Mast cells (MC) are important immune sentinels found in most tissue and widely recognized for their role as mediators of Type I hypersensitivity. However, they also secrete anti-cancer mediators such as tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, MC and their secreted cytokines play a role in the development of atherosclerotic plaque and allergic reactions. Herein, it is demonstrated that MC differentiated from adipose derived stem cells, target and induce breast cancer cell apoptosis via an IgE-mediated targeting method. It also demonstrated that the fullerene derivatives (FDs) inhibit atherosclerotic plaque formation by reducing MC cytokine release. Finally, the demonstration of a low-affinity anti-IgE antibody, that binds to and desensitizes MC, can be used for allergy therapy is exhibited herein.
STUDIES EXAMINING MAST CELLS, NANOMATERIALS, AND IMMUNOGLOBULINS TO TREAT BREAST CANCER, Atherosclerosis, and Allergies

by

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A Dissertation Submitted to the Faculty of The Graduate School at The University of North Carolina at Greensboro in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

Greensboro 2019

Approved by

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Committee Chair
DEDICATION

To my mother and father, Loulou and George Elias
and
Dr. Anthony Dellinger
This dissertation written by Michael George Elias has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

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Date of Acceptance by Committee

Date of Final Oral Examination
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. CHARACTERIZING THE DEGRANULATORY RESPONSE OF FUNCTIONAL HUMAN MAST</td>
<td>15</td>
</tr>
<tr>
<td>CELLS DIFFERENTIATED FROM ADIPOSE-DERIVED STEM CELLS IN ORDER TO</td>
<td></td>
</tr>
<tr>
<td>TARGET AND INDUCE APOPTOSIS OF BREAST CANCER CELLS</td>
<td></td>
</tr>
<tr>
<td>III. NF-κB INHIBITORS THAT PREVENT FOAM CELL FORMATION AND</td>
<td>44</td>
</tr>
<tr>
<td>ATHEROSCLEROTIC PLAQUE ACCUMULATION</td>
<td></td>
</tr>
<tr>
<td>IV. THE IMPEDIMENT OF ALLERGIC REACTIVITY WITH HUMANIZED LOW AFFINITY</td>
<td>69</td>
</tr>
<tr>
<td>ANTI-IGE ANTIBODIES</td>
<td></td>
</tr>
<tr>
<td>V. CONCLUSION AND FUTURE PERSPECTIVE</td>
<td>88</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>92</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

I.1 Introduction to Mast Cells (MC)

MC are widely regarded as key effector cells in immune responses associated with IgE. MC, among other immune cells, are responsible for wound healing, pathogen defense, vasodilation, vascular homeostasis, angiogenesis, and tumor regression[1-5]. Specifically, MC are considered cells of the innate immune system, but also regulate the adaptive immune responses [2, 4, 6-8]. In contrast to their positive role in host defense, MC and their mediators have been associated in numerous pathophysiological processes such as autoimmune disorders, allergies, anaphylaxis, asthma, tumor progression, and most notably their role in Type I hypersensitivity [3, 9-12]. Given their ubiquity in the biology of all multicellular organisms, it is of no surprise that they play a key role in protection, and sometimes harm of the host. Although relentlessly studied since their discovery by German microscopist Paul Ehrlich in the 19th century, their role in tumorigenesis is controversial and poorly understood[9, 13-15]. The controversies of whether MC facilitate or impede tumor progression could be due to their inflammatory mediators.
I.2 Pre-Formed Mediators in Secretory Granules

I.2.i Secretory Granules

MC are distinguished by their population of electron-dense secretory granules, which contain preformed and pre-activated immune mediators[16, 17]. Based on their secretory granule contents, MC secretory granule can be characterized into five general categories: 1. cytokines; such as tumor necrosis factor alpha (TNF-α), 2. biogenic amines; histamine and serotonin, 3. enzymes; β-hexosaminidase, and proteases, 4. proteoglycans, 5. peptides[17-19]. These mediators are released by either crosslinking of the FcεRI IgE receptor with a high affinity for IgE (K_a on the order of 10^{10} M^{-1}[20]) or through a variety of secretagogues that bypass FcεRI to induced mediator releases. The release of these mediators induce a wide variety of functions including bronchial intestinal smooth muscle contraction, cell adhesion, vascular permeability, cell apoptosis, regulate gut pH, and stimulate stem cell differentiation into granulocytes [21-23]. These mediators also contribute to ailments as they have been identified as key regulators in Alzheimer’s disease, inflammatory bowel disease, cytokine release syndrome, and anaphylaxis[24-27].

I.2.ii Histamine

Histamine, a biogenic amine, is a pre-formed mediator present in all MC secretory granules. Derived from the amino acid histidine, histamine is a monoamine neurotransmitter that induces bronchoconstriction, vasodilation, vascular permeability, regulates gut pH, and potentially has a role in mast-cell mediated signaling to nerve
endings[16, 28]. Most histamine in the body is generated in MC and enters the secretory granules via vesicular monoamine transporter 2 (VMAT2 or SLC18A2)[29]. Histamine is recognized for its allergy inducing properties by binding numerous histamine specific G-protein-coupled receptors H₁-H₄[30].

I.2.iii Tryptase

Tryptase and chymase are two serine-class peptidases present in MC granules that are used to identify two major subtypes of MC: tryptase-positive MC (MCₜ) and tryptase/chymase-positive MC (MCₜc). Tryptases are a diverse group of enzymes with at least six distinct forms arising from four genes in humans: βI, βII, βIII γ, δ, α – tryptases[31]. Although MC granules contain a diverse group of mediators, tryptase is the most abundant existing in oligomeric structures that resemble proteasomes and therefore used as a marker for MC activation[32]. When released into the bloodstream, tryptases are highly resistant to endogenous protease inhibitors, such as α1-anti-trypsin and α2- macroglobulin, because the active sites face a narrow central pore that is inaccessible[16, 33].

I.2.iv Chymase

Chymase located in MCₜc granules, is a chymotryptic peptidase characterized by its preferred substrate of the peptide bond following tyrosine and phenylalanine[19]. Encoded by CMA1, chymase, unlike tryptase, is only expressed in one form in human MC granules. By contrast, rodent MC express four major chymases mMCP1, mMCP2,
mMCP5, and mMCP4 which is the functional homologue of human chymase[16]. Due to its ability to convert angiotensin I to angiotensin II Human, chymase has been reported to play a role in hypertension and atherosclerosis [33].

I.2.v TNF-α

MC are unique in many different ways, but one major attribute that distinguishes them from other immune cells, is the presence of preformed TNF-α in their secretory granules[21]. TNF-α was first described in the 1968 as a lymphotoxin as it was observed as a cytotoxic factor produced by lymphocytes[34], but later identified as TNF-α[35, 36]. TNF is a type II transmembrane protein arranged in stable homotrimers consisting of 157 amino acid residues, which releases soluble homotrimeric cytokine (sTNF) by proteolytic cleavage via the metalloprotease TNF-α converting enzyme (TACE or ADAM17), to form TNF-α within the trans-Golg network[37-40].

Since its discovery, TNF-α has been extensively studied and is recognized for its pleiotropic attributes in human physiology, such as cell survival, proliferation, and differentiation[40]. After degranulation of MC secretory granules, TNF-α is released and induces expression of adhesion molecules on endothelial cells, recruiting other immune cells to the sight of inflammation. It has been demonstrated that TNF-α released for MC enhance T-cell activation, proliferation and cytokine production in T-cells that had been stimulated through the CD3/TCR complex in vitro[41]. As the name suggests, TNF-α possess the capabilities to inhibit tumor cell proliferation and shown by the studies herein, promote HER2+ breast cancer apoptosis. It has been used as an adjuvant reagent
to promote the anti-cancer effect of doxorubicin and upregulate epidermal growth factor receptors (EGFR) on carcinomas for anti-EFGR therapy[19, 40, 42, 43]. More information regarding TNF-α and its uses as an anti-cancer therapeutic can be found in the following chapter.

In contrast to TNF-α improving patient health, it has also been associated with adverse clinical outcomes. It has been documented that TNF-α levels were elevated in serum of patients who suffered fatal outcomes due to sepsis and that the cytokine is the prime mediator of the inflammatory response seen in sepsis and septic shock[44]. In fact, in 2014 a clinical trial demonstrated the reduction in mortality of patients with severe sepsis, using anti-TNF-α[45] for immunotherapy. Also, previous studies have shown that TNF-α release from activated monocytes induces foam cell formation and upregulate adhesion molecule CD54, initiating the progression of atherosclerosis[46, 47]. More information regarding cytokine release and atherosclerosis treatment can be found in Chapter 3. TNF-α has also been associated with Crohn’s[48] and Alzheimer’s[49] disease. More controversies surface in regards to TNF-α facilitating tumor regression. Several reports indicate TNF-α assisting tumor progression[50-54]. The combination of these controversial studies delineates the need to better understand TNF-α, not just its role in tumorigenesis, but its inclusive role in diseases.
I.3 Newly Synthesized Cytokines

I.3.i Interleukin 5 (IL-5)

IL-5 is an interleukin synthesized by activated MC and Th2 cells. IL-5 is synthesized in MC upon IgE, IL-33[55], LPS, and PGN[56] activation. IL-5 consists of 115-amino acids and unlike other family members of this cytokine, IL-3 and GM-CSF, IL-5 is a homodimer in its active form[57]. Binding of IL-5 to its heterodimer type I cytokine receptor[58] promotes immunoglobulin secretion in B-cells and eosinophil activation, thus playing a crucial role in inflammation and immune defense. It has been shown that IL-5 is not present in the secretory granules of MC[59].

I.3.ii Interleukin 6 (IL-6)

IL-6 is a pleotropic cytokine which is synthesized by MC during an immune defense and is an important fever mediator due to its ability to cross the blood-brain barrier[60]. IL-6 stimulates the inflammatory and auto-immune process in an array of diseases such as multiple myeloma[61], prostate cancer[62], and atherosclerosis[63]. MC will synthesize IL-6 post activation by IgE, LPS, substance-P, IL-1, or IL-33[2]. IL-6 has been associated with the production of breast cancer cells and observed in elevated levels in patients with pancreatic cancer, thus implicated to facilitate tumor progression[64]. Although it isn’t necessary, IL-6 supports MC growth and has been used in media to generate human MCs in culture[65, 66].
I.3.iii GM-CSF

GM-CSF, also known colony-stimulating factor 2, is another cytokine produced by MC post activation. As the name implies, granulocyte-macrophage colony-stimulating factor stimulates stem cells to differentiate into granulocytes, working as a white blood cell growth factor[22]. GM-CSF is a pleiotropic polypeptide glycoprotein consisting of 144 amino acids with a 17-amino acid leader sequence[67]. The human gene for GM-CSF is located in close vicinity of the IL-3 gene, within a Th2 associated cytokine gene cluster, but is separated by an insulator, permitting independent regulation[68, 69]. GM-CSF can be produced by a variety of tissues types, such as T-cells, macrophages, endothelial cells, and tumor cells[70]. Dendritic cells, an antigen-presenting cell (APC), rely highly on the presence of GM-CSF to differentiate, making GM-CSF the indirect link of T-Cell and APC interaction. Unlike TNF-α, GM-CSF is generated post MC activation but released in slightly higher concentrations[71].

Like TNF, colony-stimulating factors (CSFs) were first identified in the 1960s and after understanding their role in human physiology and potential therapeutic effects, GM-CSF was purified a decade and half after its discovery[72, 73]. The use of GM-CSF for treatment or supportive immunotherapeutic in cancers has been, and still is under investigation. A set of guidelines for the use of G-CSF or GM-CSF as an additive immunotherapeutic in patients receiving chemotherapy for solid and hematological malignancies has been provided by the American Society of Clinical Oncology[74]. There have been numerous clinical trials establishing the role in GM-CSF in prevention and treatment of neutropenia[75], acute myeloid leukemia[76], breast cancer[77-81], and
non-Hodgkin’s lymphoma[82]. Notably, the use of GM-CSF to reconstitute the immune system post-transplant or cancer therapy has been investigated in clinical trials[75]. A study by Fattorossi et al. showed that patients with ovarian and breast cancer that received GM-CSF after high dose myeloablative chemotherapy and autologous transplantation demonstrated an up-regulation of membrane proteins on phagocytic cells and increased proliferation of T-cells[83].

Although highly regarded for its clinical benefit, GM-CSF can be attributed to the cause of numerous immune and non-immune related diseases. Due to its pro-inflammatory attributes, an excess or lack of GM-CSF can be detrimental in an individual. It has been proposed that autocrine expression of GM-CSF in myeloid leukemia cells facilitated neoplasia[84] and maybe the pathophysiologic mechanism underlying leukemoid reactions in cancer[85]. One study confirmed the overexpression of GM-CSF in transgenic mice lead to blindness due to accumulation of MC and macrophages in the eyes[86]. It has also been shown that overexpression of GM-CSF lead to auto-immune gastritis[87, 88], lethal myeloproliferative syndrome[89], severe lung eosinophilia, macrophage expansion and fibrotic reactions[90]. GM-CSF has also been implicated as the underlying limitation in an emerging immunotherapy, chimeric antigen receptor T-cell (CAR T-cell) therapy[26]. CAR T-cell therapy is limited by the development of cytokine release syndrome, also regarded as a “cytokine storm”, which is the large uncontrollable release of cytokines in response to immunotherapy. The blocking of GM-CSF with a monoclonal anti-GM-CSF antibody (Lenzilumab) or disrupting GM-CSF via CRISPR/Cas9 gene editing, combined with CAR T-cell therapy
resulted in significant anti-tumor activity and CAR T-cell proliferation in acute lymphoblastic leukemia (ALL) mice[26].

