Regulators of G-protein Signaling, RGS13 and RGS16, are Associated with CXCL12-mediated CD4+ T Cell Migration

Lijin Xia
Brigham Young University - Provo

Follow this and additional works at: https://scholarsarchive.byu.edu/etd
Part of the Biochemistry Commons, and the Chemistry Commons

BYU ScholarsArchive Citation
Xia, Lijin, "Regulators of G-protein Signaling, RGS13 and RGS16, are Associated with CXCL12-mediated CD4+ T Cell Migration" (2008). All Theses and Dissertations. 1870.
https://scholarsarchive.byu.edu/etd/1870

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu, ellen_amatangelo@byu.edu.
REGULATORS OF G-PROTEIN SIGNALING, RGS13 AND RGS16, ARE ASSOCIATED WITH CXCL12-MEDIATED CD4+ T CELL MIGRATION

by

LIJIN XIA

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry and Biochemistry
Brigham Young University
December 2008
BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

LIJIN XIA

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

__________________________  ____________________________________
Date      Gregory F. Burton, Chair

__________________________  ____________________________________
Date       Barry M. Willardson

__________________________  ____________________________________
Date      David M. Belnap

__________________________  ____________________________________
Date      David L. Kooymen
As chair of the candidate’s graduate committee, I have read the thesis of Lijin Xia in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

Date

________________________  ____________________________________

Gregory F. Burton
Chair, Graduate Committee

________________________  ____________________________________

Accepted for the Department

David V. Dearden
Graduate Coordinator,
Department of Chemistry and Biochemistry

________________________  ____________________________________

Accepted for the College

Thomas W. Sederberg
Graduate Administer, College of Physical and Mathematical Sciences
ABSTRACT

REGULATORS OF G-PROTEIN SIGNALING, RGS13 AND RGS16, ARE ASSOCIATED WITH CXCL12-MEDIATED CD4+ T CELL MIGRATION

Lijin Xia
Department of Chemistry and Biochemistry
Master of Science

Chemokines are important chemical signals that guide lymphocyte movement within the immune system and promote the organization and functions of germinal centers (GCs) in the secondary lymphoid tissues. Previous studies have shown that GC T cells exhibit high expression of chemokine receptor 4, CXCR4, but that these cells are unable to migrate to the ligand for this receptor, the chemokine CXCL12. This “migratory paralysis” to CXCL12 was found to be correlated with the expression of two Regulators of G-protein Signaling, RGS13 and RGS16 in the GC T cells. The objective of my research was to determine whether RGS13 and RGS16 expression were associated with CXCL12-mediated CD4+ T cell migration. Because human GC T cells are rare and vary from one individual to another, I utilized two human neoplastic CD4+ T cell lines (i.e. Hut78 and SupT1) to facilitate and standardize my research. I also confirmed my observations using primary CD4+ T cells. Hut78 cells behaved similarly to GC T cells in
terms of CXCL12-mediated migration and RGS13 and RGS16 expression, while SupT1 cells appeared similar to CD4+ T cells that resided outside of GCs. The effect of RGS13 and RGS16 expression in the various CD4+ T cells was examined by altering the natural levels of these genes using RNA-mediated silencing and/or gene overexpression analysis after which, I examined the ability of the cells to migrate to CXCL12. RNA-mediated silencing of RGS16-, but not RGS13-, expression in Hut78 T cells resulted in a doubling of the migration rate in response to CXCL12. Overexpression of RGS13 or RGS16 in SupT1 and primary CD4+ T cells resulted in migration that was decreased by fifty percent. Because GC T cells demonstrated decreased migration to CXCL12 signals that may help them leave the GC, I reasoned that these cells may have an increased opportunity over other CD4+ T cells to become infected by the Human Immunodeficiency Virus (HIV) trapped on Follicular Dendritic Cells in the GCs of infected subjects. Examination of GC T cells obtained from HIV-infected subjects indicated that these cells were more frequently infected by HIV than other CD4+ T cells thereby confirming my postulate. My research indicated that RGS13 and RGS16 were associated with CXCL12-mediated CD4+ T cell migration and suggests that these molecules may play an important role in HIV pathogenesis within the GC.
ACKNOWLEDGEMENTS

I would like to thank Dr. Gregory F. Burton for the constant guidance, assistance, and support that he has patiently shown me during my studies here in his laboratory. I would also like to thank Dr. Keith A. Crandall and my graduate committee members, Dr. Barry M. Willardson, Dr. David M. Belnap, and Dr. David L. Kooymam for their insights into my research progress. I am grateful for having had the opportunity to work with all Dr. Burton lab members, past and present. I would like to especially thank Ann Burton, Benjamin Farnsworth, Iyas Massanet, James Gregson, Sariah Kell, In Yong Lee, Changna Wang and Xueyuan Zhou for all their generous help and friendship over the years.
TABLE OF CONTENTS

ABSTRACT ......................................................................................................................................................... iv

ACKNOWLEDGEMENTS ........................................................................................................................................ vi

LIST OF FIGURES ................................................................................................................................................ viii

INTRODUCTION ................................................................................................................................................ 1
   Chemokines ...................................................................................................................................................... 1
   Chemotaxis in lymphoid organs ..................................................................................................................... 2
   Chemokines and RGS proteins ....................................................................................................................... 4
   Chemokines, the GC and GC T cells ................................................................................................................ 5

MATERIALS AND METHODS .............................................................................................................................. 9
   Cell culture ....................................................................................................................................................... 9
   Primary CD4+ T cell preparation ................................................................................................................... 9
   Isolation of HIV+ CD4 T cells ...................................................................................................................... 10
   Plasmid construction .................................................................................................................................. 10
   Lipid-based transfection ............................................................................................................................... 11
   Electroporation ............................................................................................................................................ 12
   Cell sorting and flow cytometry analysis ..................................................................................................... 12
   DNA/RNA isolation ..................................................................................................................................... 13
   Quantitative PCR analysis .......................................................................................................................... 13
   In vitro migration assay ............................................................................................................................... 14

RESULTS .............................................................................................................................................................. 15
   RGS13 and RGS16 expression are high in GC T cells ................................................................................. 15
   RGS16 inhibition up-regulates CXCL12-induced migration in Hut78 cells ............................................. 15
   Overexpression of RGS13 and RGS16 in SupT1 down-regulates their migration to CXCL12 .................... 17
   Overexpression of RGS13 and RGS16 in primary CD4 T+ cells down-regulates CXCL12-mediated migration ........................................................................................................................................ 18
   Assessment of RGS13, RGS16 expression and migratory competence to CXCL12 in primary CD4+ T cells ................................................................................................................................. 19
   Higher concentrations of HIV DNA in GC T cells from HIV-infected patients ....................................... 20

DISCUSSION .......................................................................................................................................................... 21
   Inhibition of RGS13 and RGS16 expression in Hut78 cells ....................................................................... 22
   Overexpression of RGS13 and RGS16 in SupT1 cells ................................................................................. 23
   Overexpression of RGS13 and RGS16 in primary CD4 T+ cells ............................................................... 23
   GG T cells and CXCR5hiCD4+ T cells in blood ......................................................................................... 25
   GC T cells in HIV-infected patients ........................................................................................................... 27
   Conclusions .................................................................................................................................................... 28

REFERENCES ....................................................................................................................................................... 49
LIST OF FIGURES

Figure 1. RGS13 and RGS16 cDNA plasmids .................................................................30

Figure 2. GC T cells express high levels of RGS13 and RGS16 but demonstrate low CXCL12-induced migration .................................................................31

Figure 3. Hut78 cells express higher amounts of RGS13 and RGS16 than SupT1 cells .32

Figure 4. SupT1 cells migrate better than Hut78 cells to CXCL12 ................................33

Figure 5. Optimization of transfection with varying numbers of cells .........................34

Figure 6. Optimization of transfection with varying concentration of siRNA duplexes ...35

Figure 7. Optimization of transfection using different transfection reagents and concentrations ........................................................................................................36

Figure 8. Optimization of transfection with different electroporation programs ............37

Figure 9. Inhibition of RGS13 expression in Hut78 cells doesn’t change their migration to CXCL12 .................................................................38

