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Review

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Role of RGS proteins in regulating the migration of B lymphocytes

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Summary

The migration of B lymphocytes into distinct microenvironments in secondary lymphoid tissues and maintenance of cells in these micro-domains is strictly structured and likely supports the proper regulation of immune responses to both foreign and self-antigens. Chemokines' and other chemoattactants' signals serve as signposts to direct cell migration. They signal cells through heptahelical receptors, which couple to heterotrimeric G proteins (G protein-coupled receptors or GPCRs). The regulation of the signals transduced through these receptors ultimately determines the positioning of cells in lymphoid tissues. A variety of mechanisms regulate GPCR signaling including a family of approximately 25 proteins termed regulators of G protein signaling (RGS). These proteins act as GTPase activating proteins for Gα subunits and can also function as effector antagonists of specific Gα subunits, thereby attenuating signaling through GPCRs such as chemokine receptors. RGS proteins possess some degree of receptor and Gα subunit specificity. Thus, the particular spectrum of RGS proteins and their expression levels within a cell will determine the duration and magnitude of G protein signaling initiated by chemokines. In this review we illustrate the role RGS proteins have in regulating B cell signaling responses to chemoattractant stimuli during homeostasis as well as during an immune response.

Key words: **RGS proteins • chemokine • lymphoid tissue • germinal centers • G protein**

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INTRODUCTION

Cells use G protein-coupled receptors (GPCRs) to transduce signals generated by stimuli such as neurotransmitters, hormones, odorants, light, chemokines, and inflammatory mediators. Several of these GPCRs mediate the movement and differentiation of lymphocytes. Tissue-specific migration and recirculation of lymphocytes and their precursors is a fundamental requirement for the effective distribution of immune cells for surveillance as well as the induction of acquired immunity. It is evident from a plethora of experimental studies that the primary migration of developing lymphocytes into lymphoid tissues, the re-circulation of lymphocytes, as well as the organization of secondary immune structures such as lymphoid follicles and germinal centers results from a complex temporal configuration of adhesion molecules and chemoattractant receptors as well as a spatial overlay of multiple chemoattractant gradients[23, 36, 44, 48, 62]. Mechanisms that allow a cell, such as a B lymphocyte, to interrupt, modify and prioritize signals from such a milieu of stimuli are essential for efficient and normal immune function. Inappropriate interpretations by cells within such a complex environment is deleterious, leading to inappropriate trafficking, survival, and activation of cells. The role of such disruption of these complex paradigms in inflam0al reviews [19, 22, 42, 61]. In this review we document one mechanism of GPCR signaling regulation used by B cells focusing on the regulation of chemokine-mediated signaling during an immune response.

HOW HETEROTRIMERIC G PROTEINS PROMOTE ACTIVATION OF DOWN-STREAM EFFECTORS AND TRIGGER LYMPHOCYTE CHEMOTAXIS

GPCRs such as chemokine receptors transduce signals to down-stream signaling pathways through activation of heterotrimeric G proteins. Extensive and complete reviews of heterotrimeric G protein components and signaling are available[27, 29, 45, 53]. To briefly review, heterotrimeric G protein complexes consist of 3 subunits: α, β, and γ. Each subunit has isoforms: 23α, 5β, and 10γ. The Gα subunit exists independently or in association with the trimeric complex. The Gβ and Gγ subunits exist as either a free heterodimer or as part of the trimeric complex, but they do not exist independently. Although the Gβ and Gγ subunits do not pair indiscriminately, a significant number of distinct heterotrimeric G proteins can exist within a cell. The complex in its inactive form consists of Gβγ associated with the guanosine diphosphate (GDP)-bound Gα subunit. Upon activation of the complex Gα releases GDP and binds guanosine triphosphate (GTP) resulting in its dissociation from Gβγ (Fig. 1). Each active subunit, GTP-bound Gα and free Gβγ, independently activates a limited number of

