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Loss of Immunological Tolerance in Gimap5-Deficient Mice Is Associated with Loss of Foxo in CD4+ T Cells

H. Ibrahim Aksoylar,* Kristin Lampe,* Michael J. Barnes,†,1 David R. Plas,‡ and Kasper Hoebe*†

Previously, we reported the abrogation of quiescence and reduced survival in lymphocytes from Gimap5<sup>sph/sph</sup> mice, an ENU germline mutant with a missense mutation in the GTPase of immunity-associated protein 5 (Gimap5). These mice showed a progressive loss of peripheral lymphocyte populations and developed spontaneous colitis, resulting in early mortality. In this study, we identify the molecular pathways that contribute to the onset of colitis in Gimap5<sup>sph/sph</sup> mice. We show that CD4<sup>+</sup> T cells become Th1/Th17 polarized and are critically important for the development of colitis. Concomitantly, regulatory T cells become reduced in frequency in the peripheral tissues, and their immunosuppressive capacity becomes impaired. Most importantly, these progressive changes in CD4<sup>+</sup> T cells are associated with the loss of Forkheadbox group O (Foxo1), Foxo3, and Foxo4 expression. Our data establish a novel link between Gimap5 and Foxo expression and provide evidence for a regulatory mechanism that controls Foxo protein expression and may help to maintain immunological tolerance. The Journal of Immunology, 2012, 188: 000–000.

The family of GTPase of immunity-associated protein (Gimap) genes are expressed predominantly in lymphocytes and regulate lymphocyte survival during development, selection, and homeostasis (1). Members of this family share a GTP-binding AIG1 homology domain, which was identified originally in disease resistance genes in higher plants (2, 3). Recent crystallographic studies revealed that GDP-bound or nucleotide-free GIMAP2 exists in a monomeric configuration with an exposed guanine nucleotide binding domain (4). In the presence of GTP, GIMAP2 oligomerizes and shows similarities with the nucleotide coordination and dimerization mode observed previously for dynamin GTPase. In addition, these studies showed that GIMAP2 localized at the surface of lipid droplets, where it is thought to act as a nucleotide-regulated scaffolding protein (4). Other members of the GIMAP family appear to be localized to different subcellular compartments (5). Overall, the function of these proteins remains poorly defined.

Gimap5 was reported recently to localize in lysosomes, based on studies in human, mouse, and rat lymphocytes (5). Genetic aberrations in Gimap5 have been strongly linked to reduced lymphocyte survival and homeostasis but, importantly, also have been associated with autoimmune diseases. In humans, polymorphisms in GIMAP5 were associated with increased concentrations of Ia2 autoantibodies in type 1 diabetes (T1D) patients (6) and an increased risk of systemic lupus erythematosus (7, 8). Studies using biobreeding rats—carrying a mutation (lpy/lpy) in Gimap5—show marked lymphopenia and predisposition to the development of T1D (9–11). In addition, biobreeding rats are prone to develop intestinal inflammation on certain genetic backgrounds (12). Together, these observations suggest that, beyond lymphocyte survival, Gimap5 is essential for maintaining immunological tolerance. Interestingly, impaired lymphocyte survival and consequent lymphopenia may be linked to the loss of immunological tolerance. Specifically, CD4<sup>+</sup> T cells in a lymphopenic environment can undergo thymus-independent expansion in the periphery. This process—also referred to as homeostatic or lymphopenia-induced proliferation (LIP)—is accompanied by marked alterations in T cell phenotype and is linked to autoimmunity (13–15). Most notably, T cells undergoing LIP acquire a memory-like phenotype, exemplified by high surface expression of CD44 and low surface expression of CD62L. In addition, under lymphopenic conditions, CD4<sup>+</sup> T cells more readily adopt an effector phenotype, including the ability to robustly produce cytokines upon stimulation through the TCR. The downstream consequences can be severe, and a number of pathological conditions have been associated with CD4<sup>+</sup> T cells undergoing LIP, including colitis. Classic studies involving the adoptive transfer of naive CD45RB<sup>high</sup> CD4<sup>+</sup> T cells into lymphopenic SCID mice resulted in T cells acquiring a LIP phenotype and rapidly driving colitis when recipient mice were colonized by intestinal bacteria (16–18). Importantly, colitis could be prevented if CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells were cotransferred, suggesting that the presence or absence of Treg cells is an important determinant of immune-mediated sequelae induced by CD4<sup>+</sup> T cells undergoing LIP.

