Assessment of the effects of rosemary extract on mast cell-mediated allergic inflammation

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Submitted in partial fulfillment of the requirements for the degree
Master of Science in Applied Health Sciences
(Health Sciences)

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© August 2018
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Abstract:
The prevalence of allergic inflammatory disorders is increasing at an alarming rate, with 40-50% of school-aged children suffering today. Mast cells are immune sentinels and a driving force in both normal and pathological contexts of inflammation. Crosslinking of FcεRI by allergen-bound IgE antibodies leads to mast cell degranulation resulting in an early phase response, and the release of newly synthesized pro-inflammatory mediators, contributing to a late phase response. The mitogen-activated protein kinase (MAPK) family, phosphoinositide 3-kinase/protein kinase B (PI3K-Akt), and nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) pathways have been established to be driving mechanisms behind mast cell-induced inflammation. Rosemary extract (RE) is rich in polyphenols and has been shown to inhibit the MAPK, PI3K-Akt, and NF-κB pathways in other cellular contexts in vitro and in vivo. However, the effect of RE on mast cell activation has not been explored. Therefore, the aim of this study was to evaluate RE in modulating mast cell activation and FcεRI signaling via these pathways toward understanding the mechanism of action and functional outcomes. Mast cells were sensitized with anti-TNP IgE and were stimulated with the cognate allergen (TNP-BSA) under stem cell factor (SCF) potentiation and treated with 0 – 25 μg/ml RE. Samples were then collected for western blot analysis, quantitative polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA), β-hexosaminidase assay, and NFκB transcription factor activity assay. Western blot analysis demonstrated that RE treatment at both 5 and 25 μg/ml inhibited phosphorylation of p38-MAPK, and treatment with 25 μg/ml inhibited JNK. qPCR analysis showed that RE treatment at 25 μg/mL resulted in decreased gene expression of IL6, TNF, IL13, CCL1, and CCL3. It also reduced Rcan1, and NFκBIA
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mRNA levels. ELISA analysis further supported the qPCR data showing decreases in pro-inflammatory IL-6, TNF, IL-13, CCL1, and CCL3. The β-hexosaminidase assay demonstrated that RE treatment inhibited mast cell degranulation dose-dependently to a maximum (down to 15% of control) at 25 μg/mL RE. Finally, RE reduced NFκB activity. This work suggests that RE is capable of modulating mast cell functional outcomes, and warrants further investigation for use as a potential therapeutic.
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Acknowledgements:

Thank you to my family, my sister Mariam, my mom Mira, and my dad Emad. Thank you for constantly dealing with me for the past couple of years. Please know that without your support I would never have reached this point or achieved any of the things that I have.

To Rachel, I can’t say enough about you. You have been my rock throughout this entire experience and if it wasn’t for you supporting me, guiding me, and helping me with every stumble I had, I wouldn’t be where I am today. Thank you.

To my committee member Dr. Rebecca MacPherson, thank you for guiding me through my research experiences and for taking the time out of your busy schedule to give me advice and input on the project.

To my co-supervisor Dr. Evangelia Tsiani, I have been working with you for almost 3.5 years now, from my first summer volunteering in your lab, to my undergraduate thesis, Match of Minds, and now my master’s degree. You have always guided me and supported me in all of my research endeavors, you have helped me become a better researcher. Above that, you have always treated me as your own son, and for that I thank you.

Finally, to Dr. Adam MacNeil, thank you for guiding me throughout this entire experience. You have helped me along every step, like when you encouraged me to do more western blots or helped me refine my writing style. You have been a mentor to me, all the while allowing me the freedom to develop my own style and to learn on my own. You have also been a role model to me, you have taught me how to be a good leader, a good mentor, a good researcher, and a professional.
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List of Abbreviations:

RE – Rosemary extract
RA – Rosmarinic acid
CA – Carnosic acid
CO – Carnosol
BMMC – Bone marrow-derived mast cell
TNP – Trinitrophenyl
BSA – Bovine serum albumin
SCF - Stem cell factor
IgE – Immunoglobulin Epsilon
FceRI – Fragment crystallisable region of immunoglobulin epsilon receptor 1
c-Kit – CD117, stem cell growth factor receptor
CCL – C-C motif chemokine ligand
IL – Interleukin
TNF – Tumor necrosis factor
HI-FBS – Heat-inactivated fetal bovine serum
mRNA – Messenger ribonucleic acid
gDNA – Genomic deoxyribonucleic acid
cDNA – Complimentary deoxyribonucleic acid
DNA – Deoxyribonucleic acid
qPCR – Quantitative polymerase chain reaction
RNA – Ribonucleic acid
RT – Room temperature
SDS – Sodium dodecyl sulfate
ELISA – Enzyme linked immunosorbent assay
p-NAG - P-nitrophenyl-N-acetyl-β-D-glucosaminide
MAPK – Mitogen-activated protein kinase
ERK – Extracellular signal-regulated-kinase
p38 – Protein 38
JNK – c-Jun N-terminal kinase
IKK – Inhibitor kappa B kinase
IκBα – Inhibitor kappa B alpha
NF-κB – Nuclear factor kappa B
NFKBIA – Nuclear factor kappa b inhibitor alpha
PI3K-Akt - Phosphoinositide 3-kinase/protein kinase B
PIP2 - Phosphatidylinositol bisphosphate
PDK1 - Phosphoinositide-dependent kinase-1
Egr – Early growth response
LPS – Lipopolysaccharide
PBS – Phosphate-buffered saline
SH2 – Src Homology 2
TBS – Tris-buffered saline
TLR – Toll-like Receptor
Grb2 – growth factor receptor-bound protein 2
SOS – Son of sevenless
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**MEK** – Mitogen activated protein kinase kinase
**LAT** – Linker for activation of T cells
**GADS** – Grb2-related adaptor downstream of Shc
**SLP76** – SH2 domain containing leukocyte protein of 76 kDa
**DAG** – Diacylglycerol
**IP$_3$** – Inositol trisphosphate
**PKC** – Protein kinase C
**ER** – Endoplasmic reticulum
**SNARE** – SNAP Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor
**TCR** – T cell receptor
**COX** – Cyclooxygenase
**NOS** – Nitric oxide synthase
**PGE$_2$** – Prostaglandin E2
**ACC** – Animal care committee
**ns** – Not significant
**S473** – Serine 473
1. Introduction:

1.1 The Immune System

Living organisms are exposed daily to antigens, these include pathogens and allergens. Most of the antigens we are exposed to require an adequate and appropriate immune response to help combat the danger that they present [1]. However, some of the antigens we are exposed to only present a “perceived danger” and thus do not require an immune response; namely allergens. The immune response that is initiated upon exposure to an antigen whether appropriate or not is dependent on the immune system [1].

The immune system is the body’s defense mechanism against foreign pathogens and invaders. It is composed of two main systems, the innate and the adaptive immune systems [1]. The innate immune system is the first line of defense and is activated when pathogens are recognized by pattern recognition receptors, commonly known as toll-like receptors (TLRs) [2–4], which allows for a general response to components that are preserved among a wide range of pathogens, this also allows the innate immune system to act very rapidly [2–5]. Unfortunately, this means that the innate immune system is not specific and does not confer long-lasting immunity; therefore, inflammation and the complement system are parts of the innate immune response. The primary cells involved in the innate response are mast cells, eosinophils, natural killer cells and macrophages [2,6].

The adaptive immune response is characterized by memory which allows the immune system to “remember” a pathogen by a specific antigen leading to a much stronger and more specific immune response [7]. Activation of the adaptive immune response requires antigen presentation by cells such as dendritic cells, which act as bridges between
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the innate immune system and the adaptive immune system lymphocytes such as cytotoxic CD8+ T cells, helper CD4+ T cells and importantly B cells [7].

Inflammation is the body’s response to a harmful substance including pathogens, damaged cells or irritants such as allergens [8]. Inflammation is typically defined by four characteristic traits, heat, pain, redness and swelling [8–10]. These markers of inflammation act to help the body in defending itself by recruiting immune cells which all-together work to eliminate the original cause of inflammation [8,9]. Various immune cells are involved in both the response to and the propagation of inflammation.

1.2 Errors in the Immune Response

However, when the immune system makes an error due to its lack of specificity and initiates a response to a harmless molecule such as an allergen, the result is allergic inflammatory disorders [10]. These disorders include rhinitis (hay fever), atopic dermatitis (eczema), asthma and anaphylaxis [8]. Errors in both the innate and adaptive immune systems result in the development of allergic inflammation, the adaptive immune system incorrectly recognizes allergens as harmful and produces antibodies which then work to prime the innate immune system mast cells to more readily react upon exposure to the same allergens. The prevalence of allergic inflammatory disorders is increasing at an alarming rate, with 40-50% of school-aged children in North America suffering today [11]. $2.1 billion is spent annually in Canada on treating asthma and the cost of treating lung disease is expected to double by 2030 [12]. Mast cells are the driving force behind inflammation in these disorders [13,14].

There are two phases of the allergic inflammatory response following mast cell activation; the early and late phase [15–17]. The early phase response occurs within
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seconds to minutes upon exposure to an allergen. The characteristic trait of the early phase response is mast cell degranulation [18]. Degranulation is the release of mast cell granules which contain preformed pro-inflammatory mediators such as histamine, cytokines and chemokines [15,18,19]. These mediators affect nerve cells to stimulate itching, smooth muscle cells causing contraction of the airways, goblet cells causing mucus production and endothelial cells causing vasodilation and edema [18,19]. The late phase response occurs after several hours upon exposure to an allergen. The products of the early phase response work to increase recruitment of leukocytes to the site of insult [16,19]. The leukocytes then work to propagate the allergic response, by recruiting more mast cells and eosinophils as well as causing isotype switching of plasma cells to IgE [16–19]. Altogether, the recruitment of leukocytes and isotype switching to IgE then work to sensitize and activate mast cells so that they may react further.

1.3 Mast Cells

Mast cells are derived from progenitors in the bone marrow and are the only terminally differentiated cells which continually express high levels of the receptor for SCF, c-Kit (CD117) [20,21]. SCF signaling is required by mast cells for growth and survival but can also contribute to both normal and pathological (allergy) mast cell functions [22]. In addition to c-Kit, mast cells express high levels of the FcεRI receptor. Allergen detection by the immune system results in production of allergen-specific immunoglobulin E (IgE) antibodies [15,23]. These antibodies bind to their high-affinity receptor FcεRI, which is constitutively expressed on the surface of mast cells [15,24]. Crosslinking of FcεRI by allergen-bound IgE antibodies leads to mast cell degranulation (early-phase response) and the release of newly synthesized pro-inflammatory mediators
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(late-phase response) [15,24]. In physiological contexts, mast cell functional responses often occur in the context of both local SCF-c-Kit signaling as well as FcεRI crosslinking by allergen-bound IgE antibodies.

1.4 Signaling Pathways

There are several signaling pathways which have been shown to be activated within mast cells leading to the production of pro-inflammatory molecules. The mitogen activated protein kinase (MAPK) family, phosphoinositide 3-kinase/protein kinase B (PI3K-Akt), and nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) pathways are important examples, and have been established to be involved in mast cell induced inflammation [25–29]. Furthermore, these pathways have all been shown to be activated through FcεRI [15,24,28,30].

The RAS-MAPK, PI3K-Akt, and NFκB pathways (Figure 1) consist of different proteins in the cell that communicate a signal from the cell surface to the DNA in the nucleus of the cell. The pathways begin when a ligand binds to its tyrosine kinase receptor, this causes the receptor to become phosphorylated [31,32]. Following this phosphorylation, docking proteins such as growth factor receptor-bound protein 2 (Grb2) containing the Src homology 2 (SH2) domain bind and this promotes binding of son of sevenless (SOS) [33,34]. SOS then becomes activated which then works to promote the exchange of GDP for GTP on RAS [31–33,35]. RAS activation results in RAF activation through exchange of GDP for GTP, RAF kinase then phosphorylates and activates mitogen-activated protein kinase kinase (MEK/MKK) [31,33], MKK4/7 [36] and MKK3/6 [37]. MEK then phosphorylates and activates extracellular signal-regulated kinase (ERK) [31,33]. MKK4/7 phosphorylates and activates c-Jun N-terminal kinase (JNK) [36]. MKK3/6 phosphorylates
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and activates mitogen-activated protein kinase (p38) [37]. Following their activation, ERK, JNK and p38 work to increase transcription by promoting production and activation of transcription factors such as NFκB, c-Jun and CREB [38,39].

The PI3K-Akt pathway becomes activated when phosphoinositide 3-kinase (PI3K) phosphorylates phosphatidylinositol bisphosphate (PIP2) to produce phosphatidylinositol triphosphate (PIP3). Phosphoinositide-dependent kinase-1 (PDK1) then binds to PIP3 at the membrane and phosphorylates protein kinase B (Akt) to activate it [40]. Akt then activates many downstream signaling molecules with the ultimate outcome being protein phosphorylation, mast cell degranulation, and mediator production [28,29,41,42].

NFκB (Figure 1) becomes activated when inhibitor of κB (IκB) kinase (IKK) phosphorylates IκBα, which is bound to NFκB in the cytoplasm. [27,43–45]. When IκBα is phosphorylated it becomes ubiquinated, which targets it for degradation, this causes NFκB to become “free” to enter the nucleus where it can induce transcription of various genes [27,43–46].

There is another important signaling pathway that contributes heavily towards the early phase degranulation of mast cells, the PLCy pathway [47–50]. This pathway begins when phospholipase C binds to linker for activation of T cells (LAT), and complexes with Grb2-related adaptor downstream of Shc (GADS) and SH2 domain containing leukocyte protein of 76 kDa (SLP76) [49]. Following formation of this complex, PLCy through contributions by PI3K works to hydrolyze PIP2 to diacylglycerol (DAG) and inositol trisphosphate (IP3), which result in the activation of protein kinase C (PKC) and rise in intracellular calcium levels, respectively [49]. IP3 increases intracellular calcium stores by binding to its receptor on the endoplasmic reticulum (ER) membrane, allowing for the
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release of stored calcium into the cytoplasm where it can mediate granule docking on the plasma membrane with the assistance of SNAP Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor (SNARE) proteins, and degranulation [49,50]. When PKC is activated by DAG, it is then able to mediate protein phosphorylation events and has crosstalk with the before mentioned signaling pathways involved in the late phase response [47–49].

All of these pathways are associated with both normal and pathological function in mast cells [26,32,51,52]. Under normal conditions they promote growth, differentiation, maturation and an appropriate immune response to harmful pathogens [51]. Under pathological conditions they cause mast cell activation and ultimately result in the production of numerous pro-inflammatory mediators [53].
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Figure 1: FceRI and c-Kit receptor mediated cellular signaling pathways, resulting in degranulation and activation of mast cells through the MAPK, NFкB and PLCγ pathways. (Unpublished, M. Yousef 2018).

