Genes and Immunity (2016) 17, 228–238

www.nature.com/gene

ORIGINAL ARTICLE

The autoimmunity-associated gene *RGS1* affects the frequency of T follicular helper cells

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RGS1 (*regulator of G-protein signaling 1*) has been associated with multiple autoimmune disorders including type I diabetes. *RGS1* desensitizes the chemokine receptors CCR7 and CXCR4 that are critical to the localization of T and B cells in lymphoid organs. To explore how *RGS1* variation contributes to autoimmunity, we generated *Rgs1* knockdown (KD) mice in the nonobese diabetic (NOD) model for type I diabetes. We found that *Rgs1* KD increased the size of germinal centers, but decreased the frequency of T follicular helper (T_{FH}) cells. We show that loss of *Rgs1* in T cells had both a T cell-intrinsic effect on migration and T_{FH} cell frequency, and an indirect effect on B-cell migration and germinal center formation. Notably, several recent publications described an increase in circulating T_{FH} cells in patients with type I diabetes, suggesting this cell population is involved in pathogenesis. Though *Rgs1* KD was insufficient to alter diabetes frequency in the NOD model, our findings raise the possibility that *RGS1* plays a role in autoimmunity owing to its function in T_{FH} cells. This mechanistic link, although speculative at this time, would lend support to the notion that T_{FH} cells are key participants in autoimmunity and could explain the association of *RGS1* with several immune-mediated diseases.

Genes and Immunity (2016) **17,** 228–238; doi:10.1038/gene.2016.16; published online 31 March 2016

INTRODUCTION

RGS1, a member of the regulator of G-protein signaling (RGS) family,[1] has been associated with multiple immune-mediated diseases.[2–5] Data from genome-wide association studies show a significant association of single-nucleotide polymorphisms in the *RGS1* region with multiple sclerosis and celiac disease[2,3] ($P < 10^{-17}$), and a suggestive association with type I diabetes.[4,5] RGS proteins are GTPase-activating proteins that modulate chemokine receptor signaling.[1] Chemokine receptors depend on heterotrimeric G proteins to activate downstream effectors.[6] Upon ligand activation, the G-protein α-subunit (Gα) exchanges guanosine triphosphate for guanosine diphosphate, resulting in dissociation from the Gβγ heterodimer[7] and initiating signaling cascades that lead to cytoskeletal rearrangements and cell migration. Hydrolysis of guanosine triphosphate by Gα intrinsic GTPase activity causes signal termination. This enzymatic activity is accelerated by RGS-family proteins.[1] *RGS1* is highly expressed in lymphoid organs and serves as a negative regulator of chemokine receptor signaling in lymphocytes.[1,8] Ablation of *Rgs1* in mice was shown to modify B-cell trafficking.[9] In addition, *Rgs1* deficiency leads to aberrant architecture of germinal centers.[9–11] Although the phenotype described for *Rgs1* knockout (KO) mice was largely attributed to B-cell dysfunction, a subsequent study found that *Rgs1* also participates in chemotactic signaling in T cells.[12] *Rgs1* thus affects the migratory behavior of multiple cell types, and it is as yet unclear how *RGS1* gene variation modifies the risk of autoimmunity, and of type I diabetes in particular.

T follicular helper (T_{FH}) cells reside in the follicular areas of secondary lymphoid organs where they promote B-cell expansion and antibody affinity maturation within germinal centers.[13] T_{FH} cell maturation is a multistep process that begins in the T-cell zone with the activation of naive $CD4^+$ T lymphocytes and leads to expression of the transcription factor *Bcl6*. *Bcl6* drives the expression of the chemokine receptor CXCR5 that promotes migration from the T-cell zone toward the B-cell follicle.[14] This migration also requires downregulation of CCR7 signaling.[15] Of interest, *Rgs1* expression is markedly upregulated in T_{FH} cells,[16] and this likely contributes to desensitizing migrating cells to CCR7 ligands. Notably, several studies have recently implicated T_{FH} cells in type I diabetes.[17–19] The frequency of T_{FH} cells was found to be elevated in patients with type I diabetes. A similar increase in T_{FH} cells was observed in a mouse model for autoimmune diabetes.[19]

To investigate a possible role for *RGS1* in autoimmunity, we developed inducible *Rgs1* knockdown (KD) mice within the nonobese diabetic (NOD) mouse model for type I diabetes.[20] *Rgs1* silencing recapitulated key phenotypes described for *Rgs1* KO mice,[9] including increased lymphocyte chemotaxis and enlarged germinal centers. Although we found that *Rgs1* KD did not alter the risk of diabetes in NOD mice, we observed that loss of *Rgs1* reduced the frequency of T_{FH} cells. Furthermore, *Rgs1* KD in T cells was sufficient to modify the migration of B cells. These findings suggest that the effects of *Rgs1* KO on germinal center formation described previously may be caused in part by changes in T_{FH} cell function. In addition, our data suggest that *Rgs1* upregulation is a critical step in the migration of T_{FH} cells that enables cells to downregulate CCR7 signals and to migrate into the follicular area. A link between *Rgs1* expression and T_{FH} cell frequency, a T-cell subset implicated in type I diabetes, could explain the association of *RGS1* variants with autoimmunity.

RESULTS

Generation of *Rgs1* KD NOD mice

To study the role of *Rgs1* in autoimmune diabetes, we generated transgenic NOD mice in which *Rgs1* gene expression can be silenced by RNA interference in a doxycycline-dependent manner.[21] We first validated lentiviral constructs for *Rgs1* KD

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Received 7 January 2016; revised 19 February 2016; accepted 25 February 2016; published online 31 March 2016

in vitro. Candidate short-hairpin RNA (shRNA) sequences were cloned into the lentiviral vector pUTG[21,22] in which shRNA expression is under the control of a doxycycline-inducible promoter. The vector also contains a constitutively expressed tetracycline repressor and a green fluorescent protein (GFP) reporter. We generated an *Rgs1* luciferase reporter where *Rgs1* complementary DNA is incorporated into the 3′ untranslated region of the *Renilla* luciferase gene. We transfected the *Rgs1* luciferase reporter into HEK293 cells transduced with lentivirus encoding different shRNA sequences against *Rgs1*, and quantified *Renilla* luciferase activity as a measure of gene knockdown. We identified two shRNA sequences that potently inhibited the *Rgs1* luciferase reporter (Figure 1a). These shRNA sequences were further validated for their ability to silence expression of a FLAG-tagged *Rgs1* construct, as measured by quantitative PCR (Figure 1b) and western blotting (Figures 1c and d). The selected

