DEVELOPMENT OF A ROBUST AND IMPROVED SYSTEM FOR STUDYING INTERACTIONS BETWEEN CCL20 AND CCR6 USING BOTH RECOMBINANT AND CHEMICALLY SYNTHESIZED RHESUS MACAQUE CHEMOKINES

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DEVELOPMENT OF A ROBUST AND IMPROVED SYSTEM FOR STUDYING INTERACTIONS BETWEEN CCL20 AND CCR6 USING BOTH RECOMBINANT AND CHEMICALLY SYNTHESIZED RHESUS MACAQUE CHEMOKINES

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The chemokine CCL20 is thought to be an integral part of the communication between the innate and adaptive arms of the immune system, due to expression of the cognate receptor, CCR6, on immature dendritic cells and on memory T cells and B cells. Interest in this particular chemokine/chemokine receptor interaction has grown over time and more recently due to roles in SIV infection, mucosal immunology, and vaccinology. The need to further study the CCL20/CCR6 interactions is bolstered by our laboratory's previous findings of increased expression of CCL20 in acutely SIV infected lymph nodes and the increased expression of CCL20 in response to PAMPs in cells of lymphatic vessels. This thesis aims to develop and improve a system for studying the interaction between CCL20 and CCR6. I have found that the recombinant expression system utilized to obtain macaque chemokines provided highly pure fusion proteins. However, cleavage of the fusion protein into macaque CCL20 has been inefficient. Rhesus macaque CCL20 chemically synthesized using regioselective cyclization was highly biologically active using the chemotaxis assay and stable cell lines expressing CCR6. Chemotactic inhibition studies identified five compounds that inhibited CCL20-induced chemotaxis. The surfactant, GML, did not inhibit CCL20-induced migration. The anti-inflammatory botanicals, EGCG and gallotannin, both inhibited CCL20-driven migration at high concentrations. The three CCR6 extracellular loop mimetic peptides also partially inhibited CCL20-induced migration at high concentrations. In conclusion, I have utilized both a

recombinant protein expression system and regioselective cyclization peptide synthesis to obtain bioactive, nonhuman primate chemokines. I have also successfully developed an in vitro system to study CCL20-induced migration, and have identified a number of botanical and biochemical elements that inhibit CCL20-induced migration. The public health significance of this study is related to the fact that vaccine efficacy may be affected by anti-inflammatory compounds that inhibit CCL20-mediated chemotaxis. Another way in which public health could be affected by this study is in using the anti-inflammatory compounds studied to treat chronic inflammatory conditions in which the pathology of the disease is related to up-regulation of CCL20 and CCR6.

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1.0 Introduction

The complexity of the immune system provides the body with a method to fight pathogen associated challenges. The immune response to inflammation caused by the invading pathogens develops as infected or injured cells in the local environment produce signals to aid the local entry of specialized immune cells. A gradient of small proteins, or chemokines, drives the homing of the immune cells.

1.1 Chemokines and Chemokine Receptors

Chemokines are small, structurally related chemo-attractant cytokines, in the size range of 8-10kDa, that are involved in a wide range of biological functions, from hematopoeisis to organogenesis[3, 43]. The expression of some chemokines is required to direct the architecture of developing lymphoid tissue[42, 43]. A third functional activity of chemokines is to aid in the activation of a specific adaptive response, either a TH1 or TH2 immune response[45, 46, 47]. Chemokine biology is important in immunology and infectious disease in that in some pathogenic viral and bacterial infections, as well as cancers and autoimmune diseases, dys-regulation of chemokines and expression of chemokine receptors can contribute to the pathogenesis of the disease[9, 16, 44]. One of the main functions of chemokines is to induce the trafficking of leukocytes into sites of inflammation[3, 12].

Chemokines are defined and functionally grouped in at least two ways. First they are classified by the sequence of the amino acids near the amino-terminus of the protein, i.e. by the proximity and location of the amino-terminal conserved cysteines that form the disulfide bridges in the protein, specifically they are CC, CXC, CX3Cand XC chemokines[12, 13]. The conserved cysteines of CC chemokines are directly next to each other. The cysteines of CXC and CX3C

chemokines have as the name implies some other amino acid present in the sequence before the next conserved cysteine. XC chemokines have only one conserved cysteine in the amino-terminal region of the protein sequence[12, 13].

Chemokines are also defined functionally by their expression profile as present during either homeostasis or inflammation[3, 12]. Homeostatic chemokines are expressed under normal physiological conditions and aid in the development of secondary microstructures within lymphoid tissue[19, 42, 43]. For example, CCL21 is a homeostatic chemokine that is highly expressed in the paracortical region of peripheral lymph nodes[41, 45]. This expression aids in the cross talk, maturation, and antigen education of naïve T cells and mature dendritic cells (DCs) which both express the receptor, CCR7[41, 42, 56, 57.] Another homeostatic chemokine CXCL13 is expressed mainly in the germinal centers of lymph nodes, aiding in the development and maturation of B cells[41]. An additional function of a homeostatic chemokine is to draw immature dendritic cells (DCs) into the tissue, so that these local DCs can continually sample the local environment and be poised to respond to pathogen-associated inflammation[44, 47].

Inflammatory chemokines are up-regulated via the NFκB pathway during pathogen challenge, in response to increased IFNγ expression[9, 44]. The chemokine CXCL10 is a prime example of an inflammatory chemokine, due to findings that upon SIV infection, as IFNγ goes up, this chemokine is also highly expressed[6, 44].

1.1.1 Chemokine CCL20: Expression and Other Properties

The chemokine CCL20 or MIP-3α, (macrophage inflammatory protein) is a CC chemokine[13], with unique properties in that in some local tissue environments CCL20 is thought to have properties of both the homeostatic and inflammatory chemokines[19]. As expected for other inflammatory chemokines, CCL20 is secreted by macrophages in response to inflammation

and has been shown to be under the control of the NFκB inflammation response pathway[26, 32]. In the skin, epithelial cells secrete CCL20 at low constitutive levels under normal conditions; on the other hand, with a pathogen challenge or chronic inflammation as in atopic dermatitis, CCL20 is up-regulated[32]. Additionally, the expression of CCL20 in the skin has been related to the homeostatic influx and maintenance of Langerhan cell (LC) precursors[56, 57]. Both properties of homeostatic and inflammatory expression have been shown in the gut, where the follicular associated epithelial dome in Peyer's patches constitutively expresses CCL20[33, 34]. The importance of a homeostatic interaction of CCL20 with its receptor, CCR6, in the development of gut-associated lymph tissue (GALT) is indicated by the fact that $CCR6^{-/-}$ mice have reduced numbers and cellularity of Peyers' patches[36]. CCL20 is also up-regulated due to inflammation in the gut, as in SIV infection[6], or to chronic inflammation as in inflammatory bowel disorder or Crohns' disease[27]. In the peripheral lymph node, CCL20 is shown to be an inflammatory chemokine, where the constitutive level of CCL20 expression is increased during acute SIV infection[6]. Another indicator of the inflammatory properties of CCL20 is that lymphatic endothelial cells increase CCL20 expression with IL-1β or LPS treatment via TLR signaling[62]. In the female genital tract, there is evidence of both homeostatic and inflammatory chemokine properties[2, 7]. Recent vaccine adjuvant studies of the female genital tract define a successful adjuvant or immunogen by increases in CCL20 expression[7]. For the above reasons, immune function of CCL20 is unique depending on the local tissue environment.

1.1.2 Chemokine Receptor CCR6: Expression and Other Properties

The receptor for CCL20 is CCR6 [19]. Adaptive immune cells including memory T cells, mononuclear cells, B cells, and immature dendritic cells (DCs) express CCR6 [32]. CCL20 and CCR6 are thought to be integral parts of the communication between the innate and adaptive

arms of the immune system because of the expression of CCR6 on immature DCs and memory T cells[19, 37]. Local response to inflammation up-regulates CCL20 expression[56]. This and other signals bring the immature DCs towards the focus of increased CCL20 expression where they become mature and activated, following antigen uptake and processing [56]. DCs mature losing some level of CCR6 expression and gaining CCR7 expression, which drives the mature DCs to home to the lymph node where they interact with naïve T cells[3]. Memory T cells may migrate into the inflamed environment via some of the same signals, and as the DC mature and present antigen to the memory T cells, they can begin to display effector immunological properties and contribute to resolution of the pathogen challenge and local inflammation[28].

1.1.3 HIV and CCL20 / CCR6

The most recognized chemokine receptors in SIV or HIV immunology are CCR5 and CXCR4, which are used by gp120 protein for viral entry into CD4 positive cells[38, 39]. One commonly studied aspect of HIV immunology is the reduction of immune cells associated with the gut-associated lymphoid tissue (GALT) during acute infection[15, 20]. It has been shown that the CD4 T cells in the GALT are targeted by virus infection and these numbers do not rebound to the original percentages even with HAART treatment[20, 21]. The chemokine/chemokine receptor pair of CCL20 and CCR6 become important considerations during SIV or HIV infection for a few reasons. One reason is the CCL20/CCR6 pair are thought to be influential in regulating immune responses in both regions affected by sexual transmission of HIV, the vagina and the gut, where the primary immune response is mounted[15, 20, 21, 50]. Another reason is that DCs (which express CCR6 and respond to CCL20) have been shown to be some of the first targets of HIV replication, in addition to serving as 'trojan horses' for virus dissemination[21, 48].

CCL20 has been shown to be up-regulated in both the gut (colon) and lymph node during acute SIV infection[6]. This up-regulation is congruent during other lentiviral infections such as by human T-cell leukemia virus type 1 (HTLV) where there is up-regulation of CCL20 expression[9]. Further indication of a dysfunctional relationship of CCL20 and HIV is that HIV proteins reduce chemotaxis of B cells to CCL20, which are a cell type that expresses CCR6[1]. Ghosh, et al, further identifies anti-HIV properties of CCL20 where CCL20 directly inhibited HIV replication[55]. In the female macaque vagina, treatment with GML (glycerol monolaurate), a biochemical surfactant, inhibited SIV replication[10]. Reported in the same study, CCL20 expression in the vagina was increased with SIV infection and that upon observing inhibition of SIV replication with GML treatment, CCL20 expression was reduced as well [10]. Finally, another CCR6 mediated anti-HIV activity was observed by Lafferty, et al[49]. The authors found that signaling through the CCR6 receptor up-regulated the expression of a naturally occurring anti-HIV molecule, APOBEC3G[49].

CCR6 and CCL20 are associated with mucosal tissue, both the female genital tract and gut lymphoid tissue, including Peyers patches[59, 28, 33, 35]. Although the pathways and functional roles have not been well developed yet, there may be great importance to the CCR6/CCL20 interaction early during HIV infection. It may be that upon sexual transmission, the epithelial layers of the female genital tract or anal/rectal junction is damaged. This epithelial damage, as well as virus-infected cells, free virus and other proteins and factors present in mucus or ejaculate perpetuates a local inflammatory response, and the down-stream up-regulation of CCL20. The increased levels of CCL20 as well as other inflammatory cytokines and adhesion signals increase the number of immature DCs brought into the environment, which can serve as 'trojan horses' for virus dissemination or as target cells[21, 48]. While primary up-regulation of

CCL20 is likely not the only answer to HIV immune evasion and persistence, there is a substantial body of evidence suggesting that consideration of the CCR6/CCL20 interactions during SIV/HIV may be lucrative in developing successful HIV vaccines or therapeutics.

Models that will improve our understanding of the CCR6 and CCL20 pathways and interactions in health and disease, including macaque models, need to be developed and improved. Since most reagents commercially available are of human specificity, an efficient protocol for expression and purification of non-human primate chemokines must be developed to accommodate future studies. In particular, there are few if any reagents for the study of nonhuman primate chemokines, including CCL20. Therefore, the primary goal of these studies was to generate macaque CCL20 and develop a system to characterize of the CCR6: CCL20 interaction.

1.2 Recombinant Protein Purification

Recombinant DNA technology has developed exponentially as the use of genes and proteins in vaccines and therapeutics has developed with modern medicine. Recombinant protein expression is a method for obtaining large quantities of protein that has long been utilized commercially[75, 83]. The systems that have been used are both eukaryotic and prokaryotic, viral vector or plasmid based systems each having advantages and disadvantages in the production, yield and safety of the desired product[75, 83].

One of the first prokaryotic systems used to produce commercial recombinant proteins is the gram-negative bacteria *E. coli*[75]. Advantages to expression in *E. coli* are that cultures are grown easily to a high density in simple nutrition media and that a large body of knowledge about recombinant expression in *E. coli* has been gathered to enhance production of proteins[75]. In genetically un-manipulated *E. coli*, the reduced state of the cytosol inhibits di-sulfide bond

formation and endogenous enzymes rarely mark the protein for proteolysis[75]. Other protein modifications, such as glycosylation, do not occur in *E. coli*[75].

Recombinant protein expression in *E. coli* has been manipulated by knowledge of bacterial cellular metabolism pathways and antibiotic resistant plasmids to enhance protein production[75]. Plasmids are mutated to contain antibiotic resistance, a strong promoter, and the lacz gene linked to the recombinant gene[83]. A relatively universal manipulation utilizing these factors is that cells that have taken up plasmids encoding antibiotic resistance genes also acquire the control of the T7 promoter and the lacz gene and the recombinant protein is expressed upon the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside)[75, 83]. Other manipulations of recombinant protein expression in *E. coli* include transforming the cells with plasmids encoding genes that enhance production of a certain type of protein. In the end, based on the type of recombinant protein desired, a wide variety of *E. coli* competent cells are available and are chosen based on the protein translational properties that will ease the production of the recombinant protein.

1.2.1 Origami B DE3 and Rosettagami B DE3

The strains of *E. coli* utilized in the expression and purification protocol here are the Origami B DE3 and Rosettagami B DE3 (Invitrogen) cells, based on properties that would enhance the production of a recombinant non-human primate chemokine. Both strains are derived from a mutant cell line, DE3 lysogens, that express protein in response to IPTG under the control of T7 promoter and lacz metabolic pathway. They both also express mutant plasmids that allow for the formation of disulfide bonds in the cytosol of the *E. coli* and more efficient protein refolding in vivo[72].

Recombinant mammalian protein expression in *E. coli* is made more difficult by the rarity of certain tRNAs in *E. coli*[5]. Low expression of tRNAs that complement the codon push the cell to mis-incorporate an amino acid and therefore potentially change the structure and function of the resultant recombinant protein[5]. To combat this problem, in addition to the other plasmids contained in the Origami B DE3 cell, Rosettagami B DE3 cells encode the rare tRNAs that are lacking in the *E. coli* but are necessary for proper mammalian translation of protein[5]. In conclusion, we used strains of *E. coli* that were purported to enhance the expression of our desired recombinant proteins, chemokines.

1.2.2 SUMO Fusion Protein Expression

A proper protein expression technology is also used to express recombinant proteins of interest. The SUMO (**S**mall **U**biquitin-Like **Mo**difier) plasmid expression system (Life Sensors) provides specificity during purification of the recombinant proteins, as both the fusion protein and the protease contain 6-X-His tags allowing purification of these elements, simply and to a high degree, by metal affinity column purification. Another advantage of the SUMO expression system is that the exact n-terminal residue of the recombinant protein can be defined (Life Sensors). The SUMO protease is highly specific in that the tertiary structure of the fusion protein is the only substrate for the enzyme cleavage reaction[70, 71, 79]. In most cases, specificity of cleavage results in un-altered recombinant protein, in this study, rhesus macaque CCL20.

1.3 Peptide Synthesis

Peptides can be synthesized using organic chemistry protocols, but it is a more expensive biochemical technique used to obtain large quantities of small proteins[81, 82]. There are two major methods by which peptides are synthesized by coupling the N-terminus of an amino acid to the

C-terminus of another, beginning with the C-terminal amino acid in the peptide and ending at the n-terminal residue[82].

Liquid phase peptide synthesis is the classical method for chemically producing peptides[81]. The other method for synthesizing peptides is called solid phase synthesis, where the c-terminal amino acid is bound to insoluble beads or resin[82]. The side chains of the amino acid can be protected by tboc (using trifluoroacetic acid) or fmoc (using 9-fluorenylmethylcarbonyl) chemistry[81, 82]. The difference between each is that upon building the peptide either a neutral (tboc) or positively charged amine group (fmoc) is exposed to the next amino acids c-terminus[82]. For the synthesis of our rhesus macaque CCL20, regioselective cyclization was used. By this solid phase peptide synthesis, sulfides are protected from disulfide bond formation which occurs regularly in protein synthesis by thiol protecting groups[81, 82]. Additionally, in this type of synthesis, as the amino acid chain is synthesized, sulfides can be revealed (or de-protected) and the disulfide bond formation can be directed[82].

2.0 Statement of the Problem

Understanding the contribution of chemokines and their receptors to the complexity of the immune system is important to modeling the progression of immunologic and infectious diseases. CCL20 is constitutively and over expressed during homeostatic and inflammatory conditions, respectively. The cognate receptor CCR6 is unique in that it is one of few non-promiscuous chemokine receptors[12]. The only other known ligands for CCR6 are a family of host anti-microbial peptides, the β-defensins[8, 32]. Inflammatory expression and response to CCL20 may provide a link between the innate and adaptive immune systems. Response of immature dendritic cells to CCL20 via CCR6 signaling makes study of CCL20 as a vaccine adjuvant a likely course. Expression of CCL20 (vagina[2, 7, 10, 59], likely the anal/rectal junction, colon[19], and GALT[19]) in tissue compartments exposed to or serve as primary sites of virus dissemination HIV during sexual transmission also lends credence to study of CCR6/CCL20 in HIV vaccines and mucosal immunity. We have shown that CCL20 is expressed by lymphatic endothelial cells at the afferent face of LNs and this is upregulated during the early phase of SIV infection[48, 62]. The recently revealed anti-HIV activity of CCL20 and intracellular ligand-driven signaling through CCR6 provides additional evidence that the CCR6/CCL20 interaction is important in understanding innate immune properties that combat HIV infection[49, 55]. These findings have provided impetus for study of this chemokine/chemokine receptor interaction in vaccine adjuvant studies, mucosal immunology and HIV or SIV infection. **The overall objective of this study was to develop a robust and improved system for studying the interaction between CCL20 and CCR6 using both recombinant and chemically synthesized nonhuman primate chemokines**. In this study, generating macaque CCL20 provides a method

to study the efficacy of using CCL20 as a vaccine adjuvant in future studies. Identification of inhibitory compounds of CCL20-mediated chemotaxis may provide possible treatments for chronic inflammatory diseases to which some of the disease pathology is associated with up-regulated CCL20 expression (atopic dermatitis[32], Crohn's disease[27], etc.).

2.1 Specific Aim 1: To Develop a System to Generate Rhesus Macaque CCL20

Expression of chemokines in *E. coli* is a well-established method for obtaining large quantities of recombinant proteins. However, there are few, if any, commercially available macaque chemokines for in vitro and in vivo studies of monkey models of immunology and disease. Recombinant expression of proteins can be a flexible and cost effective way to obtain a wide variety of molecules in large quantities. In addition, such a strategy readily allows mutagenesis as a relatively simple way to obtain different forms of proteins for study.