I.4 MC Controversies in Cancer

Since MC are ubiquitously expressed in tissue and were identified in human tumors in 1879, their roles in various cancers have been comprehensively studied [11, 14, 15, 91], yet still unclear the part they have in cancer. It is reported that MC are the first immune cells to enter tumor microenvironments[92], but their contribution to tumor progression or regression still remains debated. This is in part due to the contradictory findings of MC inhibiting tumor growth in mice models, but promoting tumor growth in models with the same cancer. These contradictory reports may be due to unidentified MC subtypes, differences in tumor stages, or different methods to identify MC[19]. It has been reported that MC function varies according to the stage in prostate cancer, by supplying producing cytokines in early stages but remaining dormant in later stages[93]. Another underlying issue resulting in controversies in MC role in cancer is due to the many differences in rodent and human MC biology. It cannot be concluded that results obtained using mouse models accurately reflect the same prognosis in humans thus leading to confusion and distorted deductions[94-96]. Although well documented, but poorly understood, the role of MC in cancer needs to be further examined to bring clarity to the unanswered questions.
I.5 MC in Atherosclerosis

The development of atherosclerosis is the main cardiovascular disorder that leads to acute cardiovascular syndromes, which is one of the primary causes of death in Western societies. MC numbers have been shown to increase within the arterial wall during atherosclerosis foam cell formation[97] and even more so in ruptured human coronary plaques[98]. These findings have suggested that activated MC and their degranulated contents contribute to atherosclerotic lesions and plaque formation. MC express CCR3, a receptor for a chemokine eotaxin expressed in plaque, CCL11. It was shown that by interfering CCR3 signaling using a CCR3 antagonist in apoE deficient mice, reduced MC recruitment to adventitial tissue, thus inhibiting plaque formation[99]. MC within plaque have been found near plaque microvessels, containing basic fibroblast growth factor, inducing microvessel growth resulting in a hemorrhage[100-102]. Both chymase and tryptase have been linked to atherosclerotic plaque progression and rupture, reporting elevated levels of both in patient serum suffering acute coronary syndrome[103-105]. Activated complement has been reported to be present within atherosclerotic plaque[106] and MC express C5aR, a receptor that activates MC with the compliment system by-product C5a. MC may be a novel therapeutic target to inhibit or treat atherosclerosis.
I.6 MC in Allergies

MC activation can be classified into two categories: IgE-mediated or non IgE-mediated activation. In IgE-mediated activation, an antigen binds to an antigen-specific IgE bound to FcεRI receptors on the surface of MC. The IgEs crosslink, aggregating the surface FcεRI, triggering MC to initiate complex signaling events leading to the release of degranulatory contents (within minutes) and the synthesis of cytokines[107]. Due to its rapid response, it is termed immediate hypersensitivity. If localized in the airways, the MC activation response will increase vascular permeability, induce smooth muscle contraction, and recruit other immune cells, resulting in reduced airflow and wheezing[108]. Although most cases of allergic reactions are not life-threatening, a systemic response can result in anaphylaxis, a rapid immune response, that can quickly lead to death if untreated[109].

Allergic responses can also be elicited by non-IgE mediated MC activation. There are numerous receptors on the surface of MC that can cause a non-IgE mediated response, such as IgG binding FcγRI, C3a and C35a binding C3aR and C5aR respectively, and various allergens that bind to mas-related G-protein coupled receptor member X2 (MRGPRX2)[110]. Non-IgE mediated MC activation can cause immediate hypersensitivity, but also causes delayed hypersensitivity were symptoms can take 1-10 hours to present themselves[111].
I.7 CAR T-Cell Therapy

In a 1999 clinical trial, gene therapy was set back at least a decade with the death of 18-year-old Jesse Gelsinger. Gelsinger’s death was attributed to cytokine release syndrome, tainting the image of gene therapy and impeding future developments. The first effective CAR T-cell development in 2002 showed promising results with their ability to survive, proliferate, and kill prostate cancer in vitro[112]. Given the soiled history of gene therapy and naysayers in science, the therapy almost never saw the light of day. The therapy was speculated to cause cytokine release syndrome via IL-6, thus shunned as an effective cancer treatment[113, 114]. Scientists did not allow the naysayers[115] to impact their research and continued to improve and develop CAR T-cells as a potential to treat cancerous CD19+ B-cells[116, 117]. After much debate and speculation, the first CAR T-cell therapy (CD19 CAR T-cells) ALL proved to be successful in clinical trials, demonstrating rapid tumor eradication in all patients[118]. After facing the hurdles of the scientific realm, in 2014 the FDA recognized CAR T-cell therapy as a breakthrough, and eventually leading to FDA approval of two CAR T-cell therapies in 2017 and 2018[119]. As of now more than 400 CAR T-cell therapy clinical trials are underway (www.clinicaltrials.gov), but only two are FDA approved. Both are CD19 CAR T-cell therapies, one to treat for ALL (Kymriah®) and another to treat diffuse large B-cell lymphoma (Yescarta®).

The elegance of CD19 CAR T-cells is their ability to harness both B-cell and T-cell functions. This is achieved by combining the variable region on B-cell CD19 receptors (tagging of target) with the multimeric protein complex CD3 on T-cells (killing
component). CARs are composed of three segments: 1. extracellular antigen recognition domain: single-chain fragment variant (scFv), 2. Transmembrane domain, 3. intracellular T-cell activation domain of CD3ζ [120, 121]. To successfully engineer CAR T-cells with the suitable segments; Patients T-cells obtained via apheresis and are activated with artificial APCs[117] and are co-incubated with an inactive isoform of HIV delivery vector containing the modified CAR gene. The viral vector transfects the T-cells and leads to the expression of the CAR on the surface of T-cells. T-cells are proliferated before patient injection, where they will further proliferate while simultaneously targeting cancerous cells. Although the focus above is on CD19 targets, the scFv can be altered to recognize numerous markers[122, 123], resulting in diverse therapies. After many generations of CAR T-cells[121], the fourth generation containing all key components obtained FDA approval.

The ability of MC to pre-store TNF-α and the high affinity to IgE via FceRI sanctions many possibilities for gene and anti-IgE allergy therapies. With further developments in immunotherapy, MC becoming the next CAR T-cells is exceedingly plausible. By introducing MC to viral vectors to alter surface protein expression, the possibilities are endless[124]. In combination with viral vectors allowing for the ability to adjust cytokine level production[125], promote anti-cancer mediators[125], and the high affinity interactions with IgE[20], MC can become highly function and target specific. The aforementioned cytokines properties are seemingly controversial, but if cytokine levels are properly regulated, they offer clinical benefits. With the introduction of MC to viral vectors, the up-regulation or down-regulation of cytokines such as TNF-α
and GM-CSF can be fine-tuned to optimal clinically beneficial concentrations that would avoid cytokine release syndrome and any other adverse clinical side effects, yet still offer a proper therapy. Subsequent chapters illuminate the therapeutic benefits and detriments of MC, cytokines, and their affinity to IgE offer in regards to HER2+ breast cancer, atherosclerotic plaque formation, and allergies.
CHAPTER II
CHARACTERIZING THE DEGRANULATORY RESPONSE OF FUNCTIONAL HUMAN MAST CELLS DIFFERENTIATED FROM ADIPOSE-DERIVED STEM CELLS IN ORDER TO TARGET AND INDUCE APOPTOSIS OF BREAST CANCER CELLS

Any variations of writing style within this chapter are due to the fact that this article has been published in the journal Frontiers in Immunology, doi: 10.3389/fimmu.2019.00138, acknowledging authors who contributed to this document Jesse D. Plotkin, Mohammad Fereydouni, Tracy R. Daniels-Well, Anthony L. Dellinger, Manuel L. Penichet, and Christopher L. Kepley.

II.1 Chapter Summary

MC are important immune sentinels found in most tissue and widely recognized for their role in allergic reactivity. However, they also secrete anti-cancer mediators such as tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The purpose of this dissertation was to investigate adipose tissue as a new source of MC in quantities that could be used to study MC biology focusing on their ability to bind to and kill breast cancer cells both in and ex vivo. We tested several cell culture medias previously demonstrated to induce MC differentiation. We report here the generation of functional human MC from adipose tissue. The adipose-derived mast cells (ADMC) are phenotypically and functionally similar to connective tissue expressing tryptase, chymase, c-kit, and FcεRI and capable of degranulating after cross-linking
of FcεRI. The ADMC, sensitized with anti-HER2/ neu IgE Ab (antibody) with human constant regions (trastuzumab IgE and/or C6MH3-B1 IgE), bound to and released MC mediators when incubated with HER2/neu-positive human breast cancer cells (SK-BR-3 and BT-474). Importantly, the HER2/neu IgE-sensitized ADMC induced breast cancer cell (SK-BR-3) death through apoptosis. Breast cancer cell apoptosis was observed after the addition of cell-free supernatants containing mediators released from FcεRI-challenged ADMC. Apoptosis was significantly reduced when TNF-α blocking Abs were added to the media. Adipose tissue represents a source MC that could be used for multiple research purposes and potentially as a cell-mediated cancer immunotherapy through the expansion of autologous (or allogeneic) MC that can be targeted to tumors through IgE Abs recognizing tumor specific antigens.

II.2 Background

MC are resident tissue immune cells that play an important role in innate and acquired immunity, but are most widely recognized in their role as regulators of Type I hypersensitivity[1, 2]. Differences in MC phenotypes and functional responses between species have hampered progress in understanding their role in several disease processes[2, 94-96, 126, 127]. This incongruence has directed efforts toward obtaining sources of human MC that can be used to evaluate the role of these cells in basic mechanisms of disease without confounding differences between rodent and human systems[94, 96, 128]. For example, MC can be obtained by culturing progenitor cells from cord blood, venous blood, fetal liver, bone marrow, and skin[128-132]. However,
variations in culture conditions and the resulting MC that are phenotypically and functionally immature still result in limitations that have hindered MC research. Thus, new sources of human MC are consistently needed.

One disease in which the role of MC has been investigated is cancer [9, 14, 15]. It is controversial as to their role in this disease in light of contradictory findings between model systems and species and that studies in humans are solely correlative (i.e., an increase in MC numbers equates to poor prognosis[9-12]). Human MC contain several pro-inflammatory mediators, but are unique in their ability to pre-store and release potentially beneficial anti-cancer mediators. For example, human MC have pre-stored and releasable (through FcεRI engagement) tumor necrosis factor alpha (TNF-α) within their granules[2]. Furthermore, human MC release granulocyte-macrophage colony-stimulating factor (GM-CSF) upon FcεRI stimulation[35, 133]. Both TNF-α and GM-CSF have been used as anticancer agents[77, 134]. Correlative studies in humans cannot address if the MC are affecting tumor growth; whether their presence enhances, inhibits, or are non-participating bystanders. Thus, developing ways to use MC to target tumors will aid researchers in determining the functional role of these cells in various tumors. In addition, harnessing anti-tumor agents from MC as a potential “Trojan Horse” may represent a new form of cancer cellular immunotherapy.

Human adipose tissue is a heterogeneous tissue containing the stroma-vascular (SVF) fraction that includes a large population of immune progenitor cells[135] and is a reservoir of functional MC progenitors in mice[136]. We report here that large numbers of functional human MC can be expanded from human adipose tissue. The adipose-
derived MC (ADMC) are phenotypically and functionally similar to connective tissue MC obtained from skin as assessed through MC-specific markers and IgE- and non-IgE dependent mediator release assays. Importantly, ADMC sensitized with anti-HER2/neu IgE antibodies (Abs) are able to induce cell death in breast cancer cells overexpressing HER2/neu. Adipose tissue now provides researchers a new source of human MC that could be used for multiple research purposes and as a potential new strategy for cell-mediated cancer immunotherapy.

II.3 Materials and Methods

II.3.i Consent Statement

Tissue procurement and IRB approval including patient consent were obtained from the Cooperative Human Tissue Network.

II.3.ii Adipose Tissue Digestion

Skin and adipose tissue was obtained from patients undergoing cosmetic surgery. Adipose tissue was incubated with Hanks’ balanced salt solution (HBSS), 1% fetal bovine serum, 0.04% sodium bicarbonate, 1% HEPES, 0.5% amphotericin B, 1% streptomycin/penicillin and 0.1% collagenase type 1A. Cells were placed into a 37°C orbital shaker for 1 h with constant agitation at 4 × g. The cell slurry was centrifuged at 360 × g for 15 min and adipocytes washed, suspended in medium (DMEM with 4.5 g/L glucose, 10% fetal bovine serum, 1% streptomycin/penicillin, 1% L-glutamine, and 1%
HEPES), and cultured for up to 7 days or until the stem cells were confluent before testing of MC-differentiating media below.

II.3.iii MC Differentiation

Different cell culture media were tested for their ability to induce MC differentiation of the adipose cells using X-VIVO 15 or AIM-V (Lonza, Switzerland), plus 80 ng/ml SCF (Stemcell Technologies, Vancouver, BC) with or without non-specific (NS) psIgE (human myeloma IgE; a gift from Dr. Andrew Saxon, UCLA; 0.1μg/ml). Conditioned MC media was produced using media used to culture primary human skin MC as described [132, 137]. Briefly, skin MC cultures (>5 weeks) containing 80 ng/ml SCF in X-VIVO 15 were pelleted by centrifugation, supernatants removed, filtered through a 22μm filter (Sigma-Aldrich, St. Louis, MO) to remove any cells, and added directly to the adipose stem cells (∼15ml per 75/mm2 flask). Approximately every 7 to 10 days, viability was assessed by trypan blue exclusion and half of the media was collected and replaced with fresh media. Initial monitoring of MC differentiation was determined using toluidine blue staining of cytospins followed by further characterization as described below.
II.3.iv Flow Cytometry

Flow cytometry was performed using a FACS Arial III (Becton Dickenson, Franklin Lakes, NJ). Briefly, mouse anti-human Abs to FcεRI, c-kit, FcγRI, FcγRII, FcγRIII (Santa Cruz, Dallas, TX), or mouse IgG isotype control MOPC (Sigma-Aldrich) were added for at least 1 h on ice, washed, and F(ab’)2-FITC-goat anti-mouse Abs (BD Biosciences, San Jose, CA) added for detection [138]. All experiments were performed at least three times.

II.3.v Cytochemistry and Immunofluorescence

Immunochemistry was performed with mouse anti-human Abs to tryptase and chymase or NS mouse IgG isotype (negative) control as described[139, 140] but using Cy3-conjugated anti-mouse secondary Abs. For detection of ADMC-induced apoptosis of human breast cancer SK-BR-3 cells (ATCC, Manassas, VA), cell cytospins were incubated with 1μg/ml Alexa Fluor™ 488 dye (ThermoFisher Scientific, Walnut, CA) labeled mouse anti-human tryptase (1μg/ml; for ADMC detection; green) along with Alexa Fluor™ 647 labeled mouse anti-human Ab to the active form of caspase 3 (1μg/ml; for SK-BR-3 detection; red) or Alexa Fluor™ 647 labeled isotype control for the caspase 3 Ab. To quantify the percentage of caspase 3 positive cells observed on the cytospins a total of 200 cells were counted on each slide and the number of SK-BR-3 cells positive for caspase activation was compared to the number of those not stained for caspase 3 that were not MC.
II.3.vi Gene Expression

RNA was extracted from ADMC using the Qiagen RNeasy Plus Mini kit (Qiagen, Germany). Reverse Transcriptase PCR (RT-PCR) was performed using the Qiagen OneStep RT-PCR kit using primers previously described to amplify short fragments of the β-actin, tryptase, chymase, c-KIT, and FceRIα RNA[141]. Cycling conditions were: 50°C for 30min, 95°C for 15min, followed by 35 cycles of 94°C for 45 s, 53–63°C for 45 s (according to primer T\textsubscript{m}), 72°C for 1min and a final 10min extension at 72°C.

