DEVELOPMENT OF A MURINE MODEL TO STUDY INHIBITORS OF CXCR3-LIGAND INTERACTIONS

by

Charis G. Tjoeng

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This thesis was presented

by

Charis G. Tjoeng

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and approved by

Thesis Advisor:
Todd Reinhart, ScD
Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Tianyi Wang, PhD
Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Pawel Kalinski, MD, PhD
Associate Professor
Departments of Surgery, Immunology, and Infectious Diseases and Microbiology
School of Medicine, Graduate School of Public Health
University of Pittsburgh

DEVELOPMENT OF A MURINE MODEL TO STUDY INHIBITORS OF CXCR3-LIGAND INTERACTIONS

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CXCR3 is involved in numerous inflammatory disorders such as rheumatoid arthritis, multiple sclerosis, allograft rejection and inflammatory bowel disease. There is a strong and growing demand for novel and effective therapeutics that can mediate CXCR3 activity. In this study, a set of botanical compounds and a peptide mimetic of the second extracellular loop (ECL-2) of CXCR3 were examined for the ability to inhibit interactions between CXCR3 and its ligands in a murine model. EGCG, a green tea polyphenol, and gallotannin, derived from many plant sources, strongly inhibited the chemotaxis of stably transfected murine CXCR3-expressing L1.2 cells in response to murine CXCL9, CXCL10, and CXCL11. EGCG was also shown to bind directly to murine CXCR3 ligands with high affinities. Baicalin, a flavonoid found in the medicinal plant *Scutellaria baicalensis*, and ginkgolide A, from the *Ginkgo biloba* tree, did not significantly reduce cell migration towards murine CXCR3 ligands, nor did the peptide mimetic of the ECL-2 of murine CXCR3. Other green tea polyphenols similar in structure to EGCG were also analyzed and were less able to inhibit murine CXCR3 ligand-mediated chemotaxis than EGCG, with the following efficacies: ECG > EGC > EC. It was observed that the most effective test compounds contained more hydroxyl groups and hence were more negatively charged, similar to glycosaminoglycans, which are extracellular matrix components that bind many chemokines. It is possible that EGCG and gallotannin are able to bind the GAG-binding domains of murine CXCR3 ligands, which allows them to prevent receptor binding and inhibit their function. This possibility represents the public health relevance of this research, as EGCG and gallotannin may

be attractive candidates as lead compounds for new therapeutics for CXCR3-mediated and other inflammatory diseases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to sincerely thank Dr. Todd Reinhart and the Reinhart lab for continuous support and guidance, Dr. Shulin Qin and Dr. Jodi Craigo for their work as part of this project, and Dr. Pawel Kalinski and Dr. Tianyi Wang for their advice and thoughtful input.

CHAPTER 1: INTRODUCTION

1A. Overview

The body's immune response to a microbial pathogen can play a major role in determining disease outcome. The type of response mounted by the immune system can determine how efficiently the body clears the pathogen, if at all. Chemokines and chemokine receptors are integral parts of the immune system, as they mediate the recruitment of specific immune cells during immune induction events and to sites of infection and inflammation. For example, one of the functions of chemokine receptor CXCR3 and its ligands is to regulate the trafficking of activated T cells to sites of infection and inflammation. The ability to manipulate chemokine-chemokine receptor interactions may serve as a valuable tool for developing therapeutics to aid the host in combating certain pathogens or to ameliorate chronic inflammation. While chemokines and their receptors play significant roles in fighting infections, they also contribute to numerous pathological inflammatory conditions, such as arthritis and multiple sclerosis. Thus, compounds that can modulate chemokine-chemokine receptor interactions may also be beneficial in alleviating the consequences of excessive inflammation.

I have investigated in the studies described in this thesis two types of agents that may modulate chemokine-chemokine receptor interaction: botanically-derived compounds and chemokine receptor peptide mimetics. Specifically, I examined the effects of several plant-derived compounds like epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, which has been discovered to mediate CXCR3-ligand interactions (unpublished data). I also investigated the inhibitory effects of a peptide mimetic of the second extracellular loop segment of the CXCR3 receptor. We have named this peptide X3P3, and it may be able to alter

CXCR3-ligand interaction by binding the ligand (unpublished data). Our preliminary data suggest that both X3P3 and EGCG can bind chemokines, which may block or alter the interaction with the chemokine receptor.

These previous *in vitro* studies examined the effects of X3P3 and EGCG on human chemokines and their chemokine receptors. However, we anticipate the progression from *in vitro* to *in vivo* experiments performed in a small animal model, such as a murine model. I have performed studies using a murine chemokine receptor in order to facilitate this transition, and I have developed a model with which I tested the hypothesis that several botanically-derived compounds and X3P3 can inhibit murine CXCR3 (mCXCR3) ligand function. I used this model to accomplish my two main objectives: to identify and investigate compounds that will modulate mCXCR3-ligand interactions, and to begin shifting these experiments to an *in vivo* setting.

1B. Background

1B.1. Chemokines

Chemokines are small, structurally-related, chemotactic proteins that regulate leukocyte migration by interacting with seven transmembrane G protein-coupled receptors. They are intimately involved in physiologic processes, including inflammation[1], hematopoiesis[2], wound healing[3], angiogenesis[4], organogenesis[5], embryonic development[6] and tumor growth and metastasis[7]. Most chemokines and chemokine receptors are promiscuous in binding, as many receptors can bind multiple ligands and individual chemokines can often bind multiple receptors[8].

Chemokines can be classified into four groups based on the positioning of cysteine motifs near the N-terminus: C, CC, CXC, CX_3C, where X denotes any amino acid[9]. Alternatively, they can be described by function: homeostatic or inflammatory. Homeostatic chemokines are constitutively expressed in discrete tissues and are responsible for the homeostatic trafficking of leukocytes and hematopoietic progenitor cells[10]. For example, CXCL12 – also known as stromal cell-derived factor-1 (SDF-1) – contributes to the migration, homing and survival of hematopoietic stem cells and hematopoietic progenitor cells[11]. In contrast, inflammatory chemokines are expressed at sites of inflammation when cells are stimulated by pathogens or pro-inflammatory cytokines like IL-1β or TNF-α[12]. This set of chemokines, in turn, recruits T cells, natural killer (NK) cells, monocytes, and granulocytes to inflamed tissues. Examples of inflammatory chemokines are CXCL9, CXCL10, and CXCL11, which all share a common receptor, CXCR3.

1B.2. CXCR3

CXCR3 is a chemokine receptor expressed on a wide range of cells, mainly on activated T lymphocytes and, to a lesser extent, on resting T lymphocytes, NK cells, B cells[13], and endothelial cells[14]. Its main ligands are IFN-γ inducible chemokines CXCL9, CXCL10, and CXCL11, formerly known as monokine induced by interferon-γ (Mig), interferon-γ inducible 10-kDa protein (IP-10), and interferon-inducible T cell α chemoattractant (I-TAC)[15].

A chief function of CXCR3 and its ligands is to modulate the migration of activated T cells to sites of inflammation as part of Th1-type immune responses. These chemokines have also been shown to induce chemotaxis of NK cells[16], dendritic cells[17], and macrophages[18]. The three main CXCR3 ligands, CXCL9, CXCL10, and CXCL11, share 40% amino acid homology with each other, which is higher than any other chemokine-chemokine homology[19]. Of these three

ligands, CXCL11 binds CXCR3 with the strongest affinity[20]. CXCL13 and CXCL4 have also been suggested to bind CXCR3 and are able to activate it at high concentrations[21], also causing T-cell migration[22]. In addition, CXCR3 has also been shown to bind to CCL21, a ligand for CCR7, in mice[23].

CXCR3 is composed of 368 amino acids and is a seven-transmembrane G-protein coupled receptor[24]. It is mainly found in two alternatively spliced forms – CXCR3-A and CXCR3-B. A third splice variant, CXCR3-alt, was discovered in 2004[25]. CXCR3-A was discovered first in 1996[26]. CXCR3-B, however, was discovered in 2003 and can bind CXCL4 at a higher affinity, in addition to the main CXCR3 ligands. Studies have revealed that the differences in protein structure translate to different functions *in vitro*.

CXCR3-B was discovered while investigating the angiostatic effects of CXCL9, CXCL10, and CXCL11, as well as CXCL4[27]. When interacting with CXCR3 on human mesangial cells (HMC), the three main CXCR3 ligands promoted cell proliferation[28]. However, when binding the receptor on human microvascular endothelial cells (HMEC), they inhibited growth and induced cell death. These seemingly contradictory effects were explained by the discovery of two different forms of CXCR3. It was found that HMCs expressed CXCR3-A, while HMECs expressed CXCR3-B – and these two forms of the receptor had two opposite functions. Although both forms of CXCR3 can bind CXCL9, CXCL10 and CXCL11, the CXCR3-A form has a higher affinity for them and will outcompete CXCR3-B[29]. Thus, if a cell type expresses CXCR3-B and CXCR3-A simultaneously, its CXCR3-A receptors will predominantly interact with chemokines instead. These two forms of the receptor operate by different signaling pathways, and thus lead to different outcomes when activated[30].

CXCR3-alt encodes a truncated version of the receptor 260 amino acids in length[31] and is predicted to have four or five transmembrane domains. Chemotaxis studies show that this

change in structure abolishes the receptor's ability to interact with CXCL9 and CXCL10, but the receptor retains functional activity when interacting with CXCL11. Generally it is expressed at lower levels than full-length CXCR3.

CXCR3 and its ligands are suspected to be involved in inflammatory diseases such as atherosclerosis[32], acute allograft rejection[33], multiple sclerosis[34], inflammatory bowel disease[35], psoriasis[36], sarcoidosis[37], type I diabetes mellitus[38], and rheumatoid arthritis[39]. For instance, during development of diabetes mellitus, insulin-secreting beta cells are targeted by self-reactive CXCR3-positive T cells. Beta cells produce CXCL9 and CXCL10, which attract the T cells that ultimately destroy them[40]. A possible therapy for these numerous diseases may entail modulating the CXCR3-ligand interaction, and this possibility is of particular interest in chemokine research.

CXCR3 and its ligands have been shown also to play a role in cancer. CXCL9, CXCL10[41], and CXCL4[42] regulate tumor growth by causing damage to tumor-associated vasculature, leading to tumor necrosis[43]. Numerous studies have investigated these chemokines as possible cancer therapeutics. In a recent study using a murine model of metastatic renal cell carcinoma (RCC), treatment with IL-2 and CXCL9 greatly reduced tumor size, impaired angiogenesis, and stimulated tumor necrosis[44]. However, two studies – also using murine models – implicate that CXCR3 may also be involved in colon cancer[45] metastasis to lymph nodes and breast cancer[46] metastasis to the lung. In the breast cancer study, treatment of mice with AMG487, a CXCR3 antagonist, inhibited metastasis to the lung. Amgen's AMG487 is the first small molecule CXCR3 inhibitor to enter clinical trials[47], and this study was the first to report that a small molecular weight CXCR3 inhibitor was able to decrease tumor metastasis.

Another small molecule CXCR3 antagonist is NBI-74330, and both AMG487 and NBI74330 were included in a study by Verzijl *et al.*[48]. It was found that both compounds are

noncompetitive antagonists of CXCR3 and have a lower affinity for rodent CXCR3 compared to the human form of the receptor. The objective of this study was to enhance the understanding of these antagonists in animal models because they may be able to prevent or treat CXCR3-related diseases. Another study using NBI-74330 was undertaken by van Wanrooij *et al.*[49], and it reports that NBI-74330 treatment can reduce diet-induced plaque formation in LDL receptor-deficient mice. The complex role of CXCR3 and its ligands in biological processes and diseases makes them interesting targets of further research.

1B.3. Botanicals

Botanically-derived compounds are attractive targets for chemotherapeutic development, as they are believed to exhibit low levels of toxicity and few side effects. Such anti-inflammatory compounds may be able to inhibit CXCR3-ligand interactions. The compounds I have investigated here included epigallocatechin-3-gallate (EGCG), baicalin, gallotannin, and ginkgolide A. The structure of each compound, as well as structurally related compounds, is illustrated in Figure 1.

EGCG, the most abundant polyphenol in green tea, is known to exert a host of health benefits both on tea-drinkers and animal subjects. Among these benefits are the suppression of inflammation[50], prevention of cancer, inhibition of tumor growth, protection of DNA from damage, induction of apoptosis in tumor cells, and antioxidant effects[51]. One mechanism by which these botanicals inhibit inflammation might be by binding directly to chemokines, and preventing them from interacting with their receptor(s). Our preliminary studies support this theory, and I have examined these effects specifically on murine CXCR3 (mCXCR3) and its ligands.

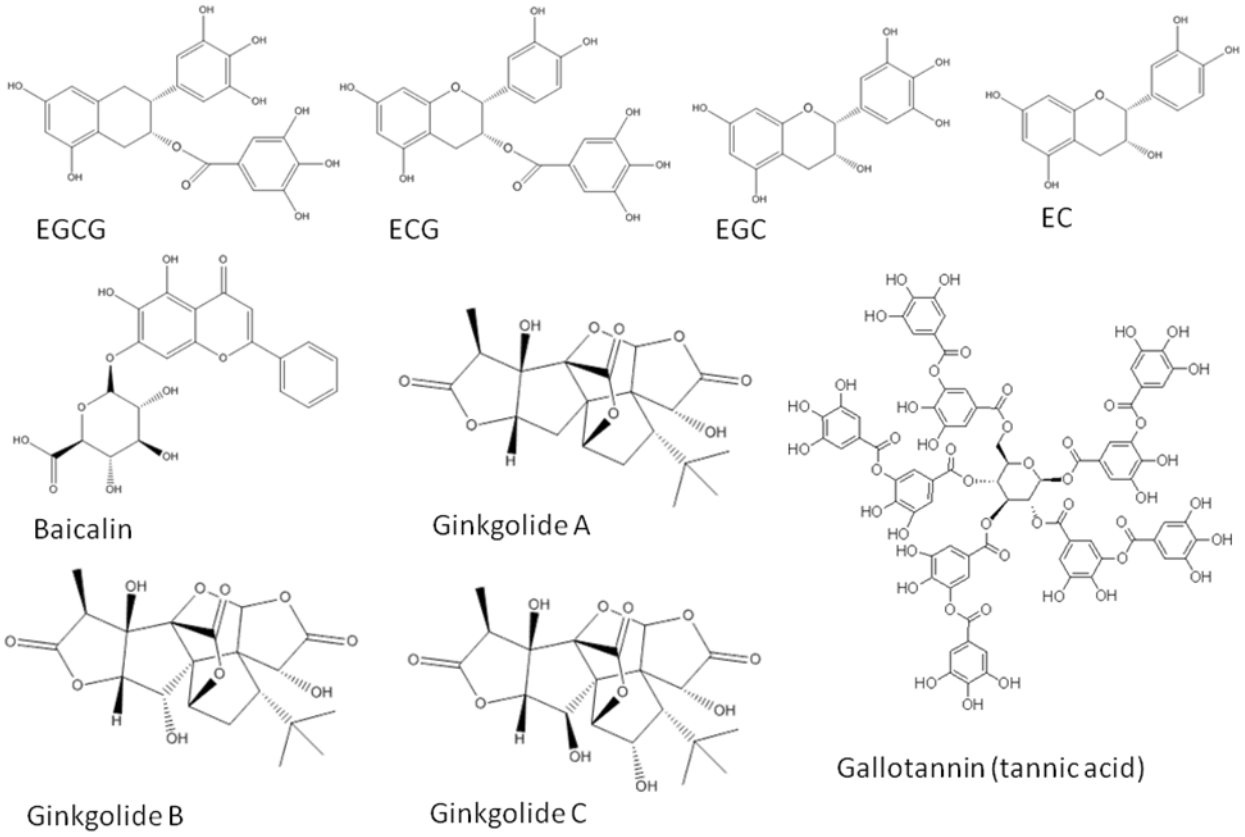

Figure 1. Illustrations of the botanical compounds used in this study. The structures for the following are shown: epigallocatechin-3-gallate (EGCG), its analogs epicatechin-3-gallate (ECG), epicatechin (EC), and epigallocatechin (EGC), baicalin, gallotannin, and ginkgolides A, B, and C. Structures were generated using ChemDraw Ultra software (Cambridge).

Baicalin, a flavonoid derived from the Chinese herb *Scutellareia baicalensis*, is a major constituent in herbal medicines used to treat chronic hepatitis in China and Japan[52]. It has been shown in one study to inhibit inflammation by binding to a subset of chemokines[53], including CXCL8, CXCL12, CCL4 (MIP-1β), and CCL8 (MCP-2). Chemotaxis assays revealed that baicalin suppresses each chemokine's ability to stimulate cell migration. Its activity, however, is limited to a subset of chemokines, as it had no effect on CX_3CL1, nor on the cytokines TNF-α or IFN-γ. Li *et al.*[54] suggest that baicalin interacts selectively with chemokines of the CXC, CC, and C classes and not CX3C chemokines or other cytokines. In this case, since CXCR3 ligands are CXC chemokines, baicalin may also bind them.