Chemical synthesis of peptides is an alternative method for obtaining highly pure proteins for experimental application. Additionally, regioselectively cyclized peptide synthesis can provide a method to produce highly pure proteins with directed di-sulfide bonding, which is thought to be required for proper tertiary structure formation of chemokines and which could contribute to more robust bioactivity of the resultant molecule. I have established systems whereby non-human primate chemokines were obtained by recombinant expression and whereby chemically synthesized non-human primate chemokines were found to be bioactive.

2.2 Specific Aim 2: To Develop and Apply a Functional Assay for Testing the Bioactivity of Recombinant and Synthetic Macaque CCL20

The importance of studying the interaction between CCL20 and CCR6 is defined by vaccine adjuvant studies, DC biology, and mucosal immunology. Due to the expression of multiple chemokine receptors on most cell types, studying specific chemokine and chemokine

receptor interaction is difficult with primary cells. A robust functional assay is important for studying the activity any specific chemokine. Therefore, I established a robust chemotaxis assay with the L1.2 murine pre-B cell line transfected both transiently and stably to express human CCR6. These stable CCR6 cells allowed me to examine specifically the outcomes of some CCL20 and CCR6 interactions.

3.0 Materials and Methods

3.1 Generation of rhesus macaque chemokine

To utilize recombinant protein expression for producing a macaque chemokine, the *E. coli* strains Origami B DE3 and Rosettagami B DE3 cells (Invitrogen) were transformed with one µl ampicillin (amp) resistant plasmid, p.SUMO.rh.CCL20 plasmid (Basu, et al[84], YT/TAR 2006). After heat shock (100°C in a water bath) the cells were rested on ice for 5 minutes. The cells were incubated for one hour at 37°C with vigorous shaking. Positive transformants were selected by plating transformations on LB (Luria Bertani) / amp (100µg/ml) plates containing kanamycin (15µg/ml) and tetracycline (12.5µg/ml) for Origami B DE3 and kanamycin, tetracycline, and chloramphenicol (34µg/ml) for Rosettagami B DE3. Plates were incubated for 24-36 hours at 37°C. A seed culture was prepared by inoculating one colony into 12-200 ml of LB media supplemented with the appropriate drugs or antibiotics for each *E. coli* strain as described above and grown overnight at 37°C with vigorous shaking. The seed culture was then added at a 1:10 dilution to 1L pre-warmed LB media supplemented with the appropriate drug or antibiotics and cultured until the optical density of the culture reached an A600 of 0.4. IPTG (isopropyl β-D-1-thiogalactopyranoside) was then added to the culture to a final concentration of 200µM to induce protein expression and the cells were further cultured at 37°C with vigorous shaking for 3 hours.

To isolate the induced SUMO-CCL20 fusion protein from the culture of bacteria, the culture was lysed with 15 ml Bugbuster protein extraction reagent (Novagen) supplemented with 15µl Benzonase (Novagen), and 67µg/ml PMSF (phenyl methyl sulphonyl fluoride). The lysate

was pelleted to separate soluble and insoluble material. The insoluble inclusion bodies were solubilized to release aggregated SUMO-CCL20 fusion protein in 50mM HEPES-NaOH pH7.5, 6M guanidine HCl, 25mM DTT (diothiothreitol). The SUMO-CCL20 fusion protein was purified by metal affinity column binding with TALON spin columns (Clontech), according to the manufacturer's recommendations.

To prepare the SUMO-CCL20 fusion protein for cleavage and the recombinant CCL20 protein for HPLC purification, the sample was randomly refolded at a ratio of one to five volumes with refolding buffer containing 50mM HEPES pH7.5, 200mM NaCl, 1mM DTT, 1M NDSB201 (3-(1-Pyridino)-1-propane sulfonate). NDSB201 acts to facilitate the renaturation of proteins in addition to preventing protein aggregation. The refolded SUMO fusion protein preparation was concentrated and further purified by size by centrifugation in the Amicon Ultra-4 30kDa cut-off centrifugal filter device (Millipore, # UFC803024). The purified fusion protein preparation was then dialyzed to remove high concentrations of chelating and reducing agents by overnight dialysis in 1L 50mM Tris-HCl, 500mM NaCl. The dialyzed SUMO fusion protein preparation was subsequently cleaved of the SUMO fusion tag by addition of SUMO protease (Life Sensors) and incubation at 37°C for from one up to three hours or overnight. Both the SUMO fusion protein and SUMO protease were purified from the cleavage reaction by metal affinity column binding by the same method as above (Talon Spin columns [Clontech]). At least 1mg of the SUMO-CCL20 fusion protein was sent for HPLC purification.

To examine the CCL20 product throughout the expression and purification steps the bacterial culture and protein preparations were sampled after performing different steps of expression and were analyzed by SDS-PAGE and immunoblotting. All samples were prepared for SDS-PAGE analysis by boiling for 5 minutes with an equal volume of 2X SDS-PAGE buffer.

Samples were then separated on 12 or 15% resolving polyacrylamide gels by electrophoresis at 85V for 30 minutes at 4ºC followed by 125V for 1 hour at 4ºC. For Coomassie brilliant blue staining, gels were then fixed with 10% acetic acid / 25% isopropanol with shaking for 15 minutes at room temperature, stained with shaking with 0.25% Coomassie blue stain overnight at room temperature, de-stained with 5% methanol / 7.5% acetic acid, and subsequently dried encased in cellulose for analysis. Immunoblotting was performed by transferring proteins to PVDF membranes and use of the Pierce Fast Western Blot Kit (Pierce, cat 35050). PVDF membrane was wetted in 100% methanol for 15 sec, then in water for 2 minutes, and finally equilibrated in Tris / Glycine transfer buffer until use. The polyacrylamide gel was also equilibrated in transfer buffer for 15 minutes. A gel sandwich was prepared with fiber pads, filter paper, PVDF membrane and polyacrylamide gel, all soaked in transfer buffer. A wet transfer was performed with the Bio-Rad Mini-Protean Cell II transfer apparatus at 4ºC for one hour at 100V 350mA. The membrane was then dried for two hours at RT, and subsequently blocked in 2% milk for 30 minutes with shaking at RT. Either anti-CCL20 (human, R&D Systems, AF360 or rhesus, ProSci, PAS 13809) or anti-HIS (RGS•His HRP conjugate (Qiagen, 34450) antibodies were diluted to the manufacturers' suggested concentrations in Pierce Fast Western Blot antibody diluent. As required, a secondary antibody (mouse anti-goat, Rockland, Inc., 105-3102) was utilized to detect the anti-CCL20 staining. The membrane was washed five times and subsequently incubated with detection reagent, then exposed to film for 30 seconds. The film was then developed and analyzed.

Peptide synthesis using regioselective cyclization was performed by providing the University of Pittsburgh Peptide Facility with rhesus macaque CCL20 sequence (70 amino acids, mature form). Two milligrams (lyophilized) of a test batch was received for biological activity

testing. The protein was resuspended in nuclease free water and quantified by BCA protein assay (Pierce, cat# 23227). After biological activity was confirmed via a chemotaxis assay, the peptide facility continued with HPLC purification of the remaining batch (35 milligrams). Pure (99%) lyophilized rhesus macaque CCL20 was resuspended in nuclease free water, quantitated by BCA protein assay and used in the functional assays of this study.

3.2 Application of chemotaxis functional assay for in vitro system development

To develop a functional assay to analyze CCL20-mediated chemotaxis, transient transfections were performed following methods developed by Fox et al. L1.2 murine pre-B cells were transfected by electroporation (300V, 975μF, Bio-Rad Gene Pulser II) with plasmid encoding the human CCR6 receptor (pcDNA3.1 human CCR6 from UMR cDNA resource center). Transfected cells were cultured in L1.2 media (Hepes modified RPMI (Sigma), 10% FBS, 1mM non-essential amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate and 0.5mM β-mercaptoethanol) overnight in the presence of 5mM sodium butyrate.

To perform chemotaxis, cells were prepared for chemotaxis by centrifugation and resuspension in chemotaxis media (0.1% BSA RPMI). Concentrations (1nM-1μM) of synthesized regioselectively cyclized rhesus (University of Pittsburgh Peptide Facility) and commercial human CCL20 (Peprotech) were diluted in chemotaxis media. The bottom reservoir of chemotaxis plates (Neuroprobe, 5um pore size) were blocked against non-specific chemokine adherence with 1% BSA RPMI for 30 minutes at room temperature. The chemokine dilutions were then loaded in the aspirated wells and the membrane placed over the plate. Cells (20ul) were loaded on to the membrane at 200,000 cells/20ul volume. The plate was incubated at 37°C/5%CO_2 for 3 hours. After incubation, the plate was scraped to remove cells off the top of the membrane and cells that had migrated to the lower wells of the chemotaxis were counted on

a hemocytometer. Data analyses of chemotactic migration were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

To generate stable cell lines for continued CCL20-mediated chemotaxis analyses, transfected L1.2 cells were selected in geneticin supplemented L1.2 media to develop stable cell lines to study subsequent preparations of macaque CCL20. A separate culture of transiently transfected cells was grown in L1.2 media overnight. Cultures were centrifuged and resuspended in L1.2 media supplemented with 1mg/ml of geneticin (Gibco) at a cell concentration of 20,000/150ul. Cells were plated in 96 well plates and cultured until foci of growing cells were evident in multiple wells. Wells with cells growing were chosen randomly and expanded in geneticin supplemented L1.2 media. Primary clones were moved forward based upon their migration to the optimal concentration of synthetic rhesus CCL20 (10nM). Secondary clones were selected from cultures that migrated potently to chemokine were cloned by diluting the culture to one cell/450ul in geneticin supplemented L1.2 media. Wells with cells growing were expanded, chemotactic ability was confirmed, and 12 stable cell clones were saved for further CCL20-mediated chemotaxis analysis.

To characterize and correlate receptor expression with functional chemotactic ability on both the transiently transfected and the stable cell line, flow cytometry was performed on parental, transiently transfected and stable CCR6 expressing cell clones. Cells that had been prepared for chemotaxis were washed and stained in blocking buffer (1X PBS with 2% BSA/200μM sodium azide) with PE-labeled anti-human CCR6 antibody (BD Biosciences, (Clone 11A9, 551773)) at 4°C for 30 minutes. Samples were washed and fixed by resuspension in 0.25% paraformaldehyde/block buffer. Data were acquired on a BD Canto using FACS Diva

Software. Data were subsequently analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

To examine the cell populations that express CCR6 in blood, primary rhesus macaque PBMCs were stained with antibodies PBMCs were then stained with a 6 color panel (BD Biosciences, Anti-CD3 Pe-Cy7 (Clone SP34.2, 557749), Anti-CD4 PerCP (Clone L200, 550631), Anti-CD20 FITC (Clone 2H7, 556632), Anti-CCR6 PE (Clone 11A9 551773), Anti-CD8 APC-Cy7 (Clone RPA-T8, 557760) and Anti-CD69 APC (Clone FN50, 555533). Data was aqcuired on the BD Canto, and analyzed with Flow Jo software (Tree Star, Inc., Ashland, OR).

To analyze the effect of botanicals, receptor peptide mimetics, and the biochemical surfactant on CCL20-mediated chemotaxis, chemotaxis was performed with stable CCR6 cell clones in the presence of the candidate inhibitors. To identify the effect of the botanicals or receptor peptide mimetic on CCL20-mediated chemotaxis, 10nM rhesus CCL20 was diluted with increasing concentrations of each. Migration towards the optimal concentration of synthetic rhesus CCL20 was considered the control for chemotactic activity, percent migration was calculated by dividing samples containing the botanicals or receptor peptide mimetic by the positive control (10nM synthetic rhesus CCL20) and setting the migration towards 10nM synthetic rhesus CCL20 at 100%. Three R6-ECL2s (described in Figure 15) were synthesized by the University of Pittsburgh Peptide facility. All R6-ECL2s were resuspended in nuclease free water and quantitated by BCA protein assay. A titration of one nM to one μM of each R6-ECL2 was added to 10nM rhesus CCL20. EGCG (Sigma-Aldrich, catalog #E4143) and gallotannin (Sigma-Aldrich, catalog #403040) were also water-soluble and treated in the same way as the R6-ECL2s. Glycerol monolaurate or Monomuls 90-L 12 (Code 56150COG, batch GR72954301, Cognis corporation, Cincinatti, OH) was not water-soluble and was resuspended in 100% ethanol

at 10 mg/ml. The final concentration of ethanol was 0.025% in the 50μg/ml preparation. All samples were normalized to 0.025% ethanol to negate any inhibitory effects that may have been attributed to ethanol. Chemokine (10nM) or cells were mixed with varying concentrations of GML and at lower concentrations, ethanol was also added to control for ethanol in every sample.

Statistical analysis was performed by the paired t-test. Values of * $p<0.05$, ** $p<0.01$, *** $p<0.001$ were found to be significant.

4.0 Results: Recombinant Protein Expression

4.1 Expression and purification of a SUMO-CCL20 fusion protein.

To identify and analyze the sequence differences between human and rhesus macaque CCL20, p.SUMO.rh.CCL20 was sent to the University of Pittsburgh Sequencing Facility after isolation of the plasmid using the Wizard Plus Miniprep Kit (Promega). Both the reference human CCL20 (NM 004591) and the rhesus macaque CCL20 nucleotide sequence were translated using Vector NTI software. Rhesus macaque CCL20 protein shared 85% identity to the human CCL20 protein (Figure 1A). To analyze the location of the differences in amino acid as it related to the structure of the protein, human CCL20 protein sequence was analyzed with Cn3d software. Secondary protein structures of an α-helix are marked by a green cylinder and β-sheets are marked by a gold bar (Figure 1C). The amino acid differences in the rhesus sequence as compared to the human sequence are marked in yellow (Figure 1C). Analysis of the location of the differences in the rhesus CCL20 revealed that changes occurred over the entire sequence of the protein (Figure 1A and 1C). Further analysis revealed that some of the amino acid differences in the rhesus macaque CCL20 protein differ in their hydrophobic or hydrophilic nature, highlighted in blue in Figure 1B. These analyses provided evidence of the differences between human and rhesus CCL20 that may cause mis-interpretation of results when using human tests (ELISA, Immunoblotting, IHC, and FC) to detect rhesus chemokine. Specifically, antibodies raised against a human antigen may cover a sequence that exhibits amino acid differences in the rhesus sequence. The binding affinity of the antibodies to antigen may be reduced by these amino acid changes. I have observed by ELISA and immunoblotting that the

amount of rhesus protein detected by human antibodies and kits was indeed less than the actual amount of rhesus protein present (data not shown).

A

```
Rhesus CCL20  ASNFDCCLRY TDRILHPKFI VGFTQQLANE TCDINAVVFH TKKGLSVCAN PKQTWVKLIV RRLSKKINKM
Human  CCL20  ........G. .......... ....R..... G.....II.. ...K...... .......Y.. .L....VKN.
```

B

Rhesus		Human	
Amino Acid	Phobic or Philic	Amino Acid	Phobic or Philic
Arginine (9)	philic	Glycine	phobic
Glutamine (25)	philic	Arginine	philic
Threonine (30)	philic	Glycine	phobic
Valine (36 & 37)	phobic	Isoleucine	phobic
Glycine (44)	phobic	Lysine	philic
Leucine (57)	phobic	Tyrosine	philic
Arginine (62)	philic	Leucine	phobic
Isoleucine (67)	phobic	Valine	phobic
Asparagine (68)	philic	Lysine	philic
Lysine (69)	philic	Asparagine	philic

C

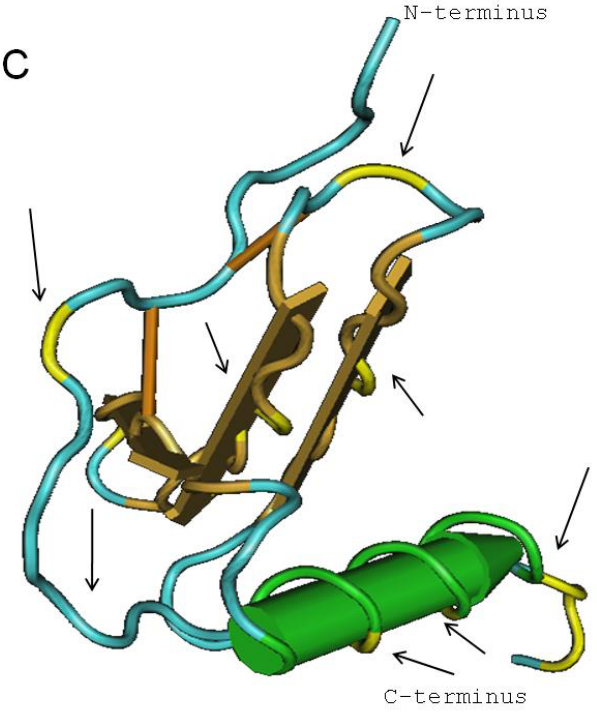


Figure 1. Amino acid differences of rhesus macaque CCL20. A. The rhesus macaque CCL20 gene was sequenced from the SUMO plasmid and the amino acid translation was aligned with the human reference sequence using Vector NTI software. **B.** Properties of the amino acid substitutions were analyzed. **C.** Cn3D (v4.1) software was utilized to view the tertiary structure of the human CCL20 chemokine. Orange bars indicate the di-sulfide bonds (Cys_6-Cys_{32} and Cys_7-Cys_{48}). Yellow highlighted regions are the amino acid residues that differ in rhesus macaque CCL20 versus human CCL20. Copper colored block arrows indicate β-sheets and the green colored block cylinder indicates an α-helix secondary protein structure.

To generate recombinant macaque chemokines, Origami B DE3 *E. coli* cells were transformed with the p.SUMO.rh.CCL20 plasmid were cultured and protein expression was induced with IPTG for 3 hours. The calculated molecular weight of the SUMO-CCL20 fusion

protein is 26-28kDa based on the addition of the molecular weight of the SUMO tag (~11 kDa, although it migrates through a denatured SDS-PAGE matrix to a size range of 18-20kDa [Lifesensors]) and CCL20 [8 kDa]). The darker staining band present only after IPTG induction in the molecular weight range between 22 and 36 kDa (lane 4, Figure 2A) indicates that IPTG induced a protein of the anticipated size, and is likely the SUMO-CCL20 fusion protein.