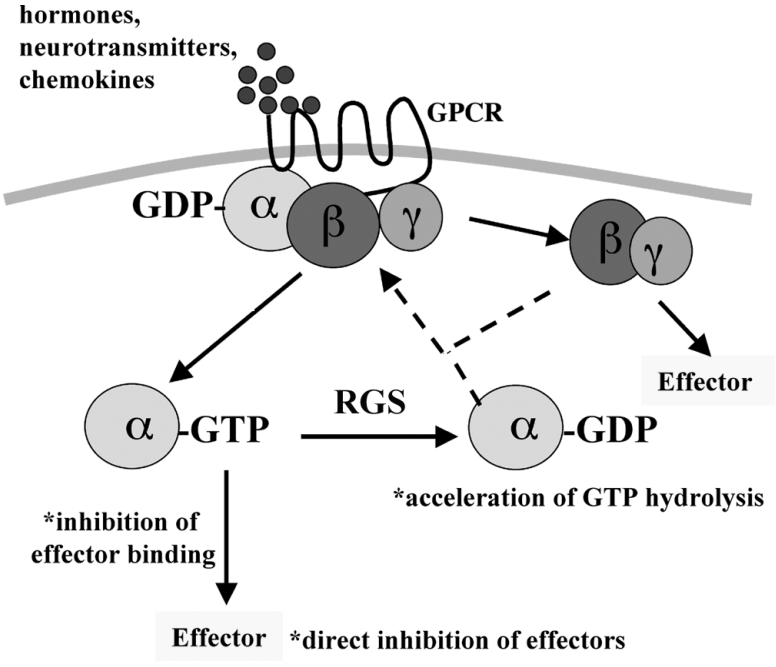

Figure 1. Schematic of GTPase cycle of heterotrimeric G proteins and role of RGS proteins. Receptor activation results in the exchange of GDP for GTP by the Gα subunit and subsequent dissociation of the GTP-Gα subunit from the Gβγ subunit. Each independent G protein subunit activates down-stream effectors, initiating an activation cascade of down-stream signaling molecules. GTP hydrolysis by the intrinsic GTPase activity of Gα results in re-association of GDP-Gα with Gβγ, terminating signaling. RGS proteins can alter the intensity of signaling activation by accelerating the rate of GTP hydrolysis by intrinsic Gα subunit, block interactions between GTP-Gα and effectors, or interact directly with down-stream effectors.

down-stream effectors. The Gα subunits bound to GTP regulate the activity of adenylyl cyclases, several protein kinases, phospholipase Cβγ, and several exchange factors for small GTPases. Free Gβγ subunits regulate the activity of phospholipase Cβγ, src family tyrosine kinases, certain ion channels, and phosphatidylinositol 3 kinase (PI3K)γ. The signal terminates when the intrinsic GTPase activity of Gα hydrolyzes GTP to GDP, which favors its reassociation with Gβγ to form the inactive heterotrimeric complex.

During chemotaxis a chemical gradient serves as a directional signal for cell motility. Directional sensing depends upon chemokine receptor-mediated Giα activation and the release of βγ subunits. Within the signal transduction pathway directional sensing is manifested after G protein activation, but prior to the localized intracellular accumulation of PI(3,4,5)P3, which in-turn leads to localized sites of actin-filled membrane extensions (reviewed in[13]). However, precisely how cells translate a shallow chemoattractant gradient into a sharp intracellular gradient of PI(3,4,5)P3 remains enigmatic. It is likely, that Gβγ signaling reciprocally regulates the activities and/or subcellular localization of PI3Ks, which phosphorylates PI(4,5)P2 and PTEN (tumor suppressor phosphatase and tensin homology deleted on choromosome 10), which dephosphorylates PI(3,4,5)P3 [1, 11, 24, 52, 58, 64, 68]. The generation of actin-filled lamellipodial protrusions also depends upon Rac activation. The lack of DOCK2 (SH3-SH2 adaptor protein dreadlocks), which functions in conjunctions with ELMO1 (hematopoietic cell specific CDM family protein) and/or ELMO2 as an exchange factor for Rac, results in a profound B and T lymphocyte migration defect[25]. In DOCK2$^{-/-}$ lymphocytes, chemokine-induced Rac activation and actin polymerization fails to occur. The generation of PI(3,4,5)P3 is likely linked to the activation of the Rac exchange factor.