Gallotannin, or tannic acid, is a polyphenol and a hydrolyzable tannin, found in many plants[55]. Multiple studies show that it, like EGCG and baicalin, possesses anti-inflammatory properties[56]. It is also suggested to have anti-viral and anti-cancer effects. Non-toxic amounts of gallotannin were found to selectively inhibit chemotactic properties of CXCL12 and decrease binding of the chemokine to THP-1 cells[57]. In addition, Erdèlyi *et al.* demonstrated that the compound reduces expression of several inflammatory chemokines and receptors in human lung adenocarcinoma (A459) cells[58]. They postulated that gallotannin targets the NF-kB pathway to inhibit expression of CXCR4 and CCL20.

Ginkgolides are terpene trilactones found in the leaves and root bark of the *Gingko biloba* tree[59]. The leaves of the *Gingko* tree have traditionally been used in Chinese herbal medicine, and *Gingko* leaf extract has recently been used to treat cerebral vascular insufficiency, or deficient blood flow to the brain[60]. Ginkgolides are known to be effective inhibitors of platelet activating factor (PAF)[61], a molecule that induces platelet aggregation. PAF can also induce inflammation, allergic reaction, and asthma[62]. Ginkgolide A, B, and C are structurally similar, and in the studies described here I focused on ginkgolide A.

1B.4. Peptide Mimetics

Peptide mimetics may also be strong candidates to modulate CXCR3-ligand interactions. They structurally resemble parts of proteins and can be designed to have extra properties that render them better drugs, such as protease resistance, targeted cellular transport, decreased toxicity, and fewer side effects[63]. Our X3P3 peptide, for instance, is likely to be structurally similar to the second extracellular loop (ECL-2) of CXCR3 (Figure 2). We have preliminary data that show that X3P3 designed based on the human CXCR3 sequence binds directly to CXCL9, CXCL10, and CXCL11 and inhibits CXCR3-mediated chemotaxis. Since it has been shown that

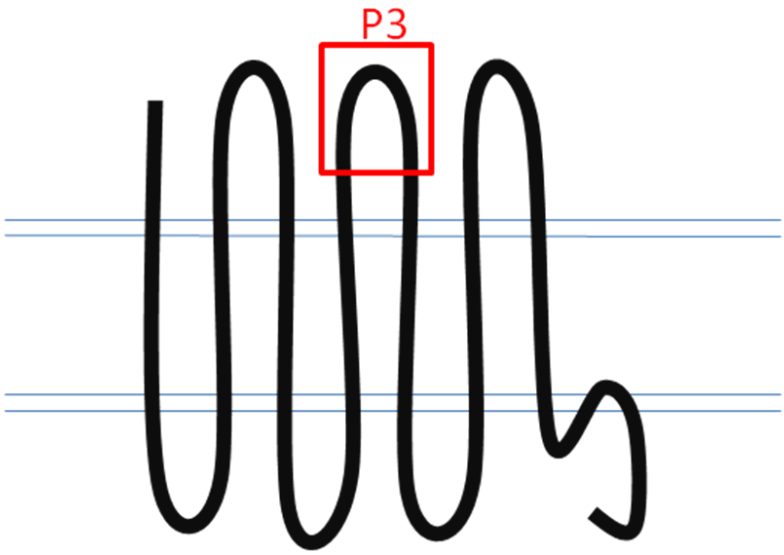

Figure 2. A simple schematic of a G-protein coupled chemokine receptor. The X3P3 peptide is designed to resemble the second extracellular loop of CXCR3. This region is noted.

human and murine CXCR3 are 86% identical[64], it seems reasonable to hypothesize that a murine X3P3 peptide might also bind murine CXCR3 ligands.

Peptide mimetics have been studied only minimally in the context of HIV. Synthetic peptides mimicking CCR5, a coreceptor for HIV-1, have been shown to inhibit viral fusion and entry[65]. The peptides studied to date are those modeled after the amino-terminal domain[66] and the extracellular loops of CCR5. Sulfotyrosine-containing peptides resembling the N-terminus of CCR5 inhibited entry of a CCR5-tropic strain, while replicas of the second and third extracellular loops of the receptor prevented entry of R5 and R5X4 strains. These peptides show potential for becoming innovative and effective HIV therapeutics.

Peptides representing N-terminal regions of CXCR3 were used by Colvin *et al*. to determine whether tyrosine sulfation of CXCR3 is necessary for ligand binding[67]. In this study, sulfated N-terminal peptides were found to inhibit CXCL10- and CXCL11-induced chemotaxis, confirming that it is possible for a peptide mimetic of an extracellular region of CXCR3 to inhibit CXCR3 function.

CHAPTER 2: STATEMENT OF THE PROJECT

2A. Overview. The main objective of this project was to develop an in vitro murine model for identifying and studying compounds that that might inhibit interactions between murine CXCR3 and its ligands. Included in these studies were natural plant-derived compounds as well as a peptide mimetic of the second extracellular loop of CXCR3. If murine cells are sensitive to chemotaxis inhibition in a manner similar to human cells, the murine model may be a suitable choice for *in vivo* studies of CXCR3 inhibition. Lead compounds may then be investigated further as possible therapeutics for CXCR3-mediated inflammatory disorders.

2B. Specific Aims.

1. *To examine the inhibitory effects of botanically-derived compounds on interactions between mCXCR3 and its ligands*. Our preliminary data indicated that EGCG, a green tea polyphenol, inhibits chemokine function in a dose-dependent manner by binding directly to human CXCR3 ligands (unpublished data). Because EGCG is able to inhibit CXCR3-mediated chemotaxis, my hypothesis was that it and other botanical compounds might also inhibit the function of murine CXCR3 ligands.

2. *To investigate the effects of the X3P3 peptide on mCXCR3 ligand function.* Our previous studies indicated that human X3P3 binds directly to chemokines and thereby inhibits their functions. However its inhibitory effects on interactions between murine CXCR3 and its ligands were previously unknown. Because of the similarities in structure and

function between murine and human CXCR3, I hypothesized that a murine X3P3 peptide will be able to modulate the mCXCR3-ligand interaction.

CHAPTER 3: MATERIALS AND METHODS

3A. Overview. To set up a system with which to examine the inhibitory effects of the botanicals or a CXCR3 peptidergic mimetic, I first generated cell lines that respond to murine CXCR3 ligands. The chosen parental cell line was the murine pre-B cell line L1.2, which did not respond chemotactically to CXCR3 ligands. An existing murine CXCR3 cDNA was transfected into L1.2 cells, and the resulting cell lines were screened via chemotaxis assay for high migratory ability towards mCXCL11. The highest migraters were then used in chemotaxis inhibition assays to determine if the peptide mimetic and botanical compounds were able to inhibit chemotaxis.

3B. Cloning. To clone the mCXCR3 cDNA into a mammalian expression, the mCXCR3 cDNA was PCR-amplified mCXCR3 using pGEMT_mCXCR3.1 previously generated by Kristi Gaus in our laboratory as a template. I constructed primers (Integrated DNA Technologies) containing a GCC clamp at the 5' end and restriction sites corresponding to sites within pcDNA3.1(+), the mammalian expression vector used for these studies. The forward primer sequence was 5'-GCCGAATTCATGTACCTTGAGGTTAGTGA-3', containing a restriction enzyme site recognized by EcoRI. The reverse primer sequence read 5'-GCCCTCGAGTTACAAGCCCAGTAGGAG-3', with an XhoI site. These primers were successfully used with Gotaq DNA polymerase (Promega) to amplify the mCXCR3 sequence and add restriction enzyme sites. The conditions for the PCR cycles were 3.5 minutes at 94° for denaturation, 30 seconds at 56° for annealing, 2 minutes at 72° for extension, and 10 minutes at 72° for final extension. Samples were run for 35 cycles. DNA in the sample was visualized by gel electrophoresis performed on 0.8% agarose gel, using GelRed (Biotium).

To obtain pcDNA3.1(+) vector and mCXCR3 insert with compatible cohesive ends, pcDNA3.1_huCCR4 (University of Missouri cDNA Resource Center) and the PCR-amplified mCXCR3 product were digested with EcoRI and XhoI (NEB) for 3 hours at 37°. The digestion products were separated by gel electrophoresis (Figure 1B), and appropriate DNA fragments were extracted (Qiagen Qiaex II Gel Extraction Kit). Then, the pcDNA3.1(+) and mCXCR3 fragments were ligated together and used to transform DH5α competent cells (Invitrogen). Digested mCXCR3 PCR product was also ligated with pGEM_T (Promega). This was a precautionary step performed in case the ligation between mCXCR3 and pcDNA3.1 was unsuccessful. Transformation products were plated on LB/ampicillin plates and incubated at 37° overnight. Subsequent colonies were picked and screened for the correct insert.

3C. Verification of the correct insert sequence in pcDNA3.1_mCXCR3. To ensure that the ligations were successful, I subjected the clones to colony PCR, and their DNA to restriction digest and DNA sequencing. To perform colony PCR, I used the primers mentioned in section D1. Colonies were picked from each of these plates: pcDNA3.1 ligated with mCXCR3, pGEM-T ligated with mCXCR3, and pGEM-T ligated with control insert. They were put into PCR mixes, and samples were run through the same steps described above. Colony PCR was performed on 18 clones, including one clone transformed with pGEM-T ligated with the manufacturer's control plasmid. PCR products were then run on a 0.8% agarose gel and visualized. Each clone appeared to yield PCR product, including the clone transformed with a plasmid that did not contain mCXCR3.

To determine whether these clones' DNA contained mCXCR3, I chose four clones based on the colony PCR results and extracted their DNA by mini prep (Promega Pure Yield DNA

Extraction Kit). I chose two clones resulting from the pcDNA3.1(+) and mCXCR3 ligation, one from the pGEM-T and mCXCR3 ligation, and one containing pGEM-T and the control insert. The DNA from the four clones was then digested with EcoR1 and XhoI, and the resulting DNA fragments were separated and visualized by gel electrophoresis.

Next, the DNA from clones #1 and #2 was further analyzed by PCR using vector-specific primers. The two sets of primers were T7 and R800, and BGH and F300. T7 and BGH are specific to the vector, pcDNA3.1(+), whereas R800 and F300 are specific to the insert sequence, mCXCR3. PCR conditions were the same as described in section 3B.

3D. Transient transfection and mCXCR3 expression confirmation. The pcDNA3.1_mCXCR3 plasmids were sequence-confirmed and transfected via electroporation (Biorad Gene Pulser II) into murine L1.2 cells. Cells were deposited into an electroporation tube (Bio-Rad) along with yeast tRNA (Ambion) and 10 μg of DNA, then pulsed for approximately 15 milliseconds seconds at 330 volts and 0.975 uF. To test for mCXCR3 expression, transiently transfected cells were stained with a PE-conjugated anti-mCXCR3 antibody (R&D, catalog #FAB1685P, clone 220803) and isotype control (R&D, catalog #IC006P) and analyzed by flow cytometry on a FACSCanto flow cytometer (Becton Dickinson). Flow data were analyzed using Flowjo software (Treestar). To test for functionality of the receptor, a chemotaxis assay was performed. After a 24-hour incubation, cells were washed and resuspended in RPMI-1640 and 0.1% bovine serum albumin (BSA) at 200,000 cells/ml. Different concentrations of murine CXCL9 (Peprotech), solubilized in water, were placed on the bottom of a chemotaxis plate (Neuroprobe, #101-5), and a membrane with 5-μm pores was placed on top of the plate. Cells were placed on top of the membrane, and incubated for 3 hours at 37° at 5% CO_2 in a

humidified box. After the incubation, cells that migrated through the membrane were counted using a hemacytometer.

3E. Establishment of stable murine cell line expressing mCXCR3. To develop a murine cell line stably expressing mCXCR3, L1.2 cells were transfected with pcDNA3.1(+) expression plasmids via electroporation with the same conditions described above. To select for cells that incorporated plasmid into their genomes, geneticin (Gibco Life Technologies) was used at 1 mg/mL. Cells were diluted to 20,000 cells per 150 μL, then aliquots of 150 μL of culture were loaded into a flat-bottom 96-well plate (Corning) and monitored for growth. Then, wells containing typically a single focus of cells were chosen for further cultivation and grown in media with geneticin in 24-well plates. These cells were tested for expression of mCXCR3 by flow cytometry and chemotaxis, and the clones that migrated at the highest levels towards 1nM mCXCL11 were chosen. To generate secondary clones, cultures of highly migratory clones were diluted, and aliquots were placed into a 96-well plate with a ratio of one cell to every three wells. This resulted in foci of cells growing from a single clone, and these single cell clones were screened for migratory responses to 1nM mCXCL11. The clones showing the highest migration were chosen as the stable cell lines used in later stages of the project.

I have also utilized an alternative way to screen for high migraters. After primary clones were cultured in 24-well plates, they were tested for migration in a chemotaxis experiment. Cells that have successfully migrated to the bottom of the membrane were removed from the chemotaxis plate and cultured. These cells were then screened for high migration, and cultures with high migraters were diluted and plated into 96-well plates as above. After foci of cells grew, single cell clones were cultured, and resulting cultures were tested for migration towards mCXCL11 at 1 nM. The clones that showed the highest levels of

Table 1. Concentrations of botanical compounds.

COMPOUND	CONCENTRATIONS USED IN PAST STUDIES (μM)	CONCENTRATIONS REACHED *IN VIVO*	CONCENTRATIONS USED IN PRESENT STUDY (μM)
EGCG	**1, 10, 100**	**Humans: 2.18 μM (6-7 cups tea)[68]**	**1, 10, 100**
Gallotannin	**11.8, 23.5, 35.3[69] 30, 100[70]**	**Unknown**	**1, 10, 50, 100**
Baicalin	**2, 22.4, 224;[71] 179.2, 717[72]**	**Rats: 2.0 μM*[73]**	**1, 50, 100, 200**
Ginkgolide A	**1, 3, 10, 30[74]; 91.8[75]**	**Humans: 181.8 μg/mL of GkA, GkB, and bilobalide**[76]**	**1, 10, 40, 80**

Table 1. A literature review yielded physiologically relevant concentrations of each test compound and also the concentrations previously used in other studies. The concentrations used in this study are based on these two parameters. *Baicalin was administered as a soy phospholipid. **Ginkgo biloba extracts were administered as a phospholipid complex (GinkgoSelect Phytosome).

migration were chosen as the stable cell lines used later in the study. Secondary clones that resulted from chemotactically-selected primary clones were designated an "s" in their name, i.e. 1E.s8 or 3E.s1.

3F. Preparation of test compounds. Test compounds were resuspended in either nuclease-free water (Ambion) or a mixture of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and nuclease-free water. Murine X3P3 (University of Pittsburgh Peptide Facility) and EGCG (Sigma-Aldrich, catalog #E4143) were water-soluble and were prepared by S. Qin and T. Reinhart, respectively. Gallotannin (Sigma-Aldrich, catalog #403040) was water soluble and therefore was also dissolved in water. Baicalin (Sigma-Aldrich, catalog # 572667) and ginkgolide A (Sigma-Aldrich, catalog # 51863) were slightly hydrophobic and thus were dissolved in a combination

of DMSO and water. Baicalin was resuspended at 11.72 mM in 75% DMSO, and ginkgolide A was dissolved in 75% DMSO at 24.5 mM.

3G. Cytotoxicity assay. To determine if the compounds studied exhibited cytotoxic effects at their working concentrations, cytotoxicity assays were performed. First, a range of test concentrations were established based on concentrations found via literature review(Table 1). These concentrations were used during chemotaxis inhibition studies, and the highest of these test concentrations was used in cytotoxicity studies. These studies were performed using the L1.2_mCXCR3.1E.s8 cell line, which was also used for the bulk of the chemotaxis inhibition studies. Cell cultures were split once a day for three days at approximately 1:1 culture:medium, and on the fourth day, one population of cells was treated with 5 mM sodium butyrate, while another population was not. The two populations of cells were used to determine if sodium butyrate treatment affects cell viability during a chemotaxis experiment. After an overnight incubation, the two populations of cells were used for two separate but identical cytotoxicity assays. Cells were placed into 96-well plates with the highest concentration of test compound to be used during chemotaxis studies, and then incubated for three hours at 37°C and 5% CO_2 to simulate incubation conditions during a chemotaxis assay. After the incubation, live cells and dead cells were counted using a hemacytometer and trypan blue (BioWhittaker), a reagent that is excluded from live cells.

3I. Chemotaxis and chemotaxis inhibition. Chemotaxis assays were performed as described[77] to measure the effects of the botanical compounds and the X3P3 peptide on mCXCR3-ligand interactions. Stable cell clones of L1.2 cells expressing mCXCR3 were treated with 5 mM sodium butyrate to induce mCXCR3 expression, and then incubated overnight.

Then, cells were washed and resuspended as described above. Aliquots of 20 µl of cells were deposited onto chemotaxis plates on top of the chemotaxis membrane. Optimal concentrations of chemokine, as well as medium-only controls, were placed on the bottom of the membrane with different concentrations of the inhibitory compound. The chemotaxis plate was left to incubate in a humid box for 3 hr at 37°C at 5% CO_2. The cells that migrated through the membrane were counted using a hemacytometer. Experiments were performed three times, with all samples in triplicate each time, unless otherwise noted. Data were visualized using Prism (Graphpad) and statistical analyses were performed using Minitab software. Paired t-tests were used to compare migration levels to chemokine only versus chemokine plus compound. If the compound was dissolved in a percentage of DMSO, the paired t-test compared migration levels towards chemokine plus DMSO and chemokine plus compound. A statistical significance of $p < 0.05$ was used.