Figure 10. Inhibition of RGS16 expression in Hut78 cells induces increased migration to CXCL12 .................................................................39

Figure 11. Optimization of SupT1 electroporation .......................................................40

Figure 12. Overexpression of RGS13 and RGS16 in SupT1 cells ...............................41

Figure 13. Overexpression of RGS13 and RGS16 in SupT1 cells decreases CXCL12-induced migration .................................................................42

Figure 14. Overexpression of RGS13 and RGS16 in primary CD4 T+ cells ...............43

Figure 15. Overexpression of RGS13 and RGS16 in primary CD4 T+ cells decreases CXCL12-induced migration .................................................................44

Figure 16. CXCR4 expression on primary CD4+ T cells after transfection .................45

Figure 17. CXCR5hiCD4+ blood T cells show an active response to CXCL12 ............46

Figure 18. HIV DNA in GC T cells and non-GC T cells ............................................47

Figure 19. Comparison of RGS13 and RGS16 mRNA expression in Hut78 cells .......48
I. **Introduction**

The human adaptive immune system is a complex network of cells and tissues (e.g. lymph nodes, tonsils, spleen) connected by the lymphatic system (1). Its chief function is to mount specific immune responses against dangerous substances. A chief component of the system is the antigen-specific cells or lymphocytes that allow it to recognize and respond to the infectious agents and substances with which it comes in contact. Antigen-specific lymphocytes are sub-divided into two major types: the B cell that is associated with the generation of antibodies, and the T cell that mediates graft rejection, fights against viral infections and interacts with the B cell to assist in the generation of some antibodies. Prior to exposure to antigen, as few as 1 in 100,000 B and T lymphocytes in our body are specific for a given antigen (2). To mount immune responses against antigens, antigen-specific T and B cells need to come together to interact. Bringing rare antigen-specific lymphocytes into physical contact occurs in secondary lymphoid tissues that include spleen, tonsils, lymph nodes (LN) and Peyer’s patches. To accomplish the localization of antigen specific B and T cells in secondary lymphoid tissues, these cells migrate to chemical signals that are present in the requisite sites. These soluble signals are referred to as “chemokines” because they facilitate the process of lymphocyte chemotaxis or migration towards a chemical gradient of these signals. Therefore, to fully understand lymphocyte interactions in the secondary lymphoid tissues, it is important to understand the chemokines, their corresponding receptors and the regulatory molecules involved in their migration into and out of those tissues.

**Chemokines**
Chemokines are small pro-inflammatory chemoattractant cytokines that bind to the class A, rhodopsin-like family of seven transmembrane G-protein-coupled receptors (GPCRs). These receptors are present on the plasma membrane of target cells (4). The chemokines are highly basic proteins; a feature that helps to establish a stable gradient by promoting interactions with sulfated proteins and proteoglycans on the cell surface (5). The binding of chemokines to their receptors activates multiple downstream signaling pathways via heterotrimeric GTP-binding proteins (G-proteins) and second messengers, including phospholipase Cβ (PLCβ) isoforms, Ser/Thr-kinases, phosphatidylinositol 3-kinase-γ (PI3Kγ) and c-Src-related non-receptor tyrosine kinases (6-9). These downstream signal transduction pathways contribute to the target cell’s different cellular and physiological activities including differentiation, activation, adhesion and migration (10-14). Emerging evidence suggests these chemokines and receptors are associated with an extraordinary number of pathologies, including rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, vascular disease, cancer, transplant rejection and acquired immunodeficiency syndrome (AIDS). Collectively, these different pathologies suggest the important roles that chemokines play in the human body (15-22).

**Chemotaxis in lymphoid organs**

The importance of chemokines in promoting the organization and function of secondary lymphoid tissue was first recognized by their role in recruiting innate immune cells and effector cells to the sites of inflammation (23, 24). Additional studies confirmed the functions of the chemical signals and identified a subset of chemokine family members involved in these processes. These members include: **CC** chemokine ligand 19 (CCL19) and CCL12 and their shared **CC** chemokine receptor 7 (CCR7); **CXC**
chemokine ligand 13 (CXCL13) and its **CXC** chemokine receptor 5 (CXCR5); and CXCL12 and its receptor CXCR4 (25-27). Although the details of lymphocyte migration are both chemokine and context dependent, a useful generic representation of the process is the migration of a leukocyte from the blood, across the endothelium, and into an affected tissue during an inflammatory response. This process starts with the interaction between selectins on the endothelium and mucin receptors on the leukocyte, causing a rolling behavior of the leukocyte along the endothelial surface (28, 29). Chemokines, secreted in response to inflammatory signals, are then bound by glycosaminoglycans on the endothelial cell surface to allow the retention of the chemokines at the inflammatory site (30). The engagement of the chemokines with their receptors triggers intracellular signals that drive firm adhesion of rolling cells. This process is mediated through integrins and the leukocyte then migrates from the blood into the secondary lymphoid tissue following the chemokine gradient (30).

The mechanism whereby B lymphocytes migrate or “home” to lymphoid follicles depends on the expression of CXCR5 on the B cells and CXCL13, a chemokine present in the follicular area of the secondary lymphoid tissue (32, 33). CXCR5 is up-regulated during B cell maturation in the bone marrow and is expressed by the mature B cells. The follicular stromal cells and follicular dendritic cells (FDCs) in the B cell follicles of secondary lymphoid tissues release the chemokine CXCL13, which in turn, recruits B cells into the follicular area (34, 35). In the follicular area, B cells undergo proliferation and movement to the CXCL13+ FDC network, and initiate the formation of germinal centers (GC). In the GC, B cells continue their development and selection, where T-cell help and contact are crucial and indispensable (36).
Naïve T cells express CCR7 and L-selectin which allow them to migrate into the CCL21/CCL19 bearing T cell zone of secondary lymphoid tissues; a process that begins in the high endothelial venules (HEVs) of secondary lymphoid tissues. Two recent studies in mice suggest that CXCR4 may co-operate in HEVs with CCR7 to recruit naïve T cells into LNs. (37, 38). The presence of CCR7 also allows antigen-bearing maturing dendritic cells (DCs) to travel into the central T cell zone of secondary lymphoid tissues. The expression of CCR7 coupled with the presence of the CCR7 ligands CCL21 and CCL19 in the T cell zone of secondary lymphoid tissues, allows T cells to co-localize with mature DCs. These DCs are required for T cell antigen priming, activation, proliferation and maturation. These interactions help produce different subsets of T “helper” (Th) cells including: Th1, Th2, GC Th (GC T) cells, and nonpolarized T cells (39-41). Th1 and Th2 cells can migrate to nonlymphoid tissues through their decreased expression of CCR7 and increased expression of sphingosine-1-phosphate receptor 1 (42). However, a subset of activated T cells has stably-increased CXCR5 expression which guides them into CXCL13+ GCs to provide T-cell “help” to the B cells. Upon entry into the GC, these T cells are referred to as “GC” T cells (40).

Chemokines and RGS proteins

Regulator of G-protein Signaling (RGS) proteins are the major regulators of GPCRs. GCPRs belong to a large family of highly diverse, multifunctional signaling proteins, which share a conserved signature domain (RGS domain). These RGS domains directly bind to activated $G_\alpha$ subunits of heterotrimeric G proteins to modulate the GPCR signaling pathways. Heterotrimeric G proteins are composed of $\alpha$, $\beta$, and $\gamma$ subunits. Each subunit has different isoforms. The $\alpha$ subunit has four different isoforms: $\alpha_i$, $\alpha_s$, $\alpha_q$ and
α_{12/13}. Ligand-bound activated receptors catalyze the exchange of GDP by GTP on the α subunit, leading to its dissociation from the βγ dimer. Both the α and the βγ components of the G-protein transduce signal to different downstream molecules thereby inducing the corresponding cellular response. Following GTP hydrolysis on G_{α}, the heterotrimeric G-protein complex reforms and the signaling process is terminated. RGS proteins work as inhibitors of G-protein signaling through their GTPase activity on the G_{α} subunit. This activity accelerates the inactivation rate of G_{α}-GTP thereby shortening the lifetime of dissociated, active G-protein subunits leading to a curtailment of GPCR signaling (43). In addition to their GTPase activity, RGS proteins may also regulate signaling by acting as effector antagonists and scaffold proteins (44, 45).