MECHANISMS THAT REGULATE GPCR SIGNALING

Several mechanisms regulate the duration and magnitude of GPCR signaling, including those that target the receptor itself, the heterotrimeric G protein, or the immediate down-stream effectors (reviewed in [4, 7, 60, 63]). Many ligand-activated GPCRs undergo phosphorylation by GPCR kinases (GRKs) or effector kinases. This leads to the recruitment of β-arrestin, which interferes with subsequent G protein activation and acts as a scaffolding protein for additional signaling molecules. Receptor de-sensitization may occur due to receptor internalization. A mechanism that directly targets the heterotrimeric G protein, and upon which this review is focused, is mediated by a family of proteins termed regulators of G protein signaling (RGS). These proteins

Table 1. Subclassificaton of RGS proteins

RGS molecular weight	Molecular weight (kDa)	Gα subunit interactions	Domains
Low:			
RGS1	24	$G_{i/o}$, G_q	+++
RGS2	24	$G_{i/o}$, G_q	+++
RGS3S	23	$G_{i/o}$, G_q	+++
RGS4	23	$G_{i/o}$, G_q	+++
RGS5	21	$G_{i/o}$, G_q	+++
RGS8	21	$G_{i/o}$	+++
RGS10	20	G_{i3},G_o,G_z	
RGS13	19	G_i,G_q	+++
RGS16	23	G_q,$G_{i/o}$,G_t	+++
RGS17	22	G_z	
RGS18	28	G_i,G_{i3},G_z	+++
RGS19	25	G_i,G_{i3},G_z	+++ C, PTB
RGS20	23	G_z	
High:			
RGS3L	57	$G_{i/o}$,G_q	A1
PDZ-RGS3	102	$G_{i/o}$,G_q	PDZ, A1
RGS6	60	G_o	DEP, GGL, R7H
RGS7	53	$G_{i/o}$,G_q	DEP, GGL, R7H
RGS9	57	$G_{i/o}$, G_t	DEP, GGL, R7H
RGS11	53	G_o	DEP, GGL, R7H
RGS12	150	$G_{12/13}$,G_{io}	PDZ,PTB, RBD GoLoco, CC
RGS14	59	$G_{12/13}$,$G_{i/o}$	RBD,GoLoco

Domain abbreviations: +++ – amphipathic helix, C – cysteine string, PTB – phosphotyrosine-binding domain, PDZ – PSD-95/Dlg/ZO-1 domain, DEP – disheveled domain, GGL – G protein γ-like domain, R7H – R7 homology domain, RBD – Rap ½-binding domain, GoLoco – Gα i/o-Loco; CC – coiled coil motif, DH – dbl homology domain, PH – pleckstrin, A1 – atrophin-1.

increase the intrinsic GTPase activity of Gα (i.e. act as GTPase-activating proteins or GAPs) as well as interfere with the ability of activated Gα subunit to interact with down-stream effectors (Fig. 1). Genetic studies in yeast, *Caenorhabditis elegans*, and *Aspergillus nidulans* first identified such proteins[14, 35, 39]. Subsequently, a mammalian protein termed GAIP (RGS19) was discovered to interact with a Giα subunit[12], and four mammalian proteins designated RGS1-4 substituted to varying degrees for Sst2p, a yeast protein involved in the desensitization of pheromone signaling[15]. Approximately 25 human RGS proteins have now been identified. When tested in standard GAP assays, most RGS proteins possess GAP activity for the α subunits of the Gi and Gq subfamilies[5, 6, 33, 69]. The co-crystal structure of the rat RGS4 and Giα-GDP-AlF-4 complex indicated that conserved epitopes in the RGS domain stabilize the switch regions of Giα1 in the transition state of GTP hydrolysis,