1.5 Roles of Pro-Inflammatory Mediators

The pro-inflammatory mediators which are produced by activation of the MAPK, PI3K-Akt, and NFкB pathways play a central and crucial role in propagating the inflammatory response. Therefore, it is important to understand their pathological functions, and the impact inhibiting them would have on the ability of mast cells to respond
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in the context of allergic inflammation. There are many cytokines and chemokines produced by mast cells, however, there are a few that play a more pivotal role. These include IL-6, TNF, IL-13, CCL1, CCL2, CCL3, and CCL9. IL-6 is responsible for inducing the growth and maturation of B cells and stimulating them to produce more antibodies, it is also capable of inducing fever by increasing prostaglandin production, and can promote specific differentiation of CD4+ T cells, thus linking the innate and adaptive immune responses [54,55]. TNF is a pleiotropic cytokine, responsible for increasing leukocyte adhesion to endothelial cells, resulting in the recruitment of various immune cells such as macrophages to the site of insult, stimulate production of IL-6 and Egr1, and can serve as a pyrogen similarly to IL-6 [56–58]. IL-13 has been shown to be crucial for stimulating IgE isotype switching of B cells, resulting in a more primed state for an allergic response [59]. IL-13 has also been shown to mediate eosinophil levels in the lungs, mucus secretion, and airway hyperresponsiveness, and can also inhibit tumor immunosurveillance [60]. CCL1 is a potent chemoattractant of monocytes, macrophages, B lymphocytes, dendritic cells, natural killer cells [61,62]. CCL2 shares many of the same functions as CCL1, but more specifically targets monocytes and memory T cells [62,63]. CCL3 has been shown to stimulate the mobilization of hematopoietic stem cells from the bone marrow to the peripheral blood where they can mature into many effector immune cells such as mast cells [64], and is responsible for recruitment of neutrophils to the site of insult [65]. CCL9 has been documented to recruit dendritic cells in peyer’s patches, which are lymphoid organs located in the small intestine and serve as housing sites for immune cells to generate and launch an immune response [66] and has been shown to increase NFκB activity [67]. Altogether, the effects of these mediators work to increase immune system activity by
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priming it for an allergic response, immune cell recruitment and activation, and induce inflammation.

1.6 Rosemary Extract

Many pharmaceutical agents have been discovered by screening natural products from plants. The exploration into natural products offers great opportunity to evaluate new chemical classes of therapeutic agents as well as study novel and potentially relevant mechanisms of action. Many labs, have reported various immunological effects of several plant-based polyphenols [68]. The plant Rosmarinus officinalis L. a member of the mint family Lamiaceae, is native to the Mediterranean region and has many culinary and medicinal uses. The main polyphenols found in rosemary extract (RE) include the diterpenes carnosic acid (CA), rosmarinic acid (RA) and carnosol (CO) [69].

RE and its main polyphenolic components RA, CA and CO have been shown to inhibit MAPK, PI3K-Akt, and NF-κB pathways and their associated pro-inflammatory mediators in vitro in various other immunological cells, as well as in vivo in animal models. The results of those studies are summarized in the literature review below.

The effect of these polyphenols on allergen-mediated FcεRI activated mast cells has not been explored. Therefore, the aim of the proposed research is to evaluate the potential of RE in modulating mast cell activation via these pathways toward establishing potentially novel future and effective interventional strategies.
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2. Literature Review:

2.1 Anti-Inflammation: Effects of RE Polyphenols

2.1.1 T Cells

Lck is a protein tyrosine kinase that is mostly found in T cells and NK cells which plays a pivotal role in T cell receptor (TCR) mediated signaling, T cell activation, maturation and proliferation. Lck deficient cells show decreased calcium influx, IL-2 expression and inhibition in development following TCR-mediated activation. Lck transmits TCR signaling through interactions with signaling effectors which occur through the Src homology 2 / 3 domains (SH2 / SH3) [70–72].

Treatment of Jurkat T cells with Rosmarinic acid in the study by Won et al., resulted in inhibition of the TCR-induced calcium mobilization and IL-2 promoter activation, however phorbol 12-myristate 13-acetate/ionomycin induced IL-2 promoter activation was not inhibited indicating its point of inhibition to be at the membrane proximal site of TCR signaling [73]. Splenocyte treatment with RA also resulted in inhibition of TCR-induced IL-2 and IFN-γ cytokine expression as well as TCR-induced proliferation [73]. Additionally, in ELISA RA treatment induced inhibition of the interaction between the Lck SH2 domain and a peptide containing its consensus binding sequence as assessed by ELISA. In addition RA treatment decreased one-way MLR elicited expression of Th1 cytokines such as IL-2 and IFN-γ indicating a shift towards an anti-inflammatory Th2 response [73]. Ahn et al., found that in ELISA RA treatment inhibited the binding of the Lck SH2 domain to a synthetic biotinylated phosphotyrosyl peptide [74]. However, interestingly when the Lck SH2 domain was pre-treated with RA for 1hr binding of the peptide was significantly decreased (IC₅₀ = 0.6 μM compared to 7 μM when the peptide was added along with RA) this suggests that RA inhibits the Lck SH2 binding domain, and
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that it is likely due to competitive blocking of the binding site of the synthetic peptide on the Lck SH2 domain [74]. RA treatment in Jurkat T cells also inhibited IL-2 promoter activity and decreased intracellular calcium release in anti-CD3/CD4 antibody activated Jurkat T cells [74].

The results from these studies suggest that RA has the potential to inhibit Lck-dependent calcium signaling, IL-2 expression as well as the increase in intracellular calcium following T cell activation through blocking of the interaction between the Lck SH2 domain and the downstream signaling effectors.

Table 1: Effects of RE polyphenols in T cells: anti-inflammatory effects

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat T cells</td>
<td>0 – 100 μM RA (IC50 = 25 – 50μM) for 15 min – 72h</td>
<td>↓ IL-2 promoter activation&lt;br&gt;↓ TCR-induced splenocyte proliferation&lt;br&gt;↓ Cytokine expression of IL-2 and IFN-γ</td>
<td>↓ interaction of Lck SH2 domain of Src family with peptides containing pYEEI binding sequence&lt;br&gt;↓ TCR-induced Ca2+ mobilization</td>
<td>[73]</td>
</tr>
<tr>
<td>Jurkat T cells</td>
<td>1.4 – 140 μM RA (IC50 = 7 – 8μM) for 10 min – 24h</td>
<td>↓ Binding of Lck SH2 domain to its associated proteins ZAP-70 &amp; PLC-γ1</td>
<td>↓ IL-2 gene expression&lt;br&gt;↓ intracellular Ca2+ release in cells stimulated with anti-CD3 and anti-CD4 antibodies</td>
<td>[74]</td>
</tr>
</tbody>
</table>

2.1.2 Macrophages

As mentioned previously, macrophages are drivers of inflammation, two main mediators of these events are the cyclooxygenase (COX) isozymes, COX-1 and COX-2, and nitric oxide synthases (NOS). The COX enzymes are responsible for the formation of prostanoids, including thromboxane and prostaglandins, which are mediators of inflammation and vasoconstriction. The NOS enzyme is responsible for catalyzing the production of nitric oxide. The inducible isoform of nitric oxide synthase (iNOS) is
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

involved in the immune response, it produces NO as an immune defense mechanism and helps drive inflammation, septic shock and may have a role in autoimmune diseases [75–77].

Treatment of peritoneal macrophages with RE resulted in significant inhibition of IL-1 and TNF-α, leading to an attenuation of the inflammatory response [78]. In the study by Cheung et al., extracts of rosemary were found to inhibit NO release in LPS-stimulated macrophages in a dose-dependent manner [79]. Additionally it was found that at the 1/500 dilution of RE there was a significant reduction in IL-1β and COX-2 and a non-significant reduction in TNF-α and iNOS mRNA expression [79]. Kuo et al., found that SC-CO2 RE extract suppressed the LPS-induced production of NO, TNF-α, iNOS, COX-2, p-IκB and NF-κB in a dose-dependent manner [80]. Treatment of macrophages with RE resulted in significant inhibition of LPS-stimulated prostaglandin E2 (PGE2) and NO levels in a dose-dependent manner, these effects were found to be mediated through decreased COX-2 and iNOS expression [81]. Interestingly, RA treatment alone only inhibited COX-2 expression in this study [81]. However, Qiao et al., demonstrated that RA treatment of macrophages did inhibit LPS-induced NO production and iNOS protein synthesis [82]. Additionally it also inhibited PMA-induced superoxide production and peroxynitrite-induced formation of 3-nitrotyrosine. Western blot analysis also demonstrated that LPS-induced phosphorylation of IκB was also inhibited [82]. Bai et al., demonstrated that among all the flavonoid and phenolic compounds extracted from Rosmarinus officinalis CA and CO were the most effective in inhibition of NO production in macrophages [83]. CO treatment of macrophages resulted in inhibition of LPS and IFN-γ induced NO production and these effects were confirmed not to be due to cellular toxicity [84]. CO and CA treatment of
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macrophages resulted in a dose-dependent reduction in NO and PGE2. Additionally they inhibited gene expression of iNOS, IL-1α, IL-6. CCL5, RANTES and CXCL10 [85]. Interestingly, CO and not CA inhibited IL-1β induced nuclear translocation of NF-κB. Treatment of macrophages with CO resulted in a significant reduction of LPS-induced NO production dose-dependently [86]. It was discovered that this was due to reduced iNOS mRNA and protein expression. In addition, CO treatment decreased NF-κB nuclear translocation and DNA binding activity, these effects were mediated through down-regulation of IκB and IKK as well as inhibition of LPS-induced phosphorylation of IκBα [86]. Furthermore, CO treatment inhibited LPS-induced ERK/MAPK activation and signaling [86]. RA treatment of Han-Wistar mice resulted in reduced CVF-induced paw oedema and inhibited passive cutaneous anaphylaxis [87]. RA administration to BALB/c mice before LPS-induced acute lung injury resulted in significant inhibition of TNF-α, IL-6 and IL-1β, it also decreased the total number of neutrophils and macrophages in the BALF, it enhanced SOD activity, thereby reducing free radical species, and finally it attenuated ERK/MAPK signaling in a dose-dependent manner [88]. Pretreatment with either CA or CO inhibited PMA-induced ear inflammation in BALB/c mice, this effect was found to be mediated through decreased expression of IL-1β and TNF-α. Interestingly, both CA and CO selectively inhibited COX-2 but not COX-1 [89]. H&E stained tissue revealed a reduction in leukocyte infiltration rate in PMA-treated ears when they were pretreated with CA. Further in vitro analysis showed that both CA and CO inhibited overproduction of NO in a dose-dependent manner [89].

Overall, the results from the studies presented above indicate that RE and its polyphenolic components can significantly inhibit macrophage activation through several
Inhibition of IgE-FceRI activated mast cells by rosemary extract mechanisms, ultimately leading to significant inhibition of inflammation and free radical release by macrophages.

Table 2: Effects of RE polyphenols in macrophages: anti-inflammatory effects

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneal macrophages</td>
<td>0.04 – 2.79 mg/ml RE for 48h</td>
<td>↓ inflammatory cytokine release  ↓ NO release</td>
<td>↓ IL-1 and TNF-α</td>
<td>[78]</td>
</tr>
<tr>
<td>RAW 264.7 macrophages</td>
<td>1/2000, 1/1000, 1/500 dilutions from RE ethanol extract</td>
<td>↓ NO production in a dose-dependent manner in LPS-stimulated cells</td>
<td>↓ iNOS gene expression  ↓ IL-1 β, COX-2 and TNF-α</td>
<td>[79]</td>
</tr>
<tr>
<td>RAW 264.7 macrophages</td>
<td>5000 psi at 40, 60 or 80°C Super-critical carbon dioxide RE extract and purified CA (IC₅₀ = 22.5 µM)</td>
<td>↓ LPS-induced NO production  ↓ TNF-α</td>
<td>↓ expression of iNOS, COX-2, P-IκB and NF-κB/p65</td>
<td>[80]</td>
</tr>
<tr>
<td>RAW 264.7 macrophages</td>
<td>30 µg/ml RE and 2.67 µM RA for 8h</td>
<td>↓ LPS-induced PGE2 and NO production</td>
<td>↓ COX2 expression</td>
<td>[81]</td>
</tr>
<tr>
<td>RAW 264.7 macrophages</td>
<td>1, 10, 50 or 100 µM RA for 24h</td>
<td>↓ NO production  ↓ LPS-induced iNOS protein synthesis  ↓ Phorbol 12-myristate 13-acetate (PMA)-induced superoxide production  ↓ Peroxynitrite-induced formation of 3-nitrotyrosine</td>
<td>↓ LPS-induced phosphorylation of IκBα</td>
<td>[82]</td>
</tr>
<tr>
<td>RAW 264.7 macrophages</td>
<td>20 or 40 µg/ml CO or CA for 24h</td>
<td>↓ LPS induced nitrite production</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td>Non-elicited peritoneal exudate macrophages obtained from male BALB/c mice</td>
<td>2.5 - 10 µM CO for 18h</td>
<td>↓ LPS and IFN-γ induced nitrite (NO₂⁻) production</td>
<td>↓ LPS-induced nitrite production</td>
<td>[84]</td>
</tr>
</tbody>
</table>
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

| RAW 264.7 macrophages | 0.16 – 50 µM CO for 24h | ↓ LPS-stimulated NO and PGE₂ production (acute inflammation) | ↓ iNOS
|↓ cytokines / interleukins (IL-1α, IL-6, IL-1β)
|↓ chemokines (CCL5/RANTES, CXCL10/IP-10)
|↓ IL-1β induced nuclear translocation of NF-κBp65
|No effect on COX2, CCL4 or MMP-9 in LPS stimulated macrophages | [85] |

| RAW 264.7 macrophages | 2.5, 5, 10 or 20 µM CO (IC₅₀ = 9.4 µM) for 24h | ↓ LPS-stimulated NO production (IC₅₀ = 9.4 µM) | ↓ LPS-induced iNOS mRNA and protein expression
|↓ NF-κB subunits translocation and NF-κB DNA binding activity
|↓ Activity of iNOS and NF-κB promoter activity
|↓ Iκκ → ↓ LPS-induced phosphorylation/degradation of IκBα (5 µM)
|↓ LPS-induced p38 and p44/42 MAPK activation | [86] |

| Han - Wistar rats | 0.316 – 3.16 mg/kg RA intramuscularly 0.5h before CVF injection; 1 – 100 mg/kg RA peritoneally 0.5h before antiserum injection | ↑ Paw oedema induced cobra venom factor (CVF)
|↓ Passive cutaneous anaphylaxis | LPS-induced TNF-α, IL-6 and IL-1β production
|↓ Total cells, neutrophils and macrophages in the BALF
|↑ Oxidase dismutase (SOD) activity
|↓ ERK/MAPK signaling | [87] |

| Male BALB/c mice with LPS-induced Acute Lung Injury | 5, 10 or 20 mg/kg RA 1h before LPS administration, samples were collected 24h after LPS administration | ↑ Anti-inflammatory effects | LPS-induced TNF-α, IL-6 and IL-1β production
|↓ Total cells, neutrophils and macrophages in the BALF
|↑ Oxidase dismutase (SOD) activity
|↓ ERK/MAPK signaling | [88] |

| Male Balb/c mice RAW 264.7 macrophages | 3.125 – 25 µg/ml CO (EC₅₀ = 10.7 µg/cm²) CA (EC₅₀ = 10.2 µg/cm²) for 24h | ↑ Phorbol 12-myristate 13-acetate (PMA)-induced ear inflammation | ↓ expression of IL-1β and TNF-α
|↓ COX-2 but not COX-1
|↓ Leukocyte infiltration and epidermal ulceration of PMA-treated ears
|↓ Overproduction of NO in macrophages | [89] |
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

2.1.3 Mast Cells

As stated previously mast cells play a pivotal role in the inflammatory response, specifically the allergic response due to their release of histamine, prostaglandins, leukotrienes and inflammatory cytokines. In addition mast cell proliferation helps amplify the inflammatory allergic response. It therefore follows that inhibition of their activation would result in a decrease in inflammation.

