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The phenylalanyl-glycyl-glycyl-alanyl-prolyl (FG-GAP) domain plays an important role in protein–protein interactions, including interaction of integrins with their ligands. Integrin-α FG-GAP repeat-containing protein 2 (Itfg2) is a highly conserved protein in vertebrates that carries two FG-GAP domains, but its role in mammalian physiology is unknown. In this article, we show that Itfg2 is an intracellular protein and it plays a critical role in B cell differentiation and development of autoimmunity. Itfg2-deficient mice displayed a phenotype consistent with retention of B cells in the spleen and had a lower concentration of IgG in the blood when compared with wild-type littermates. Itfg2-deficient splenocytes also showed a defect in cell migration in vitro. After immunization with a thymus-dependent Ag, the absence of Itfg2 caused a shift in B cell maturation from the germinal centers to the extrafollicular regions of the spleen and blocked deposition of Ag-specific plasma cells in the bone marrow. In support of hematopoietic cell intrinsic activity of Itfg2, bone marrow transplantation of Itfg2-deficient cells was sufficient to impair germinal center development in wild-type mice. Furthermore, Itfg2 deficiency exacerbated development of autoimmune disease in MRL/lpr lupus-prone mice. These results identify Itfg2 as a novel contributor to B cell differentiation and a negative regulator of the autoimmune response during lupus. The Journal of Immunology, 2013, 191: 000–000.

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Abbreviations used in this article: ANA, anti-nuclear Ab; ASC, Ab-secreting cell; BM, bone marrow; CGG, chicken γ-globulin; CHO, Chinese hamster ovary; DN, double-negative; EAE, experimental autoimmune encephalomyelitis; ESC, embryonic stem cell; FDC, follicular dendritic cell; FG-GAP, phenylalanyl-glycyl-glycyl-alanyl-prolyl; Flk04, PK506 binding protein 4; GC, germinal center; Itfg1, integrin-α FG-GAP repeat-containing protein 1; Itfg2, integrin-α FG-GAP repeat-containing protein 2; KO, knockout; LP, (4-hydroxy-3-nitrophenyl)acetyl; LP, thymus-dependent; Tfh, follicular helper T cell; WASP, Wiskott–Aldrich syndrome protein; WT, wild-type.

The phenylalanyl-glycyl-glycyl-alanyl-prolyl (FG-GAP) domain is a weak sequence homology domain that plays an important role in protein–protein interactions. It is commonly found in the N-terminal region of integrin α-chains where it plays a role in ligand binding (1–4). In integrins, FG-GAPs are usually present in up to seven repeats that fold into a β-propeller structure (4) that contains putative calcium binding motifs (3). Other proteins could have as few as one or two FG-GAP domains. One of these proteins is integrin-α FG-GAP repeat-containing protein 1 (Itfg1), also known as T cell immunomodulatory protein, which has a single FG-GAP domain. The T cell modulatory function of Itfg1 was identified during a high-throughput cell-based screening of protein products from a library of cDNAs encoding secreted and transmembrane-domain-containing proteins (5). Recombinant Itfg1 protein increased the production of IFN-γ, IL-10, and TNF-α after CD3/CD28-mediated activation of human and mouse T cells in vitro. Furthermore, i.p. injection of an Itfg1 fusion protein rescued mice from death in a graft-versus-host disease model (5). Even though Itfg1 was not detected in the supernatants of T cells, B cells, or monocytes, the results suggested that it plays an important role in regulating the immune response.

Itfg2 (also known as MDS028) is a 447-aa protein in humans that has a weak sequence homology to Itfg1. The sequence of the full-length mouse Itfg2 protein is 89% identical to the human sequence. The Uniprot protein sequence analysis (http://www.uniprot.org/uniprot/Q91W7) indicates that Itfg2 in both species contains two FG-GAP-like repeats (Fig. 1A). Further analysis by Pfam (http://pfam.janelia.org) identifies three potential BBS2 (ciliary BBBSome complex subunit 2), middle region domains. BBS subunits are so-named after their association with Bardet–Biedl syndrome and are often found at the base of flagellar microtubule structures (6). The reported transcripts of Itfg2 predict to also generate truncated isoforms as indicated in the Universal Protein Resource (http://www.uniprot.org) and GenBank (http://www.ncbi.nlm.nih.gov/genbank) databases.

To date, the role of Itfg2 in mammalian physiology remains to be described. The reported information is restricted to the expression level of Itfg2 in various experimental systems. Itfg2 was one of the highly expressed transcripts in macrophages that was modulated by the circadian clock (7) and was also significantly upregulated in human dermal microvascular endothelial cells after activation with thrombin (8). A third study has identified Itfg2 as one of the downregulated transcripts in cell lines tolerant to the neoplastic agent picropodophyllin (9).