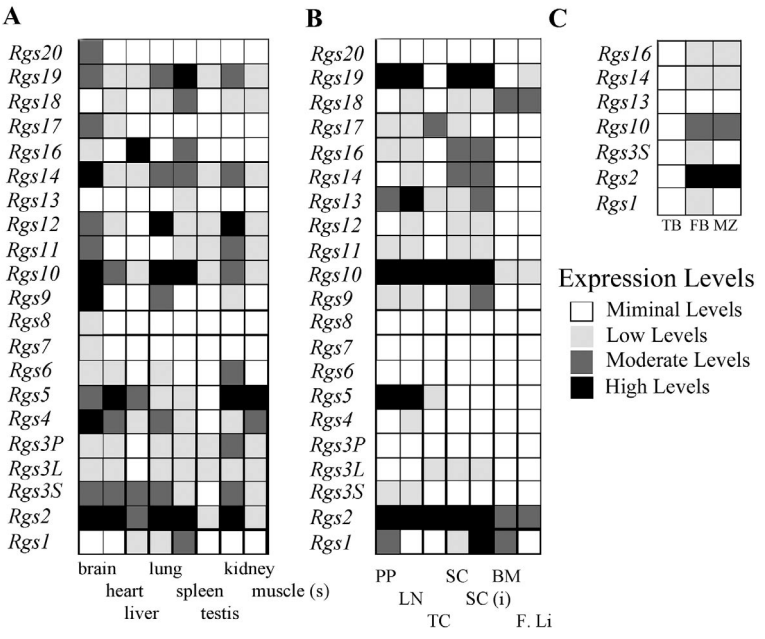

Figure 2. *Rgs* expression in various tissues. Total RNA derived from the indicated tissues was subjected to reverse transcription and amplification by polymerase chain reaction. The amount of amplified product was quantified and expressed as either absent or extremely low (blank), low (light gray), moderate (dark gray), or strong expression (black). **A** – comparison of *Rgs* expression patterns in a global tissue screen, **B** – comparison of *Rgs* expression patterns in lymphoid tissues, **C** – comparison of *Rgs* expression patterns in B cell subsets in spleen. Tissue abbreviations are: PP – Peyer's patch, LN – lymph node, TC – thymocytes, SC – splenocytes, SC (i) – 7 day post immunization B lymphocytes, BM – bone marrow, F. Li – fetal liver lymphocytes, TB – splenic transitional B cells, FB – splenic follicular B cells, MZ – splenic marginal zone B cells.

thereby boosting the intrinsic rate of GTP hydrolysis of the Giα subunit by as much as 50 fold[5, 67].

RGS EXPRESSION PATTERNS

The RGS proteins can be organized based on structural similarities, or simply divided into two general groups based on their molecular mass (Table 1). The smaller RGS proteins (group 1) range in size from 15–25 kDa and consist largely of an RGS domain without other defined domains. These proteins all contain RGS domains that act as GAPs for Giα and/or Gqα. The larger RGS proteins (group 2) consist of 50–150 kDa proteins, which contain an RGS domain and one or more additional domains, which include Dishevelled domains, which may assist in membrane localization; Ras binding domains (RID), which bind small GTPases; G protein γ subunit-like (GGL) domains, which binds Gβ5; PDZ domains, which direct the localization and assembly of multi-protein signaling complexes; and PTB domains, which facilitates protein-protein interactions[10]. These multidomain proteins likely perform more complex functions in cells other than acting as simple GAPs or effector antagonists for cell membrane GPCRs.

The tissue-specific distribution of the RGS proteins varies. Some such as *Rgs19*, *Rgs14*, *Rgs10*, *Rgs2*, and *Rgs3*, are broadly expressed and found in most tissues. Others have much more limited patterns of expression for example *Rgs5* is largely restricted to vascular smooth muscle[9], *Rgs20* is brain-specific[2], and *Rgs13* is predominantly found in germinal-center B cells[65]. Many of the RGS proteins are expressed within the brain, a result consistent with the large number of GPCRs expressed by neurons. Cell activation signals often regulate the expression levels of the group 1 RGS proteins, while the group 2 proteins tend towards a more constitutive pattern of expression.