Our laboratory previously described an ENU germline mutant, designated sphinx, which contained a recessive mutation in

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Abbreviations used in this article: Cdk, cyclin-dependent kinase; Foxo, Forkheadbox protein; LIP, lymphopenia-induced proliferation; Lp, lamina propria; MLN, mesenteric lymph node; pRb, retinoblastoma protein; Skp2, S-phase kinase-associated protein-2; T1D, type 1 diabetes; Treg, regulatory T.
Gimap5 that disrupted both lymphocyte survival and normal hematopoiesis (19). Similar to Gimap5 knockout mice, these mice lack peripheral NK cells and CD8+ T cells and exhibit dynamic changes in immune homeostasis, marked by the progressive loss of CD4+ T cells and B cells and neutrophilia (19, 20). After the collapse of lymphocyte populations, CD4+ T cells in Gimap5poph mice acquire a LIP phenotype similar to that of CD4+ T cells transferred into lymphopenic hosts (18). Around 10–12 wk of age, Gimap5poph mice develop wasting disease and colitis, limiting their survival (19). Interestingly, adoptive transfer of Rag-sufficient splenocytes into Gimap5poph mice around 5 wk of age could restore lymphocyte homeostasis and prevent colitis and wasting (19).

In this report, we show that CD4+ T cells are required for the development of colitis in Gimap5poph mice. Whereas CD4+ T cells exhibited impaired proliferation, they remained highly capable of producing proinflammatory cytokines, including IL-17a and IFN-γ. Importantly, CD4+ T cells in Gimap5poph mice exhibited a LIP phenotype and exhibited a progressive and complete loss of full-length Forkheadbox group O (Foxo)1, Foxo3, and Foxo4 expression. This loss of Foxo expression was associated with a progressive reduction in the numbers and suppressive capacity of Foxp3+ Treg cells. The development of colitis in Gimap5poph mice could be prevented by transferring wild-type Treg cells into 3-wk-old Gimap5poph mice. Because Foxo-deficient mice exhibit many of the phenotypes observed in Gimap5poph mice, including impaired Treg cell activity and colitis, our data suggest that the loss of immunological tolerance in Gimap5-deficient mice may be critically linked to the loss of Foxo expression in CD4+ T cells.

**Materials and Methods**

**Mice and reagents**

All of the experiments were performed according to U.S. National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children’s Hospital. C57BL/6J, Rag1−/−, CD45.1 congenic, and CD90.1 congenic mice were obtained from The Jackson Laboratory. Gimap5poph mice were generated as described previously (19) and bred in the vivarium of the Cincinnati Children’s Hospital. All of the mice were maintained under specific pathogen-free conditions.

All of the Abs used for flow cytometry were purchased from eBioscience or Biolegend. Abs for Western blotting (anti-Foxo3a (2497), anti-Foxo1 (2880), anti-pFoxo1(Thr24)/pFoxo3a(Thr32) (9464), anti-Foxo (9472), p27, p-retinoblastoma protein (pRb) (S807, S811), p-pRb (S780), and pan-actin Abs) were purchased from Cell Signaling Technology. Purified CD3ε (145-2C11) and CD25 (37.51) Abs (ebioScience) were used for T cell activation. PMA and ionomycin were obtained from Sigma-Aldrich.

**Real-time PCR**

CD4+ T cells were isolated from spleens and lymph nodes of 4- and 7-wk-old Gimap5poph and C57BL/6 mice using LJT4 MicroBeads (Miltenyi Biotec). RNA isolation was done with a RNeasy Micro Kit (Qiagen), and reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were amplified with LightCycler 480 SYBR Green I Master (Roche) and quantified by LightCycler 480 SYBR Green I Master (Roche). All of the experiments were performed according to U.S. National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children’s Hospital. C57BL/6J, Rag1−/−, CD45.1 congenic, and CD90.1 congenic mice were obtained from The Jackson Laboratory. Gimap5poph mice were generated as described previously (19) and bred in the vivarium of the Cincinnati Children’s Hospital. All of the mice were maintained under specific pathogen-free conditions.