As negative regulators of GPCR signaling pathways, RGS proteins has been implicated in cell development, organ physiology, neuronal behavior and chemokine-induced cell migration (46-50). Virus-mediated overexpression of RGS9-2 in the nucleus accumbens reduced the locomotor response to D2 receptor agonists and to cocaine, whereas RGS9-2-knockout mice showed increased locomotor responses to cocaine (51). Overexpression of RGS1, RGS3 and RGS4 in a pre-B-lymphoma cell line has been shown to reduce interleukin 8 (IL8)-induced activation, while RGS16 exhibited its inhibitory activity in the IL-8/CCR5 mediated signal pathway in lymphocytes (46, 52). In the RGS-mediated regulation of lymphocyte chemotaxis, RGS13 has been reported to down-regulate CXCL12-induced B cell chemotaxis in mice, while RGS16 is a negative regulator of CXCL12/CXCR4 signaling in human megakaryocytes (53, 54).

**Chemokines, the GC and GC T cells**

The GC (aka secondary lymphoid follicle) is a specialized structure that develops
within the follicles of secondary lymphoid tissue 3-4 days after antigen stimulation. It is composed of 75-85% B lymphocytes, 5-20% T cells, a small population of highly specialized tingible body macrophages (TMBs) and FDCs. The GC cells interact and appear to cooperate in the performance of significant immunological events including B cell: somatic hypermutation, class switch recombination, and affinity maturation or selection of high-affinity antigen receptors (36). The GC can be divided into two compartments, the dark and light zones, based on their immunohistochemical labeling. The light zone is occupied by FDCs, GC T cells and TMBs, while the dark zone is occupied primarily by proliferating GC B cells (55). This compartmentalization of the GC cells is important to the development of the GC reaction, the processes involved in the generation of B cell memory and antibody-forming cell formation. The specific localization of cells in the GC depends on the regulation of chemotaxis - controlled by chemokines and their cognate receptors. CXCL13 release in the GC allows the migration of CXCR5+ GC B and T cells into this site, but the accurate distribution of GC B cells within the GC is believed to be controlled by the expression of CXCR4 on their surface (55). Two studies from the same group indicated that CXLC12, the ligand for CXCR4, is more abundant in the dark zone than in the light zone (56, 57). Therefore, it is believed that the differentiating GC B cells control their migration between the two zones through the regulation of CXCR4 expression on their cell surface.

While GC B cells have been extensively investigated over the past few years, less has been reported about GC T cells; specifically the movement of GC T cells into and out of the GC. Although some studies suggest that GC T cells could be the source of peripheral blood CXCR5^Hi T cells, the fate of GC T cells following a GC response is not
definitively known (58, 59). In fact, some studies suggest that GC T cells are fully
differentiated and prone to apoptosis (because of their high expression of CD95) making
it unlikely they could migrate from the GC (60). A recent study, however, implied that
GC T cells could further differentiate to memory CXCR5+CD69+ GC T cells but that
these cells would then remain resident in the B cell follicles (61). Thus, in the last two
situations, GC T cells were not thought to migrate from the GC.

A number of studies suggest that GC T cells are enriched in the light zone of GCs
to provide T-cell help to GC B cells that are differentiating. Interestingly, CXCR4\textsuperscript{Hi} GC T
cells, despite their high expression of specific receptor, were rarely observed in the
CXCL12\textsuperscript{Hi} dark zone (62). This unexpected behavior is also observed with freshly
isolated CXCR4\textsuperscript{Hi} GC T cells \textit{in vitro}, where only a minimal migration response to
CXCL12 was observed. In contrast, CXCR4\textsuperscript{Low} non-GC T cells clearly migrated to
CXCL12 (62). The retention of GC T cells in the light zone may provide an advantage to
GC B cells that reside there. This retention would enable a high number of encounters
between the GC cells leading to the formation of stable T- and B-cell conjugates, which
are essential for B affinity maturation. In spite of the observation that GC T cells do not
migrate to CXCL12, the mechanism(s) behind this specific non-responsiveness has yet to
be resolved.

Recent studies in megakaryocytes and mature B cells have shown that
CXCR4/CXCL12 signaling was regulated by members of the RGS family (53, 54). GC T
cells also show high expression of CXCR4, while their response to the chemokine
CXCL12 is low compared to non-GC T cells from the same tissue (63). The high RGS13
and RGS16 expression in GC T cells is reminiscent of murine B cells and human
The high expression of RGS13 and RGS16 observed in GC T cells coupled with their failure to migrate to CXCL12, prompted the hypothesis that the RGS family members likely play an important role in the observed lack of migratory ability to CXCL12 (63). My current study was undertaken to test this postulate by modulating the expression of RGS13 and RGS16 in human T cell lines and primary CD4+ T cells. Short interfering RNA (siRNA)-mediated knockdown of RGS16 in neoplastic Hut78 T cells indicated that this gene may regulate CXCL12-induced chemotaxis. Moreover, overexpression of RGS13 or RGS16 in SupT1 and primary CD4+ T cells resulted in decreased CXCL12-induced migration. This study suggested that RGS13 and RGS16 may play an important role in the migration of CD4+ T cells to CXCL12. Additional studies will be needed to further define the contributions of these regulatory genes on the migratory competence of GC T cells to CXCL12. Moreover, a greater knowledge of RGS interactions should provide important understanding about the mechanism of GC T cell retention in GCs. Further work may also help us understand whether GC T cells are capable of migration at an appropriate time during or after the GC reaction.
II. Materials and Methods

Cell culture

Primary CD4+ T cells and human CD4 T cell lines (SupT1 and Hut78) were cultured in complete tissue culture medium (CM) containing RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES Buffer, 2 mM L-glutamine, 1% non-essential amino acid (all from Hyclone, Logan, UT) and with 50 μg/mL gentamicin (Gibco BRL, NY).

Primary CD4+ T cell preparation

Human peripheral blood lymphocytes (PBLs) were isolated from healthy human blood using Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) gradient separation, and CD4+ T lymphocytes were enriched by Magnetic Cell Sorting (MACS) negative selection using a CD4+ T cell isolation kit (Miltenyi Biotech, Auburn, CA). The resulting preparations were ≥95% CD4+ T cells as assessed by flow cytometry using a BD FACS Vantage (BD Biosciences, San Jose, CA). Human tonsillar CD4+ T cells were isolated following a previously published procedure (64). Briefly, tonsils were cut into ~3 mm blocks and the cells were mechanically separated from the tissue by repeated pipetting. Red blood cells (RBCs) were removed by incubation for 5 minutes at room temperature in RBC-lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) and a large percentage of the B cells were removed by incubating four times with magnetic Dynabeads CD19 (Pan B) (Invitrogen, Carlsbad, CA). The remaining cells were then purified by using MACS with a CD4+ T cell negative selection isolation kit. ≥95% purity of CD4+ T cells was achieved as assessed by flow cytometry.

For further GC and non-GC T cell isolation, isolated tonsillar CD4+ T cells were
first incubated with mouse ChromePure IgG (Jackson ImmunoResearch Lab., PA) to
block non-specific binding, then were stained with biotin-conjugated mouse anti-
hCXCR5 (R&D System, Minneapolis, MN) and anti-human inducible co-stimulator
(ICOS) conjugated with phycoertrin (PE; BD Pharmingen, CA). After 30 minutes
incubation, fluorescein (FITC)-conjugated streptavidin (BD Biosciences) was added and
the cells were incubated for another 10 minutes. Following incubation, the fluorescence
labeled cells were sorted to CXCR5^{Hi}ICOS^{Hi} GC T cells and ICOS^{Low}CXCR5^{-} non-GC T
cells using a BD FACS Vantage. Following the same procedure (no staining here with
ICOS), CXCR5^{Hi} blood T cells were isolated from blood CD4^{+} T cells.