Treatment of HMC-1 cells with RA resulted in attenuation of thymic stromal lymphopoietin (TSLP)-induced proliferation. Mouse double minute 2 homolog (MDM2) is a protein that acts as a negative regulator of p53, RA treatment lead to decreased MDM2 expression and therefore elevated levels of p53, a tumor suppressor protein [90]. Production and mRNA expression of IL-13 which acts as a growth factor for mast cells was inhibited by RA treatment [90]. Signal transducer and activator of transcription 6 (STAT6) phosphorylation which was shown to induce mast cell proliferation was significantly inhibited by RA [90]. Additionally, RA treatment significantly increased activity of caspase-3, decreased activity of procaspase-3, increased PARP cleavage and decreased levels of Bcl2 [90]. All together these results indicate that RA induced HMC-1 apoptosis and helped resist the anti-apoptotic activity of TSLP activated HMC-1 cells [90]. In addition to inducing apoptosis, RA treatment also decreased pro-inflammatory cytokine levels in TSLP activated HMC-1 cells [90]. Production of TNF-α, IL-1β and IL-6 were all significantly inhibited by RA treatment [90]. In addition to studying the effects of RA on HMC-1 cells, Yoou et al., also studied the effects of RA on short ragweed pollen-induced allergic conjunctivitis in mice and found that levels of serum IgE, TSLP and IL-4 which were elevated in the model to be significantly decreased by RA treatment [90].
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Oh et al., aimed to investigate the effects of RA treatment in an allergic rhinitis mouse model as well as in HMC-1 cells and found that RA decreased the number of nasal, ear and eye rubs after OVA challenge, IgE and histamine levels were also attenuated in OVA-sensitized mice [91]. Protein and mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were significantly inhibited by RA treatment [91]. In agreement with the reduction of IL-1β, caspase-1 OVA-induced activation, which cleaves pro-IL-1β into the secreted pro-inflammatory cytokine IL-1β, was inhibited [91]. RA also decreased mast cell and eosinophil infiltration of the nasal mucosa following OVA challenge. In HMC-1 cells PMA and calcium ionophore A23187 (PMACI)-induced NF-κB and caspase-1 activation were both significantly inhibited by RA treatment [91].

All together these results indicate that RA is capable of inhibiting mast cell activation in vitro and in vivo leading to decreases in their proliferation, pro-inflammatory cytokine production, histamine release and mucosal layer infiltration, suggesting that RA is capable of inhibiting the pro-inflammatory activity of mast cells in both the early and late phase responses.

Table 3: Effects of RE polyphenols in mast cells: anti-inflammatory effects

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMC-1 and short ragweed pollen-induced allergic conjunctivitis mouse model</td>
<td>0.1, 1, 10 or 100 μM RA for 1h</td>
<td>↓ Thymic stromal lymphopoietin (TSLP)-induced mast cell proliferation and murine double minute (MDM) 2 expression</td>
<td>↓ IL-13</td>
<td>[90]</td>
</tr>
</tbody>
</table>

| | | | ↑ Inflammatory effect on TSLP induced inflammatory reactions | ↑ Phosphorylation of the signal transducer and activator of transcription 6 (STAT6) | p53 levels | Caspase-3 activation | Poly-ADP-ribose polymerase cleavage | Procaspase-3 and Bcl2 |
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

| HMC-1 and 6 week old BALB/c | 4 mg/kg RA or 100 μM RA | ↘ Number of rubs after OVA challenge in OVA-sensitized mice | ↘ IgE in serum, spleen and nasal mucosa in OVA-sensitized mice | ↘ Serum histamine levels in OVA-sensitized mice | ↘ Protein and mRNA expression of IL-1β, IL-6 and TNF-α (Nasal mucosa and spleen of OVA-sensitized mice) | ↘ Mast cell and eosinophil infiltration | ↘ COX-2 protein expression and Caspase-1 activity (Nasal mucosa of OVA-sensitized mice) | ↘ NF-κB and caspase-1 activation (HMC-1) | [91] |

2.2 Immune System Modulation: Effects of RE Polyphenols

2.2.1 T Cells

In the study by Kang, Yun & Won, T cells treated with RA had a significant inhibition of nuclear factor of activated T cells (NF-AT) but not activating protein-1 (AP-1). This suggested that RA inhibits Ca²⁺-dependent signaling pathways only. Upstream signaling events of NF-AT were inhibited by RA, including Ca²⁺ mobilization and phosphorylation of phospholipase C-γ1 (PLC-γ1) [92]. In contrast to some previous studies, RA treatment was not found to inhibit Lck or ZAP-70 but instead inhibited the activation of Itk, which is responsible for phosphorylation of PLC-γ1 [92]. Due to the lack of activity on ZAP-70, the MAPK pathway was unaffected by RA treatment. Overall, this indicates that RA inhibits TCR signaling by blocking membrane-proximal events, specifically phosphorylation of Itk and PLC-γ1 [92]. Hur, Yun & Won treated both Lck⁺
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

and Lck+ T cells with RA and observed that only Lck+ cells underwent apoptosis in response to RA [93]. Further analysis through several mutant forms of Lck it was found that the Lck SH2 domain and not the Lck kinase activity was required for RA mediated apoptosis [93]. Alteration of mitochondrial pathways was determined to be the cause behind this induction of apoptosis, specifically increased cytochrome c release [93]. Additionally, both caspase-3 and -8 were found to be necessary for the RA mediated apoptosis [93]. In accordance with the previous study, activated T cells such as CD3+ CD25+, CD4+CD25+ and CD4+ CD45RO+ had an increased rate and susceptibility to apoptosis when treated with RA [94]. Similarly, the mechanism behind the induction of apoptosis observed was through increased release of cytochrome c from the mitochondria [94]. In the study by Urushima et al., C57BL/6 mice fed extract from Perilla frutescens (PE) which contains RA, was found to decrease serum TNF-α, IL-17A and IL-10, however these effects were found to be mediated through other active ingredients, luteolin and apigenin [95]. RA however was found to increase the regulatory T cell population, which was found to be mediated through increased FOXP3 expression. FOXP3 is a master regulator of the pathway for development and function of regulatory T cells [95].

Table 4: Effects of RE polyphenols in T cells: immune modulation

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat T cells</td>
<td>30 μM RA for 4h</td>
<td>Proliferation</td>
<td>Ca2+ dependent signaling pathway, TCR-induced tyrosine phosphorylation and subsequent activation of Itk, but not Lck or ZAP70 (MAPK pathway was intact), NF-AT activation, No effect on AP-1</td>
<td>[92]</td>
</tr>
</tbody>
</table>
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

<table>
<thead>
<tr>
<th></th>
<th>Tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1)</th>
<th>Tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lck+ Jurkat T cells and Lck- Jurkat subclone J.CaM1.6 cells</td>
<td>30 μM RA for for 24h or 48h</td>
<td>apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p56lck dependent manner</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lck SH2 domain is required and not the Lck kinase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cytochrome c release</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mitochondrial pathway is involved)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Required presence of caspase 3 and 8</td>
</tr>
<tr>
<td>T cells from Rheumatoid arthritis patients</td>
<td>10,50 or 100 μM RA for 48h</td>
<td>apoptosis of CD3+ CD25+ activated T cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Susceptibility to RA induced apoptosis of CD4+ CD25+ and CD4+ CD45RO+ T cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cytochrome c release</td>
</tr>
<tr>
<td>Mononuclear cells isolated from 8 week old C57BL/6 female mice</td>
<td>5, 25 or 50 μM RA for 48h</td>
<td>Treg cell population</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foxp3 mRNA expression compared to LPS treatment alone</td>
</tr>
</tbody>
</table>

2.2.2 Macrophages

The classic complement pathway is a key pathway in the innate immune response, which leads to lysis of antibody-coated pathogens. Immuno-lysis of antibody coated sheep erythrocytes by guinea pig serum was inhibited by RA treatment, this was found to be due to an inhibition of the C3-convertase of the classical complement pathway [87]. RA treatment was also found to inhibit activation of macrophages by heat-killed *Corynebacterium parvum*, this was determined through the reduced ability of macrophages to undergo oxidative burst following RA treatment [87].
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

### Table 5: Effects of RE polyphenols in macrophages: immune modulation

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep erythrocytes, Han-Wistar rats</td>
<td>1, 10, 100 or 1000 μM RA for 18h; 10 mg/kg RA intramuscularly 2days before and 1 day after bacterium injection</td>
<td>Immunoahaemolysis of antibody-coated sheep erythrocytes by guinea pig serum</td>
<td>Inhibition of C3-convertase of the classical complement pathway</td>
<td>[87]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In-vivo macrophage activation by heat-killed Corynebacterium parvum (i.p)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 2.2.3 Eosinophils

Treatment of mice representing a model of respiratory allergy with RA resulted in a reduction in the number of leukocytes and eosinophils, eosinophil peroxidase activity and IL-4 in the bronchoalveolar lavage (BAL) [96]. It also induced histopathological changes in the lung marked by reduced inflammatory cell infiltration within the peribronchiolar and perivascular regions and this was confirmed by a significant reduction in lung inflammation [96]. Additionally, RA treatment led to a reduction of mucus levels in the respiratory tract [96].

### Table 6: Effects of RE polyphenols in eosinophils: immune modulation

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male AJ mice model of respiratory allergy using Blomia tropicalis extract</td>
<td>2, 20 or 200 mg/kg RA</td>
<td>Number of leukocytes/eosinophils in bronchoalveolar lavage</td>
<td></td>
<td>[96]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eosinophil peroxidase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucus in respiratory tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histopathological changes in the lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-4 in BAL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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2.2.4 Dendritic Cells

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the rate-limiting step in the degradation of tryptophan. This is thought to be of immune system importance since inhibition of T cell proliferation by tryptophan catabolism protects fetuses from maternal reactions. Beyond this role, expression of IDO in different cell types is believed to have a more general role in T cell homeostasis mainly through an immunosuppressive effect and promoting tumor tolerance [97,98].

Treatment of both IFN-γ activated bone-marrow derived dendritic cells (BMDCs) and CD11c+ CD8α+ DCs in tumor bearing mice with RA was found to reduce the intracellular expression of IDO, decrease its functional activity and block IDO-dependent T cell suppression [99]. Furthermore, it was found to suppress IFN-γ induced STAT1 activation, which is required for IFN-γ mediated induction of transcription [99].

Table 7: Effects of RE polyphenols in dendritic cells: immune modulation

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMDCs and CD11c+ CD8α+ DCs tumor bearing 8-10 week old C57BL/6 mice</td>
<td>1, 10 or 100 μM RA for 2h</td>
<td>↓ intracellular expression of Indoleamine 2,3-dioxygenase (IDO) (Both models)</td>
<td>↓ Functional activity of IDO ↓ IDO-dependent T cell suppression ↓ IFN-γ induced STAT1 activation</td>
<td>[99]</td>
</tr>
</tbody>
</table>

The summary of the studies presented above indicates that RA has potent immunomodulatory effects, and works to inhibit multiple immunological cells. RA inhibits T cells involved in pathogenesis of several diseases and activates regulatory T cells which are involved in suppression of the immune response/system. RA treatment is also capable of modulating and inhibiting the innate immune response through both the classic complement pathway as well as the down-regulation of macrophage activity. It was also found to decrease respiratory pathway inflammation and decrease the number of
Inhibition of IgE-FcεRI activated mast cells by rosemary extract inflammatory cells at the site of inflammation. Finally, RA treatment was also found to modulate the immune system by decreasing the expression and activity of key enzymes involved in tumor tolerance and T cell population regulation and homeostasis.

2.3. Increased Resistance to Disease: Effects of RE Polyphenols

2.3.1 T Cells

Treatment of T cells infected with HIV with RE resulted in potent anti-HIV activity but also produced cytotoxic effects. However, treatment of the same cells with 8 μM of CO resulted in significant anti-HIV activity with no cytotoxic effects [100].

Treatment of a Balb/c mouse model of fibrosarcoma with CO was found to suppress tumor growth and deplete spleen and tumor-associated T_{reg} cells. It also caused decreased production of IL-4 and IL-10 and increased production of IFN-γ and lymphocyte proliferation [101].

The effects of CO on HIV replication as well as the decreases in IL-4 and IL-10 accompanied by the increases in IFN-γ and lymphocyte proliferation indicate that CO has a strong potential for anti-viral and anti-tumor activity by modulating immune cell numbers.

Table 8: Effects of RE polyphenols in T cells: resistance to disease

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
</table>
| C8166 human T lymphoblastoid cells infected with HIV-1MN | 0.01% (v/v) RE or 8 μM CO | ↑ anti-HIV activity  
↑ cytotoxicity | | [102] |
| Balb/c fibrosarcoma model (injected with tumor cells) | Intraperitoneal injection of 5 or 10 mg/kg/day CO for 7 days | ↓ tumor growth  
↓ Splenic and tumor-associated T_{reg} cells | ↓ IL-4  
↓ IL-10  
↑ IFN-γ  
↑ lymphocyte proliferation | [101] |
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2.3.2 Macrophages

All-trans retinoic acid (ATRA) is an established treatment for acute promyelocytic leukemia (APL), but many patients often develop resistance to the treatment [103]. Treatment of APL cells with a combination of ATRA and RA was found to increase ATRA-induced macrophage differentiation marked by increased CD11b+ expression (a marker of macrophage differentiation) [103]. In addition, these macrophages were found to be capable of phagocytosis, generating ROS and also expressed normal levels of CCR1, CCR2 and ICAM-1; indicating that these macrophages were functional [103].

