF AA
– 10min
α-pErk1/2
α-Erk2

E.

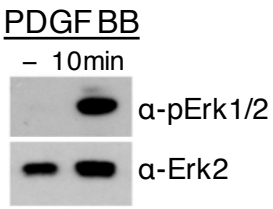

PDGF BB
– 10min
α-pErk1/2
α-Erk2

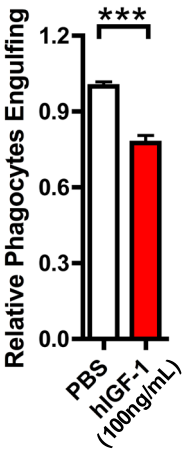
Figure 3.3 – 16HBE14o- cells and SVEC-40 cells engulf less apoptotic cells when exposed to IGF-1.

(A) Representative engulfment assay in which the uptake of apoptotic thymocytes by 16HBE14o- human airway epithelial cells was inhibited by IGF-1 treatment (n=3).

(B) Engulfment of SVEC-40 endothelial cells treated with IGF-1 and apoptotic thymocytes as targets (n=3). Data are represented as mean ± s.d.

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks).

A 16HBE14o-



B. SVEC-40

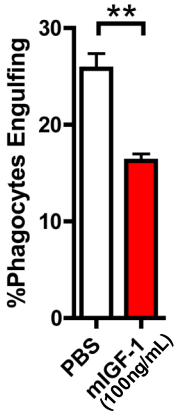


Figure 3.4 – IGF-1 signals through IGF-1R to dampen apoptotic cell engulfment in non-professional phagocytes.

(A) Histograms showing Annexin V binding to apoptotic thymocytes; thymocytes were first exposed to dexamethasone to induce apoptotic cell death and then subsequently treated with indicated concentrations of IGF-1 (n=6).

(B) Representative engulfment assay in which LR73 cells pretreated with IGF-1 were washed with plain media, and then incubated with apoptotic thymocytes in the presence or absence of IGF-1 (n=3).

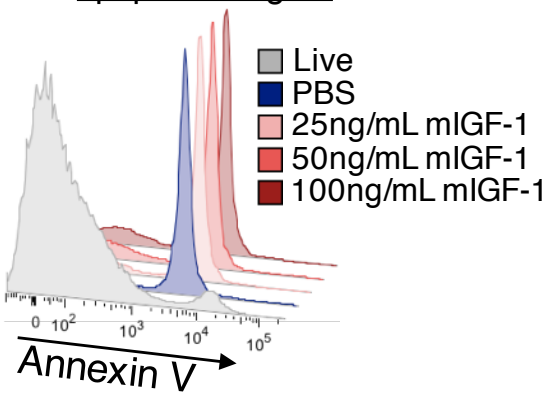
(C) Representative engulfment assay of LR73 cells treated with either PBS, IGF-1, IGFBP3 or IGF-1 preincubated with IGBFP3 using apoptotic thymocytes as targets (n=3).

(D) Addition of anti-IGF-1 receptor neutralizing antibody reversed the inhibition of engulfment due to IGF-1 in BEAS-2B cells (n=3).

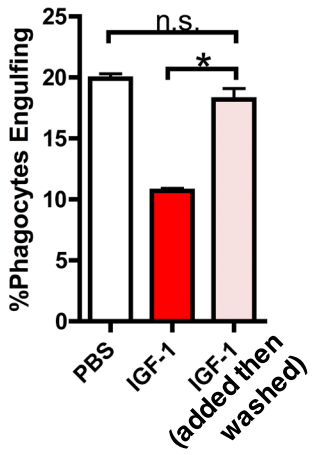
(E) (Left panel) Representative engulfment assay of LR73 cells treated with the indicated concentrations of OSI-906 in the presence or absence of IGF-1 (n=3). (Right panel) Representative Western blot of tyrosine phosphorylation of IGF-1R and Erk1/2 from serum-starved LR73 cells treated with OSI-906 and then stimulated with IGF-1 (n=3).

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks).

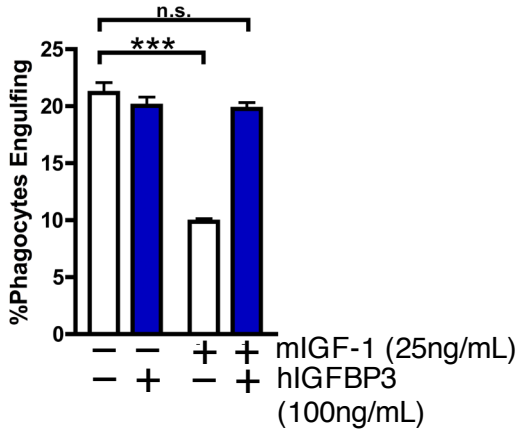
A.


PtdSer exposure on apoptotic targets
Live
PBS
25ng/mL mIGF-1
50ng/mL mIGF-1
100ng/mL mIGF-1
0
10^2
10^3
10^4
10^5
Annexin V

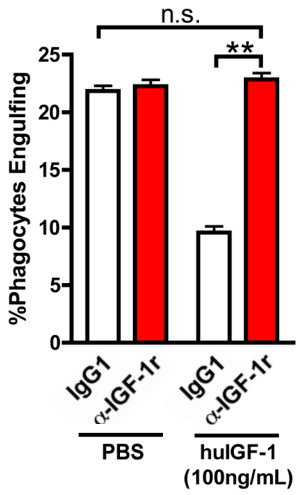
B.


LR73
n.s.
*
%Phagocytes Engulfing
25
20
15
10
5
0
PBS
IGF-1
IGF-1 (added then washed)

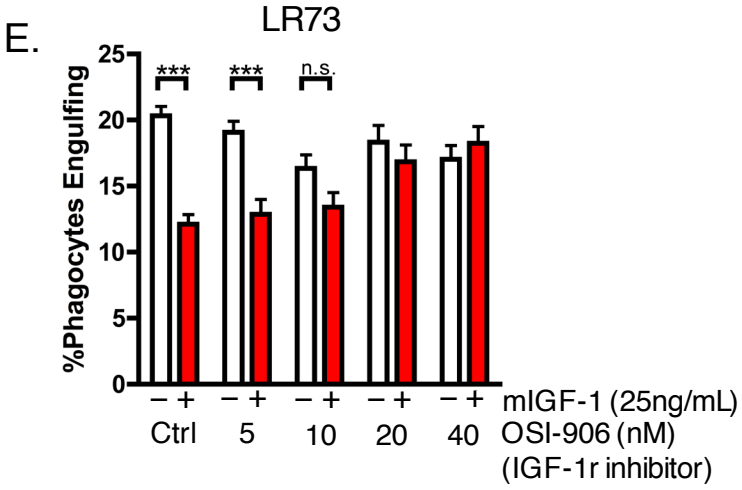
C.


LR73
n.s.
***
%Phagocytes Engulfing
25
20
15
10
5
0
– – + + mIGF-1 (25ng/mL)
– + – + hIGFBP3 (100ng/mL)

D.


BEAS-2B
n.s.
**
%Phagocytes Engulfing
25
20
15
10
5
0
IgG1
α-IGF-1r
IgG1
α-IGF-1r
PBS
huIGF-1 (100ng/mL)

E.


LR73
***
***
n.s.
%Phagocytes Engulfing
25
20
15
10
5
0
– + – + – + – + – + mIGF-1 (25ng/mL)
Ctrl 5 10 20 40 OSI-906 (nM)
(IGF-1r inhibitor)

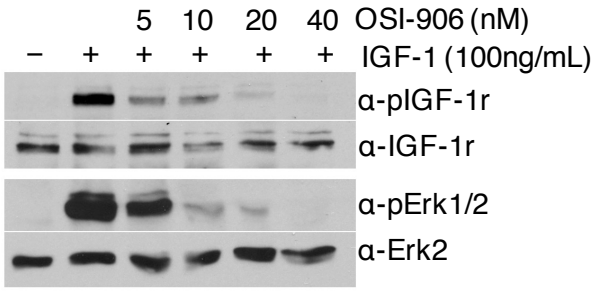

5 10 20 40 OSI-906 (nM)
– + + + + + IGF-1 (100ng/mL)
α-pIGF-1r
α-IGF-1r
α-pErk1/2
α-Erk2

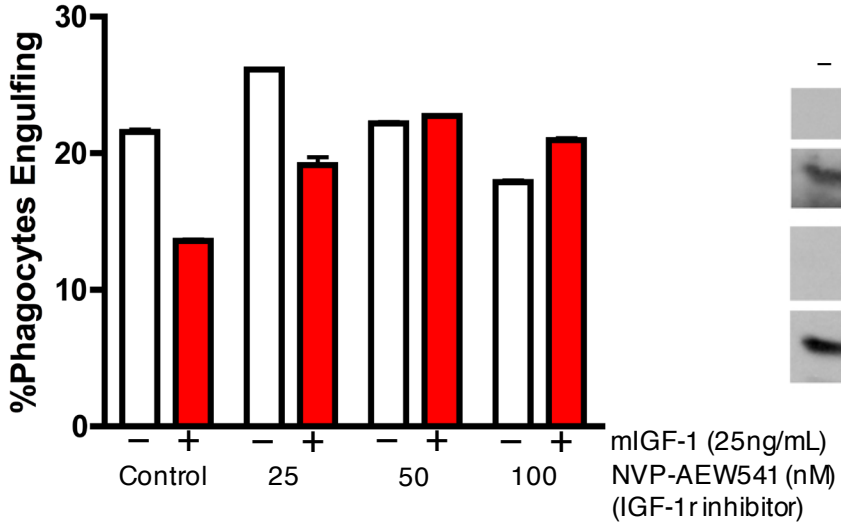
Figure 3.5 – IGF-1R inhibitor, NVP-AEW541, rescues inhibition of apoptotic cell engulfment due to IGF-1.

(A) Engulfment of apoptotic thymocytes by LR73 cells treated with various doses of NVP-AEW541, a small molecule inhibitor of IGF-1R (n=3).

(B) Representative Western blot of LR73 cells stimulated with IGF-1 and treated with increasing doses of NVP-AEW541 (n=2).

Data are represented as mean ± s.d.

A.


%Phagocytes Engulfing
30
20
10
0
– + – + – + – +
Control
25
50
100
mIGF-1 (25ng/mL)
NVP-AEW541 (nM)
(IGF-1r inhibitor)

B.

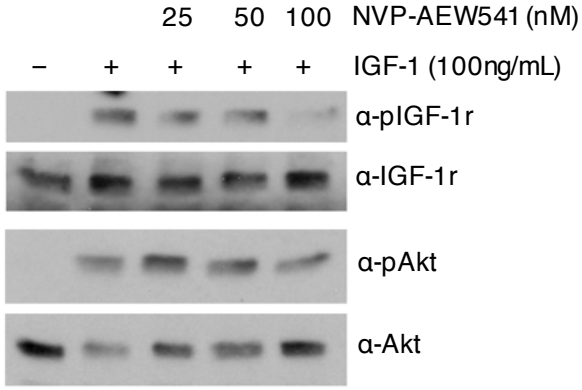
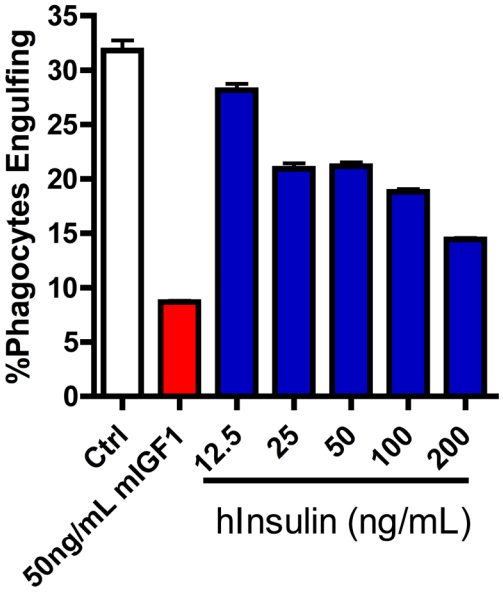

25 50 100 NVP-AEW541 (nM)
– + + + + IGF-1 (100ng/mL)
α-pIGF-1r
α-IGF-1r
α-pAkt
α-Akt

Figure 3.6 – LR73 cells treated with recombinant insulin or IGF-II engulf less apoptotic cells.

Engulfment of apoptotic thymocytes by LR73 cells that were treated with the indicated concentrations of human insulin **(A)** and human IGF-II **(B)** (n=2). Data are represented as mean ± s.d.

A.


%Phagocytes Engulfing
35
30
25
20
15
10
5
0
Ctrl
50ng/mL mIGF1
12.5
25
50
100
200
hInsulin (ng/mL)

B.

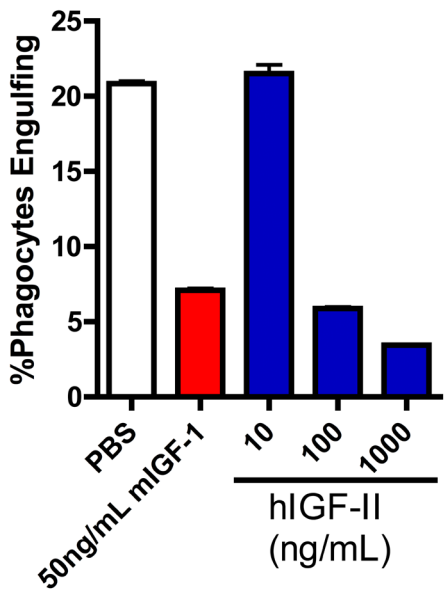
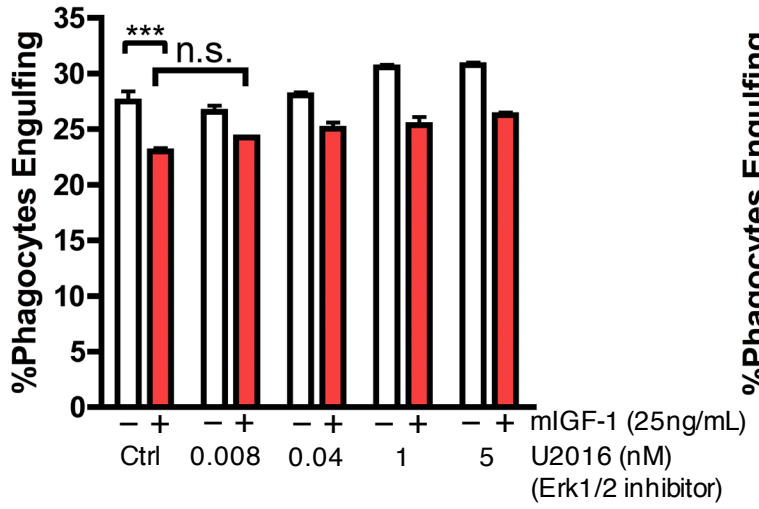

%Phagocytes Engulfing
25
20
15
10
5
0
PBS
50ng/mL mIGF-1
10
100
1000
hIGF-II
(ng/mL)

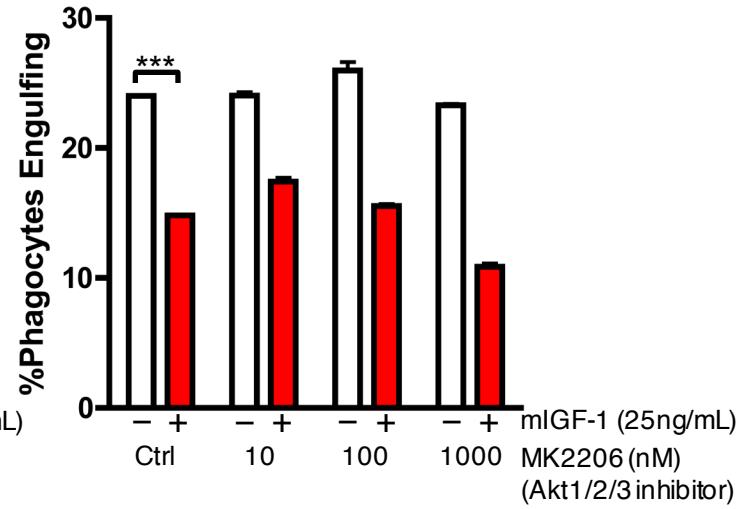
Figure 3.7 – Blocking canonical signaling intermediates downstream of IGF-1 receptor signaling does not reverse the IGF-1 mediated engulfment suppression. **(A-D)** Engulfment of apoptotic thymocytes by LR73 cells treated with U2016 (Erk1/2 inhibitor) **(A)**, MK-2206 (Akt1/2/3 inhibitor) **(B)**, Rapamycin (mTOR inhibitor) **(C)**, or Wortmannin (PI 3-Kinase inhibitor) **(D)** in the presence or absence of IGF-1 (n=2-3). Wortmannin has been previously demonstrated to inhibit phagocytosis of apoptotic cells and adding IGF-1 does not alter this inhibition. Data are represented as mean ± s.d.