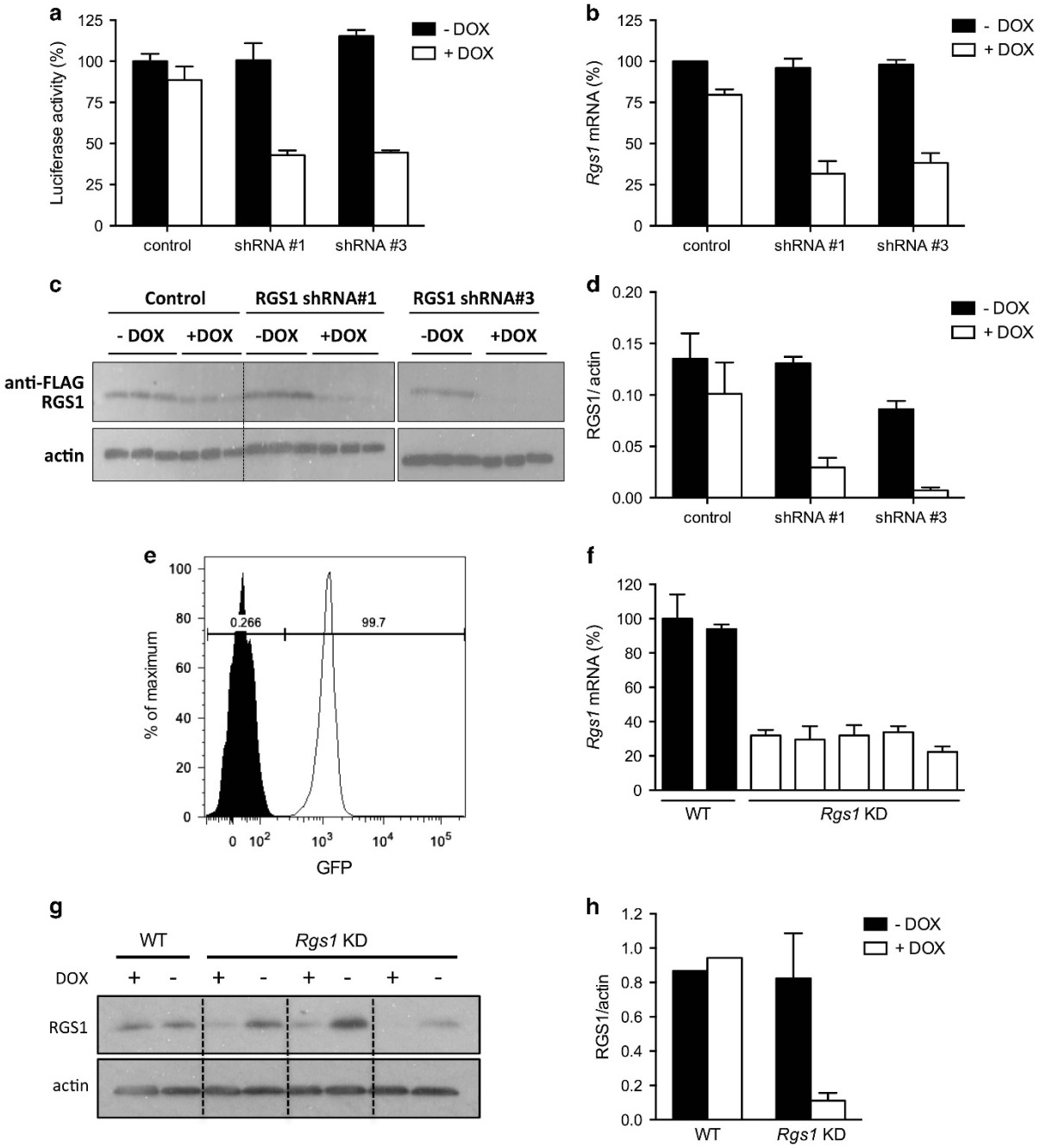


Figure 1. Generation and validation of NOD *Rgs1* KD mice. (**a–d**) HEK293 cells were transduced with lentivirus encoding *Rgs1* shRNA sequence #1 or #3 under the control of a doxycycline-inducible promoter. *Rgs1* KD or control cells were transfected with the *Rgs1* luciferase reporter (**a**) or with an expression vector containing a FLAG-tagged version of the RGS1 protein (**b–d**). *Rgs1* knockdown efficiency was then analyzed by luminescence measurement (**a**), quantitative PCR (**b**) or western blotting (**c**, **d**). Results in (**a**–**d**) are representative of three experiments. (**e**) GFP expression in blood samples from *Rgs1* KD mice. Representative histograms for *Rgs1* KD (white histogram) and WT mice (black histogram) are shown. (**f–h**) Validation of *Rgs1* silencing *in vivo*. Quantitative PCR (**f**) or western blotting (**g**, **h**) was performed with spleen mRNA or protein, respectively, from individual *Rgs1* KD (white bars) and WT mice (black bars). Rgs1 protein levels were compared in mice treated or not with doxycycline. Results in (**f–h)** are derived from three to five biological replicates and are representative of two experiments. All results show mean values ± s.e.m.

shRNA sequences were then used to generate two distinct *Rgs1* KD NOD lines by lentiviral transgenesis (Figure 1e and Supplementary Figure S1). Finally, we confirmed that doxycycline treatment (100 μg ml^{-1} in the drinking water for 2 weeks) induced *Rgs1* KD *in vivo* (Figures 1f–h and Supplementary Figure S1).

Rgs1 silencing sensitizes T cells to CCR7 ligation

Rgs1 modulates CCR7 signaling by promoting the conversion of the guanosine triphosphate-activated Gα subunit to a guanosine diphosphate-quiescent form that results in reassembly of the heterotrimeric G-protein complex,[1] thus terminating chemokine receptor signaling. Accordingly, *Rgs1* KO was shown to sensitize lymphocytes to CCR7 stimuli.[11,12] We tested the response of *Rgs1* KD T lymphocytes to CCR7 chemokine ligands in a transwell assay. Consistent with a role for *Rgs1* in diminishing CCR7 signaling, *Rgs1* KD increased T-cell migration toward CCL19 (Figure 2a) and CCL21 (Figure 2b). The chemotactic response to the CXCR4 ligand CXCL12 was also affected, though only modestly (Figure 2c). Similar results were obtained with *Rgs1* KD B cells (Figure 2d).