II.3.vii Anti-HER2/neu IgE Abs and Extracellular Domain of HER2/neu (ECD\textsuperscript{HER2})

The fully human anti-human HER2/neu IgE/kappa containing the variable regions of the human scFv C6MH3-B1 has been previously described[142]. In addition, we also developed an anti-humanHER2/neu IgE/kappa containing the variable regions of the humanized Ab trastuzumab (Herceptin®) by subcloning the variable regions of trastuzumab previously used in Ab cytokine fusion proteins [143, 144] into the human epsilon/kappa expression vectors use to the develop the C6MH3-B1 IgE. The trastuzumab IgE and C6MH3-B1 IgE bind different epitopes of human HER2/neu. They are expressed in the murine myeloma cells and the transfectomas grown in roller bottles for Ab production as described [142]. The IgE Abs are purified from cell culture supernatants on an immunoaffinity column prepared with omalizumab (Xolair ®) (Genentech, Inc. San Fransisco, CA, USA) [142]. The extracellular domain of HER2/neu (ECD\textsuperscript{HER2}) was produced as described previously [143]. All proteins were quantified using the BCA Protein Assay (ThermoFisher Scientific).
II.3.viii Degranulation and Cytokine Production From ADMC

To determine ADMC functional responses mediated through FceRI, ADMC were incubated with 1μg/ml of anti-FceRI Abs or with 1μg/ml anti-NP IgE for 1 h followed by NP-BSA. To determine ADMC functional responses mediated by non-IgE pathways, ADMC were incubated with 40μg/ml Poly-L-Lysine (Sigma-Aldrich) or 10μM A23187 (Sigma-Aldrich). Post-incubation, activation was performed for 30min (to measure degranulation) or overnight (for cytokine analysis) and β-hexosaminidase release and TNF-α and GM-CSF production were measured as described[145-147]. All experiments were performed in duplicate from four separate donors and significant differences (p <0.05) determined using the Student’s t-test.

II.3.ix HER2/neu IgE-Mediated Binding of ADMC to Breast Cancer Cells

To assess the ability of anti-HER2/neu IgE sensitized ADMC to bind to HER2/neu expressing SK-BR-3 breast cancer cells, confocal imaging was used on differentially labeled, live cells. The ADMC (1.5 × 10⁵) were sensitized with 1μg/ml of anti-HER2/neu IgE Abs or NS psIgE followed by the addition of MitoTracker™ Green (500 nM; ThermoFisher Scientific). The ADMC were washed once in warm X-VIVO 15 and added to the adherent, human HER2/neu-positive SK-BR-3 cells that were pre-stained with MitoTracker™ Red (500 nM; ThermoFisher Scientific) in a live cell incubator affixed to a confocal microscope and images acquired over 6 h.
II.3.x Breast Cancer Cell-Induced Mediator Release From ADMC

ADMC were sensitized with or without 1μg/ml of anti-HER2/\textit{neu} IgE or NS psIgE as above and added to human breast cancer cells expressing high levels of HER2/\textit{neu} SK-BR-3 or BT-474 (a gift from Dr. Hui-Wen Lo, Wake Forest University). SKBR-3 or BT-474 (high HER2/\textit{neu} expression, a gift from Dr. Hui-Wen Lo, Wake Forest University) cells for 1 h in 24 well plates. The ratio of MC to breast cancer cells varied from 1:10 to 10:1 ADMC to breast cancer cells and mediators assessed in the supernatants. In some experiments anti-HER2/\textit{neu} IgE sensitized ADMC challenged with ECD\textsuperscript{HER2} or heat-inactivated serum from patients with HER/\textit{neu} positive breast cancer (Cureline, Brisbane, CA; Table 2.1).

<table>
<thead>
<tr>
<th>Serum</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Pathological Diagnosis</th>
<th>Grade</th>
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<th>TNM Stage (N)</th>
<th>TNM Stage (M)</th>
<th>Stage</th>
<th>HER2 Status</th>
<th>Treatment History</th>
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<td>64</td>
<td>Infiltrative Ductal Carcinoma</td>
<td>G3</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
<td>IIB</td>
<td>2+</td>
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<tr>
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<td>Infiltrative Intruductal Carcinoma</td>
<td>G1</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>IA</td>
<td>3+</td>
<td>none (treatment-naive)</td>
</tr>
</tbody>
</table>
II.3.xi HER2/neu IgE-Mediated Killing of Breast Cancer Cells by ADMC and Supernatants from Activated ADMC

Three different methods were used to assess the ability of anti-HER2/neu IgE sensitized ADMC to induce cell death of HER2/neu expressing breast cancer cells. First, ADMC (1.5 × 10^5) were sensitized with 1μg/ml of anti-HER2/neu IgE or psIgE for 2 h. Breast cancer cells (5 × 10^4) on coverslips were labeled with 2μM MitoTracker™ Green (ThermoFisher Scientific) for 1 h. The washed ADMC were labeled with CellTracker™ Deep Red (which stains the cells reddish/purple under confocal; 2μM) for 1 h, washed, and added to SK-BR-3 in medium containing 25μg/ml of propidium iodide (PI; which stains the cells red) used to detect dead cells[148] and PI intensity measured over time. Second, SK-BR-3 were plated and incubated with Cellevent™ Caspase 3/7 Green (to detect activated caspase-3/7 in apoptotic cells; ThermoFisher Scientific) for 1 h according to the manufacturers protocol. ADMC, treated with Mitotracker™ red (1μg/ml), were added to the washed SK-BR-3 cells and incubated for up to 4 days. Third, cytospins of cells from separate experiments were made and used for immunofluorescence detection of apoptosis. Briefly, cytospins were fixed in methanol and incubated with Alexa Fluor™ 488 dye (ThermoFisher Scientific) labeled mouse anti-human tryptase (1μg/ml; for ADMC detection; green, for co-cultures) along with Alexa Fluor™ 647 labeled mouse anti-human active caspase 3 (BD Biosciences, 1μg/ml; for SK-BR-3 detection; red) Alexa Fluor™ 647 labeled isotype Abs were used as a control for the caspase 3 Ab.

In separate experiments, cell free supernatants from optimally activated ADMC (1.3 × 10^6) by an anti-FceRI Ab (1μg/ml for 24 h; 60–70% release) were directly added to
MitoTracker™ Green-labeled SK-BR-3 cells ($5 \times 10^4$). In some experiments, an anti-human TNF-α Ab (5μg/ml) was added to the supernatants to block TNF-α activity. Cell death was monitored over time through the quantification caspase 3/7-positive cells (>200) counted at the end of each experiment to obtain percentages. All confocal/live cell experiments were performed on three separate ADMC donors and significance ($p< 0.05$) tested using the Student’s $t$-test.

II.4 Results

II.4.i Phenotypic Characterization of ADMC

Several culture conditions were tested for their ability to induce MC differentiation[147, 149]. The conditioned media from skin derived human MC cultures was found to be optimal for ADMC differentiation (Table 2.2). In conditioned medium, ADMC were observed to emerge from large clumps of cells or tissue as shown in Figure 2.1.A. After 3–4 weeks of culture, mature MC (>90% viable) were observed as demonstrated by the classical spherical, highly granulated morphology (Figure 2.1.B) characteristic of skin-derived MC (Figure 2.1.C). In addition, the ADMC were positive for messenger RNA to the two major MC proteases, tryptase, and chymase (Figure 2.1.D). Furthermore, the ADMC expressed surface markers for tissue MC including FcεRI and the receptor for SCF, c-kit (Figure 2.1.E). As previously reported with skin MC[138], ADMC express FcγRII and not FcγRI or FcγRIII (Figure 2.1.F). As seen in Figure 2.1.G both tryptase and chymase protein was detected using immunohistochemistry. Thus, adipose tissue has MC progenitors that can be
differentiated into MC that are phenotypically similar to human connective tissue (MC_{TC})[150] based on these characteristics. Representative numbers of ADMC obtained from surgical specimens are shown in Table 2.3.

| Table 2.2. Media Used for Mast Cell Differentiation |
|------------------------|------------------------|------------------------|
| Media                  | Media Additions        | MC Numbers             |
| AIM-V                  | 80 ng/ml               | 0.1 µg/ml              |
|                        | SCF                    | +                      |
|                        | SCF                    | +                      |
| Ex vivo                | SCF                    | +                      |
|                        | SCF                    | ++                     |
| Conditioned media      | none added             | +++                     |
Figure 2.1. Phenotypic Characterization of ADMC. (A) Light microscopy of ADMC. ADMC cultures demonstrating large cell/tissue clumps from which the MC differentiate (20 × magnification). Cytospins of adipose-derived (B) or skin MC (C) were stained with toluidine blue. (D) Gene expression was measured using RT-PCR on total RNA. β-actin and 18S ribosomal subunit primers were controls (Ladder: bp). (E) Surface expression of MC-specific markers by flow cytometry. ADMC were incubated with mouse anti-c-Kit/CD117 (dashed line), FcεRIα chain (solid line), or isotype control mouse IgG (gray) for 2 h on ice, washed, and Fab’2-FITC-goat anti-mouse added for 1 h. (F) Fcγ receptor expression on ADMC. ADMC were incubated with mouse anti human FcγRI (dotted), FcγRII (solid line), FcγRIII (dashed), or isotype control mouse IgG (gray) for 2 h on ice, washed, and FITC-labeled anti-mouse F(ab)2 added for 1 h. (G) Immunohistochemistry of ADMC with MC-specific
markers. Anti-tryptase, anti-chymase, or NS IgG Ab were incubated overnight on cytospin cells, washed and incubated with Cy3-secondary Abs and Hoechst dye (blue nuclei) and visualized using confocal microscopy. Figures are representative of cells derived from three different human subjects.

<table>
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<tr>
<th>ADIPOSE SOURCE</th>
<th>STARTING GRAMS OR ML</th>
<th>MAST CELL NUMBERS AT 8 WEEKS</th>
<th>CELLS PER GRAM/ML</th>
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<td>2.0 x 10^5</td>
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<td>27 g</td>
<td>8.6 x 10^6</td>
<td>3.2 x 10^5</td>
</tr>
<tr>
<td>skin resection; WF</td>
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<td>3.4 x 10^5</td>
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<td>7.5 x 10^5</td>
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<tr>
<td>skin resection; WF</td>
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<td>750 ml</td>
<td>5.6 x 10^8</td>
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<tr>
<td>lipo; WF</td>
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<td>Average/ml</td>
<td></td>
<td><strong>5.1 x 10^5</strong></td>
</tr>
</tbody>
</table>
II.4.ii Functional Characterization of ADMC

The functional response of ADMC was compared to skin-derived MC. As seen in Figure 2.2, ADMC degranulated (Figure 2.2.A) and produced cytokines (Figure 2.2.B) in response to FcεRI engagement. Cytokine release by ADMC and skin MC was similar in response to FcεRI-dependent stimuli averaging 2,850 and 2,600 pg/ml of GM-CSF in skin MC and ADMC, respectively. A similar degranulatory response with ADMC was observed using non-FcεRI-dependent stimuli Poly-L-Lysine and A23187 (Figure 2.3). Taken together, the ADMC are functionally similar to skin-derived MC in response to FcεRI dependent and FcεRI-independent stimuli.

Figure 2.2. ADMC Functional Response. Human skin MC (black box) or ADMC (gray box; 10⁶) were challenged with or without (spontaneous release) 1μg/ml anti-FcεRI Abs or anti-NP IgE + antigen (IgE-Ag) and degranulation (A) or GM-CSF production (B) assessed in the supernatants. Error bars represent ± SD. *p < 0.05 comparing skin MC vs. ADMC release. Figure is representative of cells derived from two different donors.
Figure 2.3. Functional Response of ADMC via Non-IgE Activation. ADMC were challenged with or without (spontaneous release) optimal concentrations of non-IgE MC activators and degranulation was assessed in the supernatants. Figure is representative of cells derived from two different donors.

II.4.iii Anti-HER2/neu IgE Mediates ADMC Binding to SK-BR-3 Breast Cancer Cells

The ability of ADMC sensitized with the anti-HER2/neu IgE to bind HER2/neu–positive SK-BR-3 breast cancer cells was investigated. As seen in Figure 2.4.A, the ADMC sensitized with the anti-HER2/neu IgE (green) bound to HER2/neu-positive SKBR-3 breast cancer cells (red) as demonstrated in the time lapse pictures and video (Video 2.1). However, ADMC sensitized with a NS IgE did not target or bind to the SK-BR-3 cells (Figure 2.4.B). These results demonstrate that the anti-HER2/neu-IgE mediates the interaction between ADMC and HER2/neu-positive breast cancer cells.
Figure 2.4. Time Lapse, Confocal Microscopy of ADMC Binding to Breast Cancer Cells. ADMC (10⁵-10⁶) were sensitized with 1μg/ml of trastuzumab IgE (A, 20X) or NS IgE (B, 40X) followed by MitoTracker™ Green. The MitoTracker™ Green-loaded ADMC were added to adherent SK-BR-3 (10⁵-10⁶) that had been pre-stained with MitoTracker™ Red and time lapse video taken over 6 h. The white circular boundaries and arrows represent starting point and tracking of ADMC (green) at time 0 to SK-BR-3 (red) binding over the 6 h. 20X magnification was used to capture the cellular tracking (start and stop) that can be observed in accompanying video.

II.4.iv Anti-HER2/neu IgE-Sensitized ADMC Become Activated Through FcεRI

Upon HER2/neu-Positive Breast Cancer Cell Binding

ADMC must release their mediators upon FcεRI challenge at the site of the tumor to be effective anti-tumor agents. Thus, the ability of ADMC sensitized with the anti-HER2/neu IgE to degranulate in the presence of breast cancer cells was investigated.
ADMC were sensitized with one of two anti-HER2/neu IgE Abs recognizing different epitopes (trastuzumab IgE or C6MH3-B1 IgE). Varying ADMC cell numbers were incubated with SK-BR-3 breast cancer cells and mediator release assessed in the medium. As seen in Figure 2.5, ADMC sensitized with anti-HER2/neu IgE induced significant (p<0.05) mediator release through FcεRI when co-incubated with the HER2/neu positive SK-BR-3 breast cancer cells. The ADMC degranulated to release pre-stored mediators (Figure 2.5.A), as well as newly formed mediators TNF-α and GM-CSF (Figure 2.5.B).

Another HER2/neu-positive breast cancer cell line, BT-474, also induced degranulation and cytokine production optimally at a ratio of 1:2 (degranulation) and 1:0.5 (cytokine release) ADMC:BT-474 (Figure 2.6).

The above results suggest the possibility of using ADMC armed with IgE Abs can trigger degranulation in the presence of HER2/neu expressing cancer cells and thus, the potential of using this strategy for cancer therapy via the release of MC mediators. However, a potential concern of the systemic administration of ADMC sensitized with an anti-HER2/neu IgE is the possible induction of a systemic anaphylactic reaction as patients with HER2/neu breast cancer can have elevated levels of circulating ECDHER2 in the blood[151, 152]. The IgE Abs are not expected to induce FcεRI cross-linking when complexed with soluble antigen (ECDHER2), given the mono-epitopic nature of this interaction and the fact that ECDHER2 does not form homodimers in solution[153, 154]. To address this concern, the ability of ECDHER2 to induce FcεRI-mediator release was examined. As described previously[142], ECDHER2 in the presence of the anti-HER2/neu IgE Abs did not induce degranulation, while anti-FcεRI Abs induced release (Figure
2.5.C). Furthermore, serum from two separate HER2/neu positive breast cancer patients did not induce ADMC degranulation (Figure 2.5.D). These results suggest that the anti-HER2/neu IgE-sensitized ADMC will not induce a systemic anaphylactic response in vivo and will only release mediators upon encountering HER2/neu on breast cancer cells.