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

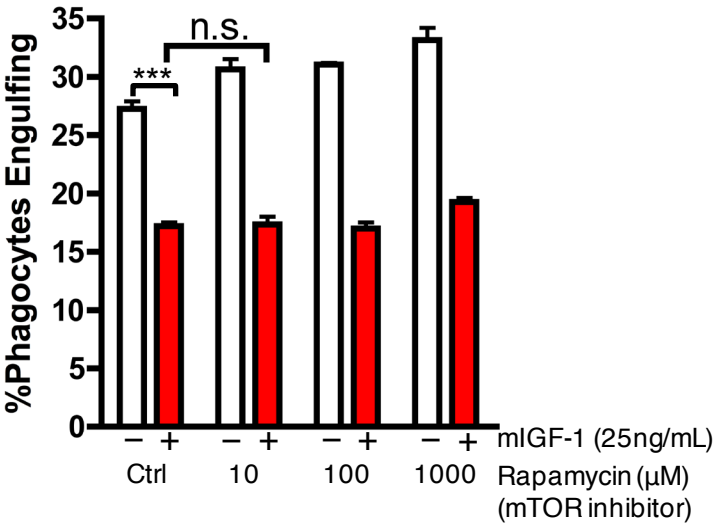
A.


%Phagocytes Engulfing
35
30
25
20
15
10
5
0
***
n.s.
− + − + − + − + − +
mIGF-1 (25ng/mL)
Ctrl
0.008
0.04
1
5
U2016 (nM)
(Erk1/2 inhibitor)

B.


%Phagocytes Engulfing
30
20
10
0
***
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl
10
100
1000
MK2206 (nM)
(Akt1/2/3 inhibitor)

C.


%Phagocytes Engulfing
35
30
25
20
15
10
5
0
***
n.s.
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl
10
100
1000
Rapamycin (μM)
(mTOR inhibitor)

D.

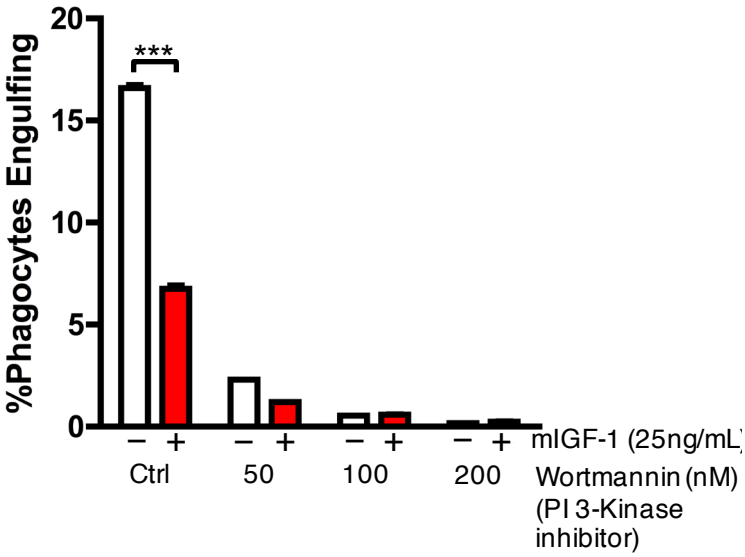
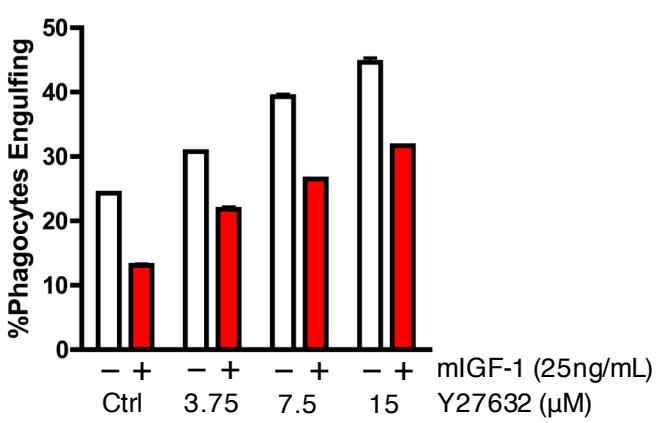
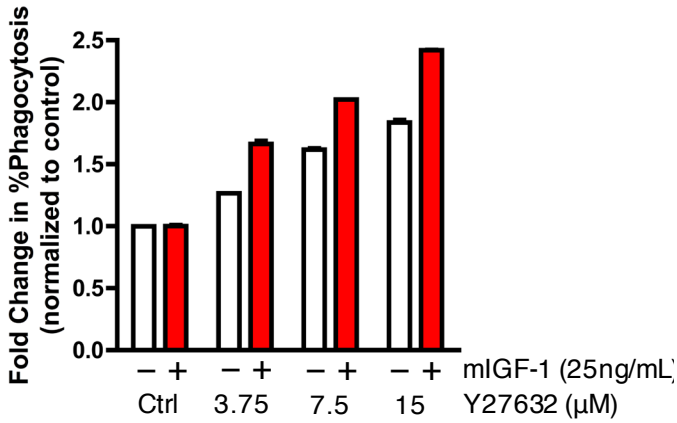

%Phagocytes Engulfing
20
15
10
5
0
***
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl
50
100
200
Wortmannin (nM)
(PI 3-Kinase
inhibitor)

Figure 3.8 – Inhibition of Rho-kinase (ROCK) does not appear to rescue IGF-1 induced engulfment suppression in LR73 cells.

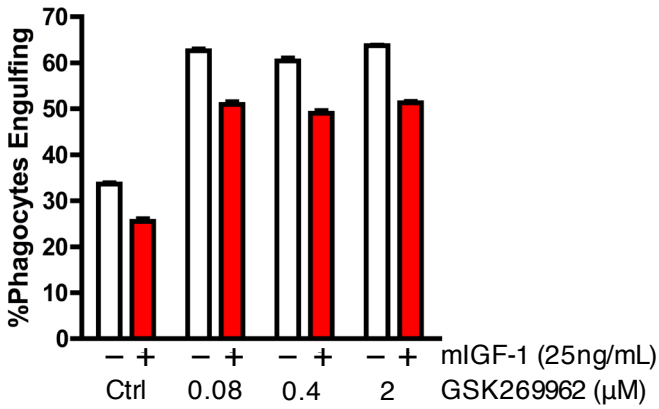
(A-B) (Left panel) Engulfment assay of LR73 cells treated with various doses of the Rho kinase inhibitors Y27632 **(A)** or GSK269962 **(B)** in the presence or absence of IGF-1 (n=2-3). Normalized data for each treatment is displayed in the right panels. Data are represented as mean ± s.d.

A.


%Phagocytes Engulfing
50
40
30
20
10
0
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl 3.75 7.5 15
Y27632 (μM)


Fold Change in %Phagocytosis
(normalized to control)
2.5
2.0
1.5
1.0
0.5
0.0
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl 3.75 7.5 15
Y27632 (μM)

B.


%Phagocytes Engulfing
70
60
50
40
30
20
10
0
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl 0.08 0.4 2
GSK269962 (μM)

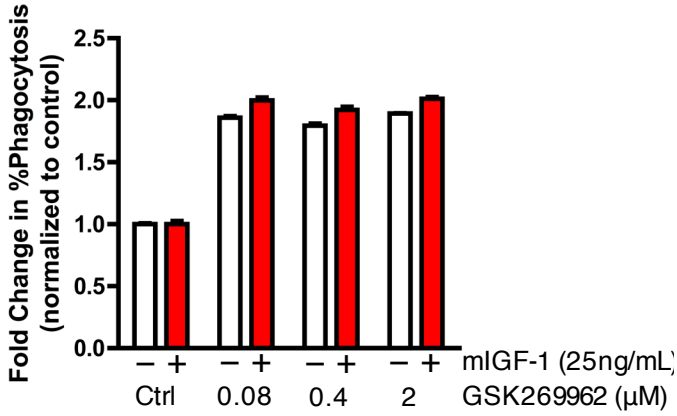

Fold Change in %Phagocytosis
(normalized to control)
2.5
2.0
1.5
1.0
0.5
0.0
− + − + − + − +
mIGF-1 (25ng/mL)
Ctrl 0.08 0.4 2
GSK269962 (μM)

Figure 3.9 – Overexpression of constitutively active Rac1 restores phagocytic capacity to non-professional phagocytes treated with IGF-1.

Representative engulfment assay of LR73 cells transfected with plasmids encoding RacG12V and incubated with apoptotic thymocytes in the presence or absence of 100ng/mL of mIGF-1 (n=3).

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

LR73

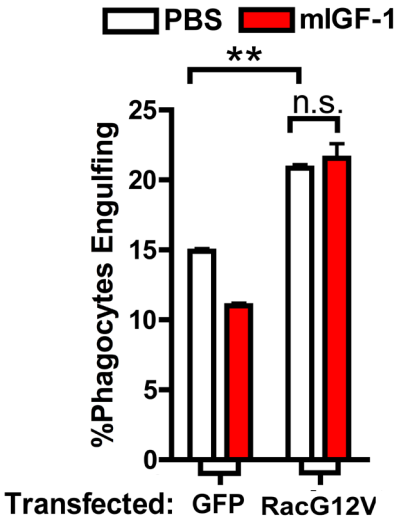

PBS
mIGF-1
**
n.s.
%Phagocytes Engulfing
25
20
15
10
5
0
Transfected:
GFP
RacG12V

Figure 3.10 – IGF-1 enhances liposome uptake in non-professional phagocytes.

(A) (Left panel) Treatment of J774 cells with various concentrations of IGF-1 did not impact their phagocytosis of apoptotic thymocytes (n=3). (Right panel) Representative Western blot of IGF-1R expression and phosphorylation of Akt in serum-starved J774 cells stimulated with IGF-1 for indicated times (n=3).

(B, C) Engulfment assay with bone marrow-derived macrophages or resident peritoneal macrophages treated with various concentrations of IGF-1, and apoptotic thymocytes as targets (n=3).

(D) Representative liposome uptake assay in which LR73 cells were treated with IGF-1 (50ng/mL), EGF (100ng/mL), or VEGF (100ng/mL) in the presence of MPs (n=3).

(E) LR73 cells were incubated with either apoptotic cells or liposome alone, with IGF-1, or with IGF-1 and OSI-906, and assessed for apoptotic cell engulfment or MP uptake (n=3).

(F) Resident peritoneal macrophages were treated with various concentrations of mouse IGF-1 and then assessed for liposome uptake (n=3).

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

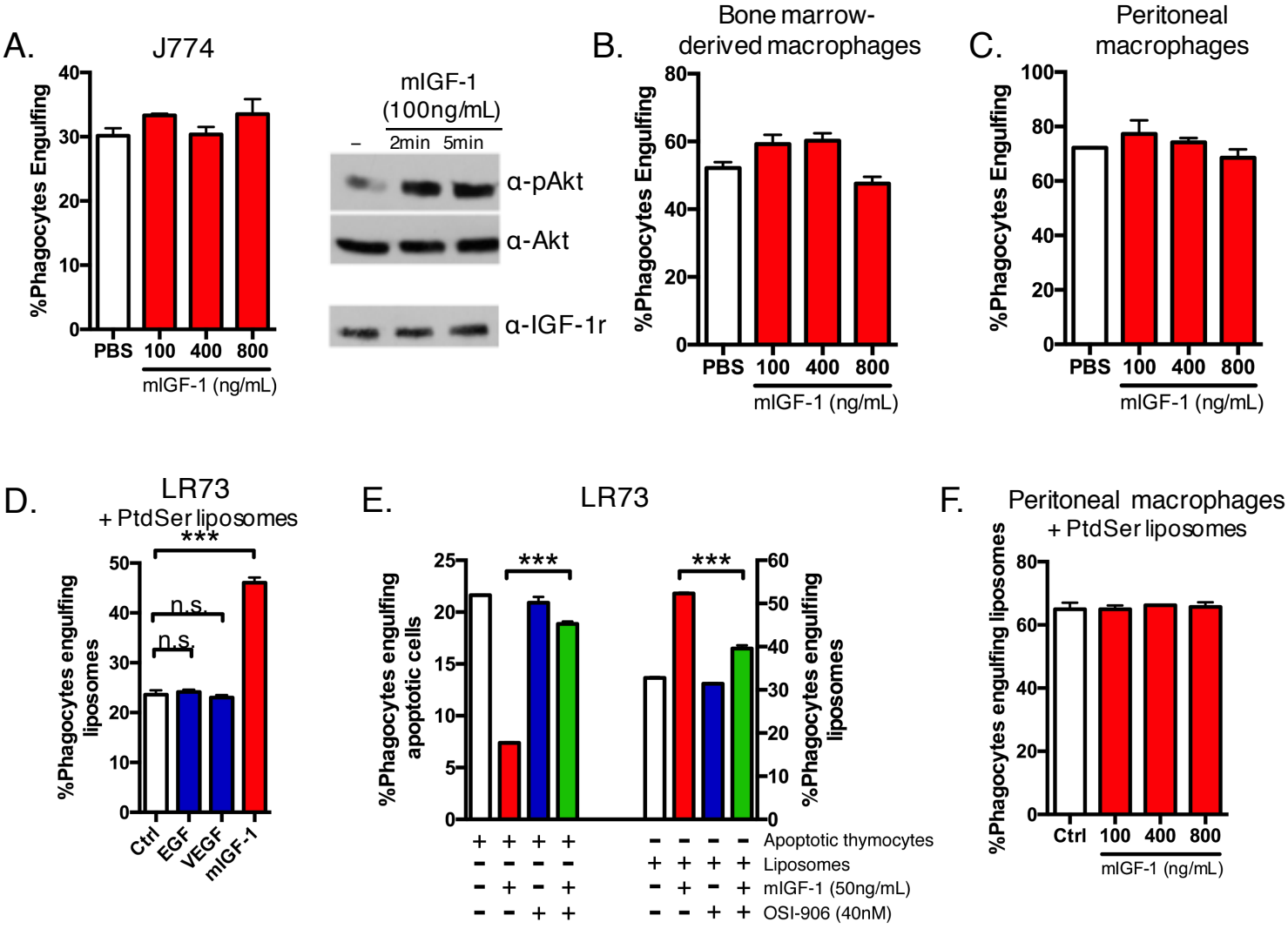
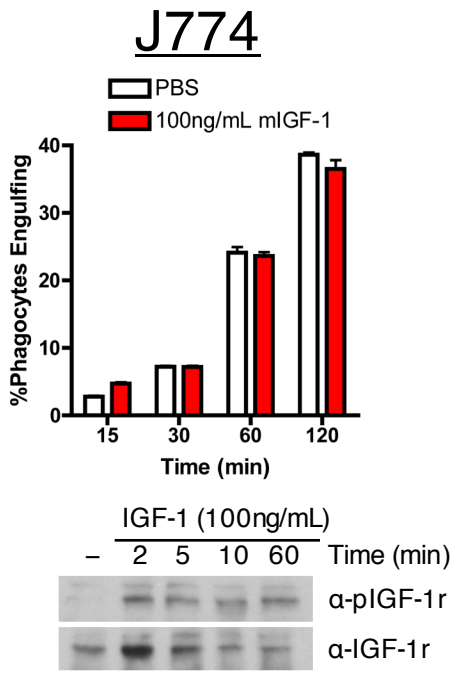

A.
J774
%Phagocytes Engulfing
PBS 100 400 800
mIGF-1 (ng/mL)
mIGF-1 (100ng/mL)
– 2min 5min
α-pAkt
α-Akt
α-IGF-1r
B.
Bone marrow-derived macrophages
%Phagocytes Engulfing
PBS 100 400 800
mIGF-1 (ng/mL)
C.
Peritoneal macrophages
%Phagocytes Engulfing
PBS 100 400 800
mIGF-1 (ng/mL)
D.
LR73
+ PtdSer liposomes
%Phagocytes engulfing liposomes
***
n.s.
n.s.
Ctrl EGF VEGF mIGF-1
E.
LR73
%Phagocytes engulfing apoptotic cells
%Phagocytes engulfing liposomes
***
***
Apoptotic thymocytes
Liposomes
mIGF-1 (50ng/mL)
OSI-906 (40nM)
F.
Peritoneal macrophages
+ PtdSer liposomes
%Phagocytes engulfing liposomes
Ctrl 100 400 800
mIGF-1 (ng/mL)

Figure 3.11 – J774 and LR73 cells phosphorylate IGF-1R upon IGF-1 stimulation similarly.

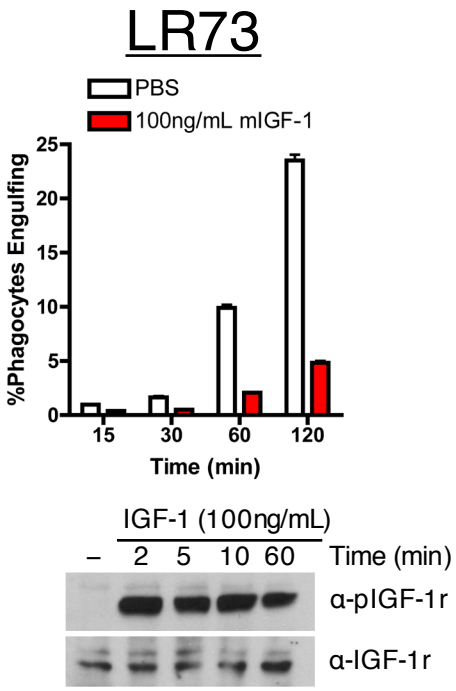
J774 cells **(A)** or LR73 cells **(B)** treated with 100ng/mL mouse IGF-1 were assessed for their ability to engulf apoptotic thymocytes (upper) or serum-starved for 6 hours and stimulated with 100ng/mL mouse IGF-1 and assessed for phosphorylation of IGF-1R by Western blot (bottom). Data are represented as mean ± s.d.