Because Itfg1 was shown to play a regulatory role in the immune response, we generated Itfg2 knockout (KO, Itfg2<sup>−/−</sup>) mice and performed comprehensive phenotypic analysis to identify its function in mouse physiology with special emphasis on the immune system. Our investigation revealed an essential role of Itfg2 in B cell maturation during thymus-dependent (TD) immune responses. In TD responses, B cells that encounter Ag differentiate
to Ab-secreting cells (ASCs) via two routes. One route involves germinal center (GC) formation, generation of plasma cells, and affinity maturation through the interaction with follicular helper T (T(FH)) cells and follicular dendritic cells (FDCs) (10–12). Some of the B cells that survive this follicular differentiation route will go on to become plasma cells (13), many of which will eventually find their niche in the bone marrow (BM) (14). Alternatively, B cells that encountered Ag may enter an extracellular pathway where they proliferate and produce extracellular foci of ASCs (15).

Dysregulation of follicular and extracellular B cell differentiation is a hallmark of certain autoimmune disorders, particularly systemic lupus erythematosus (16–19). The results reported in this article show that Itfg2 is required for development of normal GC and for an adequate humoral immune response. Absence of Itfg2 led to extracellular B cell differentiation and to promotion of autoimmunity in lupus-prone mice.

Materials and Methods

**Generation of Itfg2 mutant mice**

We generated Itfg2-null mice by gene trapping as previously described (20, 21). The Lexicon OmniBank embryonic stem cell (ESC) clone OST215121 was selected for microinjection based on sequence similarity to the mouse Itfg2 gene (GenBank accession no. NM_133927; http://www.ncbi.nlm.nih.gov/nuccore/nm_133927). The genomic insertion site of the gene trap vector in OST215121 was determined by inverse PCR. Mice carrying this mutation were generated by using standard methods of host embryo microinjection of ESCs, chimera production, and germline transmission. Genotyping of wild-type (WT; Itfg2+/+) and mutant Itfg2 alleles was performed by PCR using the following primer sequences (5′-GCC TGG CCG GGA CCT GAC GGA CTA CCT CAT and 5′-AAA TGG CGT TAC AAA TGG CGT TAC TTA AGC TAG TAT GC-3′). All experiments were performed on 2- to 6-month-old mice of mixed genetic background (129/SvEvBrd and C57BL/6J) representing littermate mutant and WT animals. In addition, to test the role of Itfg2 in a lupus-like disease, we backcrossed Itfg2 KO mice three times to MRL/MpJ-Faslpr/3 (MRL-Faslpr) background (The Jackson Laboratory, Bar Harbor, ME).

Procedures involving animals were conducted in conformity with the Institutional Animal Care and Use Committee guidelines and in compliance with the standards outlined in the Guide for the Care and Use of Laboratory Animals of the National Research Council.

**Gene expression analysis by RT-PCR**

To confirm disruption of the gene in the mutant mouse, total RNA were collected from brain and thymus of Itfg2+/+ mice and their WT littermates. RT-PCR was performed as described previously (21), using oligonucleotide primers complementary to exons 1 and 4 of Itfg2 (5′-CCA GAG TGC CAT GAG GTC GTG GTG TAT TGA AGG AGG C-3′ and 5′-AAA TGG CGT TAC AAA TGG CGT TAC TTA AGC TAG TAT GC-3′). To evaluate whether disruption of Itfg2 affected expression of the neighboring FK506 binding protein 4 (Fkbp4) gene (GenBank accession number NM_010219), we also analyzed the same RNA samples using the Fkbp4-specific primers 5′-GTGAAGGACTT- GACCTGCGAAGAGC-3′ and 5′-CATTGTTCGCCTCACCCTCATCT- CAGC-3′. Primers to the mouse β actin gene (GenBank accession no. M12481, 5′-GGC TGG CCG CCG GCT GAC CTA CCA CTT C-3′ and 5′-GCC TAG AAG CTT CCG GTG ATG GAG-3′) were used as internal control for sample handling.

**Analysis of protein expression**

Splenic B cells were enriched by negative selection using a B cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada). Proteins were purified from five different cellular compartments by using a subcellular protein fractionation kit (Thermo Scientific, Rockford, IL).

Western blot analysis was performed as described previously (21). Primary Abs to heat shock protein 90, calreticulin, histone deacetylase 2, histone H3, and cytokeratin 18 were purchased from Cell Signaling Technology (Danvers, MA), anti-Itfg2 and anti-GAPDH Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Bethyl Laboratories (Montgomery, TX), respectively. Blots were developed by using a HRP-conjugated goat anti-rabbit Ab (Bethyl Laboratories) and an ECL detection system (GE Life Sciences, Pittsburgh, PA).

**Blood cell count, flow cytometric (FACS) analysis, and histology**

The absolute number of each blood cell subset was calculated by multiplying its fractional representation, determined by FACS, by the absolute number of mononuclear cells identified by complete blood cell count, as described previously (21). All Abs were from BD Biosciences, and samples were analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA).