**Isolation of HIV+ CD4 T cells**

The handling of human immunodeficiency virus (HIV)-infected samples
complied with all relevant federal guidelines and institutional policies. After HIV-infected
secondary lymphoid tissues were received, these tissues were cut into small 1-2 mm
cubes and gently enzyme digested for 1 hour at 37°C in CM containing collegenase (10
mg/mL, Roche Applied Science, Indianapolis, IN), DNase I (1% v/v; Sigma, St. Louis,
MO) to release single cells. The released cells were collected and the tissue was subjected
to a further enzymatic digestion. The cells released from both digestions were pooled
together. After blocking non-specific binding and staining with proper antibodies [CD4-
PC5 (Beckman Coulter Immunotech, Marsellle, France), ICOS and CXCR5 stained as
above], GC and non-GC T cells were then sorted by flow cytometry based on their CD4,
ICOS and CXCR5 expression.

**Plasmid construction**

The plasmids for wild type human RGS13 and RGS16 clone were purchased from
the Missouri S&T cDNA Resource Center (Rolla, MO). The plasmids contain a complete open reading frame (ORF) region of RGS13 or RGS16 with a 3X-hemagglutinin (HA) tag at N-terminus (Figure 1A). To construct a control plasmid, RGS16 plasmid was digested with PmeI [New England BioLabs (NEB), MA] to remove the region containing the 3X-HA tag and the RGS16 ORF. The digested fragments were gel-separated and extracted to recover the larger piece. The recovered DNA was ligated back to itself using T4 DNA ligase (NEB) and was amplified in competent E. Coli. Before transfection, the amplified RGS13 and RGS16 clone plasmids and constructed control plasmid were confirmed by sequencing (data not shown) and by PmeI digestion (Figure 1B).

**Lipid-based transfection**

Hut78 or SupT1 cells were first prepared in a cell suspension containing 4-8x10^5 cells in 500 μL growth medium (CM) with serum but without antibiotics and were added to a well of a 24-well plate. siRNA-Lipofectamine 2000 (Invitrogen) complexes were prepared following the instruction manual provided by the manufacturer (Invitrogen). The prepared complexes were added to each well containing cells with medium. The plate(s) was gently rocked back and forth to mix the complexes with the cell suspension, and then incubated at 37°C in a 5% CO₂ incubator for 24-96 hours. To optimize the transfection procedure, FITC-conjugated Block-iT (Invitrogen) was used and different cell numbers, siRNA concentration and transfection reagents, including Oligofectamine, Lipofectamine and Lipofectamine 2000 (all from Invitrogen) were tested. 48 hours after transfection, the cells were washed once and analyzed by flow cytometry for transfection efficiency and cell viability. For RGS13 and RGS16 RNA silencing, the specific siRNA duplexes (Ambion, Austin, TX) for RGS13, RGS16 and non-specific negative control
siRNA were used for transfection.

**Electroporation**

The Amaxa Nucleofection System (Cologne, Germany) was used in this study for electroporation, following the description provided in the manufacturer’s instruction manual. Briefly, $2 \times 10^6$ cell line T cells ($5 \times 10^6$ for primary CD4+ T cells) were centrifuged and the resulting cell pellet was resuspended in 100 μL of the appropriate pre-warmed Nucleofector solution. The resuspended cells were then mixed with 2 μg DNA or 50 nM siRNA and transferred into a cuvette provided by the manufacturer. The appropriate Nucleofector program for electroporation was selected and applied to the cells in the cuvette. Immediately after finishing the electroporation, the cuvette was removed and 500 μL 37°C CM was added to it. The cells were then transferred into a culture well containing 1 mL CM in a pre-warmed 37°C 12-well plate. The plate(s) was then incubated at 37°C in a 5% CO₂ incubator for 24-96 hours before analysis. To optimize the procedure, different programs and Nucleofector solutions were tested. After optimization, specific plasmid DNA (for overexpression) or siRNA duplexes (for RNA silencing) were used for the corresponding studies.

**Cell sorting and flow cytometry analysis**

To ensure that only living cells were used for studies, viable cells from each sample were enriched after each transfection. Briefly, the transfected cells were collected and washed once. Then the viable cells were sorted using a BD FACS Vantage based on their forward light scatter (FSC) and side scatter (SSC) properties. The sorted cells were then analyzed for both gene expression and *in vitro* cell migration assay.

To examine CXCR4 expression, the transfected cells were collected and washed
once. After blocking non-specific binding, the cells were then stained with CXCR4-PE (R&D System, Minneapolis, MN). After 30 minutes incubation on ice, the stained cells were analyzed by flow cytometry using a BD FACS Vantage.

**DNA/RNA isolation**

Immediately upon isolation or in some cases after cell culture, cells were centrifuged and 800 µL RNA/DNA STAT 60 (Tel-Test, Friendswood, TX) was added to ≤1.0 x 10^6 cells in sterile, 1.5 mL microfuge tubes which were then stored at –80°C until testing. To isolate DNA/RNA, 160 µL of chloroform was added to the 800 µL DNA/RNA STAT cell lyses. After mixing and centrifuging the samples at 12,000 x g for 15 minutes at 4°C, 350 µL of the aqueous phase from each sample was transferred to a fresh tube containing 400 µL of isopropanol with 2-5 µL of GlycoBlue (Ambion). The contents were mixed, incubated at -20°C for 30 minutes and centrifuged at 16,000 x g for 45 minutes at 4°C. The supernatant fluid was then discarded and the DNA/RNA pellet was washed once in 1.0 mL 75% ethanol, air-dried and resuspended in 20 µL RNase/DNase-free water (Gibco).

For isolated RNA, any potential DNA contamination was eliminated by treating with RNase-free, DNA-free DNase (Ambion) according to the manufacturer’s instructions. The DNase-treated RNA was then reverse-transcribed to cDNA using Superscript III RNase H-reverse transcriptase (Invitrogen) following the manufacturer’s instructions.

**Quantitative PCR analysis**

Following reverse transcription or DNA isolation, HIV DNA or expression levels of RGS13 and RGS16 were measured by real-time PCR on duplicate reactions using an
ABI PRISM GeneAmp 5700 sequence Detection system and the TaqMan Universal PCR Master Mix (Applied Biosystems). Internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18s RNA were performed at the same time. The primers and probes were ready-to-use, commercially available gene expression assays from Applied Biosystems. A comparative threshold cycle (Ct) method was used to determine gene expression. For semi-quantitative PCR analysis of RGS13 and RGS16 expression, the primers and procedure were exactly the same as previously described (63).

**In vitro migration assay**

Cell migration was quantified *in vitro* through a 24-well, 5-µm pore Transwell culture system (Costar Corp., Cambridge, MA). Cells were washed once in chemotaxis medium [RPMI-1640 containing 20 mM HEPES buffer and 50 µg/mL gentamicin (Gibco BRL)] and adjusted to 5 × 10^6 cells/mL in the same medium. Then, a 100 µL aliquot of the above cell suspension containing 5 × 10^5 cells was placed in the upper well of the Transwell. In the lower chamber, 600 µL medium containing 1 µg/mL CXCL12 (Upstate, Inc.) was added. After 4 hours incubation (or 12 hours for transfected primary T cells) in a 37°C incubator, the upper inserts were removed and the wells with migrated cells were first checked briefly under a microscope to judge whether a dilution was needed for cell counting using a hemacytometer. After counting, the migrated cells were determined according to the following formula: the total number of cells migrating in the presence of chemokine minus the total number of cells migrating in the absence of chemokine. The migrated cells divided by the total input cells multiplied by 100 was used to determine migration efficiency.
III. Results

RGS13 and RGS16 mRNA expression are high in GC T cells

Previous studies reported that GC T cells expressed higher amounts of CXCR4 on their surface than did other CD4+ T cells obtained from the same tissues and that these cells were readily infected \textit{in vitro} with doses of X4-tropic HIV that did not infect other cells (64). Remarkably, in spite of this increased chemokine receptor level, these GC T cells lost responsiveness to the CXCL12. At the same time the GC T cells lost the ability to migrate to CXCL12, they began expressing two regulators of G-protein signaling, RGS13 and RGS16, suggesting that these molecules might be involved in the regulation of GC T cell migration (63).