Treatment of cultures with RA inhibited osteoclast formation and receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastic differentiation in bone marrow-derived macrophages in cocultures of mouse bone marrow cells and osteoblasts [104]. Furthermore, RANKL-induced p38 MAPK and expression of nuclear factor of activated T cell (NF-AT), c-Jun and c-Fos were inhibited with RA in bone marrow macrophages [104]. Interestingly, oral administration of RA was found to increase bone mass in a soluble RANKL-induced bone loss mouse model, which indicated the capability of RA to affect bone homeostasis and help protect against osteoporosis [104].

Table 9: Effects of RE polyphenols in macrophages: resistance to disease

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose and Duration</th>
<th>Findings</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB4, acute promyelocytic leukemia cells (APL)</td>
<td>40 μM RA + 10 nM all trans retinoic acid (ATRA) for 72h</td>
<td>↑ ATRA induced macrophage differentiation in APL cells</td>
<td>↑ CD11b expression (marker of differentiation)</td>
<td>[103]</td>
</tr>
<tr>
<td>Bone marrow macrophages derived from four – six week old Std ddY male mice</td>
<td>1 mg/kg sRANKL daily for 4 days, followed by 1, 10 or 100mg/kg/day RA</td>
<td>↓ Osteoclast differentiation</td>
<td>↓ RANKL-induced p38 mitogen-activated protein kinase and expression of nuclear factor of activated T cell, c-Jun and c-Fos ↓ Receptor activator of</td>
<td>[104]</td>
</tr>
</tbody>
</table>
## 2.4 Conclusion

There is evidence suggesting the potential of RE and its polyphenolic components RA, CA and CO to modulate the immune system in various ways. These polyphenols have been shown to inhibit the inflammatory response, to regulate the immune response by limiting its activation in situations where it is inappropriate and to increase resistance to several diseases which affect immune system activity. However, the effect of these polyphenols on mast cells has not been documented and there is a gap in the literature regarding the effects of these polyphenols on allergic inflammatory disorders.

The use of these polyphenols in other important immune cells has shown great promise. They have been shown to inhibit the same pathways which drive mast cell activation in other cells, such as the MAPK, NFκB and the COX pathways. They have also been shown to decrease the production and secretion of various pro-inflammatory mediators that are responsible for initiating and propagating the inflammatory response, such as IL-4, -6, -10, -13 and TNF-α. Furthermore, they have also been shown to decrease activity of immune cells, such as increasing Foxp3 mRNA expression to increase T_{reg} cell populations which work to suppress the immune response.

The proposed research will help determine if the effects of these polyphenols on other immune cells is conserved when they are used to treat allergically activated mast cells.
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and to evaluate their potential as future therapeutics in the treatment of allergic inflammatory disorders, which present an ever-increasing risk to the public.
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3. Objectives:

The objectives of the proposed research are:

1. To determine the effect of treatment with RE and its polyphenolic components on IgE-FcεRI allergen-mediated mast cell activation and protein modulation of the MAPK, PI3K-Akt, and NFκB pathways.

2. To determine the effect of treatment with RE and its polyphenolic components on transcription factors bridging protein signaling to gene expression.

3. To determine the effect of treatment with RE and its polyphenolic components on the late phase response following IgE-FcεRI allergen-mediated mast cell activation:
   a. Effects on gene expression of various pro-inflammatory mediators.
   b. Effects on pro-inflammatory mediator release.

4. To determine the effect of treatment with RE and its polyphenolic components on degranulation of IgE-FcεRI allergen-activated mast cells during the early phase response.
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4. Hypothesis:

The hypotheses for the corresponding objectives are:

1. RE and its polyphenolic components will inhibit IgE-FcεRI allergen-mediated activation of the MAPK, PI3K-Akt, and NFκB pathways and therefore will decrease the phosphorylation of their components including ERK, p38, JNK, Akt, and IκBα.

2. RE and its polyphenolic components will inhibit IgE-FcεRI allergen-mediated activation of transcription factors.

3. RE and its polyphenolic components will inhibit IgE-FcεRI allergen-mediated activation of the late phase response:
   a. Gene expression of several important pro-inflammatory mediators will decrease following treatment.
   b. Pro-inflammatory mediator release will decrease following treatment.

4. RE and its polyphenolic components will play a minor role in degranulation since degranulation occurs very rapidly and time is required for RE and its components to enter the cell and exert their effects.
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5. Materials and Methodology:

**Figure 2:** Summary of methodology.

5.1 Mice

Female C57BL/6 (~ 9 weeks old) (Charles River Laboratories) maintained with normal diet and living conditions. All protocols were approved by the Animal Care Committee (ACC) at Brock University.

5.2 Bone Marrow Isolation

All bone marrow isolation surgeries were carried out in a ThermoForma biological safety cabinet (Class II A2 BSC, 100660-2888) to ensure sterile conditions. Preparation for surgery was done by autoclaving surgical tools, including two pairs of forceps and 2 pairs of scissors. Tools were removed from autoclave bag inside BSC and placed in 50 ml conical tube (Sarstedt, #62.547.205) filled with a 70% ethanol (Commercial Alcohols, #P016EA95) solution. To begin the bone marrow isolation the mice were first anaesthetized with isoflurane and immediately transferred to CO₂ for euthanization. Euthanized animals were transferred to the BSC and placed on a square of paper towel.
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The entire animal was sprayed with a 70% ethanol solution to wet the fur and enhance sterility. The first incision was made around the entire circumference of the animal at sternal level, another incision was made around the base of the tail and the final incision was made to connect these two incisions up the abdominal midline. All incisions were only through the skin and care was taken to not cut into any internal organs. Following the main 3 incisions were made the feet were removed just below the ankle joint, and the skin was carefully pulled to remove it entirely from the lower half of the body. Each leg was removed by carefully cutting into the muscle around the base of the femur to expose the femoral-pelvic joint. Once the joint was located the forceps were used to pull the leg upwards while downward pressure was placed on the rest of the body with the scissors and the leg was removed with a cut through the center of this joint, being careful to leave the entire femur intact. All muscle tissue was removed from the legs and the femur and tibia were isolated and placed in sterile RPMI-1640 medium until all bones were isolated. To isolate the bone marrow, both ends of the bones were cut off with a pair of scissors to expose the bone marrow. Bone marrow derived mast cell complete (BMMC complete) medium was used to flush marrow from bones and is made by mixing 500 ml of RPMI-1640 (Life Technologies Inc., 11875119) with 10% (63 ml) of heat-inactivated fetal bovine serum (HI-FBS), 10% (63 ml) of WEHI supernatant (collected from WEHI-3B cell line cultures; American Type Culture Collection, ATCC), 1% (6.3 ml) penicillin/streptomycin (PenStrep) (Gibco, 15140-122), 0.002 % (12.6 µl) prostaglandin E2 (Sigma, #P5640) and 0.005% (31.6 µl) of 2-mercaptoethanol (Sigma, #M3148) [105] and filter sterilized through a 0.2 µm pore diameter 500 ml bottle top filter (Filtpour BT50, Sarstedt, #83.1823.101). A 30 ml Luer-Lok Tip syringe (BD, #302832) was filled with BMMC complete medium.
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and was loaded with a 30-gauge needle (BD PrecisionGlide, #305106). The needle was inserted into the bone shaft and constant pressure was placed on the syringe plunger to flush all the bone marrow from inside the bone into a sterile 50 ml conical tube (using a new tube for each mouse). Once both femurs and tibias from a specific mouse were flushed into a tube, the medium remaining in the syringe was expelled into the tube and the entire solution was mixed by repeated suction and expulsion of the solution with a serological pipette. The recovered cells were then strained into another sterile 50 ml conical tube through a 40 µm nylon cell strainer (Falcon, #352340) to remove any debris from the flushing process. The new solution was then centrifuged in a Thermo Electron Corporation Centrifuge (CentraCL3R, #3755-1851) at 1300 rpm for 10 minutes at 4°C to pellet the cells. The supernatant was discarded and the cell pellet was resuspended in 10 ml of BMMC complete media. The resuspended cells were then added to 40 ml of fresh medium in a sterile T75 vented cap cell culture flask (Sarstedt) and placed in the cell culture incubator at 37°C with 5% carbon dioxide (CO₂) and 95% humidity to begin proliferation.

5.3 Rosemary Extract Preparation

Whole dried rosemary (*Rosmarinus officinalis* L.) leaves (purchased from Compliments/Sobey’s, Mississauga, ON, Canada- product of Turkey) were ground and passed through a mesh sieve and extracted following protocols established by the National Cancer Institute of the U.S. The ground plant (5g) was steeped overnight (16 hours) in dichloromethane-methanol (1:1) (30ml). The filtrate was collected under slight vacuum followed by methanol (30ml) extraction for 30 minutes. The solvent was removed by rotary evaporation. Aliquots were prepared in dimethyl sulfoxide (DMSO) (100mg/ml) and stored at -20°C [106]. It is understood in the literature that 0.1% is the threshold value.
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5.4 BMMC Culturing

Following the bone marrow isolation, as described above, the medium in the new cell cultures was changed twice per week, approximately every 3 or 4 days. To begin, the cell culture medium is drawn out of the flask with a serological pipette and transferred to 50 ml conical tubes. The tubes were then centrifuged at 1300 rpm for 10 minutes at 4°C to pellet the cells. The supernatant was then discarded and the cell pellet was resuspended in 10 ml of BMMC complete medium (depending on the counts from previous culturing protocols the volume of resuspension medium may be altered). A small amount of the cells are transferred to a 1.5 ml and then 46 μl of this sample is mixed with 4 μl of NucBlue stain (Life Technologies, R37605) and incubated at room temperature for 20 minutes. Following the incubation a 12-15 μl sample of the cells is placed on a hemocytometer slide and the number of cells was counted on a Countess II FL (Life Technologies Inc., AMQAF1000). The cell count number is then used to determine the volume of medium needed to culture the cells at a density of 0.5 x10⁶ cells/ml. The completed cultures are then placed back in the cell culture incubator at 37°C with 5% carbon dioxide (CO₂) and 95% humidity.

5.5 Mast Cell Sensitization, Treatment, and Stimulation

BMMCs were collected and sensitized in 25% IgE supernatant (prepared from TIB-141 cell line cultures, ATCC) and incubated overnight at 0.5 x10⁶ cells/ml. Samples were centrifuged in a Thermo Electron Corporation Centrifuge (CentraCL3R, #3755-1851) at 1300 rpm for 10 minutes at 4°C and resuspended in 30 ml of sterile RPMI-1640 medium three times to wash them. Cells were subsequently resuspended at a density of 1.0 x10⁶
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cells/ml in RPMI-1640 containing 10% HI-FBS (Sigma, F2442) and 1% PenStrep. The cells were then separated into appropriate treatment numbers (variable depending on specific experiment) and placed into T25 vented cap cell culture flasks (Sarstedt). Cells were subsequently stimulated with either 100 ng/ml of TNP-BSA (Biosearch Technologies) in combination with 100 ng/ml of SCF (PeproTech Inc. #250-03) for various times (depending on the protocol, indicated for each protocol below). The total cell volume was divided in three, one third of the cells received 0 µg/ml RE, one third of the cells received 5 µg/ml RE and the final third received 25 µg/ml RE, based upon each protocol (details included under each protocol), and was incubated for at 37° C, 95% humidity and 5% CO₂.

5.6 Protein Extraction

10 x 10⁶ BMMCs per condition were sensitized, washed, treated and stimulated as described above. At each time point (0, 5, 20 and 60 mins), cells were centrifuged at 1500 rpm for 5 minutes at 4° C to pellet the cells. The supernatant was aspirated off and the cell pellet was lysed with 30 µL of RIPA lysis buffer per 5 x10⁶ cells (473 µL sterile water (Baxter, JF7624), 500 µL 2X RIPA buffer (BioBasic, #RB4477), 2 µL leupeptin (5 mg/ml, Bio Basic Canada Inc., LDJ691), 2 µL pepstatin (5 mg/ml, Bio Basic Canada Inc., PDJ694), 2 µL aprotinin (5 mg/ml, Bio Basic Canada Inc., AD0153), 4 µL iodoacetamide (0.5 M, Bio Basic Canada Inc., IB0539), 5µL NaF (200 mM, Sigma, S-7929, 1:2000 dilution), 5 µL Na₃VO₄ (200 mM, Sigma, S-6508, 1:2000 dilution), 5 µL PMSF (100 mM, Bio Basic Canada Inc, PB0425). The lysate was vortexed for 10 seconds and then incubated on ice for at least 20 minutes. Following the incubation, the lysate was vortexed for another 10 seconds and subsequently centrifuged at 13,500 rpm for 10 minutes at 4° C in a Labnet
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International Inc. Centrifuge (Prism-R, # C2500-R). Supernatant was isolated from pellets and stored in a 1.5 ml tube at -80°C.

**Table 10:** Representative experimental design for protein modulation through western blot.

<table>
<thead>
<tr>
<th>Western Blot</th>
<th>Control</th>
<th>5 µg/ml RE</th>
<th>25 µg/ml RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 4</td>
<td>NT</td>
<td>TNP + SCF</td>
<td>NT</td>
</tr>
<tr>
<td>Time (mins)</td>
<td>0</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

### 5.7 Protein Quantification

The protein samples were quantified according to the following protocol. Protein samples were diluted at 1:20 in sterile water in a separate 1.5 ml tube by adding 2 µL of each corresponding protein to 38 µL of sterile water. The dilute samples were then gently vortexed, with 10 µL/well added to a 96-well Tissue Culture Plate (Sarstedt, 83.1835) in triplicate. BSA protein standards (0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml) and a blank containing 2 µL of RIPA (same buffer used for lysis) in 38 µL of sterile water were also run in duplicate to ensure accuracy of the assay. Protein assay dye reagent was prepared by mixing one part 5x Protein Assay Dye Reagent Concentrate (Bio-Rad, #500-0006) with 4 parts of sterile water in a volume appropriate for 200 µL to be added to each well. The absorbance of the protein samples, BSA standards and blanks were measured through a spectrophotometer (BIO-TEK, Synergy HT-1, #191356) set at a wavelength of 595 nm. The average absorbance of each blank duplicate was subtracted from the average absorbance value across each protein sample triplicate and subsequently compared to the standard curve generated by the BSA standards to calculate the actual protein concentrations of each sample (See Fig. A.1 in the appendix). From these values the required volume of protein, RIPA buffer and 4x SDS buffer (Bio-Rad) needed to create a
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sample with 30 μg of protein were calculated. Each gel loading protein sample was created using the recipe determined by the previous calculations and in newly labeled 1.5 ml tube the appropriate volumes of RIPA lysis buffer, protein solution, SDS buffer were combined. The tubes were then placed in an 85°C water bath for at least 20 minutes to ensure total denaturation of proteins in and the samples were stored at -20°C until further analysis.