(C) Flow cytometry histograms of IGF-1R expression on J774 cells (left), BMDM (middle), and peritoneal macrophages (right) (n=3-4).

A.


J774
PBS
100ng/mL mIGF-1
%Phagocytes Engulfing
0
10
20
30
40
15
30
60
120
Time (min)
IGF-1 (100ng/mL)
–
2
5
10
60
Time (min)
α-pIGF-1r
α-IGF-1r

B.


LR73
PBS
100ng/mL mIGF-1
%Phagocytes Engulfing
0
5
10
15
20
25
15
30
60
120
Time (min)
IGF-1 (100ng/mL)
–
2
5
10
60
Time (min)
α-pIGF-1r
α-IGF-1r

C.

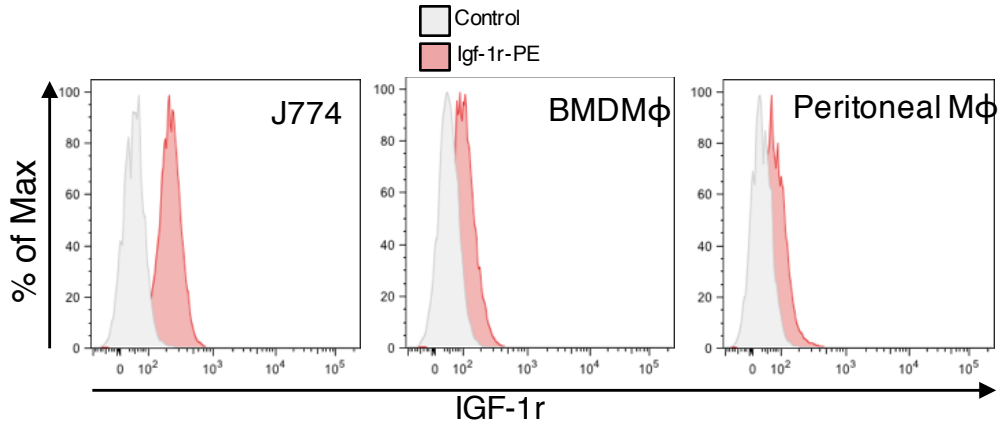
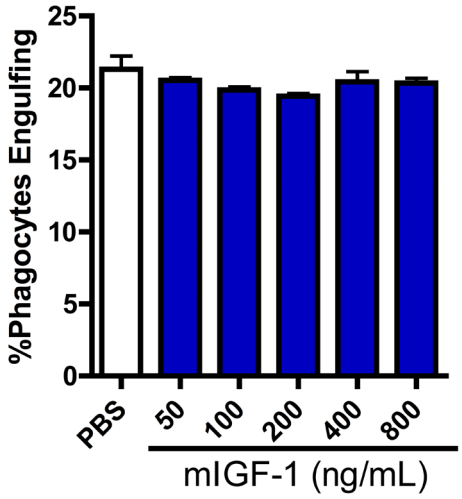

Control
Igf-1r-PE
J774
BMDMϕ
Peritoneal Mϕ
% of Max
IGF-1r

Figure 3.12 – IGF-1 and insulin do not modulate apoptotic cell uptake in the IC-21 macrophage cell line.

IC-21 cells treated with the indicated concentrations of mouse IGF-1 **(A)** or human insulin **(B)** were assessed for their ability to engulf apoptotic thymocytes (n=2-3). Data are represented as mean ± s.d.

A.


%Phagocytes Engulfing
25
20
15
10
5
0
PBS
50
100
200
400
800
mIGF-1 (ng/mL)

B.

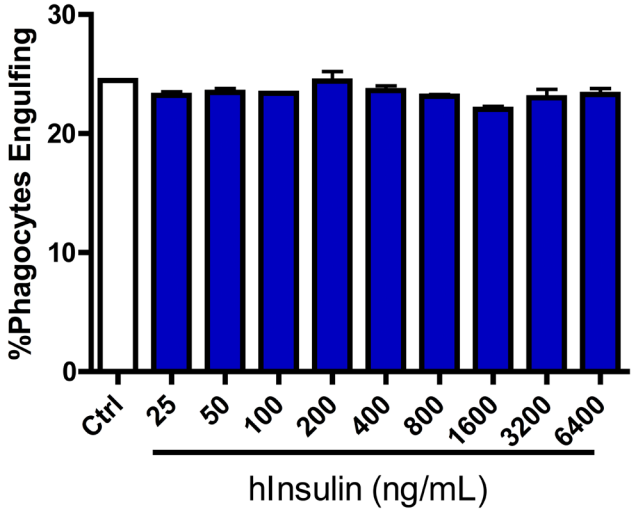

%Phagocytes Engulfing
30
20
10
0
Ctrl
25
50
100
200
400
800
1600
3200
6400
hInsulin (ng/mL)

Figure 3.13 – IGF-1 appears to affect actin dynamics in non-professional phagocytes engulfing liposomes.

LR73 cells were treated with Cytochalasin D **(A)**, Latrunculin A **(B)**, Jasplakinolide **(C)**, or CK-666 **(D)** at the concentrations indicated and then assessed for uptake of liposomes in the presence of IGF-1. Promoting actin depolymerization (A, B) suppressed the IGF-1 mediated enhancement of liposome uptake; stabilizing actin filaments (C) had minimal effect. Mechanistically, the IGF-1 modulation of phagocytosis does not seem to require Arp2/3-mediated actin nucleation (D). Data are represented as mean ± s.d.

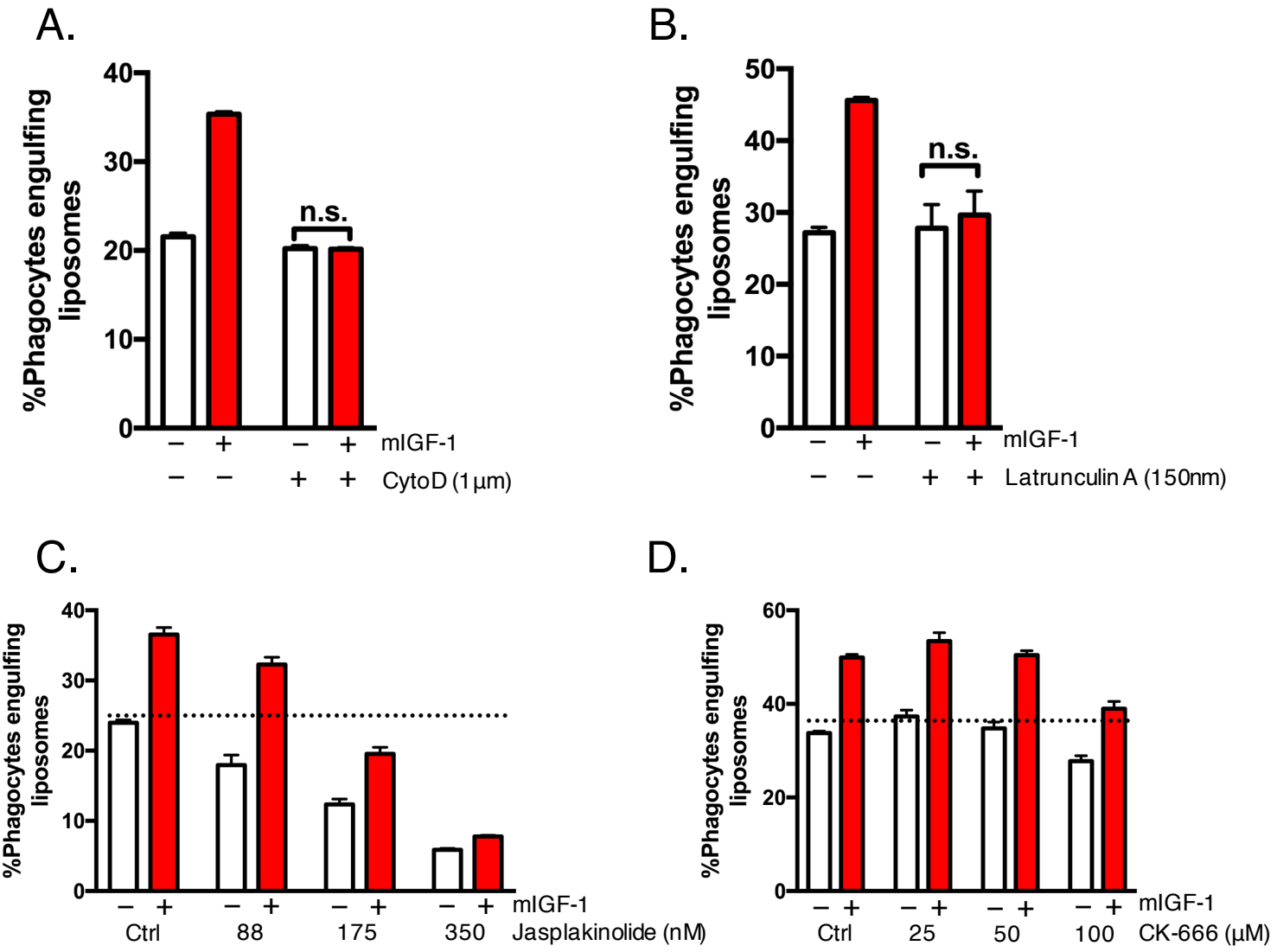

A.
%Phagocytes engulfing liposomes
40
30
20
10
0
n.s.
– + – + mIGF-1
– – + + CytoD (1 µm)
B.
%Phagocytes engulfing liposomes
50
40
30
20
10
0
n.s.
– + – + mIGF-1
– – + + Latrunculin A (150nm)
C.
%Phagocytes engulfing liposomes
40
30
20
10
0
– + – + – + – + mIGF-1
Ctrl 88 175 350 Jasplakinolide (nM)
D.
%Phagocytes engulfing liposomes
60
40
20
0
– + – + – + – + mIGF-1
Ctrl 25 50 100 CK-666 (µM)

Figure 3.14 – Macrophages produce IGF-1 during apoptotic cell clearance.

(A) (Left panel) Concentration of IGF-1 secreted by peritoneal macrophages stimulated with recombinant IL-4, apoptotic Jurkat cells or live Jurkat cells were measured by ELISA. (Right panel) The apoptotic Jurkat cells and live Jurkat cells alone did not produce IGF-1 (n=3). Represented as mean ± s.d.

(B) (left panel) Schematic of an experiment in which J774 cells were treated with PBS or rIL-4. Twenty-four hours later, the supernatant was harvested and half of it was used for ELISA to measure the secreted IGF-1 concentration (top right panel) and the other half was used in an engulfment assay to assess its effect on regulating apoptotic cell engulfment by LR73 cells (bottom left panel) (n=3). To confirm that the inhibitory effect of the supernatants was mediated through IGF-1, the supernatants were pre-incubated with PBS or IGFBP3 prior to use in the phagocytosis assay as indicated. Because J774 cells produce low levels of IGF-1 even without adding the IL-4 stimulus, addition of IGFBP3 to that supernatant was able to increase phagocytosis of apoptotic cells by LR73 cells. Data are represented as mean ± s.d.

(C) (Top panel) CCSP-Cre mice in which YFP is expressed in Club cells were first pre-treated with either PBS or 1μg of IGF-1 intranasally. One hour later, 100 million fluorescently labeled (CypHer5E) apoptotic thymocytes were given intranasally with or without IGF-1. After 3.5 hours, BAL fluid was collected and the lungs harvested for alveolar macrophages and airway epithelial cells. The cells were subsequently stained with appropriate markers and assessed for their engulfment of the instilled apoptotic

cells. Alveolar macrophages were identified as $CD11c^{+}$ Siglec F^{+}, while airway epithelial cells are identified as $EpCAM^{hi}$ YFP^{+}.

(D) Engulfment of apoptotic thymocytes by alveolar macrophages (left panel) and CCSP-YFP cells (right panel) from the same mice, as described in (C) are shown (n=6 per group). Data are represented as mean ± s.e.m.

(E) Engulfment of liposomes by alveolar macrophages (left panel) and CCSP-YFP cells (right panel) from the same mice, treated as described in (C) (n=3 per group). Data are represented as mean ± s.e.m.

(F) Schematic for intranasal IGF-1 analysis. Mice were given PBS, IL-4, IL-13, apoptotic Jurkat cells, or IL-5 intranasally for 2 consecutive days. On the third day, BAL fluid was collected and the IGF-1 concentrations in the lavage assessed by ELISA.

(G) Wild-type mice were given PBS, IL-4, apoptotic cells, IL-5, or IL-13 intranasally and the BAL fluid was assessed for IGF-1 concentration (n=2-3 mice per group for cytokines, n=5, 8 for apoptotic cell instillation). Data are represented as mean ± s.e.m.

(H) Mice from LysM-Cre/$Igf1^{wt/wt}$ or $Igf1^{fl/fl}$ were given PBS, IL-4 or IL-13, or apoptotic cells intranasally. BAL fluid was harvested and assessed for IGF-1 using ELISA (n=6, 6, 4 mice per group for rIL-4; n=6, 4, 4 for rIL-13; n=6, 9, 9 mice per group for apoptotic cell instillation). Data are represented as mean ± s.e.m.

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

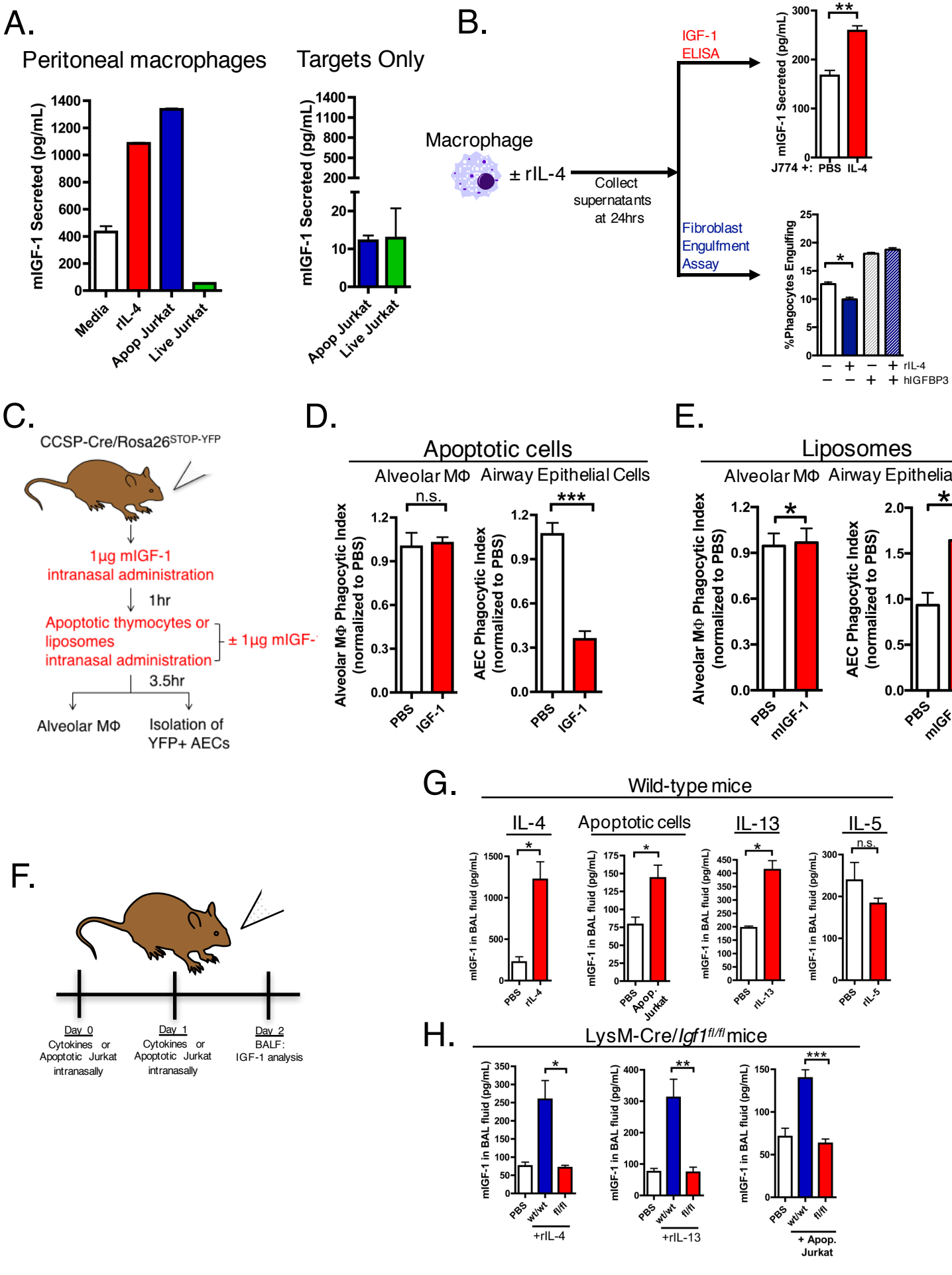

A.
Peritoneal macrophages
mIGF-1 Secreted (pg/mL)
Media
rIL-4
Apop Jurkat
Live Jurkat
Targets Only
Apop Jurkat
Live Jurkat
B.
Macrophage
± rIL-4
Collect supernatants at 24hrs
IGF-1 ELISA
mIGF-1 Secreted (pg/mL)
J774 +: PBS IL-4
Fibroblast Engulfment Assay
%Phagocytes Engulfing
rIL-4
hIGFBP3
C.
CCSP-Cre/Rosa26STOP-YFP
1µg mIGF-1 intranasal administration
1hr
Apoptotic thymocytes or liposomes intranasal administration
± 1µg mIGF-
3.5hr
Alveolar MΦ
Isolation of YFP+ AECs
D.
Apoptotic cells
Alveolar MΦ
Airway Epithelial Cells
n.s.
***
Alveolar MΦ Phagocytic Index (normalized to PBS)
AEC Phagocytic Index (normalized to PBS)
PBS
IGF-1
E.
Liposomes
Alveolar MΦ
Airway Epithelial Cells
*
*
Alveolar MΦ Phagocytic Index (normalized to PBS)
AEC Phagocytic Index (normalized to PBS)
PBS
mIGF-1
F.
Day 0
Cytokines or Apoptotic Jurkat intranasally
Day 1
Cytokines or Apoptotic Jurkat intranasally
Day 2
BALF: IGF-1 analysis
G.
Wild-type mice
IL-4
Apoptotic cells
IL-13
IL-5
mIGF-1 in BAL fluid (pg/mL)
PBS
rIL-4
Apop. Jurkat
rIL-13
rIL-5
n.s.
H.
LysM-Cre/Igf1fl/fl mice
mIGF-1 in BAL fluid (pg/mL)
PBS
wt/wt
fl/fl
+rIL-4
+rIL-13
+ Apop. Jurkat

Figure 3.15 – Production of IGF-1 by peritoneal macrophages after apoptotic cell or IL-4 stimulation is mostly likely new transcription.