H&E and immunofluorescence tissue staining was done as described previously (21, 22). All primary Abs and streptavidin were purchased from BD Biosciences, with the exception of the anti-IgG1 Ab (eBioscience, Novato, CA), Ig isotypes (Southern Biotech, Birmingham, AL), chicken OVA (BD Biosciences, San Jose, CA), anti-nuclear Abs (ANA), anti-dsDNA (both from Alpha Diagnostic International, San Antonio, TX), and Baff (R&D Systems, Minneapolis, MN). Humoral immune responses to NP and OVA were elicited by i.p. injection of 100 μg NP coupled to chicken γ-globulin (CGG) in alum and 100 μg OVA in CFA, respectively. For the anti-OVA response, mice were also given an additional i.p. injection of 100 μg OVA in IFA 12 wk after the initial immunization. The anti-NP response was measured using NP6 µg BSA (16 molecules of NP coupled per molecule of BSA) as Ag, coated on ELISA plates.

ELISPOT assays were performed as described previously (23) using 96 multiscreen plates (Millipore, Billerica, MA) coated with 5 μg/ml rat anti-mouse IgG (BD Biosciences) or with 10 μg/ml NP6 µg BSA (Biosearch Technologies).

**BM transplantation**

Recipient mice were irradiated as described previously (24) with the following exception: 2 × 10⁶ donor BM cells were injected 8 h after the second dose of irradiation. Reconstitution was monitored by performing quantitative PCR for the presence of the neomycin resistance gene (Neo, present in the trapping cassette) in peripheral blood leukocytes 12–16 wk after transplantation. The average percentage of Neo+ cells derived from donor Itfg2+/+ BM was >85% in the reconstituted animals.

**Induction of experimental autoimmune encephalomyelitis**

Mice were immunized s.c. in the flanks with 300 μg mouse myelin oligodendrocyte glycoprotein peptide (aa 35–55, MEGGWYRSFSROVH-LYRNGK, Sigma Genosys, The Woodlands, TX) emulsified in CFA containing 250 μg Mycobacterium tuberculosis strain H37RA (Difco Laboratories, Detroit, MI) and i.v. injected with 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA). Mice were scored in a blinded manner for clinical symptoms of experimental autoimmune encephalomyelitis (EAE) using the following scale: 0 = no symptoms, 1 = light weakness of the tail/floppy tail and/or slumped headquarts; 2 = definite total paralysis of the tail; 3 = mild waddle or distorted gait and total paralysis of the tail and/or mild impaired righting reflex; 4 = heavy waddle with impaired control, weakness of hind limbs, and/or impaired righting reflex; 5 = paralysis of one of the hind limbs possibly with mild forelimb weakness; 6 = paralysis of both hind limbs and/or moderate-to-severe forelimb weakness; 7 = quadriplegia/paralysis of all limbs; and 8 = moribund or dead.

**Evaluation of lupus development**

Parameters of lupus development were evaluated as described previously (25, 26). Sera were collected at the indicated time points and analyzed for autoantibodies as described earlier. Urine samples were examined using a Cobas Integra 400 chemistry analyzer (Roche Diagnostics, Indianapolis, IN).
Cell migration assay
Migration of splenocytes was assessed after a 4-h incubation in 5-μm pore-size 96-well plates (NeuroProbe, Gaithersburg, MD), with 100 ng/ml of the indicated mouse chemokines (PeproTech, Rocky Hill, NJ) according to the manufacturer’s instructions.

Statistical analyses
Statistical significance of group differences was evaluated by the unpaired, two-tailed, Student t test. A p value <0.05 was considered significant.

Results
Itfg2 is a cytoplasmic and nuclear-soluble protein
Public databases and our own data (not shown) indicated that Itfg2 is widely expressed across different tissues, including the lymphoid organs. However, the cellular localization of Itfg2 was not known. Therefore, we enriched splenic B cells from mice and isolated proteins from five different subcellular compartments. The purity of these preparations was confirmed using organelle-specific markers. Western blot analysis revealed that Itfg2 is present in the cytoplasmic and nuclear-soluble fractions of the cells (Fig. 1B). Furthermore, Itfg2 was not detected in the supernatants of CHO cells transfected with an Itfg2 construct (Fig. 1C). Consistent with the Western blot analysis of B cells, the GFP signal in the Itfg2-GFP–transfected CHO cells localized to the cytoplasm and nucleus as evidenced by counterstaining of cell nuclei and cytoskeleton with Hoechst 33342 and Alexa Fluor 568 phallolidin, respectively (Fig. 1D).

Itfg2−/− mice do not show gross abnormalities
We generated Itfg2−/− mice from the Lexicon OmniBank ESC clone OST215121 (20). Inverse PCR of genomic DNA isolated from Itfg2−/− ESCs identified a gene trap vector insertion within the intron before the second exon of the Itfg2 gene (Fig. 2A). Mice heterozygous for the Itfg2 deletion (Itfg2+/-) were fertile and were intercrossed to obtain homozygous progeny. Successful targeting of the Itfg2 gene in KO mice was confirmed by expression analysis of the gene transcript (Fig. 2B) and Western blot analysis of the Itfg2 protein (Fig. 2C). The Itfg2 gene transcript was detected only in WT tissues and not in tissues derived from KO mice. Furthermore, disruption of the Itfg2 gene did not impact expression of the neighboring gene Fkbp4 (Fig. 2B). An Itfg2-specific mAb detected a protein with an apparent molecular mass of 50 kDa in cell lysates prepared from WT, but not Itfg2−/− spleens (Fig. 2C), which is consistent with the calculated molecular mass (49.8 kDa) of the mouse Itfg2.