Figure 2.5. Breast Cancer Cell-Induced ADMC Mediator Release. ADMC were sensitized with 1μg/ml anti-HER/neu IgE (clone C6MH3-B1 IgE or trastuzumab IgE), washed, and incubated with SK-BR-3 cells and degranulation (A) or cytokine release (B) assessed. Data are from a single experiment representative of experiments performed on cells derived from four separate donors. Error bars represent ± SD. *p < 0.05 Compared with non-IgE (spontaneous) release. All values in (B) are significant between spontaneous and the given conditions. (C) ECD^{HER2} does not induce ADMC mediator release. ADMC were sensitized with 1μg/ml anti-HER/neu IgE as in (A), washed, and incubated with ECD^{HER2} and mediator release assessed. As a control, optimal concentrations of anti-FcεRI Ab were tested in parallel. Each condition was tested in triplicate and is representative from two separate ADMC donors. Error bars represent ± SD. (D) Sera from HER2/neu
positive breast cancer patients does not induce ADMC degranulation. Heat inactivated sera from two separate HER2/neu positive breast cancer patients (P1 and P2; see Table 2.1) or normal control serum (Ctrl) was used to challenge anti-HER/neu IgE (C6MH3-B1 IgE or trastuzumab IgE) sensitized ADMC and β-hexosaminidase release measured as described. Background levels of β-hexosaminidase naturally found in the sera was subtracted from values. Experiment is representative of two separate ADMC preparations each done in duplicate. Error bars represent ± SD.

Figure 2.6. Anti-HER2/neu IgE-Sensitized ADMC Degranulation with BT-474 Breast Cancer Cells. ADMC were sensitized with 1μg/ml anti-HER2/neu clone C6MH3-B1 IgE, washed, and incubated with BT-474 cells and degranulation (A) or cytokine release (B) assessed. As a control, optimal concentrations of 1μg/ml anti-FcεRI Ab were tested in parallel. Data are from a single experiment representative of experiments performed on cells derived from four separate donors.

II.4.v IgE Sensitized ADMC Induce SK-BR-3 Cell Death

The ability of ADMC to induce breast cancer cell death was investigated. ADMC sensitized with the anti-HER2/neu IgE were added to SK-BR-3 cells in medium containing PI to discriminate dead cells from live cells[148]. As seen in Figure 2.7.A, binding of anti-HER2/neu-sensitized (trastuzumab IgE) ADMC to SK-BR-3 cells induced significant cell death of the breast cancer cells as assessed by the uptake and visualization (red) of the PI in the SK-BR-3 cells but not the ADMC. Quantification of
the PI signal in Figure 2.7.B demonstrated significant breast cancer cell killing after 4 days (p = 0.0003). Sensitization of the ADMC with NS IgE did not result in significant SK-BR-3 cell death. Similarly, anti-HER2/neu IgE C6MH3-B1 sensitized ADMC induced significant (p = 0.032) SK-BR-3 cell death (data not shown). In addition, ADMC added to the SK-BR-3 over 4 days revealed significant (p = 0.003) breast cancer cell death, but not ADMC death (anti-tryptase labeled), as indicated by immunostaining of the SK-BR-3 with an Ab specific for the apoptotic enzyme caspase 3 (Figures 2.7.C,D). Lastly, a significant (p = 0.0004) increase in caspase 3/7-positive breast cancer cells was confirmed at day 4 when anti-HER2/neu–sensitized ADMC were co-incubated (Figures 2.7.E,F). The tumor cell specificity of the responses was verified as the NS isotype control IgE did not affect breast cancer cell viability. These experiments indicate ADMC binding to SK-BR-3 results in ADMC activation through FcεRI capable of inducing significant SK-BR-3 cell death.
Figure 2.7. ADMC Killing of Human Breast Cancer Cells Measured as Evidence by the Uptake of PI. (A) CellTracker™-red labeled ADMC (7.5 × 10⁴; shown here as purple cells) were sensitized with 1μg/ml anti-HER2/neu IgE (clone trastuzumab), washed, and incubated with MitoTracker™-green-stained SK-BR-3 (10⁵) in culture medium containing PI and images taken before (A; left) and after (A; right) 96 h. The call out box shows a representative ADMC being activated through loss of granularity over time. (Mag 20x). (B) Quantification of overall PI fluorescence before and after incubation. The percent of PI-positive cells was counted in culture. The *p = 0.0003 SK-BR-3 cell death at day 4 compared to day 0 when ADMC where sensitized with anti-HER2/neu IgE. No cell death was observed with the NS IgE. (C) ADMC-induced breast cancer cell apoptosis. Anti HER2/neu IgE-sensitized ADMC (7.5 × 10⁴) were incubated with SK-BR-3 (1 × 10⁵) for 72 h, cytopsins made, fixed, and incubated with Alexa Fluor™ 488 labeled, mouse anti human tryptase (left; green) along with Alexa Fluor™ 647 labeled, mouse anti-human caspase 3 (red) or Alexa Fluor™ 647 labeled, isotype control IgG for caspase 3 Ab (right). (D) Quantification of overall Alexa Fluor™ 647 fluorescence before and after incubation. *p = 0.002 SK-BR-3 apoptosis comparing psIgE and anti-HER2/neu IgE-sensitized cells at day 4 from three experiments. (E) ADMC killing of human breast cancer cells measured by caspase 3/7 activation Mitotracker™-red labeled ADMC (7.5 × 10⁴; shown here as red cells) were sensitized with 1μg/ml anti-HER2/neu IgE (clone trastuzumab), washed, and incubated with caspase 3/7 green-labeled SK-BR-3 (10⁵ and images taken before (A, left) and after (A; right) 96 h. (F) Quantification of overall caspase 3/7 fluorescence before and after incubation. The
percent of caspase 3/7-positive cells was counted in culture. *p=0.004 SK-BR-3 cell death at day 4 compared to day 0 when ADMC where sensitized with anti HER2/neu IgE. No SK-BR-3 cell death was observed with the NS IgE.

II.4.vi Mediators Released from ADMC Through FcεRI Induce SK-BR-3 Cell Death

As shown above, ADMC produce mediators that induce significant breast cancer cell death upon FcεRI cross-linking using a tumor-tagged IgE. The ability of the mediators obtained from FcεRI-activated ADMC alone to induce SK-BR-3 cell death was then examined. As seen in Figures 2.8.A,B, medium alone (not containing ADMC) from optimally activated ADMC incubated with SK-BR-3 cells induced significant (p = 0.009) SK-BR-3 cell death when incubated for 4 days. Further, when the media from optimally activated FcεRI ADMC were added to the SK-BR-3, there was a significant (p = 0.01) increase of apoptotic cells as evidenced by the increase in active caspase 3 (Figures 2.8.C,D) indicating cell death of the breast cancer cells as in Figure 2.7. A significant (p = 0.0002) increase in activated caspase 3/7-positive breast cancer cells was confirmed at day 4 when SK-BR-3 cells were incubated with supernatants from ADMC activated through FcεRI (Figures 2.8.E,F). Blocking TNF-α activity significantly prevented SK-BR-3 cell death (Figure 2.8.G).
Figure 2.8. Mediators from FcεRI-Challenged ADMC Induce SK-BR-3 Cell Killing. (A) ADMC (1.3 × 10⁶) were challenged with optimal concentrations of anti-FcεRI stimuli (70% release) for 24 h and supernatants (XL media; no cells) from these ADMC were incubated with the MitoTracker™ green stained SK-BR-3 (10⁵) in culture medium containing optimal concentrations of PI and images taken before (left) and after (right) 96 h. (B) Quantification of overall PI fluorescence before and after incubation. The increased number of red cells indicates breast cancer cell death as indicated by the PI (red) and quantified in showing overall PI fluorescence before and after incubation. Graph represents average PI intensity from two separate experiments (SD; *p = 0.0008). (C) Mediators from FcεRI-challenged ADMC induce human breast cancer cell apoptosis. The same media from anti-FcεRI challenged ADMC were incubated with SK-BR-3 (10⁵) for 72 h, cytospins prepared, fixed, and incubated with Alexa Fluor 647 labeled, anti-human caspase 3 (left) or Alexa Fluor 647 labeled, isotype control Ab for caspase 3 (right). Representative panels are shown. (D) Quantification of overall Alexa Fluor™ 647 fluorescence before and after incubation with supernatants from FcεRI activated ADMC. *p = 0.01 SK-BR-3 apoptosis comparing anti-HER2/neu IgE-sensitized cells at day 0 vs. day 4 from three experiments. (E) Mediators from FcεRI-challenged ADMC induce SK-BR-3 cell killing measured by caspase 3/7. ADMC (1.8 × 10⁶) were challenged with optimal concentrations of anti-FcεRI stimuli (63% release) for 24 h and supernatants (no cells) from these ADMC were incubated with SK-BR-3 (10⁵) and caspase 3/7 green images taken before (left) and after (right) 96 h. The
increased number of green cells indicates breast cancer cell death as indicated by the caspase 3/7 and quantified by counting live vs. dead cells before and after incubation. (F) Graph represents average percentage of cells from two separate experiments (±SD; *p = 0.002). (G) Blocking TNF-α significantly reduces SK-BR-3 cell death. SK-BR-3 were treated and quantified as in (C) except anti-TNF-α Ab were added during the incubation time. *p = 0.028 Decrease in SK-BR-3 cell death when anti-TNF-α Abs are added to the supernatants from anti-FcεRI stimulated ADMC.

II.5 Discussion

Here we report that functional MC can be differentiated from adipose tissue obtained from human subjects undergoing cosmetic surgery procedures. This research discovery is notable as there is an ever-present need for new sources of human MC for research, given the differences between human and rodent MC phenotypes and functional responses[96, 126, 127]. This incongruence has led to confusion and inconsistent findings in the field of MC biology and allergic mechanisms[128, 155, 156], especially in Fc receptor expression and function[138]. While a plethora of human “mast cell” lines exist, each is wrought with phenotypic and functional anomalies compared to primary human MC[128]. Primary human MC can be obtained from cord blood[157, 158], bone marrow[157], fetal liver[159], peripheral blood[160], and human tissue (e.g., skin)[131]. For autologous applications, MC can be obtained from CD34+ hematopoietic progenitor cells in the blood[161], but not in sufficient numbers for most applications. For example, the total MC number generated from 1.0 × 10⁸ lymphocytapheresis or peripheral blood mononuclear cells averaged 2.5 × 10⁶ and 2.4 × 10⁶, respectively[162]. Large numbers of enriched CD34+ cells can also be obtained commercially to increase the quantities of subsequent MC following GM-CSF injection, apheresis, and subsequent positive
Selection with magnetic beads has been described[160]. However, given the various protocols for differentiation the MC obtained from these methods are not fully mature and functional. In this report, approximately $5.1 \times 10^5$ ADMC were obtained per ml of liposuction compared to $4.8 \times 10^3$ MC per gram of skin. Thus, ADMC can be utilized as a relatively rapid, more cost effective, and efficient method for studying MC biology and function. Current efforts are focused on identifying the molecule(s) in the conditioned media that are responsible for the ADMC differentiation and maturation.

The role of MC in cancer is controversial as to whether they are beneficial, harmful, or innocuous and is dependent on the tumor type and location within the tumor in humans and animal models[10, 12, 92, 163, 164]. Animal models, mostly MC deficient mice, have suggested that MC and their mediators play a pro-tumorigenic role[163]. Yet, MC-deficient mouse models have paradoxically indicated that in certain tumors, and even in the same models, MC appear to play a protective role[93, 163]. These contradictory results might reflect differences in the stage, incongruences between animal models (i.e., MC knockout through kit mutation vs. Cre mutation) and/or rodent MC lines vs. human MC, grade, and subtypes of tumor; as well as the different methods to identify MC. While in certain human cancers the presence of MC is associated with poor prognosis, in other malignancies, such as breast and colorectal cancer, the presence of MC has been associated with a favorable clinical prognosis depending on their location[165-168]. Currently, multiple questions remain as to the nature of the role of MC in cancer pathogenesis.
Human MC are unique in that they have pre-stored TNF-α within their
granules[169, 170]. Furthermore, human MC release copious amounts (2,500–4,000
pg/ml from 10^5 cells) of GM-CSF upon FcεRI stimulation[35, 133]. Indeed, the above
blocking experiments suggest TNF-α activity is the major component in FcεRI-activated
ADMC supernatants that induces SK-BR-3 apoptosis (Figure 2.8.G). TNF-α is an
anticancer agent shown to suppress tumor cell proliferation, induce tumor regression, and
used as an adjuvant that enhances the anti-cancer effect of chemotherapeutic agents[171-
173]. GM-CSF is also being investigated as an anti-breast cancer therapeutic, including
its use in combination strategies with other immunotherapies[77-81]. There are over 50
clinical trials completed or underway examining the beneficial clinical effects of GM-
CSF (www.clinicaltrials.gov). In addition to GM-CSF and TNF-α, MC also store and
release several other potential anti-tumor mediators including reactive oxygen species
(ROS), prostaglandin D_2 (PGD_2), interleukin-9 (IL-9), and heparin[2, 9]. In one study
cord blood-derived MC and eosinophils, sensitized with an anti-CD20 IgE, were shown
to kill CD20+ cancer cells[174]. Thus, it may be possible that even in cases where MC
may act favoring the tumors in certain cases through a controlled release of certain
agents, they may have anti-tumor activity upon an IgE-mediated strong and immediate
release of their granular content. Given that these MC mediators may have unwanted side
effects, further in vivo studies are needed to address this topic.

There are 21 FDA approved Abs on the market to treat various cancers[175].
While all are of the human IgG class, IgE has several potential advantages over Abs of
the IgG class, such as the IgE-FcεRI high affinity interaction, which allows a more
effective arming of effector cells without losing surface-bound Abs[142, 176, 177] and the low serum levels of IgE that result in less competition for FcR occupancy[176-178]. The first clinical trial (www.clinicaltrials.gov; clinical trial number NCT02546921) is currently underway in patients with advanced solid tumors to examine the safety of a mouse/human chimeric IgE Ab (MOv18 IgE), specific for the tumor-associated antigen folate receptor-α, which has exhibited superior anti-tumor efficacy for IgE compared with IgG1 in animal models[179, 180].

Three separate experimental approaches were used above to demonstrate ADMC, and mediators from FceRI-challenged ADMC, have anti-tumor activity and suggests the possibility of using autologous (or allogeneic) MC in cancer immunotherapy. There are several advantages for this potential technology. First, mature, functional, autologous or allogeneic MC can be obtained in quantities necessary for patient infusion. Second, the availability of IgE Abs with human constant regions (chimeric, humanized, and fully human) targeting tumor antigens has grown substantially[176, 178]. Third, the high affinity binding between IgE and FceRI is very stable with a long half-life resulting in an effective arming of MC, which would be able to target the tumor and doing so induces tumor cell death. The presence of dead tumor cells would facilitate their uptake and presentation of tumor antigens by antigen presenting cells (APC), eliciting an adaptive broad-spectrum anti-tumor immunity. This would increase due to MC local release of GM-CSF[35, 133] and potentially the release of suppressors of regulatory T-cell (Tregs) function as reported for IgE degranulation in murine MC[181]. Lastly, unlike other immune cells currently being used for cancer immunotherapy[182], ADMC sensitized
with anti-HER2/\textit{neu} IgE are equipped to kill tumor cells without genetic reprogramming, which is time consuming and expensive[182].