Peritoneal macrophages were either untreated, stimulated with rIL-4 or apoptotic Jurkat cells and *Igf1* mRNA *(top panels)* and IGF-1 protein in the supernatant *(bottom panels)* were assessed in a time course (n.d. refers to not detected). Data are represented as mean ± s.d.

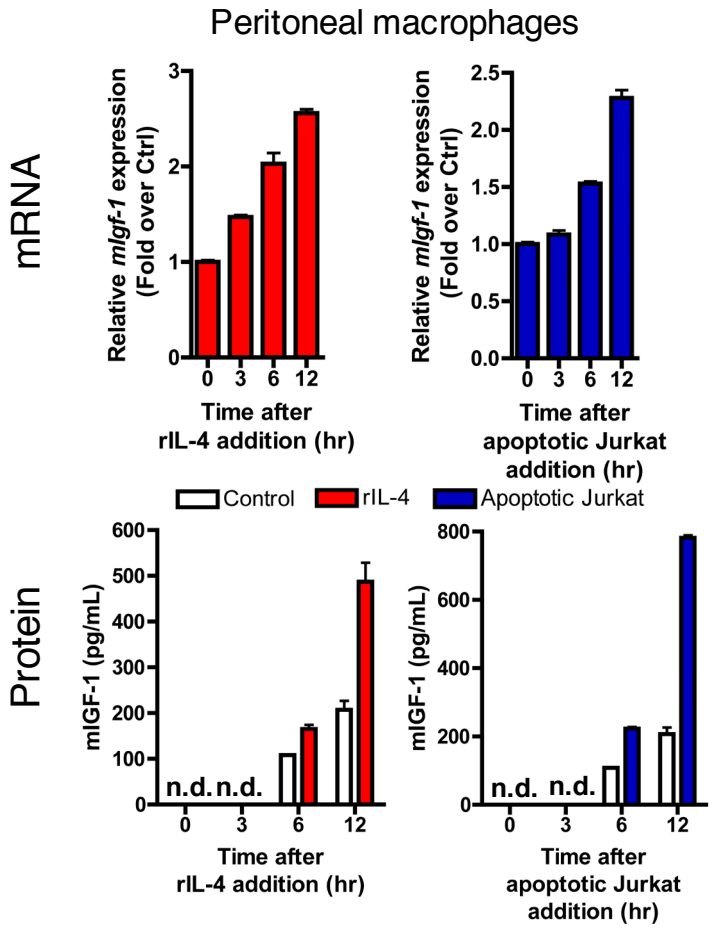
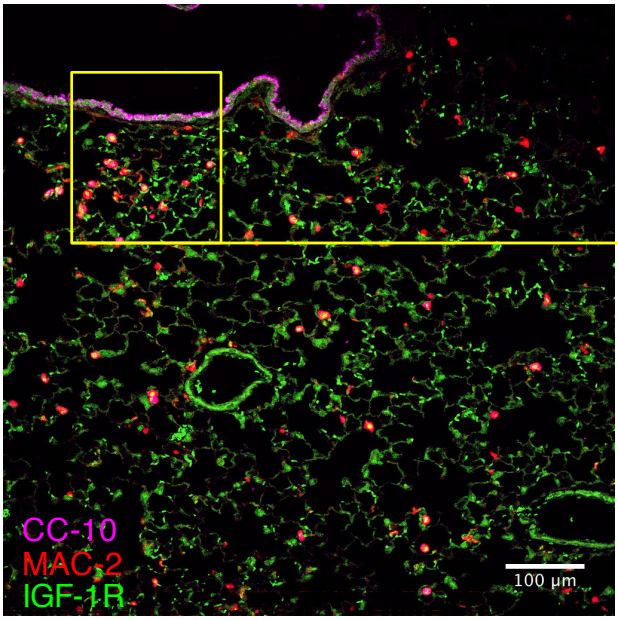

Peritoneal macrophages
mRNA
Protein
Relative mlgf-1 expression (Fold over Ctrl)
Time after rIL-4 addition (hr)
Time after apoptotic Jurkat addition (hr)
Control
rIL-4
Apoptotic Jurkat
mIGF-1 (pg/mL)
n.d.
n.d.

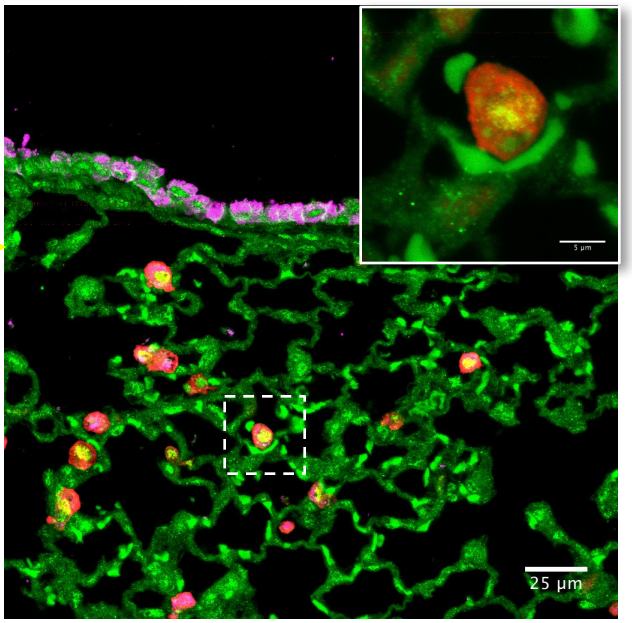
Figure 3.16 – IGF-1R expression in alveolar macrophages and airway epithelial cells.

(A, B) Lung sections from wild type mice were stained with antibodies against alveolar macrophages (MAC-2), airway epithelial cells (CC-10), and IGF-1R. **(C)** is another field showing colocalization of IGF-1R and MAC-2 staining.

A.



B.



C.

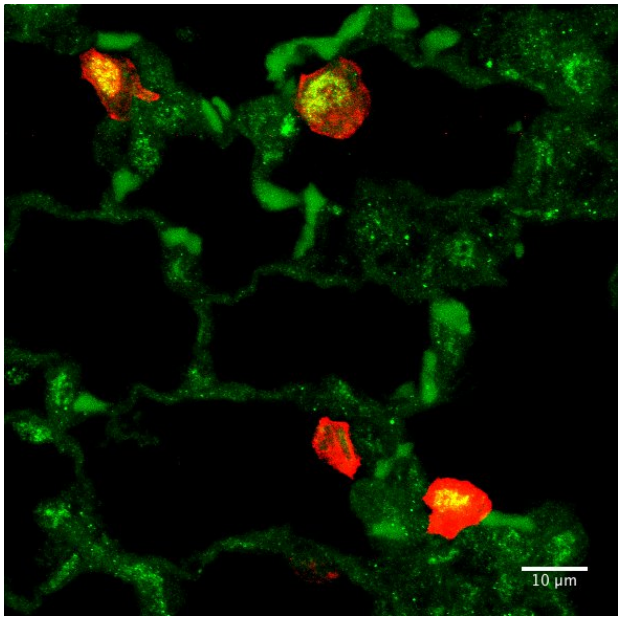


Figure 3.17 – Alveolar macrophages from LysM-Cre/*Igf1*$^{fl/fl}$ mice have no detectable *Igf1* transcript.

Alveolar macrophages isolated from LysM-Cre/*Igf1*$^{fl/fl}$ and littermate controls were assessed for *Igf1* mRNA expression (n=2 per group). Data are represented as mean ± s.d.

LysM-Cre/*Igf1*$^{fl/fl}$ mice

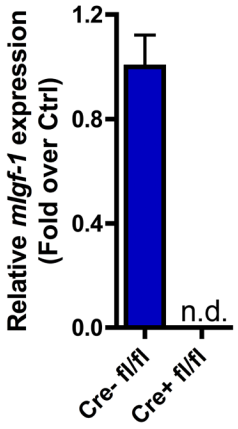


Figure 3.18 – Mice lacking IGF-1R in airway epithelial cells have exacerbated airway inflammation.

(A) Schematic of HDM sensitization scheme. Mice were given doxycycline 7 days prior to first allergen administration in the drinking water. Three doses of low endotoxin HDM were given intranasally every other day, followed by 5 days of rest, and 3 challenge doses of HDM, every other day. Mice were analyzed 1.5 days following the last challenge for the indicated parameters.

(B) Representative immunohistochemistry images (from one control and one CCSP-$_{rtTA/tetO}$-Cre /*Igf1r*$^{fl/fl}$ mouse) showing IGF-1R expression is primarily seen in the bronchial epithelial cells and that the signal for IGF-1R is lost in CCSP-$_{rtTA/tetO}$-Cre /*Igf1r*$^{fl/f}$ mice treated with doxycycline (to induce Cre expression in epithelial cells).

(C) Total cell counts of eosinophils, alveolar macrophages, and CD4^{+} T cells in the BAL of CCSP-Cre/*Igf1r*$^{wt/wt}$ and *Igf1r*$^{fl/fl}$ mice administered PBS or HDM.

(D) (Left panel) Representative images of lung draining lymph nodes harvested from CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice that were given PBS or HDM. (Right panel) Total CD4 T cell counts from the lung draining lymph nodes harvested from these mice. Each data point shown represents analysis from one mouse.

(E) (Top) Representative hematoxylin and eosin (H&E) images of lung sections CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given PBS or HDM. Two examples of the HDM-treated conditions are shown to highlight the greater infiltration of mononuclear cells in the CCSP-Cre/*Igf1r*$^{fl/fl}$ mice (n=3-4 mice per condition). (Bottom)

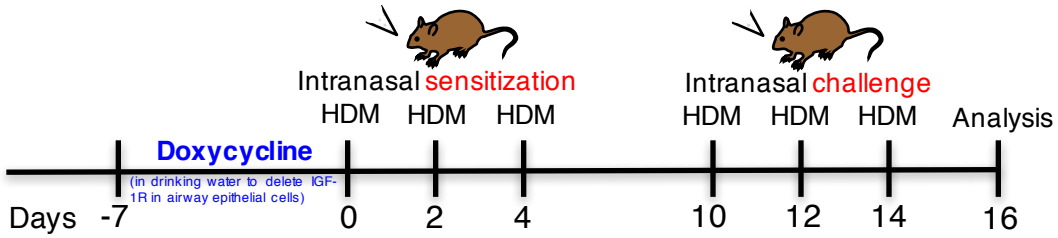
Representative histological scoring of inflammation (by two independent blinded scorers) (n=6-10 sections per at least 3 mice per condition).

(F) (Top) Representative periodic acid-Schiff (PAS) staining of lung sections from CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given PBS or HDM. Two examples of the HDM-treated conditions are shown to highlight greater mucus accumulation in the CCSP-Cre/*Igf1r*$^{fl/fl}$ mice. (Bottom) Representative histological scoring of PAS staining (by two independent blinded scorers) (n=6-10 sections per at least 3 mice per condition).

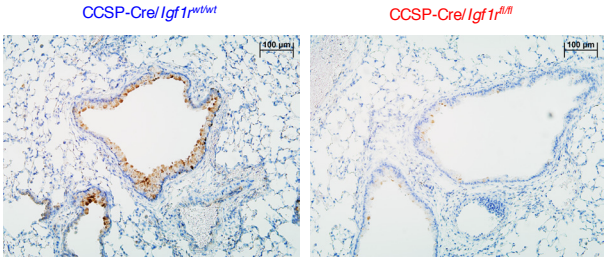
(G) Airway hyper-responsiveness to methacholine (another measure of allergen sensitivity) in the in CCSP-Cre/*Igf1r*$^{fl/fl}$ mice compared to control CCSP-Cre/*Igf1r*$^{wt/wt}$ mice treated with HDM (n=6-8 mice per group).

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

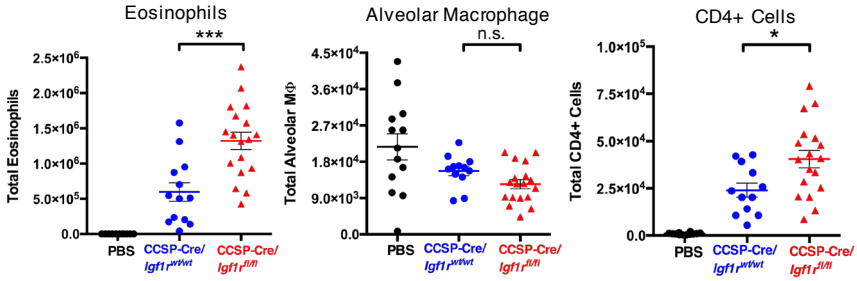
A.



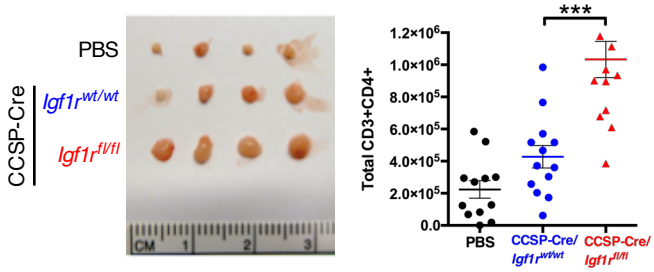
B. Immunohistochemistry staining for IGF-1 receptor in airways



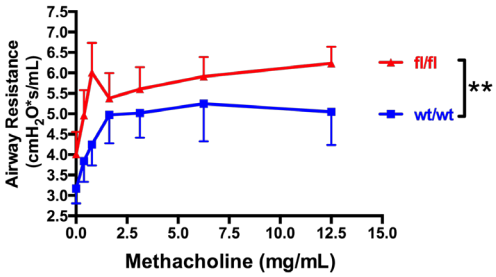
C. Bronchoalveolar lavage



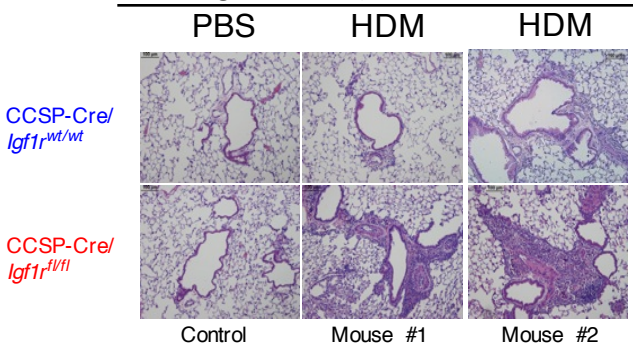
D. Draining lymph nodes

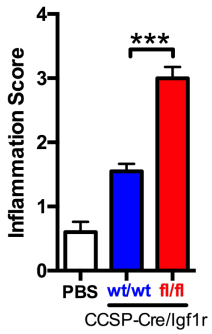


G. Airway hyper-sensitivity

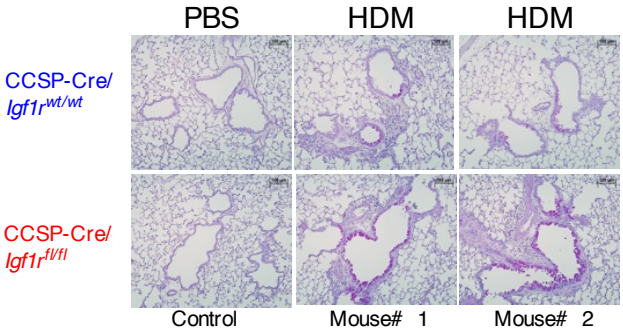


E. Histological analysis of inflammation





F. Periodic acid-Schiff (PAS) staining



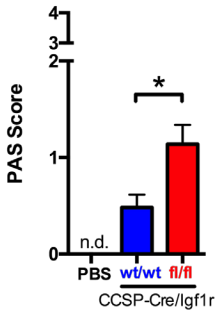
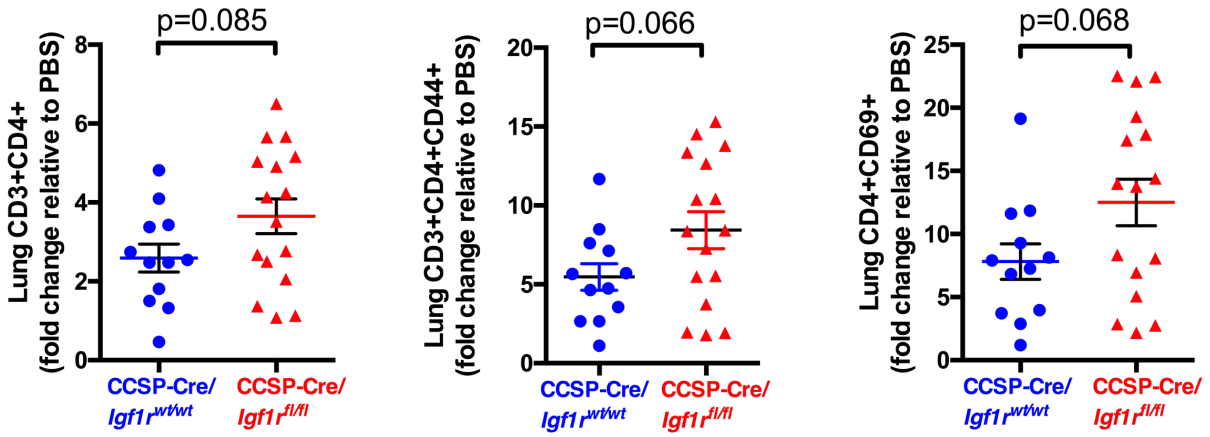


Figure 3.19 – CCSP-Cre/*Igf1r*$^{fl/fl}$ mice exposed to HDM show a trend toward greater immune cell infiltration in the lungs and greater apoptotic cells.