CHAPTER 4: RESULTS

4A. Overview. The objective of these studies was to identify agents that could modulate interactions between mCXCR3 and its ligands and mCXCR3-mediated chemotaxis. To accomplish this, I have designed a system in which the effects of such agents on mCXCR3 can be observed. I first constructed a mammalian expression plasmid containing the mCXCR3 cDNA, and used this plasmid to generate cell lines expressing mCXCR3. These cell lines were screened for high migratory capacity and a highly migratory cell clone expressing wild type mCXCR3 was used for the majority of the chemotaxis inhibition studies. Use of these cells to study the chemotactic inhibitory properties of different compounds revealed that the botanicals EGCG and gallotannin were effective at inhibiting chemotaxis at concentrations that were not cytotoxic. Baicalin and ginkgolide A were less effective at inhibiting chemotaxis. Murine X3P3, which is a peptidergic mimetic of ECL2 of mCXCR3, was ineffective at inhibiting chemotaxis at the concentrations used. Results for all test compounds were consistent for three cell clones: L1.2_mCXCR3.1E.s8 (wild type mCXCR3), L1.2_mCXCR3.2B.s5 (wild type mCXCR3), and L1.2_mCXCR3.3E.s4 (mutant mCXCR3).

4B. Cloning. The mCXCR3 DNA was amplified by PCR, and the resulting PCR product was visualized by gel electrophoresis (Figure 3A). Analysis of the gel confirmed that the primers were successful in amplifying the mCXCR3 sequence and revealed that the PCR product was of the correct size – approximately 1.6kb. The murine CXCR3 ORF contains 1,608 base pairs. The PCR product was gel extracted and digested, as was the vector pcDNA3.1(+) (Figure 3B). The digestion products were ligated, and the resulting plasmid was used to transform the DH5α strain of *E. coli*. Colony PCR was performed, and all clones appeared to contain the insert

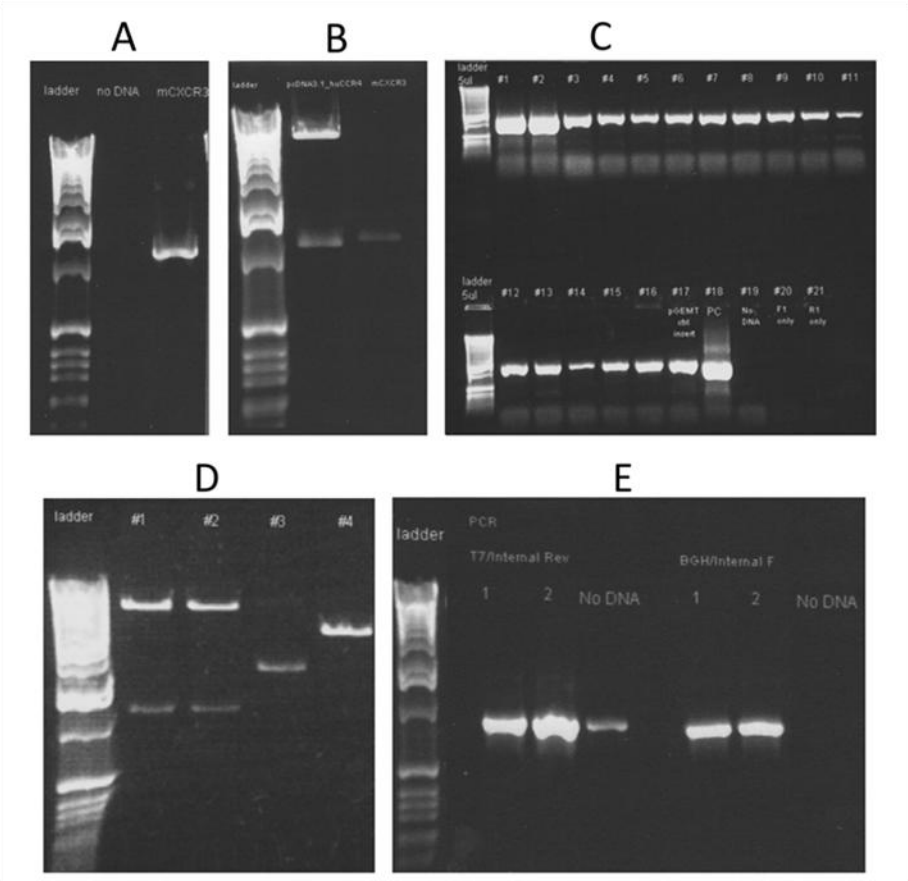

Figure 3. *A*, PCR amplification of mCXCR3. The correct size band (1.6 kbp) for mCXCR3 was detected after 35 cycles of PCR. ***B*, Gel electrophoresis of restriction digest products**. The PCR product containing mCXCR3 and pcDNA3.1_huCCR4 were digested with EcoRI and XhoI. Plasmids were digested for 3 hours at 37°. ***C*, Figure 3. Colony PCR of 18 transformed clones**. DH5α cells were transformed with ligated plasmids and were plated on LB/ampicillin plates. Colony PCR was performed on 18 clones (#1-17, 22). Lane #18 contains the PCR product of pGEM-T_mCXCR3. ***D*, Restriction digests of DNA from clones 1, 2, 3, and 17**. DNA samples were digested with EcoRI and XhoI for 3 hours at 37°. Fragments were separated via gel electrophoresis and visualized using GelRed. ***E*, PCR screening of plasmid DNA from clones 1 and 2 using vector-specific primers**. DNA samples from clones 1 and 2 were subjected to PCR using two sets of primers: T7 and an internal reverse primer, and BGH and an internal forward primer. T7 and BGH are specific to pcDNA3.1 vector.

(Figure 3C). Four clones were expanded for small-scale DNA isolation. Clones #1 and #2 resulted from the pcDNA3.1 and mCXCR3 ligation, clone #3 resulted from the pGEM-T and mCXCR3 ligation and was included as a ligation control, and clone #17 contained pGEM-T and the control insert. The plasmids were restriction digested and run on a gel (Figure 3D). The gel revealed that clones #1 and #2, expected to be pcDNA3.1_mCXCR3, both contained an insert that was of the appropriate size expected for the mCXCR3 cDNA insert. Clones #3 and #4 did not release a correctly sized insert, verifying that these plasmids did not contain mCXCR3.

To further verify the identities of clones #1 and #2, I screened them via PCR using vector-specific primers. I used two sets of primers: T7 and R800, and BGH and F300. T7 and BGH are specific to the vector, pcDNA3.1(+), whereas R800 and F300 are specific to the insert sequence, mCXCR3. Thus, PCR amplification of the template would only occur if the plasmid contained both vector and insert sequence. For both samples, PCR product of the correct size was detected (Figure 3E). The small band in the No DNA control is likely a result of spillover.

To confirm that clones #1 and #2 contained the correct insert, DNA sequences were obtained through with the assistance of the the Genomics and Proteomics core laboratory. The primers used were F300 and F700, which are mCXCR3-specific and were designed previously by Kristi Gaus, as well as the vector-specific primer T7. The DNA sequences from the two clones were confirmed to contain mCXCR3 ligated to pcDNA3.1(+). Restriction enzyme recognition sites corresponding to those within the pcDNA3.1 multiple cloning site (MCS) were identified, as was the ORF of mCXCR3. The sequences derived here were compared to the mCXCR3 ORF sequence present in the GenBank database (GI: 118129823).