In conclusion, it is shown that adipose tissue represents an alternative source for human MC that are phenotypically and functionally similar to primary MC. This new source of MC, ADMC, can be used in research to address fundamental questions in MC biology and to study IgE Abs including those targeting tumor antigens. Importantly, ADMC exhibit tumoricidal activity when armed with IgE Abs specific for a tumor antigen. Future studies are needed to evaluate the utility of ADMC, sensitized with tumor targeting IgE, to examine anti-tumor activity and toxicity in \textit{in vivo} cancer models to further validate this potential new cancer immunotherapy strategy.
CHAPTER III

NF-κB INHIBITORS THAT PREVENT FOAM CELL FORMATION AND AThERSCLEROTIC PLAQUE ACCUMULATION

Any variations of writing style within this chapter are due to the fact that this article has been published in the journal *Nanomedicine: Nanotechnology, Biology and Medicine*, doi: 10.1016/j.nano.2017.04.013, acknowledging authors who contributed to this document Jesse D. Plotkin, Anthony L. Dellinger, and Christopher L. Kepley.

III.1 Chapter Summary

The transformation of monocyte-derived macrophages into lipid-laden foam cells is one inflammatory process underlying atherosclerotic disease. Previous studies have demonstrated that fullerene derivatives (FD) have inflammation-blunting properties. Thus, it was hypothesized that FD could inhibit the transformation process underlying foam cell formation. Fullerene derivatives inhibited the phorbol myristic acid/oxidized low-density lipoprotein-induced differentiation of macrophages into foam cells as determined by lipid staining and morphology. Lipoprotein-induced generation of TNF-α, C5a-induced MC activation, ICAM-1 driven adhesion, and CD36 expression were significantly inhibited in FD treated cells compared to non-treated cells. Inhibition appeared to be mediated through the NF-κB pathway as FD reduced expression of NF-κB and atherosclerosis-associated genes. Compared to controls, FD dramatically inhibited plaque formation in arteries of apolipoprotein E null mice. Thus, FD may be an
unrecognized therapy to prevent atherosclerotic lesions via inhibition of foam cell formation and MC stabilization.

III.2 Background

Atherosclerotic disease begins when monocytes are exposed to oxidized low-density lipoprotein (ox-LDL) which form macrophage-derived foam cells that initiate plaque formation on blood vessels [183]. The plaque can suddenly rupture and cause thrombosis; leading to myocardial infarction or stroke. Given the severe implications of this process, new strategies that inhibit this progression before it occurs would reduce patient mortality and morbidity.

MC are ubiquitously expressed in tissue and have been shown to be involved in the pathogenesis of atherosclerotic plaque buildup, erosion and rupture [99, 184]. These cells are uniquely positioned to release their vast content of inflammatory mediators in and around affected vessels and into the circulation. MC accumulate in human atherosclerotic lesions [185] and chronic activation of MC in the atherosclerotic lesions may be a pre-disposing stimulus leading to plaque rupture [184].

The mechanisms leading to the transformation of monocytes into foam cells is complex, involving MC activation, oxidative stress, NF-κB activation, localized cytokine secretion, and the up-regulation of scavenger and cell adhesion receptors/ligands [186, 187]. Thus, a therapy that would block macrophage-to-foam cell transformation, stabilize MC activation, and reduce MC noxious mediator secretion may serve as a platform for new treatment strategies aimed at preventing atherosclerosis.
Fullerenes are carbon spheres that have a wide range of potentially beneficial biological properties. We recently showed water soluble FD are non-toxic [188] and inhibit allergic [35, 189], pulmonary, and dermatological inflammation [145]. Previous studies examining the biodistribution of FD have shown them to be efficiently excreted from the body with negligible accumulation in major organs [133, 190-194]. Our studies suggest the anti-inflammatory effects of FD may be due to their ability to interfere with several molecules involved in NF-κB-mediated activation, cytokine secretion, and oxidative stress responses [35]. Given the link between inflammation, oxidative stress, NF-κB activation and atherosclerosis, we hypothesized that FD could inhibit the underlying mechanisms of atherosclerosis and foam cell formation. We show that certain FD inhibit foam cell formation and inflammatory mediator release associated with atherosclerosis through a NF-κB-dependent mechanism in vitro and can prevent the morbidity and mortality associated with a high fat diet in plaque-susceptible mice in vivo.

III.3 Materials and Methods

III.3.i Fullerene Derivatives

A panel of approximately 30 anti-inflammatory FD [35] were initially tested for their ability to inhibit foam cell formation in vitro. From this initial screen, both Amphiphilic Liposomal Malonylfullerene (ALM) and Tris-malonate fullerene (C$_3$) were chosen for further analysis. The FD were bought from Luna Innovations and their formulation is described elsewhere [190, 195].
III.3.ii Generation of Monocyte-Derived Macrophage Foam Cells

Low-density lipoproteins from human plasma were oxidized as previously described to produce ox-LDL [196]. To obtain macrophage-derived foam cells U937 monocyctic cells (0.5 - 1.0 x 10^6 cells/ml) were treated with 100ng/mL phorbol myristilic acid (PMA) for 24 hours, washed 1 time with media, and ox-LDL (10μg/mL) added for 48 hours as described previously [197]. Confirmation of macrophage and foam cell development was determined using surface markers to CD68, CD36, and oil red O staining (ORO), respectively (data not shown) as described [35] [198, 199]. TNF-α levels were measured using a kit from Sigma.

III.3.iii Scanning Electron Microscopy

U937 cells were challenged with PMA as above, followed by FD (5μg/mL) for 24 hours before addition of ox-LDL. Following treatment cells were fixed on an aluminum foil substrate, and dehydrated using a gradient of ethanol with increasing percentages of 60%, 70%, 80%, 90% and 100% for 10 min. each. After air-drying, the foil substrate was mounted on standard Hitachi 15mm aluminum specimen mount with standard double-sided carbon tape. Images were obtained using a Hitachi S-4800 FESEM with a beam setting of 1.0kV and 5.0μA.
III.3.iv Effect of FD on ICAM-1 and CD36 Expression and NF-κB Signaling

Intermediates

U937 cells were treated with PMA and ox-LDL as above followed by various concentrations of FD for 24 hours before the ox-LDL was added. Treated cells were subjected to FACs analysis with ICAM-1 and CD36 specific mouse antibodies (Santa Cruz) and FITC-conjugated anti-mouse antibodies. For Western blotting to examine concentration and time-dependent effects on macrophages signaling molecules, cells with or without FD treatment were lysed, subjected to SDS-PAGE, and probed with the indicated mouse anti-human antibodies as described previously [35, 198]. As a negative control a non-specific mouse antibody was used in all experiments.

III.3.v Analysis of NF-κB Pathway and Related Molecules by qPCR

U937 cells were treated with PMA and ox-LDL as above followed by various concentrations of FD for 24 hours before the ox-LDL was added. Total cellular RNA was extracted from FD treated or untreated cells as per manufacturer’s protocol using the RNeasy® mini kit (Qiagen, Valencia CA). Gene expression was determined using Applied Biosystems Human NF-κB TaqMan® Array Gene Signature Plates and TaqMan® RNA-to-CT™ 1-Step Kit (Life Technologies, Carlsbad CA) using an Applied Biosystems 7500 Fast Real-Time PCR System. In addition, custom primers for ICAM-1, CCL2, NF-κB1, TRAF2, IKK-β and IL-8 where designed and purchased from Integrated DNA Technologies [187, 200]. Experiments with the custom primer sets were performed using the AMV First Strand Synthesis Kit (New England Biolabs, Ipswich, MA) to
produce cDNA followed by the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) for the qPCR. Fold change in expression was obtained by the ΔΔCt method which gives a comparative analysis of gene expression between two samples. Briefly, to obtain the up or down regulation of those genes affected by FD control cell RNA (no FD treatment) were probed with housekeeping and target-specific primers to establish a baseline expression. The RNA expression from cells treated with FD was then normalized to the controls in the non-treated cells. Data is presented as the fold change of expression between these two conditions.

III.3.vi Human MC Preparation and Mediator Release

MC from human skin and lung [137, 198] were incubated with or without FD for 16 hours at 37°C (optimal for cellular uptake) [35, 139]. The next day, cells were stimulated with 500μg/mL C5a for 30 minutes (degranulation) or overnight (cytokine production). TNF-α levels were measured as described above and degranulation was measured as previously described [35].

III.3.vii In vivo Assessment of Plaque Formation

Homozygous male B6.129P2 Apolipoprotein E knockout (ApoE -/-) and wild-type C57BL/6J mice (n=6/group) were treated by i.p. with ALM or C₃ (i.p. 5mg/kg/100μL per injection) or fed ad libitum (2.5μg/ml) in drinking water. There was no observed difference in the amount of water intake by the mice given control water (no FD) and mice given FD. Given an average water intake of 5.8mL/day, each mouse
consumed approximately 14.5μg (0.2μg/gm body weight) of FD each day. At day eight (after four injections) mice were fed a Teklad custom atherogenic rodent high fat diet (HFD; 02028) for up to nine weeks with FD or PBS injected every other day. Mice were maintained in a temperature and humidity-controlled room with a 12 hour light/dark cycle and were given free access to food and water. At week 10 mice were sacrificed and tissue sample prepared as described [145] and serum cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and lactate dehydrogenase (LDH) levels evaluated using kits from Cayman Chemical. All studies were approved by the UNCG Animal Welfare Committee.

III.4 Results

III.4.i Fullerene Derivatives

The size distribution profile of ALM and C₃ was 0.251μm and 0.117μm, respectively while the zeta potentials were -59.02mV and -95.50mV, suggesting stability of FD in solution. The size distribution and zeta potential did not significantly change after vigorous vortexing or sonication. Nanosight nanoparticle tracking of ALM and C₃ revealed a mean particle size of 0.142 μm for ALM and 0.082 μm for C₃. The smaller mean size from the Nanosight is a result of the individualized nature of particle size measurement. No cellular (U937 and THP-1) toxicity was observed with either FD when using up to 100μg/mL (data not shown).
III.4.ii Fullerene Derivatives Prevent Morphological Changes in PMA/ox-LDL-Challenged Cells

Treating monocytes with PMA and ox-LDL induces their transformation into lipid-laden foam cells[201]. SEM was used to examine the morphological changes associated with foam cell formation under these conditions with or without FD pre-incubation. As seen in Figure 3.1.A.B, untreated U937 cells have a relatively smooth plasma membrane with minor areas of surface irregularity and are spherical in shape. The addition of PMA/ox-LDL induces a drastic phenotypic change characterized by an amorphous shape, cellular elongation, and ruffling of the plasma membrane (Figure 3.1.C.D) as described previously [197]. However, the addition of ALM prevented this phenotypic change in the U937 cells (Figure 3.1.E.F). Similar results were observed with C3 (data not shown).
Figure 3.1. FD Prevent Morphological Changes Associated with Foam Cell Formation. U937 cells not treated with ALM (A,B) show the characteristic smooth plasma membrane and spherical shape of a monocyte. The PMA/ox-LDL-challenged U937 cells transformed to foam cells exhibit amorphous shape, elongation, and roughing of the plasma membrane (C,D). However, when the PMA/ox-LDL-challenged U937 cells were treated with ALM (5μg/mL), the phenotypic changes were prevented (E,F).
III.4.iii Fullerene Derivative Pre-Incubation Prevents Cytokine Release, Foam Cell Formation, and Cell Adhesion

Previous studies have shown that TNF-α release from activated monocytes induces foam cell formation [46]. Given that FD have been shown to prevent TNF-α release in other cell types [35, 189, 202], we hypothesized that FD would also inhibit the production of TNF-α from monocytes/macrophages. To test this hypothesis, we examined the effects of ALM on TNF-α release from PMA activated U937 monocytes. As shown in Figure 3.2.A, cells were treated for 24 hours with PMA and ox-LDL, and those untreated with FD showed a significantly higher amount of TNF-α than FD treated U937 cells. However, when cells were pretreated with 5.0μg/mL of FD for 24 hours there was statistically significant inhibition of TNF-α release, ranging from 58% to 77%. Similar statistically significant inhibition of TNF-α release was seen with ALM and C3 when levels were monitored at 12 hours (data not shown). Thus, FD may inhibit foam cell formation through inhibition of TNF-α release.

TNF-α activated monocytes initiate cellular clumping and lipid uptake as part of the foam cell formation process [203]. PMA-treated cells showed significant clumping and ORO staining (Figure 3.2.B; middle) consistent with previous studies [203]. However, the ALM-treated cells showed no significant clumping or ORO staining (Figure 3.2.B; right) compared to non-treated U937 cells (Figure 3.2.B; left). In order to quantify the observations in Figure 3.1 and Figure 3.2.B, the uptake of lipids was assessed. Quantification of the ORO staining demonstrated significant inhibition of lipid uptake in FD treated vs. untreated cells (Figure 3.2.C).
The clumping induced by activated monocytes in atherogenesis is mediated through the up-regulation of ICAM-1 [203]. We hypothesized that FD inhibited the clumping of activated monocytes through ICAM-1 inhibition. To test this hypothesis, we treated cells with PMA (100ng/mL), ox-LDL (10μg/mL), and varying concentrations of FD and examined for the up-regulation of ICAM-1 using flow cytometry. As seen in Figure 3.2.D, the same FD treatment conditions that prevented cell clumping also prevented the up-regulation of ICAM-1, in a dose-dependent manner. Although a trend can be observed, the data was not statistically significant. Thus, FD prevents activation-induced monocytic cell-cell adhesion possibly through the inhibition of ICAM-1.

Up-regulation of CD36 scavenger receptors on foam cells is another key event in ox-LDL uptake and foam cell formation during atherosclerosis. We investigated the effect of ALM and C3 on CD36 receptor expression. As seen in Figure 3.2.D, CD36 receptor expression was down-regulated with varying concentrations of both FD. Taken together, FD inhibit several foam cell specific functional and phenotypic characteristics.
Figure 3.2. (A) TNF-α Release From U937 Cells is Inhibited by FD. U937 cells were incubated with PMA, 5μg/mL FD [ALM (dark grey bars), C₃ (light grey bars), or no FD (black bars)], and ox-LDL as described in the Materials and Methods. Cells were centrifuged and supernatants assayed for TNF-α via ELISA. Significance (P<0.05) indicated by an * was determined using the Student’s t test. Results are the average of three separate experiments, each done in triplicate. (B) and (C) Fullerene derivatives inhibit cell adhesion and lipid uptake in U937 cells. U973 cells (in duplicate) were treated with PMA, ox-LDL, followed by various concentrations of FD. Treated cells were assayed via FACS using antibodies against ICAM-1 (a) and CD36 (b). The graph shows the average mean fluorescent intensity (MFI) of the
signal (±SD) from three separate experiments. As a control MOPC was substituted for the primary antibodies and demonstrated no staining (not shown). All values with an * are significantly different compared to non-FD cells (P<0.05).