Mating of Itfg2+/- mice generated pups of the three possible genotypes with ratios that fit well within normal (1:2:1) Mendelian frequencies (26% WT, 51% Itfg2+/-, 23% Itfg2−/−; n = 1807; χ² = 3.7, p = 0.16). This result indicated that Itfg2 is not essential for neonatal viability. Itfg2−/− mice exhibited no substantial differ-

FIGURE 1. Itfg2 is a cytoplasmic and nuclear-soluble protein. (A) Protein sequence alignment of the two murine Itfg2 isoforms reported in GenBank. FG-GAP–like domains are highlighted. (B) Immunoblot of indicated cellular fractions purified from mouse splenocytes and analyzed for presence of Itfg2 and organelle-specific markers. (C) Immunoblot of supernatants and lysates of CHO cells collected 48 h after transfection with Itfg2 or mock control. Itfg2 was detected using an anti-Itfg2 polyclonal Ab. (D) Immunofluorescence staining of CHO cells transfected with Itfg2-GFP. Green: Itfg2; red: cytoskeleton; blue: nucleus. Scale bar, 50 μm.
ence in growth rate and size. Likewise, comprehensive clinical
diagnostic and pathologic analysis, including tests of behavior,
obesity, diabetes, bone, skin fibroblast proliferation, and cardio-
vascular function (described in detail in Ref. 27), did not dem-
onstrate significant differences in Itfg2−/− mice relative to their
WT littermates (data not shown).

Itfg2−/− mice exhibit a B cell retention phenotype and
decreased levels of circulating Igs

Blood cell analysis revealed significant lymphopenia caused by re-
duced B cell numbers in Itfg2−/− mice compared with WT littermates
(Fig. 3A). Furthermore, an Ig panel analysis of sera obtained from
Itfg2−/− and WT mice showed significantly reduced concentration of
circulating Ig subclasses, with the exception of IgA and IgM, in the
mutant mice (Fig. 3B). Neutrophil, monocyte, NK cell, RBC, and
platelet counts in the Itfg2−/− mice were not significantly different
from those of WT mice (Fig. 3A). Expression of a single allele of
Itfg2 was sufficient to prevent the B lymphopenia as demonstrated by
the normal blood cell composition of Itfg2+/− mice (data not shown).

Contrary to the decrease in peripheral B cell counts, the spleen of
the Itfg2 KO mice showed increased cellularity, primarily because of
significantly elevated B cell numbers. The B cell content of the
Itfg2−/− spleen significantly increased when compared with WT
spleen (40.4 ± 3.6 × 10⁶ WT versus 50.3 ± 2.6 × 10⁶ Itfg2−/−; n =
25–26 each genotype; p = 0.029), whereas the difference in T cell
numbers did not reach statistical significance (20.4 ± 2.8 × 10⁶ WT

**FIGURE 2.** Generation of Itfg2-deficient mice. (A) Schematic showing the gene trap mutation strategy used
to generate Itfg2−/− mice. (B) Indicated gene transcripts
were detected in the designated tissues by RT-PCR. (C)
Immunoblot of mouse spleen lysates with Abs to the
indicated proteins.

**FIGURE 3.** Itfg2−/− mice exhibit B lymphopenia and decreased levels of circulating Igs. (A) Peripheral blood cell profile
in WT (n = 47) and Itfg2−/− (n = 52) mice, and (B) serum concentration of Ig isotypes
WT (n = 70) and Itfg2−/− (n = 72) mice. Data are presented as mean ± SEM. Num-
bers near bars depict p values compared
with WT mice. (C) T and B cell development appears to be normal in naive Itfg2−/−
mice. The representative FACS plots show normal CD4/CD8 mAb-staining patterns in
the thymus and spleen, as well as comparable CD21/IgM mAb staining patterns of
splenic B cells of the indicated genotype. Representative fraction of cells in different
maturation stages is indicated. (D) Fraction of splenocytes migrating to the different stim-
uli. Study shown is representative of three
experiments with similar findings (n = 3 each
genotype). Data are presented as in (A). Lymph, Lymphocytes; Mono, monocytes;
Neutro, neutrophils.
versus $26.5 \pm 3.7 \times 10^6 \text{Itfg2}^{2-/-}; n = 22$ each genotype; $p = 0.19$).

Such redistribution of the B cell population suggests that the B lymphopenia in the blood is the consequence of retention of B cells in the spleen rather than deficiency in B cell development. Indeed, FACS analysis of the BM, as well as thymus, lymph node, and Peyer’s patches, showed no significant alteration in the distribution and absolute number of various cell subsets characterized by expression of cell-surface markers B220, IgM, IgD, TCR, CD4, CD5, CD8, CD11b, CD21, CD23, CD44, CD62L, and IL-7R$\alpha$ (Fig. 3C and data not shown). In addition, histopathological examination of mesenteric, mammary, and cervical lymph nodes, spleen, and thymus taken from 13-wk-old mice showed no structural differences between Itfg2$^{2-/-}$ mice and WT littermates (data not shown).