The full amino acid sequence of pcDNA3.1_mCXCR3.1, aligned with the amino acid sequence of the mCXCR3 present in GenBank is shown in Figure 4. At the amino acid sequence level, the clones generated here are identical to the previously determined mCXCR3 sequence. Dots (•) denote identity with the amino acid in the reference sequence.

```
mCXCR3 protein      ---MYLEVSE RQVLDASDFA FLLENSTSPY DYGENESDFS DSPPCPQDFS LNFDRTFLPA LYSLLFLLGL LGNGAVAAVL LSQRTALSST
pcDNA3.1_mCXCR3.1   ---....... .......... .......... .......... .......... .......... .......... .......... ..........

                    DTFLLHLAVA DVLLVLTLPL WAVDAAVQWV FGPGLCKVAG ALFNINFYAG AFLLACISFD RYLSIVHATQ IYRRDPRVRV ALTCIVVWGL
                    .......... .......... .......... .......... .......... .......... .......... .......... ..........

                    CLLFALPDFI YLSANYDQRL NATHCQYNFP QVGRTALRVL QLVAGFLLPL LVMAYCYAHI LAVLLVSRGQ RRFRAMRLVV VVVAAFAVCW
                    .......... .......... .......... .......... .......... .......... .......... .......... ..........

                    TPYHLVVLVD ILMDVGVLAR NCGRESHVDV AKSVTSGMGY MHCCLNPLLY AFVGVKFREQ MWMLFTRLGR SDQRGPQRQP SSSRRESSWS
                    .......... .......... .......... .......... .......... .......... .......... .......... ..........

                    ETTEASYLGL *
                    .......... .
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Figure 4. Protein sequence alignment of pcDNA3.1_mCXCR3.1 and mCXCR3 (GenBank, reference sequence #NP_034040.1). Dots (.) indicate an identical amino acid. The asterisk (*) denotes a stop codon.

4C. Generation of mutant mCXCR3. The first set of primers designed for PCR amplification of mCXCR3 cDNA contained two nucleotide substitutions that led to the creation of a mutant form of mCXCR3, designated mCXCR3_mut (Figure 5). The two base pair substitutions, both guanine to cystidine, were made at amino acid positions 4 and 5 of the mCXCR3 ORF. When translated, these substitutions will result in two amino acid changes, E4D to V5L. Both human and murine CXCR3 contain glutamic acid and valine at positions 4 and 5, respectively, and they are part of the first 16 amino acid sequence at the N-terminus. This sequence is thought to be required for binding to CXCL10 and CXCL11, but not to CXCL9[78]. Thus, the mutant mCXCR3 may not bind

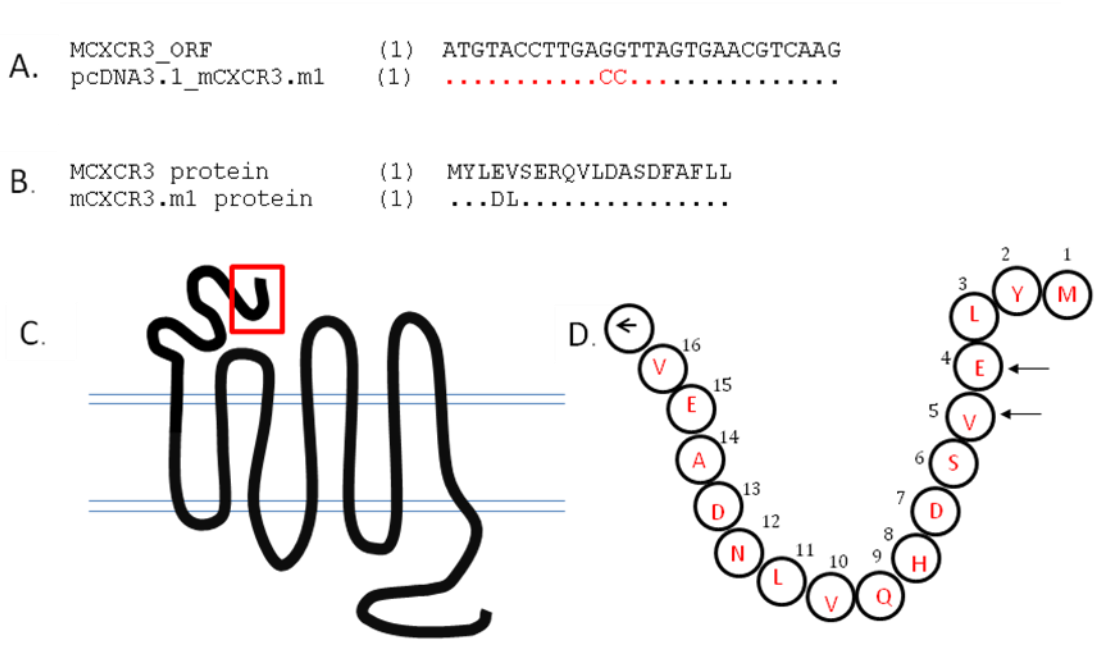

Figure 5. *A*, Partial DNA sequences of mutant clone #1 aligned with the mCXCR3 ORF. Samples were purified by mini prep (Promega) and sequenced. A portion of the priming site appears in red, and the two substitution mutations are indicated. Dots (•) denote a base identical to that of the mCXCR3 ORF. **B, Partial protein sequences of mutant clone #1 aligned with the protein sequence of mCXCR3.** The mCXCR3 reference sequence was taken from GenBank, reference sequence #NP_034040.1. **C, Predicted structure of human CXCR3.** Figure is adapted from Colvin *et al*., 2006. The region of the protein necessary for binding to CXCL10 and CXCL11 is in the red box. **D, Amino acids in human CXCR3 necessary only for CXCL10 and CXCL11 binding, according to Colvin *et al*., 2006**. Arrows point to the glutamine and valine affected by the mutations. These two amino acids in the mutant form of mCXCR3 are encoded as aspartate and leucine.

mCXCL9 or may have reduced affinity for it. If so, this mutant mCXCR3 could be used to examine ligand-specific modulation of mCXCR3.

4D. Transient transfection and verification of mCXCR3 expression. To confirm that the engineered pcDNA3.1 plasmids expressed mCXCR3 and that a commercially available antibody would detect it on cell surfaces, L1.2 cells were transiently transfected. L1.2 cells are murine pre-B lymphoma cells that can be stimulated to migrate at high levels and can be transfected with high efficiencies. The plasmids that were examined included pcDNA3.1_mCXCR3.1, pcDNA3.1_mCXCR3.2, pcDNA3.1_mCXCR3.m1, and pcDNA3.1_mCXCR3.m2. The plasmids pcDNA3.1_mCXCR3.1 and pcDNA3.1_mCXCR3.2 encoded wild type mCXCR3 and were derived from separate bacterial colonies following transformation of *E. coli* with a sequence-confirmed pcDNA3.1 clone containing the mCXCR3 cDNA . Similarly, the other two plasmids encoded the mutant form of mCXCR3 and were also products of two colonies from the same bacterial transformation. As controls, L1.2 cells were transfected in parallel with just the vector pcDNA3.1(+), with pEGFP-N1, or without DNA. All transfected cells were treated with sodium butyrate four to five hours after transfection. After culturing cells overnight, cells were stained with an anti-mCXCR3 antibody or isotype control and examined by flow cytometry. The resulting data were analyzed using Flowjo software. Transfections were successful, as evidenced by successful staining with antibodies specific for mCXCR3 (Figure 6). Unexpectedly, cells transfected with pcDNA3.1_mCXCR3.m2 did not stain for mCXCR3 at high levels in multiple experiments (not shown).

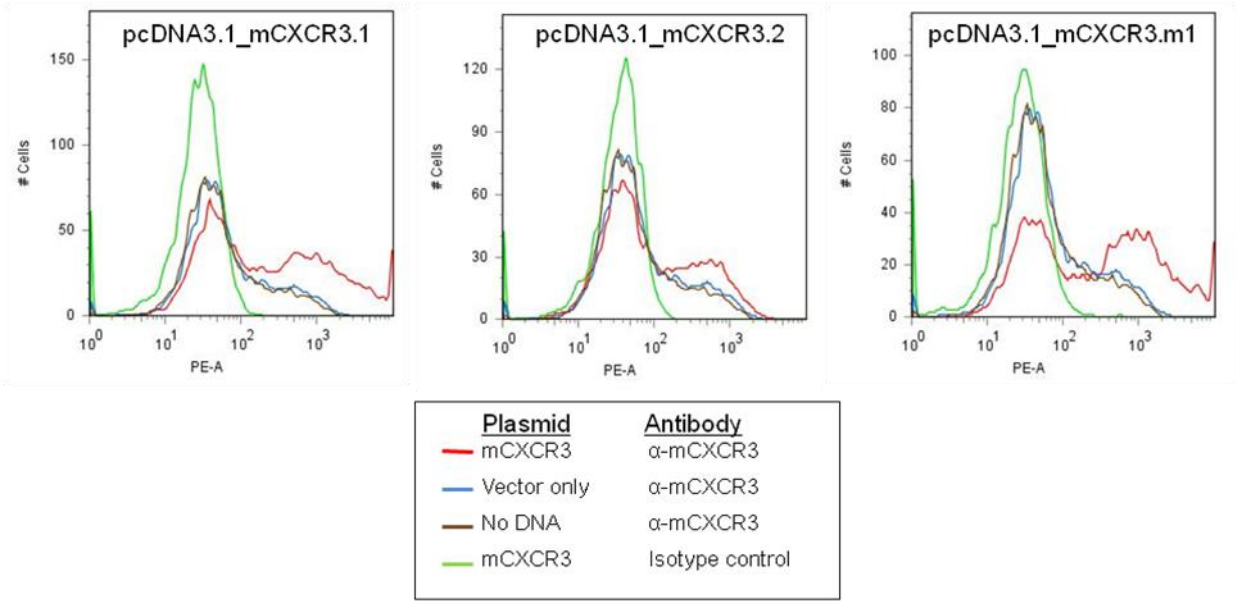

Figure 6. Flow cytometry histogram analysis of three mCXCR3 clones. L1.2 cells were transiently transfected with pcDNA3.1_mCXCR3.1 (left histogram), pCDNA3.1_mCXCR3.2 (middle), pcDNA3.1_mCXCR3.m1 (right), or pcDNA3.1 only. A no DNA control was also included. Cells were treated with 500mM sodium butyrate and cultured for 24 hours. Then, cells were stained with anti-murine CXCR3 antibody or isotype control (R&D Systems) and analyzed by flow cytometry. In the histogram, the red line denotes L1.2 cells transfected with a plasmid encoding mCXCR3 and stained with α-mCXCR3 antibody. The green line represents these cells stained with an isotype control. The blue line indicates cells transfected with pcDNA3.1(+) only, and the brown line represents cells that were electroporated but did not receive any DNA. These data are representative of five identical experiments.

The flow cytometric analyses indicated that subpopulations of cells transiently transfected with pcDNA3.1_mCXCR3.1 and pcDNA3.1_mCXCR3.m1 expressed higher levels of mCXCR3 as compared to cells transfected with the other two plasmids and negative controls. Altogether these data confirm that the transfections were successful, that a significant proportion of transfected cells are expressing the mCXCR3 protein, and that the commercially available mCXCR3 antibody binds to this protein.

To examine the functionality of the receptor, chemotaxis assays were performed with cells transfected with pcDNA3.1_mCXCR3.1, pcDNA3.1_mCXCR3.2, pcDNA3.1_mCXCR3.m1, or plasmid vector only. After 24 hours after of incubation, cells were washed and resuspended in 0.1% BSA in RPMI medium. Different concentrations of murine CXCL9 were placed on the

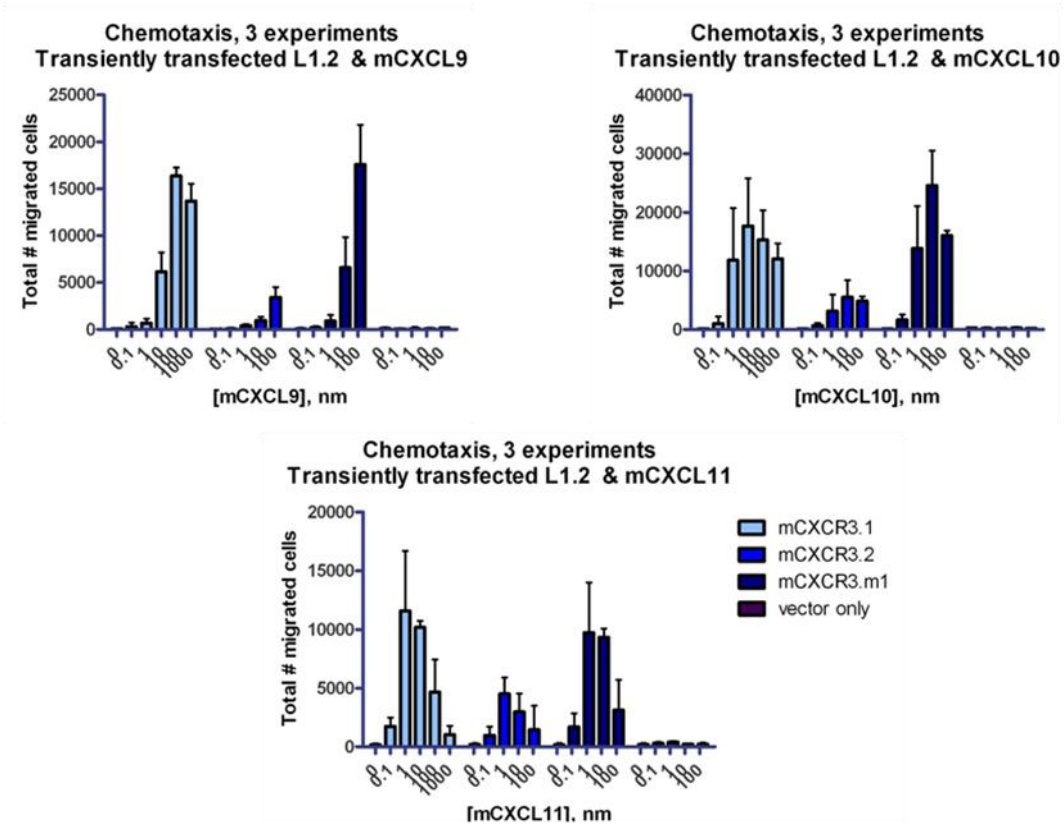

Figure 7. Chemotactic response of L1.2 cells transiently transfected with mCXCR3 constructs. L1.2 cells were transfected with pcDNA3.1_mCXCR3.1, pCDNA3.1_mCXCR3.2, pcDNA3.1_mCXCR3.m1, or pcDNA3.1 only. 200,000 cells were treated with 500mM sodium butyrate and cultured for 24 hours, then placed on top of a membrane over a chemotaxis plate containing different concentrations of chemokine. The plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments.

bottom of the chemotaxis plate, and the membrane was placed over the plate. Cells were placed on top of the membrane and and the entire unit was incubated at 37°C. Cells that migrated through the membrane were counted using a hemacytometer. Only cells transfected with pcDNA3.1_mCXCR3.1 were tested with 1,000nM chemokine to conserve on reagents. Cells were found to successfully migrate to each chemokine (Figure 8). The optimal concentration of mCXCL9 was 100 nM, whereas mCXCL10 stimulated chemotaxis optimally at 10 nM. mCXCL11 was more bioactive per unit mass and was most effective at 1 nM.

4E. Generation of L1.2 cell lines stably expressing mCXCR3. To establish cell clones stably expressing mCXCR3, parental L1.2 cells were transfected via electroporation as described above. These cells were not treated with sodium butyrate and were cultured in 10 mL of complete RPMI-1640 medium. After four to five days of incubation in 37°C at 5% CO_2, cells were pelleted and resuspended in RPMI medium containing geneticin (1 mg/mL). Cells that incorporated the mCXCR3-containing plasmid will have also gained the neomycin resistance gene (*neo*^{r}*)*, which confers resistance to geneticin. Cells were then diluted, seeded into each well of a 96-well plate, and left to incubate for one week. The resulting foci of cells were collected and expanded in 24-well plates. These primary clones were screened for functionality and expression of mCXCR3 by chemotaxis and flow cytometry, respectively. Primary clones resulting from cells transfected with pcDNA3.1_mCXCR3.1 were designated clones "1A", "1B", etc.. Cells grown from cells that were transfected with pcDNA3.1_mCXCR3.2 were similarly named "2A", "2B", etc., and cells transfected with pcDNA3.1_mCXCR3.m1 were named "3A", "3B", etc. The clones showing the highest levels of migration were used in a second round of single cell cloning. They were diluted to 1 cell per every 3 wells of a flat-bottom 96-well plate and cultured in the presence of geneticin for two weeks. The resulting secondary clones were screened for migratory ability (Figure 8) and mCXCR3 levels (Figure 9), and the highest migrating clones were chosen as the stable cell clones to be used in the chemotaxis inhibition studies. Secondary clones' names incorporated the primary clone designation and a number, such as "1A.1" or "3E.4".

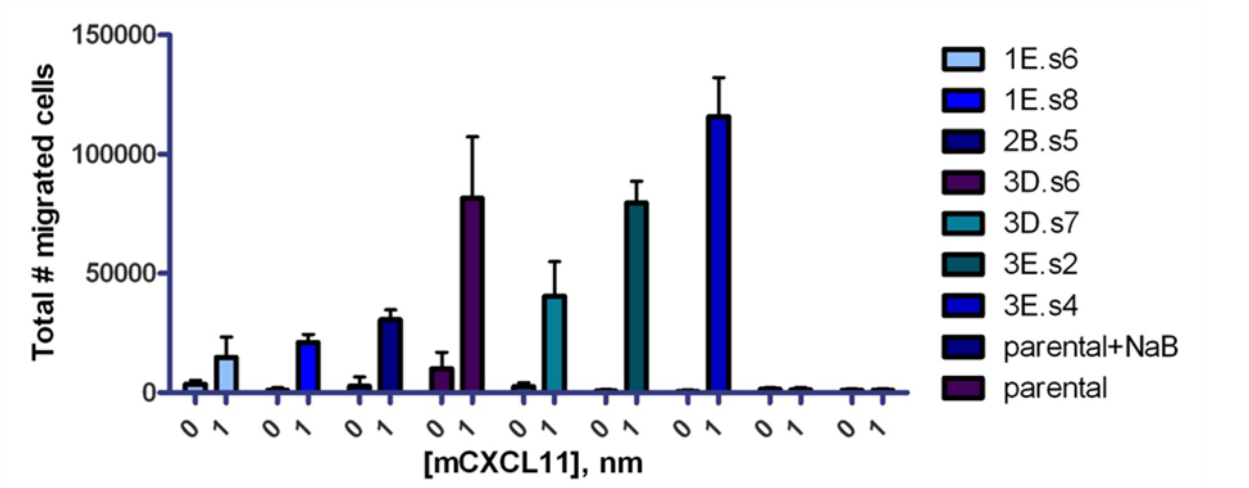