III.4.iv Fullerene Derivatives Effects on Atherosclerosis-Associated RNA Transcripts, Protein Expression, and NF-κB-Signaling Intermediates in Foam Cells

Given the observation that TNF-α release was significantly inhibited by FD, we focused our efforts on the NF-κB pathway using qPCR. As seen in Figure 3.3.A, ALM caused a significant reduction in several atherosclerosis-related intermediates including CCL2 (91% reduction), IL-8 (90% reduction), VCAM (89% reduction), IL-1 (73% reduction), and TNFS15 (75% reduction). To further validate how FD affected these gene products, we performed more focused studies on several genes including TRAF2, ICAM1, IKBKB, CCL2, IL8, and NF-κB. As seen in Figure 3.3.B, those genes that were observed to be downregulated by FD were again observed to be downregulated using separate primers thus validating those observations in Figure 3.3.A.

NF-κB is involved in TNF-α induced foam cell formation [204], which involves the signal transducer TRAF2 [205] and IκB kinase (IKK) [206]. Since both C₃ and ALM appear to similarly inhibit foam cell formation, we chose ALM for the signaling studies. We hypothesized that ALM was inhibiting foam cell formation through the inhibition of the NF-κB pathway through RIP, IKK, TRAF, or Nf-κB1 intermediates. As seen in Figure 3.3.C.D, ALM/PMA/ox-LDL-treated U937 cells that were pretreated with ALM had a significant reduction of NF-κB1 and TRAF2 expression at the protein level. This effect was dose (3.3.C) and time (3.3.D) dependent. The ALM-mediated inhibition of
NF-κB was maximal at two hours after ox-LDL challenge and total levels remained significantly lower at 16 hours compared to non-ALM treated cells. Similar results were observed with TRAF2, however little expression or change was observed with other signaling intermediates involved in the NF-κB signaling pathway. Antibodies to detect phospho-IKK-α/β and IKK-α revealed a slight reduction in expression after two hours of ox-LDL treatment in ALM treated cells. Thus, ALM apparently inhibits the ox-LDL-induced increases in foam cell formation by affecting NF-κB1, TRAF2, and IKK proteins.
Figure 3.3. Fullerene Derivatives Effects on Atherosclerosis-Related mRNA Transcripts in Macrophages (A, B). U937 cells (in duplicate) were treated as above with PMA and ox-LDL with or without ALM (5μg/mL). RNA was extracted via the RNeasy mini kit (Qiagen, Valencia CA) and qPCR was preformed using the Applied Biosystems Human NF-κB TaqMan® Array Gene Signature Plates per manufacturer’s instructions. Values above the bars represent the percent downregulation of each gene as compared to non-treated cells and FD treated cells. Fullerene derivatives differentially affect NF-κB associated signaling intermediate...
proteins in a dose (C) and time (D) dependent manner. In (C) U937 cells treated with PMA followed by treatment with or without varying concentrations of FD and ox-LDL for 2 hours. In (D) U937 cells treated with or without PMA followed by FD treatment and ox-LDL for 2 hours or 16 hours. Cells were lysed, subjected to SDS-PAGE, and Western blotted using the indicated antibodies as described previously [192].

III.4.v Stabilization of Human Connective Tissue MC by FD

The complement system is activated in atherosclerosis [207, 208] and patients with the disease have elevated serum levels of complement activation products (e.g. C5a) [209]. MC are activated by complement byproducts such as C5a [210], which may be one initiating or amplifying mechanism of atherosclerotic disease. As seen in Figure 3.4, human connective tissue MC pre-treated with ALM showed significant inhibition of C5a-induced degranulation (3.4.A) and cytokine production (3.4.B).
Figure 3.4. Fullerenes Reduce C5a-Induced Noxious Mediator Release From Connective Tissue MC. MC were cultured with ALM or C3 at various concentrations, then washed and stimulated with C5a (500 µg/ml) for 30 min for degranulation (A) or 16 hours for TNF-α release (B). Cells were centrifuged and β-hexosaminidase release or TNF-α production was determined via ELISA. Data shown are means ± SE of triplicate samples that is representative of three separate experiments with separate MC cultures. All values with an * are significantly different compared to non-FD cells (P<0.05).

III.4.vi Effect of FD Derivatives on Atherogenesis in vivo

Given that FD inhibit several underlying mechanisms leading to atherosclerosis in vitro, we hypothesized that they could prevent atherosclerosis in vivo. As seen in Figure 3.5.A, 80% of ApoE -/- mice fed a high HFD for ten weeks and injected with PBS did not survive. In contrast, the FD-treated mice appeared healthy, had no obvious abnormalities,
and had weights similar to the age-matched WT mice (Figure 3.5.A). Upon further examination of the aortic arches, both ALM and C₃ significantly reduced the size of the vessel-clogging plaque lesions (Figure 3.5.B). The non-treated, ApoE -/- mice had extremely large, vessel-blocking lesions in the aortic arch (Figure 3.5.C,D-arrows) that were virtually absent when mice were treated with FD (Figure 3.5.E-H) over the 9 weeks. Thus, FD prevent the plaque build-up associated with atherosclerosis in mice fed a HFD.

Figure 3.5. Fullerenes Derivatives Prevent Atherosclerosis in vivo. Mice were treated orally with 2.5µg/mL FD and i.p. with 5mg/kg/100µL. After 10 weeks, the percent of ApoE -/- or WT mice living was assessed (A). The aortas from ApoE -/- treated with PBS and fed a HFD, ApoE -/- treated with ALM or C₃ plus HFD, were stained with hematoxylin and lesion sizes assessed as mean ±SD (B). Representative images of PBS treated (C, D), ALM oral (E, F), or C₃ oral (G, H) are shown in the panel image. Note the large fatty streak lesions (arrows) in the PBS treated mice
that are absent in the ALM or C3 treated mice. Pictures were taken using a 4X magnification. Pictures are representative of each of the six mice and were examined for each condition in a blinded fashion. All values with an * are significantly different compared to the PBS vehicle control group (P<0.05).

III.4.vii Fullerene Derivatives Prevent Liver Toxicity Associated with HFD and do not Affect Kidney Function

Several toxicological markers in ALM and C3 treated mice were examined following administration via i.p. or oral administration. There were no morphological changes in the livers of ALM or C3-treated mice compared to ApoE -/- mice on HFD (data not shown). Serum cholesterol levels were significantly elevated in the non-FD treated animals fed a HFD compared to controls (p<0.05). ALM and C3 did not affect total cholesterol, LDH, or AST levels (Figure 3.6.A.B.E), but did reduce the HFD-induced elevations in creatinine and ALT levels (Figure 3.6.C.D). Thus, FD do not induce toxicity in animals challenged for up to 10 weeks.
Figure 3.6. Fullerenes Derivatives Effect on *in vivo* Toxicity. Mice (n=6/group) were treated as described in Materials and Methods. After ten weeks, blood was collected from hearts of ApoE -/- mice fed a HFD and ApoE -/- treated via i.p. or oral (p.o.) administrations of ALM or C3 plus HFD. The total serum Cholesterol (A), Lactate dehydrogenase (B), Creatinine (C), ALT (D), and AST (E) levels were measured in triplicate.

### III.5 Discussion

The aim of our study was to determine if FD could prevent the development of atherosclerosis based on the observation that certain FD could attenuate other chronic inflammatory diseases [133, 146, 211]. After initial testing of several FD, two were chosen for further studies based on their ability to inhibit foam cell formation *in vitro*. Monocyte U937 cells treated with FD had a significant decrease in total ORO staining under conditions that transform them into foam cells compared to untreated cells. Second, SEM analysis clearly demonstrated drastic morphological changes that occur in PMA/ox-LDL challenged U937 cells are prevented with FD pre-incubation. Third, the clumping
process, which occurs upon the monocyte-to-foam cell transformation [212], is prevented, possibly through the down-regulation of ICAM-1. Lastly, inflammatory mediator release from macrophages (TNF-α and MC-derived) leading to atherosclerosis was inhibited by FD.

Macrophages and MC release a wide variety of inflammatory mediators involved in the initiation and progression of atherosclerosis including TNF-α which can upregulate ICAM-1 [47]. Reducing serum levels of TNF-α improves patient outcomes [213]. Our findings show that FD inhibit the production of TNF-α up to 72% compared to non-treated cells and reduce cellular adhesion in U937 cells through reduction of CD11 expression.

The CD36 receptor mediates the ox-LDL induction of atherogenic foam cells making it a therapeutic target for atherosclerosis [214]. For example, cholesterol lowering statins significantly reduce CD36 receptor expression [215]. Our results are similar as it is shown FD can inhibit CD36 expression and thus interfere with a critical step involved in foam cells formation.

Several studies have demonstrated that the induction of foam cell formation in atherosclerosis is mediated in part through oxidative stress [216], For example, CD36 expression in monocytes is increased by oxidative stress [47, 217]. Previous reports have proposed that membrane expression of CD36 involves redox signaling pathway via NADPH oxidase activation and the administration of antioxidants leads to a reduction in CD36 expression in monocytes derived from humans [217]. Given that certain FD may
possess anti-oxidant properties, these molecules may interfere in the progression of atherosclerotic disease at several levels.

Furthermore, it has been demonstrated that oxidative stress influences the expression of NF-κB [218]. NF-κB is a key regulator of cell survival and proliferation and previous studies have shown NF-κB inhibition reduces foam cell formation [219]. TRAF2 is part of a group of adaptor proteins involved in signal transduction by most members of the TNF receptor family. TRAF2 has a key role in mediating TNFR1 induced activation of NF-κB [220]. As seen in Figure 3.3, ALM was capable of dramatic reductions in TRAF2 and NF-κB expression in cells activated with ox-LDL. The activation of NF-κB via TRAF2 has been shown to increase foam cell formation. [221].

Relatedly, we show for the first time that several atherosclerosis-related genes are up-regulated in foam cells are dramatically inhibited in ALM-treated cells. For example, CCL2 is a chemokine found in the serum of hypercholesterolemic patients as well as atherosclerotic lesions [222]. We found ox-LDL/PMA caused a 40-fold induction in its cellular expression, which dramatically decreased in FD-treated cells. Similarly, ICAM-1 expression was dramatically reduced at both the gene (Figure 3.3) and protein levels (Figure 3.2) in foam cells.

MC have traditionally been associated with the initiation and proliferation of allergic responses, but a role for MC in atherosclerosis has now been clearly demonstrated [99]. MC numbers are greatly increased in the intima at sites of arterial plaque rupture [97], in advanced plaque lesions in the carotid artery [223], and in
vulnerable plaque [224]. Clinically, patients who died of acute myocardial infarction have an increased number of degranulated MC at the site of plaque erosion or rupture [98]. Animal studies demonstrate that MC-deficient mice develop less atherosclerosis when fed a HFD compared to WT mice [225]. The complement system is extensively activated in human atherosclerosis and lesions [207, 208]. There also appears to be a positive correlation between serum levels of complement activation products (i.e. C5a) and human patients with atherosclerosis [209]. Similar to other studies examining FD, inhibition of MC mediator release through IgE and non-IgE pathways, [35, 146, 226] we show for the first time that human MC activation through C5a is also inhibited by FD. Thus, FD may not only affect foam cell formation but may also prevent atherosclerosis (via C5a stimulation) through the stabilization of MC and inhibition of the release of their noxious mediators.

ALM and C3 demonstrated an almost complete inhibition of atherosclerotic plaque lesions in the aortic arches of ApoE -/- mice fed a HFD. The ALM and C3 treated groups showed significant inhibition of plaque lesions when compared to the PBS vehicle group. This inhibition of plaque lesions by the FD most likely explains the striking difference in survival rates between the two groups; 80% of the non-treated mice did not survive at the 10 week HFD regimen. None of the ALM-treated mice died in these studies. In both treated groups body weights did not change when compared to controls. Thus, rationally designed FD may represent a novel way for the prevention of complications associated with the accumulation and rupture of atherosclerotic plaque.
The studies examining the toxicity of FD are still the subject of debate [227-229]. Our studies using highly purified and well characterized FD support studies demonstrating a lack of toxicity [188, 230, 231]. Here, it is demonstrated that the two anti-atherogenic FD are not toxic to the liver and kidney under the conditions tested.

In conclusion, we have shown that certain FD inhibit the formation of U937-derived foam cells possibly through the reduction of inflammatory cytokine release and adhesion molecule membrane expression through a NF-κB dependent mechanism. These compounds also significantly inhibit C5a-induced MC activation. The same FD also attenuated the accumulation of plaque lesions in vivo. These results further extend the utilization of FD and suggest they may be used as a platform for developing new therapeutics for the treatment of atherosclerosis.
CHAPTER IV

THE IMPEDIMENT OF ALLERGIC REACTIVITY WITH HUMANIZED LOW AFFINITY ANTI-IGE ANTIBODIES

IV.1 Chapter Summary

Options for effective prevention and treatment of epidemic allergic diseases remain limited and particularly so for IgE mediated food allergies. It has previously been found that mouse low-affinity anti-human IgE antibodies with dissociation constant in $10^{-6}$M to $10^{-8}$ M range were capable of profoundly blocking allergic reactivity[232]. In collaboration with Zhang K, Zhang H, Saxon A. et al. (under review), humanized mouse anti-human IgE mAbs, Low affinity Allergic Response Inhibitor (LARI) were characterized for their biological and immunological features. The mechanisms of action responsible for their therapeutic effects were explored. Zhang K et al., demonstrated LARI profoundly blocked human peanut- and cat-allergic IgE-mediated basophil activation and inhibited the acute release of both pre-stored and newly synthesized mediator from human MC and MC line (under review). We report at both high and low concentrations, LARI failed to trigger the acute release of the pre-stored mediator histamine and β-hexosaminidase, did not induce secretion of newly synthesized lipid mediator leukotriene C₄ and prostaglandin D₂. We also demonstrated that LARI did not promote cytokine/chemokine production. Mechanistic studies by Zhang et al. revealed
that LARI functioned as a partial antagonist for FcεRI signaling to reduce the FcεRI signaling component phosphorylation of syk, ERK, p38 MAPK and AKT; promoted CD203c expression level on basophils, and induced a piecemeal-like degranulation phenotype in skin MC (under review). Here, we confirmed their findings by assessing the interaction LARI has on FcεRI/IgE on primary human skin and lung MC. These studies demonstrate that targeting surface-bound IgE with LARI can profoundly suppress human allergic reactivity.

IV.2 Introduction

Given their increased prevalence and incidence, IgE-mediated allergic disorders have become a major worldwide public health concern[233-235]. This epidemic of IgE-mediated allergic diseases has led to a corresponding interest in developing new therapeutic approaches, including novel immune based biologic therapies[232, 236-245]. In an effort to develop a more effective therapy that would inhibit all IgE mediated reactions and in particular, severe food allergic reactions, Zhang et al. has proposed the use of specifically designed low affinity anti-IgE antibody to directly target IgE bound to its high affinity receptor on allergic effector cells[232].