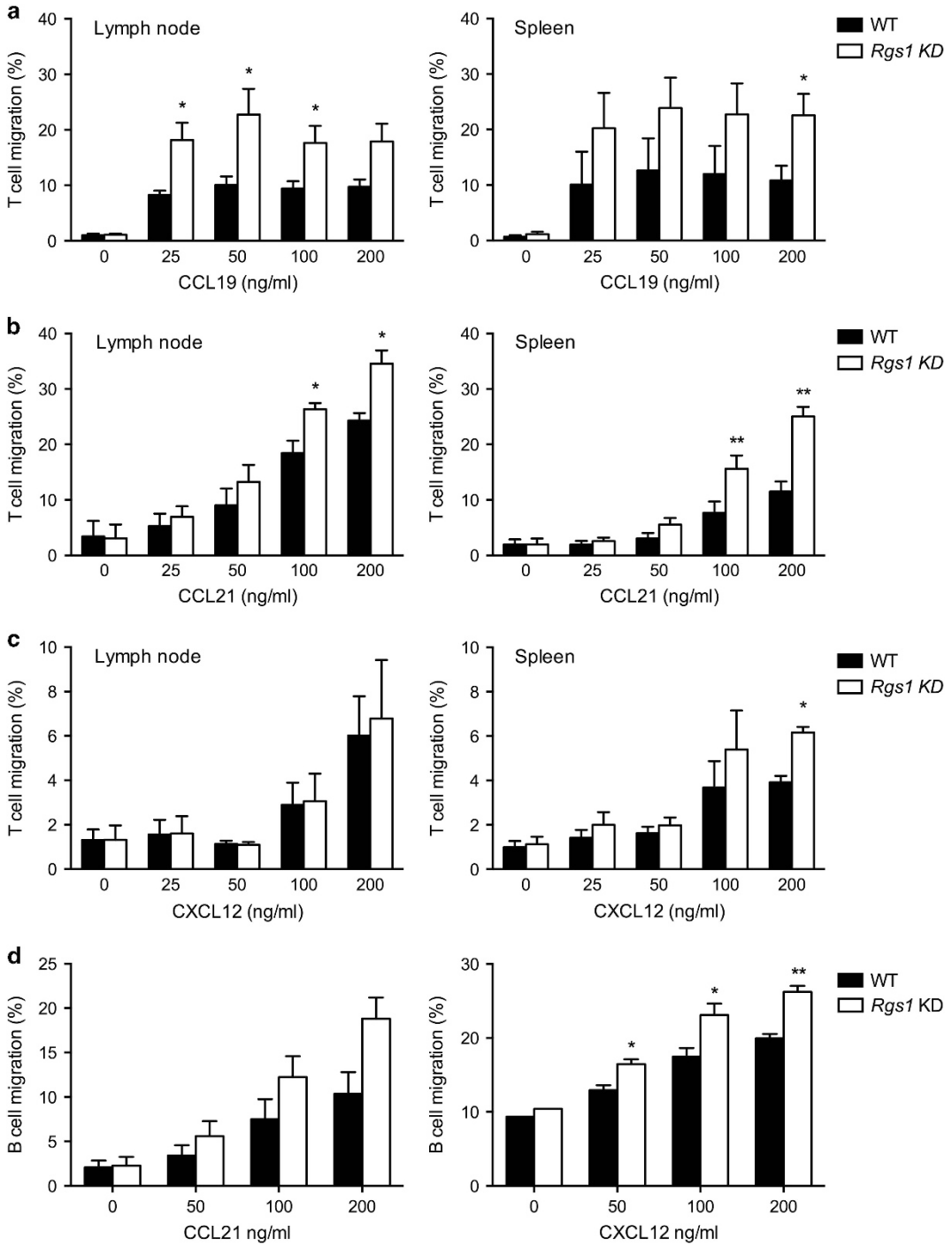


Figure 2. *Rgs1* KD sensitizes T cells to CCR7 and CXCR4 stimulation. Migration of T cells from the spleen or lymph nodes (**a–c**) or splenic B cells (**d**) from WT (black) and *Rgs1* KD (white) in response to CCL19 (**a**), CCL21 (**b**, **d**) or CXCL12 (**c**, **d**). Data represents mean ± s.e.m. of three biological replicates. *$P < 0.05$, **$P < 0.01$ (unpaired *t*-test).

These data are consistent with a previous report showing that *Rgs1* KO sensitized T cells to CCR7 and CXCR4 ligation.[12] We concluded that *Rgs1* KD modifies lymphocyte chemotaxis *in vitro* in a manner consistent with observations made with *Rgs1* KO cells.

Rgs1 KD protects against colitis, but does not change the risk of autoimmune diabetes

A study by Hayday and colleagues[12] reported that T cells derived from *Rgs1* KO mice were less pathogenic than their wild-type (WT) counterparts in an experimental colitis model based on the transfer of $CD4^+CD45RB^{high}$ T cells into immunodeficient mice. The authors speculated that the absence of *Rgs1* increases the propensity of $CD4^+$ T cells to home to secondary lymphoid organs, reducing their dwell time in the gastrointestinal tract and diminishing colitis severity. To test whether *Rgs1* KD would recapitulate this phenotype, we transferred sorted $CD4^+$ $CD45RB^{high}$ T cells from *Rgs1* KD or WT mice into NOD.SCID recipient mice. The results of this experiment suggested that *Rgs1* KD T cells were less colitogenic than WT cells. Colons were lighter and longer, and histological scores lower in mice transplanted with *Rgs1* KD cells (Supplementary Figure S2). With an indication that *Rgs1* KD was able to alter T-cell function *in vivo*, we proceeded to ask whether *Rgs1* silencing would affect the development of autoimmune diabetes in NOD mice. However, we found no difference in the frequency of spontaneous diabetes between WT and *Rgs1* KD mice, whether treated or not with doxycycline (Figure 3a). In addition, diabetes onset was tested by two alternative approaches: by administering cyclophosphamide (Figure 3b) that is thought to disproportionally deplete the regulatory T-cell population, leading to a breakdown in immune regulation,[23] and by transfer of splenocytes from overtly diabetic *Rgs1* KD mice into immunodeficient NOD.SCID mice (Figure 3c). Both approaches indicated again that *Rgs1* silencing was insufficient to alter the risk of diabetes in the NOD model.

Rgs1 silencing affects the differentiation of T_{FH} cells

To further dissect the role of *Rgs1*, we explored its function within secondary lymphoid organs where *Rgs1* may fine-tune local migratory signals. Reports from *Rgs1* KO mice previously linked loss of *Rgs1* to aberrant splenic architecture, characterized by enlarged germinal centers.[9,10] This observation was replicated in *Rgs1* KD mice: transgenic mice treated with doxycycline had significantly larger germinal centers after immunization (Figures 4a and b). Accordingly, both the frequency and absolute number of IgD^{lo} B cells were increased by *Rgs1* silencing (Figure 4c). Although the proportion of proliferating (Ki-67^+) IgD^{lo} cells was not affected by *Rgs1* KD, transgenic mice harbored more proliferating B cells overall (Figures 4d and e). When we analyzed different T-cell populations in lymph nodes collected from immunized mice, we found that the frequency of T_{FH} cells, characterized as $CD4^+PD1^{high}CXCR5^{high}$ cells, was diminished in *Rgs1* KD mice (Figures 5a–c). Further analysis of the T_{FR} population, the regulatory subpopulation of follicular T cells that expresses the transcription factor *Foxp3*,[16] suggested that the frequency of T_{FR} cells may be elevated (Figure 5d), though this difference did not reach statistical significance ($P = 0.07$) and the absolute number of T_{FR} cells was instead decreased by *Rgs1* KD (Figure 5e) similarly to the number of T_{FH} cells. Although *Rgs1* KD T_{FH} cells were reduced in numbers, they resembled WT T_{FH} cells in their expression of ICOS (Inducible T-cell COStimulator) (Figure 5f) and Bcl6 (Figure 5g). Immunohistochemical staining further confirmed that the numbers of $CD4^+$ T cells within germinal centers were reduced in *Rgs1* KD animals (Figure 5h). Collectively, these data show

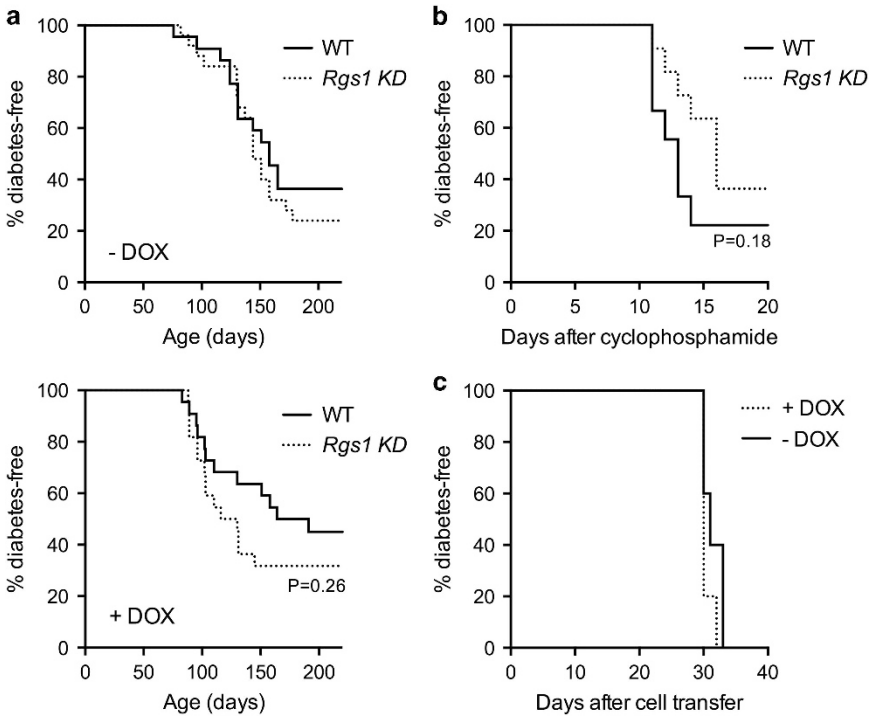


Figure 3. *Rgs1* KD does not change the risk of autoimmune diabetes in the NOD model. (**a**) Spontaneous diabetes incidence in WT and *Rgs1* KD mice treated (bottom panel, $P = 0.26$) or not (top panel, $P = 0.42$) with doxycycline continuously from birth ($n = 22$ mice per group). None of the differences between genotypes or between treated and untreated groups were statistically significant. (**b**) Cyclophosphamide-accelerated diabetes in WT and *Rgs1* KD mice treated with doxycycline for the duration of the experiment ($n = 7$ mice per group, $P = 0.18$). (**c**) Diabetes in NOD.SCID mice ($n = 5$ per group, $P = 0.17$) treated or not with doxycycline following transfer of splenocytes from an overtly diabetic *Rgs1* KD NOD mouse not treated with doxycycline.

that *Rgs1* KD modifies germinal center formation, as shown previously, and indicate that loss of *Rgs1* reduces the frequency of follicular T cells.