Figure 8. Chemotactic response of L1.2 cell lines stably expressing mCXCR3. Secondary stable clones expressing mCXCR3 were split in a 1:2 ratio every day for three days, then treated with 500 mM NaB. 200,000 cells were placed on top of a membrane over a chemotaxis plate 1 nM mCXCL11. The plate was incubated at 37° at 5% CO_2 in a humidified chamber for three hours, and then migrated cells were counted. The presented data result from one experiment, with each sample performed in triplicate.

Three cell clones were chosen for chemotaxis studies: 1E.s8, 2B.s5, and 3E.s4. The 1E.s8 and 2B.s5 cell clones both expressed wild type mCXCR3, and these two cell lines were chosen because they originated from different primary clones. The 3E.s4 cell line expressed mutant mCXCR3. All three expressed higher levels of mCXCR3 than other cell clones and responded chemotactically to mCXCL11.

4F. Cytotoxicity assays. To determine whether the compounds studied for potential chemotaxis inhibition were cytotoxic, the compounds were cultured with L1.2_mCXCR3.1E.s8 cells and the effects on cell viability were measured. The standard conditions for a chemotaxis assay were replicated to examine if the highest concentrations of each test compound would exert cytotoxic effects during a chemotaxis experiment. Cells were split each day for three days to keep them healthy, and then on the fourth day, a population of cells received an overnight sodium butyrate treatment. Another population was not treated with sodium butyrate to test if overnight sodium butyrate treatment affects cell viability during a chemotaxis assay. Cells

were incubated with these concentrations of compounds for three hours at 37°C in 5% CO_2, and then were counted using trypan blue exclusion.

Both the sodium butyrate-treated (Figure 10A) and untreated cells (Figure 10B) showed similar results, as neither population of cells showed evidence of cytotoxic effects from the highest test concentrations of EGCG, gallotannin, murine X3P3, baicalin, and ginkgolide A. The baicalin and ginkgolide A samples contained 1.28% and 0.25% DMSO, respectively, and the vehicle controls, 0.25% DMSO and 1.28% DMSO, were also not cytotoxic. However, cell viability significantly declined in the presence of 10% DMSO, or 20% DMSO, which served as positive controls for cytotoxicity, yielding approximately 80% dead cells after the incubation.

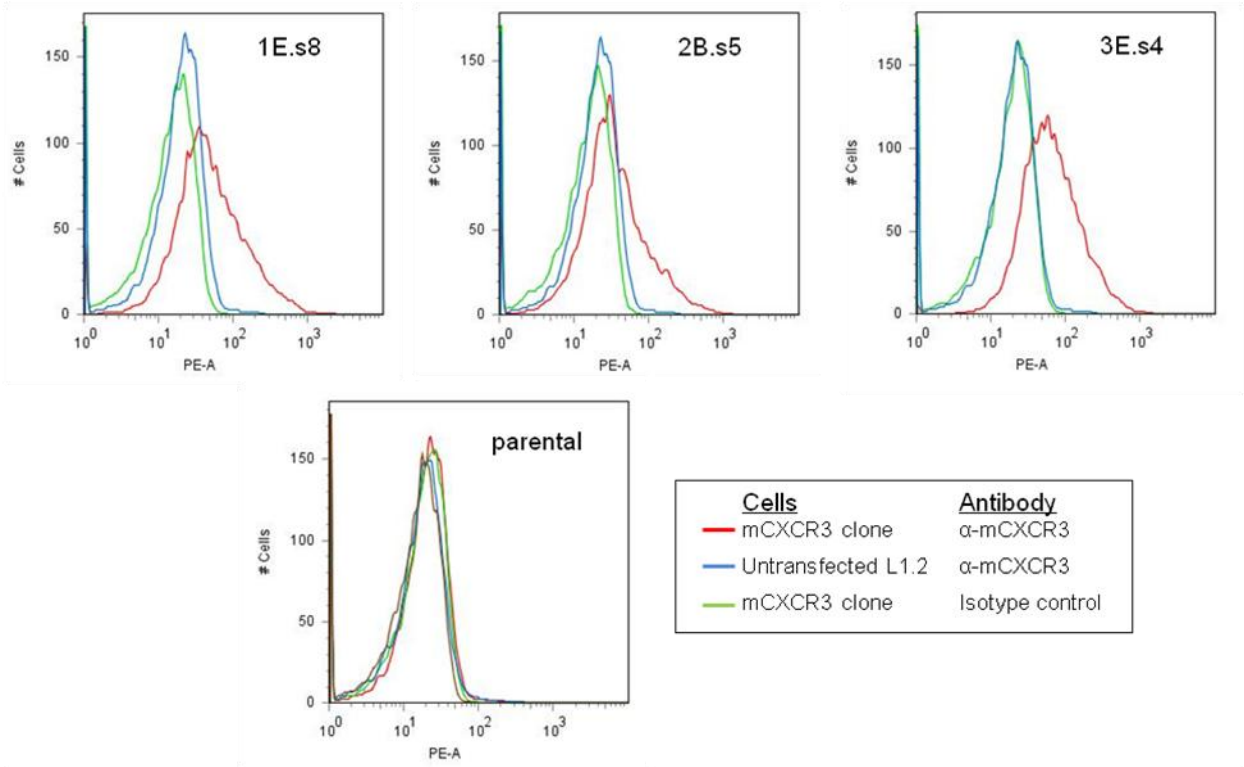

Figure 9. Flow cytometry histogram analysis of three mCXCR3 secondary clones and parental L1.2 cells. Secondary clones as well as parental cells were treated with 500mM sodium butyrate and cultured for 24 hours. Then, cells were stained with anti-murine CXCR3 antibody or isotype control (R&D Systems) and analyzed by flow cytometry. In the histogram, the red line represents mCXCR3-expressing cells stained with α-mCXCR3 antibody, and the green line indicates the same cells stained with isotype control. The blue line shows the control cells: untransfected, parental L1.2 cells treated with NaB and stained with α-mCXCR3 antibody. These data are representative of one experiment.

From these data, it was concluded that none of the compounds examined exerted cytotoxic effects at the highest test concentration, and any chemotactic inhibition that each compound might exhibit was unlikely to be the result of direct cell killing. The data also indicate that overnight incubation with sodium butyrate did not render cells more prone to cell death during the chemotaxis assay on the day after incubation.

4G. Chemotaxis inhibition assays. . To investigate the test compounds' abilities to inhibit chemotaxis, each compound was examined in a chemotaxis inhibition assay. Each assay was completed three separate times, with each sample performed in triplicate. In the first set of chemotaxis experiments, the highest concentration of each compound was tested, based on the concentrations found via literature review. Next, to examine dose-dependent effects, another

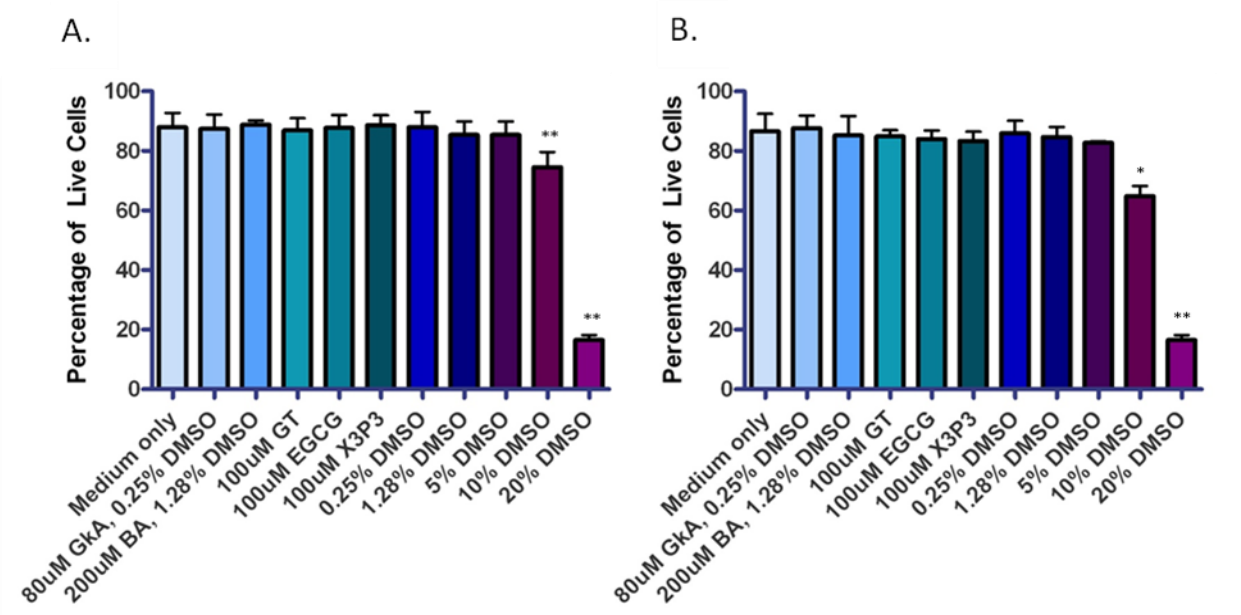

Figure 10. Cytotoxicity assays using sodium butyrate-treated and un-treated cells. *A*, percentage of live sodium butyrate-treated cells after incubation with test compounds, media, or DMSO. *B*, percentage of live sodium butyrate-untreated after incubation with test compounds, media, or DMSO. Cells were split each day for three days, and incubated during the fourth day. The next day, cells were incubated with the indicated concentrations of compounds for three hours at 37°C, the standard conditions for a chemotaxis assay, and then live and dead cells were counted using Trypan Blue.

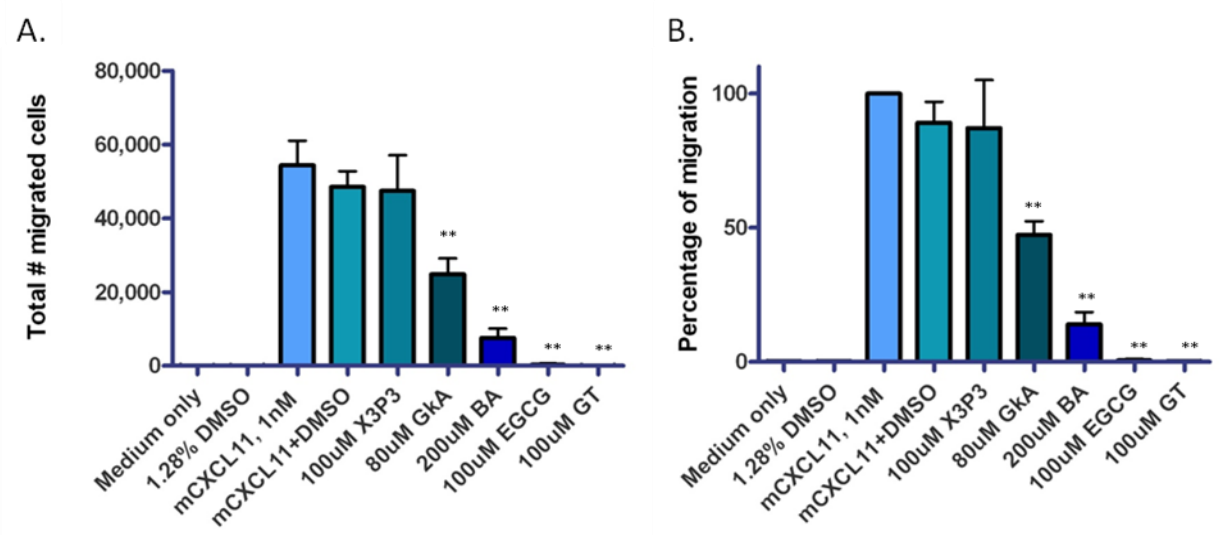

Figure 11. Chemotaxis inhibition using high concentrations of each compound. L1.2_mCXCR3.1E.s8 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing chemokine or chemokine plus test compound. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The wells containing ginkgolide A and baicalin both contained 1.28% DMSO total, as did the sample containing mCXCL11 + DMSO. The data are representative of one experiment.

group of chemotaxis assays included a dilution series of each compound and an optimal concentration of chemokine. These optimal concentrations were determined in earlier studies, as described above. In the third series of assays, the highest concentration of each test compound was used with a titration of each chemokine to observe the effects of the compound on each concentration of chemokine. .

During the first round of chemotaxis inhibition assays, the highest test concentration of each compound was used to inhibit chemotaxis towards mCXCL11, using the cell line L1.2_mCXCR3.1E.s8 (Figure 11). EGCG and gallotannin both appeared to be potent inhibitors of chemotaxis. These high concentrations of EGCG and gallotannin were able to prevent approximately 99% of migration. Because baicalin and ginkgolide A samples contained 1.28% DMSO, their migration values were compared to the chemokine plus DMSO control. Baicalin was slightly less effective than EGCG and gallotannin, as it inhibited about 80% of migration at 200 μM, as compared to the migration towards mCXCL11 plus 1.28% DMSO. Ginkgolide A

appeared to be even less effective, inhibiting chemotaxis by about 50% at the highest test concentration. The difference between the numbers of cells that migrated to mCXCL11 only and mCXCL11 plus 100 μM X3P3 was not statistically significant, and because murine X3P3 failed to show inhibitory properties at 100 μM, I did not include it in following studies.

Titrations of each compound next were used to evaluate their ability to inhibit chemotaxis to each of the three mCXCR3 ligands in a dose-dependent manner. Then a constant concentration of compound and a titration of chemokine was used. All of these experiments were performed using the L1.2_mCXCR3.1E.s8 cell line. These studies showed that EGCG and gallotannin strongly inhibited chemotaxis in a dose-dependent manner and were more potent than baicalin and ginkgolide A. Baicalin showed moderate inhibitory effects at high concentrations, and ginkgolide A appeared to have slight inhibitory effects at its highest concentration.

EGCG has recently been determined to be an effective inhibitor of human CXCR3 ligands by our laboratory (Dr. Shulin Qin, unpublished data). Here, I have found that it is also a potent inhibitor of murine CXCR3 ligands, as shown in Figure 12. At 100 μM, it prevented approximately 98-99% of migration towards the optimal concentration of each of the three chemokines. For example, the average number of cells that migrated towards 1nM mCXCL11 was approximately 50,000 cells, but only approximately 300 cells were observed to migrate towards 1nM mCXCL11 in the presence of 100 μM EGCG. At a 10-fold lower concentration, EGCG was still able to prevent approximately 50% of migration towards the optimal concentration of each chemokine. EGCG was also effective at preventing chemotaxis towards different concentrations of chemokine. When three different concentrations of each chemokine were tested, EGCG was able to inhibit almost all migration towards each chemokine, regardless

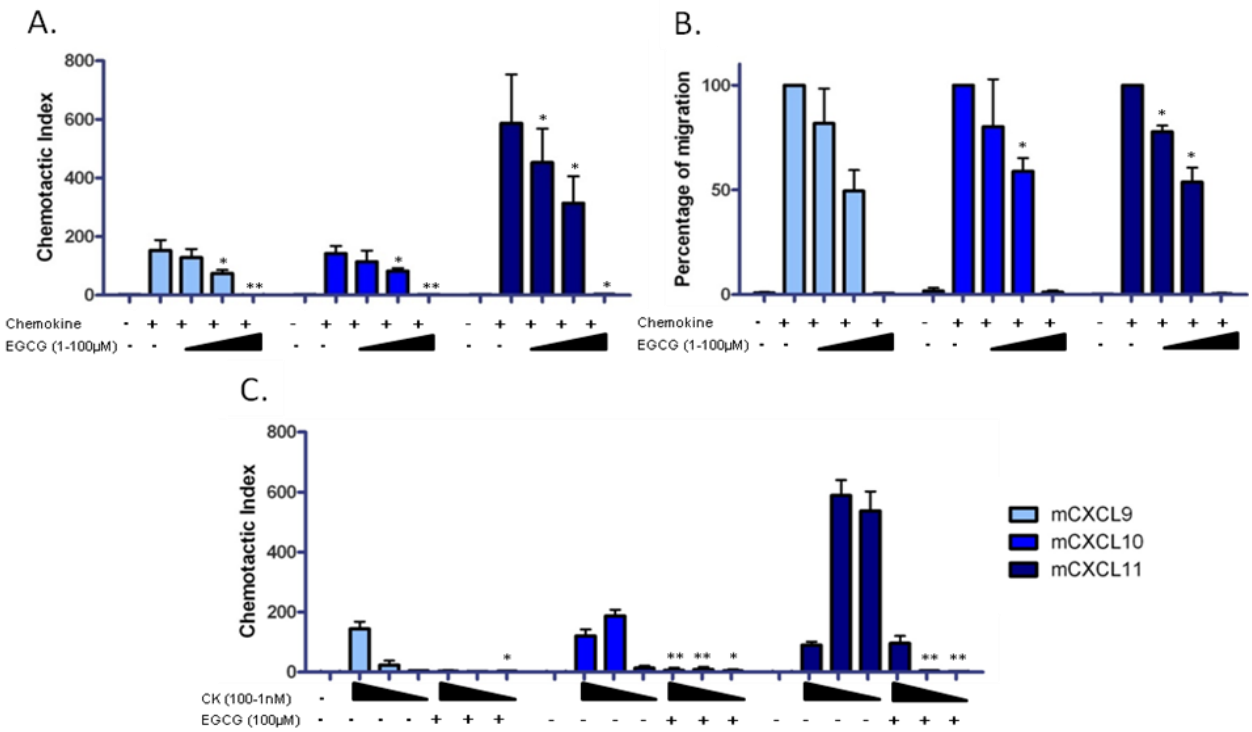

Figure 12. Chemotaxis inhibition using titrations of EGCG and mCXCL9, mCXCL10, and mCXCL11. L1.2_mCXCR3.1E.s8 cells were split at a 1:3 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, or chemokine plus EGCG. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A* and *B*, chemotaxis inhibition using constant chemokine concentrations and titrations of EGCG.** Chemotactic indices are presented in ***A***. Chemokine concentrations were 100 nM mCXCL9, 10 nM mCXCL10, and 1 nM mCXCL11. Paired t-tests were performed with Minitab software using chemotactic index values. Percentage of migration is presented in ***B***. Paired t-tests were performed using cell numbers. ***C*, chemotaxis inhibition using 100 uM EGCG and titrations of each mCXCR3 ligand.** Paired t-tests were performed using chemotactic index values.

of concentration. To determine whether EGCG might bind directly to chemokines, a binding assay performed by Dr. Jodi Craigo (University of Pittsburgh, Center for Vaccine Research) and revealed that EGCG binds directly to murine CXCR3 ligands (Figure 13), with apparently higher affinity for mCXCL11 than mCXCL9 and mCXCL10. This direct binding by EGCG offers one potential mechanism by which it inhibits CXCR3 ligand driven chemotaxis.

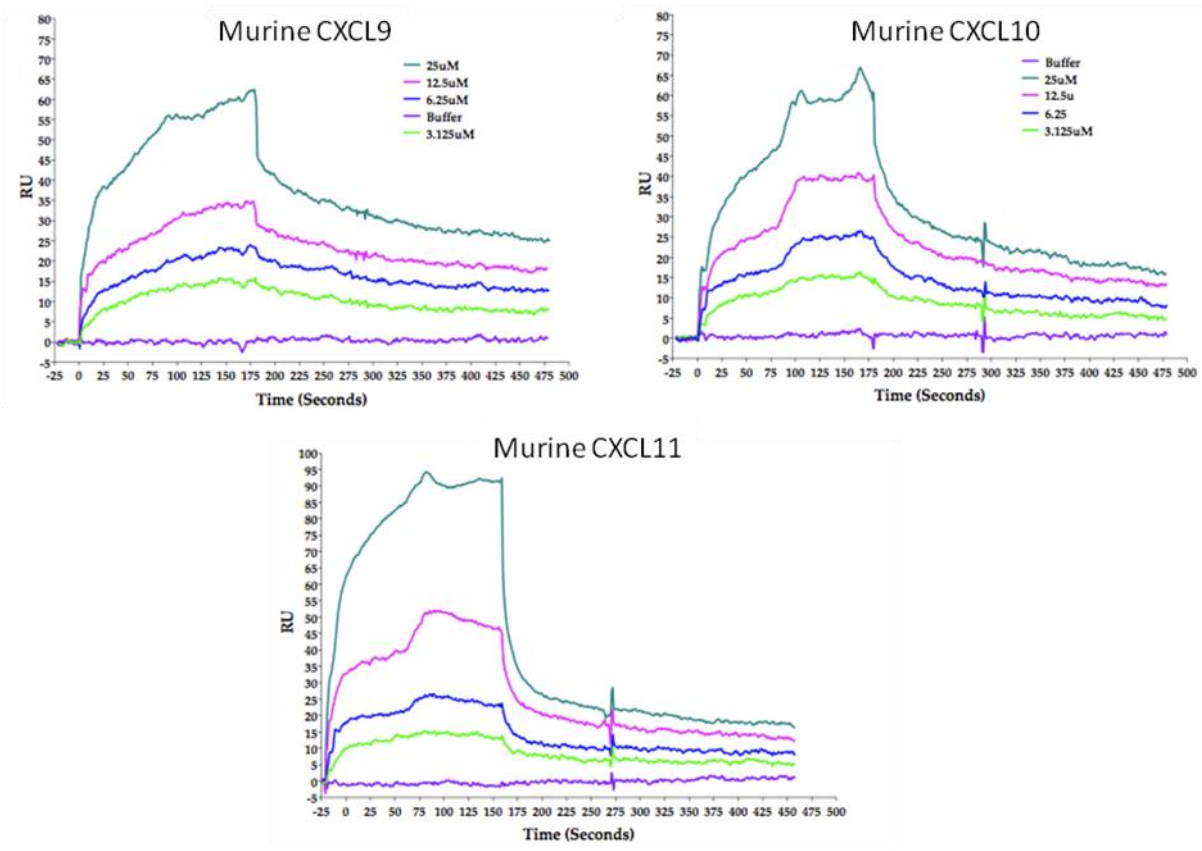

Figure 13. Binding of EGCG to murine CXCL9, CXCL10, and CXCL11. This portion of the study was performed by Dr. Jodi Craigo at the Center for Vaccine Research, using Biacore technology to analyze binding between CXCR3 ligands and EGCG. Murine CXCL9, CXCL10, and CXCL11 were used as ligands and were immobilized on the surface of a CM5 sensor chip via amine coupling. The inhibitory compound EGCG was diluted in sodium phosphate buffer and used as the analytes. Various concentrations of EGCG, as well as buffer only as a negative control, were passed over the chip at a flow velocity of 10µl/min for 3 minutes. Dissociation of binding was measured for five minutes, and adherence of analyte to ligand was measured in resonance units (RU)