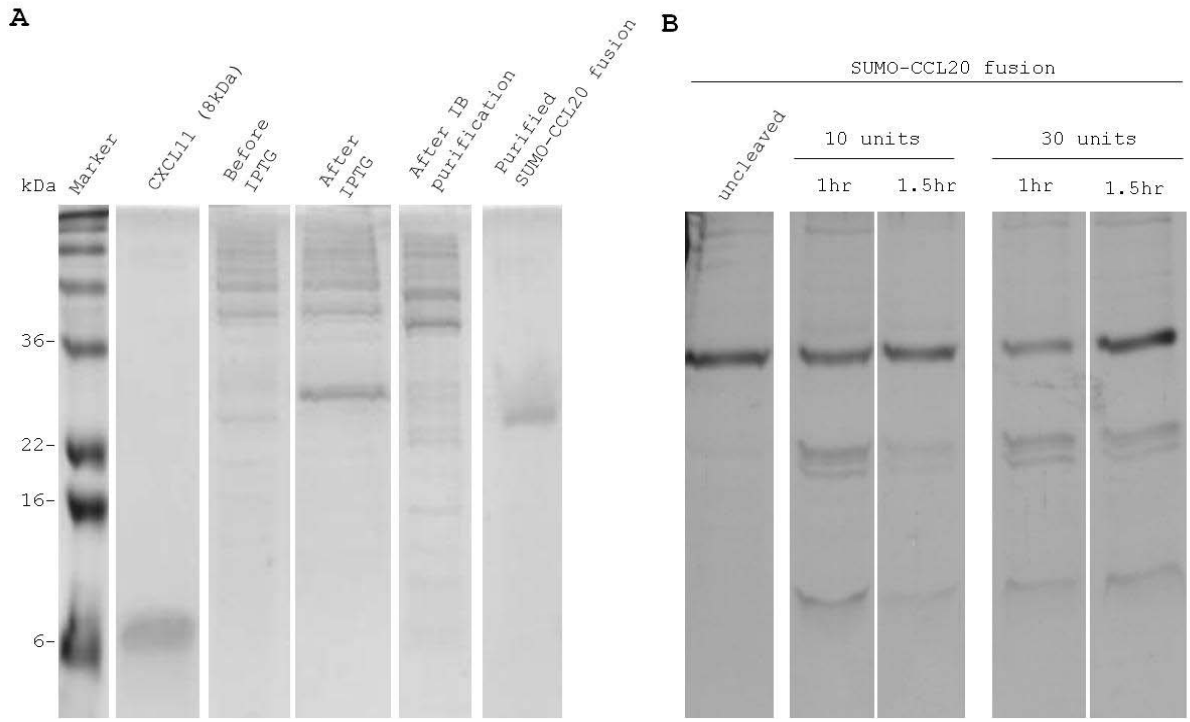


Figure 2. SUMO-CCL20 fusion protein purification and cleavage. A. Origami B DE3 cells transformed with p.SUMO.rh.CCL20 were cultured in the presence of IPTG to induce protein expression. The SUMO fusion protein was then purified by lysis of the culture and subsequent centrifugation of inclusion bodies. Purified SUMO-CCL20 fusion was obtained by metal affinity column purification via the 6X-his tag in SUMO fusion. Shown is a representative SDS-PAGE gel of numerous purification protocols. The culture was sampled before and after IPTG induction and prepared for SDS-PAGE analysis by boiling at 100°C for 5 minutes with an equal volume of 2X SDS-PAGE buffer. Samples taken after IPTG induction were normalized to the OD of the before culture to verify the over production of the SUMO fusion protein. **B.** Metal affinity column purified SUMO-CCL20 fusion protein was dialyzed with 50mM TrisHCl 500mM NaCl to remove harsh reagents. The dialyzed protein was cleaved at 37°C with varying amounts of SUMO protease as described and aliquots were removed and prepared as above for SDS-PAGE analysis in order to analyze the efficiency of cleavage. All prepared samples were separated by electrophoresis on 15% resolving polyacrylamide gel, fixed and stained with 0.25% Coomassie brilliant blue.

Some inducible or over produced proteins in *E. coli* aggregate in insoluble inclusion bodies (IB)[17, 65]. Based on this limitation, further steps need to be taken to purify recombinant proteins, if they are insoluble. To purify the induced protein and determine whether the SUMO–CCL20 fusion protein was soluble or insoluble, the *E. coli* culture was pelleted and lysed. Centrifugation separated soluble and insoluble material including inclusion bodies. The insoluble pellet was then solubilized with reducing and chaotropic agents to disaggregate protein by disrupting and protecting free charged atoms from inter- and intra- molecular bonding. After another round of centrifugation, the supernatant containing solubilized inclusion body proteins was analyzed for the presence of the SUMO-CCL20 fusion protein. The lack of a 26-28kDa band present in the soluble fraction after cell lysis, shown in lane 5 [After IB Purification], Figure 2A indicated that the induced SUMO-CCL20 fusion protein was located nearly exclusively in inclusion bodies.

The SUMO fusion tag contains a hexahistidine (6X-His) tag that allows the SUMO-CCL20 fusion protein to be purified from cell lysate via metal affinity column purification, by binding to nickel or cobalt ions in the column (Life Sensors). In lane 6 [Purified SUMO-CCL20 fusion] Figure 2A, only one protein of size 26-28kDa was abundant, which confirmed that only the 6X-His tagged SUMO-CCL20 fusion protein was purified by metal affinity column binding.

In summary, sequence differences between the human and rhesus CCL20 warrant the production of non-human primate chemokines to use as reagents in monkey models studying immunology and infectious disease. The recombinant protein expression system using SUMO fusion technology to express rhesus macaque CCL20 in the Origami B DE3 *E. coli* strain produced a protein of the anticipated size of SUMO-CCL20 fusion upon IPTG induction. The induced protein was aggregated in inclusion bodies, but was purified by centrifugation and

solubilization. The solubilized SUMO-CCL20 fusion protein was abundant after metal affinity column purification, indicating that a 6X-His tag protein was purified. The success of the expression and purification of the SUMO-CCL20 fusion means that this system is a good candidate protocol for obtaining recombinant SUMO-rhesus chemokines.

4.1.1 Cleavage of the SUMO-CCL20 fusion protein

To obtain bioactive chemokine from recombinant protein expression using the SUMO fusion technology, the chemokine (CCL20) must likely be cleaved from the fusion protein. The SUMO fusion system is ideal for obtaining recombinant proteins because the SUMO protease recognizes only the tertiary structure of the SUMO tag in the protein rather than recognizing cryptic or inappropriate target sequences for cleavage. Additionally, the SUMO protease was mutated to contain a 6X His tag and can therefore be removed from preparations via the same metal affinity column purification (Talon Spin Columns [Clontech]). These facts facilitate the resultant desired recombinant chemokine from being cleaved inside the protein and that after a second round of metal affinity column purification, all that should flow through is the final cleaved recombinant chemokine.

Depicted in Figure 2B, is the pattern of a typical cleavage reaction with the SUMO-CCL20 fusion protein. The same amount of purified SUMO-CCL20 fusion protein was cleaved with SUMO protease, 10 or 30 units, and the reaction was incubated for 1.5 hours at 37°C. The appearance of two lower molecular weight proteins in lanes 2 through 5 of Figure 2B showed that the SUMO-CCL20 fusion protein was indeed cleaved by the SUMO protease. Comparison of the reduced intensity of staining of the substrate (the largest staining protein band in lanes 2

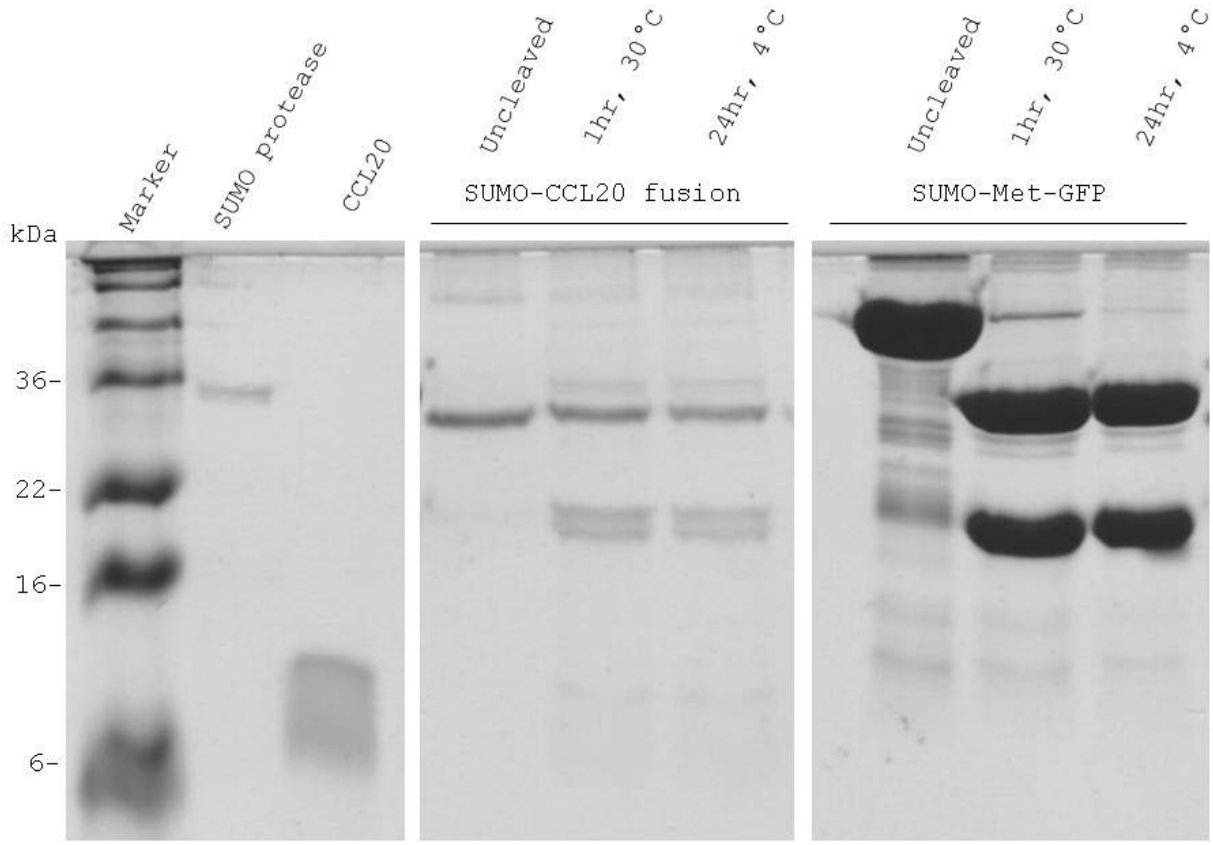


Figure 3. SUMO-CCL20 is cleaved inefficiently, although SUMO protease is highly efficient at cleaving control SUMO fusions. 150ug/ml of purified SUMO-CCL20 fusion protein was cleaved with 10 units of SUMO protease at 30˚C for 1 hour. As a control for protease activity, 50ug/ml of SUMO-met-GFP was cleaved with 3 units of protease under the same conditions. Both cleavage reaction samples were then stored at 4˚C overnight to allow for complete cleavage of fusion proteins. The first panel shows the MW marker, SUMO protease control (26kDa) and synthetic CCL20 (8kDa). Samples were prepared for SDS-PAGE analysis by boiling at 100°C for 5 minutes with an equal volume of 2X SDS-PAGE buffer. All prepared samples were separated by electrophoresis on 15% resolving polyacrylamide gel, fixed and stained with 0.25% Coomassie brilliant blue.

through 5) to the input substrate (lane 1), revealed that after 1 hour, the SUMO protease was only able to cleave about 10-30% of the SUMO-CCL20 fusion protein. Additionally, there appeared to be little difference in the efficiency of the cleavage reactions, even upon increasing SUMO protease 10-30 times the recommended amount (1U protease cleaves 100µg [Lifesensors]). Increasing the time of the reaction by a short time also did not appear to have an effect on increasing the efficiency of the cleavage reaction (lanes 3 and 5, Fig. 2B).

One of the first steps taken toward optimizing the cleavage of SUMO-CCL20 fusion was to verify the ability of the SUMO protease to cleave SUMO fusions. To clarify this, the SUMO-CCL20 fusion and the control SUMO-met-GFP fusion (LifeSensors) were cleaved with SUMO protease at 30°C for 1 hour and then at 4°C overnight. The second panel of Figure 3 confirms in an independent experiment, as previously described in Figure 2B, there was inefficient cleavage of the SUMO-CCL20 fusion. Cleavage of the SUMO-CCL20 fusion was not completed even with overnight incubation with SUMO protease. However, the control fusion SUMO-met-GFP was cleaved nearly completely after only one hour, which verified that the SUMO protease was active and efficient. Complete cleavage of the control SUMO fusion protein was achieved with incubation overnight, proving that as described by the manufacturer, SUMO protease is also active at 4°C.

Since there appeared to be problems with cleavage of the SUMO-CCL20 fusion protein (Figure 2B and 3), it was decided that a strategy to improve cleavage efficiency was necessary. Development of a multi-parameter experiment may solve the problem of identifying optimal cleavage conditions. In the next section, I will describe my efforts at increasing the efficiency of cleavage of the SUMO-CCL20 fusion.

4.2 Strategies to increase efficiency of cleavage

After demonstrating that the SUMO protease was active (Figure 3) and that the SUMO-CCL20 fusion protein was not efficiently cleaved (Figure 2B and Figure 3), I reasoned that there were many possibilities for observing inefficient cleavage of the SUMO-CCL20 fusion protein. I developed a multi-parameter experiment to optimize the efficiency of cleavage of the SUMO-CCL20 fusion.

4.2.1 Multi-parameter cleavage experimental design

Optimization of the cleavage reaction was required for obtaining cleaved recombinant chemokine prior to HPLC purification and use in bioassays. A multi-parameter experiment was performed with the goal of increasing efficiency of cleavage of the SUMO-CCL20 fusion. To work towards identifying parameters that would increase cleavage of the SUMO-CCL20 fusion, the parameters analyzed were the use of differing dialysis buffers, refolding techniques and temperature or concentration dependent variables. The specific parameters analyzed were the time at which the protein was refolded, the dialysis reagents and concentrations of cleavage reaction reagents and the temperature at which the cleavage reaction was performed. A flow chart, shown in Figure 4, depicts the variables analyzed in the multi-parameter experiment.

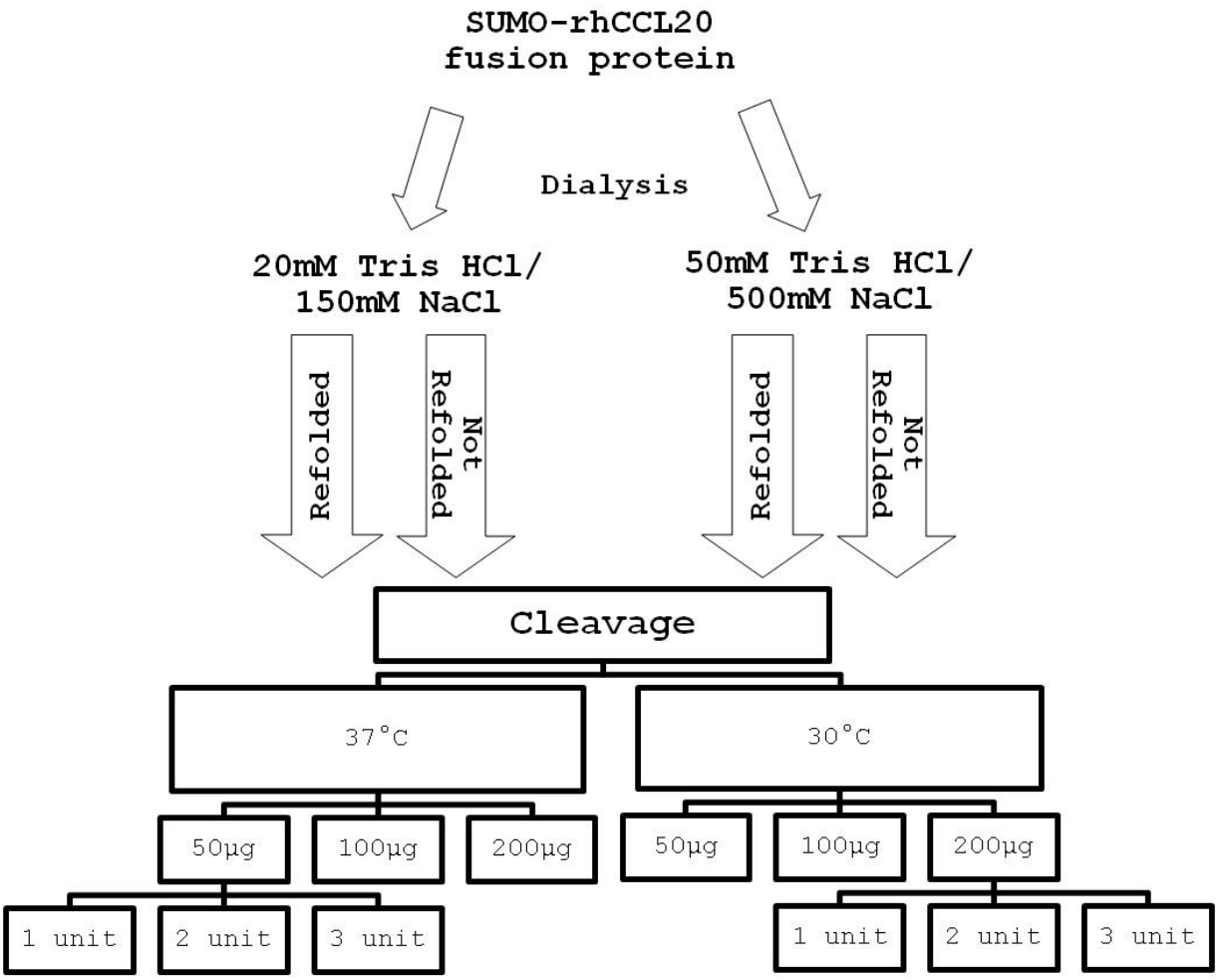
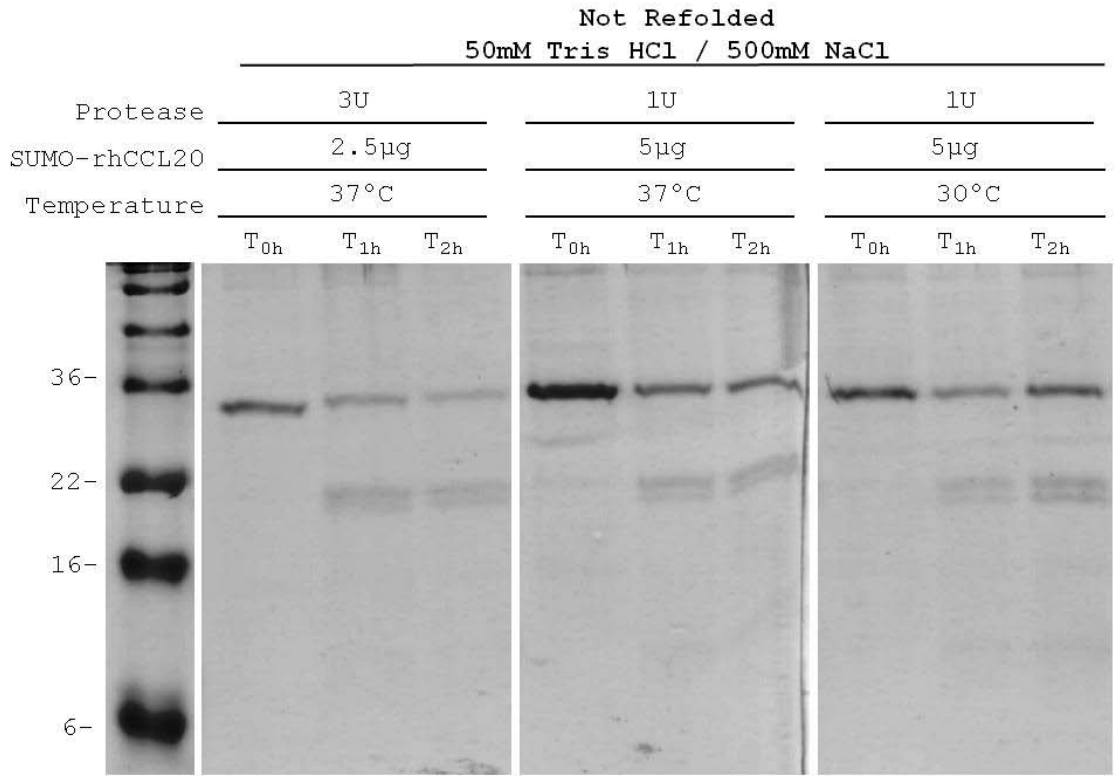


Figure 4. Multi-parameter cleavage analysis. Optimal conditions for cleavage of the SUMO-CCL20 fusion protein were analyzed in a multi-parameter experiment. Metal affinity column purified SUMO-CCL20 fusion protein was dialyzed against solutions containing 20mM Tris HCl/150mM NaCl or 50mM Tris HCl/500mM NaCl. Fusion proteins collected after dialysis in either solution were then separated again based upon whether or not the protein existed in the nascent form (not refolded) or in a chemically refolded state (refolded). Each of the samples was quantified by BCA protein assay. Fusion proteins were resuspended at the concentrations shown above (50, 100 and 200µg/ml). Cleavage was then carried out using 1, 2, or 3 units of protease at the above temperatures (30°C and 37°C).