Rgs1 KD increases B-cell migration in a B cell-extrinsic manner

Data from the *Rgs1* KO mouse model had demonstrated that *Rgs1*-dependent chemokine signals modify germinal center formation.[9–11] The increase in germinal centers in the absence of *Rgs1* had been attributed to a B cell-intrinsic effect. We sought to investigate whether this phenotype is also influenced by *Rgs1* function in T lymphocytes. To this end, we transferred B cells from NOD *Raspberry* mice that express the mRaspberry fluorescent reporter[24] into WT or *Rgs1* KD mice. Migration of *Raspberry*$^+$ B cells into the spleen was analyzed by flow cytometry 4 days later. As observed in previous experiments, T_{FH} cell frequency and number was decreased in the spleen of *Rgs1* KD mice (Figures 6a and b). Notably, transferred *Raspberry*$^+$ B cells were more numerous in the spleen of *Rgs1* KD recipient animals (Figures 6c

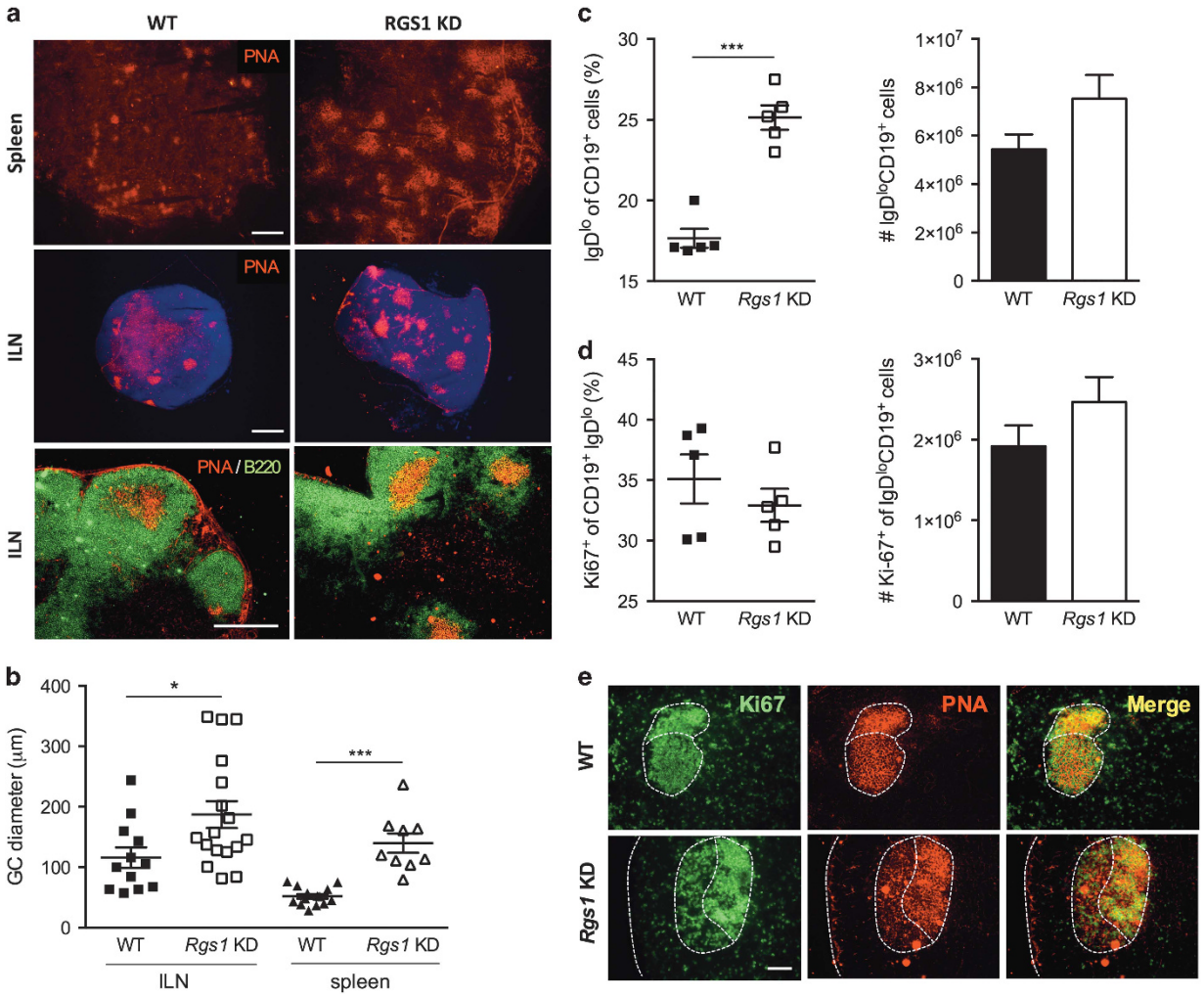
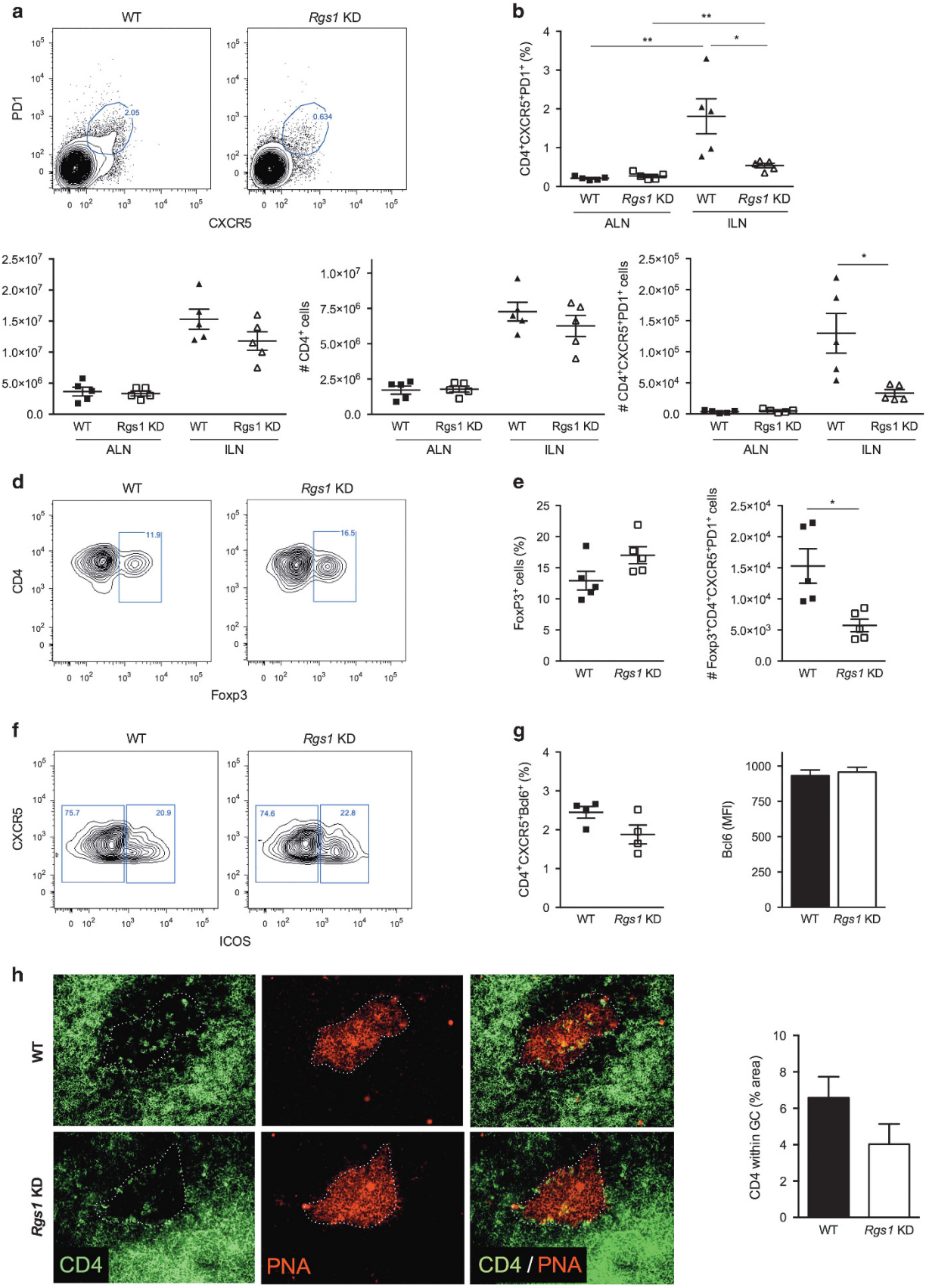