The alteration in B cell populations in the blood and spleen, as well as the previous reports suggesting a potential association of Itfg2 with cytoskeletal proteins (28), led us to examine cell migration in Itfg2$^{2-/-}$ and Itfg2$^{+/+}$ mice in response to in vitro signals elicited by the chemokines SDF-1, CXCL13, and CCL19. SDF-1 is produced in the red pulp of the spleen and is involved in lymphocyte migration (29, 30), whereas CXCL13 and CCL19 are required for the localization of B cells in the spleen (31–33). The migration of Itfg2-deficient splenocytes to the three chemokines tested was significantly reduced when compared with WT cells (Fig. 3D), suggesting a potential explanation for the altered B cell distribution in the blood and spleen of Itfg2$^{2-/-}$ mice.

**Absence of Itfg2 leads to impaired GC formation**

The observed defect in the B cell compartments of Itfg2$^{2-/-}$ mice led us to investigate the impact of Itfg2 expression on the humoral immune response. Immunization of WT mice with NP-CGG, a TD Ag (34), resulted in induction of GCs, identified by the presence of cells expressing the GC marker GL7 (35). Maximum expansion of GCs was observed on day 9 after immunization (Fig. 4A, 4B). In contrast, only a few GCs were formed in the absence of Itfg2. However, the Itfg2$^{2-/-}$ splenocytes did contain normal B cell areas as evidenced by distribution of CD23$^+$ follicular B cells that showed no clear difference between WT and KO animals (Fig. 4A). Furthermore, a significant reduction in GC cells with the B cell phenotype B220$^+$GL7$^+$IgD$^+$/CD95$^+$ (35) was verified by FACS analysis of single-cell suspensions of WT and Itfg2$^{2-/-}$ splenocytes (Fig. 4C, 4D). Accordingly, there was a significant reduction in the NP-specific ASCs in Itfg2$^{2-/-}$ spleens immunized for 14 d with NP-CGG compared with WT littermates determined by ELISPOT analysis (Fig. 4E).

Under normal circumstances, the GC reaction leads to class switching by ASCs and gives rise to long-lived BM plasma cells (14). To examine whether these Ag-specific processes were affected by the absence of Itfg2, we performed ELISPOT assays to determine the frequency of NP-specific, IgG-secreting cells in the BM of mice 45 d after immunization with NP-CGG. In accordance with the impaired GC development, the BM of Itfg2$^{2-/-}$ mice contained significantly reduced numbers of total and NP-specific ASCs compared with WT littermates (Fig. 4F).

**Itfg2 deficiency in hematopoietic cells is sufficient to impair GC development**

To investigate the cellular source that is responsible for the immune deficiency in the absence of Itfg2, we have transplanted cells from

![FIGURE 4. Itfg2 deficiency disrupts GC development in the spleen.](http://www.jimmunol.org/)

- **A** Representative images of immunofluorescence staining of spleen sections from mice with the designated genotypes, obtained at the indicated time points after immunization with NP-CGG. Green: GL7$^+$ GCs; red: CD23$^+$ follicular B cells.
- **B** Graph summarizes the number of GCs in the spleens of mice described in (A) ($n = 5–7$). Two additional independent studies provided similar results.
- **C** Representative FACS plots of B220 and GL7 expression on splenocytes from NP-CGG–challenged mice 2 wk after immunization. Rectangles and associated values indicate the proportion of B220$^+$GL7$^+$ cells. **D** Proportion of GC cells (B220$^+$GL7$^+$IgD$^+$/CD95$^+$) in the same spleen samples as in (C), determined by FACS analysis ($n = 4–5$ each genotype). **E** NP-specific ASCs in the spleen and BM of the indicated mice 2 wk after immunization with NP-CGG ($n = 4–5$ each genotype). **F** ASCs in the BM of WT and mutant mice that were immunized with NP-CGG 45 d before analysis ($n = 4$ each genotype). Data are presented as in Fig. 3.
WT and Itfg2<sup>−/−</sup> mice into irradiated recipients. When the recipients were assessed at 16–20 wk after transplantation with KO BM, they exhibited a trend of reduced serum concentration of most Ig subclasses compared with the control cohort, although the difference reached statistical significance only for IgG1 (Supplemental Fig. 1A). Peripheral blood B cell counts were normal in the chimeric mice, but immunization with NP-CGG revealed decreased GC formation in the spleen, similar to the phenotype of the Itfg2 KO mice (Supplemental Fig. 1B). In contrast, WT BM cells transferred to Itfg2<sup>−/−</sup> mice rescued GC development in their host animal.