Previously, the direct targeting of surface-bound IgE with bivalent anti-IgE antibodies as an allergy therapeutic was thought not to be feasible due to the common assumption that anti-IgE Abs would trigger immediate allergic reaction by crosslinking the surface IgE/FcεRI. However, it was recently reported that murine prototype low affinity anti-IgE mAbs, with dissociation constant in the $10^{-6}$ to $10^{-8}$ M range, that bound
surface IgE/FcεRI, did not trigger the anaphylactic reactivity but instead, the weak binding of the surface IgE actively inhibited the reactivity of human allergic effector cells[232].

In order to understand the mechanism of targeting human surface-bound IgE with low affinity anti-IgE mAbs as a novel allergy therapeutic, a set of humanized low affinity anti-human IgE mAbs designated as Low affinity Allergic Response Inhibitor(s) (LARI) was developed and gifted from Ke Zhang and Andrew Saxon (UCLA) for these studies (E59, E23, E14, E17, S91; mouse founder clones; P6.2, F11 and mE17 were humanized to human IgG1 (γ1, κ/λ)). Zhang et al. characterized their biological and immunological features, determined their therapeutic effect, and further examined their effects related to their therapeutic mechanism of action (under review).

Two major subtypes of MC have been identified, tryptase-positive (MC_T) cells which are found mainly in the gut/lung mucosa and tryptase/chymase (MC_TC) cells, located in the skin[33]. Our collaborators used CD34+ hematopoietic stem cell derived MC (CMC)[162], which are MC_T[246]. In order to verify their findings, we used an alternate source of MC_TC (from skin) and our own source of MC_T (lung MC). Our contribution to the findings was assessing the effects LARI had on primary human skin and lung MC. Overall, we demonstrated that the defined LARI has a profound inhibiting effect on allergic reactivity and could be a candidate for future clinical development.
IV.3 Materials and Methods

IV.3.i Histamine, Leukotriene C₄ (LTC₄), Prostaglandin D₂ (PGD₂) and β-

Hexosaminidase Release Assay

β-hexosaminidase measurement in triplicate in cell supernatants and cell lysate was used as the indicator of MC degranulation. To determine the triggering capacity of LARI, we used approximately $4 \times 10^4$ to $5 \times 10^4$ IgE sensitized lung and cultured skin tissue MC[247, 248] and Zhang et al. used CMC[162], washed them, pre-incubated in them a 37°C water bath for 5 min, following by stimulation of LARI, PAE or an anti-FceRIα mAb as the positive control and IgG isotype as the negative control, for 30 minutes in stimulation buffer (10mM HEPES, 137mM NaCl, 2.7mM KCl, 0.38mM Na₂HPO₄.7H₂O, 5.6mM glucose, 1.8mM CaCl₂.H₂O, 1.3mM MgSO₄.7H₂O, 0.4% BSA, pH 7.4) (under review). The released β-hexosaminidase was quantified by hydrolysis of 45µl p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich) solution in 0.1M sodium citrate buffer (pH 4.5) for 90 min at 37 °C. After the reaction was stopped with the addition of 150µl of 0.2 M Glycine (pH=10.7), the reaction plates were read at 405nM[249]. The reaction supernatants were assayed for β-hexosaminidase, prepared by lysing the cells by freeze-thawing 3 times and used to calculate the percentage of the β-hexosaminidase released with media alone as the background.

To determine the inhibitory effects of LARI on MC degranulation, Zhang et al. cultured various concentrations of LARI or a corresponding isotype control mAb with CMC or LAD2 MC already sensitized with myeloma IgE, biotinylated myeloma IgE or recombinant dansyl-specific IgE, for 48 hours in Stempro-34 SFM medium supplemented
with SCF and IL-6 (under review). To confirm their results, we used the same experimental conditions except with skin MC were pre-sensitized with NP-IgE in X-VIVO 15 supplemented with SCF. All the MC where were challenged with appropriate cross-linkers, e.g. NP-BSA at 10µg/ml (for skin MC), PAE at 1µg/ml, dansyl-BSA at 22µg/ml, streptavidin at 0.1µg/ml and mastoparan (Anaspec Inc., Fremont CA) at 7µM, in the reaction simulation buffer for 30 minutes (skin MC and CMC), and 1 and 4 hours for LAD2[250]. We assayed the skin MC reaction supernatants β-hexosaminidase, while Zhang et al. assayed the CMC and LAD2 cells for LTC₄ and PGD₂. Lysates, were prepared by lysing the cells in pure water and frozen-thawed 3 times, and were used as the measure of the total content of β-hexosaminidase to calculate the percentage released (under review).

IV.4 Results
IV.4.i LARI Suppresses Allergic Basophil Activation

Using a Basophil Activation Test (BAT) as previously described[232], Zhang et al. demonstrated ex vivo allergic basophil activation from both peanut and cat allergic donors was profoundly suppressed by all five LARI tested, ranking them as E59=E23>E14>E17>S91 in terms of their efficacy for BAT inhibition (under review).

Zhang et al. also tested whether LARI’s effect would be altered by high levels of free IgE, as was the case for Omalizumab (Xolair®), a FDA approved allergy therapeutic which is intended to work by neutralizing and depleting free serum IgE (under review). They report high serum IgE levels as commonly seen in patients are not predicted to be a
significant limiting factor for the therapeutic efficacy of LARI (Zhang et al.; under review). Basophils and MC share many functionally similar characteristics. They both contain secretory granules and express FcεRI[251, 252], resulting in a high affinity to IgE. In order to comprehensively examine LARIs effect on allergic reaction inhibition, MC were examined in parallel with basophils.

**IV.4.ii LARI Suppresses the Immediate Release of the Pre-Stored Allergic Mediator from Human MC**

The ability of LARI to inhibit the burst release of the pre-stored allergic mediator was tested by Zhang et al. using CMC and LAD2, and our group, using cultured skin MC (under review). The MC were first sensitized with myeloma IgE, biotinylated IgE, or NP-IgE (skin MC) then treated with LARI or various controls for 48 hours, followed by challenging with the FcεRI pathway cross-linker PAE or streptavidin, or the MRGPRX2 pathway agonist mastoparan[253] to determine the effect of LARI on inhibiting degranulation.

As reported by Zhang et al. treatment for 48 hours with LARI E14, E17, E23 or E59, culture medium or omalizumab, significantly blunted the CMC and LAD2 β-hexosaminidase release of the FcεRI pathway when the cells were then challenged with PAE, streptavidin (Figure 4.1.A,B), but not the non-IgE mediated pathway MRGPRX2 using mastoparan (Figure 4.1.A,B) (under review). To further of verify their findings, we tested LARIs ability to suppress antigen-specific IgE mediated degranulation using cultured human skin MC sensitized with NP-IgE followed by a 48-hour treatment with
LARI. As shown in Figure 4.1.C, LARI significantly inhibit the NP-BSA and although without significance, also inhibited NP-LPS activated β-hexosaminidase release but only at higher concentration (10µg/mL) than in other systems.

IV.4.iii LARI Causes Loss of Pre-Stored Allergic Mediator from Human MC

It was reported by Zhang et al. that LARI induced a phenotypic picture of piecemeal degranulation in skin MC in FcεRIα Tg mice (under review). It was hypothesized that LARI induced the gradual release of the pre-stored mediators. To test this hypothesis, our collaborators used IgE and biotinylated IgE sensitized CMC/LAD2 cells and incubated them with LARI, along with the corresponding controls for 48 hours, followed by assaying the supernatant histamine level. Zhang et al. report that 48-hour unstimulated cultured CMC/LAD2, LARI, but omalizumab, slightly but reproducibly increased the supernatant histamine level to about 10% of that seen with full activation by PAE (Figure 4.1.D.F.) (under review). After examining total β-hexosaminidase contents of the lysates, it was shown that they indeed show a decrease β-hexosaminidase levels (Figure 4.1.E). Taken together, these results revealed that LARI promotes slow release of mediators while rendering the cells less responsive to strong FcεRI crosslinking but not to MRGPRX2 pathway activation.
Figure 4.1 Effects of LARI on Pre-Store Mediator Release from Human MC. (A) β-hexosaminidase release from 48-hour cultured CMC challenged with stimulation buffer, PAE, streptavidin and mastoparan in the presence and absence of LARI. (B) β-hexosaminidase release from 48-hour cultured LAD2 challenged by stimulation buffer, PAE and streptavidin in the presence and absence of LARI. (C) β-hexosaminidase release from 48-hour cultured skin MC challenged with PAE in the presence and absence of LARI. (D) Histamine level in 48-hour cultured CMC supernatants. (E) The total β-hexosaminidase level in 48-hour cultured CMC.
lysate in the presence and absence of LARI.  (F) The total β-hexosaminidase level in 48-hour cultured LAD2 cell lysate in the presence and absence of LARI.  * P<0.05;  ** P<0.01, Student’s t test.

IV.4.iv LARI Blunts Lipid Allergic Mediator Release from CMC

The effects of LARI on rapidly induced lipid allergic mediator synthesis were assessed by Zhang et al. using CMC in conjunction with the histamine and β-hexosaminidase release experiments presented in Figure 4.1 (under review). They report in 48-hour culture supernatants, PAE, as the positive control, induced robust LTC₄ and PGD₂ secretion, while LARI, but notomalizumab, only slightly enhanced the LTC₄ and PGD₂ compared to spontaneous production, indicating LARI treatment promoted low level spontaneous release of the newly synthesized lipid mediators. Upon challenging CMC with PAE or an anti-IgE post-LARI treatment, LTC₄, and PGD₂ were significantly blunted, but had no effect on mastoparan stimulation (Zhang et al; under review).

IV.4.v LARI Itself Fails to Trigger Burst Release of Pre-Stored or Newly Synthesized Allergic Mediators ex vivo

The inability of LARI to activate MC and basophils, trigger allergic mediator release, and result in acute allergic reactivity is paramount to the safety of this approach. Thus, this possibility was evaluated using multiple systems including basophils, CMC, lung and skin MC and LAD2 cells with high concentrations of LARI (Figure 4.2). In the BAT assay conducted by Zhang et al., the positive control high affinity mAbs E4.15 and E7.12[254] at 1µg/ml, and fMLP at 50 nM all induced strong basophil CD63 expression,
indicative of cell activation with release of pre-stored allergic mediators (under review). In contrast, LARI E14, E17, E23, E59 and S91 at concentration up to 50µg/ml, failed to increase basophil CD63 expression in any blood donors tested (n=32, including 24 non-allergic and 8 allergic donor, Figure 4.2.A). No activation was observed with the negative non-IgE binding control LARI E4. Similarly, all five selected LARI at concentrations up to 50µg/ml failed to induce basophil histamine release (n=6, cells from 3 non-allergic and 3 allergic donors, Figure 4.2.B). With MC degranulation, conducted by our group, LARI at 20-100µg/ml did not trigger β-hexosaminidase release from (a) the freshly isolated human lung MC (n=3, Figure 4.2.C), (b) cultured human skin MC (n=2, Figure 4.2.D), or (c) CD34+ hematopoietic stem cell derived CMC (Figure 4.2.E). For LARI, only E23 induced any but minimally increased β-hexosaminidase release in LAD2 cells (Figure 4.2.F), a notable exception given that E23 has the highest LARI affinity for IgE (Zhang et al; under review). As the positive control for the assays, PAE (1µg/ml) triggered robust β-hexosaminidase release.

To test the acute effects of LARI on rapidly synthesized lipid mediator release, our collaborators sensitized CMC with IgE and challenged them with LARI for 1 hour, followed by measurement of LTC₄ and PGD₂ in the supernatant. LARI at 5µg/ml and 50µg/ml did not promote higher background levels of LTC₄ and PGD₂ secretion (Figure 4.2.G.H), while PAE, the positive control, at 1µg/ml, drove robust LTC₄ and PGD₂ (Figure 4.2.G.H) secretion.
Figure 4.2. Safety Profiles of LARI ex vivo. (A) Effects of LARI on basophil activation, using CD63 expression as the activation marker. The high affinity anti-IgE mAb E4.15 and non-receptor stimulating agent fMLP were used as the positive BAT controls (n=32). (B) Effects of LARI on basophil histamine release, with PAE as a positive control (n=6). (C) Effects of LARI on the lung MC degranulation. β-hexosaminidase release was served as the degranulation marker of MC, with a PAE as a positive control (n=3). (D) Effects of LARI on skin MC degranulation (n=2). (E) Effects of LARI on CMC degranulation, with PAE as a positive control. The
data is the representative of three similar experiments. (F) Effects of LARI on LAD2 degranulation, with E7.12 and PAE as the positive controls. The data is the representative of four similar experiments. (G) Effects of LARI on LTC4 production from CMC, with PAE as the positive control. The data is the representative of two similar experiments. (H) Effects of LARI on PGD2 production from CMC, with PAE as the positive control. The data is the representative of two similar experiments.

IV.4.vi Effects of LARI on Slowly Synthesized Cytokines and Chemokines

Some cytokines and chemokines have been reported to be preferentially induced by low affinity engagement of FcεRI receptor with chemically conjugated multivalent ligands using mouse MC[255]. Thus, Zhang et al. examined the potential effects of LARI on slowly synthesized cytokine/chemokine release from human CMC using a multiplex luminex assay to simultaneously measure 38 cytokines/chemokines (under review). CMC were sensitized with 10µg/ml of recombinant dansyl-specific IgE, followed by incubating with various LARI, or the high affinity stimulation controls of PAE or dansyl-BSA for 1, 4 and 48 hours. Their results indicate low affinity engagement of FcεRI by some LARI would weakly induce selected types of cytokines/chemokines in human CMC, implying that LARI can preferentially activate pathway(s) distinct from those of the high affinity crosslinking, resulting in differential outcomes for cytokine/chemokine production.

IV.5 Discussion

Direct targeting the IgE bound to FcεRI on human basophils and MC, the primary effector cells of the immediate allergic response, with an anti-IgE mAb as an allergy therapeutic has been deemed unlikely due to the consensus expectation of triggering
anaphylactic reactions. However, it has been previously shown by Zhang et al. that murine anti-IgE Abs specifically designed with low affinity for human IgE are capable of binding surface-bound IgE without triggering anaphylactic degranulation while simultaneously having potent anti-allergic effects[232]. That seminal discovery suggested the possibility of using a low-affinity IgE targeting approach as a novel allergy therapeutic. To move this concept to a practical allergy therapeutic, shown herein the characterization of the biological and immunological features of a humanized low affinity anti-IgE mAbs, LARI, tested their safety profiles, determined their therapeutic ability, and explored the potential effects that are responsible for LARI mechanism of action. The results confirmed that low affinity humanized mAbs for human IgE can be designed such that they fail to trigger anaphylactic degranulation even at high concentration, and are able to rapidly and profoundly blunt allergic reactivity by human MC and basophils.