Figure 4. *Rgs1* KD alters germinal center formation. WT and *Rgs1* KD mice were treated with doxycycline and immunized subcutaneously with ovalbumin in complete Freund's adjuvant (CFA). The draining inguinal lymph node (ILN), non-draining axillary lymph nodes (ALNs) and spleen were analyzed 4 days later. (**a**) Representative images of germinal centers stained with peanut agglutinin (PNA) in the spleen (top) and ILN (middle) of WT and *Rgs1* KD mice. The bottom panels show co-staining of PNA with B220 to control for specificity of PNA staining in B-cell areas. Scale bar: 200 µm. (**b**) Quantification of the area of germinal centers in three (spleen) and five (ILN) mice per group. (**c**, **d**) Frequency and absolute number of IgD^{lo} B cells (**c**) and of Ki-67$^+$ cells within the IgD^{lo} population (**d**) in immunized mice ($n = 5$ mice per group). (**e**) ILNs were stained for PNA and Ki-67 to visualize proliferating B cells within germinal centers. Scale bar: 100 µm. *$P < 0.05$, ***$P < 0.001$ (unpaired *t*-test).

Figure 5. *Rgs1* KD decreases the frequency of T_{FH} cells. Representative flow cytometry results (**a**), frequency (**b**) and number (**c**) of CD4$^+$ PD-1$^+$CXCR5$^+$ T_{FH} cells in immunized WT and *Rgs1* KD mice. Cells shown in (**a**) are gated on the CD4$^+$ population. (**d**, **e**) Representative flow cytometry results (**d**) and frequency (**e**) of FoxP3$^+$-expressing cells within the CD4$^+$PD-1$^+$CXCR5$^+$ population gated as shown in (**a**). (**f**) Representative flow cytometry results for ICOS expression within the CD4$^+$PD-1$^+$CXCR5$^+$ population as gated in (**a**). (**g**) Frequency of CD4$^+$ CXCR5$^+$Bcl6$^+$ cells and mean fluorescence intensity (MFI) of Bcl6 staining within this population in immunized WT and *Rgs1* KD mice. (**h**) Spleen sections were stained with PNA and anti-CD4 to quantify CD4$^+$ T cells within germinal centers of immunized WT and *Rgs1* KD mice. Representative images and summary data for five WT and three *Rgs1* KD mice are shown. Results are representative of two or more experiments. All data show means ± s.e.m., *$P < 0.05$, **$P < 0.01$ (unpaired *t*-test).


a
WT
Rgs1 KD
PD1
CXCR5
2.05
0.634
b
CD4+CXCR5+PD1+ (%)
WT
Rgs1 KD
ALN
ILN
c
total cells in LN
CD4+ cells
CD4+CXCR5+PD1+ cells
d
CD4
Foxp3
11.9
16.5
e
FoxP3+ cells (%)
Foxp3+CD4+CXCR5+PD1+ cells
f
CXCR5
ICOS
75.7
20.9
74.6
22.8
g
CD4+CXCR5+Bcl6+ (%)
Bcl6 (MFI)
h
CD4
PNA
CD4 / PNA
CD4 within GC (% area)

and d), suggesting that loss of *Rgs1* leads to active recruitment and/or retention of circulating B cells into secondary lymphoid organs owing to B cell-extrinsic effects.

Rgs1 deficiency in T cells modifies B-cell migration

In order to investigate whether *Rgs1* KD in T cells alone can influence the migratory behavior of B cells, we co-transferred B and T cells from WT and *Rgs1* KD mice into NOD.SCID mice in all four possible combinations (Supplementary Figure S3). Mice reconstituted with *Rgs1* KD T cells had fewer T_{FH} cells (Figures 7a and b) irrespective of the genotype of co-transferred B cells, indicating that the effect of *Rgs1* KD on T_{FH} cell frequency is T-cell intrinsic. The frequency and number of B cells in the spleen of recipient mice was increased in the presence of *Rgs1* KD T cells (Figures 7c and d); in contrast, the frequency and number of $CD4^+$ T cells was comparable in all experimental groups (Figure 7e). These results suggest that *Rgs1* deficiency in T cells is sufficient to modify B-cell migration. Collectively, the data indicate that *Rgs1* KD modifies B-cell migration through both B cell-intrinsic and T cell-mediated effects.

Rgs1 KD sensitizes T_{FH} cells to CCR7 ligands

The reduced frequency of T_{FH} cells in *Rgs1* KD mice could be because of impaired proliferation after immunization. Alternatively, compromised migration into the follicular area may hinder T-cell differentiation toward a T_{FH} cell phenotype. We found that *Rgs1* was upregulated after T-cell activation (Supplementary Figure S4). However, reduced *Rgs1* expression in transgenic T cells did not affect their proliferation (Supplementary Figure S4). To test whether *Rgs1* KD had a direct effect on T_{FH} cell migration, $CD4^+$ T cells isolated from immunized mice were subjected to a migration assay toward CCL19, CCL21 and CXCL13. T_{FH} cells from WT mice migrated in response to CXCL13 but were irresponsive to CCR7 ligands (Figure 8). In contrast, we found that *Rgs1* silencing sensitized transgenic T_{FH} cells to CCL19 and CCL21, without affecting migration toward CXCL13. It was shown previously that the expression of CXCR5 is not sufficient for T_{FH} cell migration into the germinal centers.[15] T_{FH} cells must also downregulate CCR7 signaling to skew chemotactic signals in favor of CXCL13. Our results show that silencing *Rgs1* in T cells potentiates CCR7 responsiveness, a feature that may compromise the differentiation of T_{FH} cells by positioning them outside of their follicular niche.