(A) Total cell counts of lung CD3+ CD4+ T cells (left), CD3+ CD4+ CD44+ T cells (middle), and CD3+ CD4+ CD69+ T-cells (right panel) in the lungs of CCSP-Cre/*Igf1r*$^{wt/wt}$ and *Igf1r*$^{fl/fl}$ mice given the full HDM course. CCSP-Cre/*Igf1r*$^{fl/fl}$ mice exposed to HDM clearly show a trend in increased T- cells in the lung compared to *Igf1r*$^{wt/wt}$ mice, but due to the spread in the data among the many mice analyzed, the data did not achieve statistical significance.

(B) (left) Representative histology images of cleaved caspase (CC3) staining in lung sections of mice given the full HDM course. Average CC3-positive cells per mouse are quantified on the right (n=3 per group). Black arrowheads indicate positive staining. Data are represented as mean ± s.e.m.

A.


p=0.085
Lung CD3+CD4+ (fold change relative to PBS)
0
2
4
6
8
CCSP-Cre/ Igf1r wt/wt
CCSP-Cre/ Igf1r fl/fl
p=0.066
Lung CD3+CD4+CD44+ (fold change relative to PBS)
0
5
10
15
20
CCSP-Cre/ Igf1r wt/wt
CCSP-Cre/ Igf1r fl/fl
p=0.068
Lung CD4+CD69+ (fold change relative to PBS)
0
5
10
15
20
25
CCSP-Cre/ Igf1r wt/wt
CCSP-Cre/ Igf1r fl/fl

B.

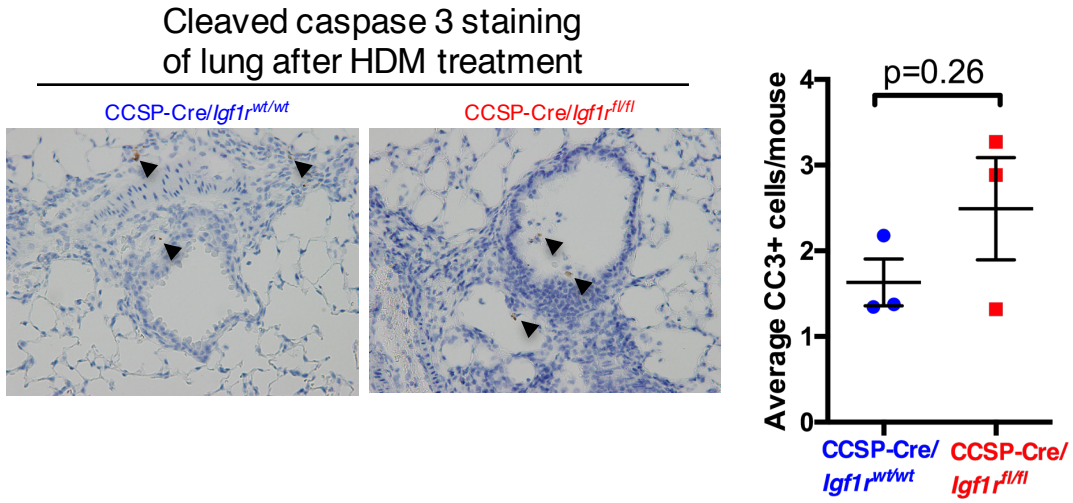

Cleaved caspase 3 staining of lung after HDM treatment
CCSP-Cre/Igf1r wt/wt
CCSP-Cre/Igf1r fl/fl
p=0.26
Average CC3+ cells/mouse
0
1
2
3
4
CCSP-Cre/ Igf1r wt/wt
CCSP-Cre/ Igf1r fl/fl

Figure 3.20 – IGF-1R expression in airway epithelial cells is required in the sensitization phase of airway inflammation.

(A) Schematic of IGF-1R deletion during the sensitization phase to assess its effect on early stages of inflammation. Mice are given doxycycline 7 days prior to first allergen administration in the drinking water. Then, three doses of low endotoxin HDM are given intranasally every other day; 1.5 days after the last dose, the BAL fluid as well as other parameters of inflammation are assessed in these mice.

(B) Total cell counts of eosinophils, alveolar macrophages, and $CD4^+$ T cells in the BAL fluid of CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice sensitized with PBS or HDM.

(C) Luminex analysis of IL-4, IL-5 and eotaxin-1 in the BAL fluid from representative CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice sensitized with PBS or HDM (n=3).

(D) ELISA of TSLP levels in the BAL fluid from representative CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice sensitized with PBS or HDM (n=2, 7, 9 mice per group).

(E) Luminex analysis of IL-6 in the BAL fluid from representative CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice sensitized with PBS or HDM (n=3).

(F) Schematic of generation and isolation of MPs released from mouse alveolar macrophages. Briefly, supernatants from macrophages were harvested and spun at 5000xg to remove cell debris and apoptotic bodies. Then clarified supernatants were filtered through a 0.8μm filter and spun again at 17,000xg to pellet the MPs. After washing, pelleted MPs were then analyzed or used in assays.

(G) Representative images from electron microscopy of MPs isolated from mouse alveolar macrophages. Images show collapsed particles.

(H) Representative images from electron cryomicroscopy of MPs isolated from mouse alveolar macrophages. Images show a range of sizes of spherical membrane-bound structures (yellow arrows).

(I) ImageStreamX™ analysis of MPs isolated from mouse alveolar macrophage cell line and primary mouse alveolar macrophages and stained for representative alveolar macrophage markers. In both cell types, MPs are single positive for CD11c, SiglecF or AnnexinV, or double positive for either CD11c and AnnexinV or SiglecF and AnnexinV.

(J) Tunable resistive pulse sensing analysis of MPs from alveolar macrophages using qNano pore size 400nm to determine frequency and sizing of MPs.

(K) BEAS-2B cells were treated with IGF-1 (100ng/mL) and assessed for MP uptake (n=4)

(L) BEAS-2B cells were treated with HDM either in the presence or absence of MPs isolated from mouse alveolar macrophages for 3 hours and then assessed for expression of *TSLP, CSF2, IL6, IL8* (n=4).

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

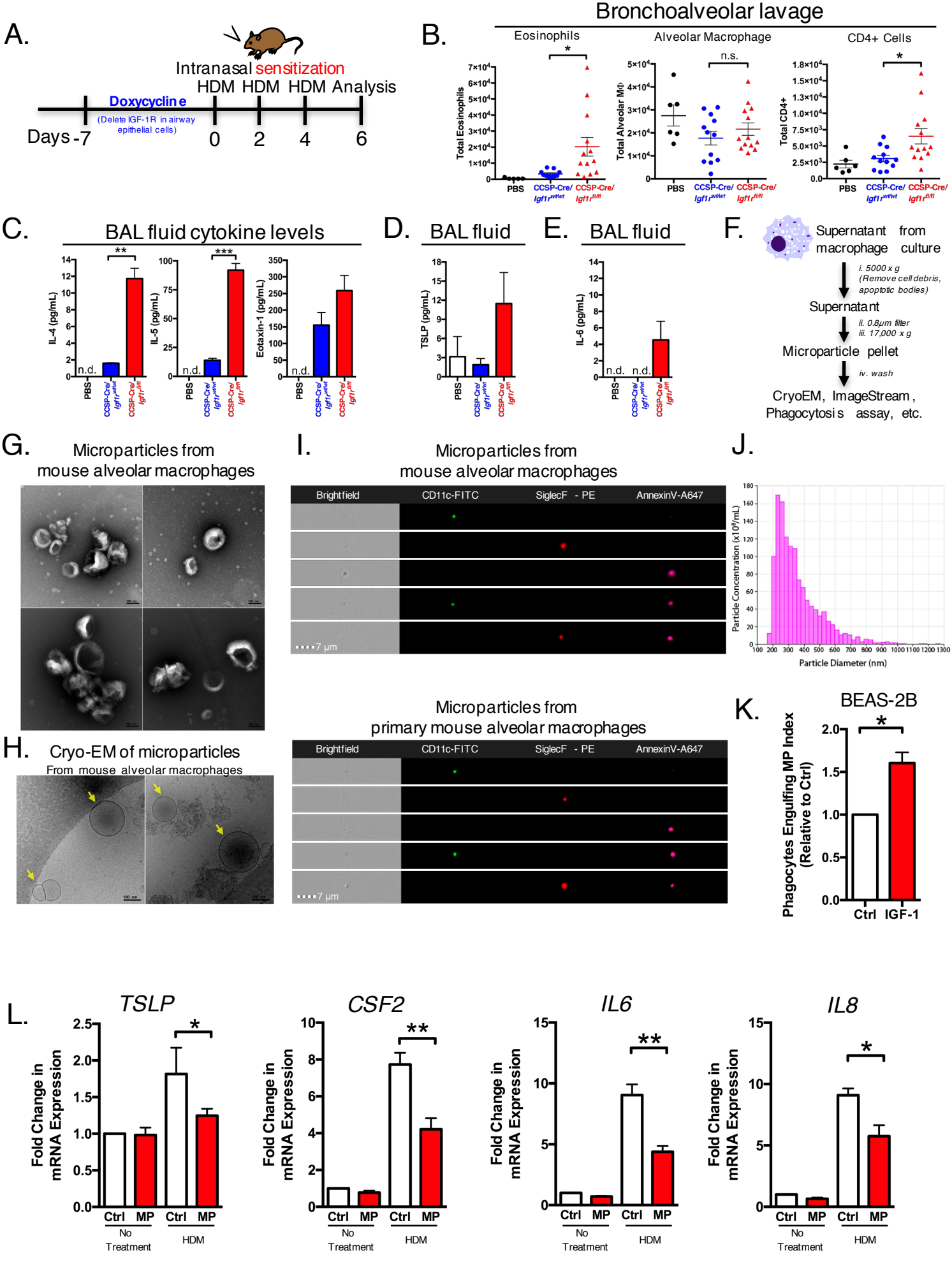
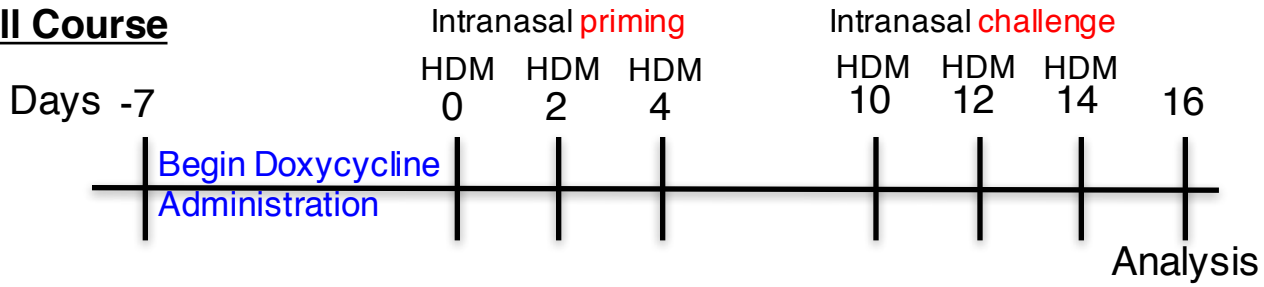
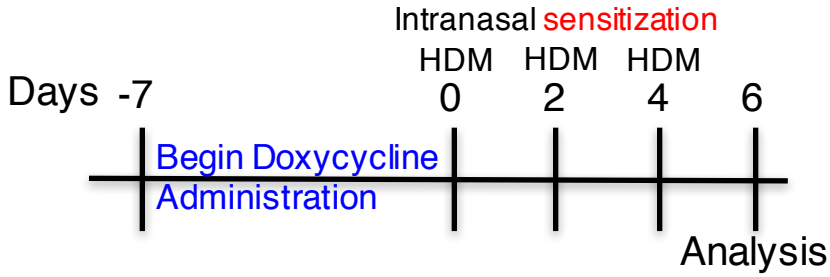

A.
Intranasal sensitization
HDM HDM HDM Analysis
Doxycycline
(Delete IGF-1R in airway epithelial cells)
Days -7
0
2
4
6
B.
Bronchoalveolar lavage
Eosinophils
Alveolar Macrophage
CD4+ Cells
Total Eosinophils
Total Alveolar Mϕ
Total CD4+
n.s.
PBS
CCSP-Cre/ Igf1r wt/wt
CCSP-Cre/ Igf1r fl/fl
C.
BAL fluid cytokine levels
IL-4 (pg/mL)
IL-5 (pg/mL)
Eotaxin-1 (pg/mL)
n.d.
D.
BAL fluid
TSLP (pg/mL)
E.
BAL fluid
IL-6 (pg/mL)
F.
Supernatant from macrophage culture
i. 5000 x g (Remove cell debris, apoptotic bodies)
Supernatant
ii. 0.8μm filter
iii. 17,000 x g
Microparticle pellet
iv. wash
CryoEM, ImageStream, Phagocytosis assay, etc.
G.
Microparticles from mouse alveolar macrophages
H.
Cryo-EM of microparticles
From mouse alveolar macrophages
I.
Microparticles from mouse alveolar macrophages
Brightfield
CD11c-FITC
SiglecF - PE
AnnexinV-A647
7 μm
Microparticles from primary mouse alveolar macrophages
J.
Particle Concentration (x10⁹/mL)
Particle Diameter (nm)
K.
BEAS-2B
Phagocytes Engulfing MP Index (Relative to Ctrl)
Ctrl
IGF-1
L.
TSLP
CSF2
IL6
IL8
Fold Change in mRNA Expression
Ctrl
MP
No Treatment
HDM

Figure 3.21 – Schematic detailing various HDM administration timelines tested. Schematic describing the different time courses for *Igf1r* deletion from Club cells (induced through administration of doxycycline) and for the allergen HDM exposure.