A summary of the tissue expression of *Rgs1–20* is presented in Fig. 2. Because of some confusion in the nomenclature, no *Rgs15* exists. The levels of expression were determined by RT-PCR of cDNA generated from isolated RNA from tissues. Although this analysis is not quantitative, it presents a general expression pattern within the tested tissues (panel A), including some of the primary and secondary lymphoid tissues (panel B). *Rgs2*, *Rgs10*, and *Rgs19* are strongly expressed in secondary lymphoid tissues. The GGL domain containing RGS proteins, encoded by *Rgs6*, *Rgs7*, *Rgs9*, and *Rgs11*, tend

to be poorly expressed in lymphoid tissues although low levels of *Rgs9* and *Rgs11* can be detected. *Rgs8* and *Rgs20* were not found in the immune tissues tested, and *Rgs3* and *Rgs4* at very low levels or not at all. *Rgs5* expression in lymphoid tissues likely represents blood vessel expression. *Rgs18* is well expressed in fetal liver and bone marrow, likely reflecting its high levels in megakaryocytes. *Rgs1* and *Rgs13* are found in splenocytes and upregulated following immunization. *Rgs14* is also found at relatively high levels in splenocytes.

However, this overall view does not provide information specific to individual B cell developmental subsets or differentiation stages, some of which are shown in panel C. Nothing is know about the expression of RGS proteins in developing B cells in fetal liver and there is limited information on developing B cells in the bone marrow. Low levels of *Rgs1*, *Rgs13*, *Rgs14*, and *Rgs18* can be detected by RT-PCR in bone marrow-derived immature B cells (Han and Kehrl, unpublished observation). B cell subpopulations in the spleen consist of transitional, follicular and marginal zone B cells. Analysis of RGS protein mRNA expression indicated that transitional B cells are relatively deficient in their RGS proteins profile. In contrast, follicular and marginal zone B cells express a rich variety of RGS proteins. *Rgs2* and *Rgs10* are both well expressed, while *Rgs16* is weakly present in follicular and marginal zone cells. *Rgs1* is expressed at moderate levels in follicular B cells, but only weakly so in marginal zone B cells. *Rgs3* has a similar pattern. *Rgs13* is not detected in these B cell subsets, being largely restricted to germinal- center B cells[65]. In addition, germinal-center B cells strongly express *Rgs1* and based on germinal-center-derived ESTs also *Rgs10*, *Rgs18*, and *Rgs19*. Expression of *Rgs1* and *Rgs10* in plasma cells has been reported[28]. Further studies are needed to fully document the changes in RGS protein expression that occur during B lymphocyte development and maturation in peripheral tissues as well as following stimulation with various ligands.

RGS PROTEINS MODULATE THE RESPONSE OF B LYMPHOCYTES TO CHEMOATTRACTANTS

By virtue of their GAP ability for Giα, the presence of an RGS protein will shorten the duration that the Giα subunit remains GTP bound following ligand stimulation. This in-turn facilitates the recombination of the Gα subunits with free Gβγ subunits to reconstitute the inactive heterotrimer and thereby limit the duration that Gβγ subunits activate down-stream effectors in the signaling pathway. Gβγ subunits released from GTP-bound Giα stimulate phospholipase Cβ activity, triggering a rapid although short-lived increase in intracellular Ca^{+2} levels. CXCL12, a chemokine needed for normal B cell development and proper plasma cell trafficking, transiently increases intracellular Ca^{+2} levels in primary B cells and many B cell lines. The introduction of RGS1 into a human B cell significantly reduced the magnitude and duration of CXCL12-induced increase in intracellular Ca^{+2} [50]. Conversely, RGS1-deficient mouse B cells have an enhanced Ca^{+2} flux following exposure to CXCL12 compared with wild-type mouse B cells[49]. Not only is the magnitude of the Ca^{+2} response enhanced, the duration of the signal is prolonged. Besides Ca^{+2} signaling, CXCL12-induced mitogen-activated protein kinase (MAPK) activation is also significantly attenuated in RGS1-overexpressing cells compared with control cells. Mirroring the changes in proximal signaling, a significantly lower percentage of the RGS1- transfected B cells migrate in standard transwell chemotaxis assays than do control cells while a higher percentage of the *Rgs1*$^{-/-}$ B cells migrate compared with wild-type B cells. However, the dose response curve, percemtage of cells migrating versus concentration of chemokine, is not significantly changed. A higher concentration of chemo-kine cannot overcome the effects of RGS1, nor are the *Rgs1*$^{-/-}$ cells supersensitive to chemokine. The failure to observe a clear shift in the dose response curve may be accounted for by a threshold effect and the varying concentrations of RGS1 present in the B cell populations.