DISCUSSION

In this study, we describe a functional link between *Rgs1* and T_{FH} cells. *Rgs1* had previously been implicated in chemokine receptor signaling in both T and B cells.[9–12] In particular, *Rgs1* deletion was shown to modify the migratory behavior of B cells within follicular areas and to increase germinal center formation.[9–11] Even the partial loss of *Rgs1* in heterozygous *Rgs1* mutant mice was demonstrated to affect chemokine responses,[11] and this was confirmed in our experiments with *Rgs1* KD mice. Germinal centers are structured into the so-called dark and light zones.[13] These zones are functionally distinct, and their organization is strictly dependent on CXCR4 signals that retain centroblasts—dividing B cells undergoing somatic hypermutation—within the dark zone.[25] In CXCR4-deficient mice, germinal centers lack distinguishable dark and light zones. The cycling of germinal center B cells from the dark to the light zone and back thus requires sequential up- and downregulation of CXCR4 signals. Expression of CXCR4 itself modifies these signals. In addition, increased *Rgs1* expression is required to desensitize B cells to CXCR4 ligation. Consistent with this notion, germinal centers in *Rgs1* KO mice feature a more prominent dark zone,[9] likely because germinal center B cells stay sensitive to CXCL12 even after decreasing CXCR4 expression. Consequently, the abnormal size and structure of germinal centers in *Rgs1*-deficient mice had been attributed primarily to a change in B-cell chemotaxis. However, *Rgs1* is also expressed in T cells.[12] *Rgs1* is upregulated following

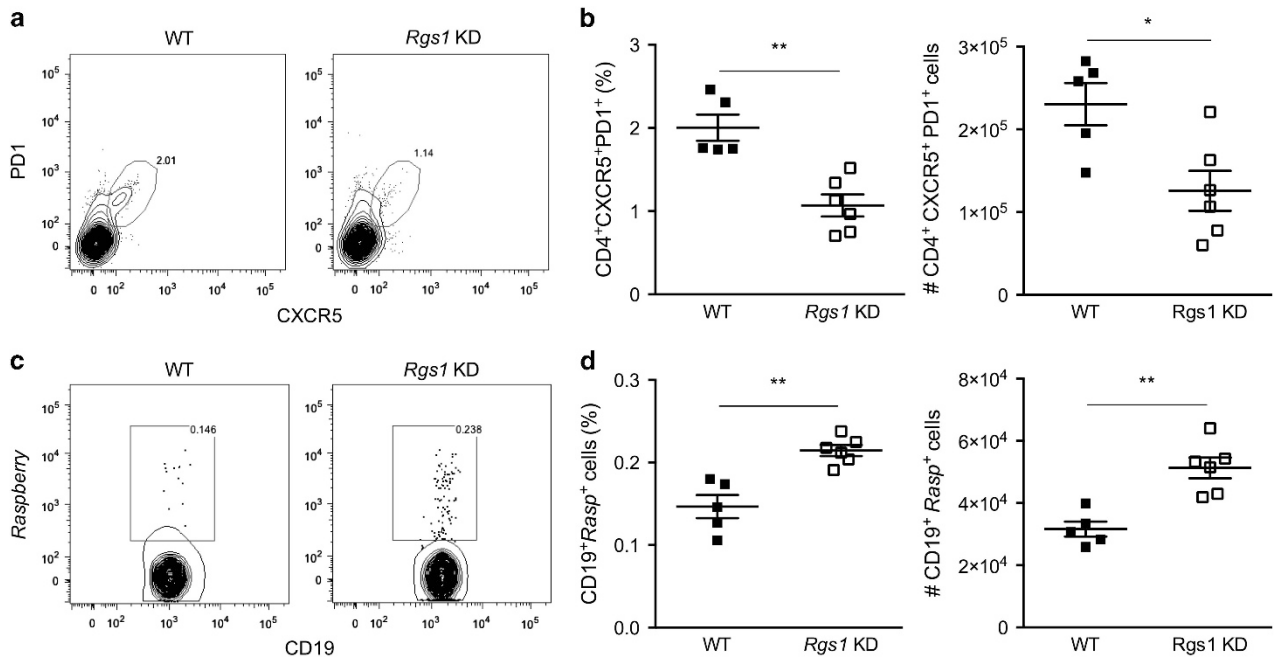


Figure 6. The migratory behavior of WT B cells is modified in *Rgs1* KD mice. WT NOD *Raspberry* B cells were transferred into WT or *Rgs1* KD mice treated with doxycycline. Recipient mice were immunized, and spleens were analyzed 4 days later. Representative flow cytometry data (**a**, **c**), frequency and absolute number (**b**, **d**) of host $CD4^+PD1^{high}CXCR5^{high}$ T cells (**a**, **b**) and donor WT *Raspberry* B cells (**c**, **d**). Data show mean values ± s.e.m. in five WT and six *Rgs1* KD mice. $^*P < 0.05$, $^{**}P < 0.01$ (unpaired *t*-test).