Gallotannin appeared to be an even stronger inhibitor of chemotaxis. It was most potent at 50 and 100 µM, at which it prevented approximately 99% of cell migration towards optimal concentrations of each chemokine. At 10 µM, it reduced the number of cells migrating towards 10 nM mCXCL10 and 1 nM mCXCL11 by 80-85%. The average number of cells migrating towards 1 nM mCXCL11 was about 55,000, and the average number of observed cells migrating towards 1 nM mCXCL11 plus 50 or 100 µM gallotannin was 300 and 100,

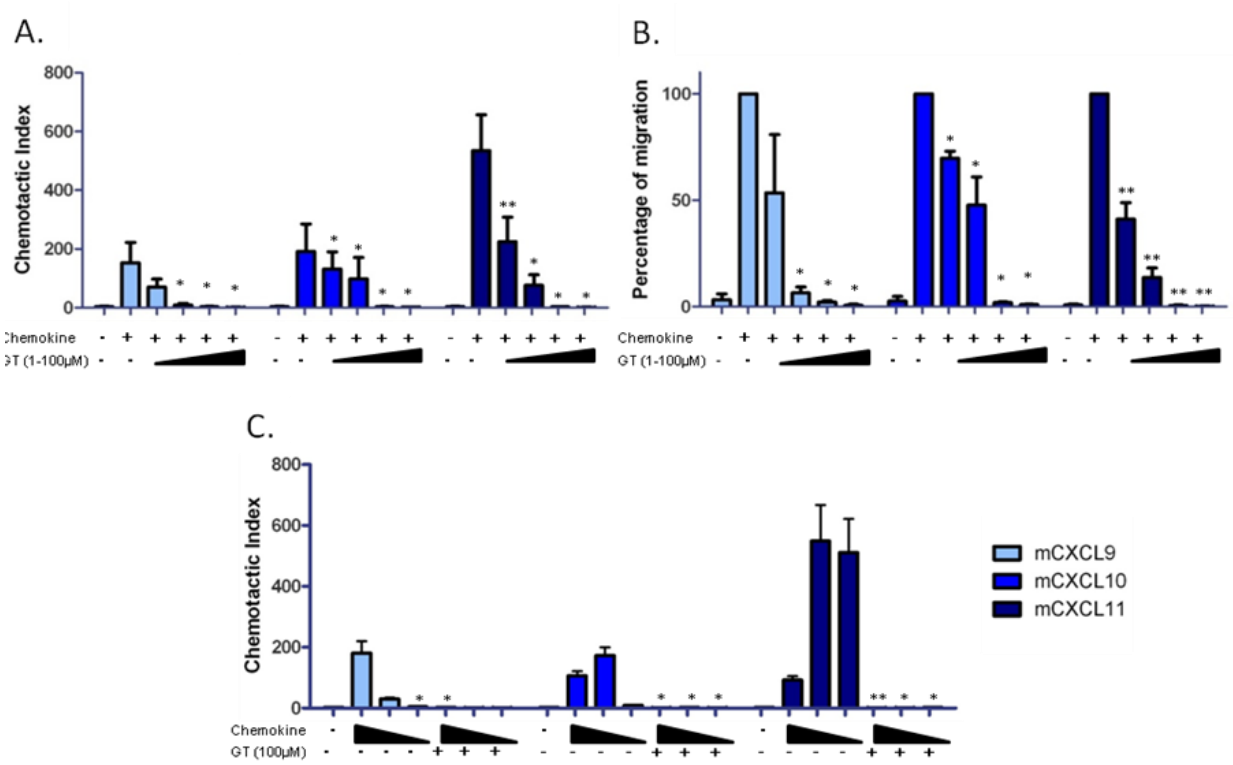

Figure 14. Chemotaxis inhibition using of gallotannin (GT) and mCXCL9, mCXCL10, and mCXCL11. L1.2_mCXCR3.1E.s8 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, or chemokine plus GT. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A* and *B*, chemotaxis inhibition using constant chemokine concentrations and titrations of GT.** Chemotactic indices are presented in ***A***. Chemokine concentrations were 100 nM mCXCL9, 10 nM mCXCL10, and 1 nM mCXCL11. Paired t-tests were performed with Minitab software using chemotactic index values. Percentage of migration is presented in ***B***. Paired t-tests were performed using cell numbers. ***C*, chemotaxis inhibition using 100 uM GT and titrations of each mCXCR3 ligand.** Paired t-tests were performed using chemotactic index values.

respectively. Similar to EGCG, 100 μM gallotannin inhibited almost all chemotaxis towards three different concentrations of each mCXCR3 ligand.

The botanical baicalin showed moderate inhibitory effects on cell migration in a dose-dependent manner. It appeared to affect CXCL11 in a differently dose-dependent manner than with mCXCL9 and mCXCL10. Murine CXCL11 appeared to be more sensitive to baicalin than mCXCL9 and mCXCL10. At 200 μM, baicalin prevented about 85% of cell migration towards 1

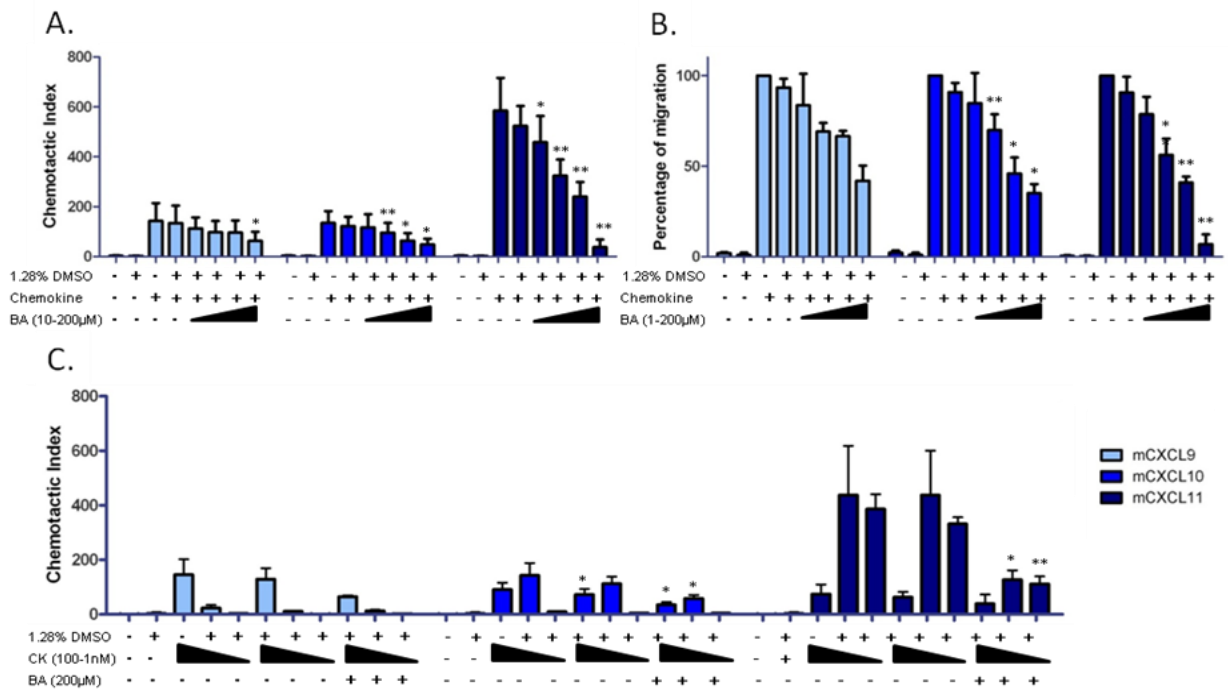

Figure 15. Chemotaxis inhibition using baicalin (BA) and mCXCL9, mCXCL10, and mCXCL11. L1.2_mCXCR3.1E.s8 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, or chemokine plus BA. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A* and *B*, chemotaxis inhibition using constant chemokine concentrations and titrations of BA.** Chemotactic indices are presented in ***A***. Chemokine concentrations were 100 nM mCXCL9, 10 nM mCXCL10, and 1 nM mCXCL11. Paired t-tests were performed with the Minitab software package using chemotactic index values. Percentage of migration is presented in ***B***. Paired t-tests were performed using cell numbers. ***C*, chemotaxis inhibition using 100 uM BA and titrations of each mCXCR3 ligand.** Paired t-tests were performed using chemotactic index values.

nM mCXCL11, and 40-50% of migration towards optimal concentrations of mCXCL9 and mCXCL10. Baicalin showed similar effects on the three different concentrations of chemokine, as it inhibited mCXCL11 more than the other two chemokines. These data suggest that if baicalin exerts inhibitory effects by binding chemokines, it may have a higher affinity for mCXCL11.

The fourth botanical, ginkgolide A, was shown to be the weakest inhibitor of the four test compounds. At the highest concentration of 80 μM, ginkgolide A inhibited approximately

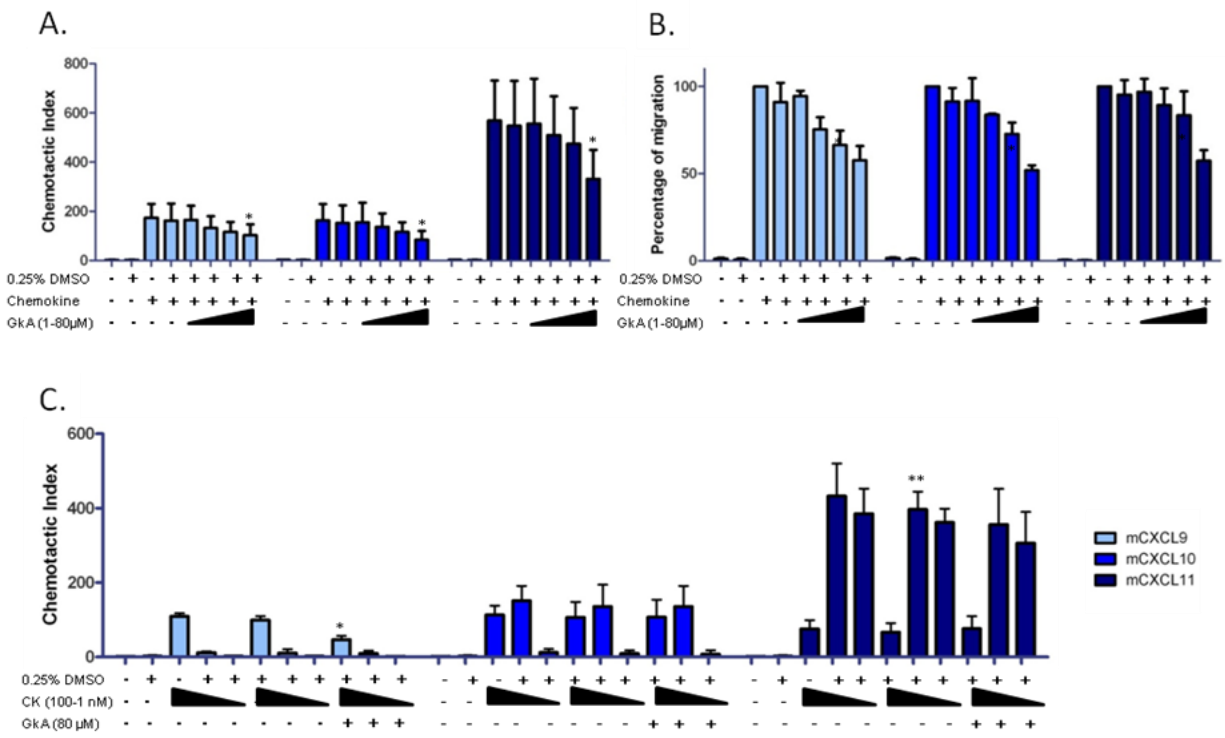

Figure 16. Chemotaxis inhibition using ginkgolide A (GkA) and mCXCL9, mCXCL10, and mCXCL11. L1.2_mCXCR3.1E.s8 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, or chemokine plus GkA. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A* and *B*, chemotaxis inhibition using constant chemokine concentrations and titrations of GkA.** Chemotactic indices are presented in ***A***. Chemokine concentrations were 100 nM mCXCL9, 10 nM mCXCL10, and 1 nM mCXCL11. Paired t-tests were performed with the Minitab software package using chemotactic index values. Percentage of migration is presented in ***B***. Paired t-tests were performed using cell numbers. ***C*, chemotaxis inhibition using 80 uM GkA and titrations of each mCXCR3 ligand.** Paired t-tests were performed using chemotactic index values.

60% of chemotaxis towards each of the three mCXCR3 ligands. At 40 μM, it prevented only 20-30% of cell migration towards each chemokine, and it showed no significant effects at reduced concentrations. Its effects were consistent between each chemokine and for each concentration of chemokine.

4H. Chemotaxis inhibition with other cell lines. To ensure that the test compounds' effects were not unique to the L1.2_mCXCR3.1E.s8 cell clone, two other cell clones were used in chemotaxis inhibition studies: L1.2_mCXCR3.2B.s5 and L1.2_mCXCR3.3E.s4.

L1.2_mCXCR3.2B.s5 expressed wild-type mCXCR3, whereas L1.2_mCXCR3.3E.s4 expressed the mutant form of mCXCR3 described previously. The highest test concentration of each botanical compound was used to test the compound's ability to inhibit cell migration towards mCXCL9, mCXCL10, and mCXCL11. Each test compound affected both cell clones similarly to the L1.2_mCXCR3.1E.s8 cell line (Figures 17 and 18). EGCG and gallotannin strongly inhibited chemotaxis towards the three chemokines, and baicalin and ginkgolide A had moderate effects.

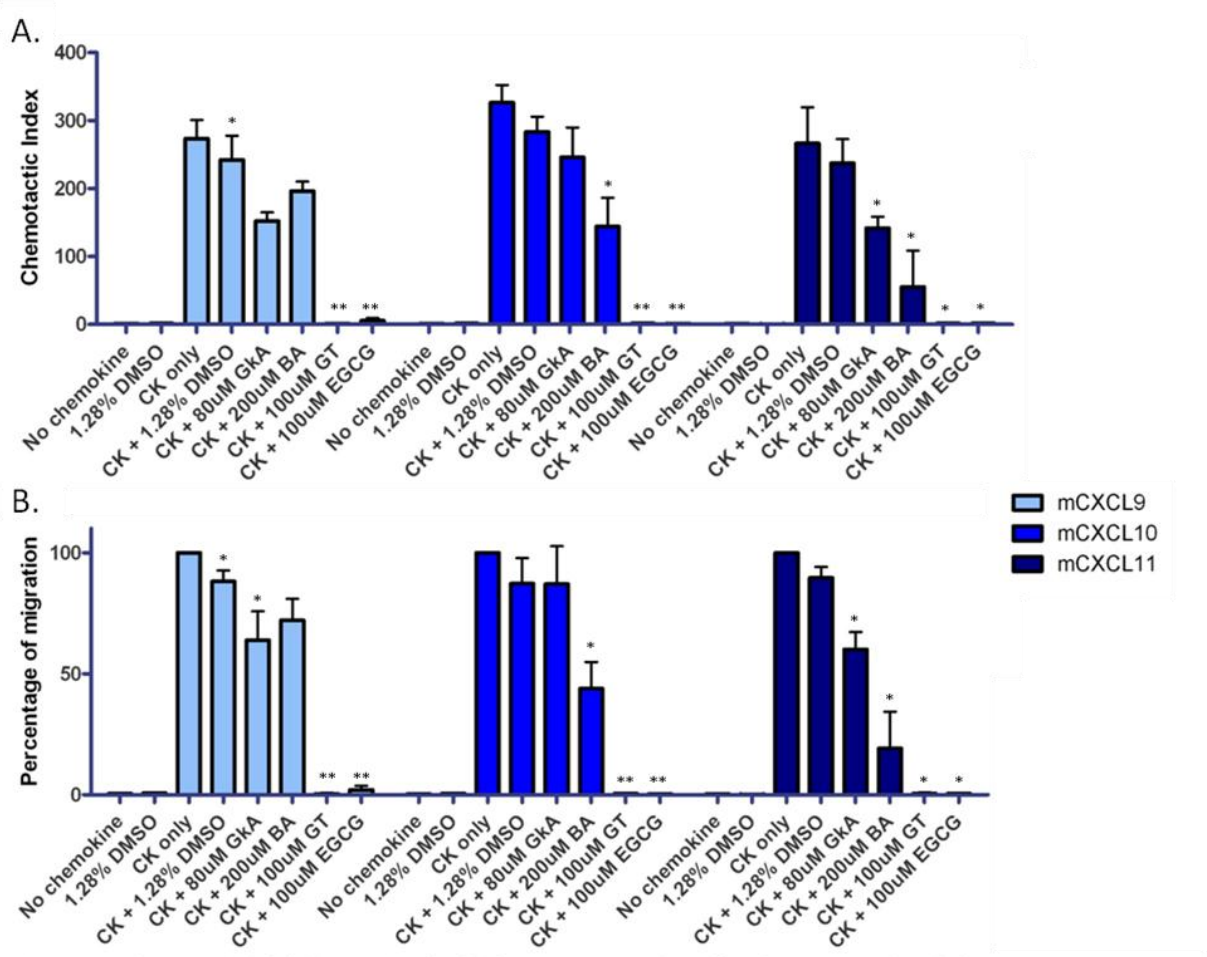

Figure 17. Chemotaxis inhibition using the highest concentration of each compound and the L1.2_mCXCR3.2B.s5 cell line. L1.2_mCXCR3.2B.s5 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, chemokine plus DMSO, or chemokine plus compound. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A,* chemotactic indices** and ***B,* percent of migration.** ***A,*** paired t-tests were performed with Minitab software using chemotactic index values. ***B,*** paired t-tests were performed using cell numbers.

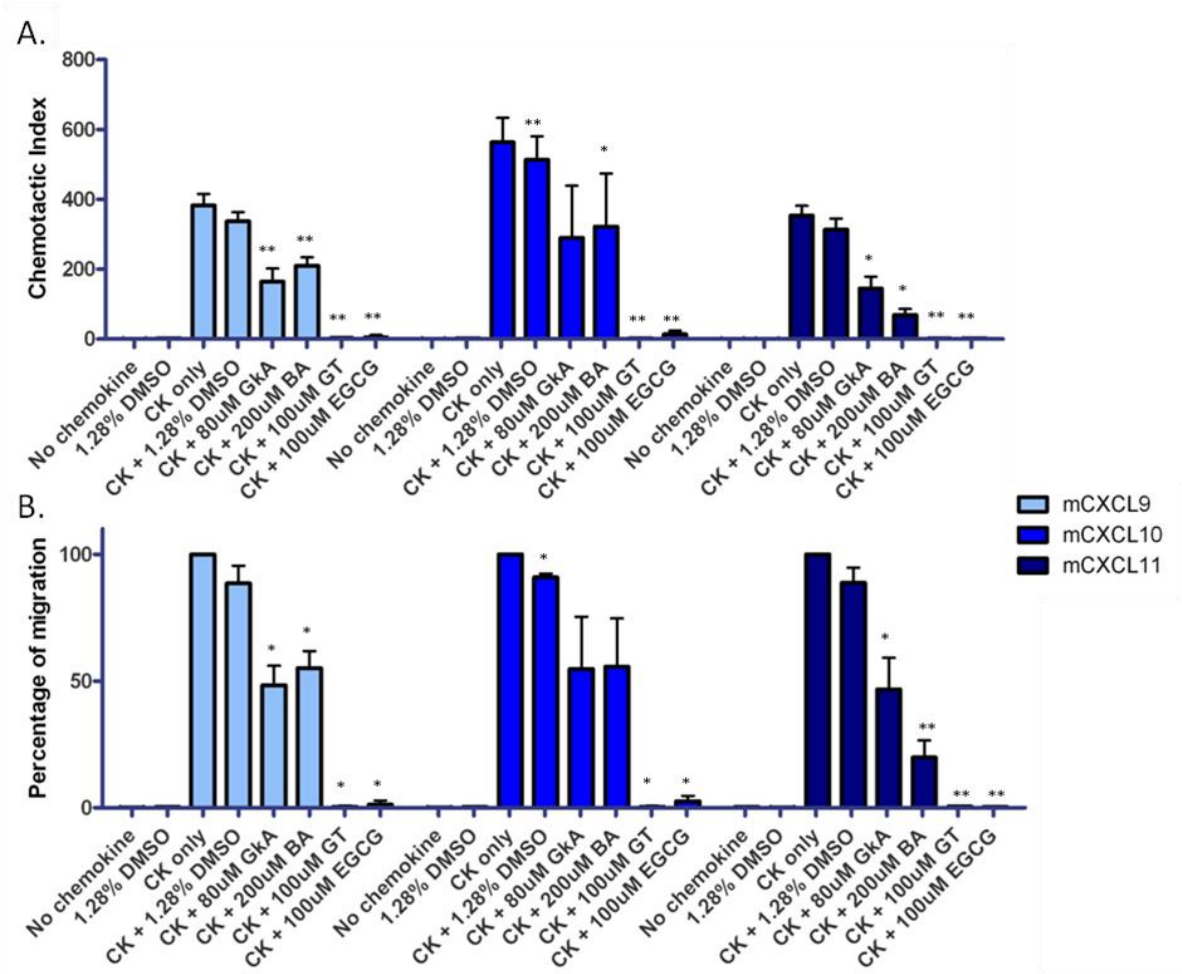

Figure 18. Chemotaxis inhibition using the highest concentration of each compound and the L1.2_mCXCR3.3E.s4 cell line. L1.2_mCXCR3.3E.s4 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, chemokine plus DMSO, or chemokine plus compound. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A,* chemotactic indices** and ***B,* percent of migration.** ***A,*** paired t-tests were performed with Minitab software using chemotactic index values. ***B,*** paired t-tests were performed using cell numbers.

For both cell clones, EGCG and gallotannin inhibited approximately 98-99% of cell migration to each chemokine, as they did in previous chemotaxis experiments using L1.2_mCXCR3.1E.s8. Murine CXCL11 also appeared to be more sensitive to baicalin compared to mCXCL10 and mCXCL11, as 200 μM baicalin prevented 70-80% of migration towards mCXCL11 for each cell line. It inhibited approximately 40-50% of migration towards mCXCL9

and mCXCL10 for both of the cell clones. The effects of ginkgolide A were consistent between each of the cell clones tested, as it inhibited at most 50% of migration towards each chemokine. The cell clone expressing mutant mCXCR3 migrated at higher levels to each chemokine, as compared to the L1.2_mCXCR3.1E.s8 and L1.2_mCXCR3.2B.s5 cell lines. For example, the average number of L1.2_mCXCR3.3E.s4 cells migrating towards 1 nM mCXCL11 was 70,000, whereas the corresponding number of L1.2_mCXCR3.2B.s5 cells was 53,000. An average of 51,000 cells from the mutant mCXCR3-expressing cell line migrated towards 100 nM mCXCL9, compared to an average of 27,000 cells from the L1.2_mCXCR3.2B.s5 cell line. These results suggest that the mutant mCXCR3 protein does not have diminished capacity to bind or respond to mCXCL9. However, only one mutant mCXCR3-expressing cell clone was examined, and other such cell clones must be examined

4I. Chemotaxis inhibition with EGCG analogs. To investigate which functional groups of EGCG might be important for inhibition of chemotaxis, three compounds with similar structures were used in chemotaxis inhibition assays. These analogs of EGCG are epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC), and their structures are depicted in Figure 1.

The most effective compound of these three green tea polyphenols was ECG, which, at 100 μM, was able to inhibit approximately 50% of migration toward mCXCL9 and mCXCL10 and about 75-80% of chemotaxis toward mCXCL11 in a dose-dependent manner (Figure 19). In Figure 19, the light blue bars on the right represent mCXCL9, the set of royal blue bars in the middle correspond to mCXCL10, and the navy blue bars on the right denote mCXCL11. Murine CXCL11 appeared to be the most sensitive to the inhibitory effect of each compound. The highest concentration of EGC and EC prevented approximately 50% of migration toward

mCXCL9 and mCXCL10, and about 60% of chemotaxis toward mCXCL11.

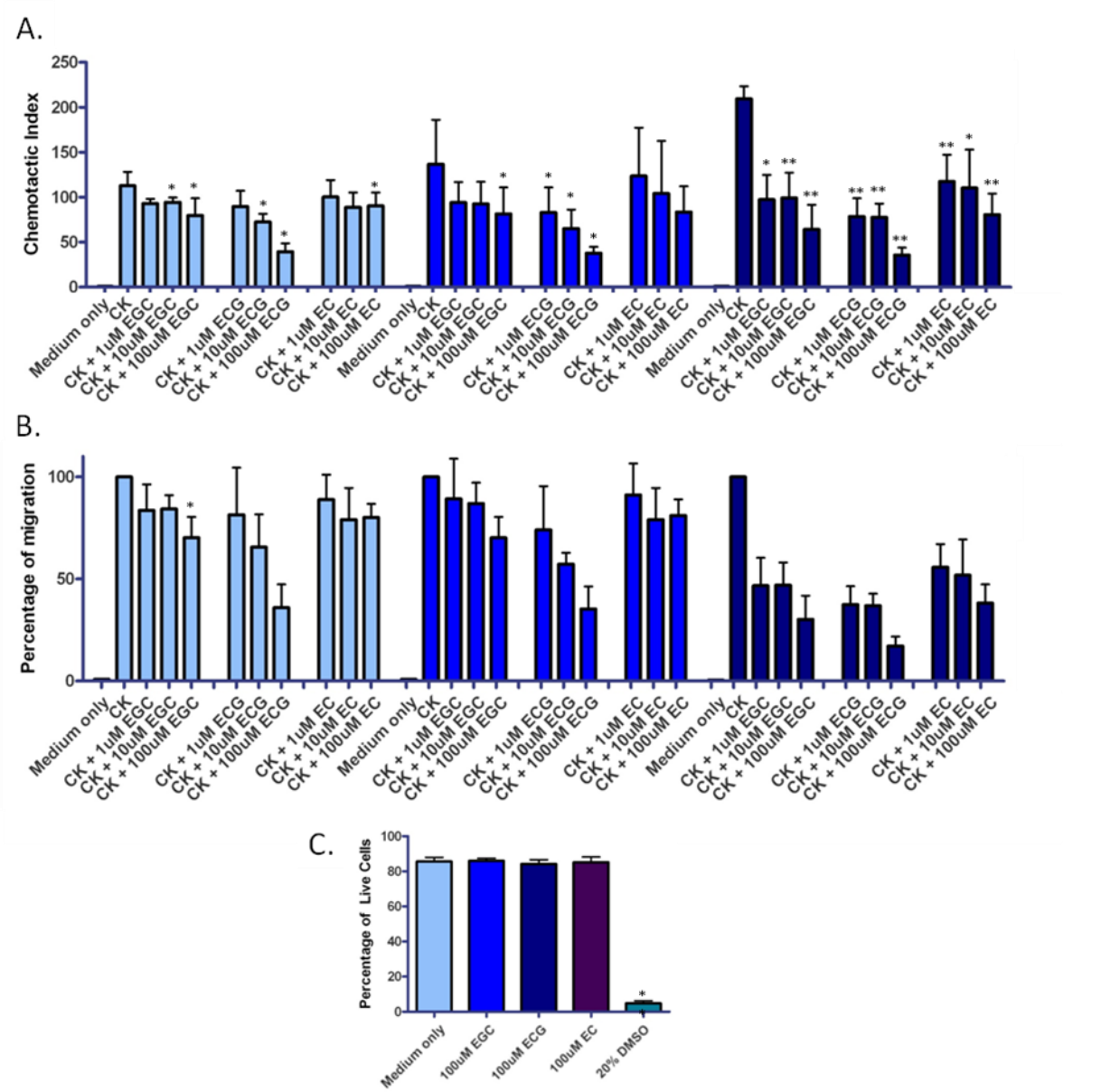

Figure 19. Chemotaxis inhibition using 100 µM of EGC, ECG, and EC and the L1.2_mCXCR3.1E.s8 cell line, and cytotoxicity assay. *A,* chemotactic indices and ***B*, percent of migration.** L1.2_mCXCR3.1E.s8 cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, chemokine plus DMSO, or chemokine plus compound. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are the averages of the means of three experiments. ***A,*** Paired t-tests were performed with the Minitab software package using chemotactic index values. ***B,*** paired t-tests were performed using cell numbers. ***C,* A set of cytotoxicity assays were performed using 100 µM of each compound.** The same cells used in the chemotaxis studies were incubated for three hours with media only, 20% DMSO, or 100 µM of each EGCG analog. After a three-hour incubation at 37°C and 5% CO_2, live and dead cells were counted using Trypan Blue. Paired t-tests were performed using total cell numbers.