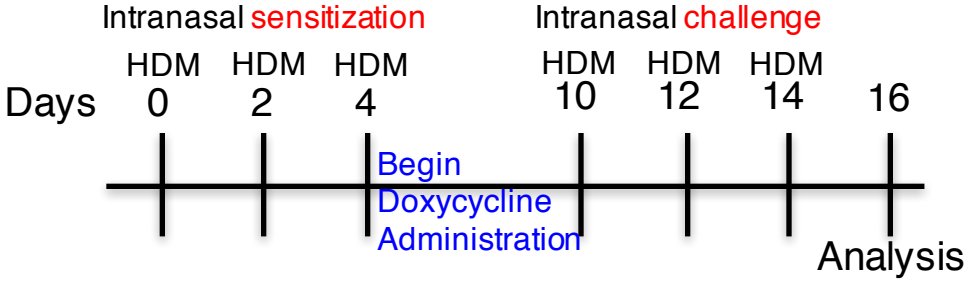
Full Course



Regimen #1: Sensitization Phase



Regimen #2: Challenge Phase



Regiment #3: Recovery Phase

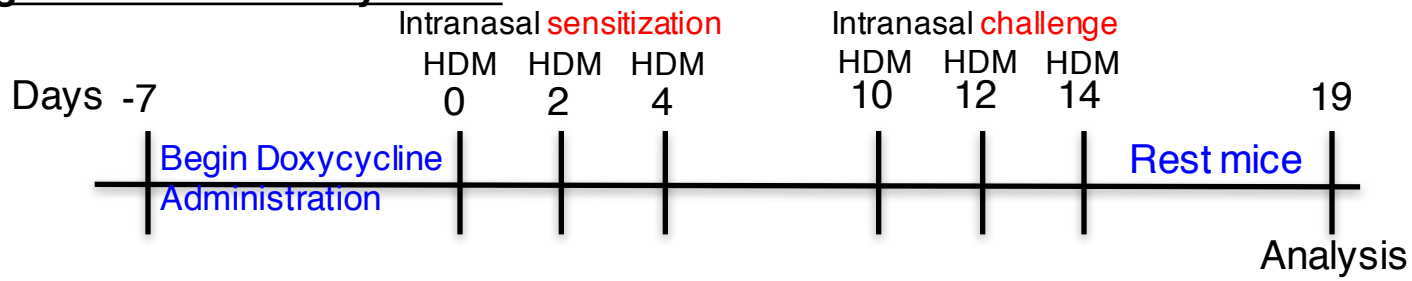
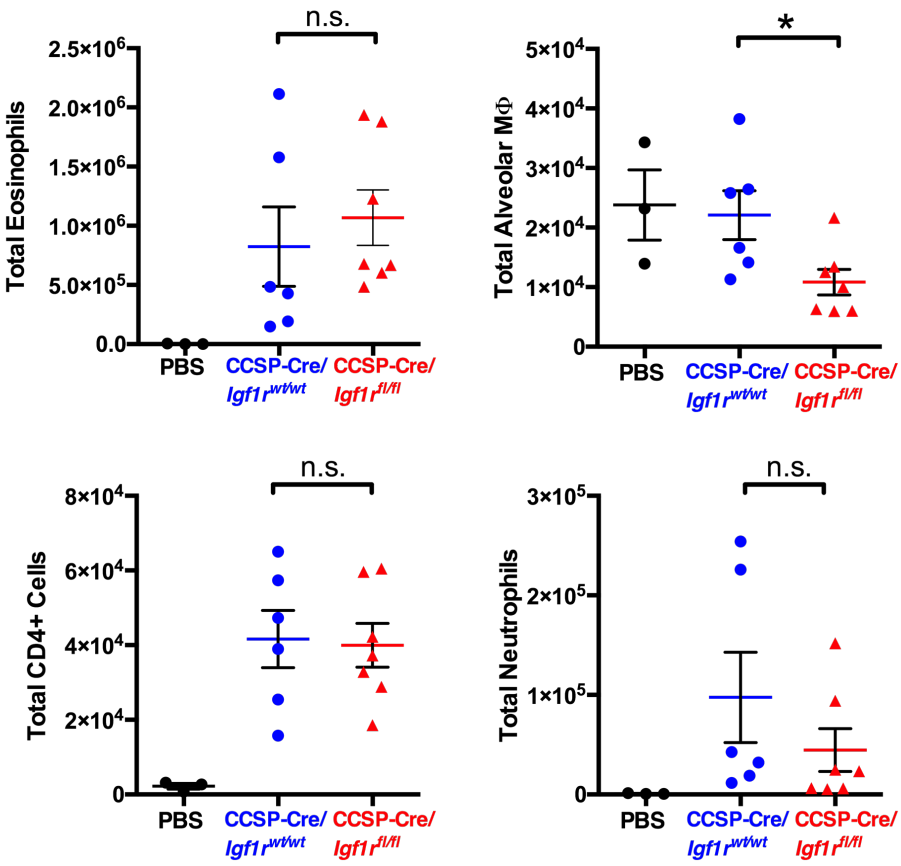


Figure 3.22 – CCSP-Cre/*Igf1r*$^{wt/wt}$ and *Igf1r*$^{fl/fl}$ mice exposed to HDM for regimen #2 (the challenge phase) have no significant differences in airway inflammation.

(A) Total cell counts of various populations in the BAL fluid of CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given HDM for regimen #2 (the "challenge phase" time course). **(B)** Total cell counts of CD3+ CD4+ T cells of draining lymph nodes of CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given HDM for regimen #2 (the "challenge phase" time course). Data represented as mean ± s.e.m.

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

A. Bronchoalveolar lavage



B. Draining lymph nodes

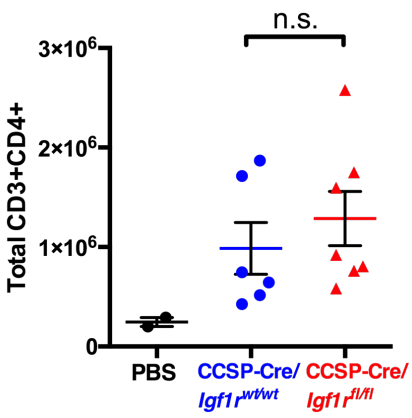


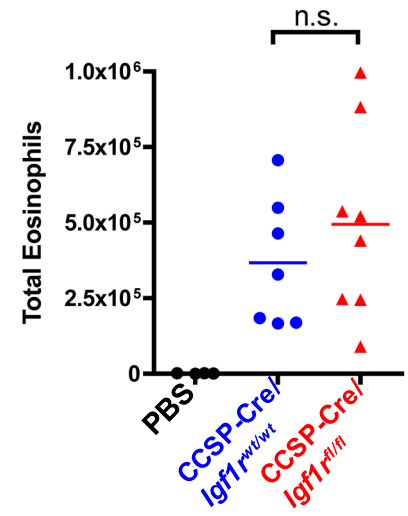
Figure 3.23 – CCSP-Cre/*Igf1r*$^{wt/wt}$ and *Igf1r*$^{fl/fl}$ mice exposed to HDM for the recovery phase have no significant differences in airway inflammation.

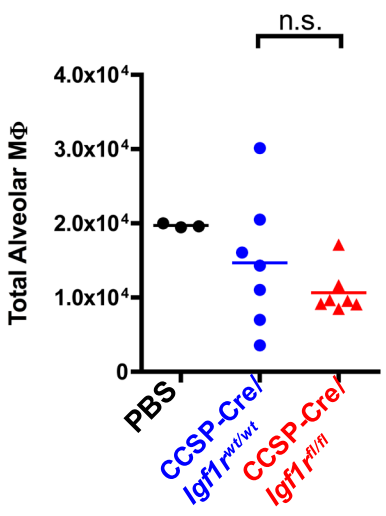
(A) Total cell counts of various populations in the BAL fluid of CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given HDM for the "recovery phase" time course.

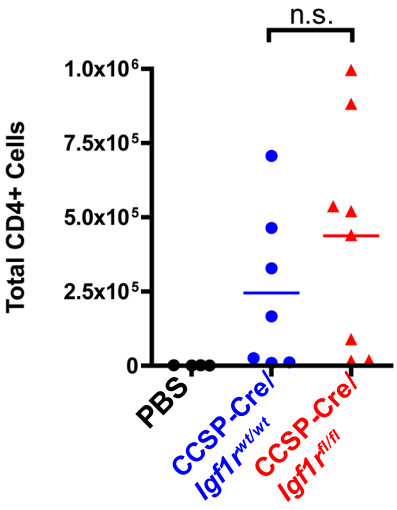
(B) Total cell counts of CD3+ CD4+ T cells of draining lymph nodes of CCSP-Cre/*Igf1r*$^{wt/wt}$ and CCSP-Cre/*Igf1r*$^{fl/fl}$ mice given HDM for the "recovery phase" time course. Data represented as mean ± s.e.m.

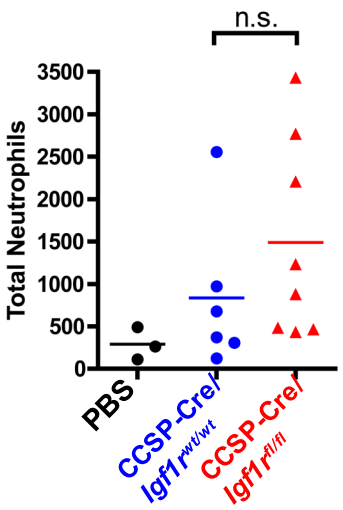
Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

A. Bronchoalveolar lavage









B. Draining lymph nodes

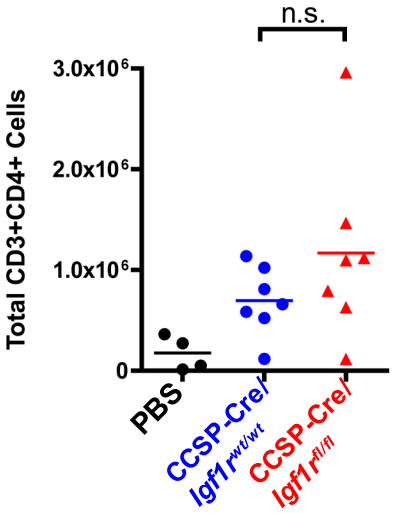


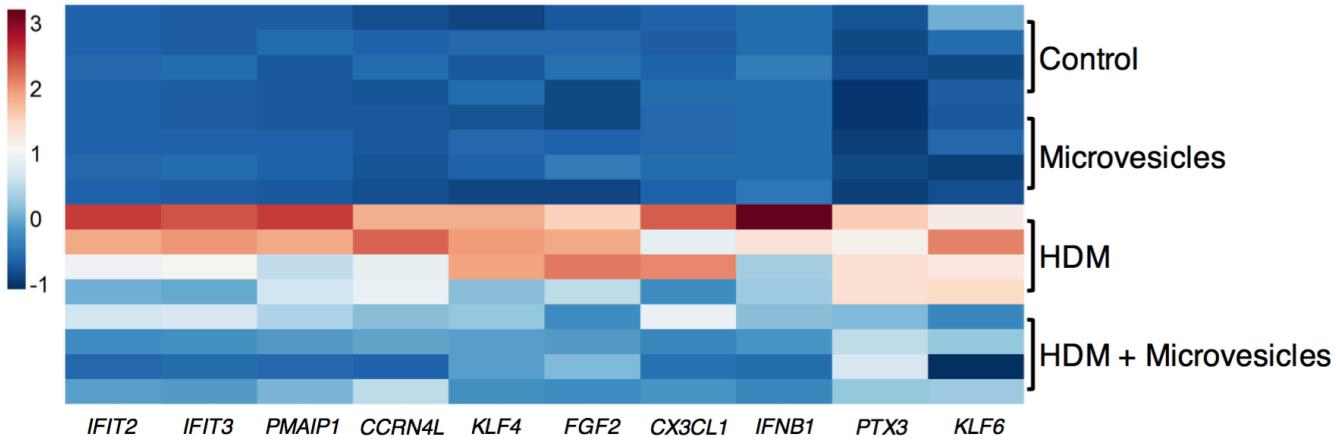
Figure 3.24 – Gene expression changes in BEAS-2B cells treated with HDM and/or macrophage-derived microparticles.

BEAS-2B cells were treated with HDM in the presence or absence of alveolar macrophage-derived MPs and total RNA was extracted. RNA-Seq was performed on the extracted RNA. **(A)** Heatmap representing the top 10 differentially expressed genes in BEAS-2B cells exposed to HDM either in the presence or absence of alveolar macrophage-derived MPs. Changes in gene expression of four genes were later validated by real-time qPCR (n=6) **(B).**

Data represented as mean ± s.e.m.

Asterisks represent a *p*-value of <0.05 (indicated by one asterisk), <0.01 (indicated by two asterisks), or <0.001 (indicated by three asterisks). n.s. is not significant.

A.


3
2
1
0
-1
Control
Microvesicles
HDM
HDM + Microvesicles
IFIT2
IFIT3
PMAIP1
CCRN4L
KLF4
FGF2
CX3CL1
IFNB1
PTX3
KLF6

B.

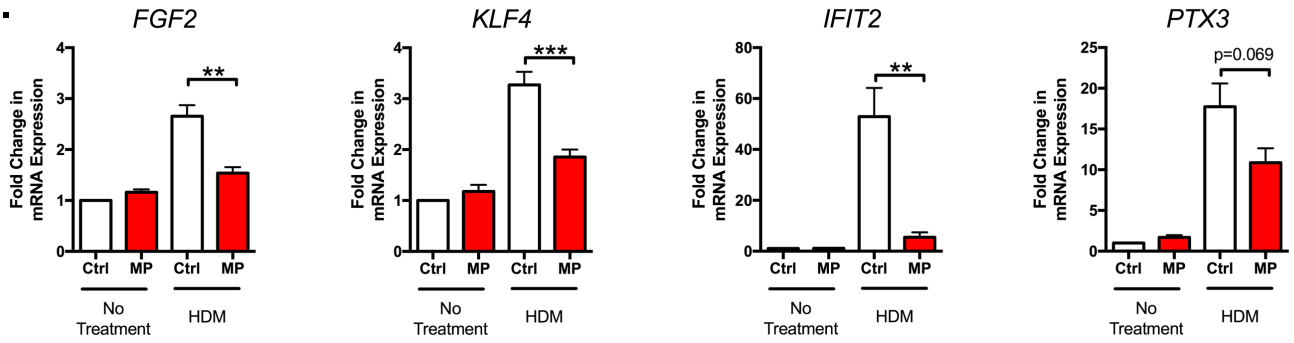
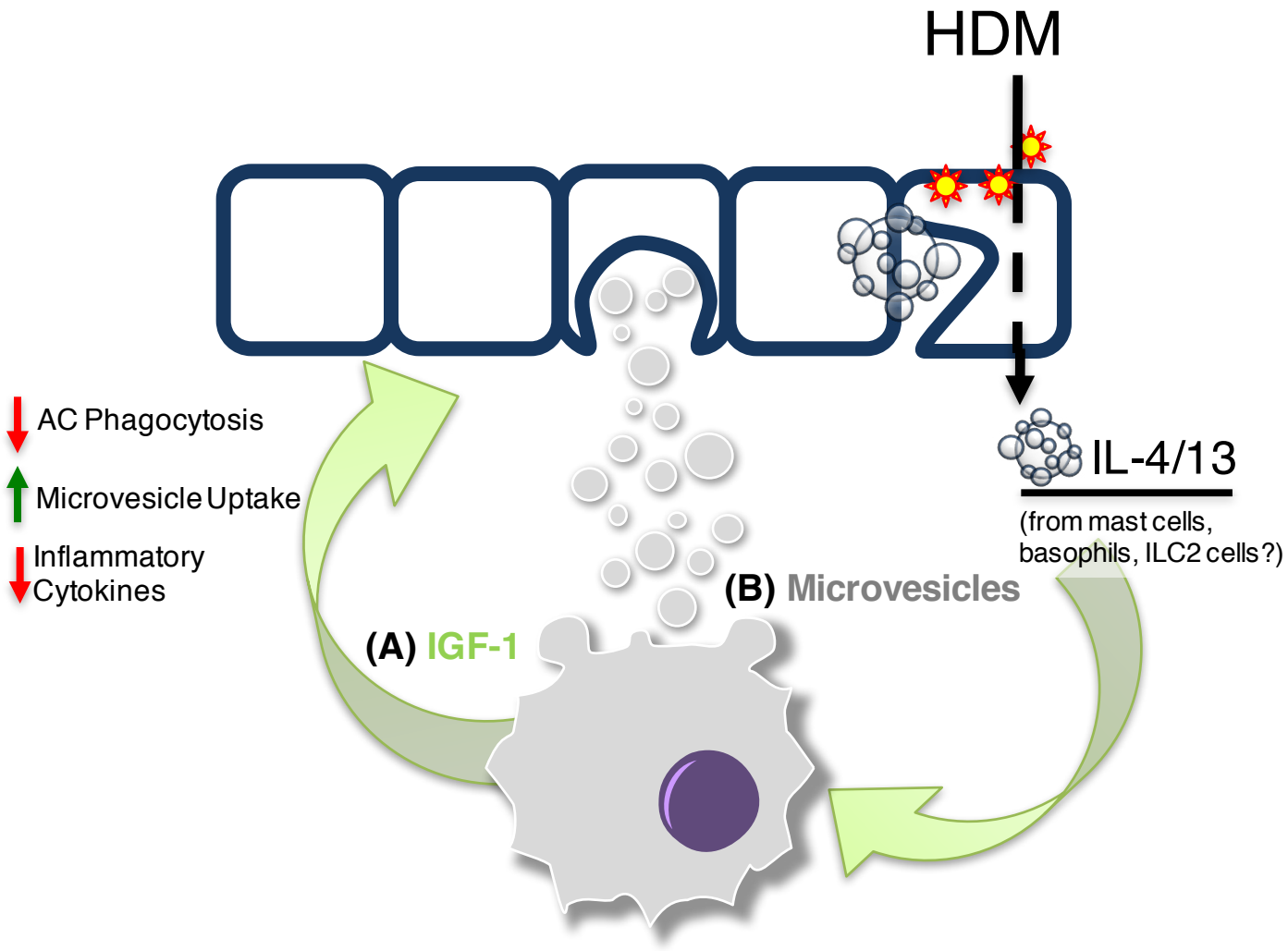

FGF2
KLF4
IFIT2
PTX3
Fold Change in mRNA Expression
**
***
**
p=0.069
Ctrl
MP
Ctrl
MP
No Treatment
HDM

Figure 3.25 – Potential model of alveolar macrophage and airway epithelial crosstalk.

Exposure of airways to allergens, such as HDM, can cause apoptotic cell death as well as IL-4 and IL-13 production, from mast cells and type 2 innate lymphoid cells (ILC2). These cytokines, along with apoptotic cells, trigger alveolar macrophages to produce IGF-1. The released IGF-1 **(A)** then acts on the airway epithelium to elicit two actions: first, to decrease the uptake of apoptotic cells and second to enhance the uptake of macrophage-derived MPs. These MPs **(B)** dampen inflammatory cytokine production by the airway epithelial cells.