To determine the role of RGS13 and RGS16 in the migratory competence of GC T cells to CXCL12, GC T cells were isolated utilizing a new procedure reported to yield a higher percentage of GC T cells than previously used in our laboratory (described in Material and Methods). These cells were then examined for RGS13 and RGS16 specific mRNA using quantitative, reverse transcriptase (RT) PCR (Figure 2A). The data indicated that GC T cells expressed more than a four-fold increase in RGS13 mRNA and over five-fold more RGS16 mRNA than non-GC T cells obtained from the same tissue. I next assessed the ability of these GC T cells to migrate to CXCL12 (Figure 2B). The GC T cells were largely unresponsive to CXCL12 while the non-GC T cells migrated well to this same chemokine, confirming and extending the previously published data of Estes \textit{et al} (63).

RGS16 inhibition up-regulates CXCL12-induced migration in Hut78 cells

I next sought to examine the individual role(s) of RGS13 and RGS16 expression
in the observed failure of cells to migrate to CXCL12. Because GC T cells are rare (<3%) and have the potential to vary from donor to donor, I sought to establish a model system where I could use human T cell lines to determine the contributions of the specific RGS genes that were expressed. I selected two commonly used neoplastic T cell lines, Hut78 and SupT1, for study based upon their ready availability and their extensive use in HIV infection studies. Semi-quantitative PCR analysis suggested that SupT1 cells produced much less RGS13 and RGS16 than did Hut78 cells (Figure 3A). To ensure that the observed qualitative differences in RGS13 and RGS16 mRNA expression in the above cell lines were quantitative, RT-PCR was also performed and normalized to the expression of the housekeeping gene, GAPDH (Figure 3B). Migration analysis indicated that Hut78 cells migrated with a lower frequency to CXCL12 than did SupT1 cells (Figure 4), consistent with the higher level of expression of RGS13 and RGS16 in the Hut78 cells. Because these two cell lines behaved in a similar manner to GC and non-GC T cells in both RGS13 and RGS16 expression and migration to CXCL12, I selected these cells for further study.

To begin to determine the role of RGS13 and RGS16 in Hut 78 and SupT1 migration to CXCL12, I first chose to use RNA interference to specifically knockdown these genes in the Hut78 cells after which I monitored their migration. To optimize the transfection of siRNA duplexes, I used commercially available, FITC-conjugated, siRNA duplexes to determine the most efficient method for my study. I first examined the efficiency and cell viability when lipid-based transfection agents were used on varying numbers of cells (Figure 5). Cell viability and transfection efficiency peaked when 350,000 cells were used. I next used this number of cells to determine the optimal siRNA
concentration, and found that 33.3nM of siRNA duplexes was ideal for transfection of Hut78 cells (Figure 6). I also compared different lipid-based transfection reagents and found that Lipofectamine 2000 achieved the best results in terms of both transfection efficiency and cell viability (Figure 7). Because my migration studies normally require 500,000 or more cells for each assay, and because electroporation can be very efficient at introducing nucleic acid into a large number of cells, I next examined this technique for Hut78 transfection. Since electroporation can be performed using a number of different signal intensities and times, I analyzed five different programs to determine which would be best for my studies (Figure 8). The V-001 program coupled with solution R from the Amaxa electroporation system was selected for the Hut78 cell transfections (electroporation) because of the high efficiency and low toxicity to the cells.

Hut78 cells were next transfected with siRNA duplexes specific for RGS13 or RGS16 or a non-specific negative control siRNA to determine if the expression of these genes could be modulated (Figure 9A, 10A). This procedure resulted in 85% efficiency in the knockdown of RGS13 mRNA expression and 67% efficiency in the knockdown of RGS16 mRNA expression. These cells were then examined for their ability to migrate to CXCL12 (Figure 9B & 10B). Decreasing the expression of RGS16 in Hut78 cells led to a doubling of their migration compared to controls. In contrast, no obvious change in the migration response was observed when RGS13 knockdown occurred.

**Overexpression of RGS13 and RGS16 in SupT1 down-regulates their migration to CXCL12**

Because my results indicated that decreasing the expression of RGS16 resulted in increased migration, I next determined whether increasing RGS13 and RGS16 expression
in SupT1 cells would decrease their migration to CXCL12. I selected electroporation-based transfections for introduction of the overexpression plasmids and then determined the optimal conditions for this procedure using SupT1 cells (Figure 11). After optimization, SupT1 cells were transfected with RGS13 or RGS16 expression plasmid or control plasmid and analyzed for gene expression and migration. The level of RGS13 mRNA in RGS13-transfected cells was 95-fold higher than in the same cells transfected with the control plasmid (Figure 12A). In the RGS16-transfected cells, a 1900-fold increase in RGS16 mRNA expression was observed (Figure 12B). Importantly, overexpression of RGS13 didn’t alter the expression of RGS16 and vice versa. I then evaluated the ability of the transfected cells to migrate to CXCL12 (Figure 13). Decreased SupT1 cell migration to CXCL12 occurred with overexpression of either RGS13 or RGS16.

**Overexpression of RGS13 and RGS16 in primary CD4+ T cells down-regulates CXCL12-mediated migration**

Because it appeared that RGS13 and RGS16 affected the CXCL12-mediated migration of Hut78 and SupT1 neoplastic cells, I determined whether a similar effect occurred in primary CD4+ T cells. Freshly isolated CD4+ T cells were enriched from the peripheral blood of normal donors or from the tonsils. These cells were transfected with the same RGS13 or RGS16 (or control) plasmid as utilized in the above experiments. In contrast to the transfection of neoplastic cell lines, a specific transfection reagent designed for human primary CD4+ T cells and program V024 (Amaxa) were used for transfection. This procedure resulted in a 60% transfection efficiency (data not shown).

Following electroporation of the primary CD4+ T cells with RGS13 and RGS16,
viable cells were obtained by cell sorting and the resulting changes in expression of the RGS genes were determined. Electroporation of the RGS expression plasmids into primary CD4+ T cells resulted in the overexpression of RGS13 and RGS16 (Figure 14). As shown in Figure 14A, RGS13 mRNA expression was increased 30–fold in cells transfected with RGS13 compared to cells transfected with control DNA. Transfection of primary CD4+ T cells resulted in a 7-fold increase in RGS16 mRNA compared to the same T cells transfected with control DNA (Figure 14B). Overexpression of RGS13 didn’t alter the expression of RGS16 neither did over expression of RGS16 alter that of RGS13.

To determine the effect of RGS13 and RGS16 overexpression in primary CD4+ T cells, CXCL12-mediated migration was assessed (Figure 15). A 60% decrease in the response to CXCL12 was observed in RGS13-transfected CD4+ T cells. Moreover, overexpression of RGS16 also inhibited CXCL12-mediated migration in a similar manner. To exclude the possibility that the change in migration of the transfected cells comes from different levels of CXCR4 expression, the transfected cells were also examined for their CXCR4 expression (Figure 16). I did not see any evidence of alteration in the level of CXCR4 expressed at the cell surface indicating that this was not a contributor to the observed results.

**Assessment of RGS13, RGS16 expression and migratory competence to CXCL12 in primary CD4+ T cells**

I was unable to obtain sufficient GC T cells to perform siRNA-mediated gene down-regulation and the following migration studies. However, I reasoned that it might be possible to compare the expression of RGS13 and RGS16 in cells in the blood that had
successfully migrated from the GC. The rationale for this experiment is that GC T cells with high levels of RGS13 and RGS16 do not migrate effectively to CXCL12. However, there are cells in the blood that appear to be derived from the GC and these cells express a GC T cell-like phenotype (i.e. CD4+, CXCR5+) (58, 59). If, as postulated by others, these blood cells (i.e. those with the GC-like phenotype) migrated from the GC, then I reasoned that they must have decreased levels of the inhibitory RGS13 and RGS16 genes that blocked their initial migration. To determine the level of RGS13 and RGS16 expression in peripheral blood GC T-like cells, I sorted these cells and subjected them to quantitative, RT-PCR to determine their RGS13 and RGS16 expression (Figure 17A). I observed that these CXCR5+CD4+ blood T cells showed a 93% decrease in RGS16- and a 30% decrease in RGS13- mRNA expression, compared to GC T cells obtained at the same time. I also analyzed the cells for their ability to migrate to CXCL12 (Figure 17B). As expected, these cells exhibited higher migration to CXCL12 than did GC T cells.