4.2.1.1 Differences in buffer, quantity of protease or protein, time and temperature do not increase the efficiency of cleavage of SUMO-CCL20.

To analyze the effects of varied conditions of the multi-parameter optimization experiment, SUMO-CCL20 fusion protein was dialyzed against one of two different neutral salt buffers. The protein was then placed under conditions thought to facilitate refolding or allowed to remain in the form after dialysis. Cleavage reactions containing differing amounts of substrate ranging from 50-200µg/ml were carried out at different temperatures with different amounts of protease. SDS-PAGE with Coomassie brilliant blue staining was performed to analyze the results of the different cleavage reactions.

To analyze the difference in dialysis buffers on the cleavage of not-refolded SUMO-CCL20 fusion, different amounts of protease were added to varying amounts of SUMO-CCL20 fusion. The cleavage reaction was incubated at either 30°C or 37°C for 1 (1h) or 2 hours (2h) to identify possible temperature or time dependent differences in efficiency of cleavage. In contrary to the manufacturer's reference that one unit of SUMO protease cleaves 100µg of SUMO fusion protein (Lifesensors); all gels shown below in Figure 5 revealed that none of the conditions examined lead to efficient cleavage of the SUMO-CCL20 fusion.


Not Refolded
50mM Tris HCl / 500mM NaCl
Protease
SUMO-rhCCL20
Temperature
3U
2.5µg
37°C
1U
5µg
37°C
1U
5µg
30°C
T0h T1h T2h T0h T1h T2h T0h T1h T2h
36-
22-
16-
6-

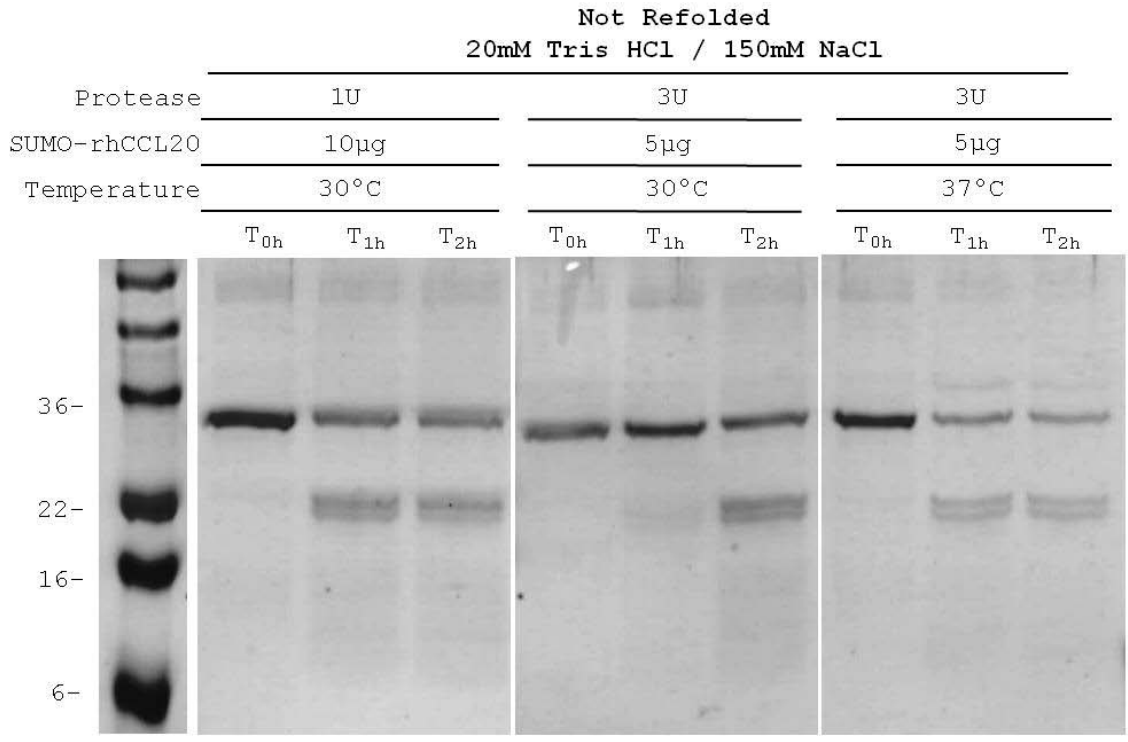

Not Refolded
20mM Tris HCl / 150mM NaCl
Protease
SUMO-rhCCL20
Temperature
1U
10µg
30°C
3U
5µg
30°C
3U
5µg
37°C
T0h T1h T2h T0h T1h T2h T0h T1h T2h
36-
22-
16-
6-

Figure 5. Changing four different variables did not increase cleavage efficiency of the 'not refolded' SUMO-CCL20 fusion. SUMO-CCL20 fusion protein was quantified by BCA assay to achieve the final concentrations in the cleavage reaction as shown. Metal affinity column purified SUMO-CCL20 fusion protein that was not chemically refolded prior to cleavage was aliquotted into 50µg/ml (2.5µg), 100µg/ml (5µg), 200µg/ml (10µg) total protein concentrations, cleaved with varying quantities of SUMO protease and the reaction was allowed to continue for 1 hour at either 30° or 37°C. Samples were prepared for SDS-PAGE analysis by boiling at 100°C for 5 minutes with an equal volume of 2X SDS-PAGE buffer. All prepared samples were separated by electrophoresis on 15% resolving polyacrylamide gel, fixed and stained with 0.25% Coomassie brilliant blue.

Under the conditions analyzed, where 3U (or 3 units of SUMO protease) was the largest amount of protease added to the cleavage reaction, and 50µg (2.5µg) total SUMO-CCL20 fusion was the least amount of substrate added to the cleavage reaction, increasing the amount of protease did not increase the extent of cleavage the SUMO-CCL20 fusion protein, first gel panel, Figure 5. Additionally, the middle panel and far right panel of the lower gel, where 3 units of protease was used to cleave 100µg (5µg) of SUMO-CCL20 fusion revealed that increasing the amount of protease did not increase the efficiency of cleavage. All other gel panels lend additional support to the overall conclusion of increasing the amount of protease does not increase the efficiency of cleavage by a significant degree.

Comparison of the top and bottom 2 right gels (1U, 5µg, 30ºC and 37ºC with dialysis buffer containing 50mM TrisHCl and 3U, 5µg, 30ºC and 37ºC with dialysis buffer containing 20mM TrisHCl) indicates that temperatures of 30°C vs. 37°C yielded similar, incomplete levels of cleavage. Based on the decreased intensity of staining of the SUMO-CCL20 fusion protein (lane T_{0h} of each gel) compared to the staining in lane T_{1h} of each gel and also given the appearance of 3 smaller proteins (2 bands at ~22kDa and 1 very light band at ~8kDa), the percentage of SUMO-CCL20 fusion that was cleaved was about 20-30%. Additionally, there was little to no increased detection of cleavage products after another hour of cleavage (lane T_{2h} of each gel).

In summary, the multi-parameter experiment showed that the SUMO protease did cleave the SUMO-CCL20 fusion. However, the cleavage of the SUMO-CCL20 fusion protein was not efficient. The composition of the dialysis buffer in which the protein was equilibrated did not appear to have a significant effect on the efficiency of cleavage. Therefore, it was decided that the 50mM TrisHCl dialysis buffer would be sufficient to use in the protocol. Neither temperature analyzed (30ºC or 37ºC) provided any improved cleavage. No ratio of substrate to protease yielded better cleavage than the others, however, based on data from the multi-parameter experiment, Figures 2B and 3, as well as additional references[70, 71, 73], it was decided that a more concentrated substrate provide additional opportunity for diagnosing the efficiency of a cleavage reaction. Overall, the multi-parameter experiment was useful in narrowing down the variables to analyze to increase efficiency of cleavage of the SUMO-CCL20 fusion.

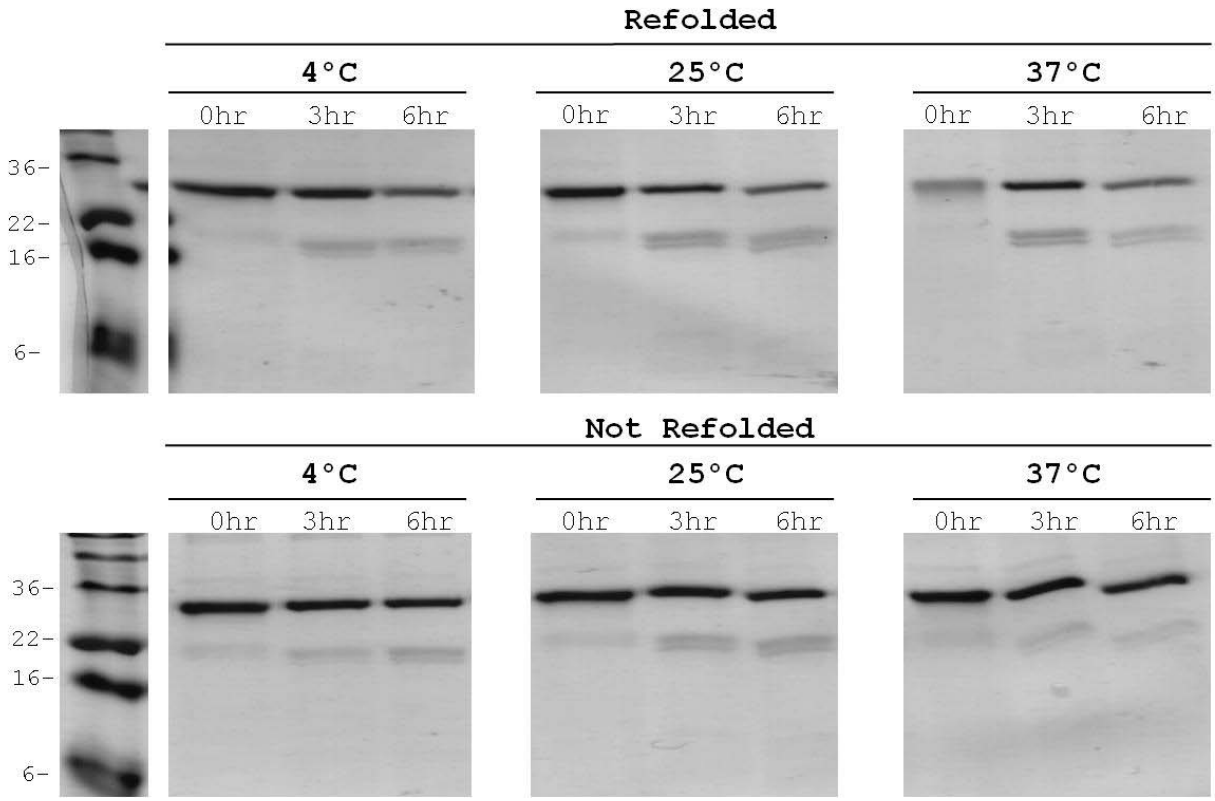


Figure 6. Varying temperature and incubation time did not increase the efficiency of cleavage of SUMO-CCL20 fusion. Metal affinity column purified SUMO-CCL20 fusion protein that was either refolded or not refolded prior to cleavage was cleaved with SUMO protease and the reaction was allowed to continue for 3 or 6 hours at 4°, 25° or 37°C. Samples were separated by electrophoresis on 15% resolving polyacrylamide gel, fixed and stained with 0.25% Coomassie brilliant blue.

A broader range of temperatures and incubation periods were then examined, as enzymatic activity of SUMO protease was evidenced at a wide range of temperatures (Lifesensors). As shown in Figure 6, the same quantity of refolded or not-refolded SUMO-CCL20 fusion was cleaved with 10 units SUMO protease at 4°C, 25°C and 37°C. Samples were collected after 3 and 6 hours of incubation and analyzed by SDS-PAGE and Coomassie brilliant blue staining, as previously described.

Neither temperature nor longer incubation appeared to have an effect on increasing the efficiency of cleavage. Comparing only the top 3 gels, based upon the reduced intensity of the

un-cleaved SUMO-CCL20 fusion protein between 22-36kDa and the appearance of staining of products of the cleavage reaction, bands staining between 16-22kDa (SUMO fusion tag) and only slightly visible band at ~8kDa (CCL20), efficiency of cleavage was approximately 30% at most. The additional three hours of incubation did not increase cleavage, as seen by the band pattern in lane '6hr' in each of the same three top gels. The same conclusion can be drawn by results shown in the bottom 3 gels.

There does not appear to be a difference in cleavage of either the chemically refolded or not-refolded SUMO-CCL20 fusion, since the ratios of input substrate to products are all about the same or a significant amount of SUMO-CCL20 fusion protein appears to be left un-cleaved after the endpoint of the reaction.

Based on the continued findings of inefficient cleavage, it was possible that something inherent to the SUMO-CCL20 fusion prevented it from being cleaved by SUMO protease. It was then hypothesized based on a study done by Calderone, et al, that because the CCL20 sequence is mammalian there may be mis-incorporated amino acids that could affect the tertiary structure of the resultant protein[5], which could potentially affecting the ability of the SUMO protease to cleave the SUMO-CCL20 fusion protein.

Two approaches were utilized to address these questions. First, a SUMO fusion protein that previously had been successfully cleaved in our laboratory, SUMO-CXCL11, was analyzed for the capacity of the SUMO protease to cleave that fusion protein. It does appear that SUMO-CXCL11 was cleaved more efficiently (data not shown). This, as well as the success of previous experiments by others that showed almost complete cleavage of the SUMO-CXCL11 fusion protein (data not shown, Yu-jen Tung), suggests that the specific chemokine attached to the SUMO fusion tag affects the efficiency of cleavage. The efficient cleavage of the SUMO-

CXCL11 fusion protein suggests that the cleavage of SUMO fusions may be better suited for some SUMO chemokine fusion proteins and not others.

The method utilized to resolve the other postulate that due to the expression of the fusion protein in *E. coli* there may be mis-incorporated amino acids conferring a different tertiary structure that prohibits the SUMO protease from cleaving the fusion protein was the use of another strain of *E. coli* (Rosettagami B DE3). This was done to examine whether synthesis of the protein in this strain, which has been modified to express mammalian tRNAs rare in *E. coli*, increased the efficiency of cleavage of the SUMO-CCL20 fusion protein.

4.2.2 Rosettagami and Origami B DE3 strain of *E. coli*

Calderone et al[5]. showed that *E. coli* used to express fusion proteins off mammalian cDNA sequences could mis-incorporate a lysine residue for an arginine residue, when arginine is coded for by 'AGA' [5]. Rhesus CCL20 does not contain any 'AGA' codons (data not shown). The SUMO fusion tag however, contains four 'AGA' codon arginine residues (data not shown). As postulated previously, mis-incorporation of lysines for these arginine residues might be a factor in the poor efficiency of cleavage. There may be different intramolecular interactions due to the amino acid substitution that would affect the structure of the fusion protein causing the SUMO protease to be unable to recognize and cleave the fusion protein appropriately.

To address this we utilized the Rosettagami B DE3 strain of *E. coli* because of its expression of tRNAs that are rarely seen in *E. coli*. The p.SUMO.rh.CCL20 plasmid was transformed into Rosettagami B DE3 cells and subsequently sequenced. Analysis of the sequences revealed that the p.SUMO.rh.CCL20 plasmid aligned with the reference rhesus CCL20 sequence except for one nucleotide substitution (Figure 7). There was a non-synonymous 'T' to a 'G' nucleotide substitution, causing the cysteine at position 32 to be

changed to a tryptophan (see Figure 1A for the reference rhesus CCL20 protein sequence). Although the SUMO CCL20 expression plasmid had been isolated and sequenced after passing through multiple *E. coli* strains (DH5 alphas and Origami B DE3) the output sequence had no nucleotide substitutions. We then sub-cloned and sequenced five p.SUMO.rh.CCL20 plasmids from the Rosettagami B DE3 strain of *E. coli*. Each of the sequences of the five isolates of p.SUMO.rh.CCL20 showed the same tryptophan substitution at position 32; leading to a conclusion that it was possible that the *E. coli* strain the plasmid was isolated from was affecting the sequence output.