HDM
ILC2
AC Phagocytosis
Microvesicle Uptake
Inflammatory Cytokines
IL-4/13
(from mast cells, basophils, ILC2 cells?)
(B) Microvesicles
(A) IGF-1

Chapter IV

Summary and Future Directions

1: Summary

In numerous tissues, professional and non-professional phagocytes reside in close proximity. While both populations clear apoptotic cells, non-professional phagocytes, such as epithelial cells and fibroblasts, have additional essential functions, such as proliferation to facilitate wound closure. During tissue injury and inflammation, both phagocytic clearance and wound healing need to occur for proper resolution, leading us to ask how professional and non-professional phagocytes might co-regulate the apoptotic cell clearance process. To begin our investigations, we focused on secreted factors, specifically those involved in cellular proliferation and wound healing, as they are present during inflammation resolution. We found that insulin-like growth factor 1 (IGF-1) could significantly decrease phagocytosis of apoptotic cells by non-professional phagocytes (**Figure 3.1A, B, C; 3.3A, B**). Additionally, we examined whether IGF-1 would affect phagocytosis of smaller sized particles and found that IGF-1 treatment of non-professional phagocytes enhanced the uptake of small particles of 150-200nm in size (**Figure 3.10D**). Interestingly, both primary macrophages and macrophage cell lines were unaffected by IGF-1 in their phagocytic capacity (**Figure 3.10A, B, C, F**). To confirm our results *in vivo*, we examined phagocytic clearance in the lung and found that airway epithelial cells, when exposed to IGF-1, exhibited decreased apoptotic cell engulfment and increased uptake of small liposomes (**Figure**

3.14D, E). Alveolar macrophages, however, showed no change in their phagocytic capacity in the presence or absence of IGF-1 addition (**Figure 3.14D, E**).

Next, we investigated the production of IGF-1. The liver is a key source of circulating IGF-1, but mice with deletion of IGF-1 in the liver were surprisingly normal compared to controls, with only minimal retardation in growth, despite having much lower levels of serum IGF-1, suggesting that there were other sources of IGF-1 in peripheral tissues[170]. Interestingly, macrophages, when treated with IL-4, IL-13 or apoptotic cells, robustly produced IGF-1, which could suppress apoptotic cell uptake by fibroblasts (**Figure 3.14A, B**). Furthermore, we generated mice with *Igf1* deletion in myeloid cells, and showed that the resident myeloid population in the lung are the primary source of IGF-1 in response to IL-4, IL-13, or apoptotic cells (**Figure 3.14G**).

Having established a potential source of IGF-1, we then examined the physiological role of this communication between macrophages and non-professional phagocytes in a model of allergic airway inflammation, a Th2 disease. First, we crossed mice carrying floxed alleles of IGF-1 receptor (*Igf1r*$^{fl/fl}$) to CCSP-$_{rtTA/tetO}$-Cre mice to target inducible deletion of *Igf1r* in airway epithelial cells via the administration of doxycycline (**Figure 3.18B**). We administered the allergen house dust mite (HDM) intranasally to mice and then examined disease severity by several parameters, such as total cellular counts in the BAL fluid, draining lymph node size, pulmonary function, and histological analysis. Our laboratory had previously shown that inhibition of phagocytosis in airway epithelial cells via genetic ablation of Rac1 leads to exacerbated airway inflammation[141]. As IGF-1 signaling suppresses apoptotic cell clearance by airway epithelial cells, we had hypothesized that by inhibiting IGF-1

signaling and allowing airway epithelial cells to better clear apoptotic cells, CCSP-Cre/*Igf1r*$^{fl/fl}$ mice would have better disease outcomes. To our surprise, in all parameters measured, mice in which *Igf1r* was deleted in airway epithelial cells had exacerbated disease (**Figure 3.18C-F**).

To determine the mechanism for this phenotype, we performed a kinetic dissection of disease (**Figure 3.21**), in which a) *Igf1r* was deleted and mice were sensitized with allergen, b) mice were sensitized with allergen and then *Igf1r* was deleted for the challenge phase, or c) *Igf1r* was deleted and the full time course of HDM exposure was given to mice and then the mice were allowed to rest for an additional 5 days to measure the impact of IGF-1 signaling in the recovery phase. Using the same parameters as assessed above, we found that IGF-1 signaling in airway epithelial cells was critical for controlling airway inflammation only during the sensitization phase (**Figure 3.20A-C**). As IGF-1 also enhances the uptake of small particles in epithelial cells, we wondered whether this phenomenon had a physiological role in allergic airway inflammation.

Interestingly, these small particles, termed microparticles (MPs), have been shown to be important mediators of inflammation in other contexts. We found that alveolar macrophages produced MPs basally and that IGF-1 treatment of lung epithelial cells also enhanced the uptake of these MPs. During the sensitization period, airway epithelial cells, after encountering allergen, produce a variety of inflammatory cytokines, such as thymic stromal lymphopoeitin (TSLP) that prime the innate immune response. Inhibition of IGF-1 signaling in airway epithelial cells would also abolish the ability of airway epithelial cells to enhance uptake of these MPs. Therefore, we

hypothesized that these MPs may dampen the response of airway epithelial cells to allergen. Using an *in vitro* assay of HDM stimulation, we showed that treatment of lung epithelial cells with HDM increased transcription of *TSLP, CSF2, IL6, and IL8* (**Figure 3.20L**). Co-treatment with alveolar macrophage-derived MPs significantly suppressed transcription of these inflammatory cytokines (**Figure 3.20L**). Collectively, we propose a model describing the impact of phagocytic communication on inflammation (**Figure 3.25**). Specifically, in a model of allergic airway inflammation, IL-4 or apoptotic cells, produced as a result of allergen encounter, stimulates alveolar macrophages to secrete IGF-1; this IGF-1, in turn, signals in airway epithelial cells to decrease engulfment of apoptotic cells and to increase uptake of MPs derived from alveolar macrophages. Uptake of these MPs then dampens epithelial cell production of inflammatory cytokines. These results not only ascribe a new function for IGF-1, but also provide insight as to how phagocytic communication can impact tissue inflammation.

In the following section, I will discuss the future directions that this work can lead to, specifically focusing on the mechanism of IGF-1 mediated suppression of apoptotic cell clearance, MPs as a potential therapeutic tool for allergic asthma, and additional physiological areas in which phagocytic communication maybe important.

2. Future directions

2.1. *What are the molecular mechanism(s) of how professional and non-professional phagocytes respond to IGF-1 stimulation with respect to phagocytosis?*

Non-professional and professional phagocytes differ in several respects, including the types of targets ingested, the phagocytic receptors expressed, and downstream cellular and immunological consequences. We show here that they also respond differently to IGF-1 stimulation with IGF-1 modulating phagocytosis in non-professional phagocytes while professional phagocytes have normal phagocytic capacity. However, it is unclear exactly how IGF-1 elicits an effect in non-professional phagocytes and why the two phagocytic populations differ in their response. Four key area for further investigation are a) the molecular mechanism by which IGF-1 influences phagocytosis, b) the effect of IGF-1 on other professional phagocytes, c) the effect of IGF-1 on the uptake of other targets, such as bacteria, and d) the molecular differences between professional and non-professional phagocytes in their response to IGF-1.

2.1.1 Molecular mechanism of IGF-1 mediated redirection of phagocytosis in non-professional phagocytes

In our analyses to date, we have found that the effect of IGF-1 on non-professional phagocytes with respect to phagocytosis most likely does not involve canonical downstream signaling effectors of IGF-1R, including Akt, mTOR, PI-3K and Erk1/2. However, activation of IGF-1R kinase activity is essential as treatment of non-professional phagocytes with a small molecule inhibitor of receptor activation was able to rescue/reverse the IGF-1 mediated change in phagocytosis of apoptotic cells or

MPs. Furthermore, IGF-1 signaling has been shown to activate the small GTPases Rho and Rac in different cell types. We observed that inhibition of ROCK, a downstream effector of Rho, did not rescue IGF-1 mediated suppression of apoptotic cell clearance in non-professional phagocytes in a specific manner. Interestingly, overexpression of constitutively active Rac1 was able to restore phagocytic capacity of non-professional phagocytes treated with IGF-1 to levels comparable to control, suggesting the regulation via IGF-1 might occur at a step upstream or parallel to Rac activation.

Phagocytosis of apoptotic cells requires actin polymerization and de-polymerization. To use an alternative approach to further understand the change in actin dynamics in non-professional phagocytes following IGF-1 treatment, we used pharmacological tools to assess liposome uptake. Curiously, we found that treatment of phagocytes with Cytochalasin D and Latrunculin A, reagents that promote depolymerization of actin by preventing addition of G-actin to the positive end of actin filaments, was able to suppress IGF-1 mediated enhancement of liposome uptake. Treatment of phagocytes with Jasplakinolide, a promoter of actin polymerization through actin nucleation, had minimal effect on the IGF-1 mediated enhancement. Based on these observations, it appears that IGF-1 may be modulating the actin polymerization, rather than nucleation during the process of phagocytosis in non-professional phagocytes. Additional experiments imply that, in the case of liposome uptake, IGF-1 may affect actin elongation rather than actin nucleation, as inhibiting Arp2/3, a key actin nucleation factor, did not suppress IGF-1 mediated enhancement of liposome uptake.

To further extend these studies, it would be useful to first perform live-cell imaging using non-professional phagocytes expressing fluorescently-labeled actin[370,371]. In conjunction with the use of the LSM 880 with AiryScan, available in the UVa Advanced Microscopy Core, which provides greater resolution and signal-to-noise ratio over traditional spinning disk confocal microscopes, this methodology would allow visualization and temporal quantification of actin dynamics in cells exposed to IGF-1 in the presence or absence of apoptotic cells or liposomes. Further, addition of fluorescently labeled G-actin, incorporated into the cells through a mild saponin treatment, would allow tracking of actin polymerization[372]. Potential cellular targets involved in actin elongation and polymerization include profilin, the formin family, and Ena/VASP (vasodilator-stimulated phosphoprotein)[373]. Briefly**,** profilin binds to monomeric actin and then interacts with formin and VASP to promote the addition of actin monomers to existing F-actin[374-378]. By use of siRNA knockdown or transfection of dominant negative mutants, modifying functional levels of these proteins in non-professional phagocytes and then assessing their phagocytic capacity in the presence of IGF-1 could reveal a new downstream effector of IGF-1. Additionally, via live cell-imaging, it may also be possible to identify what phagocytic processes, such as phagocytic cup formation, are disrupted by IGF-1. Formin family members and Ena/VASP maybe more promising candidates, as it has been shown that VEGF signaling in endothelial cells induces phosphorylation of profilin, which in turn can affect actin dynamics[379,380]. In our observations, VEGF was shown not to affect phagocytosis of liposomes or apoptotic cells in non-professional phagocytes.

An alternative possibility is that, upon IGF-1 binding to IGF-1R, transient endocytic membrane structures, termed circular dorsal ruffles (CDR), may form. CDRs are enriched in F-actin and form minutes after stimulation with growth factors and require small Rho GTPases[381]. Currently, EGF[382], HGF[383], and PDGF[384] have been shown to induce CDR formation. Additionally, CDRs are dynamic and transient and their purpose is two-fold: to induce receptor internalization and cell motility[381,385]. For example, fibroblasts stimulated with PDGF rapidly internalize integrins via macropinocytosis; these integrins then are recycled and redistributed on the cell surface[386]. Therefore, it is possible that upon IGF-1 stimulation, two things occur: a) rapid membrane ruffling in CDR causing uptake of small particles and b) internalization of receptors involved in apoptotic cell uptake. This mechanism could potentially explain the differential effect of IGF-1 on apoptotic cell and microparticle phagocytosis. PI3K, Arp2/3, N-WASP and WAVE have been shown to be important for the classical pathway of CDR formation[381], which may be at odds with our preliminary data concerning the involvement of PI3K and Arp2/3 in IGF-1 mediated enhancement of microparticle uptake. Furthermore, growth factors that do not affect phagocytosis in non-professional phagocytes can also induce CDR formation. Nevertheless, the transient nature of these structures makes them an interesting avenue to explore. Use of live-cell imaging coupled with fluorescently labeled actin and fluorescently tagged IGF1r can reveal whether CDRs are formed following IGF-1 stimulation. Additionally, we can measure phagocytic receptor internalization by isolating cell surface receptors via biotinylation, followed by western blot. The caveat of this latter experiment is that it is a relatively slow process, as each individual phagocytic receptor will have to be

analyzed by immunoblot and subsequently tested for significance (e.g. using a knockdown system). Furthermore, CDRs may cause the internalization of multiple phagocytic receptors, complicating downstream studies.

2.1.2. The effect of IGF-1 on other professional phagocytes

We find that macrophages, a professional phagocyte, exposed to IGF-1 have similar phagocytic capacity as untreated cells. First, it is important to extend our observations from macrophages to other professional phagocytes, such as dendritic cells and neutrophils. Each cell type has distinct roles in shaping the innate immune response, with immature dendritic cells serving as the predominant antigen presenting cells and neutrophils functioning to clear microbial pathogens and release anti-microbial enzymes. In looking at apoptotic cell clearance solely, immature dendritic cells that phagocytose apoptotic cells have a reduced capacity to stimulate T-cells[387,388], possibly due to impaired maturation. However, in the context of ingested infected apoptotic cells, immature dendritic cells can cross-present and activate CD8+ T cells[389]. Thus, if IGF-1 does modulate phagocytosis by dendritic cells, it may greatly affect the adaptive immune branch in both homeostatic and infectious states, by either promoting an immunologically silent environment or augmenting the T-cell response, respectively. In the field of apoptotic cell clearance, neutrophils have generally served as a source of apoptotic targets, especially during inflammation. However, they have been shown to clear their apoptotic brethren[390]. Like other cell types, apoptotic cell engulfment by neutrophils elicits an anti-inflammatory response with decreased TNF-alpha production[391]. Interestingly, one study showed that neutrophils must first be

activated, by LPS or CSF2, in order to phagocytose apoptotic cells[391]. If neutrophil phagocytosis is affected by IGF-1, it could influence their role in inflammation resolution. Both of these populations can be tested by using neutrophil cell lines, such as HL-60 cells, isolating primary neutrophils or dendritic cells, or using bone marrow-derived dendritic cells, as phagocytes in engulfment assays. If the phagocytic capacity of either population is affected by IGF-1 treatment, then their *in vivo* relevance can be tested by crossing the Igf1r$^{fl/fl}$ mice to the appropriate tissue specific transgenic Cre mouse lines, such as zDC(*Zbtb46*)-Cre, that targets conventional dendritic cells[392], the CD11c(*Itgax*)-Cre[393,394] (dendritic cells and some macrophage subsets), and the Mrp8(*S100a8*)-Cre[395,396] or the *GE*-cre[397] (both target neutrophils). Additionally, it could be possible that these cells are not responsive to IGF-1, but to other growth factors, such as VEGF and EGF. Therefore, it would also be interesting to determine if phagocytosis is affected using the panel of growth factors initially tested in Chapter III.

Second, another phagocytic population to consider is the semi-professional phagocytes, such as Sertoli cells of the testis and retinal epithelial cells (RPE) of the eye. IGF-1 has been shown to be important for the proliferation of immature Sertoli cells; deletion of IGF-1 results in reduced testis size and decreased sperm production[398]. Preliminary studies suggest that Sertoli cells are refractory to the IGF-1 mediated modulation of apoptotic cell engulfment. However, it is possible that Sertoli cells may respond differently when exposed to apoptotic germ cells as opposed to apoptotic thymocytes. In the retina, overexpression of IGF-1 results in retinopathy, due to increased proliferation of RPEs[399,400]. Given the pleiotropic effects of IGF-1 signaling, *ex vivo* phagocytosis assays would be important in separating the growth-associated

effects of IGF-1 versus any potential impact of IGF-1 on clearance. These confounding aspects highlight the importance of using inducible tissue-specific transgenic Cre mouse lines for *in vivo* studies.

Third, peritoneal macrophages, bone marrow-derived macrophages, and alveolar macrophages were utilized in our studies. Recent transcriptome analyses showed that macrophage subsets have distinct gene expression and enhancer profiles as defined by their tissue environment[401-403]. It would be important to extend our observations to other macrophage subsets, such as microglia and Kupffer cells, to not only assess their phagocytic capacity when exposed to IGF-1, but also their ability to produce IGF-1 in response to IL-4, IL-13, and/or apoptotic targets. Potential physiological applications of these potential experiments are discussed in more detail below.

2.1.3. <u>The effect of IGF-1 on uptake of microbial pathogens and other targets</u>

The current work examines only the uptake of apoptotic cells and MPs. However, because IGF-1 appears to affect the cytoskeleton, it is also important to investigate if uptake of other particles, such as bacteria, are affected. Bacteria are especially important, as they can be highly pathogenic and can range in size from submicron to several hundred microns[404], reflecting size properties of apoptotic cells and MPs. Bacteria manipulate the actin cytoskeleton of non-professional and professional phagocytes, and many species can invade the cell. As IGF-1 modifies the actin cytoskeleton in non-professional phagocytes, these changes may actually benefit bacterial invasion. Furthermore, if CDRs are formed, they may promote the

nonselective uptake of smaller sized bacteria. In a model of bacterial infection, such as *Mycoplasma pneumoniae* (<1um in size), disrupting the IGF-1/IGF-1R signaling axis, may actually be protective as IGF-1 may no longer cause disturbances in the actin cytoskeleton and aid in bacterial invasion. Alternatively, professional phagocytes, such as macrophages and neutrophils, are highly essential for clearing microbial pathogens. It would be critical to determine whether or not IGF-1 affects uptake of these bacteria and other pathogens by professional phagocytes. In fact, in a model of sepsis, IGF-1 treatment was found to enhance neutrophil clearance of bacteria[405,406]. The results of these investigations would have great implications in inflammation and resolution in infectious diseases.