4J. Summary. In summary, each botanical compound examined had different inhibitory effects on the cell migration of three cell lines expressing mCXCR3. Gallotannin seemed to be the most potent inhibitor, followed closely by EGCG. Baicalin was able to inhibit 50% of migration towards mCXCL9 and mCXCL10 at the highest test concentration, but it inhibited migration to mCXCL11 more strongly. The fourth compound, ginkgolide A, was somewhat effective at the highest test concentration, with at most 40% inhibition of chemotaxis. These results were consistent for the three cell lines tested. Among the EGCG analog compounds tested, ECG was the strongest inhibitor of chemotaxis, although it was not as potent as EGCG. It was able to inhibit migration induced by mCXCL11 by about 80%. EGC and EC were both weaker inhibitors than ECG, as 100 μM of each compound prevented 50% of migration toward mCXCL11.

CHAPTER 5: DISCUSSION

5A. Overview. CXCR3 is thought to be involved in numerous inflammatory disorders. In an effort to research new therapies for such diseases, I investigated several compounds that may modulate CXCR3-ligand interactions using an in vitro mouse model. I used this model in preparation for establishing an *in vivo* model that would seek to accurately reflect the effects of the inhibitory compounds in humans. The main purpose of this study was to study the inhibitory properties of botanical compounds and a murine CXCR3 peptidergic mimetic on murine CXCR3 ligand function.

5B. Murine X3P3. In this project, I sought to determine if a peptide mimetic of the ECL-2 of murine CXCR3 would inhibit the activity of mCXCR3 ligands. A small number of previous studies have examined peptide mimetics of chemokine receptor extracellular domains. CCR5 mimetics are known to bind the HIV glycoprotein gp120, a CCR5 ligand, as demonstrated by Agrawal *et al.*[79]. The second extracellular loop (ECL-2) of CCR5 was shown to bind the gp120 of an R5 HIV strain and inhibit viral fusion and infection in a dose-dependent manner. However, the effects of this mimetic on the natural ligands for CCR5 was not examined. Human X3P3, an ECL-2 mimetic of CXCR3, has also been determined by our group to bind CXCR3 ligands and exert dose-dependent inhibition of chemotaxis (S. Qin, unpublished data). Thus, it has been established some ECL-2 peptide mimetics are able to bind several chemokine receptor ligands. However, whereas human X3P3 is efficient at inhibiting chemotaxis towards CXCR3 ligands, its murine counterpart mX3P3 did not significantly inhibit migration of murine CXCR3+ cells. This incongruity may be attributed to the possibility that that murine X3P3, when presented separately from the chemokine receptor as a whole, has a different structure. Whereas human

X3P3 retains a conformation that is able to bind ligands, murine X3P3 likely assumes a structure that is unable to bind CXCR3 ligands. Murine X3P3 may not bind murine CXCR3 ligands with as much affinity as human X3P3 binds human CXCR3 ligands, and perhaps murine CXCR3 ligands bind other ECLs with higher affinity due to a difference in chemokine receptor structure. In any case, the *in vitro* data suggest that the current form of murine X3P3 likely will not effectively inhibit mCXCR3 ligands *in vivo*. More research is needed to discover if an ECL-2 peptide mimetic or other ECL-based mimetics will inhibit CXCR3-ligand interactions.

5C. Botanical compounds. Some of the botanical test compounds, however, successfully inhibited mCXCR3 ligand function, and the data suggest that EGCG and gallotannin will be strong inhibitors of mCXCR3 ligands in mice. EGCG has been tested in chemotaxis inhibition assays using human cells (S. Qin), and the pattern of inhibition in murine and human cells is similar (Figure 20). Thus, it may be anticipated that EGCG will have anti-inflammatory effects in mice similarly to humans *in vivo*. Gallotannin, baicalin, and ginkolide A have not yet been examined in our hands with human cells, and these studies may be pursued in the future to determine if these botanicals inhibit the migration of human cells to the same extent as they inhibited murine cells. If so, the murine model may be suitable for *in vivo* studies of infection and inflammation.

To understand why some compounds inhibited chemotaxis and others did not, it will be helpful to compare their structures. When comparing the structures of successful versus ineffective compounds, a pattern emerges. The more hydroxyl groups a compound has, the more potent it is at inhibiting chemotaxis. Gallotannin has by far the most hydroxyl groups and was the strongest inhibitor, followed by EGCG, then baicalin, then ginkoglide A. Ginkgolide A is the weakest inhibitor, and as the pattern predicts, has the least hydroxyl groups. The mCXCR3

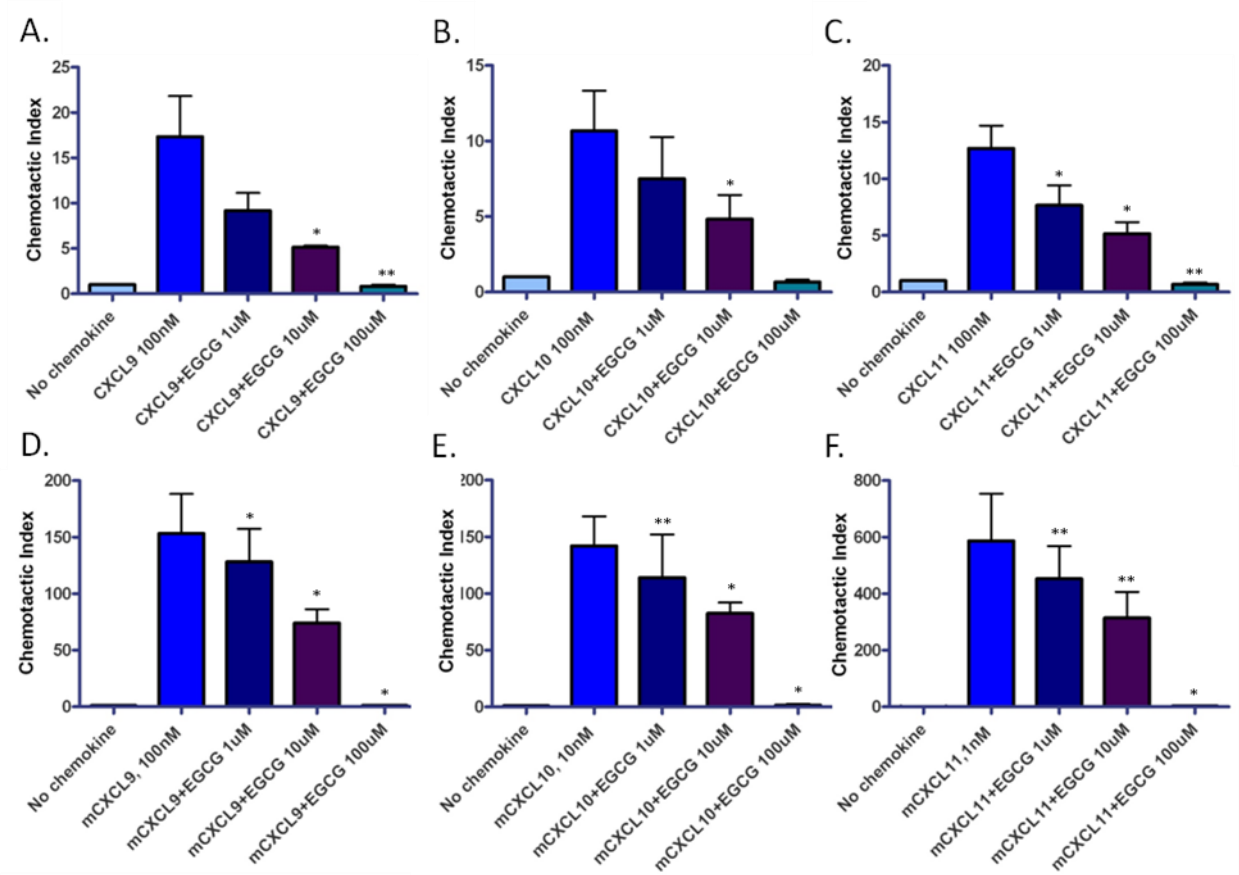

Figure 20. The effects of EGCG on the chemotaxis on human versus murine cells. Cells were split at a 1:2 ratio each day for three days, and then treated with sodium butyrate. Cells were incubated overnight, and then placed on top of a chemotaxis membrane over wells containing media only, chemokine only, or chemokine plus EGCG. The chemotaxis plate was incubated at 37°C at 5% CO_2 in a humidified chamber for three hours, and migrated cells were counted. The presented data are averages of three experiments. ***A, B, C,*** Chemotaxis inhibition assays were performed using human CD4+ cells (Dr. S. Qin). EGCG was used to inhibit migration toward ***A,*** human **CXCL9**, ***B,*** **CXCL10,** and ***C,*** **CXCL11.** The presented data are averages of three experiments, with each experiment using cells from a different donor. ***D, E, F,*** **chemotaxis inhibition assays using mCXCR3-expressing murine cells**. EGCG was used to inhibit migration toward ***A,*** murine **CXCL9**, ***B,*** **mCXCL10,** and ***C,*** **mCXCL11.** Paired t-tests were performed on the chemotactic indices using the Minitab software package.

ligands may prefer to bind these groups due to structural complementarity or perhaps due to their negative charge. These data are consistent with past studies' findings that chemokines generally must be able to bind to glycosaminoglycans (GAGs) to function *in vivo*[80]. GAGs are linear polysaccharides found on cell surfaces and within the extracellular matrix, and they are acidic, very negatively-charged, and highly-sulfated with the exception of hyaluronan. GAGs are long chains made up of disaccharide units, also called disaccharide repeating regions[81]. A representative disaccharide unit of each type of GAG is shown in Figure 21. It is generally believed that GAGs selectively bind most chemokines and help establish chemokine gradients[82].

Notably, GAGs and the botanicals that most potently inhibited chemotaxis – gallotannin and EGCG – share similar properties. In solution, the hydroxyl groups on gallotannin and EGCG would donate their hydrogens. Gallotannin and EGCG would become negatively charged and acidic, as are GAGs, which are known to bind chemokines. There is also a dose-dependency of sorts, as gallotannin has more hydroxyl groups and more of a negative charge, it is able to inhibit migration more efficiently. Thus, it is a plausible theory that EGCG and gallotannin are able to bind mCXCR3 ligands on GAG-binding domains due to their hydroxyl groups and negative charge. They form complexes with the chemokines and subsequently prevent them from binding with their receptors. This is similar to the activity of soluble GAGs, as described by a study by Kuschert *et al*[83]. Soluble GAGs were shown to bind to IL-8 and MIP-1α and inhibit binding to their respective receptors. Furthermore, soluble heparin was able to inhibit IL-8 induced intracellular calcium mobilization in neutrophils in a dose-dependent manner.

To investigate the hypothesis that EGCG and gallotannin inhibit chemokine function similarly to soluble GAGs, it must be determined if they bind mCXCR3 ligands. EGCG has been shown to bind directly to human and murine CXCR3 ligands (J. Craigo, Figure 13), but such studies have not yet been performed with gallotannin. These studies may be performed in the

future to verify the binding of gallotannin to mCXCR3 ligands. There is a caveat in assuming gallotannin is inhibiting the function of mCXCR3 ligands simply by binding them in solution. During cytotoxicity studies, a slightly grainy residue was observed when examining cells under the microscope, suggesting that gallotannin was precipitating out of solution when dissolved in chemotaxis medium. Although this residue was not observed when examining samples after a chemotaxis assay, the compound may be coming out of solution and drawing the chemokines out of solution as well, instead of simply binding them free in solution. Further studies are required to determine the mechanism by which gallotannin inhibits chemokine function.

If EGCG and gallotannin bind the GAG-binding domains on mCXCR3 ligands, it is possible that these compounds may prevent chemokine binding to GAGs and subsequently

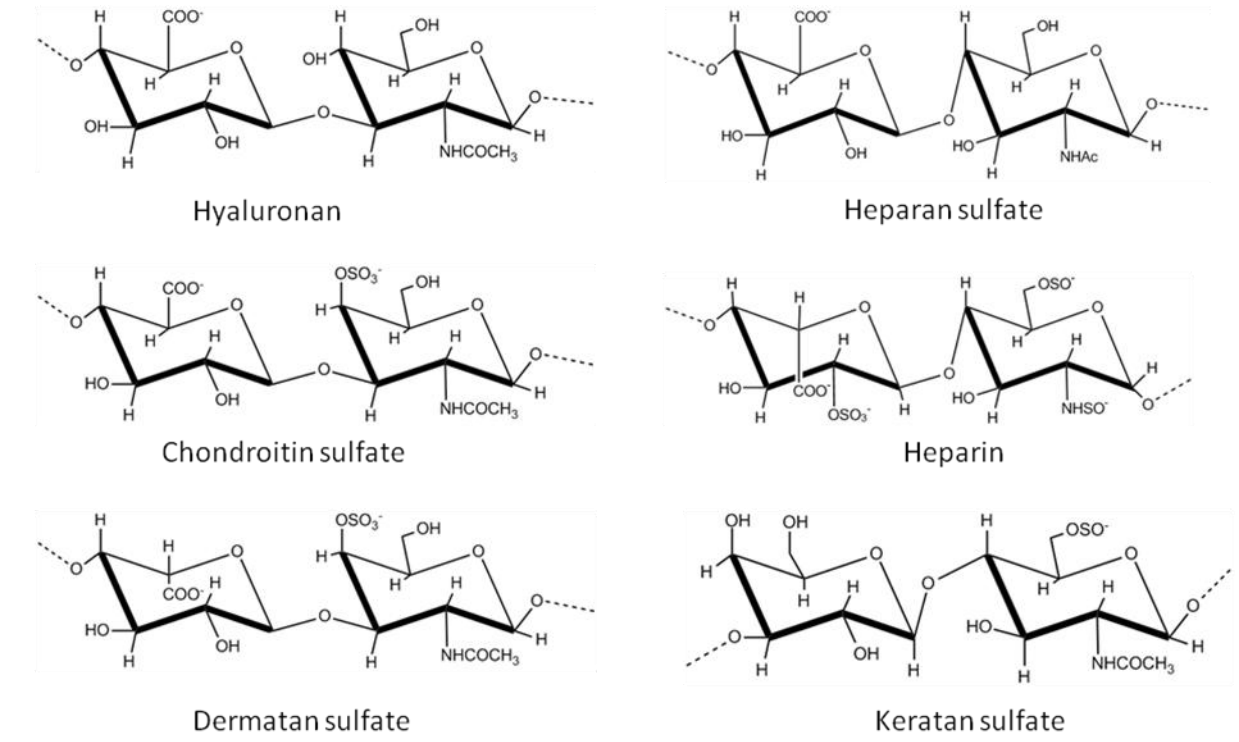

Figure 21. Structures of glycosaminoglycans (GAGs). A representative disaccharide unit of each is shown: hyaluronan, heparan sulfate, heparin, chondroitin sulfate, dermatan sulfate, keratan sulfate. Structures were generated using ChemDraw Ultra software (Cambridge) and were adapted from Gandhi and Mancera, 2008.