**Itfg2<sup>−/−</sup> mice generate a reduced number of TFH cells**

Failure to generate GCs can be a consequence of intrinsic signaling defect in lymphocytes or inadequate support of B cell differentiation from T<sub>FH</sub> cells and the FDC network. The proliferation rate of splenocytes from Itfg2<sup>+/+</sup> and Itfg2<sup>−/−</sup> mice was similar after activation with various polyclonal B and T cell stimuli in vitro (Supplemental Fig. 2A). Furthermore, splenic B cells isolated from WT and Itfg2 KO mice and activated in vitro with LPS or a mixture of CD40 mAb and IL-4, produced similar levels of Ig (data not shown). Serum concentration of Baff, one of the factors that is required for formation and maintenance of GCs (36), was also normal in the absence of Itfg2. In fact, Itfg2<sup>−/−</sup> mice showed a modest yet statistically significant increase in Baff levels compared with WT littermates that could reflect a compensatory response (Supplemental Fig. 2B). In addition, Itfg2<sup>−/−</sup> mice also responded equally to WT mice in a B cell–independent disease model of multiple sclerosis (EAE) when immunized with the encephalitogenic myelin oligodendrocyte glycoprotein (35–55) peptide (Supplemental Fig. 3). The latter data indicate that T cell and APC functions are largely intact in the absence of Itfg2. However, FACs analysis of splenocytes demonstrated that the reduced GC formation in Itfg2<sup>−/−</sup> mice after NP-CGG immunization was associated with decreased representation of T<sub>FH</sub> cells that express the markers CD4, CD44, PD1, and CXCR5 (37) (Fig. 5A, 5B). Immunohistochemical staining for the complement receptor CD35 (38) and FDC-M1 (39) showed that the architecture of the primary reticulum and the mature FDC network did not show significant differences between immunized Itfg2<sup>−/−</sup> and WT mice (Fig. 5C, 5D).

**Itfg2<sup>−/−</sup> mice show increased extrafollicular B cell differentiation and continue to mount TD Ab responses**

We evaluated the serologic response of Itfg2<sup>−/−</sup> and WT mice 45 d after NP-CGG immunization by measuring the NP-specific IgG concentration in the serum. Despite the significant reduction in GC formation, the IgG responses for all the IgG subclasses were within normal range in the Itfg2<sup>−/−</sup> mice; in fact, the IgG1 response trended higher (p = 0.07; Fig. 6A). More detailed time-course analysis of the Ab response was performed with another TD Ag, OVA. This study confirmed with statistical significance that Itfg2<sup>−/−</sup> mice displayed an increased serological response. Over a course of 25 wk, Itfg2<sup>−/−</sup> mice produced significantly higher levels of anti-OVA IgG1 and IgG2b Abs than WT littermates even after a booster immunization at 12 wk (Fig. 6B). The IgG2a and IgG3 isotypes of the anti-OVA response showed no significant difference. These results indicated that despite the observed deficiencies in GC formation, alternative mechanisms continued to function in Itfg2<sup>−/−</sup> mice, which allowed the animals to mount a humoral immune response. We did not observe a significant difference in the IgG immune response in WT and Itfg2 KO mice 14 d after immunization with the thymus-independent Ags TNP-LPS and TNP-Ficoll (data not shown).

An intact or increased humoral immune response can be the result of exaggerated extrafollicular B cell activity that can occur despite a deficient GC response (19). Indeed, immunofluorescence staining showed that IgG1<sup>+</sup> cells in the spleens of NP-CGG–immunized Itfg2<sup>−/−</sup> mice were more numerous in areas outside of the follicles, indicating that extrafollicular Ab class switching was under way in these mice. In contrast, IgG1<sup>+</sup> cells were mainly located in the GC region of the B follicles in Itfg2<sup>+/+</sup> mice (Fig. 6C, 6D).

**Itfg2 deficiency exacerbates the autoimmune disease in MRL-Fas<sup>−/−</sup> mice**

Pathogenic autoantibodies can arise from self-reactive B cells undergoing class switching within the extrafollicular environment (16–19). This process is exemplified by the lupus-prone MRL-Fas<sup>−/−</sup> mouse model, which displays a dominance of the extrafollicular pathway over the GC pathway of B cell differentiation (16). Therefore, we have investigated the potential effect of Itfg2 deficiency on lupus development in the MRL-Fas<sup>−/−</sup> mouse.