**Higher concentrations of HIV DNA in GC T cells from HIV-infected patients**

I reasoned that if RGS13 and RGS16 expression in GC T cells caused them to remain in the GC environment for extended periods, then these cells should be more likely to become infected with FDC-trapped HIV. To test this hypothesis, I isolated ICOS$^{\text{Hi}}$CXCR5$^{\text{Hi}}$ GC T cells and ICOS$^{\text{Low}}$CXCR5$^{\text{Low}}$ non-GC T cells from HIV patients, and compared the levels of HIV DNA in these two cell populations (Figure 18). The data indicated that all three patient samples had higher amounts of HIV DNA in GC T cells compared to non-GC T cells.
IV. Discussion:

The GC is a unique tissue microenvironment that brings together CD4+ T lymphocytes, B cells and FDCs. This site plays an important role in the generation of specific, T cell dependent antibody responses and the transmission and replication of HIV-1 (36, 65). Previous research determined that CD4+ GC T cells expressed high levels of CXCR4, the receptor recognizing the chemokine, CXCL12. Because GC T cells expressed higher levels of CXCR4 than other CD4+ T cells, it was originally postulated that these cells would migrate more efficiently to CXCL12 than other CD4+ T cells. Remarkably, despite their high expression of CXCR4, GC T cells failed to migrate to CXCL12. Coincident to the block in migratory ability, GC T cells expressed two RGS proteins, RGS13 and RGS16 and it was postulated that these GTPase-activating proteins blocked the ability of GC T cells to migrate to CXCL12 (63). I therefore sought to determine the role of RGS13 and RGS16 in the failure of GC T cells to migrate to CXCL12.

I began my studies by analyzing human CD4 T cell lines that could be used in place of primary GC T cells. A surrogate for the GC T cell was desired because of the rare nature of these cells (i.e. less than 3% of the total cells in the lymph node). Moreover, primary cells are donor dependent in that they possess a variable state of activation as well as different levels of specific receptors. I selected Hut78 and SupT1 neoplastic T cells for use based on their ability to mimic the RGS expression and migratory competence of GC and non-GC T cells. Importantly, these two cell lines were readily available, easy to propagate and susceptible to infection with HIV-1, an important criterion for much of the research that is ongoing in the laboratory.
Inhibition of RGS13 and RGS16 expression in Hut78 cells

Hut78 cells expressed higher levels of RGS13 and RGS16 than did SupT1 cells and, as noted above, migrate less efficiently to CXCL12 than do SupT1 cells. Down-regulation of RGS13 by a specific RGS13 siRNA duplex decreased the expression of the gene by over 80%. Interestingly, in spite of this alteration, there was no apparent effect on this cell’s ability to migrate to specific ligand. In contrast, decreasing the expression of RGS16 in Hut78 cells by over 60% significantly increased the migratory response. These data are consistent with the postulate that RGS16 plays a dominant role in the failure of cells to migrate to CXCL12 and suggest that RGS13 may play little to no role in the process. It should be noted, however, that this result appears in only a single human T cell line and thus the general applicability of this observation to all CD4+ T cells was not demonstrated. Comparison of the relative quantities of RGS13 and RGS16 mRNA in Hut78 cells indicated that RGS13 was expressed at a much lower level than RGS16 (Figure 3, 19). It seems plausible that due to the already low quantities of RGS13 mRNA expressed in Hut78 cells, a further reduction would only have a negligible effect and perhaps this accounts for the observed outcome. RGS proteins have been reported to have different half-lives (66). Thus, it is also possible that there is a difference in the half-lives of RGS13 and RGS16 proteins in Hut78 cells. If this latter reasoning is correct, the transcriptional impact of transfecting RGS13- and RGS16-specific siRNA duplexes may be temporally separated with RGS16 siRNA-mediated effects occurring before those of RGS13. The half-life of these two proteins is currently unknown and needs further investigation.

I noted that the transfection of the two siRNA duplexes did not have a profound
effect upon the basal ability of the cells to migrate as observed by comparing the rate of migration of Hut78 cells transfected with the control DNA to that of non-transfected cells (compare Figures 9B and 10B with Figure 4). It is also interesting to note that in contrast to my observations in Hut78 T cells, RGS13 has been implicated in regulating CXCL12-induced chemotaxis in other cell types suggesting that the overall impact of RGS expression in different cell types may differ (53).

**Overexpression of RGS13 and RGS16 in SupT1 cells**

In contrast to the results observed in Hut78 cells, SupT1 T cells have low expression of RGS13 and RGS16 and actively undergo chemotaxis to CXCL12. When RGS13 and RGS16 were overexpressed in SupT1 cells, decreased cell migration to CXCL12 was readily apparent compared to the control cells. Thus, in SupT1 human cells, both RGS13 and RGS16 appear to play roles in the migration to CXCL12. It is interesting to note the difference in cell migration capability of Hut78 and SupT1 cells after transfection. In contrast to the results observed with Hut78 cells where the introduction of foreign DNA did not have much of an effect upon migration, transfection of SupT1 cells resulted in a 50% decrease in the percentage of cells capable of migration. The reason for this observed transfection sensitivity of SupT1 cells is unknown but may be related to differences in membrane stability and/or cell viability. The important observation, however, is that despite different impacts of transfection, the overexpression of RGS13 or RGS16 led to a significant decrease in the ability of the cells to migrate to CXCL12 thus further implicating these two RGS family members in the impaired migratory competence of CD4+ T cells to CXCL12-induced migration.

**Overexpression of RGS13 and RGS16 in primary CD4+ T cells**
While the use of human CD4 T cell lines greatly facilitated my research studies, I am aware that these cells may possess different activities compared to primary CD4+ T cells, or for that matter, GC T cells. This concern prompted my examination of primary CD4+ T cells obtained from the blood or tonsils. It was interesting to note that overexpression of RGS13 or RGS16 also decreased migration by about 50%, a feature consistent with the observations obtained using SupT1 cells. Because the expression of CXCR4 in primary CD4+ T cells is reported to change with time in vitro (64), it was especially important to monitor the expression of this chemokine receptor on the transfected cells used in the migration assays. Flow cytometry confirmed a similar level of CXCR4 expression on all the cells indicating that the transfection procedure did not in itself cause alterations in CXCR4 density that could contribute to the observed migration differences (Figure 16).

My studies using both human T cell lines and primary CD4+ T cells indicate that RGS13 and RGS16 can play an important role in the regulation of CXCR4/CXCL12 associated migration. While some differential effects were apparent related to the ability of RGS13 and RGS16 in specific cells to affect cell migration, it is apparent that these RGS family members can both play important modulating roles on CXCL12-mediated lymphocyte migration. RGS13 has already been described as an important regulator for lymphocyte trafficking by attenuating the signaling of chemokine receptor CXCR4 (53). In that study, it was proposed that RGS13 possesses GTPase-activating protein (GAP) activity towards G\textsubscript{ia} of CXCR4 signaling pathway. RGS13 binds tightly to this subunit in a state that mimics the transition state of GTPase reactions (67). It seems likely that RGS13 and RGS16 share the same type of activity in the regulation of the CXCR4
signaling pathway because of their conserved RGS domains and shared GAP activities (66). It will be important in follow up studies to assess the differences in signaling within the CXCR4 pathway evoked by the two RGS genes that I examined. Perhaps this understanding may allow eventual modulation of cellular migration based on altering the ratio of RGS13 and RGS16, although this may be well into the future.