5.8 Western Blotting

30 μg samples (~15 μl) in SDS (Bio-Rad) loading buffer were loaded onto a 10% TGX FastCast acrylamide gel (Bio-Rad, #161-0173) and electrophoresed for ~35 mins at 200 V. Gels were then transferred to PVDF membrane using a Trans-blot Turbo Transfer System (Bio-Rad) for 7 mins (using the Mixed MW* setting) using Transfer stacks (Bio-Rad) and Transfer buffer (Bio-Rad). Membranes were then blocked in 5% skim milk powder (Zehrs) in TBS-Tween (tris-buffered saline with 0.1% tween-20) for 2 hours at room temperature on an orbital shaker. Blocking buffer was then washed away with distilled water and membranes were re-equilibrated in TBS-Tween before addition of primary antibody (Cell Signaling Technology) at a 1:1000 dilution in a solution of 5% BSA (Sigma, #A7906-500G) in TBS-T buffer with 0.2% sodium azide (Sigma, 26628-22-8). Primary antibody was applied overnight on a rocking platform at 4°C. The following day, membranes were washed with TBS-Tween three times for 15 mins, and subsequently incubated in anti-rabbit or mouse HRP-linked secondary antibody (Cell Signaling Technology) at a 1:2000 dilution in 5% skim milk powder in TBS-T buffer. Three more washes were performed as above and 2 ml of Clarity ECL substrate (Bio-Rad, #170-5060) was prepared per membrane and applied in a drop-wise manner to fully coat the membrane. The protein marker was illuminated using the Li-Cor WesternSure Pen (Mandel Scientific,
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#LIC-926-91000). Membranes were placed face down on a C-Digit blot scanner (Li-Cor) and scanned for 6 mins (standard sensitivity). Images were exported as .tif files and cropped in Photoshop (Adobe). Images from multiple experiments were combined and quantitatively analyzed by densitometry (ImageJ) comparing phosphor/total protein. Briefly, densitometry values of phosphorylated protein were divided by densitometry values of total protein, thus providing a measure of phosphorylated protein compared to total amount of that protein in the cell. Quantified band density values were further analyzed in Microsoft Excel (Microsoft Inc.), and statistical significance was determined using a two-tailed t-test with a $p \leq 0.05$ considered significant.

**Table 11:** Western Blot Antibody List.

<table>
<thead>
<tr>
<th>Type</th>
<th>Target</th>
<th>Model</th>
<th>Cell Signaling Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Phospho-Erk (Thr 202 / Tyr 204)</td>
<td>Rabbit</td>
<td>9101</td>
</tr>
<tr>
<td>Primary</td>
<td>Erk</td>
<td>Rabbit</td>
<td>4695</td>
</tr>
<tr>
<td>Primary</td>
<td>Phospho-p38 (Thr 180/Tyr 182)</td>
<td>Rabbit</td>
<td>9211</td>
</tr>
<tr>
<td>Primary</td>
<td>p38</td>
<td>Rabbit</td>
<td>8690</td>
</tr>
<tr>
<td>Primary</td>
<td>Phospho-JNK (Thr 183 / Tyr 185)</td>
<td>Rabbit</td>
<td>9251</td>
</tr>
<tr>
<td>Primary</td>
<td>JNK</td>
<td>Rabbit</td>
<td>9252</td>
</tr>
<tr>
<td>Primary</td>
<td>Phospho-Akt (S473)</td>
<td>Rabbit</td>
<td>9271</td>
</tr>
<tr>
<td>Primary</td>
<td>IκBα</td>
<td>Mouse</td>
<td>4814</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td>Rabbit</td>
<td>7074</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td>Mouse</td>
<td>7076</td>
</tr>
</tbody>
</table>
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5.9 RNA Isolation

2-3 x10^6 BMMCs per condition were sensitized, washed, treated and stimulated as described above; however time points were 15, 30, 60, 180 and 300 mins and RNA was isolated using the RNeasy Plus kit (Qiagen, 74136). When each subsequent time period finished the tubes were centrifuged in a Thermo Electron Corporation Centrifuge (CentraCL3R, #3755-1851) at 1500 rpm for 5 minutes at 4° C to pellet the cells. The supernatant was aspirated off and the cell pellet was lysed with 350 µL of RLT Plus RNeasy Plus lysis buffer with 10 µl/ml 2-mercaptoethanol added. Following lysis the homogenous lysate was transferred to a gDNA elimination column, to ensure any genomic DNA is removed from the sample and centrifuged in a Labnet International Inc. Centrifuge (Prism-R, # C2500-R) for 30 sec at 10,000 rpm. The flow through was kept and mixed with 350 µL of 70% ethanol and this entire volume was transferred to the RNeasy spin column.
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included in the kit and processed with a series of wash buffers as described by the manufacturer. The RNeasy spin column was then moved to a new 1.5 ml tube and 40 µl of RNase-free water was added directly to the membrane to act as an elution buffer and RNA was eluted by centrifugation for 1 min at 10,000 rpm. RNA samples generated were stored at -80°C until future use.

**Table 12:** Representative experimental design for RNA expression through qPCR.

<table>
<thead>
<tr>
<th>qPCR</th>
<th>Control</th>
<th>25 µg/ml RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 3</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Time (mins)</td>
<td>TNP + SCF</td>
<td>TNP + SCF</td>
</tr>
<tr>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
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<td>300</td>
<td>120</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>300</td>
</tr>
</tbody>
</table>

5.10 Quantitative Polymerase Chain Reaction (qPCR)

Following extraction, the RNA content was quantified using the NanoVue Plus spectrophotometer (General Electric Healthcare, #28956016). The concentration determined by spectrophotometer was used to calculate the appropriate volumes of RNA sample and molecular grade water (MgH₂O)(Sigma, #W4502) required for cDNA generation. The calculated volumes were combined into double-primed EcoDry RNA to cDNA reaction tubes (Clontech, #639549) and placed into a SimpliAmp Thermal Cycler (Applied Biosystems, #228001548) to facilitate the generation of cDNA. The resultant cDNA was diluted to 1:20 in molecular grade H₂O (mgH₂O). qPCR reaction solutions were made by combining 5 µl of the KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, #KM4103), 0.2 µl of each of the forward and the reverse primers (designed using Primer-BLAST from NCBI and optimized by testing in a serial dilution of cDNA to have 90-110% amplification efficiency; stored at -20°C in 10 µM stocks), and 3.6 µl of mgH₂O. The solution was then distributed into a MicroAmp 96-well plate (Applied Biosystems, 4346907) with a volume of 9 µl per well. Subsequently 1 µl of 1:20 diluted
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cDNA generated from each time point was added to each well resulting in each well containing a 10 μl final reaction volume. The qPCR reaction plate was then sealed with a MicroAmp optical adhesive film (Applied Biosystems, #201602-363) and placed in a StepOnePlus Real-Time PCR System (Applied Biosystems, #272004476) and the included software was used to run the plate using the following cycling conditions: 3 min @ 95°C followed by 5 sec @ 95°C and 30 sec @ 60°C; this protocol was carried out for 40 cycles, followed by a melting curve stage to test amplicon specificity. Briefly, the melt curves are used to determine whether a single product is being amplified or if multiple unspecific products are produced. For melt curves representing a single product then a single peak will be observed which is based on the temperature required to break the C:G and A:T bonds in the target DNA; this ratio will be different for each product. However, during early timepoints where often times little expression of a gene of interest is seen, primer dimerization or unspecific amplification due to unspecific binding is observed. This will appear as multiple bands on the melt curve (See Fig. A.2-A.15 in the appendix). The threshold value of the amplification plot was adjusted accordingly prior to exporting the threshold cycle (Ct) values. The threshold value was set at 0.2, which was based on approximately a 50% cut-off in the linear range of the amplification plots for the genes of interest. Using the Ct values generated for the primers of interest as well as the Ct values for HPRT housekeeping gene a ∆∆Ct analysis [108] was performed to determine the fold change of expression for a given gene of interest (See Fig. A.16 in the appendix).
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Table 13: qPCR Primer List.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Direction</th>
<th>Sequence</th>
<th>NCBI Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIL6_439</td>
<td>IL-6</td>
<td>Forward</td>
<td>AGACAAAGCCAGAGTCCTTCAGAGA</td>
<td>NM_031168</td>
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<tr>
<td>mIL6_599</td>
<td>IL-6</td>
<td>Reverse</td>
<td>TGGTCTTTGGTCTTTAGCCACTCC</td>
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<tr>
<td>mTNF_319</td>
<td>TNF</td>
<td>Forward</td>
<td>TGAACCTCCGGGGTGATCGGTCC</td>
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<td>TNF</td>
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<td>IL-13</td>
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<td>IL-13</td>
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<tr>
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<td>IkBa</td>
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<td>CAGTCCAGCAATGGGCACATCA</td>
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<td>HPRT</td>
<td>Forward</td>
<td>CTTGCTGGTGAAAAGGACCTCTCG</td>
<td>NM_013556.2</td>
</tr>
<tr>
<td>mHPRT_811</td>
<td></td>
<td>Reverse</td>
<td>CGCTCATCTTACGCTTTGGATTTGG</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4: Representative figure for qPCR experimental protocol. (Unpublished, M. Yousef 2018).

5.11 Enzyme Linked Immunosorbent Assay (ELISA)

2 x 10^6 BMMCs per condition were sensitized, washed, treated and stimulated as described above, however time points were 1, 3, 6, 12, and 24 hrs. At the end of each time point cells were centrifuged in a Labnet International Inc. Centrifuge (Prism-R, # C2500-R) at 3500 rpm for 10 mins to pellet the cells, the supernatant (~ 1 ml) was then collected and placed into a 1.5 ml tube. Samples were stored at -80°C. 50 µL of capture Antibody was added to each well on a Nunc MaxiSorp flat-bottom 96-well plate (Thermo Fisher
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Scientific, #44-2404-21). The plate was then sealed and incubated at 4° C overnight. The following day the entire volume of liquid in each well was discarded, and the wells were washed three times by adding 200-300 µL of 0.01% PBS-T using a manual plate washer (VWR) followed by the full volume being discarded. To ensure all liquid was removed the plate was inverted and blotted against a clean paper towel. The plate was then blocked by adding 100 µL of blocking buffer and incubated for 1 h at RT followed by the same discard/wash procedure. Following plate preparation 50 µL of sample or standard solutions was added to each well and the plate was sealed and incubated overnight at 4°C (See Fig. A.17-A.22). Following incubation, the discard/wash procedure was carried out and 100 µL of Detection Antibody was added to each well and the plate was again sealed and incubated for 1 h at RT followed by another discard/wash cycle. 50 µL of Streptavidin-HRP was then added to each well and the plate was sealed, covered from direct light and incubated for 30 mins at RT, followed by another discard/wash cycle. Following this final plate wash 100 µL of BD OptEIA TMB Substrate (BD Biosciences, #555214) was added to each well and the plate was again sealed, covered from direct light and incubated for at least 30 mins at RT. Immediately following this incubation 50 µL of 0.3 M H₂SO₄ was added to each well, the plate was tapped to gently mix and then the optical density was measured on a spectrophotometer (BIO-TEK, Synergy HT-1, #191356) at a 450 nm wavelength.

Table 14: Representative experimental design for mediator release through ELISA.

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Control</th>
<th>25 µg/ml RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 6</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Time (hours)</td>
<td>0 1 3 6 12 24</td>
<td>0 1 3 6 12 24</td>
</tr>
</tbody>
</table>
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5.12 Gel Electrophoresis

To generate a qualitative representation of primer specificity, DNA electrophoresis was carried out. A 1.8% agarose gel was prepared by combining 1.8 g of agarose (Sigma, 9012-36-6), to 100 ml of sodium boric acid buffer (8 g NaOH, (Sigma, S2770), 47 g Boric Acid (Bio Basic Canada Inc., BB0044), Millipore H₂O was then added until a final volume of 1L was reached, pH adjusted to 8.0). The agarose solution and flask was weighed before being microwaved for ~2 min until the solution became homogenous and all visible agarose particles were dissolved. Once allowed to cool, the total weight was returned to its initial weight before heating with Millipore water (Baxter, JF7624) to ensure the finished gel maintained 1.8% agarose. Once the volume was corrected 5 µl of 10 mg/ml ethidium bromide (EtBr, Bio Basic Canada Inc., D0197) was added and the solution was poured into the gel mold apparatus with appropriate well combs in place. Visible bubbles were removed with a needle and the gel was allowed to solidify for ~30 mins at RT. Once the gel was ready, the combs were removed, and the gel was placed into the electrophoresis apparatus (Bio-Rad) which was pre-filled with sodium boric acid buffer. To prepare the qPCR samples for electrophoresis 1.1 µl of 10X DNA load dye (Biobasic, GM303) was added to each well and mixed thoroughly. 6 µl of each sample and DNA ladder was then loaded into the wells of the gel and it was electrophoresed 17 min at 200V. Ethidium bromide fluorescence was detected on an Alpha GelDoc instrument (FluChem, #5500)

5.13 β-hexosaminidase Assay: Measurement of Mast Cell Degranulation

Preparation of the required solutions was completed: 0.1 M citrate buffer was made by dissolving 1.114 g of citric acid (Bio Basic Canada Inc., #C2123) and 1.368 g of sodium citrate (Bio Basic Canada Inc., #CB0035) into 100 ml of Millipore water and adjusting the
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pH to 4.5, for p-NAG, 100 ml of the 0.1 M citrate buffer was added to 34.2 mg of P-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG)(Sigma, #N9376); 0.1 M carbonate buffer was made by dissolving 1.060 g of sodium carbonate (Bio Basic Canada Inc., #SDB0615) and 0.840 g of sodium bicarbonate (Bio Basic Canada Inc., #SB0482) into 100 ml of Millipore water, testing to ensure pH ~10.5. Hanks Balanced Salt Solution (HBSS) buffer-BSA was made by dissolving 0.5 g of BSA into 500 ml of HBSS (gibco by Life Technologies, #14025-092) and filter sterilizing. 1% Octyl Phenoxypolyethoxyethanol-40 (NP-40) solution was made by combining 50 µl of NP-40 (Bio Basic Canada Inc., #NDB0385) with 5 ml of HBSS buffer. To begin the experiment BMMCs were sensitized with IgE overnight and washed as described above except cold HBSS buffer was used instead of RPMI-1640. Following the wash cycles the cells were resuspended to a final density of 2 x10^6 cells/ml in HBSS buffer and 100 µl aliquots were added to a sterile 1.5 ml tubes in duplicate. TNP-BSA and SCF were diluted to a concentration of 200 ng/ml and 100 µl and 10 µl, respectively, were added to each treatment condition, or 100 µl HBSS buffer for control reactions. The tubes were incubated for 20 mins in the cell culture incubator at 37°C, 95% humidity and 5% CO2 and following incubation they were centrifuged in a Labnet International Inc. Centrifuge (Prism-R, # C2500-R) for 10 min at 1500 rpm to pellet cells. The supernatant was then drawn away from the pellet and was transferred to a separate 1.5 ml tube and 200 µl of 1% NP-40 buffer was used to resuspend the pellet and all tubes were incubated for 10 mins at room temperature. Following incubation tubes were centrifuged for 5 mins at 3400 rpm at room temperature and then 50 µl of each sample was transferred to a 96-well Tissue Culture Plate (Sarstedt, 83.1835) along with 4 wells containing only HBSS to use as blanks. 50 µl of 1mM p-NAG solution
Inhibition of IgE-FceRI activated mast cells by rosemary extract was then added into each well and the plate was incubated in the cell culture incubator for 2 hours. Following incubation 200 µl of the 0.1 M carbonate buffer was added to each well to stop the reaction and the plate was then analyzed on a spectrophotometer (BIO-TEK, Synergy HT-1, #191356) at 405 nm wavelength. The absorbance values were used in the following equation to determine the percent granule release by the BMMCs.