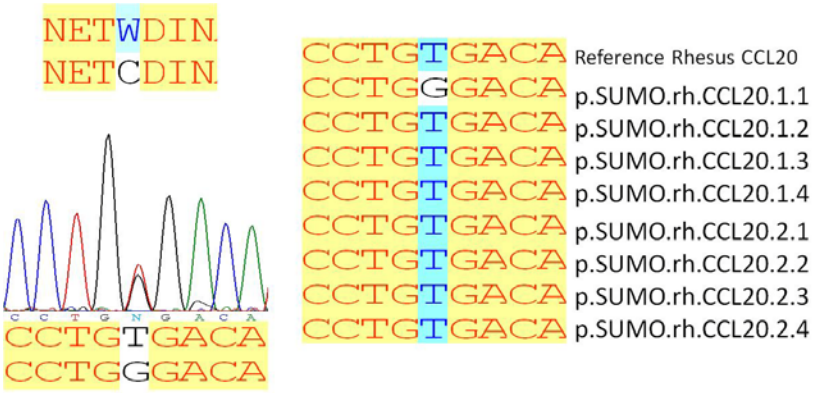


Figure 7. Isolation of clones of the plasmids p.SUMO.CCL20.1 and p.SUMO.CCL20.2. The p.SUMO.CCL20.1 plasmid preparation was shown to have a mixed population of plasmids, with either a 'T' or a 'G' at the base pair in question. The p.SUMO.CCL20.2 plasmid preparation did not have evidence of a mixed population of plasmids.

Since we had not observed this nucleotide substitution before in other *E. coli* strains, and to analyze whether the two original clones of the p.SUMO.rh.CCL20 plasmids had evidence of this same base pair change, all past sequence trace files of the p.SUMO.rh.CCL20 plasmid were then analyzed (data not shown). This revealed that in the clone 1 of p.SUMO.rh.CCL20 plasmid used in all protein expression, there were two peaks present at the base pair in question (Figure 7). Results of double peaks seen in a sequencing trace file could mean at least two things. It

could mean that the gene that was sequenced and used for cloning had a point mutation or that there was a mixed population of plasmids in clone 1 of the p.SUMO.rh.CCL20 plasmid. The other original clone (2) of the p.SUMO.rh.CCL20 plasmid showed no evidence of this ambiguity at the base pair in question (Figure 7).

To identify a sub-clone of the p.SUMO.rh.CCL20 (clone 1) plasmid preparation that did not show evidence of two peaks in the sequence trace file. Plasmid p.SUMO.rh.CCL20 (clone 1) was re-transformed into the DH5α *E. coli* strain, and individual colonies were sequenced. Out of the five clones (named simply 1.1, 1.2, 1.3, 1.4. 1.5), only plasmid p.SUMO.rh.CCL20.1.1 was revealed to have ambiguity at the base in question (Figure 7 and data not shown). Five clones sequenced from plasmid p.SUMO.rh.CCL20 (original clone 2) all aligned with 100% identity to the reference rhesus CCL20 sequence.

After sequencing of five retransformed clones for original clones 1 and 2 of p.SUMO.rh.CCL20, revealed that only one retransformed clone named p. SUMO.CCL20.1.1 showed the base-pair change which translated to a tryptophan substitution at position 32 (from cysteine) in the protein, it was hypothesized that the translation of pSUMO.rh.CCL20.1.1 C_{32} to T_{32} might be the fusion protein left uncleaved. To address this hypothesis, it was assumed that it would be possible to see differences in the cleavage of the sub-clones that translated either SUMO-CCL20 fusion with the cognate $cysteine_{32}$ or mutated $tryptophan_{32}$.

4.2.2.1 Differences in SUMO-CCL20 sequence at position 32 do not affect Origami B DE3 expression or cleavage of protein.

Because of the differences in the rhesus macaque CCL20 sequence in the plasmid that was used in the protein expression system, we sought to determine if these sequence differences had an effect on the efficiency of the cleavage of the SUMO-CCL20 protein. Three different

sub-clones of the SUMO-CCL20 fusion protein were sequence verified and transformed into the Origami B DE3 *E. coli* strain. Protein was induced with the addition of IPTG; the protein was purified from the inclusion bodies by centrifugation and metal affinity column purification, as previously described. Purified protein was normalized to total protein concentration and cleaved with SUMO protease. There were few differences in the amounts of protein purified (data not shown). As shown in Figure 8, cleavage of the purified SUMO-CCL20 fusion protein was inefficient (Figure 8). Only 25-50% cleavage of the SUMO-CCL20 fusion protein was achieved, as depicted by the reduced staining of the SUMO-fusion protein and the appearance of staining of two bands lower in molecular weight. With cleavage of the SUMO-CCL20 fusion protein, the SUMO fusion tag of the protein stains in the area of about 22kDa, and rhesus CCL20 stains in the area of 8kDa. The low molecular weight protein marker of synthetic rhesus CCL20 shown in lane 2 (CCL20 (8kDa)) confirms that upon cleavage of the SUMO-CCL20 fusion protein a protein of similar molecular weight appears and is assumed to be the recombinant CCL20. Differences in cleavage of the retransformed SUMO-CCL20 fusion proteins could not be attributed to a reduced activity of the SUMO protease as the protease was added at the same time in each reaction and the control fusion protein, SUMO-met-GFP, was cleaved within one hour (Figure 9, SUMO-met-GFP cleavage).

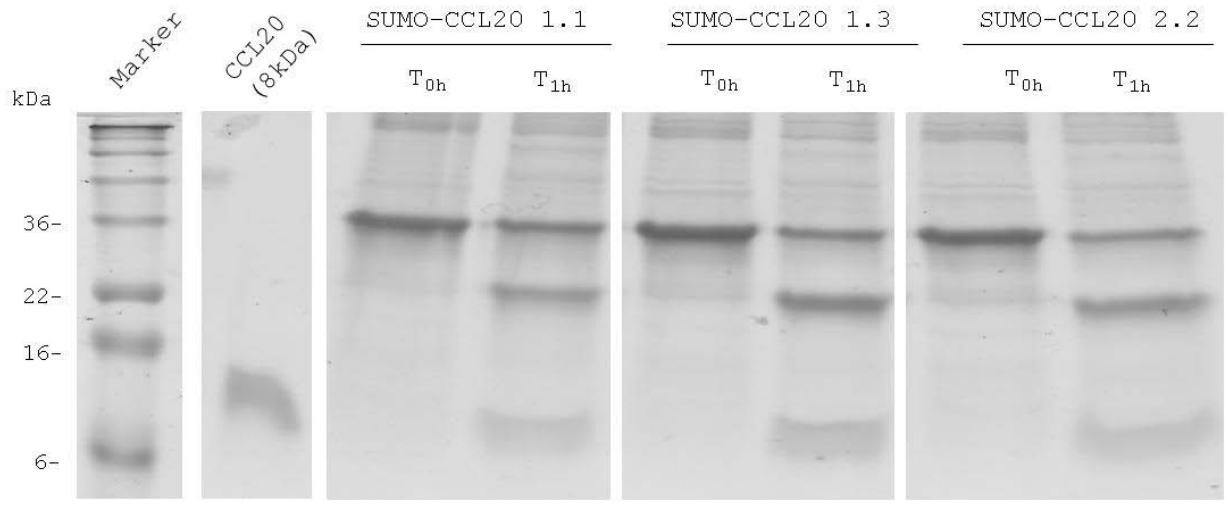


Figure 8. There is no difference in the expression or cleavage of different isolates of SUMO-CCL20 fusions purified from the Origami B DE3 strain of *E. coli*. Recombinant protein expression was performed for each of 3 different cultures of transformed Origami B DE3 sequences that conferred both non-synonymous base pair mutations and reference rhesus CCL20 sequences with different isolates of the SUMO-CCL20 fusion plasmid. SUMO-CCL20 fusion proteins were cleaved with SUMO protease. Samples from different conditions were separated by electrophoresis on 15% resolving polyacrylamide gel, fixed and stained with 0.25% Coomassie blue staining.

4.2.2.2 Expression and purification of SUMO-CCL20 isolates are different in Rosettagami B DE3 cells.

In the same way as with the Origami B DE3 strain of *E. coli*, a different strain of *E. coli*, Rosettagami B DE3, was analyzed for differences in efficiency of cleavage of the three different p.SUMO.rh.CCL20 retransformed plasmids (sequences shown in Figure 7). Although the purified SUMO-CCL20 fusions were normalized with respect to the total concentration of protein, there are apparent differences in the intensity of staining of the protein at the size of the anticipated SUMO-CCL20 fusion (22-36kDa, T_0, Figure 9). As shown in Figure 9, there were differences in the purity in total protein purified via the metal affinity column purification when comparing the banding patterns in the molecular weight range of 22-36 kDa. The protein

induced by the retransformed clone of p.SUMO.rh.CCL20 2.2 was more abundant and cleavage of this protein was more efficient, as indicated by the reduced staining of the top most band (between 22 and 36 kDa) and the appearance of bands staining in the molecular weight range of about 22kDa (SUMO-fusion tag) and 8kDa (recombinant rhesus macaque CCL20). Cleavage of the SUMO-CCL20.2.2 from the Rosettagami B DE3 *E. coli* strain appeared to be similar in efficiency to the cleavage observed for the SUMO-CCL20 fusion produced in the Origami B DE3 *E. coli* strain, (Figure 9). This suggests cleavage of SUMO-CCL20 fusions containing wild-type rhesus CCL20 sequence does not differ depending on the strain of *E. coli* utilized to express the fusion protein. Additionally, there was no increase in cleavage efficiency in using a different strain of *E. coli*, Rosettagami B DE3 for expression of the SUMO-CCL20 fusion.

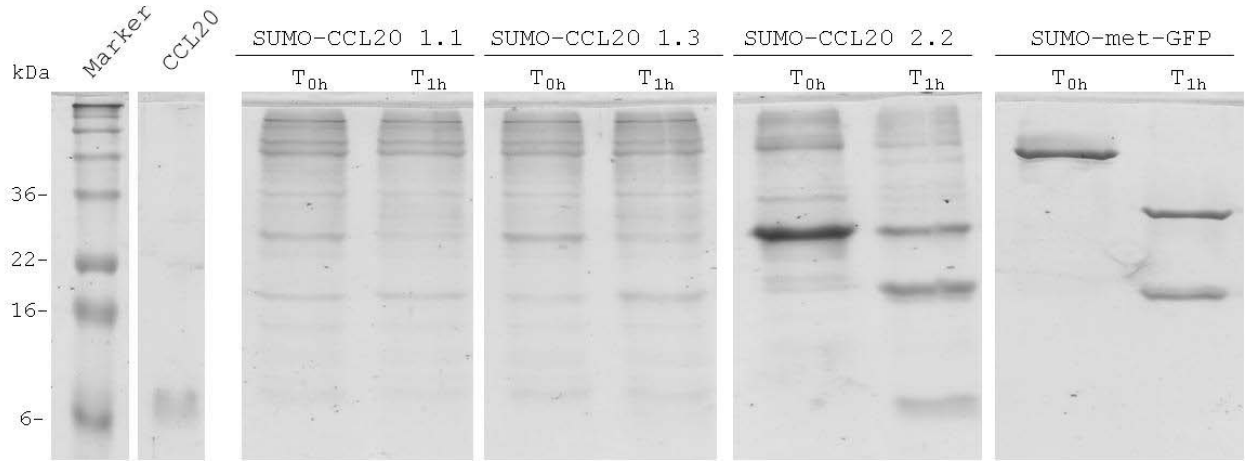


Figure 9. Rosettagami B DE3 cells appear to differ in the expression and cleavage of different isolates of SUMO-CCL20 fusions. Recombinant protein expression was performed for each of 3 different cultures of transformed Rosettagami B DE3 SUMO-CCL20 fusion proteins with sequences that conferred both non-synonymous base pair mutations and reference rhesus CCL20 sequences. SUMO-CCL20 fusion proteins were cleaved with SUMO protease. Samples from different conditions were separated by electrophoresis on 15% resolving polyacrylamide gel, fixed and stained with 0.25% Coomassie brilliant blue.

4.3 Peptide synthesis

As there were continued problems with obtaining recombinant protein for HPLC purification and subsequent biological activity testing, rhesus macaque CCL20 was synthesized using regioselective cyclization. Mature signal peptide cleaved (amino acids 1-70, Figure 1A) rhesus macaque CCL20 sequence was forwarded to the University of Pittsburgh Peptide Facility. The peptide was synthesized using solid phase peptide synthesis with regioselective cyclization, where a peptide is synthesized from the c-terminal amino acid up to the n-terminal amino acid, with thiol groups protecting side groups of cysteines to prevent disulfide bond formation, intermediately. When the second cysteine amino acid of a disulfide bond is added to the growing peptide, the cysteines are de-protected and allowed to form the intended disulfide bond. For CCL20, disulfide bonds are formed between Cys_6-Cys_{32} and Cys_7-Cys_{48}. CCL20 was then purified by HPLC and analyzed with NMR. Pure lyophilized peptide was resuspended in nuclease free water and quantitated by BCA protein assay. This protein was then used in the second aim of this study to analyze the CCL20/CCR6 interaction.

4.4 Overall Protein Expression System Summary

SUMO-CCL20 fusion was expressed upon IPTG addition and was found to be aggregated in inclusion bodies. The protein was purified and detected by the 6X His-tag in the SUMO-tag. Only 30-50% efficiency of cleavage of the SUMO-CCL20 fusion was achieved overall. Chemically refolding or not refolding in different dialysis buffers did not increase efficiency of cleavage. Varying temperature and changing the ratios of SUMO-CCL20 fusion: SUMO protease did not increase efficiency of cleavage. There was no difference in the cleavage of the SUMO-CCL20 in two different *E. coli* strains, Origami B DE3 or Rosettagami B DE3s. Repeated purifications of SUMO-CCL20 fusion from up to 12 L of culture did not yield enough

SUMO-CCL20 fusion or cleaved recombinant rhesus CCL20 for bioactivity testing. To generate non-human primate chemokine, rhesus CCL20 was synthesized with regioselective cyclization by Univ. Pitt. Peptide Facility for bioactivity testing.

5.0 Results: Application of a Functional Chemotaxis Assay to Develop for Vitro System Development

For the generation of non-human primate chemokines to be useful, the purified resultant protein must be proven bioactive in vitro. To find out whether the nonhuman primate CCL20 preparation that I had chemically synthesized was bioactive, a cell population that responds to the CCL20 had to be identified or developed for testing this factor.

5.1 Primary Cells

With the goal of identifying primary cell populations that express CCR6 and could serve as responder cells in CCL20 chemotaxis assay, uninfected primary macaque PBMCs were stained with antibodies and flow cytometry was utilized to analyze the expression of CCR6. As shown in Figure 10, a low percentage of cells expressed CCR6. Only 2% of the total CD3+ lymphocyte population expressed CCR6, whereas only 0.7% of CD3- lymphocyte population expressed CCR6. Of the T cells (CD3+) 61% of CD4+ T cells expressed CCR6, and 24% of CD8+ T cells expressed CCR6. A large percentage (80%) of B cells, which are CD3- and CD20+, expressed CCR6.

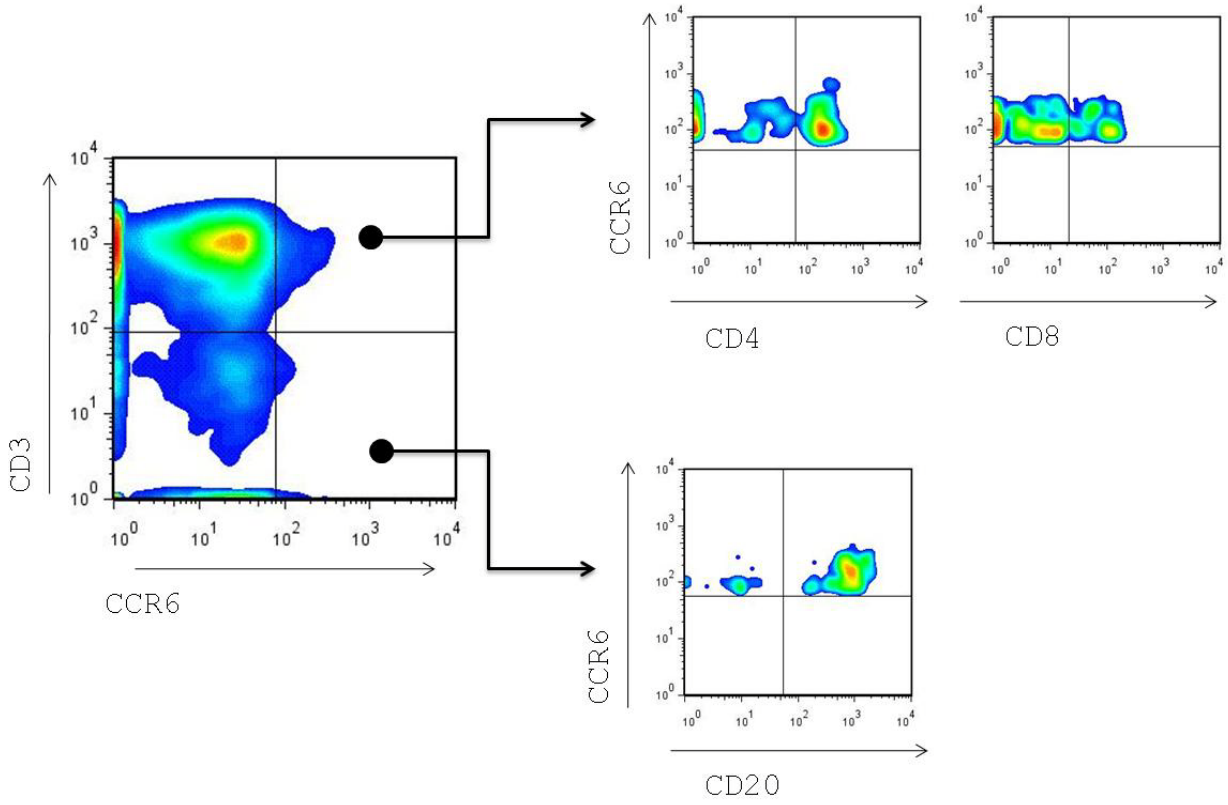


Figure 10. CCR6 is expressed on both B- and T-lymphocytes. Flow cytometry was utilized to analyze the expression of CCR6 on B and T cells by isolating primary rhesus macaque PBMCs. PBMCs were then stained with a 6 color panel (Anti-CD3 Pe-Cy7, Anti-CD4 PerCP, Anti-CD20 FITC, Anti-CCR6 PE, Anti-CD8 APC-Cy7 and Anti-CD69 APC). Data was aqcuired on the BD Canto, and analyzed with Flow Jo software.

To determine whether uninfected primary PBMCs would be a good population of cells to test the bioactivity of the regioselectively cyclized rhesus macaque CCL20 (abbreviated synthetic rhesus CCL20), the isolated PBMCs were placed in a functional chemotaxis assay. To determine whether the small population of PBMCs that express CCR6 (see Figure 10) were able to migrate toward to the synthetic rhesus CCL20, freshly isolated normal rhesus PBMCs were placed in a chemotaxis assay with a titration of synthetic rhesus CCL20, as well as 2 other chemokines, CXCL11 and CCL21. After incubation at 37ºC with 5% CO_2 for 1.5 hours, cells that had migrated through the membrane toward the chemokine were counted on a hemocytometer.