**GC T cells and CXCR5^{Hi}CD4+ T cells in blood**

The latter portion of my research begins to address the concept of whether RGS13 and RGS16 may completely inhibit the migration of GC T cells from lymphoid follicles or just block migration for a defined period. It is interesting to note that GC B cells, which share many similarities to GC T cells, also are known to block migration and this effect is mediated by RGS proteins (53). In the instance of B cells, ligation of the immunoglobulin receptor (antigen receptor) is sufficient to down-regulate RGS3 and RGS14 (68). After appropriate antigen-induced activation, GC B cells are known to migrate to the bone marrow where they complete differentiation into plasma cells and produce high levels of specific antibody (69). This migration is mediated by CXCR4/CXCL12 interactions. While it might be postulated that GC T cells could also migrate to the bone marrow, the rationale for such a migration is not clear. Both B and T lymphocytes undergo significant, random gene rearrangements to arrive at the expression of a functional antigen-specific receptor. During this process, two-thirds of the cells are lost to apoptosis or programmed cell death (70). Perhaps the RGS13 and RGS16 mediated block to GC T cell migration to CXCL12 plays a role in the selection process. After expending significant cellular resources in developing antigen-specific receptors on the T cells in GCs, it may be important to “test” their ability to provide needed signaling
in that microenvironment. This signaling may then allow them to continue to live or revert into a resting state for prolonged survival. Thus after participating in a GC reaction and interacting with resident cells, the block to migration may be lifted and the cells migrate to the bone marrow or another site rich in CXCL12 where they obtain a survival signal. In this manner, only cells that are actually capable of functioning properly in the GC would survive to participate in future memory immune responses.

If the above postulate is correct, one could presumably find cells with a GC T cell phenotype migrating in the blood. Based on the premise that to migrate, RGS13 and RGS16 would need to be down-regulated, I examined T cells from the peripheral blood that had the GC T cell-like phenotype of CD4+ and CXCR5+. When the expression of RGS13 and RGS16 in these cells was examined, it was consistently lower than that seen in actual GC T cells. A potential problem with my data is that the GC T cells and the blood cells were derived from different donors. This was mandated by the inability to obtain tonsillar tissue and blood cells from the same donors. The tonsils that I used were obtained from routine tonsillectomies. Unfortunately, I could not obtain a blood sample at the same time. However, I reasoned that if sufficient numbers of GC T cells were compared with a number of different blood samples (i.e. samples from unrelated donors), a statistically relevant average expression value for each of the RGS genes could be obtained and compared. At the time of writing this thesis, I have only been able to examine 2 tonsil samples and 2 blood samples. It is intriguing to note that initially, the above stated premise appears to be correct. Obviously these data are preliminary in nature, but they support the major postulate of my work that RGS13 and RGS16 play important roles in controlling the migration of GC T cells to the chemokine CXCL12.
In the previous published study of GC T cell migration, GC T cells were selected based on their CD57 expression (63). Since the time when this initial work was published, additional studies have suggested that GC T cells may be isolated in a more functional state if obtained using the markers ICOS and CXCR5 (71). I therefore re-assessed the RGS13 and RGS16 expression in ICOS^{Hi}\text{CXCR5}^{Hi} GC T cells and found these cells also demonstrated low migratory competence to CXCL12. A study from Hutloof et al documented that CD4 T cells expressing CD57 show a similar distribution to ICOS^{+} T cells in the GC. These latter cells are found to be largely confined to the apical light zone of the GC (72). Although differently defined GC T cells have different B cell stimulatory activity, they are similar in their responses to the chemokine CXCL12 in vitro. My results indicated that ICOS^{Hi}\text{CXCR5}^{Hi} GC T cells also exhibited high expression of RGS13 and RGS16 compared to the ICOS^{Low}\text{CXCR5}^{-} CD4 T cells from the same tissue. These observations in GC T cells are consistent with the notion that dynamic control of specific RGS expression acts as a general mechanism that shortens the duration of GPCR signaling.

**GC T cells in HIV-infected patients**

GC T cells have high expression of RGS13 and RGS16 but exhibit a down-regulated responsiveness to chemokine CXCL12 signaling that occurs outside the light zone of the GC. Therefore, CD4+ T cells from the light zone are relatively sessile within the CXCL13+ FDC network compared to other CD4 T+ cells that traffick through the FDC network. This previous observation provides a potential advantage to GC T cells allowing them to remain for longer periods in the GC to offer essential help with B cell differentiation. However, in HIV-infected patients, I expect that these GC T cells
experience longer exposure to the HIV-rich FDC-virus reservoir because of the prolonged retention in the surrounding of FDC network. This would provide GC T cells more chance to become infected by the HIV present on FDCs. In contrast, migrating non-GC T cells would only have a limited contact with FDC trapped HIV. In the three HIV patients from whom I obtained secondary lymphoid tissues, I observed a higher amount of HIV DNA in the GC T cells compared to the non-GC T cells from the same tissue.

While my studies suggest that GC T cells remain in the lymphoid follicles for long periods, the possibility that these GC T cells can migrate out of the GC can’t be excluded. Migration of GC T cells has the potential to allow the recirculation of these “GC T” cells throughout the body including into the CXCL12-rich bone marrow. If this happens in HIV-infected patients, it is possible that HIV DNA positive GC T cells could propagate HIV infection by carrying virus from the FDC reservoir through the recirculation. The FDC-HIV reservoir is possibly the largest virus reservoir in the human and infectious virus persists at this site (73, 74). Moreover, virus on FDCs is more genetically diverse than virus in other tissue sites and even contains “archived” including variants with drug resistance that has not been observed elsewhere. Thus trafficking of T cells infected in the GC, may increase the diversification of virus in infected subjects. Importantly, the actual GC T cell recirculation in humoral immunity as well as in HIV pathogenesis needs further study and this could be done using RGS13 and/or RGS16 deficient mice. Continued understanding of the processes involved in the GC T cell trafficking may be important in our understanding of why HIV infection persists for many years.

Conclusions
FDCs have been shown to contribute to HIV pathogenesis by maintaining a major reservoir of highly infectious HIV and providing an attractive microenvironment for HIV transcription and replication (73, 75). Understanding how this reservoir is established and how it may contribute to propagation of HIV infection in the body will be important to understanding this important cellular reservoir. Thus, an understanding of FDC contributions to surrounding target cells and their ability to migrate is of great importance. In this study, I elucidated the mechanism that underlies the nonresponsiveness of GC T cells to CXCL12-induced migration. Expression of two regulators of G-protein signaling, RGS13 and RGS16 were associated with the down-regulation of GC T cell migration. Moreover, increased HIV infection was observed in GC T cells. Future studies will confirm and extend this work with the hopes of finally uncovering how FDCs, the GC microenvironment and CXCL12 migration contribute to continued HIV infection in the body.
Figure 1. RGS13 and RGS16 cDNA plasmids. (A) Structure of the plasmids for RGS13 and RGS16 overexpression studies. An N-terminal 3X-hemagglutinin tagged human RGS13 or RGS16 open reading frame was cloned into pcDNA3.1+ at KpnI (5') and XhoI (3'). The open reading frame was derived from human RGS13 or RGS16 by PCR. (B) Gel analysis of Pmel-digested plasmids for RGS13 cDNA clone, RGS16 cDNA clone and control plasmid DNA. The control plasmid was constructed by removal of the RGS16 insert from the vector as described in “Materials and Methods”.
Figure 2. GC T cells express high levels of RGS13 and RGS16 but demonstrate low CXCL12-induced migration. GC and non-GC T cells were purified from tonsillar CD4+ T cells based on their ICOS and CXCR5 expression. (A) RGS13 and RGS16 mRNA expression levels were analyzed by quantitative, RT-PCR for freshly isolated GC and non-GC T cells. GAPDH was used as an internal reference. (B) CXCL12-induced cell migration. Chemotaxis of freshly isolated GC and non-GC T cells was assessed by *in vitro* migration to 1 μg/mL CXCL12. The graphs represent the mean ± standard deviation (SD) of three independent experiments.
Figure 3. Hut78 cells express higher amounts of RGS13 and RGS16 than SupT1 cells. mRNA from the two cell lines was extracted and reverse-transcribed into cDNA. (A) Semi-quantitative PCR was then performed with specific primers to human RGS13, RGS16 and β-Actin. The amplified PCR products were then examined by gel electrophoresis. (B) Quantitative, RT-PCR was performed with GAPDH used as an internal reference. The graph shows the mean ± SD of three independent experiments.
Figure 4. SupT1 cells migrate better than Hut78 cells to CXCL12. Chemotaxis of Hut78 and SupT1 cells was assessed by in vitro migration to 1 μg/mL CXCL12. The data represent the percentage of migrated cells compared with the total input cells. The graph shows the mean ± SD of three independent experiments.
Figure 5. Optimization of transfection with varying numbers of cells. With 1 ul Lipofectamine 2000 used as transfection reagent, Block-iT RNA (FITC labeled) at a final concentration of 40 nM was transfected into different numbers of Hut78 cells. Forty-eight hours after transfection, the cells were analyzed using flow cytometry. The percentage of viable cells was determined by forward and side light scatter. The transfection efficiency was determined by comparing FITC positive to the total cells present.
Figure 6. Optimization of transfection with varying concentrations of siRNA duplexes. Using 1 ul Lipofectamine 2000 as transfection reagent, different concentrations of Block-iT siRNA duplexes were transfected to 350,000 Hut78 cells. Forty-eight hours after transfection, the cells were collected for fluorescence analysis using flow cytometry. The percentage of viable cells was determined by forward and side light scatter. The transfection efficiency was determined by comparing FITC positive cells to the total number of cells present.
was determined by comparing FITC positive cells to the total number of cells present.