In addition, the β-hexosaminidase assay was utilized to measure whether RE or its polyphenolic components were interacting with either TNP-BSA or mSCF (See Fig. A.23 in the appendix). Increasing concentrations of TNP-BSA and mSCF were used to stimulate BMMCs, following which 10 µg/ml RE were added then degranulation was measured, indicating that the percent inhibition with RE treatment under increasing concentrations of TNP-BSA and mSCF was unaffected.

\[
\text{% Release} = \frac{[\text{O.D. supernatant} - \text{O.D. background}]}{[\text{O.D. supernatant} - \text{O.D. background}] + [\text{O.D. pellet} - \text{O.D. background}]} \times 100
\]

**Table 15:** Representative experimental design for degranulation through β-Hexosaminidase assay.

<table>
<thead>
<tr>
<th>β-Hex</th>
<th>Control</th>
<th>1, 5, 10, 25, 40, or 50 µg/ml RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 3</td>
<td>NT</td>
<td>TNP + SCF</td>
</tr>
<tr>
<td>Supernatant</td>
<td>x x</td>
<td>x x</td>
</tr>
<tr>
<td>Pellet</td>
<td>x x</td>
<td>x x</td>
</tr>
</tbody>
</table>

**5.14 Flow Cytometry**

0.5 x10^6 BMMCs were collected per staining condition and transferred to a 15 ml conical tube. The cells were centrifuged for 10 min at 1300 rpm and 4°C and the supernatant was discarded. The BMMCs were then washed with 6 ml IF buffer (PBS with 0.2% NaN₃ and 1% BSA) and centrifuged again for 5 mins at 1500 rpm and 4°C. Supernatant was discarded and cells were resuspended in 1 ml of IF buffer and transferred
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to a 1.5 ml tube and centrifuged in a microcentrifuge for 5 min at 3500 rpm at 4°C. Cells were resuspended at a density of 0.5 x10⁶ cells/ml and 100 µl per staining condition were aliquoted into a 1.5 ml tubes. Lights were turned off and fluorochrome conjugated antibodies were added as per manufacturer concentration guidelines, and the tubes were incubated on ice for 1 hr. Following incubation, the cells were centrifuged at 3500 rpm for 5 min at 4°C, and the supernatant was discarded. The cells were then washed in 600 µl of IF buffer and centrifuged again at 3500 rpm for 5 min at 4°C. Supernatant was discarded and cells were fixed in 400 µl of 0.37 % formalin (1:100 dilution of 37% formaldehyde in 1x PBS). Cells were shielded from light and analyzed on a Sony SH800S cell sorter (Sony Biotechnology).

Table 16: Flow Cytometer Antibody List.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Laser</th>
<th>Amount Used / 0.5 x 10⁶ cells</th>
<th>Sony Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse FceRIα</td>
<td>PE</td>
<td>0.2 µg (1 µl)</td>
<td>1271535</td>
</tr>
<tr>
<td>Anti-mouse c-Kit</td>
<td>FITC</td>
<td>0.5 µg (1 µl)</td>
<td>1129025</td>
</tr>
<tr>
<td>Armenian Hamster IgG (Isotype Control)</td>
<td>PE</td>
<td>0.2 µg (1 µl)</td>
<td>2604535</td>
</tr>
<tr>
<td>Rat IgG2b (Isotype Control)</td>
<td>FITC</td>
<td>0.5 µg (1 µl)</td>
<td>2603025</td>
</tr>
</tbody>
</table>

5.15 Nuclear & Cytoplasmic Fraction Extractions

10 x10⁶ BMMCs per condition were sensitized, treated and stimulated as described above; however, time points were 1, 3, and 6 hrs and nuclear and cytoplasmic fractions were extracted using Active Motif Nuclear Extract Kit (Active Motif, 100946). Solutions required were prepared. Cells were washed with 3 ml ice-cold PBS/phosphatase inhibitors. Cells were then centrifuged (CentraCL3R, #3755-1851) for 5 mins at 200 x g at 4 °C to pellet. The supernatant was then discarded, and the pellets were placed on ice. Pellets were
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gently resuspended in 500 µl 1X hypotonic buffer, transferred to a pre-chilled 1.5 ml Eppendorf tube and incubated for 15 mins on ice. 25 µl detergent was then added and the cells were vortexed on the highest setting for 10 s. Then the cells were centrifuged (Prism-R, # C2500-R) for 30 s at 14,000 x g at 4°C. The supernatant (Cytoplasmic fraction) was then transferred to a pre-chilled 1.5 ml Eppendorf tube and stored at -80°C till further use. The pellets were then lysed in 50 µl complete lysis buffer and vortexed on the highest setting for 10 s. The lysates were then incubated in a cold room at 4°C on a vortex for 30 mins at ~150 rpm. After which the cells lysates were vortexed on the highest setting for 30 s, followed by a centrifuge spin (Prism-R, # C2500-R) for 10 mins at 14,000 x g at 4°C. The supernatant (Nuclear fraction) was then transferred to a pre-chilled 1.5 ml Eppendorf tube and stored at -80°C till further use. The protein concentration of the nuclear fractions was then determined using the same protein quantification protocol described previously.

5.16 NFκB Transcription Factor Activity Assay

The transcription factor assay was performed using the TransAM NFκB activity assay kit (Active Motif, 102092). Solutions required were prepared. To begin, 30 µl complete binding buffer was added to each well, which is coated with an oligonucleotide sequence that the activated transcription factor would bind to, then 20 µl of sample (2-20 µg of nuclear fraction protein diluted in complete lysis buffer) was added to the appropriate wells, blank wells had 20 µl complete lysis buffer added. The plate was sealed with an adhesive cover and incubated for 1 hr at RT on a rocking platform at 100 rpm. Following the incubation, the plate was washed 3 X with 200 µl 1X wash buffer/well. Then 100 µl of diluted NFκB antibody (1:1000 DF in antibody binding buffer) was added to each well. The plate was once again covered and incubated for 1 hr
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at RT without agitation. The plate was washed 3 X with 200 µl 1X wash buffer/well. Then 100 µl of diluted HRP-conjugated antibody (1:1000 DF in antibody binding buffer) was added to each well. The plate was once again covered and incubated for 1 hr at RT without agitation. The plate was washed 3 X with 200 µl 1X wash buffer/well. Then 100 µl developing solution was added to each well. The plate was then incubated at RT and covered from direct light. Blue color development was monitored until it began turning a medium to dark blue, at which point 100 µl stop solution was added to each well and the absorbance of the plate was read at 450nm on a spectrophotometer (BIO-TEK, Synergy HT-1, #191356). The absorbance values were then used to determine activity of NFκB.

5.17 Statistics

Quantified data of various readouts (band densities for Western blots, ∆∆Ct values for qPCR etc.) were analyzed and compared between the untreated (NT) cells and various time points in both control and RE treated groups. The difference in values between control and treated groups was compared using two-tailed paired t-test and a $p \leq 0.05$ was considered significant. A two-tailed paired t-test was used in order to not infer a direction (increase or decrease) with RE treatment.
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6. Results:

6.1 Mast Cell Isolation, Differentiation, and Growth

Mast cells are derived from hematopoietic stem cell progenitors in the bone marrow and enter various tissues where they become terminally differentiated. Terminally differentiated mast cells continually express high levels of c-Kit (CD117), the receptor for SCF, and FcεRI, the high affinity immunoglobulin E receptor. The expression of both of these is well established to be a marker of a mast cell population, as discussed previously. Therefore, we sought to determine whether our hematopoietic stem cell isolation, differentiation and culture conditions were in fact producing a healthy and robust population of mast cells. Cell staining protocol and flow cytometer use was performed as described above. Flow cytometry analysis demonstrated that there is a shift to the right, indicative of an increase in cell surface levels of both FcεRIα and c-Kit, while the controls demonstrated relatively low levels of expression indicating that there is a minimal level of antibody isotype cross-reactivity (fluorescence). Analysis demonstrated that 98.53% of cells expressed high levels of FcεRIα and 98.44% of cells expressed high levels of c-Kit (Fig. 5). These results demonstrate that our isolation, differentiation and culture conditions are in fact producing a healthy and robust population of mast cells.
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**Figure 5:** Representative flow cytometer histograms used to assess cell surface expression of FcεRIα and c-Kit in C57BL/6J BMMCs.

- IgG Antibody Isotype Control
- Stained Cells
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6.2 RE Effects on the MAPK Pathway

Rapid MAPK activation is crucial for mast cell function in the context of both normal and pathological responses. Thus, modulation of the MAPK pathway would be an advantage to a therapeutic agent. To assess the effects of RE on the MAPK pathway, mature BMMCs were sensitized with allergen-specific IgE and stimulated through the FcεRI and c-Kit receptors with their cognate ligands TNP-BSA and SCF respectively, in the presence of RE at 0, 5 or 25 µg/ml for 0, 5, 20, and 60 mins. At each time point, protein lysates were prepared, quantified, and western blotting protocol was followed as described above. As expected from the literature, rapid phosphorylation of all MAPK proteins under normal stimulation was seen. ERK and p38 (Fig. 6, 7, 8, 9) phosphorylation peaked by 5 mins, whereas JNK (Fig. 10, 11) phosphorylation peaked by 20 mins. BMMCs treated with 5 or 25 µg/ml RE had no significant reduction in ERK phosphorylation across all time points (Fig. 6, 7). p38 phosphorylation was significantly reduced at 5 mins by both 5 (p < 0.05) and 25 (p < 0.05) µg/ml RE but had no significant differences at 20 or 60 mins (Fig. 8, 9). JNK phosphorylation was reduced at 20 mins by treatment with 5 (ns) and 25 (p < 0.01) µg/ml RE but had no significant differences at 5 or 60 mins (Fig. 10, 11). In addition, RE treatment did not alter the phosphorylation of different isoforms of either ERK or JNK within time-points (Fig. 6, 7, 10, 11). These findings indicate that RE is capable of modulating the activity of MAPK in FcεRI-IgE and c-Kit activated mast cells.
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**Figure 6:** Representative western blots used to assess ERK phosphorylation in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF with or without the addition of 5 or 25 µg/ml RE for the indicated times. Total ERK protein levels were used for analyses.
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Figure 7: Relative phosphorylation of ERK as calculated by band densitometry (ImageJ) in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 5 or 25 µg/ml RE to control samples. Results are expressed as mean relative phosphorylation ±SEM compared to BMMCs not treated with RE. n = 3.
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**Figure 8:** Representative western blots used to assess p38 phosphorylation in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF with or without the addition of 5 or 25 µg/ml RE for the indicated times. Total p38 protein levels were used for analyses.
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Figure 9: Relative phosphorylation of p38 as calculated by band densitometry (ImageJ) in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 5 or 25 µg/ml RE to control samples. Results are expressed as mean relative phosphorylation ±SEM compared to BMMCs not treated with RE. n = 3. * p < 0.05.
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**Figure 10:** Representative western blots used to assess JNK phosphorylation in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF with or without the addition of 5 or 25 µg/ml RE for the indicated times. Total JNK protein levels were used for analyses.
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Figure 11: Relative phosphorylation of JNK as calculated by band densitometry (ImageJ) in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 5 or 25 µg/ml RE to control samples. Results are expressed as mean relative phosphorylation ±SEM compared to BMMCs not treated with RE. n = 3. ** p < 0.01.
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6.3 RE Effects on the NFκB and PI3K-Akt Pathways

IkBα protein levels can be used as a proxy measure of NFκB pathway activity. IkBα typically sequesters NFκB in the cytoplasm, however when IkBα is phosphorylated by IKK, it becomes degraded and thus releases NFκB. Once NFκB is released it is then free to enter into the nucleus where it works as a transcription factor for pro-inflammatory mediator production. The PI3K-Akt pathway is another major contributor to pro-inflammatory mediator production and has cross-talk with both the MAPK and NFκB pathways. To assess the effects of RE on the NFκB and PI3K-Akt pathways, mature BMMCs were sensitized with allergen-specific IgE and stimulated through the FcεRI and c-Kit receptors with their cognate ligands TNP-BSA and SCF respectively, in the presence of RE at 0, 5 or 25 µg/ml for 0, 5, 20, and 60 mins. At each time point, protein lysates were prepared, quantified, and western blotting protocol was followed as described above.