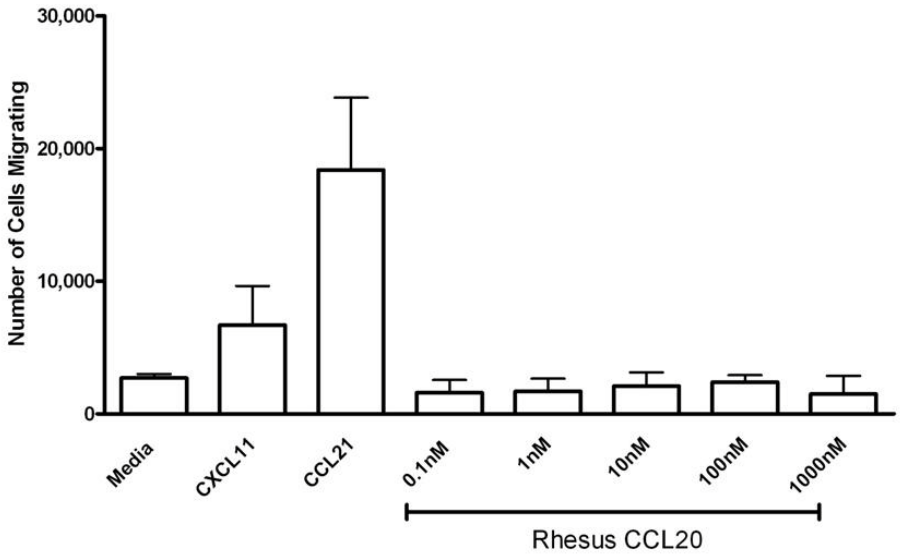


Figure 11. Normal macaque PBMCs do not respond to synthetic macaque CCL20. Freshly isolated normal rhesus macaque PBMCs were placed in a chemotaxis assay. A titration of synthetic rhesus macaque CCL20 was prepared in chemotaxis media. Human CXCL11 and CCL21 were prepared in the same manner and used as positive controls for migration. 200,000 cells (PBMCs) were loaded on the top of the membrane, and the plate was incubated for 1.5 hours at 37°C and 5% CO_2.

As shown in Figure 11, the cells were able to migrate towards certain chemokines, CXCL11 and CCL21, with the highest number of cells migrating towards CCL21. These results are consistent with expression (data not shown), in that, 60-80% of lymphocytes express CCR7, the receptor for CCL21, and 30-60% of lymphocytes express CXCR3, the receptor for CXCL11. No cells migrated towards the increasing concentrations of CCL20 (Figure 11), suggesting that either the synthetic rhesus CCL20 was not bioactive, or the receptor, although expressed, was biologically inactive, or that the population tested did not have sufficient numbers of cells that expressed CCR6.

Given the finding that primary PBMCs do not migrate towards CCL20 despite expression of the receptor on both T- and B-lymphocytes, a cell line that expressed CCR6 had to be developed to test whether the rhesus macaque CCL20 produced the regioselectively cyclized synthesis was bioactive. The cell line should be one that proliferates quickly, are easily

transfectible and do not respond to many human chemokine receptor ligands, including CCL20. Murine L1.2 pre-B cells were found to be a cell line that possessed these properties and were therefore utilized in developing a CCR6 cell line[63].

5.2 Regioselectively cyclized synthetic rhesus CCL20 is highly bioactive

As there were continued problems with obtaining recombinant chemokine for in vitro study, rhesus macaque CCL20 was synthesized by regioselective cyclization, described in material and methods and above (section 4.3). To examine the bioactivity of rhesus macaque CCL20 synthesized by regioselectively cyclization, L1.2 murine pre-B cells were transfected with a mammalian expression vector, pcDNA3.1 containing the cDNA sequence for human CCR6. After transfection, the cultures were treated with 5mM sodium butyrate overnight to increase expression of the receptor. To determine the expression profiles, cells were then isolated, washed and stained with CCR6-specific antibody. Data were acquired with either the Coulter XL or the BD Canto flow cytometers. CCR6 expression data were further analyzed with FlowJo software. To determine the functionality of the CCR6 receptor in transiently tranfected L1.2 cells, the cells were also placed in a traditional chemotaxis assay. A titration of both human and synthetic rhesus macaque CCL20 was prepared was and after three hours incubation at 37°C in 5% CO_2, cells that had migrated into the bottom well were then counted on a hemocytometer and total migrated cells was calculated.

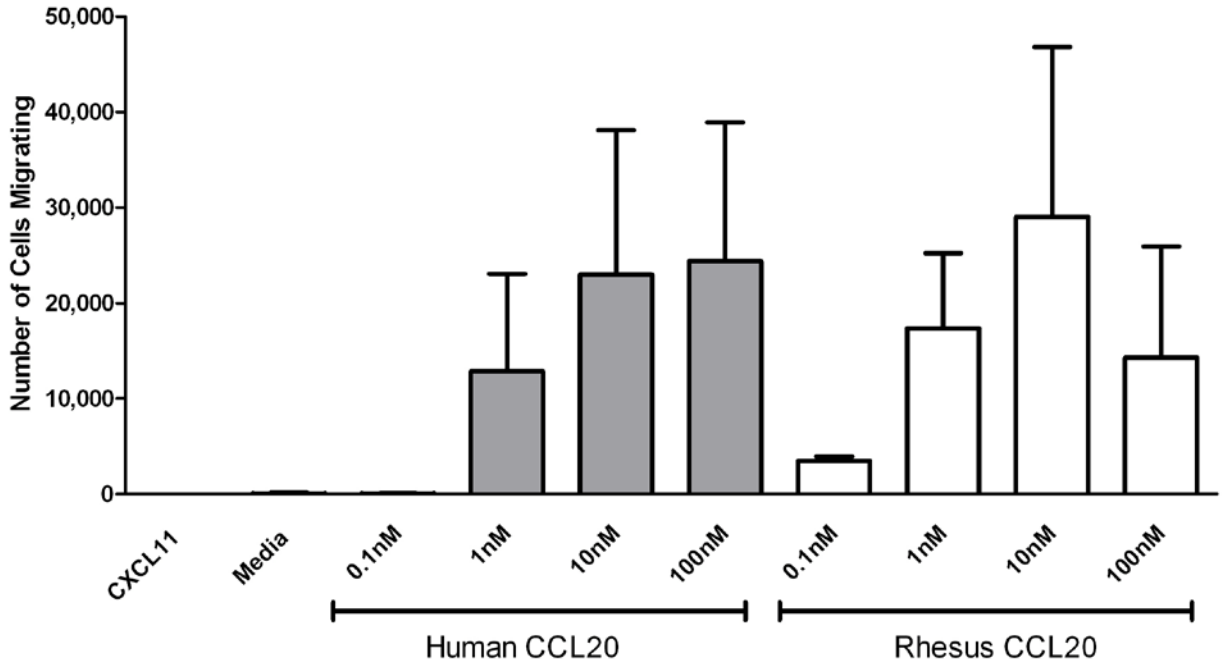


Figure 12. Peak migration of transiently transfected L1.2 cells is towards 10nM synthetic macaque CCL20. Murine L1.2 pre-B cells were transiently transfected with mammalian expression vector containing human CCR6 and placed in a chemotaxis assay. A titration of regioselectively cyclized rhesus macaque CCL20 (white bars) and human recombinant CCL20 (Peprotech) (grey bars) was diluted with chemotaxis media at the concentrations depicted. Chemotactic responsiveness was measured by counting cells in the bottom well after a three hour incubation at 37°C in 5%CO_2. Data represents an experiment performed in triplicate +/- SD. The average number of cells migrating to the positive control was 30,000 cells.

As shown in Figure 12, the transiently transfected cells migrated towards both human and synthetic CCL20, demonstrating that in vitro synthetic rhesus CCL20 was bioactive. Parental (untransfected) and mock transfected L1.2 cells did not migrate towards human or synthetic rhesus CCL20, indicating that migration was driven by CCR6 (data not shown). In addition, the cells did not migrate towards a non-cognate ligand (CXCL11) or the media only control, demonstrating that the migration was specific and CCL20-mediated. The highest number of cells migrated towards 10nM synthetic rhesus CCL20. It appeared from the dose titration curve observed for synthetic rhesus CCL20, that the synthetic rhesus CCL20 was bioactive at a lower

concentration than the recombinant human CCL20, since a lower concentration induced more cells to migrate (compare 10nM of human and synthetic rhesus CCL20).

5.2.1 Stable Cell Line Development

There was variability in the transfection efficiency with the CCR6 expression plasmid, with efficiency varying from 8-60% of the input cells (data not shown). With a transient transfection that is not 100% efficient, it is likely not all the cells loaded on the top of the membrane in chemotaxis express CCR6 or respond chemotactically to CCL20. In addition, to complete the study of inhibitory compounds on CCL20-mediated-chemotaxis, it is necessary to control these other variables (such as transfection efficiency) with a stable cell line so as not to attribute inhibition to these other factors.

To obtain a more efficient and controlled population of cells to test CCL20-mediated chemotaxis and chemotactic inhibition, stable cell lines were developed. CCR6 transfected L1.2 cells were cultured for 48 hours at 37°C in 5% CO_2 then resuspended with 1mg/ml G418 (geneticin). The cells were then aliquotted into a 96-well plate and cultured in the presence of G418 until cell foci were apparent, typically 5-7 days. These primary clones were cultured, treated with sodium butyrate and placed in a CCL20-mediated chemotaxis assay to measure their chemotactic responses to CCL20. Reserved cultures of primary clones that migrated in high numbers towards synthetic rhesus CCL20 were further cultured, single cell cloned and confirmed for CCL20-mediated chemotaxis. To this end, 12 stable cell clones were identified and moved ahead for further characterization and analysis of CCL20-mediated chemotaxis and inhibition studies.

5.2.2 A new approach for obtaining highly functional CCR6 expressing cells

Stable cell clone selection was performed with two criteria in mind, clones that had both high receptor expression and high migratory capacity. Despite these stable clone selection criteria, in some chemotaxis assays, only 5-10% of input stable cells migrated towards the optimal concentration of synthetic CCL20, despite greater than 90% of the cells demonstrating receptor expression as examined by flow cytometry. Additionally, since these cells were to be used to study possible inhibitors of CCL20-mediated chemotaxis, it would be ideal to study inhibition of chemotaxis with cells that displayed high efficiency (80-100%) of migration. To increase the percent of stable cells that would migrate, it was postulated that it might be possible to use another method to select highly efficient migrating stable cell clones, so-called "Chemotactic Selection". This is based on the idea that stable cell clones that had already been selected based on receptor expression and migratory capabilities may not need sodium butyrate pre-treatment to upregulate the chemokine receptor to drive migration.

To this end, three different stable cell clones were pre-treated with sodium butyrate or not and were placed in a traditional chemotaxis assay. As shown in Figure 13, none of the stable CCR6 clones showed CCR6 expression without sodium butyrate treatment, whereas with sodium butyrate treatment all of the stable clones, detection of CCR6 expression was 85-95%. Additionally, the functional capacity of these cells was dependent on sodium butyrate pretreatment, where only the pre-treated cells migrated towards the optimal concentration of synthetic CCL20 (Figure 13).

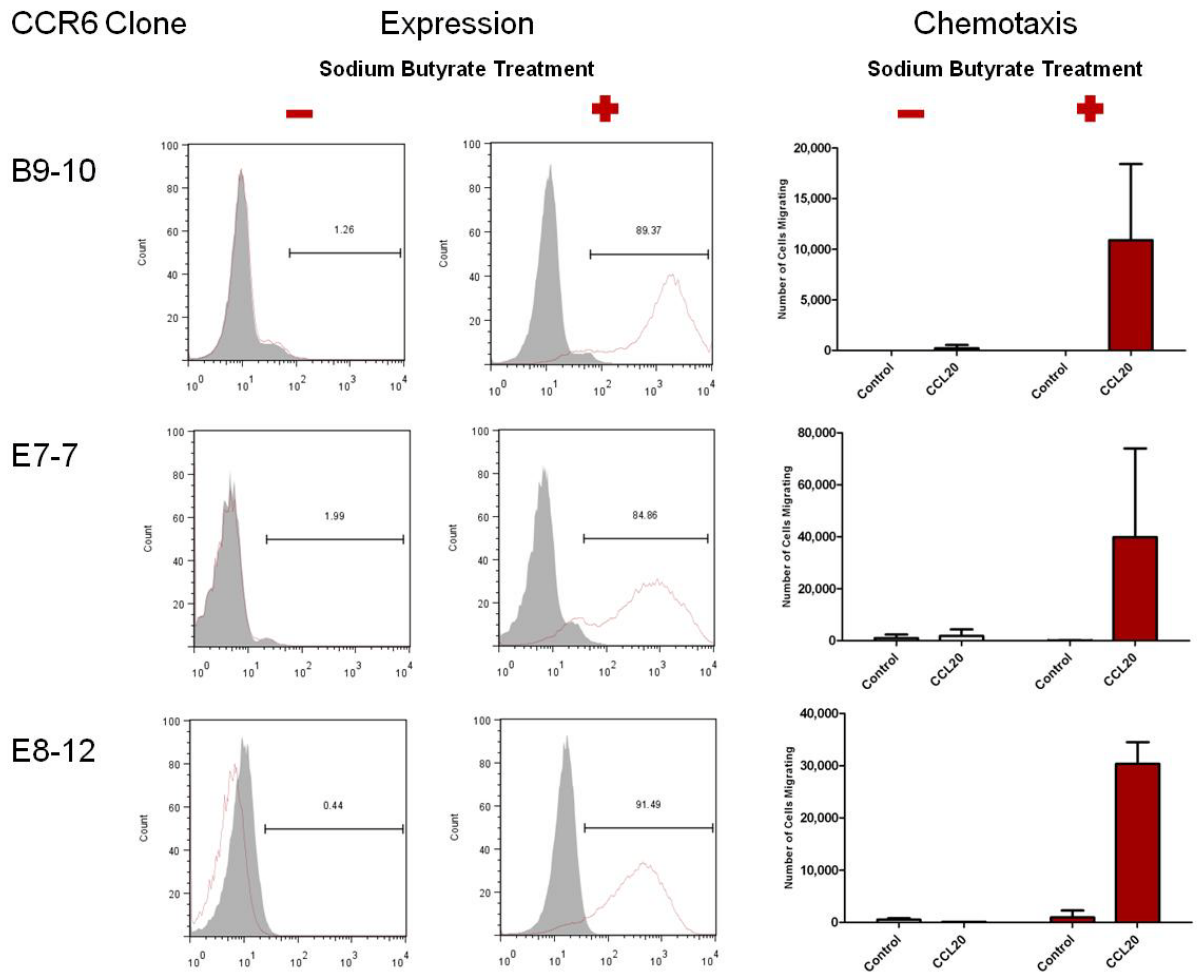


Figure 13. Only pre-treated stable CCR6 L1.2 cells express CCR6 and migrate towards synthetic macaque CCL20. Stable CCR6 L1.2 pre-B cells were treated (+) or not (-) with 5mM sodium butyrate overnight. Cells were then stained with anti-human CCR6-PE (BD biosciences) and flow cytometry data was acquired on the BD Canto. Cells were also placed in a chemotaxis assay. Regioselectively cyclized rhesus macaque CCL20 was diluted with chemotaxis media to 10nM. Chemotactic responsiveness was measured by counting cells in the bottom well after a 3 hour incubation at 37°C/5%CO_2 and normalized to the positive control (rhesus CCL20). Data represents an experiment performed in triplicate +/- SD.

In summary, a low percentage of uninfected primary macaque PBMCs expressed CCR6 but did not respond functionally by chemotaxing towards synthetic rhesus CCL20. Transient transfection yielded only 8-60% efficiency as analyzed by flow cytometry, and only 5-20% migration of input cells as analyzed by chemotaxis. Twelve (12) stable cell lines were

established to increase CCR6 expression and functional response to CCL20. Even with 85-95% CCR6 expression, only 10-40% of input cells migrated towards synthetic rhesus CCL20. A chemotactic selection strategy of the stable cell clones failed to yield 80-100% migration of input cells, but revealed that pre-treatment with sodium butyrate was required to observe a positive result of CCR6 expression and CCL20-induced migration.

5.3 Inhibiting the CCL20/CCR6 Interaction

Modifying the CCL20/CCR6 may prove to be important in studying mucosal immunology, HIV transmission and vaccinology, so it follows that identifying compounds that do inhibit CCL20-mediated chemotaxis is important. To study the effects of a biochemical surfactant, anti-inflammatory botanicals, and receptor peptide mimetics on CCL20-mediated chemotaxis, the stable cell lines were utilized.

5.3.1 GML does not inhibit CCL20-mediated chemotaxis

GML (glycerol monolaurate) is an FDA approved fatty acid surfactant used recently to inhibit SIV replication. GML has been shown to have anti-microbial and virucidal properties without cytotoxic effects on normal macaque vaginal flora [76, 77]. Based on a study showing that inhibition of SIV-induced CCL20 up-regulation observed with GML treatment in the female macaque vagina [10], we sought to analyze whether CCL20/CCR6 chemokine/chemokine receptor pair was affected by GML. We used cell clones stably expressing CCR6 L1.2 cells to test this. GML at increasing concentrations was added to the chemokine, the cells, or both. Cytotoxicity of the cells was tested separately, and 50µg/ml GML was about 30% cytotoxic when mixed with the cells (data not shown).

A significant variability was observed among the triplicate repeated experiments, and may have been due to the slight cytotoxic effects of GML observed at higher concentrations.

However, as shown in Figure 14, GML had little effect on CCL20-mediated chemotaxis, measured by the percent of migration relative to the positive control (CCL20 with no GML). The slight inhibition of migration observed when 50µg/ml of GML was present with both the cells and the chemokine was not statistically significant. In conclusion, we determined that within the constraints of this chemotaxis assay, there was not a direct effect of GML on the CCL20/CCR6 interaction.

Since GML did not appear to interact with either CCL20 or CCR6 and higher concentrations (50µg/ml) induced cytotoxic effects on the cells, further study was performed with the goal of identifying an inhibitor of CCL20 or its receptor CCR6.