A general property of GPCR-initiated signaling pathways is that prolonged stimulation decreases responsiveness, i.e. desensitization. While a major role has been ascribed for receptor phosphorylation and arrestin binding in chemokine receptor desensitization, the recruitment of an RGS protein to the vicinity of the ligand-activated receptor/G protein complex provides an additional mechanism to desensitize the signaling pathway. Several studies have demonstrated that GPCR signaling can alter the intracellular localization of certain RGS proteins, resulting in their recruitment to the plasma membrane[16, 17]. The availability of *Rgs1*$^{-/-}$ B cells allowed a direct test of the role of RGS1 in the desensization of the CXCR4 and CXCR5 signaling pathways[49]. The re-exposure of wild-type B cells to CXCL12 or CXCL13 shortly after a primary exposure led to a significantly reduced Ca^{+2} flux; however, a similar experiment, but with *Rgs1*$^{-/-}$ B cells, revealed little or no attenuation of the Ca^{+2} flux on the second exposure. Thus, *Rgs1* plays an important role in the ability of B cells to desensitize the CXCL12 and CXCL13 signaling pathways.

RGS PROTEINS CONTRIBUTE TO THE APPROPRIATE MIGRATION OF B LYMPHOCYTES

B cells and their precursors originate in the omentum and fetal liver during fetal ontological development and later (neonatal and adult) in the bone marrow[26, 30, 34, 47, 66]. From these sites of origin, B lineage cells migrate to sec-

ondary lymphoid tissues such as spleen, lymph nodes, gut-associated tissues, bronchus associated tissue and non-lymphoid sites such as skin. Antigen stimulation further alters B cell trafficking, resulting in the development of germinal centers, the movement of antigen-activated B cells from mucosal sites to draining lymph nodes, and the eventual targeting of antibody-forming cells to bone marrow and lamia propria. Multiple chemokines have been shown to have a role in the recruitment of cells to primary lymphoid tissues and their ontological organization[21, 23, 32, 40, 43, 51]. B cell trafficking between secondary lymphoid tissues is also dependent on chemokine gradients, as well as expression patterns of integrins and selectins (reviewed in [18, 41, 54, 55, 57, 59, 61, 70]).

Rgs4, *Rgs5*, *Rgs6-9*, *Rgs11*, *Rgs12*, *Rgs17*, and *Rgs20* are poorly expressed or not at all in B lymphocytes, therefore, unlikely to influence B cell migration. Although present in B cells, RGS2 largely affects Gq signaling, thereby making it an unlikely candidate to regulate chemokine signaling. B lymphocytes contain significant amounts of *Rgs19*. RGS19, also termed GAIP, functions as an essential part of the membrane budding machinery for a subset of vesicles derived from the trans-Golgi network, but in addition it localizes at the plasma membrane, where it may affect chemokine receptor signaling and/or receptor endocytosis[71]. Further study of RGS19 in B lymphocytes is warranted. *Rgs10* may also serve as an important regulator of chemokine signaling in marginal zone and splenic plasma cells; however, no functional studies relevant to immune cells have been reported. Both RGS3 and RGS18 potently inhibit chemokine receptor signaling when introduced into cell lines, however, their low expression level in most types of B cells likely preclude them from having a significant role[8] (Shi and Kehrl, unpublished observation). Mature B cells also express *Rgs16*, although mature $CD4^+$ T cells express higher levels[3, 20]. Functional studies of RGS16 on chemokine receptor signaling also have not been reported. Although B lymphocytes express significant levels of *Rgs14*, much of RGS14 localizes in centrosomes (Cho and Kehrl, unpublished data). Since microtubule minus-end capture and release from the centrosome is critical for efficient cell migration, RGS14 could contribute indirectly to B lymphocyte chemotaxis. The two RGS proteins most likely to regulate B cell chemotaxis are RGS1 and RGS13.