BMMCs treated with 5 µg/ml RE had no significant reduction in total IkBα levels across all time points, however there was a reduction at 20 and 60 mins (Fig. 12, 13). Whereas treatment with 25 µg/ml RE reduced total levels of IkBα across all time points and reached significance (p < 0.05) at 5, 20, and 60 mins (Fig. 12, 13). Consistent with the literature, Akt phosphorylation in the control samples was significantly induced by 20 mins. Treatment with 5 µg/ml RE reduced Akt phosphorylation across all time points, but never reached significance (Fig. 14, 15). However, treatment with 25 µg/ml RE reached significant levels of Akt inhibition at 5 (p < 0.05), 20 (p < 0.001), and 60 (p < 0.05) mins (Fig. 14, 15). These findings indicate that RE is not only capable of modulating the activity of MAPK, but also both the NFκB and PI3K-Akt pathways in FcεRI-IgE and c-Kit activated mast cells.
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**Figure 12:** Representative western blots used to assess total levels of IκBα in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF with or without the addition of 5 or 25 µg/ml RE for the indicated times. Total IκBα protein levels were used for analyses.
Figure 13: Total levels of IкBα as calculated by band densitometry (ImageJ) in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 5 or 25 µg/ml RE to control samples. Results are expressed as mean relative phosphorylation ±SEM compared to BMMCs not treated with RE. n = 3. * p < 0.05.
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**Figure 14:** Representative western blots used to assess Akt (S473) phosphorylation in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF with or without the addition of 5 or 25 µg/ml RE for the indicated times. Ponceau staining was used for analyses.
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**Figure 15:** Relative phosphorylation of Akt as calculated by band densitometry (ImageJ) in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 5 or 25 µg/ml RE to control samples. Results are expressed as mean relative phosphorylation ±SEM compared to BMMCs not treated with RE. n = 3. * p < 0.05, *** p < 0.001.
6.4 RE Effects on the Gene Expression of Pro-Inflammatory Cytokines and Chemokines

Following the significant inhibitory effects RE exhibited on the MAPK, NFκB, and PI3K-Akt pathways at the protein level, we sought to determine whether the gene-expression of the cytokines and chemokines downstream of those signaling pathways were affected. Those mediators included IL-6, TNF, IL-13, CCL1, CCL2, CCL3, and CCL9. To assess the effects of RE on the gene expression of pro-inflammatory mediators, mature BMMCs were sensitized with allergen-specific IgE and stimulated through the FcεRI and c-Kit receptors with their cognate ligands TNP-BSA and SCF respectively, in the presence of RE at 0 or 25 µg/ml for 0, 15, 30, 60, 120, and 300 mins. At each time point RNA was collected for cDNA generation and qPCR protocol was followed as described above. mRNA levels of IL-6, TNF, IL-13, CCL2, and CCL3 were significantly induced by 60 mins, mRNA levels of CCL1, and CCL9 were significantly induced by 120 mins (Fig. 16–22). Treatment with 25 µg/ml RE reduced IL-6 mRNA at 60 (p < 0.05), and 120 (p < 0.05) mins (Fig. 16), IL-13 mRNA at 60 (ns), and 120 (p < 0.05) mins (Fig. 17), TNF mRNA at 60 (ns), and 120 (p < 0.05) mins (Fig. 18), CCL1 mRNA at 60 (p < 0.01), and 120 (p < 0.05) mins (Fig. 19), CCL2 mRNA was unaffected (Fig. 20), CCL3 mRNA at 60 (p < 0.05), and 120 (p < 0.05) mins (Fig. 21), CCL9 mRNA at 60 (ns), 120 (p < 0.01), and 300 (ns) mins (Fig. 22). These findings demonstrate that the results observed by RE on protein modulation of the MAPK, NFκB, and PI3K-Akt pathways translate into the gene expression of several mast cell-derived pro-inflammatory cytokines and chemokines involving these pathways.
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Figure 16: qPCR quantification of IL-6 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3. * p < 0.05.
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Figure 17: qPCR quantification of TNF mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3. * p < 0.05.
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**Figure 18:** qPCR quantification of IL-13 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3, * p < 0.05.
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Figure 19: qPCR quantification of CCL1 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3, * p < 0.05, ** p < 0.01.
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Figure 20: qPCR quantification of CCL2 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3.
Figure 21: qPCR quantification of CCL3 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3. * p < 0.05.
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**Figure 22:** qPCR quantification of CCL9 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3. **p < 0.01.**
6.5 RE Effects on the Gene Expression of Transcription Factors

Transcription factors play a crucial role in pro-inflammatory responses by linking protein signaling to gene expression and eventual mediator production. Therefore, we sought to measure the gene expression of transcription factors involved in linking the MAPK, NFκB, and PI3K-Akt pathways to the production of the pro-inflammatory cytokines and chemokines presented above. mRNA levels of c-Jun, Egr1, and Egr2 were significantly induced by 30 mins. mRNA levels of NFκBIA, and Rcan1 were significantly induced by 60 mins (Fig. 23 – 27). Treatment with 25 µg/ml RE appeared to reduce c-Jun mRNA at 30 (ns), 60 (ns), and 120 (ns) mins (Fig. 23), Egr1 and Egr2 mRNA (Fig. 24, 25) was unaffected by RE treatment, NFκBIA mRNA was reduced at 60 (ns), and 120 (p < 0.05) mins (Fig. 26), Rcan1 mRNA at 60 (ns), and 120 (ns) mins (Fig. 27). These findings were disappointing considering the significant effects observed at the protein level and pro-inflammatory mediator mRNA levels, however it was noted that NFκBIA (the gene for IκBα) as well as Rcan1 (a negative regulator of mast cells) were both drastically inhibited.
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**Figure 23**: qPCR quantification of c-Jun mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3.
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Figure 24: qPCR quantification of Egr1 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3.
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**Figure 25:** qPCR quantification of Egr2 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3.
Figure 26: qPCR quantification of NFκBIA mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3. * p < 0.05.
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Figure 27: qPCR quantification of Rcan1 mRNA expression in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean fold change ±SEM RE-treated compared to untreated BMMCs. n = 3.
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6.6 RE Effects on the Production and Release of Pro-Inflammatory Cytokines and Chemokines

Given that significant modulation of protein signaling, and pro-inflammatory mediator gene expression was observed with RE treatment, we decided to measure if those results translated into the actual production and release of those mediators. To assess the effects of RE on the production and release of pro-inflammatory mediators, mature BMMCs were sensitized with allergen-specific IgE and stimulated through the FcεRI and c-Kit receptors with their cognate ligands TNP-BSA and SCF respectively, in the presence of RE at 0 or 25 µg/ml for 0, 1, 3, 6, 12, and 24 hrs. At each time point the cytokine supernatant was collected and ELISA protocol was followed as described above. In the control samples, significant levels of IL-6, TNF, IL-13, CCL1, CCL2, and CCL3 were observed by 3 hrs (Fig. 28 – 33). Treatment with 25 µg/ml RE reduced IL-6 levels at 3 (ns), 6 (ns), 12 (p < 0.05), and 24 (p < 0.05) hrs (Fig. 28), TNF levels at 6 (ns), 12 (ns), and 24 (p < 0.05) hrs (Fig. 29), IL-13 levels at 3 (ns), 6 (p < 0.05), 12 (ns), and 24 (p < 0.05) hrs (Fig. 30), CCL1 levels at 3 (ns), 6 (ns), 12 (p < 0.05), and 24 (p < 0.05) hrs (Fig. 31), CCL2 levels at 1 (p < 0.01), 6 (ns), 12 (ns), and 24 (ns) hrs (Fig. 32), CCL3 levels at 1 (p < 0.05), 3 (p < 0.01), 6 (p < 0.001), 12 (p < 0.05), and 24 (p < 0.05) hrs (Fig. 33). These results altogether align with the data presented above demonstrating that RE is capable of inhibiting not just protein and mRNA, but also production and release of the MAPK, NFκB, and PI3K-Akt pathway driven pro-inflammatory mediators. In addition, all of the results presented thus far indicate that RE is responsible for mediating impairments in mast cell responses and functions that drive late-phase allergic inflammation.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

**Figure 28:** ELISA assay quantification of IL-6 concentration in the supernatant of C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 6. * p < 0.05.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

Figure 29: ELISA assay quantification of TNF concentration in the supernatant of C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 6. * p < 0.05.
Inhibition of IgE-FceRI activated mast cells by rosemary extract

**Figure 30:** ELISA assay quantification of IL-13 concentration in the supernatant of C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 6. * p < 0.05.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

Figure 31: ELISA assay quantification of CCL1 concentration in the supernatant of C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 6. * p < 0.05.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

**Figure 32:** ELISA assay quantification of CCL2 concentration in the supernatant of C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 6. **p < 0.01.**
Inhibition of IgE-FceRI activated mast cells by rosemary extract

**Figure 33:** ELISA assay quantification of CCL3 concentration in the supernatant of C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 6. * p < 0.05, ** p < 0.01, *** p < 0.001.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

6.6 RE Effects on Degranulation

Following the profound effects of RE on the late-phase reaction of mast cells, we sought to determine if RE is also capable of modulating the early-phase reaction. To assess the effects of RE on the early-phase, mature BMMCs were sensitized with allergen-specific IgE and stimulated through the FcεRI and c-Kit receptors with their cognate ligands TNP-BSA and SCF respectively, in the presence of RE at 0, 1, 5, 10, 25, 40 or 50 µg/ml for 20 mins. After 20 mins the β-hexosaminidase assay protocol was followed as described above. In the control samples, mast cells degranulated robustly (20 – 40% degranulation), each WT was normalized and set to 100%, treatment samples were then compared back to their respective normalized control. Treatment with all concentrations of RE was capable of significantly inhibiting mast cell degranulation (p < 0.001) (Fig. 34). Interestingly, the dose-dependent response of RE on mast cell degranulation seemed to reach a plateau around 25 µg/ml RE (Fig. 34), which prompted us to use that same concentration in our study of the late-phase response of mast cells all while maintaining relatively low concentrations of RE and physiological relevance. These results demonstrated that RE is capable of significantly inhibiting the early-phase response of mast cells.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

**Figure 34:** β-hexosaminidase assay showing the percent granule release in C57BL/6J BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with various concentrations of RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 3. * p < 0.05, *** p < 0.001.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

6.7 RE Effects on NFκB Transcription Factor Activity

Thus far, RE has demonstrated a potent and significant effect on both the early-phase degranulation of mast cells and the late-phase protein signaling, gene expression, and cytokine production and release that help drive pro-inflammatory responses in allergen activated mast cells. However, the effect on transcription factors which serve as bridges between protein signaling and mediator production did not represent the profound effects we had observed. Therefore, we decided to measure the transcription factor activity of NFκB, to determine whether the reduction in total IκBα levels observed at the protein level (Fig. 12, 13) and mRNA (Fig. 26) was resulting in more NFκB release and translocation into the nucleus, thus working as a compensatory mechanism to counteract the other significant effects RE was exhibiting on allergen activated mast cells. To assess the effects of RE on NFκB transcription factor activity, mature BMMCs were sensitized with allergen-specific IgE and stimulated through the FcεRI and c-Kit receptors with their cognate ligands TNP-BSA and SCF respectively, in the presence of RE at 0 or 25 µg/ml for 0, 1, 3, and 6 hrs. At each time point the nuclear and cytoplasmic fractions were extracted and the NFκB transcription factor assay protocol was followed as described above. In the control samples, NFκB activity was significantly upregulated at 1 hr. Treatment with RE at 25 µg/ml resulted in a significant inhibition in NFκB activity at 1 (p < 0.01) hr (Fig 35). These results demonstrated that although transcription factor levels may not be significantly impacted by RE treatment, activity of NFκB, a vital pro-inflammatory transcription factor, was significantly inhibited. In addition, these data can provide a strong rationale for the drastic effects observed at the protein, mRNA and mediator release levels described above.
Inhibition of IgE-FceRI activated mast cells by rosemary extract

**Figure 35:** Transcription factor activity assay showing the activity of NFκB in the nuclear fractions of C57BL/GJ BMMCs stimulated with 100 ng/ml TNP-BSA and 100 ng/ml SCF comparing samples treated with 25 µg/ml RE to control samples. Results are expressed as mean concentrations ±SEM RE-treated compared to untreated BMMCs. n = 3. ** p < 0.01.
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7. Discussion:

7.1 RE Modulates the MAPK, and PI3K-Akt Signaling Pathways in Allergen Activated Mast Cells

The effects of RE and its polyphenolic components on the MAPK pathway have been well documented in the literature, showing significant modulation in various other cell types and disease contexts, such as in hepatocytes, melanoma, and macrophages [86,109–111]. Similarly, the PI3K-Akt pathway has also been shown to be inhibited by RE and its polyphenolic components in other contexts, such as lung cancer, breast cancer, hepatocellular carcinoma, and inflammation [41,112–114]. Given the vital role of these pathways in mediating inflammation, it was hypothesized that if RE was to work as a therapeutic agent, then it would be vital for it inhibit these central pathways in allergen activated mast cells. The results demonstrated above provide strong evidence to indicate that RE is capable of inhibiting the MAPK pathway, shown through a significant inhibition of p38 and JNK following allergen stimulation in mast cells (Fig. 8 – 11). Akt phosphorylation was significantly inhibited as well by RE treatment in our study (Fig. 14, 15). The inhibition of the MAPK and PI3K-Akt pathways is of crucial importance to inhibiting the pro-inflammatory process that occurs in allergen activated mast cells. Both signaling pathways are involved in activation of downstream transcription factors, cytokines, and chemokines which work to drive inflammation, tissue damage, and allergy at the site of insult [26,28,29,115]. To our knowledge, this is the first study to determine the effects of RE on the MAPK and PI3K-Akt pathways in the context of IgE-FceRI activated mast cells. Thus far RE has demonstrated a potent ability to inhibit pro-inflammatory signaling in allergen activated mast cells and thus warranted further investigation into its potential as a therapeutic agent.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

7.2 RE Reduces Gene Expression of Key Pro-Inflammatory Mediators in Allergen Activated Mast Cells

Given the significant effects RE exhibits on the MAPK and PI3K-Akt pathways, we hypothesized that their downstream pro-inflammatory mediator genes would also be significantly inhibited. Gene expression is commonly used as a screening tool for disease, such as in obesity, cardiovascular disease, anemia, chronic kidney disease, and breast cancer [116–119]. Given the power of gene expression as a proxy measure of disease prognosis and severity, we sought to measure important cytokines and chemokines in the pro-inflammatory process using qPCR. All pro-inflammatory genes which are induced following allergen stimulation of cells measured, with the exception of CCL2 (Fig. 20), including IL-6, TNF, IL-13, CCL1, CCL3, and CCL9 (Fig. 16 – 19, 21, 23) were significantly inhibited by treatment with RE. CCL2 expression was unaffected by RE treatment, however this could be due to compensatory mechanisms in the cell, or through differential control by various signaling pathways. There is literature that indicates that CCL2 is more directly under the influence of ERK compared to the other MAPKs such as p38 and JNK. Given that ERK phosphorylation was unaffected by RE treatment, this could be another mechanism behind the stabilization of CCL2 levels [120–122]. All together, these results indicate that the majority of pro-inflammatory mediators influenced by the MAPK and PI3K-Akt pathways are inhibited, demonstrating the translation of protein level modulation to gene expression modulation by RE. Next, we sought to measure whether the effects observed thus far at the gene expression level would translate to the actual production and release of those same mediators.
7.3 RE Inhibits Production and Release of Key Pro-Inflammatory Mediators in Allergen Activated Mast Cells

Thus far RE has demonstrated an ability to inhibit pro-inflammatory protein signaling and gene expression, but arguably the ultimate test is whether RE is capable of inhibiting the production and release of induced pro-inflammatory mediators in the context of allergen activated mast cells. Therefore, we measured the concentrations of important pro-inflammatory mediators in the supernatant of allergen activated mast cells using ELISA. Treatment with RE was capable of translating gene expression modulation to mediator release. As previously mentioned, gene expression of IL-6, TNF, IL-13, CCL1, and CCL3 (Fig. 16 – 19, 21, 22) was significantly inhibited, the concentrations of these same mediators was also significantly inhibited in the supernatants of allergen activated mast cells (Fig. 28 – 31, 33). The gene expression results seen with CCL2 (Fig. 20) were also mirrored in ELISA (Fig. 32) with both showing no changes between the control and RE treated groups. These results together indicate that RE is capable of inhibiting the pro-inflammatory process of allergen activated mast cells beginning at protein signaling and ending in the actual production and release of pro-inflammatory mediators and that RE has a strong potential for use as a therapeutic agent. The results presented thus far are profound and novel. However, we decided to look further into the effects of RE on transcription factors to better understand REs mechanism of action and the direct translation of inhibition at protein signaling to gene expression to mediator production and release by RE.