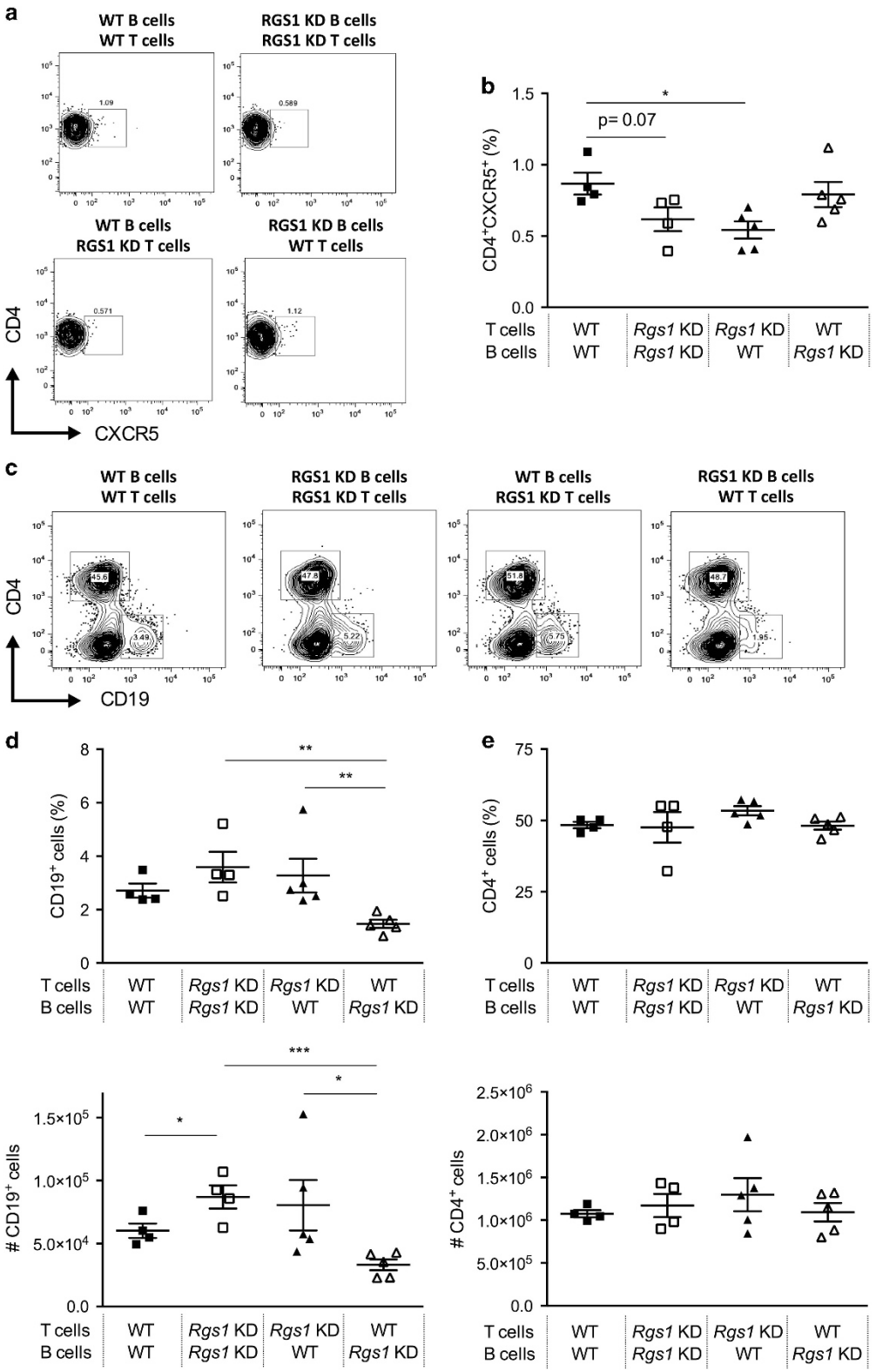


Figure 7. *Rgs1* KD in T cells is sufficient to modify B-cell migration. T cells and B cells from WT and *Rgs1* KD were co-transferred into NOD.SCID mice in all four combinations. Recipient mice were immunized with ovalbumin in complete Freund's adjuvant (CFA), and draining lymph nodes and spleen were analyzed 4 days later. Representative flow cytometry data (**a**, **c**) used to measure the frequency of $CD4^+CXCR5^+$ T_{FH} cells (**b**), and the frequency and number of $CD19^+$ B cells (**d**) and total $CD4^+$ T cells (**e**). Data show mean values ± s.e.m. from four to five mice per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired *t*-test).

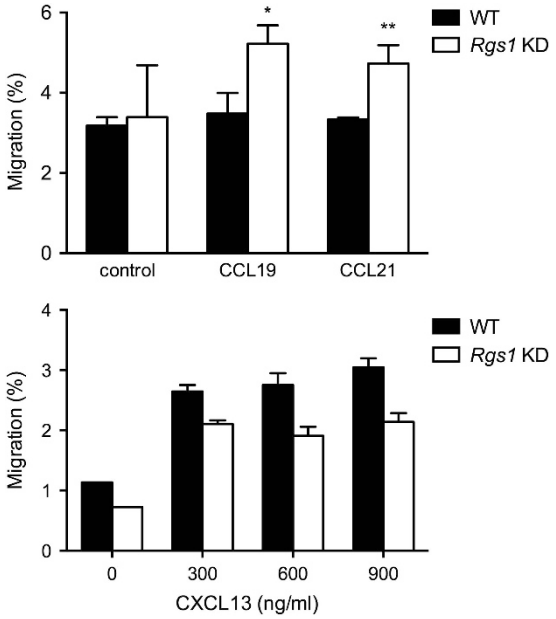


Figure 8. *Rgs1* KD increases T_{FH} cell sensitivity to CCR7 but not CXCR5 ligation. $CD4^+$ T cells were purified from WT (black bars) and *Rgs1* KD mice (white bars) and subjected to a transwell migration assays toward CCL19 and CCL21 (100 ng ml^{-1}, top panel) or CXCL13 (concentration as indicated, bottom panel). After 4 h of incubation, transmigrated cells were labeled for CD4 and CXCR5 and analyzed by fluorescence-activated cell sorting (FACS). Data show the percentage of $CD4^+CXCR5^+$ cells within the $CD4^+$ population and represent means ± s.e.m. of three biological replicates. $^*P < 0.05$, $^{**}P < 0.01$ (unpaired *t*-test).

T-cell activation, and is expressed at particularly high levels in T_{FH} cells.[16] T_{FH} cells localize to the follicular areas of secondary lymphoid organs, and participate in germinal center reactions. Their correct localization requires not only upregulation of CXCR5 that draws T_{FH} cells towards B-cell areas, but also simultaneous downregulation of CCR7 signals.[15] Expression of CXCR5 alone was shown to be insufficient to promote T-cell migration into follicles. Notably, CCR7 expression is reduced but not absent in T_{FH} cells.[15] Furthermore, Kehrl and colleagues[10] have reported that partial loss of CCR7 is insufficient to significantly diminish CCR7-dependent chemotaxis. *Rgs1* expression may thus serve to further desensitize activated T cells to CCR7 ligands that would otherwise retain them in the T-cell zone. We propose that loss of *Rgs1* impairs the migration of activated T cells into the B-cell zone and thereby inhibits the full differentiation of T_{FH} cells.[26] In a situation analogous to *Rgs1* KO B cells being retained in the dark zone by hypersensitivity to CXCL12, *Rgs1*-deficient T cells are presumably retained in the T-cell zone by hypersensitivity to CCL19 and CCL21. This idea is supported by our observation that *Rgs1* KD T_{FH} cells migrated in response to CCR7 ligation, whereas WT T_{FH} cell did not. Of note, CCR7 expression itself was not affected by *Rgs1* KD (data not shown). Our data thus indicate that *Rgs1* is a key modifier of T-cell localization within lymphoid organs, and that *Rgs1* plays a role in the differentiation of T_{FH} cells.

Interestingly, our data suggest that *Rgs1* silencing in T cells alone had an effect on B-cell migration. We posit that the germinal center phenotype described for *Rgs1* KO mice may be exacerbated by a change in T-cell function, and that this phenotype is not solely caused by B cell-intrinsic effects. Both previously reported data[9] and our own experiments support the notion that loss of *Rgs1* directly affects B-cell migration. However, T cells appear to contribute to altered B-cell trafficking in *Rgs1*-deficient mice. Mice devoid of T_{FH} cells fail to form germinal centers. The observations that loss of *Rgs1* both reduces T_{FH} cell frequency and increases germinal center formation may seem contradictory. This unexpected correlation could be explained first by the fact that the B cell-intrinsic effects of *Rgs1* deficiency are dominant, and second because T_{FH} cells are reduced in number but not entirely absent in *Rgs1* KD mice. *Rgs1* KD had a moderate impact on T_{FH} cells overall, and it will be of interest to revisit the role of *Rgs1* in T_{FH} cells in the context of mice completely deficient in *Rgs1*.[9] Exactly how and to what extent the loss of *Rgs1* in T cells might contribute to increased germinal center size remains to be explained. Notwithstanding, our findings that *Rgs1* plays a role in T_{FH} cells may provide a possible explanation for the association of *RGS1* with several autoimmune disorders including type I diabetes.[2–5] T_{FH} cells have been implicated in multiple sclerosis[27,28] and type I diabetes.[17–19] Conceivably, *RGS1* variants that promote T_{FH} cell formation could increase the risk of autoimmunity. In this regard, a disease-associated *RGS1* variant has been associated with elevated levels of CXCL13 in the cerebrospinal fluid of multiple sclerosis patients, thus linking a T_{FH}-relevant chemokine to *RGS1* gene variation in the context of autoimmune disease.[29] Whether an analogous relationship can be found between the type I diabetes-associated variants and T_{FH} cell frequency warrants further investigation.

In sum, we have shown that *Rgs1* is a critical modifier of T-cell migration within lymphoid tissue, and that this disease-associated gene participates in the development and function of T_{FH} cells. In light of multiple recent publication reporting an association between elevated T_{FH} cell frequency and type I diabetes,[17–19] our findings raise the intriguing possibility that *RGS1* variation affects the risk of autoimmunity owing to its role in T_{FH} cells.