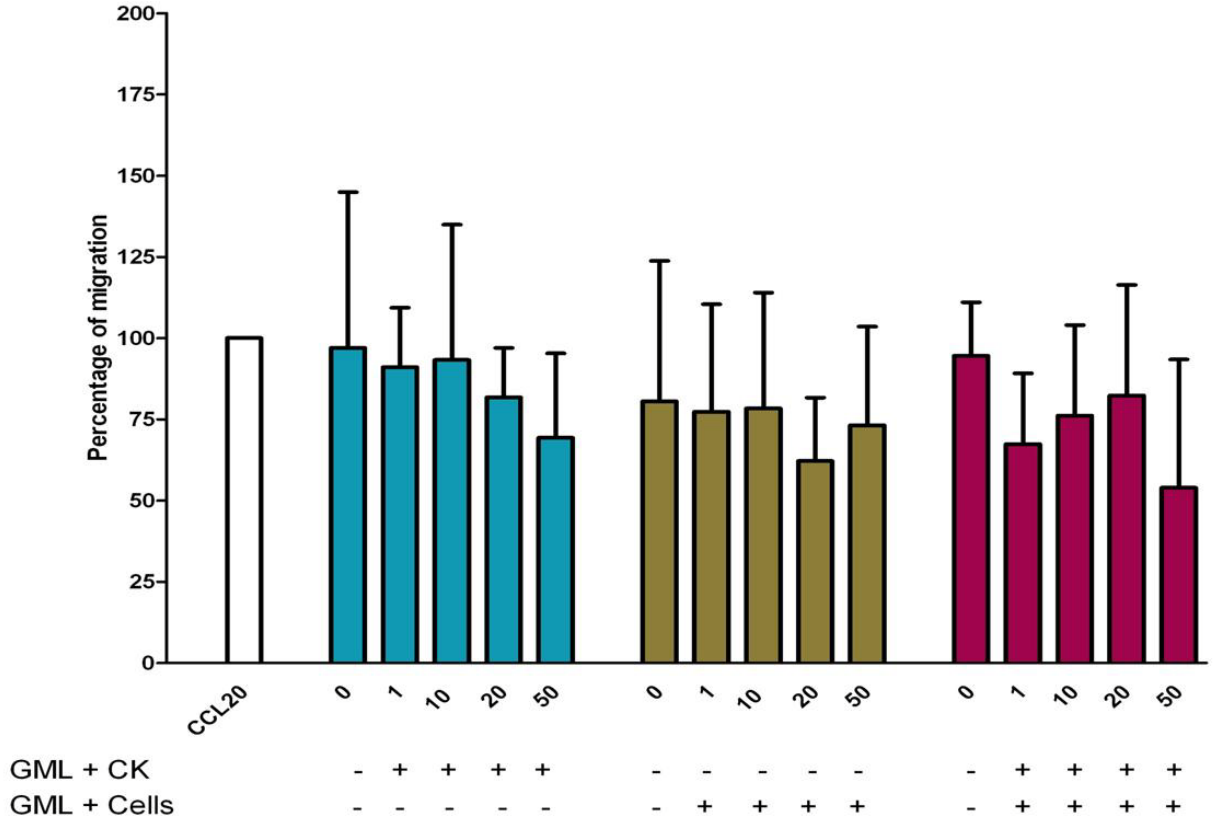


Figure 14. GML does not inhibit CCL20-mediated chemotaxis. Stable CCR6 L1.2 cells were prepared as for chemotaxis, with overnight pre-treatment with sodium butyrate. 10nM synthetic rhesus CCL20 was prepared and increasing concentrations (μg/ml) of GML were added to the chemokine or to the cells. After a three hour incubation at 37°C in 5% CO_2, cells that had migrated through the membrane were counted on a hemocytometer and percent migration total was calculated as the total number of cells that had migrated towards the positive control, 10nM CCL20. Data represents 3 independent repeats, +/- SD.

5.3.2 CCR6 receptor peptide mimetics ECL-2 inhibit CCL20-induced migration

Our laboratory has shown that the extracellular loops of chemokine receptors, so called receptor peptide mimetics or ECL-X, where X refers to the number of the sequential extracellular loop, can bind directly to chemokine[64] and inhibit chemokine-induced migration. The method of using the second extracellular loop to affect outcomes of signaling through chemokine receptors has also been used to inhibit HIV entry by using the second extracellular loop of CCR5 [85]. Interest in this property of the ECL-X was applied here to CCL20-induced migration. To this end, the second extracellular loop, or ECL-2, of CCR6 (R6 ECL-2) was synthesized by the University of Pittsburgh Peptide Facility. Due to the possibility that the cognate rhesus R6 ECL-2 CYS (containing a cysteine residue) could form disulfide bridges in solution, two other R6 ECL-2s were also synthesized. Amino acid substitutions for the cysteine residue were decided upon based on weight and isoelectric point, striving for comparable behaviors in solution and similar overall molecular weights. To this end, alanine or threonine were substituted for the cysteine during peptide synthesis producing R6 ECL-2 ALA and R6 ECL-2 THR. A graphic representation of the location of the extracellular loop with respect to the structure of a G protein coupled receptor and the location of the amino substitutions used is shown in Figure 15.

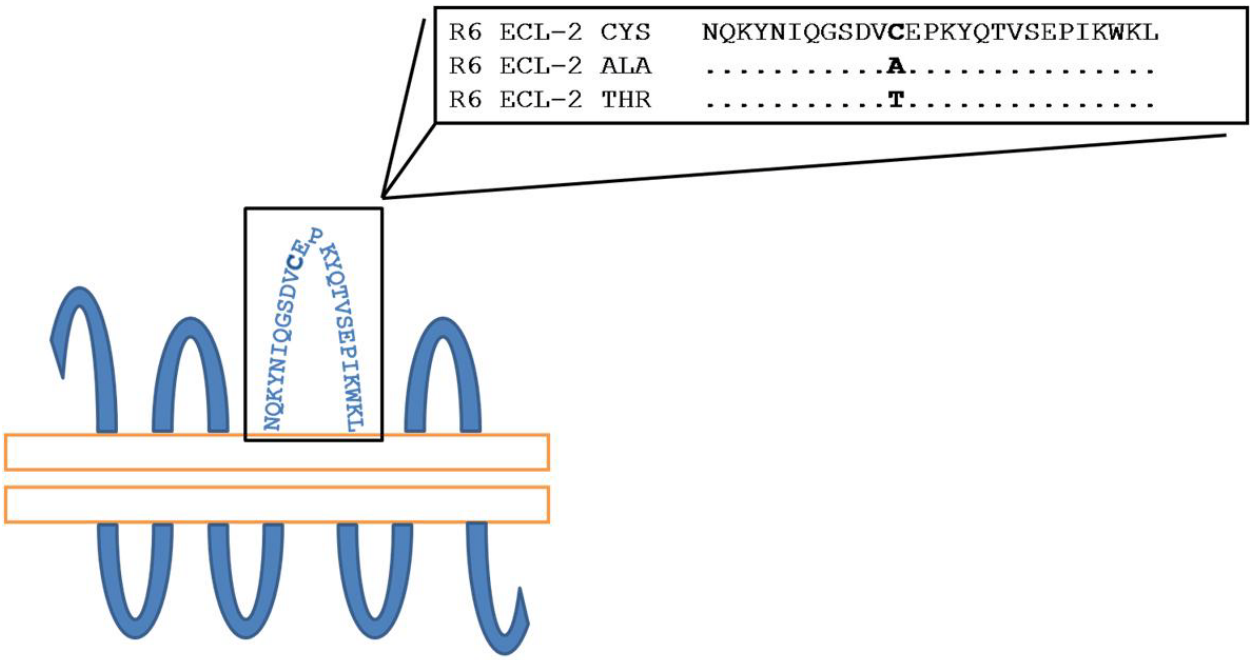
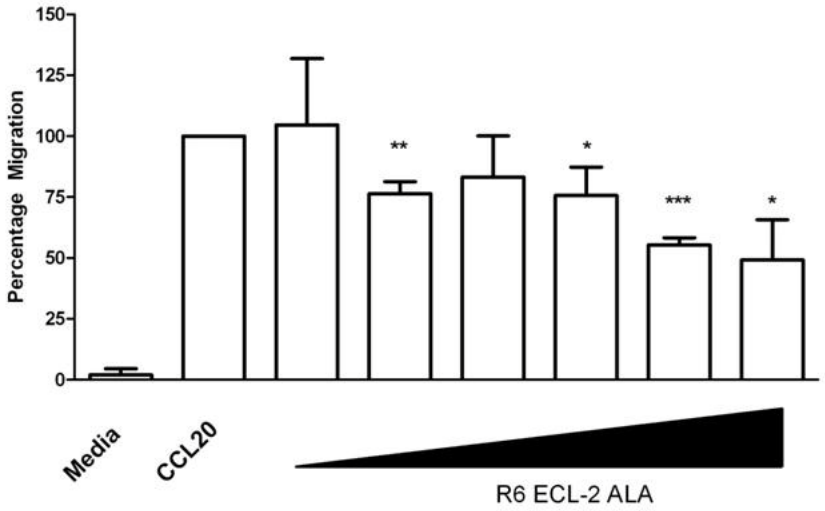


Figure 15. Design of the second extracellular loop of CCR6 (ECL-2). The regions of the sequence of the rhesus macaque were referenced and analyzed to identify inter-, intra-, and extra-cellular membrane regions. Three sequences of the second extracellular loop (ECL-2) were synthesized with amino acid substitutions in two of the peptides to negate di-sulfide bridge formation in solution.

The R6 ECL-2 receptor peptide mimetics R6 ECL-2 CYS, ALA and THR were analyzed for inhibition of CCL20-mediated chemotactic response in a traditional chemotactic inhibition assay. As shown in Figure 16, R6 ECL-2 with the alanine substitution (R6 ECL-2 ALA) significantly inhibited CCL20-mediated chemotaxis 15-50% of the migration observed to the optimal 10nM rhesus macaque CCL20 at concentrations of 10nM ($p<0.01$), 1µM ($p<0.05$), 10µM ($p<0.001$) and 100µM ($p<0.05$). R6 ECL-2 CYS (100µM) significantly inhibited CCL20-mediated chemotaxis to about 50% migration to the optimal 10nM synthetic rhesus CCL20 ($p<0.01$). Interestingly, 1nM R6 ECL-2 CYS significantly increased CCL20-mediated chemotaxis ($p<0.05$). Significant chemotactic inhibition was only observed at 100µM of R6 ECL-2 THR ($p<0.01$). None of the concentrations of the receptor peptide mimetics were

cytotoxic (data not shown), so it can be concluded that the inhibition observed was due to an interaction of R6 ECL-2 with either CCL20 or CCR6.


Percentage Migration
150
125
100
75
50
25
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*
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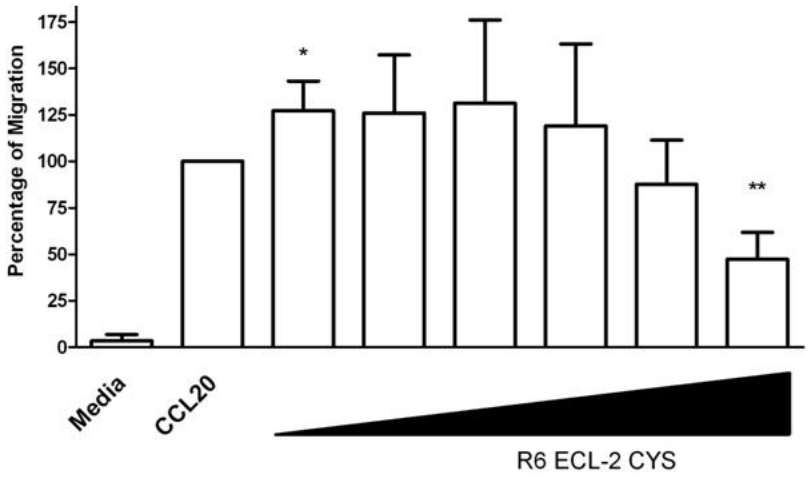
Media
CCL20
R6 ECL-2 ALA


Percentage of Migration
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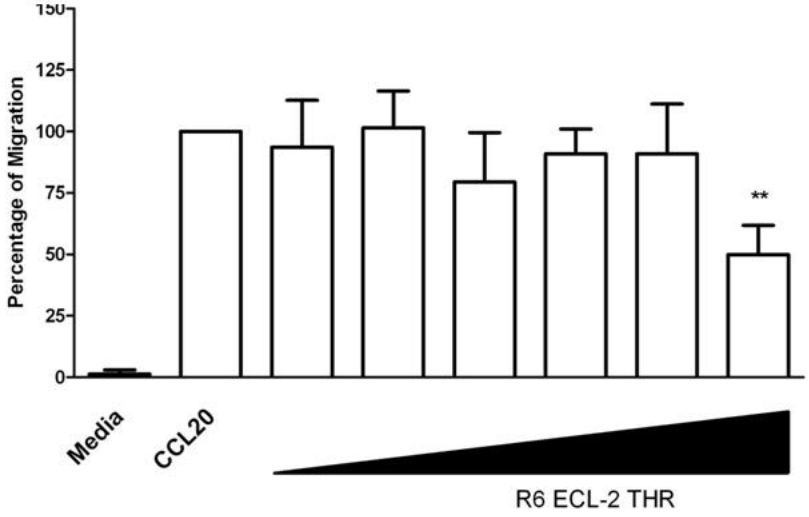
Media
CCL20
R6 ECL-2 CYS


Percentage of Migration
125
100
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25
0
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Media
CCL20
R6 ECL-2 THR

Figure 16. R6 ECL-2 (receptor peptide mimetics) inhibit CCL20-induced migration. Stable L1.2 human CCR6 cells were placed in a chemotaxis inhibition assay. 10nM synthetic rhesus CCL20 served as a positive control for migration and was mixed with 10 fold increasing concentrations (range of 1nM to 100μM) of the 3 receptor peptide mimetics, R6 ECL-2 ALA, R6 ECL-2 CYS and R6 ECL-2 THR. Data represents 3 repeat experiments +/- SD. Paired t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3.3 Anti-Inflammatory Botanical Inhibitors

Botanical elements have long been studied for their medicinal and biochemical properties. Our laboratory has recently been interested in a polyphenol compound found in green tea, epigallocatechin gallate, EGCG, due to its anti-inflammatory properties[52]. Additionally, our laboratory has observed that low concentrations of EGCG can inhibit chemotaxis of CXCR3+ cells [64]. Biacore binding assays revealed that EGCG bound CXCR3 ligands with high affinity (ref.[64] and Qin, et al, in preparation). Our laboratory has observed another anti-inflammatory property of EGCG in a mouse model of asthma, where there were a reduced number of T cells and airway inflammation with EGCG treatment (Qin et al, in preparation)**.** For these reasons, EGCG seemed an excellent candidate inhibitor that may affect CCL20-mediated chemtoaxis.

5.3.3.1 EGCG Inhibition of CCL20-mediated Chemotaxis

To determine whether EGCG inhibited CCL20-mediated chemotaxis as was observed by our laboratory for CXCR3 ligands, stable L1.2 human CCR6 cells were placed in a chemotactic inhibition assay with increasing concentrations of EGCG. A significant (10-20%) inhibition of CCL20-mediated chemotaxis was observed at concentrations of 1nM and 10μM EGCG (Figure 18, $p<0.01$). At the 100μM EGCG concentration, 75% inhibition was observed ($p<0.001$). EGCG induced inhibition of CCL20 chemotaxis was at a much higher concentration than we have previously observed with EGCG inhibited CXCR3 mediated chemotaxis (Tjoeng, 2009).

Given that inhibition was observed at different concentrations of EGCG, this botanical element may interact with either CCL20 or CCR6.

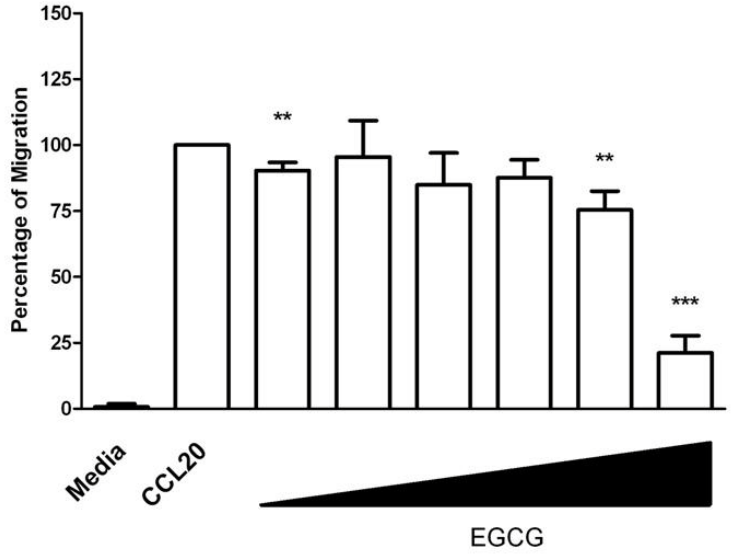

Figure 17. EGCG inhibits CCL20-mediated migration. Stable L1.2 human CCR6 cells were placed in a chemotaxis inhibition assay. 10nM synthetic rhesus CCL20 served as a positive control for migration and was mixed with 10 fold increasing concentrations (range of 1nM to 100μM) of EGCG. Data represents 3 repeat experiments +/- SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3.3.2 Gallotannin Inhibition of CCL20-mediated Chemotaxis

In an extensive study of naturally occurring botanical anti-inflammatory compounds, our laboratory has recently identified gallotannin as an inhibitor of CXCR3-mediated chemotaxis [64]. Gallotannin is a phenolic metabolite (tannin) that occurs naturally in some foods, chick and cow peas, mangos and rhubarb, as well as some nuts [29]. Tannins have been associated with scavenging of free radicals, and have been associated with interference of both herpes virus and HIV [29]. For these reasons, gallotannin was a prime candidate to test whether it displayed CCL20 inhibitory properties.

To determine whether gallotannin exhibited the same inhibitory properties as was observed for another chemokine/chemokine receptor interaction (CXCR3 controlled

chemotaxis); a chemotactic inhibition assay was performed with synthetic rhesus CCL20 and the stable CCR6 L1.2 cells. Significantly, 100% inhibition of CCL20-mediated chemotaxis was observed with 100μM gallotannin (Figure 18). This level of inhibition was observed in the absence of any cytotoxic effects (data not shown). Due to the chemotactic inhibition, it can be concluded then that gallotannin had an effect on CCL20-induced chemotaxis.

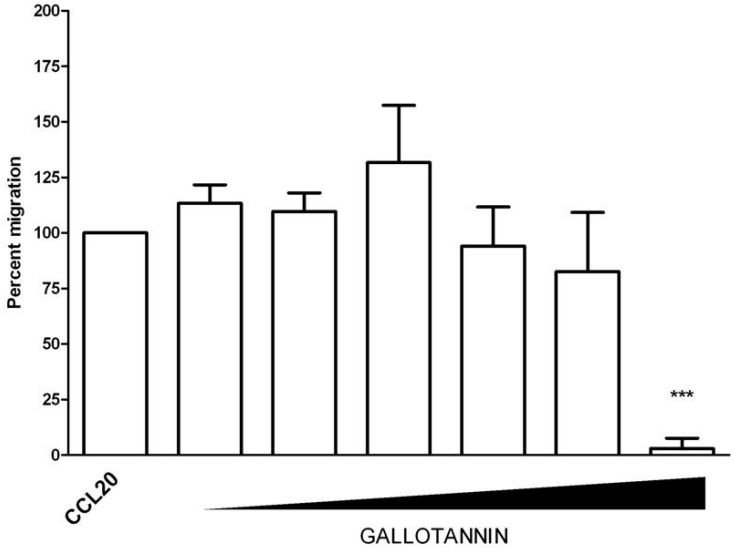

Figure 18. Gallotannin inhibits CCL20-mediated migration. Stable L1.2 human CCR6 cells were placed in a chemotaxis inhibition assay. 10nM synthetic rhesus CCL20 served as a positive control for migration and was mixed with 10 fold increasing concentrations (range of 1nM to 100μM) of EGCG. Data represents 3 repeat experiments +/- SD. *** $p<0.001$.