7.4 RE Effects on Transcription Factor Levels and Activity in Allergen Activated Mast Cells

Transcription factors act as links between protein signaling and gene expression, which ultimately controls the production and release of pro-inflammatory mediators.
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Therefore, we sought to measure the levels of various transcription factors which may play a role in the context of allergen activated mast cells. We began with c-Jun which has been shown to be under the influence of MAPK activity [123], RE treatment did seem to reduce c-Jun mRNA levels (Fig. 23) however the inhibition never achieved statistical significance. Next, we sought to measure Egr1 transcription factor mRNA levels. Egr1 and TNF have been shown to be involved in the regulation of each other [58,124] and Egr1 has been shown to be responsible for IL-13 production in SCF activated mast cells [124,125], thus we hypothesized that Egr1 mRNA levels would be inhibited similarly to TNF (Fig. 17, 29) and that the inhibition of IL-13 (Fig. 18, 30) was due to a reduction in Egr1 levels. However, Egr1 levels were unaffected by RE treatment. This could be due to cross-talk between various pathways that differentially control the levels of Egr1, such as Egr1 levels and activity being mediated through ERK signaling [126] which was unaffected by RE treatment (Fig. 6, 7). Next, we decided to measure Egr2 mRNA levels which have been linked to increases in CCL1 levels in allergen activated mast cells [127]. However, once again Egr2 mRNA levels were unaffected which did not reflect the significant downregulation of CCL1 mRNA (Fig. 19) and ELISA concentrations (Fig. 31). Finally, we decided to measure the levels and activity of NFκB, a potent transcription factor which has been shown in the literature to be correlated with increased levels of IL-6, TNF, IL-13, CCL1, and CCL3 [128–134]. As seen in Figure 35, RE significantly reduced nuclear levels of NFκB. Furthermore, RE was able to reduce NFκB transcription factor activity in the nucleus. This finding serves as the link between the potent modulation at the protein level to the inhibition of pro-inflammatory mediator mRNA levels and ultimately to the reduction in production and release of those mediators.
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Understanding the timeline of events is also crucial at this point, protein signaling occurred in the first few mins following allergen stimulation around 5 – 20 mins (Fig. 6 – 11, 14, 15), protein signaling then caused alterations at the transcription factor level which spiked around 30 – 60 mins (Fig. 35), following which mRNA levels of their downstream targets began to be significantly expressed around 60 – 120 mins (Fig. 16 – 27), and finally ELISA cytokine supernatant concentrations were elevated around 3 hrs (Fig. 28 – 33).

7.5 RE Blocks the Activation of Allergen Activated Mast Cells

In addition to the data presented thus far, we also measured total protein levels of IκBα, mRNA levels of NFκBIA, the gene for IκBα, and mRNA levels of Rcan1, a negative regulator of mast cells [124,135]. Regularly IκBα sequesters NFκB in the cytoplasm and prevents it from translocating into the nucleus where it can increase expression of pro-inflammatory mediators. When IκBα is phosphorylated it becomes degraded (total protein decreases) and NFκB is released [43–45]. Considering the decrease in NFκB transcription factor levels and activity we hypothesized that total protein and mRNA levels of IκBα would be elevated in the RE treated groups compared to the control groups to reflect that inhibition. However, both protein (Fig. 12, 13) and mRNA levels (Fig. 26) of IκBα were inhibited by RE. Following these results, we measured mRNA levels of Rcan1, which was also inhibited by RE (Fig. 27). When looking at these results together we determined that the apparent decreases in negative cellular controls (IκBα and Rcan1) was due to the mast cells being stimulated less potently in the RE treated groups than the control groups. Another explanation could be that the protein and mRNA levels of IκBα and Rcan1 were measured using whole cell lysates whereas the NFκB levels and activity assay was performed on nuclear fractions. IκBα has been shown in the literature to be an unstable
Inhibition of IgE-FcεRI activated mast cells by rosemary extract protein, easily degraded in its free form, and only stabilized by NFκB interactions [136,137], which may explain why lower levels of IκBα have been observed given that NFκB levels and activity were also inhibited (Fig. 35).

7.6 RE Attenuates Early Phase Degranulation of Allergen Activated Mast Cells

So far all the data presented has thoroughly dissected the late phase reactions of allergic inflammation and has indicated the ability of RE to potently inhibit the late phase response in allergen activated mast cells. However, the early phase response is just as important in the context of allergic inflammation. Therefore, we measured early phase degranulation through the βhexosaminidase assay to determine if RE would have a similar effect in the early phase as it had on the late phase. Degranulation of mast cells is a very rapid event, which involves the release of pre-formed granules that contain various pro-inflammatory mediators such as histamine, TNF, and β-hexosaminidase [15,18,19]. Degranulation in the literature has been linked to activity of the PI3K-Akt pathway, PLC pathway as well as the release of intracellular calcium stores [138–140]. Although, RE demonstrated protein modulation of the PI3K-Akt pathway, we still hypothesized that degranulation would be unaffected due to the rapid timeline in which it occurred. When the β-hexosaminidase assay experiments were performed we observed that RE dose-dependently inhibited allergen mediated mast cell degranulation (Fig. 34) significantly at concentrations as low as 1 µg/ml. These results are the first to determine the effects of RE in mast cell degranulation and provide a strong rationale for the potential use and continued investigation of RE as a therapeutic agent for the treatment of allergic inflammation.
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8. Conclusions:

In summary, the presented data provided strong evidence for the ability of RE to modulate mast cell functional processes and outcomes with direct relevance to innate immunity, allergy, and inflammation as evidenced by the ability of RE to inhibit levels of major pro-inflammatory cytokines and chemokines.

1. RE and its polyphenolic components inhibited IgE-FcεRI allergen-mediated activation of the MAPK, and PI3K-Akt pathways demonstrated through a decrease in phosphorylation of p38, JNK, and Akt.

2. RE and its polyphenolic components inhibited IgE-FcεRI allergen-mediated activation of transcription factors demonstrated through decreased c-Jun mRNA levels as well as decreased NFκB activity and levels in the nucleus.

3. RE and its polyphenolic components inhibited IgE-FcεRI allergen-mediated activation of the late phase response demonstrated through

   a. Decreased gene expression of IL-6, TNF, IL-13, CCL1, CCL3, and CCL9.

   b. Decreased production and release of IL-6, TNF, IL-13, CCL1, and CCL3.

4. RE and its polyphenolic components inhibited IgE-FcεRI allergen-mediated activation of mast cell degranulation.

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**Figure 36:** Brief depiction of the effects of RE in IgE-FcεRI-c-Kit activated mast cells.
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**Figure 37:** Summary of the impact of RE treatment in IgE-FcεRI-c-Kit activated mast cells. (Unpublished, M. Yousef 2018).

In conclusion, the results presented in this thesis warrant further research into the many mechanisms of action of RE and provide a strong basis from which to build future research upon. However, as with all studies there are several limitations that should be discussed.
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9. Limitations:

Although the work done in this thesis is novel and powerful, through that novelty and rather complex nature of mast cells and their activation pathways there are a few limitations which will be discussed below.

1. RE is a complex mixture of polyphenols which can vary slightly in relative composition depending on where the original plant is from, the type of extraction performed, growth conditions, and many other factors. Therefore, we cannot make any claims as to whether a specific polyphenol in RE is producing these effects or if the results observed are due to a combination of various polyphenols.

2. The data shown in Fig. 12, 13, and 26 looking at protein and mRNA levels of IкBα are performed on whole cell extracts, and do not line up with the other data (Fig. 35) showing potent inhibition of NFкB transcription factor activity in the nucleus. Therefore, more work needs to be performed to better understand the events occurring in the NFкB pathway.

3. Akt is a protein that requires phosphorylation at two sites (S473 and T308) for its full enzymatic activity, western blot analysis was only performed on S473. Therefore, we cannot with 100% certainty claim that Akt activity is fully inhibited, even though there is a strong indication that it is.

4. There are many other cytokines and chemokines which were not measured in this study that play an important role in inflammation.

5. The final limitation to this study is that it was carried out in an in vitro murine model. There are correlations between animal and human models, however often times results observed in vitro fail to be reproduced in vivo. Luckily, our model
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utilized various different mice with each acting as a different ‘individual’ and therefore provides an extra level of confidence than typical when discussing animal vs. human models. In addition, \textit{in vitro} models allow for a more detailed examination of specific components and therefore allowed us to study the mechanism of action by RE.

Though these limitations may affect the current research presented, many of them have began to be addressed through future studies. These will be discussed below.
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10. Future Directions:

1. Through the help of an undergraduate student, Rob Crozier, work has begun to study each of the three main polyphenolic components of RE being RA, CA, and CO. Our aim is to perform the same experiments and measures but through each polyphenol alone or perhaps a combination of multiple to determine which component is exerting which effects.

2. More western blots should be performed to determine levels of phosphorylated IкBα, IKK, phosphorylated Akt (T308), and cytoplasmic vs. nuclear translocation of NFκB.

3. Further investigation into other important cytokines and chemokines should be performed to more completely understand the effect of RE on mast cell activity.

4. In vivo animal models should be used next to determine if the effects observed in vitro will translate to an in vivo model, with the ultimate goal and hope of reaching clinical trials.

Many more experiments and years of hard work lie ahead of our group if we aim to achieve our goal of finding a novel, accessible, and strong treatment for allergic inflammation not just by treating symptoms but by stopping them at their core. However, we believe that we are on the path to a great discovery and cannot wait to see what is yet to come.
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References:
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Inhibition of IgE-FcεRI activated mast cells by rosemary extract


Inhibition of IgE-FceRI activated mast cells by rosemary extract


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Appendix:

Appendix I: Sample Protein Quantification

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Standard values used to generate standard curve

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Trendline equation used to determine protein concentration in the samples

\[ Y = 0.9904x + 0.036 \]

\[ x = \frac{(y-0.036)}{0.9904} \]

Averages values for each sample

| 0.334 | 0.298 | 0.297 | 0.269 | 0.298 | 0.261 | 0.238 | 0.227 | 0.280 | 0.261 | 0.231 | 0.241 |

Adjusted concentrations after account for 20x dilution factor


**Figure A.1:** Sample protein quantification using a standard curve generated using BSA as a protein control. Samples quantified were used for western blotting analysis, proteins were loaded at 30 µg / 15 µl.
Appendix II: qPCR Melt Curves and DNA Gel Electrophoresis

**Figure A.2:** Melt curve and DNA gel electrophoresis image for IL-6 (160 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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Figure A.3: Melt curve and DNA gel electrophoresis image for Egr1 (115 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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**Figure A.4:** Melt curve and DNA gel electrophoresis image for Egr2 (124 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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**Figure A.5:** Melt curve and DNA gel electrophoresis image for IL-13 (139 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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Figure A.6: Melt curve and DNA gel electrophoresis image for CCL1 (132 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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Figure A.7: Melt curve and DNA gel electrophoresis image for CCL2 (114 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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Figure A.8: Melt curve and DNA gel electrophoresis image for CCL3 (225 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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**Figure A.9**: Melt curve and DNA gel electrophoresis image for TNF (117 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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Figure A.10: Melt curve and DNA gel electrophoresis image for CCL9 (88 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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Figure A.11: Melt curve and DNA gel electrophoresis image for NFkBIA (196 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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**Figure A.12:** Melt curve and DNA gel electrophoresis image for Rcan1 (103 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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**Figure A.13:** Melt curve and DNA gel electrophoresis image for c-Jun (140 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
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**Figure A.14:** qPCR data depicting HPRT housekeeping gene used as a control for all qPCR genes measured.
Figure A.15: Melt curve and DNA gel electrophoresis image for HPRT (180 bp) used as quality controls to ensure amplicon specificity in qPCR reaction.
## Inhibition of IgE-FcεRI activated mast cells by rosemary extract

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### Figure A.16: Sample ΔΔ CT calculations used in qPCR analysis
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

**Example ΔΔ CT Calculation:**

AVG Tech Reps = (Rep 1 + Rep 2)/2
= (25.34 + 25.30) / 2 = 25.32

Δ CT = AVG Tech Reps - Reference Gene AVG Tech Reps
= 25.32 – 23.97 = 1.35

Ref AVG = Average Δ CT NT (NT1 + NT2 + NT3) / 3
= (1.35 + 0.60 + 2.60) / 3 = 1.52

ΔΔ CT = Δ CT - Ref AVG
= 1.52-1.35 = -0.17

Fold Change = 2^(- ΔΔ CT)
= 2^(-0.17) = 1.13

AVG Biol Reps = Average Fold Change (NT1 + NT2 + NT3) / 3
= (1.11 + 1.89 + 0.47) / 3 = 1.16

ST DEV = STDEV (Fold Change NT1 + NT2 + NT3)
= STDEV(1.11 + 1.89 + 0.47) = 0.71

SEM = ST DEV / SQRT(3)
= 0.41
Inhibition of IgE-FceRI activated mast cells by rosemary extract

Appendix III: ELISA Standard Curves

**Figure A.17:** ELISA standard curve used to calculate IL-6 concentrations, utilizing a fifth order polynomial equation.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

Figure A.18: ELISA standard curve used to calculate TNF concentrations, utilizing a fifth order polynomial equation.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

Figure A.19: ELISA standard curve used to calculate IL-13 concentrations, utilizing a fifth order polynomial equation.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

Figure A.20: ELISA standard curve used to calculate CCL1 concentrations, utilizing a fifth order polynomial equation.
Inhibition of IgE-FceRI activated mast cells by rosemary extract

**Figure A.21:** ELISA standard curve used to calculate CCL2 concentrations, utilizing a fifth order polynomial equation.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

Figure A.22: ELISA standard curve used to calculate CCL3 concentrations, utilizing a fifth order polynomial equation.
Inhibition of IgE-FcεRI activated mast cells by rosemary extract

**Figure A.23:** β-Hexosaminidase assay depicting titrations performed using increasing concentrations of TNP-BSA and SCF.