MATERIALS AND METHODS

Mice

NOD mice were maintained under specific pathogen-free conditions at the Joslin Diabetes Center. Transgenic mice were generated as described previously.[30] Briefly, lentiviral vectors encoding a shRNA that targets the sequences 5′-CGCAAATAACAGTTGCTATTA-3′ (#1) or 5′-GCATAACAAAGCA GAGAATAT-3′ (#3) in the *Rgs1* gene were used in which shRNA expression is under the control of a tetracycline-inducible promoter.[21] The same lentiviral construct also encodes the tetracycline repressor and a GFP reporter, both driven by a constitutive promoter.[21] Lentiviral particles were microinjected into the perivitelline space of NOD zygotes. Transduced embryos were reimplanted into pseudo-pregnant NOD mice. Transgenic animals were identified by GFP expression, and bred several generations to establish stable lines carrying a hemizygous transgene with Mendelian inheritance. Transgenic mice were treated with 100 μg ml^{-1} doxycycline in the drinking water to silence *Rgs1* in all experiments unless specified. Data for shRNA #1 mice are shown in all experiments, unless otherwise noted. All experiments were approved by the institutional committee for the care and use of animals at the Joslin Diabetes Center (protocol #2014-01).

Luciferase assay

The *Rgs1* cDNA (GenBank: BC028634.1) was cloned into the dual-luciferase reporter plasmid psiCheck2 (Promega, Fitchburg, WI, USA). HEK293T cells were transfected using Fugene 6 transfection reagent (Promega) combining 100 ng psiCheck2 plasmid together with 300 ng lentiviral vector pUTG containing a doxycycline-inducible shRNA sequences against *Rgs1*. Luminescence in cell lysates was measured with a SynergyMx luminometer (Biotek, Winooski, VT, USA) after 48 h to assess reporter silencing.

Western blotting

To validate the knockdown efficiency of selected shRNA sequences *in vitro*, HEK293T cells were co-transfected with 1 μg of pcDNA3 plasmid containing the *Rgs1* gene fused to a N-terminus FLAG-tag peptide and 1 μg of pUTG vector containing a shRNA sequence against *Rgs1* transcript. Cell lysates were resolved in a 15% SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Proteins were visualized using the following antibodies: HRP-anti-FLAG antibody (Sigma, St Louis, MO, USA) and rabbit anti-actin antibody (Santa Cruz Biotech,

Dallas, TX, USA). Endogenous *Rgs1* expression in lymphocytes was also analyzed using an anti-rabbit Rgs1 antibody (Thermo Scientific, Waltham, MA, USA).

Quantitative PCR

RNA isolation was performed using TRIzol reagent (Life Technologies, Waltham, MA, USA). Subsequently, cDNA was synthesized using the Superscript III (Life Technologies) according to the manufacturer's protocol. Quantitative *Rgs1* PCR was performed using the following primers: forward 5′-TTTTCTGCTAGCCCAAAGGA-3′ and reverse 5′-TGTTTTCACGTCCATTCCAA -3′. Results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Lymphocyte migration assays

T and B cells were isolated by magnetic sorting using Pan T cell isolation kit II or CD43 (Ly48) Microbeads respectively (Miltenyi Biotech, Bergisch Gladbach, Germany). Cell purity following magnetic sorting was $>95\%$ for all experiments. Cells derived from *Rgs1* KD (GFP^+) and WT (GFP^-) mice were mixed in a 1:1 ratio and placed in the upper chamber of transwell plate. In the lower chamber, CCL19, CCL21, CXCL12 or CXCL13 were added to the media at the indicated concentrations. Cells that transmigrated to the lower chamber over the period of 4 h were analyzed by flow cytometry. Percentage of migration = (number of cells in the lower chamber after migration/input cell number in the upper chamber) $\times 100$.

Diabetes measurements

Diabetes incidence in transgenic mice was compared with non-transgenic littermates. The 4-week-old mice were fed 100 μg ml^{-1} of doxycycline in drinking water *ad libitum* and were either left to develop diabetes spontaneously over the course of 30 weeks or administered 200 mg kg^{-1} cyclophosphamide intraperitoneally to accelerate diabetes onset. In additional experiments, adoptive transfer of diabetes was performed by injecting intravenously 2×10^6 splenocytes from overtly diabetic NOD *Rgs1* KD mice into 6-week-old NOD.SCID mice. Cell recipients were then either treated or not with doxycycline. Diabetes was tested by glycosuria measurements using Diastix (Bayer, Leverkusen, Germany). Mice with two consecutive readings of 250 mg dl^{-1} were considered diabetic.

Histology and immunofluorescence

At 5 days after subcutaneous immunization in the leg with ovalbumin in complete Freund's adjuvant, mice were killed and spleen, axillary lymph nodes and inguinal lymph nodes were dissected and frozen in optimal cutting temperature medium. Sections mounted on slides were stained with the following antibodies: anti-CD45R (B220) Alexa fluor 488 (eBioscience, Santa Clara, CA, USA), anti-Ki67 (eBioscience), anti-CD4 (Serotec, Raleigh, NC, USA) or biotinylated peanut agglutinin (Sigma), followed by the secondary antibody anti-rat Alexa Fluor 488 or Alexa Fluor-594 streptavidin (Invitrogen, Carlsbad, CA, USA), and analyzed by fluorescent microscopy. Germinal centers size was determined using the measuring tool in the CellSense software (Olympus, Tokyo, Japan).

Flow cytometry

Lymphocytes were purified from the spleen or lymph nodes of WT and *Rgs1* KD mice and labeled with the following antibodies: anti-PD1 PE-Cy7, anti-CXCR5 APC, anti-CD4 PE, anti-IgD PE, anti-ICOS PE or anti-CD19 PB (BioLegend, San Diego, CA, USA). Foxp3 intracellular staining kit (eBioscience) was used to label follicular regulatory T cells. Fixation/Permeabilization buffers were used to stain intracellular Bcl6 and Ki-67. Intrinsic GFP and *Raspberry* fluorophores were also analyzed in specific experiments. Data were acquired on an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the FlowJo software (TreeStar Inc., Ashland, OR, USA).

B-cell transfer assay

B cells were isolated from NOD *Raspberry* donors by negative selection using CD43 (Ly-48) magnetic beads (Miltenyi Biotech). Cell purity following magnetic sorting was $>95\%$. 2×10^7 cells were injected intravenously into age- and gender-matched WT or *Rgs1* KD NOD mice. After 2 weeks, mice were immunized via intradermal injection in the leg with 160 μg of ovalbumin in complete Freund's adjuvant. Mice were killed for analysis 4 days later.

Lymphocyte transfer into NOD.SCID mice

T and B lymphocytes were isolated by magnetic sorting from WT or *Rgs1* KD mice by negative selection using the Pan T cell isolation kit or CD43 (Ly-48) microbeads (Miltenyi Biotech). WT and *Rgs1* KD T and B cells (each 1×10^7) were mixed in a 1:1 ratio in all four combinations. A total of 2×10^7 cells were injected intravenously into age- and gender-matched NOD.SCID recipient mice. After 2 weeks, mice were immunized as described previously. Mice were killed for analysis of lymph nodes and spleens 4 days later.

Statistical analyses

Experimental groups were compared by two-tailed unpaired Student's *t*-test using the GraphPad Prism software (Treestar Inc.). A *P* value of <0.05 was considered significant. Sufficient sample size was estimated without the use of a power calculation. No samples were excluded from the analysis. No randomization was used for animal experiments. Data analysis was not blinded.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge support from the Joslin DRC Flow Cytometry Core facility. This work was supported in part by a Career Development Award (2-2010-383) and an Innovative Award (1-INO-2014-169-A-V) from JDRF to SK, and by a DRC award (NIH Award Number P30DK036836) to the Joslin Diabetes Center.

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Supplementary Information accompanies this paper on Genes and Immunity website (http://www.nature.com/gene)