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**FIGURE 5.** Itfg2<sup>−/−</sup> mice fail to generate a normal number of T<sub>FH</sub> cells. (A and B) FACS analysis showing reduced representation of PD1<sup>+</sup>CD4<sup>+</sup>CXCR5<sup>+</sup> T<sub>FH</sub> cells among the CD4<sup>+</sup> CD44<sup>+</sup> spleen cell population in Itfg2<sup>−/−</sup> mice 14 d after NP-CGG immunization. (A) Representative FACS plots and rectangles show gating strategy. Circles and associated values indicate the proportion of T<sub>FH</sub> cells. (B) Proportion of indicated cell subsets in the same spleen samples as in (C), determined by FACS analysis. Data are presented as in Fig. 3; n = 4 each genotype. (C and D) Representative images of immunofluorescence staining of spleen sections from WT and Itfg2<sup>−/−</sup> mice, obtained 9 d after immunization with NP-CGG. Green: GL7<sup>+</sup> GCs; red: CD35<sup>+</sup> and FDC-M1<sup>+</sup> FDC network as indicated. Scale bars, 100 μm.
strain. We generated MRL-Faslpr-Ifg2+/+ and MRL-Faslpr-Ifg2−/− mice, and examined disease development over a period of 19 wk. Consistent with the results described earlier for Ifg2−/− mice in normal background, the Ifg2-deficiency in MRL-Faslpr background led to a decrease in circulating B cells for the first 13 wk of age when compared with MRL-Faslpr-Ifg2+/+ mice (Fig. 7A). However, the differences became insignificant by 16 wk of age. Total Ig panel on sera from 19-wk-old mice revealed a significant increase in IgM and IgG2a in the sera of the MRL-Faslpr-Ifg2−/− mice compared with control MRL mice (Fig. 7B). Histopathological evaluation revealed significantly more severe autoimmune disease development in the absence of Ifg2, which was characterized by inflammatory cell infiltration of the kidney and hypertrophy in the lymphoid organs (Fig. 7C, Supplemental Fig. 4). Although histopathological scoring did not reach statistical significance for readouts of kidney damage because of the inherent variability of disease development, they all showed a trend of increased prevalence in the absence of Ifg2. Furthermore, glomerular protein leakage was evident by the significantly increased protein secretion in the urine of MRL-Faslpr-Ifg2−/− mice compared with MRL-Faslpr-Ifg2+/+ littermates (Supplemental Fig. 4). Accordingly, MRL-Faslpr-Ifg2−/− mice produced increasingly higher amounts of ANA, anti-dsDNA, and Baff than MRL-Faslpr-Ifg2+/+ littermates during the course of the study (Fig. 7D). In addition, the fraction of the CD4−CD8− (double-negative [DN]) T cell population in the blood increased faster and to higher proportions in the Ifg2-deficient, lupus-prone mice than in their WT counterparts (Fig. 7D). Increased representation of DN T cells

FIGURE 6. Robust Ig response and extrafollicular B cell differentiation in Ifg2−/− mice. (A and B) Serum concentration of Ag-specific Abs in WT and Ifg2−/− mice 45 d after immunization with NP-CGG (A), and at the indicated time points after injection with OVA (B). Data were pooled from 2–3 independent experiments providing similar results (n = 5–15 each genotype per study). Arrows depict the time point when mice got a booster injection of OVA. Data are presented as in Fig. 4. (C and D) Representative images of immunofluorescence staining of spleen sections from WT and Ifg2−/− mice, obtained 14 d after immunization with NP-CGG. Green: GL7+ GCs; red: IgG1-producing B cell; blue: B220 B cell marker (C) or CD35 (D). Scale bars, 100 μm.
MRL-Itfg2 development, which resembles the TD response of Itfg2-prone mice contain extrafollicular foci of ASCs in the absence of autoantibody production in mouse lupus models (16, 18). Lupus- and spontaneous activation of extrafollicular ASCs can lead to exclusion of autoreactive B cells from GCs is defective in human lupus (46), suggesting that B cells are normally excluded from the follicular differentiation process (43, 44). Under normal circumstances, such autoreactive B cells are nonresponsive but can develop extracellular ASC foci when T cell help is provided (45). Exclusion of autoreactive B cells are nonresponsive but can develop extrafollicular differentiation in mice of the indicated genotype. Data are presented as in (A). An additional independent study gave similar results.

Discussion

Itfg2 controls the B cell response to TD Ags and during lupus development

Inactivation of the Itfg2 gene resulted in marked reduction in GC formation but increased extrafollicular B cell differentiation in mice immunized with a TD Ag. This conclusion is supported by immunohistochemistry and FACS analysis of GCs, and by the reduced number of ASCs present in the spleen on day 14, as well as in the BM on day 45, in Itfg2−/− mice compared with WTs. In contrast, IgG1+ ASCs were mainly located outside of the follicles in Itfg2−/− mice, which could explain the normal and, in some cases, increased Ag-specific serum Ab response in these mice. Exaggerated extrafollicular B cell activity is also the likely cause of the increased production of ANA and accelerated lupus development in MRL-Itfg2−/− mice in the absence of Itfg2. Self-reactive B cells are normally excluded from the follicular differentiation process (43, 44). Under normal circumstances, such autoreactive B cells are nonresponsive but can develop extracellular ASC foci when T cell help is provided (45). Exclusion of autoreactive B cells from GCs is defective in human lupus (46), and spontaneous activation of extrafollicular ASCs may lead to autoantibody production in mouse lupus models (16, 18). Lupus-prone mice contain extrafollicular foci of ASCs in the absence of GC development, which resembles the TD response of Itfg2−/− mice. In addition, the phenotype of the lupus-prone mice in the absence of Itfg2 is similar to what has been reported for MRL-Itfg2−/− mice carrying a transgene for the rheumatoid factor IgH chain (16, 17). It is worth noting that we have investigated whether Itfg2 KO mice on a 129/C57Bl6 background develop autoimmune. These experiments involved young (~12 wk old) and aged (~1 y old) Itfg2 KO mice and their WT littermates. Histopathological examination revealed no consistent differences between the two groups, and serum analysis showed no significant differences in the level of autoantibodies produced (unpublished data).