Figure 7. Optimization of transfection using different transfection reagents and concentrations. Lipofectamine, Lipofectamine 2000 and oligofectamine were used to transfect 40 nM (final concentration) Block-iT siRNA duplexes into 350,000 Hut78 cells. Forty-eight hours after transfection, the cells were collected for fluorescence analysis using flow cytometry. The percentage of viable cells was determined by forward and side light scatter. The transfection efficiency was determined by comparing FITC positive cells to the total number of cells present.
Figure 8. Optimization of transfection with different electroporation programs. A GFP-expressing plasmid was electroporated into Hut78 cells using different programs within the Amaxa Transfection System. Forty-eight hours after transfection, the cells were harvested and analyzed for FITC using a BD FACS Vantage. The percentage of viable cells was determined by forward and side light scatter. The transfection efficiency was determined by comparing the number of FITC positive cells to the total cells present.
Figure 9. Inhibition of RGS13 expression in Hut78 cells doesn’t change their migration to CXCL12. Hut78 cells were transfected with RGS13 specific siRNA or negative control siRNA duplexes. Forty-eight hours after transfection, viable cells were obtained by cell sorting and, (A) analyzed by quantitative RT-PCR for RGS13 expression; (B) assessed by in vitro migration to 1 μg/mL CXCL12 for their migration activity. The percentage represents the proportion of migrated cells compared with the total input cells. The graph shows the mean ± SD of three independent experiments. ND refers to not statistically significant different as determined by Student’s t-test (i.e. p>0.05).
Figure 10. Inhibition of RGS16 expression in Hut78 cells induces increased migration to CXCL12. Hut78 cells were transfected with RGS16 specific siRNA or negative control siRNA duplexes. Forty-eight hours after transfection, viable cells were obtained by cell sorting and, (A) analyzed by quantitative RT-PCR for RGS16 expression; (B) assessed by *in vitro* migration to 1 μg/mL CXCL12 for their migration activity. The percentage represents the proportion of migrated cells compared with the total input cells. The graph shows the mean ± SD of three independent experiments. *P<.01
Figure 11. Optimization of SupT1 electroporation. A GFP-expressing plasmid was transfected (electroporation) into 2,000,000 SupT1 cells with different programs using the Amaxa Nucleofection System. Forty-eight hours after transfection, the cells were harvested and analyzed for FITC using flow cytometry. The percentage of viable cells was determined by forward and side light scatter. The transfection efficiency was determined by comparing the number of FITC positive cells to the total cells present.
Figure 12. Overexpression of RGS13 and RGS16 in SupT1 cells. SupT1 cells were transfected with RGS13, RGS16 clone or control plasmid. Forty-eight hours after transfection, the viable cells were sorted by flow cytometry, and were analyzed by quantitative RT-PCR for RGS13 expression (A) and RGS16 repression (B). GAPDH was used as an internal reference to which RGS expression values were compared. The graphs show the mean ± SD of three independent experiments.
Figure 13. Overexpression of RGS13 and RGS16 in SupT1 cells decreases CXCL12-induced migration. SupT1 cells were transfected with RGS13, RGS16 clone or control plasmid. Forty-eight hours after transfection, the viable cells were obtained by flow cytometry, and their chemotactic ability was assessed by in vitro migration to 1 µg/mL CXCL12. Data represent the percentage of migrated cells compared with total input cells. The graph shows the mean ± SD of three independent experiments. *$P<.01$
**Figure 14. Overexpression of RGS13 and RGS16 in primary CD4+ T cells.** Primary CD4+ T cells were transfected with RGS13, RGS16 clone or control plasmid. Forty-eight hours after transfection, viable cells were obtained by flow cytometry, and were analyzed by quantitative, RT-PCR for RGS13 expression (A) and RGS16 repression (B). GAPDH was used as internal reference. The graphs show the mean ± SD of three independent experiments.
Figure 15. Overexpression of RGS13 and RGS16 in primary CD4+ T cells decreases CXCL12-induced migration. Primary CD4+ T cells were transfected with RGS13, RGS16 clone or control plasmid. Forty-eight hours after transfection, viable cells were obtained by flow cytometry, and their chemotactic ability was assessed by in vitro migration to 1 μg/mL CXCL12. Data represent the percentage of migrated cells compared with total input cells. The graph shows the mean ± SD of three independent experiments. *P<.01
Figure 16. CXCR4 expression on primary CD4+ T cells after transfection. Primary CD4+ T cells were transfected (electroporation) with plasmid DNA for RGS13, RGS16 or control clone. Forty-eight hours after transfection, CXCR4 expression on the transfected cells [RGS13 (a), RGS16 (b) and control (c)] was analyzed by flow cytometry.
Chemotaxis of freshly isolated cells was assessed by in vitro migration to 1 \( \mu \)g/mL CXCL12. The graphs represent the mean ± SD of two independent experiments.

**Figure 17.** CXCR5\textsuperscript{Hi}CD4\textsuperscript{+} blood T cells showed an active response to CXCL12. CXCR5\textsuperscript{Hi} GC T (ICOS\textsuperscript{Hi}CXCR5\textsuperscript{Hi}) cells were obtained by cell sorting from tonsillar CD4\textsuperscript{+} T cells based on CXCR5 and ICOS expression. CXCR5\textsuperscript{Hi} blood T cells were sorted from peripheral blood CD4\textsuperscript{+} T cells based on CD4 and CXCR5 expression. The isolated cells were then analyzed for gene expression and cell migration. (A) RGS13 and RGS16 mRNA expression were analyzed by quantitative RT-PCR for freshly isolated cells. GAPDH was used as internal reference. (B) CXCL12-induced cell migration. Chemotaxis of freshly isolated cells was assessed by *in vitro* migration to 1 \( \mu \)g/mL CXCL12. The graphs represent the mean ± SD of two independent experiments.
Figure 18. HIV DNA in GC T cells and non-GC T cells. GC T (ICOS^{Hi}CXCR5^{Hi}) and non-GC T (ICOS^{Low}CXCR5^{Low}) cells were obtained by cell sorting from secondary lymphoid tissues of three different HIV-infected patients (A. ERBR, B. LN113, C. UCSHC), and HIV DNA in these cells was assessed by quantitative, DNA-PCR using 18s RNA genomic DNA as an endogenous control.
Figure 19. Comparison of RGS13 and RGS16 mRNA expression in Hut78 cells. The displayed data represent the quantitative RT-PCR amplification curves for RGS13, RGS16 and GAPDH in Hut78 cells. Rn, the reporter signal normalized to the Passive Dye (Rox) Reference for a given reaction; Delta Rn, Rn value of the specific gene minus the Rn value for the No Template Control.
Reference List


Chemokine requirements for B cell entry to lymph nodes and Peyer’s patches. *J. Exp. Med.* 196:65–75


51