Based on its expression pattern and rapid upregulation following antigen stimulation, RGS1 might be expected to alter chemokine signaling following antigen activation rather than during the development and initial organization of immune tissues. In fact, while disruption of *Rgs1* does not perturb B cell development or B cell homeostatic positioning, $Rgs1^{-/-}$ immunized mice have significant defects in B cell trafficking[49]. Immunization with many antigens causes the formation of germinal centers in B cell follicles. Antigen-activated B and T lymphocytes along with follicular dendritic cells localize in the primary lymphoid follicle to form a cluster of blast–like cells. This cluster eventually organizes into separate regions termed the dark zone, which contains centroblasts that proliferate and whose Ig genes undergo somatic hypermutation, and the light zone, which contains centrocytes that undergo selection for high-affinity antigen receptors. In order to acquire Ig receptors with the highest affinities B cells likely need to recirculate between the two zones. B cells emerge from the germinal-center as either memory cells or plasma cell precursors[40, 46, 56]. As indicated above germinal-center B cells prominently express *Rgs1* and *Rgs13*. Why germinal-center B cells should deploy both *Rgs13* and *Rgs1* is unknown although their expression levels respond differently to CD40 and antigen receptor signaling. Both proteins have similar *in vitro* GAP activities toward Gi and Gq and nearly similar abilities to inhibit CXCL12 and CXCL13 induced signaling. While their expression patterns overlap in the germinal-center, individual cells may preferentially express one versus the other. Antigen receptor signaling preferentially induces *Rgs1* expression, while CD40 signaling preferentially triggers *Rgs13*[52, 65, 69]. $Rgs1^{-/-}$ mice form and dismantle germinal centers aberrantly, which results in persistent germinal centers in these mice. However, affinity maturation of the humoral immune response seems largely intact[49]. Base on the restricted expression pattern of *Rgs13* in the germinal-center, a more compelling phenotype may arise in $Rgs13^{-/-}$ mice or in mice deficient for both *Rgs1* and *Rgs13*. Unfortunately, because of the close proximity of *Rgs1* and *Rgs13* on chromosome 1, the interbreeding of $Rgs1^{-/-}$ and $Rgs13^{-/-}$ mice to generate the double knock-out will not be possible.

The ultimate destiny of some antigen-activated B cells is to differentiate into antibody- secreting plasma cells. In contrast to mature B cells, these cells do not recirculate, but lodge in the splenic red pulp, lymph node medullary cords, lamia proprina, or bone marrow. IgG and IgM plasma cell precursors exhibit a high degree of sensitivity to CXCL12, while IgA precursors respond well to mucosal epithelial chemokine (MEC), a ligand for CCR10[31, 36–38]. Furthermore, CXCL12 and MEC-producing cells reside at the sites where IgG and IgA plasma cells accumulate. While the generation of plasma cell precursors in the $Rgs1^{-/-}$ mice appears normal, IgG-secreting plasma cells fail to appropriately localize in the bone marrow and IgA-secreting plasma cells do not localize in the lamina propria[49]. As a result following immunization there is accumulation of plasma cell pre-

cursors in the peripheral blood. Further studies are needed to resolve whether this represents a failure of the cells to enter the target tissue or to remain lodged there.

CONCLUSIONS

While it is evident that RGS1 has a role in the regulation of B cell chemotaxis, the importance of the other RGS proteins found in B cells awaits the analysis of mice deficient in one or more of them. RGS2$^{-/-}$ mice apparently have no defect in lymphoid chemotaxis. Besides acting as stop signals, RGS proteins likely have additional functional roles in B cell chemotaxis. For example, in multiple overlapping chemoattractant gradients neutrophils use a step-by-step navigation method, whereby they migrate towards a primary source, desensitize, and then migrate towards a secondary source. Presumably, B cells use a similar strategy and RGS proteins may contribute to the desensitization mechanism that allows the cells to turn away from the primary gradient. This would suggest some degree of receptor specificity or the ability to selectively recruit an RGS protein to an activated receptor/G protein complex. Complicating the study of RGS proteins, post-translational modifications can alter their intracellular localization as well as their GAP activity. Signaling through other pathways for example via the B cell antigen receptor or CD40 may trigger these post-translational modifications and/or directly impinge on RGS protein expression. Thus, providing a mechanism whereby signaling through these receptors can alter the responses to chemoattractants.

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