2.1.4. Differences between professional and non-professional phagocytes in their response to IGF-1

Our observations show that IGF-1 treatment in non-professional phagocytes decreases apoptotic cell phagocytosis and enhances uptake of MPs while phagocytic capacity is not affected in macrophages, a professional phagocyte. One key question is why do professional phagocytes and non-professional phagocytes respond differently? One important step in investigating this question is to determine the molecular mechanism of IGF-1 in mediating changes in phagocytosis in non-professional phagocytes, as discussed above, and then testing whether these mechanisms apply in macrophages. A parallel approach is to conduct a proteomics analysis and assess changes in phosphorylation status of proteins between professional and non-professional phagocytes exposed to IGF-1. As the effect of IGF-1

on non-professional phagocytes is rapid, most likely there is a difference in downstream signaling effectors or their activation status between non-professional phagocytes and macrophages that may explain why only one population is responsive to IGF-1.

2.2. *Can microparticles be used as a therapeutic agent for allergic airway diseases?*

2.2.1. Profiling microparticles

Microparticles (MPs) and other extracellular vesicles (EVs) have recently evolved from being simple biomarkers of disease to being agents that can serve as communicators between cells, transferring proteins and nucleic acids, which then can affect the status of the cellular recipients[250]. We found that MPs, secreted by alveolar macrophages, can dampen inflammatory cytokine production by bronchial epithelial cells exposed to house dust mite (HDM). Due to the technical difficulty in generating enough MPs, it was not feasible to test the MPs as a possible therapy as this point. Furthermore, as shown by our ISX data, our isolated MP fraction is heterogeneous in terms of surface protein and lipid expression. However, it may be possible to preferentially select for certain MPs or even engineer artificial MPs that can then be used to treat airway inflammation or any other pathologies in which EVs have a beneficial impact. To fully understand the effect of these MPs on pathology, and, again, to potentially test them for therapeutic benefit, it is crucial to first improve the isolation methods and to completely characterize this fraction in their size distribution, marker expression, and cargo load. While we used differential centrifugation and filtration to isolate the particles and then confirmed their morphology using cryo-electron

microscopy (EM), it has recently been shown that purification can be improved through the use size-exclusion chromatography, predominantly to remove contaminating protein aggregates and lipoproteins[407]. These protein aggregates and lipoproteins can also impact cell signaling and confound the response.

Second, using the qNano system, a tunable resistive pulse sensor, we analyzed the size distribution and concentration of the isolated particles. One limitation of the qNano system is that measurements are taken with the use of differently sized pores, with each pore having a specific range of detectable sizes. Due to the high potential of clogging, it is common to only use a certain pore size for the type of EV isolated. It is possible that the current isolation methodology does not completely separate "exosomes" from "microparticles" (Erdbrugger, personal communication). Further, while sizing is currently one of the predominant ways to classify EVs into MPs (100nm to 1μm), exosomes (<100nm) or apoptotic blebs (>1μm), there is emerging evidence that suggests there is greater overlap between the three populations than originally perceived. Hence, to fully size this MP fraction, it will be important to use a combinatorial method of ImageStreamX (ISX) and cryo-EM. Artificial liposomes are used as sizing guides for ISX and large-scale MP preparations allow for statistical analysis to be performed on cryo-EM images. Unfortunately, due to the enormous (currently experimentally unfeasible) number of macrophages needed to produce enough MPs, the cryo-EM methodology can currently only be performed using cell lines.

Third, it is critical to determine the cargo content as well as the surface expression of proteins on the MPs as these components may be involved in

dampening inflammatory cytokine production in epithelial cells. A blanket approach to characterize MP cargo can be tandem mass spectrometry followed by validation via Western blot analysis and ISX (antibody labeling, to separate surface versus "cytosolic" fractions). Recently, it was shown that tumor cells, when bathed in chemotherapy drugs, could package these drugs into MPs. In a mouse model of ovarian cancer, mice receiving these packaged MPs had greater survival over control treated mice[408]. A similar principle can be applied to protein packaging in that macrophages can be engineered to overexpress the functionally relevant proteins. In addition to tandem mass spectrometry, miRNA expression analysis should also be investigated, as many MPs and exosomes have been shown to contain different RNA species[409-411].

In addition to cargo content, the membrane profile of MPs is another essential component to understanding how they are taken up by target cells. Unlike exosomes, MPs bud from the plasma membrane and therefore will carry signatures of the parental cell, including any surface proteins that can serve as ligands. Currently, we have characterized our MP fraction by their expression of CD11c, Siglec F, and Annexin V with CD11c and Siglec F being markers of alveolar macrophages and Annexin V as a detector of phosphatidylserine (PtdSer). Furthermore, it may be possible to sort MPs using flow cytometry, a technique under development by Joanne Lannigan and Dr. Uta Erdburgger here at UVa. Sorting MPs into Annexin V positive and negative fractions and then testing the efficacy in reducing inflammatory cytokine expression by bronchial epithelial cells can provide a better understanding of the relevance of PtdSer exposure on MPs. For example, certain PtdSer receptors, such as Tim4, most likely function more as tethering receptors[66] than bonafide engulfment receptors. Perhaps, IGF-1

mediates the selective uptake of PtdSer positive MPs through increasing tethering of these MPs, which may not require the full apoptotic cell engulfment pathway for internalization. Additionally, it is also possible that the MP content between the PtdSer-positive and PtdSer-negative fractions are different, in that the PtdSer-positive fraction are more efficacious at suppressing inflammatory cytokine transcription in bronchial epithelial cells. With the combination of these different methodologies, and through, albeit tedious sorting and functional validation, it may be possible to design MPs that are uniquely poised to suppress inflammation. Furthermore, it may be possible to manipulate existing cells *ex vivo* to produce MPs that are more physiologically relevant than engineered nanoparticles.

Lastly, it will be important to determine the molecular mechanism by which MPs mediate cytokine suppression in epithelial cells. To first examine changes on the chromatin landscape, we can perform chromatin immunoprecipitation (ChIP-Seq) to examine DNA methylation patterns and profile histone modifications at the promoters and potential enhancers of the genes that are differentially expressed upon microparticle treatment. For example, it is possible that the promoter of *TSLP* and *CSF2* become marked by trimethylation at histone 3 lysine 27, indicative of repression[412], upon treatment with MPs in the presence of HDM. Further, it has been shown in intestinal epithelial cells that the *TSLP* promoter has multiple putative binding sites for transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1)[413]. Abolishment of one NF-κB binding site reduced the ability of cells to upregulate *TSLP* expression in response to IL-1[413]. Additionally, we can investigate changes in chromatin accessibility by performing

transposase-accessible chromatin (ATAC)-Seq, with closed chromatin suggesting gene repression.

In our model, we assay cytokine mRNA expression at 3 hours post-treatment with HDM and MPs. As this is a relatively short time course, it would also be useful to examine protein changes in epithelial cells. Recently, it was shown that some macrophage derived EVs contain suppressor of cytokine signaling (SOCS) proteins which then decrease activation of signal transducer and activator of transcription (STAT) proteins in cells treated with inflammatory stimuli[354]. A similar mechanism can apply in our system in which the MPs dampen the signaling induced by HDM treatment, resulting in decreased cytokine transcription. We can assess for changes in protein activation, such as phosphorylation, by conducting a proteomics screen on airway epithelial exposed to HDM and MPs.

2.2.2. Microparticle and other EV production *in vivo*

One important question that arises from this work is whether alveolar macrophages produce MPs *in vivo.* MPs have been detected in the BAL fluid from patients with acute respiratory distress syndrome, but are hypothesized to be of alveolar epithelial origin[414]. We can assess for alveolar macrophage-derived MPs in naïve mice as well as mice given HDM using ISX and cell specific surface markers. Another method is to use *in vivo* imaging. Two-photon microscopes have already been applied to the lung[415,416]; however, the current resolution may not be fine enough to capture the entire breadth of MPs and exosomes produced. An alternate microscope could the lattice light sheet, which would provide resolution to approximately 230nm in

the x-y plane, catching the predominant sizes of MPs. The use of transgenic animals expressing a cytoplasmic fluorescent protein, such as GFP under the LysM promoter, would allow visualization of MPs released from myeloid cells. The ability to trace the production of MPs in real time would allow for the kinetic dissection of MP release during HDM administration and investigation into the distance that MPs can travel.

As a corollary, another question is whether alveolar macrophage-derived MP composition changes following different stimuli. In our assays, we examined basal production of MPs by alveolar macrophages, which occurs at very low levels. However, it has been shown that, in response to inflammatory stimuli, cells can rapidly increase MP secretion[417,418]. For example, isolated neutrophils stimulated with TNF-α increase their MP secretion within 45 minutes[419]; astrocytes, in culture, rapidly release MPs upon ATP treatment, with production reaching a plateau 20 minutes after initial stimulation[420]. During allergic airway inflammation, alveolar macrophages encounter a variety of different soluble mediators, such as cytokines and leukotrienes; it would be important to investigate how the number and composition of MPs change throughout the course of disease. It is possible that some cytokines, such as IL-4, stimulate greater production of MPs or skew the MP cargo towards a more anti-inflammatory phenotype, whereas soluble mediators like histamine, promote a more inflammatory MP phenotype. Understanding the dynamics of MP production during airway inflammation would benefit therapy development as to when and what types of compounds can be delivered into the lung for optimal response.

Lastly, while these studies focused on the contribution of MPs, other EVs should not be ignored. Exosomes are another type of EV that can carry cargo and affect

nearby cells. Alveolar macrophage-derived exosomes can be isolated in a similar process to the procedure for isolating MPs and assessed for their efficacy in dampening inflammatory cytokine production as well as in phagocytosis assays.

2.3. *Is the communication between phagocytes important in other physiological contexts?*

Our current model hypothesizes that during allergic airway inflammation, airway exposure to HDM leads to cellular apoptosis and production of Th2 cytokines, such as IL-4. Apoptotic cells and IL-4 then trigger local macrophages to secrete IGF-1, which in turn signals lung epithelial cells to decrease phagocytosis of apoptotic cells and increase uptake of alveolar macrophage-derived MPs. These MPs then suppress inflammatory cytokine production in lung epithelial cells. As apoptosis occurs during development and other pathologies, the IGF-1 mediated redirection of phagocytosis may be applicable in other physiological settings. I will briefly describe two potential areas of exploration below.

2.3.1. Phagocytic clearance in the brain

Defects in cell clearance have been linked to numerous pathologies, such as atherosclerosis and autoimmune diseases. One organ in which both professional and non-professional phagocytes have been shown to be important in the clearance of apoptotic cells is the brain[5]. Microglia, the resident macrophages of the brain, are one of the main populations involved in synaptic pruning during development[421], clearing debris after acute injury[422,423], and phagocytosing myelin in experimental autoimmune

encephalomyelitis (EAE)[424,425], a mouse model of multiple sclerosis. In the developing brain, satellite glial cell precursors, which are not of myeloid lineage, have been shown to be the predominant population that clears apoptotic neurons in the dorsal root ganglia[426]. Additionally, astrocytes have also been shown to contain TUNEL-positive corpses following brain injury[427] and engulf synapses via MER[131]. Furthermore, microglia have been shown to produce IGF-1, an important neurotropic and pro-survival factor, following IL-4 exposure[428]. As for the MP production, a variety of glial cells, neurons, and microglia have been shown to release MPs and exosomes that can be detrimental or beneficial for disease progression[429]. For example, the cerebral spinal fluid of Alzheimer's disease patients contains greater amounts of microglia-derived MPs, which when co-incubated with neurons, were shown to cause more neuronal death[430].

To begin investigating the potential relevance of this phagocytic cross-communication, an appropriate model is adult neurogenesis, specifically examining clearance in the subventricular zone (SVZ). In the SVZ, immature neurons, arising from stem cells, migrate through the rostral migratory stream, culminating in their integration into the olfactory bulb[431]. During neurogenesis, numerous progenitors undergo programmed cell death if the correct neurotropic factors are not received[432]. It was recently shown that impairment of phagocytosis in microglia, through genetic ablation of TAM receptors, resulted in an increase in caspase-3 positive corpses in the SVZ[433]. Astrocytes have also been shown to phagocytose apoptotic cells derived from the SVZ *in vitro*; however, astrocytes were much less efficient at degrading apoptotic targets compared to macrophages[427]. Interestingly, astrocytes in the SVZ serve another

purpose: they promote neurogenesis either by acting as neural stem cells[434] or through cell-cell contact with neuronal precursors[435]. These physiological properties provide a possible research model in which regular turnover of neural progenitors in the SVZ can induce IGF-1 production from microglia. This IGF-1 then could potentially inhibit phagocytosis in astrocytes, allowing the astrocytes to function as potential neural stem cells. If this is the case, then disrupting IGF-1/IGF-1R signaling could lead to decreased neurogenesis and subsequently, decreased neurons in the olfactory bulb. Physiologically, these changes may translate into poor odor memory[436,437] and decreased pheromone-associated social interactions[438]. Experimentally, this hypothetical model can be tested by crossing the *Igf1r*$^{fl/fl}$ mice to the GFAP-CreERT2 mice, in which tamoxifen inducible Cre is expressed specifically under the glial fibrillary acidic protein, marking astrocytes[439]. An inducible Cre bypasses any developmental issue that may be associated with a lack of IGF-1 signaling, especially given that IGF-1 is a prominent neurotropic factor. Measurements indicative of perturbations are olfactory bulb neuronal density, corpse accumulation in the SVZ, and olfactory memory behavioral tests. MPs may not necessarily be as important for this model, as it involves homeostatic development as opposed to an inflammatory state. However, MPs could potentially contain factors that aid astrocytes in neurogenesis process.

2.3.2. Phagocytosis during tumorigenesis

Many tumors have associated necrosis and in some cancers, the presence of necrosis or a necrotic core is negatively correlated with prognosis[440-442]. Necrosis typically occurs as a result of tumors growing faster than blood vessel formation,

stripping the tumor cells of oxygen. While hypoxia can directly lead to necrosis, it is appreciated that hypoxic conditions also induce apoptotic cell death[443,444]. In other models of hypoxia, such as ischemic stroke[445] or myocardial infarction[446], dampening apoptotic cell clearance is associated with poorer outcomes[447]. One rationale is that failure to clear apoptotic cells leads to secondary necrosis of the dying tumor cells, eliciting an inflammatory response. Further, many tumors arise from transformed epithelial cells, which are non-professional phagocytes. Thus, these tumor cells are surrounded by neighboring epithelial cells and fibroblasts that may have a currently unexplored physiological role in clearing apoptotic tumor cells. As in homeostasis, professional phagocytes, like macrophages, are in proximity.

IGF-1 has been and still remains an essential promoter of tumor growth in a variety of cancers[448], such as ovarian[449], melanoma[450], and breast cancer[214], due in part to its well-documented roles in epithelial-mesenchymal transition, tumor cell proliferation, and pro-survival signaling. In numerous clinical studies, serum levels of IGF-1 correlate positively with increased risk of developing breast cancer[451], colorectal cancer[452], and others. A Pubmed search with “IGF-1” and “cancer” results in over 7700 citations. The work presented here provides a new function for IGF-1 in modulating phagocytosis by non-professional phagocytes. Hence, it can be hypothesized that the IGF-1/IGF-1R axis (with IGF-1 produced by local macrophages encountering apoptotic cells) negatively regulates clearance of apoptotic tumor cells by surrounding non-professional phagocytes, thereby potentially contributing to inflammation and secondary necrosis. Additionally, tumor cells are also known to secrete MPs containing proteins and RNA species that aid in cancer progression and metastasis[453]. In this

instance, IGF-1 signaling may deliver a second hit in that signaling not only decreases clearance of apoptotic tumor cells, but also promotes the uptake of pro-tumorigenic MPs. Experimentally, it is possible to study this relationship again by crossing the *Igf1r*$^{fl/fl}$ mice to an epithelial cell type, using either a xenograft model or transgenic mice expressing oncogenes. However, interpretation of the results would be difficult given the pleotropic effects of IGF-1 on tumor development and growth. New mouse lines that target downstream molecular effectors of IGF-1 on phagocytosis most likely need to be created and tested to corroborate initial *in vivo* findings.

2.4. *Summary*

Collectively, the data presented here provide several key insights on phagocytic crosstalk and the control of inflammation in tissues. Our data suggest that when apoptotic cells are present in the proximity of both professional and non-professional phagocytes, macrophages produce IGF-1 that can control the engulfment capacity of neighboring non-professional phagocytes. This cross-communication has important implications for numerous physiological contexts, such as organogenesis and development, when cell turnover is commonly seen. Further research on these dynamics could offer great insight on how cell communication and soluble mediators contribute to homeostasis and tissue inflammation. Additionally, we also find that macrophages secrete MPs that subsequently can dampen inflammatory responses by non-professional phagocytes. Understanding how IGF-1 modulates preferential uptake of these MPs by non-professional phagocytes and how MPs influence tissue

inflammation can lead to the development of therapies for a variety of pathologies, such as atherosclerosis and cancer.

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