Which cellular functions are affected by Itfg2 expression?

Our comprehensive phenotypic screen indicated that absence of Itfg2 in mice primarily affected immune function and not other physiological systems. Furthermore, transplantation of Itfg2−/− BM into WT mice largely reproduced the KO phenotype, showing that the deficiency originates from hematopoietic cells. The deficiency was also specific to the humoral immune response because the B cell–dependent autoimmune response of Itfg2−/− mice was normal in the EAE model. Itfg2−/− splenocytes did not have an intrinsic broad defect in cell signaling because they proliferated normally in the presence of polyclonal T and B cell stimuli in vitro. Moreover, when immunized with thymus-independent Ags TNP-LPS and TNP-Ficol, Itfg2−/− mice mounted normal Ab response, further indicating that development of plasmablasts and Ab generation outside of the GCs is not inherently deficient in the absence of Itfg2. Rather, in vitro cell migration assays and histological changes indicated that defective migration of lymphoid cells and possibly cell–cell interaction with Tfh cells in the spleen microenvironment may be responsible for a shift in B cell differentiation pathways in the absence of Itfg2. The redistribution of B cells from the blood to the spleen in naive Itfg2−/− mice points to a defect in trafficking pathways that are used both during movement of B cells from the spleen to the periphery, and during follicular and extrafollicular B cell development within the spleen.

What pathways can contribute to Itfg2 function?

The Itfg2 sequence contains two FG-GAP domains. These domains are usually expressed in multiple repeats in α-integrin proteins, assembled into a β-propeller structure (4). It has been suggested...
that most FG-GAP domains contain calcium-binding propeller blades that regulate the folding, activation status, and heterodimerization of integrins, and presumably affect ligand binding (47–49). Indeed, the second FG-GAP domain of Iftg2 contains the sequence Dx[D/N]xDG (aa 134–139), which is the consensus sequence of such calcium blades (48). However, our data showed that Iftg2 is an intracellular protein; therefore, it cannot be directly involved in extracellular ligand binding like the majority of cell-surface α-integrin molecules. Only two other intracellular proteins have been identified to date that contain FG-GAP domains with the calcium blade sequence, kaprin, and the BB52 protein (48). Interestingly, both proteins are involved in processes that regulate dynamic change in cell shape and locomotion, and require actin rearrangements and polymerization. Kaprin is an actin-binding protein thought to mediate actin dynamics during shape change in activated platelets, as well as in motile fibroblasts and elongating stereocilia (50). Similarly, mutation in BB52 leads to motile dysfunction and misshaped appearance in cilia (51). Although we have not observed cytoskeletal localization of Iftg2 in naïve B cells, our results do not exclude the possibility that Iftg2 regulates actin function after cell activation.

Intriguingly, actin-binding kaprin is one of only two proteins identified as interacting partners of Iftg2 in a high-throughput analysis of protein–protein interactions (28). The other associated protein is activated Cdc42 kinase (Ack1), a nonreceptor tyrosine kinase that is activated in response to multiple cellular signals, including cell adhesion, and can also mediate cell migration (52). Our preliminary evaluation of Ack1-deficient mice revealed a decrease in serum Ig levels similar to what we observed in Iftg2−/− mice (unpublished results). Importantly, Ack1 has been shown to phosphorylate the Wiskott–Aldrich syndrome protein (WASP), which regulates the formation of actin filaments in hematopoietic cells. Phosphorylation of WASP enhanced its ability to stimulate actin polymerization (53). In addition, WASP has been shown recently to play an important role in peripheral B cell development and function in mice, including the immune response to the TD Ag TNP-keyhole limpet (54).

A plethora of other molecules have also been implicated in GC formation and/or extrafollicular B cell differentiation and homing. However, only one protein has been identified to date that influences GC formation and extrafollicular ASC differentiation in opposing ways. This protein is SWAP70, a multidomain molecule that is highly expressed in GCs (55). Analogous to the phenotype of Iftg2-deficient mice, the KOs of SWAP70 display deficient GC formation but increased extrafollicular B cell differentiation and Ab production after immunization with a TD Ag (19). Remarkably, similar to Iftg2-interacting proteins, SWAP70 also binds actin and is required for the normal process of actin polymerization, formation of lamellipodia in motile cells, and B cell migration (19).

In summary, we showed that Iftg2 is a novel intracellular protein that plays a unique and specific role in determining the fate of B cells responding to TD Ags and influences the magnitude of lupus inflammatory immune responses. Proc. Natl. Acad. Sci. USA 94: 65–72.


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Disclosures

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