impede the establishment of haplotactic chemokine gradients in the extracellular matrix. While it is not necessary for chemokines to form a gradient to induce chemotaxis[84], the disruption of a chemokine gradient would interfere with or reduce cell migration induced by that particular chemokine. Without the establishment of a gradient, chemokines can also stimulate chemotaxis by a process called chemokinesis, if they are matrix-bound and are presented close together in concentric rings[85]. The interference of GAG-chemokine binding by EGCG and gallotannin will also interfere with chemokinesis, as they will prevent chemokines from becoming matrix-bound.

5D. EGCG analogs. The data here suggest that amongst the botanicals examined, the more hydroxyl groups or negative charge a compound has, the more potently it inhibits chemotaxis. This pattern is also seen when comparing the inhibitory ability of EGCG and its analogs, which are also green tea polyphenols. EGCG is the strongest inhibitor, followed by ECG, then EGC and EC. EGCG has eight hydroxyl groups, ECG has seven, EGC has six, and EC has five. However, this observation is not enough to fully explain why EGCG is a much stronger inhibitor of mCXCR3 ligands than is ECG, with a difference of only one hydroxyl group. The difference in potency is likely due to differences in structure, although EGCG and ECG are identical in structure except for the one hydroxyl group. Thus, EGCG may be better able to bind chemokines because of a certain conformational advantage.

5E. Mutant mCXCR3.

I hypothesized that the mutant form of mCXCR3 may be unable or less able to efficiently bind mCXCL9 due to two amino acid substitutions located in its N-terminus. The 16 amino acids located in the N-terminus of human CXCR3 are thought to be important for binding

CXCL9[86]. However, the L1.2_mCXCR3.3E.s8 cell line was not impaired in its ability to respond to mCXCL9. This observation suggests that the glutamine and valine at positions 4 and 5 are not essential for binding mCXCL9 and responding to it, or that the two amino acid substitutions did not substantially change the folding of the N-terminus of mCXCR3. This may be possible, as that substituted amino acids have the same charge as the original amino acids. However, only one mutant mCXCR3-expressing cell clone was analyzed in this study and analysis of additional clones will help to confirm this finding.

5F. Future studies. Several studies that may helpful for understanding the data generated in the current study have already been mentioned. Other cell clones expressing the mutant form of mCXCR3 could be analyzed to see if the results observed in this study are consistent with these other cell lines, and to further understand the effect, if any, of the two point mutations in the extracellular C-terminal region. Other mutation studies may include forms of mCXCR3 that respond specifically to each chemokine and determine if each mutant is affected differently by mCXCR3 inhibitors like EGCG. These mutants would contain specific mutations in multiple regions that bind mCXCL9, mCXCL10, and mCXCL11. A third type of mutant study can include GAG-binding domains, such as the study by Ali et al.[87]. In this study, a mutant form of CCL7 that did not bind heparin was constructed. This mutant was able to generate a chemotactic response *in vitro*, but failed to do so *in vivo*. One of the hypotheses discussed earlier is that EGCG and gallotannin are effective at binding mCXCL9 ligands due to their negative charges, which GAGs also have. GAG-binding mutants may be analyzed to determine if they are refractory to the inhibitory properties of EGCG and gallotannin. If so, this will confirm that negative charges are important properties for mCXCR3 inhibitors.

An additional study may be performed to follow up on the negative data concerning murine X3P3. Since it did not appear to inhibit mCXCR3 ligand function efficiently, future studies may focus on finding a peptide mimetic of mCXCR3 that is more effective at inhibiting mCXCR3-ligand interactions. Perhaps it would be useful to study how murine X3P3 can be modified to become a more effective inhibitor. A hypothesis about mX3P3 discussed previously is that it may not fold correctly when separated from the mCXCR3 protein, thus future studies may address how mX3P3 can be induced to fold in different ways that are more conducive to binding mCXCR3 ligands.

Lastly, an approach that may be useful in illuminating the method by which EGCG and gallotannin inhibit mCXCR3 ligand function is a binding study. This has been accomplished in the past using Biacore technology (Figure 13), which measures the binding of a molecule bound on a chip to another molecule passed over the chip in a flow channel. Another binding study uses radioactive ^{125}I to label chemokines, and these chemokines are incubated with the compound in question[88]. Cells are then centrifuged, and cell-associated radioactivity is counted with a gamma counter.

5G. Public health relevance. EGCG and gallotannin are able to modulate CXCR3-ligand interactions, and they may be novel candidates for development into therapeutics for CXCR3-mediated inflammatory diseases, and possibly other chemokine-related inflammatory diseases. Currently there is a demand for naturally-derived therapies with fewer side effects, and botanicals are excellent sources for such therapies.

While EGCG and gallotannin may be able to regulate inflammation, they may also non-specifically bind other chemokines due to their negative charges or through their GAG-binding domains. Most chemokines in addition to CXCL9, CXCL10, and CXCL11 are positively charged,

including chemokines involved homeostatic and inflammatory processes. Thus, EGCG and gallotannin may interfere with important physiological processes such as organogenesis and normal inflammatory responses to combat pathogens. However, it must be recognized that green and black teas have been consumed for millennia with no clear detriment to health or fetal development. To prevent this interference, dosages of EGCG and gallotannin must be further researched, and low dosages that will allow these processes to occur should be used. EGCG and gallotannin, may also interfere with vaccination. Inflammatory responses are necessary for an effective vaccination, and inhibition of inflammatory chemokines would be counterproductive to the purposes of vaccination. It may be a useful recommendation for a patient to refrain from taking EGCG, gallotannin, or foods and supplements that contain these compounds while being vaccinated.

5H. Model of inhibition. It has been discovered that EGCG and gallotannin can inhibit chemotaxis driven by mCXCR3 ligands, and it has been shown that EGCG can bind mCXCR3 ligands. The model of inhibition proposed in this study, then, is that compounds like EGCG and gallotannin inhibit mCXCL9 ligand function by binding the chemokines and preventing them from interacting with their receptor (Figure 22). This inhibitory action may be beneficial in treatments for inflammatory disorders such as rheumatoid arthritis and Crohn's disease. In rheumatoid arthritis, the lining of the joints – called the synovium – become inflamed due to the recruitment of Th-1 type T lymphocytes. Because CXCR3 is preferentially expressed on these cells, a CXCR3 inhibitor such as EGCG may prevent the migration of Th-1 cells to the synovium by binding CXCR3 ligands, thereby reducing the severity of inflammation and alleviating the symptoms of rheumatoid arthritis.

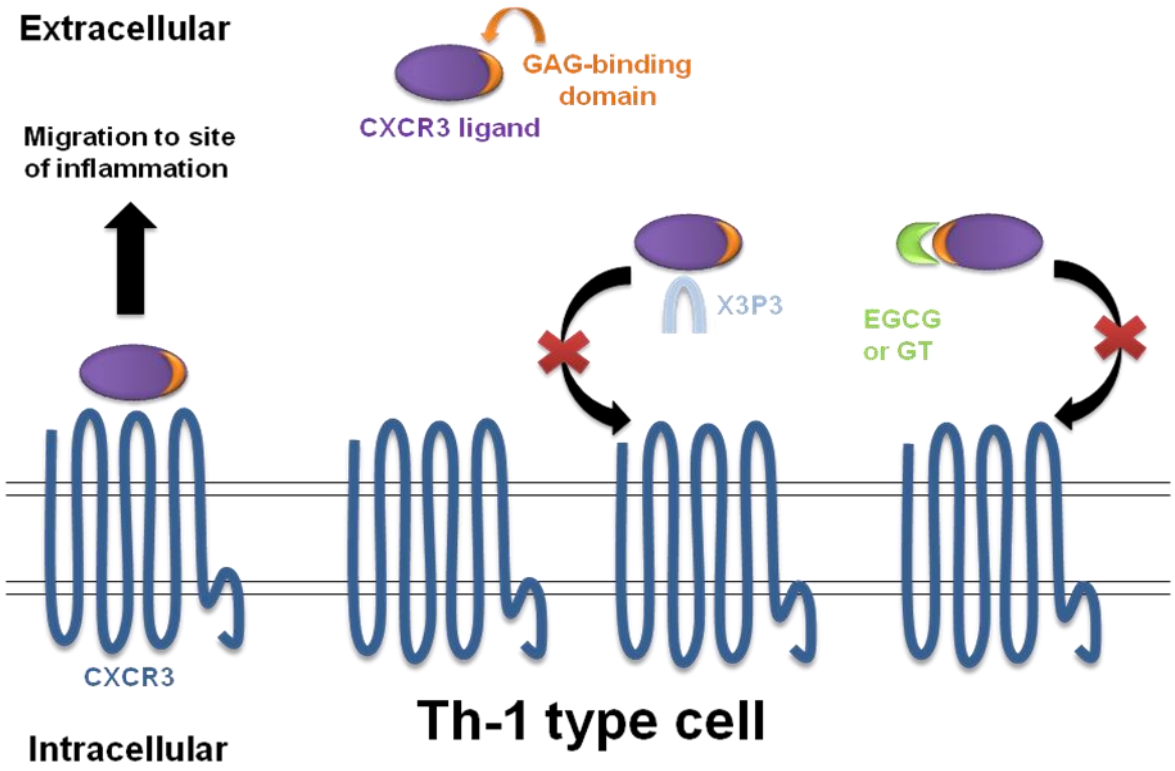

Figure 22. Model of inhibition. This model depicts a Th-1 type cell bearing CXCR3 (in blue) on its surface. One receptor is binding a CXCR3 ligand, in purple. The GAG-binding domain on the CXCR3 ligand is depicted in orange. Binding of CXCR3 and its ligand sends a signal to the cell to migrate toward a site of inflammation. An inhibitory compound such as EGCG or gallotannin, in green, is binding to a CXCR3 ligand, preventing the chemokine from binding its receptor. X3P3, shown in light blue, is binding to a chemokine and preventing it from binding to CXCR3. Because not many receptors bind their ligands, the cell's migration is inhibited, so it does not migrate toward the site of inflammation.

The data generated in this study suggest that inhibitors like EGCG and gallotannin may bind the GAG-binding domains of CXCR3 ligands, not only preventing their interaction with CXCR3 but also with GAGs in the extracellular matrix. This could lead to interference with the formation of haptotactic chemokine gradients, as the matrix would not be able to efficiently immobilize CXCR3 ligands.

Human X3P3 studies performed in our laboratory indicate that X3P3 may also be a strong CXCR3 inhibitor. It has been demonstrated to bind CXCR3 ligands and thereby prevent CXCR3-ligand binding. Murine X3P3 has not been shown to have this ability. More studies are necessary to determine why it is ineffective at inhibiting chemotaxis, how mX3P3 may be

modified to become a strong inhibitor, and if other ECL-based peptide mimetics of murine CXCR3 will be effective.

5I. Conclusion. In this study, EGCG and gallotannin have been revealed to be strong inhibitors of mCXCR3 ligand function. Because of their inhibitory properties, they are strong candidates for the development of therapies for CXCR3-mediated inflammatory disorders. The results observed in this study suggest that the mouse model may be used in future *in vivo* studies to examine EGCG and gallotannin as CXCR3 inhibitors, and such studies are in progress. Other futures studies may include binding assays and the use of GAG-binding domain mutants, which may help determine how EGCG, gallotannin, and other compounds inhibit murine and human CXCR3 ligand function, and how other compounds such as mX3P3 can be modified to more efficiently inhibit mCXCR3- and CXCR3-ligand interactions.

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