5.4 Overall Functional Application Summary

Low CCR6 expression of uninfected primary PBMCs did not translate to functional chemotaxis towards synthetic rhesus CCL20. The synthesized regioselectively cyclized rhesus macaque CCL20 was found to be highly bioactive, inducing more CCL20-mediated chemotaxis at a lower concentration (10nM) than a commercial control, human CCL20 (100nM). Stable cell lines were produced to test the bioactivity of both the recombinant and synthetic rhesus macaque CCL20 and the inhibitory properties of different biochemical elements. The surfactant

compound that has been linked with reduced SIV replication in the macaque vagina[10] where CCL20 is expressed, GML, was not found to affect migration towards CCL20. The receptor peptide mimetic, R6 ECL-2 peptides, R6 ECL-2 ALA, CYS and THR were all found to inhibit CCL20-mediated chemotaxis significantly at a concentration of 100μM. R6 ECL-2 ALA was also found to significantly inhibit CCL20-mediated chemotaxis at 10nM, 1μM and 10μM. n R6 ECL-2 CYS significantly enhanced CCL20-mediated chemotaxis at 1nM. The anti-inflammatory botanical EGCG inhibited chemotaxis at 1nM, 10μM and 100μM, with almost complete inhibition observed at 100μM. Another botanical anti-inflammatory, gallotannin, significantly inhibited chemotaxis to 100% at 100μM. Inhibitory concentrations of all anti-inflammatory elements that inhibited CCL20-mediated chemotaxis were all higher than has been observed previously in our laboratory.

6.0 Discussion

Chemokines are important factors in immunology in that they exhibit multiple functional roles, including lymph organogenesis, polarization of immune responses and leukocyte trafficking. Non-human primate chemokines are unavailable for use in studies modeling immunological responses, infectious disease and pathogenesis. My project provided our laboratory with two methods by which to obtain non human primate chemokines to use in vitro and in vivo. Additionally, I improved our understanding of the affect of our repertoire of anti-inflammatory compounds, in addition to studying new compounds to study (GML), on the CCL20/CCR6 interaction.

6.1 Protein Expression

Both the Origami B DE3 and Rosettagami B DE3 strains of *E. coli* were utilized due to their ability to over-express proteins via IPTG addition and plasmids encoding a gene that aided in the di-sulfide bond formation in the cytosol that may disallow over-expressed proteins to aggregate in inclusion bodies. The SUMO fusion technology was utilized due to the ease of purification of the SUMO-fusion proteins and the specificity of the SUMO protease. I found that both strains of *E. coli* expressed the protein upon IPTG induction. However, the resultant SUMO-CCL20 fusion protein aggregated in inclusion bodies.

Aggregation may have affected the ability to extract and purify abundant SUMO-CCL20 fusion proteins as large scale batch (up to 12L) protein expression did not yield sufficiently high quantities of SUMO-CCL20 fusion protein, since after HPLC purification of the yield of 1.7 milligrams of SUMO-CCL20, 10μg of SUMO-CCL20 fusion was available for additional cleavage testing and biological activity testing. Factors affecting the yield of SUMO-CCL20 are

potentially that all inter-molecular bonding of aggregated proteins might not be completely dispersed and some of this protein may not be a proper substrate for metal affinity column purification. Evidence to this fact is that it has been shown that once proteins have aggregated in proteins, these same proteins could have a tendency to aggregate in solution despite reducing and chaotropic agents that should disrupt inter-molecular aggregation.

Reasons for the aggregation of SUMO-CCL20 in inclusion bodies are as follows. It may be that SUMO-fusion proteins have a propensity to aggregate upon over-production. It may also be that the sequence and structure of the chemokine-here CCL20-contribute to the formation of inclusion bodies. Disulfide bonding in the cytosol has long been identified as a difficulty in *E. coli* due to the reduced state of the cytosol [75]. This may be a reason why inclusion bodies are still formed despite the expression of a thioredoxin reductase and glutathione reductase, two factors that increase the formation of disulfide bonds in the cytosol [72]. Additionally, it has been shown that proteins that are cytotoxic to *E. coli* are aggregated in inclusion bodies [72, 74]. CCL20 has been proven to have anti-microbial properties which may contribute to its aggregation in inclusion bodies upon overproduction[23].

The SUMO fusion technology was successful in providing relatively low quantities of pure protein. However, when the time came to remove the SUMO-fusion tag by cleavage with the SUMO protease, the cleavage of the SUMO-CCL20 fusion protein was only about 30% efficient and precluded the ability to obtain enough recombinant protein for bioactivity analysis.

The main issue with the recombinant protein expression system here was the poor yield of the desired, final protein. Cleavage of the SUMO-CCL20 protein, despite efforts at optimizing the yield and cleavage of the protein, remained at an efficiency of only 30-50%. The multi-parameter experiment (Figure 4) did not reveal conditions optimal for cleavage. None of

the reaction conditions showed complete cleavage of the SUMO-CCL20 fusion protein (Figure 5 and 6), despite the protease being shown to be active (Figure 3). Use of two different strains of *E. coli* did not change the efficiency of cleavage (Figure 8 and 9).

It may be that the cleavage site in the SUMO-CCL20 fusion protein is unavailable for the protease. Because the recognition 'site' of the SUMO protease is the tertiary structure of SUMO tag, the fusion protein requires refolding through chemical agents and dialysis in a neutral buffer, so that both the chemokine and fusion tag of the protein have the best chance to be in the proper tertiary structure(s) to allow efficient cleavage. Refolding of the fusion protein could make the cleavage site of the fusion protein unable to be recognized by the SUMO protease, in that the tertiary structure of the SUMO-CCL20 fusion would be disrupted as the chemokine may fold over the SUMO protease recognition sequence. Additionally, or even in conjunction, there might be different co-existent entities of partially folded fusion proteins, misfolded fusion proteins and properly folded fusion proteins that influence the level of cleavage that can be detected. It may be that the SUMO-CCL20 fusion protein that is evidenced as being cleaved (Fig 2B and Figure 3) may be only the properly folded fusion protein and other mis-folded SUMO-CCL20 fusions are left uncleaved.

Inefficient cleavage may be the result of SUMO protease may be more active in cleaving the SUMO-CCL20 fusion protein at a different concentration or temperature than was examined in the multi-parameter analysis. It may also be that despite using Rosettagami B DE3 cells to control the incorporation of rare tRNAs, there were mis-incorporations of amino acids in the SUMO tag protein versus mis-incorporation in the rhesus CCL20 protein since, as stated in section 4.2.2, SUMO tag contains four 'AGA' targets for mis-incorporation[5]. Mis-incorporation

could again cause disrupted proper tertiary structure of SUMO tag protein hindering its recognition by SUMO protease.

Despite the problems with cleavage of SUMO-CCL20 fusion, a bioactive form of rhesus macaque CCL20 was chemically synthesized. Regioselective cyclization peptide synthesis provided a method to obtain a large batch (35mgs) of highly pure protein measured by HPLC. In vitro studies revealed the rhesus macaque CCL20 was highly bioactive.

The assessment of the recombinant expression protocol utilizing the SUMO technology is that further optimization and study may reveal the steps by which large quantities of a recombinant rhesus macaque CCL20 can be purified and used in vitro or in vivo as a therapeutic agent or vaccine adjuvant. Although peptide synthesis provided a method by which highly pure bioactive protein was produced, mutation or amino acid substitution analysis of the protein sequence to identify important binding sequences of recombinant chemokine would be much more expensive and therefore limited than utilizing the recombinant expression system. Both recombinant expression and chemical synthesis of a rhesus macaque chemokine is definitely needed because very few if any macaque chemokines are available commercially.

6.2 Application of a functional chemotaxis assay for in vitro system development

A cell line expressing stable CCR6 was produced to study the bioactivity of both the recombinant and synthesized rhesus macaque CCL20. Enough SUMO-CCL20 fusion or cleaved recombinant rhesus CCL20 was not produced, and therefore bioactivity of the recombinant proteins was not measured. Rhesus macaque CCL20 was generated for bioactivity testing by peptide synthesis using regioselective cyclization. The synthetic rhesus macaque CCL20, in contrast, was shown to be highly bioactive in a chemotaxis assay. Multiple compounds were analyzed for inhibition of CCL20-mediated chemotaxis. The biochemical anti-microbial

surfactant, GML, did not have a direct effect on CCL20-mediated chemotaxis. Second extracellular receptor peptide mimetics R6 ECL-2 ALA, R6 ECL-2 CYS and R6 ECL-2 THR were shown to inhibit chemotaxis at high concentrations and were determined to have some effect on CCL20-mediated chemotaxis. Both anti-inflammaotry botanicals, EGCG and Gallotannin, analyzed potently inhibited chemotaxis at high concentrations.

In vivo studies of the response of immune cells to intradermal injections with synthetic rhesus CCL20 did not show that an inflammatory response to the chemokine. After further analysis, it may be that the concentration of CCL20 administered may have been at a concentration too high to elicit an appropriate localized immune response. CCL20 may have been marked for degradation due to the over-abundance by the 'chemokine sink' receptors which act to sequester over abundant chemokine[25]. Another possible explanation is that the time at which samples were taken for analysis was too soon to observe the effects of the CCL20 skin injections.

The importance of the development of a cell line stably expressing CCR6 is that the specific response to CCL20 and how that response may be affected by potential inhibitors can be studied. For example, on primary cells isolated from the blood or other tissues, there are a variety of chemokine receptors expressed on any cell type[3]. While the varied chemokine expression is no doubt immunologically indispensable, study of the influence of an individual chemokine response (i.e. CCL20/CCR6) would be difficult to interpret and define functionally. Therefore defining the specific effect of any inhibitory element on the CCL20/CCR6 interaction is warranted in chemokine/chemokine receptor in vitro studies.

6.3 Conclusion

In conclusion, the utilization of the recombinant protein expression system to produce chemokines can sufficiently produce pure SUMO-CCL20 fusion proteins, but the quantity of culture needed to produce enough SUMO-CCL20 fusion or recombinant CCL20 for bioactivity testing was not feasible. Additionally, the most efficient cleavage of the SUMO-CCL20 fusion protein achieved was about 30-50% cleavage.

In addition, the CCR6 stable cell lines were highly efficient at responding to synthetic rhesus CCL20 and represents a good tool for performing CCL20 chemotaxis inhibition. Using this assay, I determined that the CCR6/CCL20 interaction was modulated by high concentrations of several compounds, R6 ECL-2 peptides and the botanical elements, EGCG and gallotannin.

The model of chemotactic inhibition induced by EGCG and gallotannin has been described elsewhere[64]. Briefly, the high number of hydroxyl groups on the gallate groups of both EGCG and gallotannin may mimic glycosaminoglycans (GAGs)[60] and bind to GAG binding domains on the chemokine, in this case, CCL20. The fact that only high concentrations of both botanicals inhibited chemotaxis may mean that gag binding domains within rhesus CCL20 are resistant to binding of EGCG or gallotannin gallate groups.

6.3.1 Next Steps and Future Studies

In taking these studies to the next level, the following studies will be important to execute. The expression and purification protocol needs further optimization and adaptation to be useful for any future in vitro or in vivo studies with recombinant rhesus macaque CCL20. Also, the bioactivity of the SUMO-CCL20 and the recombinant CCL20 needs to be tested. To optimize the yield of SUMO-CCL20, the plasmid containing both the fusion protein tag and rhesus CCL20 could be codon optimized for *E.coli* expression. This may help to determine

whether the problems of cleavage could be attributed to the mis-incorporations of amino acids in both the SUMO fusion protein tag and rhesus CCL20. Expression of the protein could be carried out at different temperatures to analyze whether the solubility of the fusion protein could be increased. A soluble protein may be cleaved more efficiently and this would increase the yield of recombinant rhesus CCL20 from the expression system.

The stable CCR6 cell line could be utilized to test the effects other anti-inflammatory compounds, such as curcumin, other green tea polyphenols, EGC, ECG, and EC, on CCL20-mediated chemotaxis. To further confirm the model of inhibition of CCL20-mediated chemotaxis via gallate groups acting as GAG binding domains, the GAG binding domain of CCL20 could be mutated, as suggested in Tjoeng, 2009[64].

In vitro dendritic cell studies could be performed to analyze whether addition of CCL20 and HIV immunogens would enhance antigen processing and subsequent T cell priming and direction in vitro. Specifically, a prime and boost strategy of vaccination using CCL20 as an adjuvant for HIV immunogens could be performed to identify whether CCL20 is in fact a candidate adjuvant for vaccine studies.

Since constitutive CCL20 expression has been shown in the vagina and colon, but likely is also present at the anal/rectal junction, and since it has been suggested that chemokine receptor expression is or may be involved in the directing of immune cells to different tissue environments, it could be tested whether using CCL20 as a vaccine adjuvant also directed memory T cells to the correct tissue compartment (i.e. vagina, anal/rectal junction, or colon) for fighting initial HIV/SIV challenge. The synthetic rhesus CCL20 could be used as a vaccine adjuvant in mucosal delivery (vaginal or anal/rectal) of highly expressed SIV or HIV immunogens to study whether the delivery of CCL20 with SIV/HIV immunogens boosts the

initial immune response to sexual transmission of SIV/HIV in that tissue compartment. Specifically, another prime and boost vaccination strategy, but where both the immunogen (HIV/SIV proteins) and adjuvant (CCL20) were applied to the mucosal interface where initial challenge occurs in transmission (vagina, anal/rectal junction, or colon). If CCL20 were found to be a candidate adjuvant, stronger memory T cell responses would be observed with the use of CCL20 and immunogen versus immunogen alone upon HIV/SIV challenge.

Since inhibition of CCL20-mediated chemotaxis was observed, any one of the compounds could be used in vivo to study the effects of the compound on vaginal SIV transmission, similar to the study done by Li, et al with GML[10]. Specifically, the question would be whether the mucosal delivery of anti-inflammatory compounds inhibits SIV/HIV transmission. Although, this may seem contradictory to a desired immunological outcome of a fast and strong response to the invading pathogen (HIV), it may be that despite chronic immune activation it appears that the virus evades the immune system, turning the favor to a slower inflammatory response may give the immune system the foothold it needs to overcome virus replication.

Other immunological diseases that may be studied based on this study are those in which at least some of the pathology of the disease is associated with up-regulation of CCL20 expression. Examples of diseases to consider with respect to CCL20 expression are chronic inflammatory diseases, such as some skin diseases, i.e. atopic dermatitis and psoriasis, or gut associated diseases, inflammatory bowel disease (IBD) and Crohn's disease[27] and some autoimmune diseases, such as colorectal cancer. If the up-regulation of CCL20 expression causes tissue destruction, application of the anti-inflammatory compounds that inhibited CCL20 migration may be useful as treatments of these diseases. For the skin diseases, the inhibitory

dose of EGCG or gallotannin could be applied in a cream to psoriatic lesions to test whether the treatment reduced the size and longevity (i.e. how long until the lesion disappears) of the lesion. Use of the anti-inflammatory compounds for gut associated diseases (IBD, Crohn's disease) would be a little more complicated as the metabolism of EGCG and gallotannin may break down or alter the structures (gallate groups) that inhibit CCL20-mediated chemotaxis. A way in which the delivery to more internal tissue compartments of these anti-inflammatory compounds could be made easier may be through something like biodegradable microspheres. Microspheres are similar to micelles, in that the desired compound is encased in polylactic acid (PLA)[11]. As the microsphere degrades, increasing levels of the desired compound are released, in a so-called time-release fashion. Biodegradable microspheres have been used to time deliver insulin in a mouse model of diabetes[11]. The time-released insulin did appear to aid in leveling the blood glucose levels[11]. Additionally, while working out their own protocol, the University of Pittsburgh Peptide Facility (UPPF) encased our rhesus macaque CCL20 in PLA microspheres. Although the protocol was not optimized by UPPF, this at least gives a possible avenue for studying the time-release drug delivery hypothesis for anti-inflammatory botanicals. In the same fashion, biodegradable microspheres could be utilized to deliver EGCG or gallotannin to the gut essentially unperturbed by metabolic and enzymatic processing or breakdown. It could then be analyzed whether these treatments produced desirable prognosis or outcomes of the disease, i.e. reduction of the chronic inflammation of the gut in IBD or Crohn's disease.

6.3.2 Public Health Revelance of this Study

The public health relevance of studying ways to produce recombinant bioactive non-human primate chemokines is that studies of human immunity and treatment for diseases are sometimes modeled in monkeys. In monkey models of infections and immunologic diseases,

most reagents used are of human specificity. The species difference may alter the immune response generated or the ability to detect the antigen, thereby skewing the interpretation of the results, which then translate to poor modeling of human immunity and disease. The use of species specific reagents will provide more confidence in the results and interpretations of non-human primate studies.

Prevention is a main public health consideration and vaccination is one way prevention of transmissible diseases is achieved. If an anti-inflammatory compound affects the immune response to a vaccine, the efficacy and longevity of the vaccine may then be affected. One reason studying how CCL20/CCR6 interaction is modulated by anti-inflammatory compounds is that to mount an efficient immune response to vaccinations, a main player is immature DCs which express CCR6. Other factors affecting the efficacy of vaccination are the immunogen and the inflammatory immune response that is mounted to a vaccination. Therefore, identifying anti-inflammatory compounds that potentially cause a stinted inflammatory response to the vaccine and inhibit the influx of CCR6+ immature DCs is important in developing efficacious vaccines.

An additional reason studying the CCR6/CCL20 interaction is important to public health is in relation to the HIV epidemic. The ultimate goal to eradicate the world-wide HIV-1 epidemic will require a vaccine to or treatment of HIV that either prevents HIV transmission, or cures the prolonged immune activation and also inhibits virus replication. Recent HIV studies have linked CCR6 and CCL20 with both enhanced and reduced HIV replication[49, 51, 53, 54, 55] . The simple fact that a chemokine/chemokine receptor pair has potentially host-beneficial properties is reason to consider the pair in transmission and treatment of HIV. The CCR6/CCL20 interaction may be important in developing 'a site of entry' mucosal vaccine to HIV because of CCL20 expression in the mucosal tissues involved by sexual transmission of

HIV and CCL20's role in maintaining and recruiting immature DCs within the tissue. Another indication that CCR6/CCL20 should be considered to combat HIV transmission and replication is that a naturally occurring anti-HIV molecule, APOBEC3G, is up-regulated by CCR6 signaling, thereby protecting the cell expressing APOBEC3G from HIV replication[49]. These findings indicate that CCR6/CCL20 may be important in combating HIV transmission and persistence, and therefore important to consider in developing methods to treat and prevent HIV.

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