Although a sufficiently low affinity for IgE is critical so that the LARI fail to trigger burst release of early or late allergic mediators, this needs to be balanced by an appropriate affinity capable of induced efficient inhibition of allergic effector cell function. Clearly too low an affinity, although displaying greater safety, will destroy the therapeutic value as seen with the p6.2 derived humanized mAbs. Thus, the balance between safety and efficacy needed be carefully evaluated along with some other features such as the predicted in vivo half-life and lack of predicted immunogenicity in order to define the most suitable candidate for further development. These studies determined that LARI with affinity for IgE in the K_D range of 10^{-7}M to 10^{-8}M had the best therapeutic index of safety/efficacy. After extensive safety and efficacy testing ex vivo
and in vivo (Zhang et al; under review) of multiple LARI, LARI E59, with a $K_D$ of $1.2 \times 10^{-7}$M, was chosen as the candidate for further development based on its therapeutic index, long predicted in vivo half-life (Zhang et al; under review), and relatively poor potential for inducing late cytokine/chemokine synthesis.

Two other key characteristics of LARI in general and LARI E59 in particular make this approach appealing. First, is that cross-linking LARI in vivo or ex vivo (Zhang et al; under review), failed to induce allergic reactivity. This is critically important, as some individuals will make antibodies to all humanized mAbs. For LARI, in addition to the usual concern of decreased efficacy from drug neutralization, there was also the specific concern that such anti-drug antibodies and their resulting complexes might provide a full activation signal that would give rise to anaphylaxis. Second, is that the high levels of free IgE did not alter the therapeutic efficacy of LARI. This is not surprising given the low affinity of LARI, e.g. E59 has an affinity about 500 times less or even lower than most mAb therapeutic agents currently in use (Zhang et al; under review). It is expected that LARI binds to and rapidly releases from IgE in a catalytic like fashion in contrast to high affinity antibodies that remain bound to their target antigen for prolonged periods. This lack of sensitivity to the levels of free IgE will permit a simplified dosing regimen in contrast to what is necessary for omalizumab.

The overall mechanism of action of LARI is unique compared with other allergy therapeutic biologics on the market or under development[236-245]. LARI’s weak binding to the FcεRI-bound IgE as a partial agonist results in several downstream effects. Initially, LARI induces weak FcεRI signaling that antagonizes the ability of strong
activation signaling to drive allergic reactivity. Such a mechanism was previously observed with low affinity ligands interacting with IgE on murine MC[256]. This occurs within minutes and acts at the most proximal aspects of the FcεRI signaling. LARI’s ongoing weak activation signaling appears to rapidly act to produce active suppression by curbing amplifying signaling cascades initiated from the high affinity FcεRI crosslinking. This is similar to what has been observed with low dose allergen desensitization[257-261] or low dose high affinity anti-IgE and anti-FcεRI[261]. Additionally, within an hour, LARI selectively activated slow piecemeal-like degranulation, a process that appeared to be ongoing for at least 48 hours. This process gradually depleted pre-stored and newly synthesized allergic mediators from granules and vesicles by mediator leak outpacing newly synthesized mediator production following short-term LARI treatment. The long-term effects of this process remain to be elucidated although reduction of content of allergic mediators in effector cells is expected to lead to blunting of allergic reactivity. Finally, over 1-2 days, low affinity binding of FcεRI bound IgE leads to internalization of the IgE/FcεRI complexes[232]. While the internalized receptors may be denatured or recycled, any future FcεRIs expressed, upon binding IgE, should be immediately engaged by LARI, which will again induce their internalization. As the therapeutic benefit of the loss of FcεRI expression has been validated by studies with omalizumab, internalization related decreased expression of IgE/FcεRI also likely contributed to the therapeutic effects of LARI. Overall, the combined effects for LARI’s mechanism of action discussed above are likely responsible for its rapid and potent therapeutic benefit seen in the multiple experimental systems employed in this study.
Piecemeal degranulation in basophils and MC deserves specific consideration as it is an “orphan” concept. It was developed by Drs. Ann and Harold Dvorak more than forty-five years ago to describe an ultrastructural basophil granule degranulation pattern that completely distinct to that of anaphylactic degranulation[262, 263]. It remains poorly understood in basophils and MC in terms of its role in their physiological and pathological functions, particularly as related to allergic responses. This was due to co-existence of piecemeal and anaphylactic degranulation patterns in basophils and MC in allergic responses, and the lack of reagents/methods that selectively activate piecemeal degranulation. However piecemeal degranulation has been carefully investigated in eosinophils because they lack the FceRIβ chain in their FceRI complex and therefore only piecemeal degranulation upon activation[264]. Using eosinophils as a model, it has been demonstrated that piecemeal degranulation was a general mediator secretory pathway regulating the physiological function of eosinophils[265].

Although speculative, it has been suggested that piecemeal degranulation in MC and basophils represents a transient or intermediate degranulation, e.g. a forme fruste, or incomplete, degranulation[263, 266, 267]. These studies with LARI and basophils/MC provide some intriguing insight into this process. At cellular level, potent FceRI cross-linking occurs with high affinity anti-IgE mAb, PAE, or anti-FceRI mAb that cause typical anaphylactic degranulation, simultaneously induced both CD63 and CD203c expression/up-regulation on basophils[232]. In contrast, the weak/transient interaction of LARI with FceRI-bound IgE only selectively up-regulated CD203c but not CD63[232], indicating that CD203c expression/up-regulation, an alternative basophil activation
marker, was not associated with typical anaphylactic degranulation. Thus, CD203c may serve as a marker of piecemeal degranulation for basophils. Such a hypothesis is supported by Zhang et al. producing electron microscopy examination showing that PAE induced both compound anaphylactic degranulation and piecemeal degranulation, whereas LARI only triggered piecemeal degranulation-like phenotypic changes (under review). The fact that LARI treatment promoted the “leak” of mediators but not burst degranulation of large amounts of pre-stored allergic mediators (histamine and β-hexosaminidase, Figure 4.1) and newly synthesized lipid mediators (LTC₄ and PGD₂) from cultured MC further support the notion that LARI is capable of triggering a pathway leading to release of small packets of mediators. One can envisage that an ongoing mediator “leaking” process triggered by LARI would regulate the cellular content of allergic mediators, and function as a mechanism to regulate the mediator release in a controlled manner as for defined piecemeal degranulation in eosinophils.

Since low affinity engagement of FcεRI pathway in mouse MC by artificially conjugated multivalent ligands was reported to trigger preferential slowly synthesized cytokine and chemokine production without affecting pre-stored mediator release[255], the effect of LARI were surveyed on cytokine/chemokine production using human CMC. These studies revealed that LARI, at relatively high concentrations, displayed the capacity to selectively promote production of certain cytokines and chemokines. Thus, low affinity engagement of FcεRI pathway in human CMC also showed a similar cellular response as seen in mouse MC and distinct from that observed with high affinity crosslinking. LARI E23 showed greater stimulation of cytokines/chemokines compared
to other LARI. This suggests that E23’s relatively higher affinity for IgE ($K_D = 8.09 \times 10^{-8}$ M) (Zhang et al. under review) is a major determinant as other LARI with same complementarity-determining regions bound the same epitope but with lower affinity for IgE (with $K_D$ in $10^{-7}$ M ranges) showed less or no induction of cytokines/chemokines. The relatively weak effects of LARI on inducing cytokines/chemokines, compared with the stronger inducing effects driven by the multivalent ligands in mouse MC, also suggest that the level of low affinity engagement contributes to the strength of the induced cytokine/chemokine response. The degree of cross-linking also is likely a contributor as LARI, which are only bivalent, appear to have weaker ability to drive this late cytokine/chemokine production compared to low affinity multivalent (antigens and/or allergens) ligands. Even so, the potential of eliciting cytokine/chemokine induction needs consideration in LARI development.

Omalizumab derives its therapeutic effect solely by decreasing circulating unbound (free) IgE by greater than 99% with the indirect result of lowering the level of Fc\(\varepsilon\)RI expression on allergic effector cells[268, 269]. Thus, it takes weeks or longer for omalizumab to have its therapeutic benefit, as Fc\(\varepsilon\)RI-bound IgE remains stable for weeks. In contrast, given LARIs direct active effects on signaling, granule content and receptor expression, LARI rapidly blocks allergic responses. Thus, in all the experimental systems used in this study, it was shown that LARI exerted profound basophil suppression within 48 hours, and blocked MC-mediated allergic reactions in 2 to 4 days; systems in which omalizumab as a comparison control, as anticipated, failed to show any beneficial effects.
The observed inhibition of allergic reactivity induced by low affinity interaction of LARI with FcεRI bound IgE appears mimicking the low dose allergen administration. In the classic low-dose allergen desensitization protocol, the small amount of allergen injected is insufficient to cross-link enough specific IgE antibody to reach the degranulation threshold. The resulting subthreshold activation signaling renders the cells transiently desensitized. The partial activation signal - desensitization effect is rapidly lost following the internalization of the allergen engaged IgE/FcεRI complex[270, 271]. As the triggering threshold gradually increases, increased allergen dosing can be given, eventually achieving longer-term desensitization. Compared to the low dose allergen desensitization, LARI rapidly induces allergic desensitization in an allergen non-specific manner, functionally acting as a “low dose pan-allergen” but having a persistent rather than transient effect due to its continuous presence. This rapid allergen non-specific desensitization will not only allow LARI to be a stand-alone therapy, e.g. in IgE food allergy, but when combined with allergen immunotherapy, its use could significantly shorten the time span and improve the safety of attempted allergen tolerance induction protocols.
CHAPTER V
CONCLUSION AND FUTURE PERSPECTIVE

The studies herein demonstrate the heterogeneous and context specific nature of MC/IgE and their role in immune defense, cancer treatment, atherosclerosis, and IgE-mediated allergic disorders. With the introduction of viral vectors into cells, MC like most cells, can be fine-tuned to up-regulate or down-regulate specific surface markers or cytokines[124]. MC novelty of storing and releasing anti-cancer mediators such as GM-CSF, TNF-α, ROS, PGD$_2$, IL-9, and heparin[2, 9] may offer many anti-cancer therapies. Although the role of MC in tumorigenesis is poorly understood and seemingly controversial, proper regulation of MC mediators may provide an insightful resolution. In atherosclerosis, reducing serum levels of TNF-α improves patient outcomes [213] due to its ability to promote ICAM-1 production[47]. Thus, like CAR T-cells, MC are capable of gene manipulation via transfection offering the ability to regulate proteins for clinical benefit in but not limited to, cancer, atherosclerosis, and allergy therapy.

Currently the FDA has approved, Trastuzmab® and Pertzumab® which are monoclonal IgGs commercially available and are currently being used in combination to treat HER2+ breast cancer. Cetuximab®, another FDA approved monoclonal IgG, targets the epidermal growth factor receptor overexpressed in breast cancer and epidermoid carcinoma patients. Although the human IgG class of Ab is generally recommended for cancer therapies, IgE offers many benefits when compared to the IgG class. For example,
the high affinity of IgE to FcεRI allows for a more effective arming of effector cells, while maintaining surface-bound Abs[142, 176, 177]. In addition, the high affinity leads to IgE rapidly binding FcεRI expressing cells, resulting in low serum levels of IgE (.02% of serum immunoglobulins), thus less competition for FcR occupancy [176-178]. Low serum levels of IgE can also be attributed to the short half-life (2-3 days) of free floating IgE (23 days for IgG)[272]. The half-life of IgE is significantly extended by weeks-months when bound to FcεRI[272]. Currently, there are more than 20 FDA approved Abs commercially available to treat cancers[175], all of which are from the human IgG class. The advantages of IgE over IgG led to the first clinical trial using a mouse/human chimeric IgE Ab specific for the tumor-associated antigen folate receptor-α. This ab, MOv18 IgE, has demonstrated superior anti-tumor efficacy compared with IgG1 in animal models[179, 180].

Other than MOv18, there are numerous recombinant cancer-targeting IgEs undergoing investigation. Chapter II demonstrates that C6MH3-B1 is an effective IgE in allegro-oncology. Similar studies using MC as effector cells for allegro-oncology are under investigation: anti-HCD20 targeting human B-cell non-Hodgkin lymphoma[174], anti-hMUCI targeting epithelial antigen MUC1[174], anti-PSA targeting specific antigen[273], and anti-dog albumin treating anaphylactic shock in vivo[274]. The continuous research in immunotherapy will lead to improvements and developments in the field, facilitating allegro-oncology to move from its infancy into the forefront of cancer treatment.
The use of MC in allegro-oncology has been controversial for a plethora of reasons. As previously stated, MC offer unique anti-tumorigenic properties such as containing pre-formed cytotoxic mediators, synthesizing anti-tumor cytokines, and their high affinity to IgE. There are some concerns raised in using MC for cancer treatment as well. Similar to CAR T-cell therapy, there is a concern that activated MC will lead to an unintended immune response resulting in cytokine release syndrome. There are reports that present evidence that MC cytokines promote tumor growth as well as secrete proteases permit the formation of new blood vessels and metastases[275]. By permitting angiogenesis, the MC are promoting tumor growth by providing a route of more nutrients and growth factors. It is understood that MC role in cancer is tumor and tumor stage dependent, thus understanding their role in cancer is extremely challenging. MC either promote tumor growth, inhibit tumor growth, or are just innocent bystanders.

The advantages of IgE are not limited to cancer treatment. The direct target of IgE bound to FceRI on human basophils and MC with an anti-IgE as an allergy therapeutic was deemed unsafe due to unexpected MC/basophil activation triggering anaphylactic reactions. However, studies have shown that targeting surface bound IgE with a low affinity anti-IgE can bind IgE offering anti-allergic effects and while not triggering anaphylactic shock[232]. Similarly, a study targeting the transmembrane form of IgE (mIgE) with a mutant lower-affinity FceRIα expressing CAR T-cells did not induce anaphylaxis while simultaneously re-directing T-cell specificity to cells expressing mIgE[276]. mIgE is present on the surface of all IgE producing cells, re-directing T-cells to mIgE expressing cells causes a decrease in IgE production, ultimately
decreasing clinic risks associated with IgE production. This holds promise for a highly effective cell-based therapy to treat severe allergic diseases.

In conclusion, with the combination of gene therapy and MC unique qualities, MC could become the next CAR T-Cell, or CAR M-Cell, therapy. Given their ubiquity and multitude of cell types they encounter and interact with in vivo, their potential to provide therapeutic benefits isn’t just plausible, but demonstrated with the studies herein. T-cells are renowned for their capabilities of cell killing, and excitingly enough it has been shown that MC, like dendritic cells, act as APCs for T-cells[277]. MC also contribute to the development and magnitude of T-cell associated responses such as those elicited by exogenous Ags; contact hypersensitivity[278-280], delayed-type hypersensitivity[281], and asthma [282, 283], and autoimmune diseases such as encephalomyelitis[284] and Ab-induced arthritis[285]. As previously mentioned, CAR T-cell therapy is under deep investigation and on the forefront of science, and with the combination of CAR T-cell and CAR M-cell therapy, goals of improving patient care are achievable. Influencing MC to express CARs via transfection would eliminate the MC IgE sensitization step, thus allowing for a more rapid and intimate interaction with the target. Herein, we have demonstrated the therapeutic effects of MC and their cytokines. With proper regulation of immune cell surface proteins and cytokine release via gene therapy and the disregard of the naysayers, immunotherapy is reducing and will continue to reduce morbidity and mortality rates in patients.
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