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**Flow cytometry and T cell analyses**

To quantify T cell proliferation, MACS-purified (Miltenyi Biotec) CD4+ T cells were labeled with 5 μM CFSE in PBS with 0.1% FCS for 10 min. Cells were cultured in supplemented IMDM media containing 10% FCS and 1% penicillin/streptomycin and were either left unstimulated or stimulated with PMA (50 ng/ml/ionomycin (1 μg/ml). After 3 d of incubation, proliferation was measured by analyzing CFSE dilution using flow cytometry. To assess the capacity of T cells to produce cytokines, MACS-purified CD4+ T cells were incubated for 6 h with or without PMA (10 ng/ml/ionomycin (10 μg/ml) and subsequently fixed and analyzed for intracellular IL-17A, IL-4, or IFN-γ production using flow cytometry. To measure surface markers ex vivo, CD4+ T cells from spleen, mesenteric lymph node (MLN), or lamina propria (LP) were isolated and stained with fluorochrome-labeled Abs specific for mouse CD44, CD62L, and CD69.

Foxp3 expression was analyzed by intracellular staining.

**BrdU staining**

T cell-specific BrdU incorporation was measured as follows: during an interval of 24 h, wild-type or Gimap5poph mice received three i.p. injections with 100 μl of a 10 mg/ml BrdU solution in sterile PBS. Incorporation of BrdU in CD4+ T cells was measured 8 h after the last injection using flow cytometry.

**In vitro Treg cell suppressor assays**

The Treg cell suppressor assay was performed under the conditions described previously (21, 22). Briefly, spleens were isolated, and Treg cells were MACS-purified using the CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec). Subsequently, Treg cells were harvested and cocultured at the indicated ratios with 5 × 10^6 MACS-purified CFSE-labeled CD8+ T cells or CD4+ T cells. Also included were 1 × 10^6 T cell-depleted, gamma-irradiated (1500 rad) splenocytes as bystander cells and 0.5 μg/ml soluble CD3 Ab. CFSE dilution was assessed by flow cytometry after 3 d of coculture.

**Histology**

Colon tissue was collected and immediately fixed in 10% buffered formalin solution overnight, followed by routine paraffin embedding. H&E staining was performed on 4-μm sections from the paraffin-embedded tissue blocks for conventional light microscopy analysis. Histological scoring was performed as described previously (23). Briefly, scoring parameters included quantification of the area of distal colon involved, edema, erosion/ ulceration of the epithelial monolayer, crypt loss/damage, and infiltration of immune cells into the mucosa. Severity for the area involved (erosion/ ulceration and crypt loss) was graded on a scale of 0 (normal), 1 (0–10%), 2 (10–25%), 3 (25–50%), and 4 (>50%). Immune cell infiltration was scored as: 0, absent; 1, weak; 2, moderate; 3, severe. Total disease score was expressed as the mean of all of the combined scores per genotype.

**Adoptive transfer and survival assays**

For adoptive transfer studies, Gimap5poph mice at 25–35 d of age were injected i.v. with 3 × 10^6 Treg cells isolated from C57BL/6d mice using a Treg cell isolation kit (Miltenyi Biotec). Purity was confirmed by Foxp3 staining using flow cytometry, and cells were >90% Foxp3+. Mice were monitored and weighed every week after cell transfer.

**Statistical analysis**

Data were analyzed using Prism4 software (GraphPad). Unless indicated otherwise, statistical significance of the differences among groups was determined from the mean and SD by Student’s two-tailed test or by ANOVA followed by Dunnett’s test for three or more groups. Data were analyzed using Prism4 software (GraphPad). Unless indicated otherwise, statistical significance of the differences among groups was determined from the mean and SD by Student’s two-tailed test or by ANOVA followed by Dunnett’s test for three or more groups. Data were considered statistically significant if p values were <0.05.

**Results**

Gimap5poph CD4+ T cells from MLN are Th1/Th17 polarized

In previous work, we determined that NK, NKT, CD8+, CD4+, and B lymphocyte survival are impaired in Gimap5poph mice. In addition, they developed spontaneous colitis that required the presence of microbiota and survived poorly, with most mice succumbing by 150 d of age (19). Because several mouse models have linked impaired lymphocyte function with colitis development, we further explored the contribution of lymphocytes to the immunopathology observed in Gimap5poph mice. First, we investigated the survival and functional capacity of CD4+ T cells at different ages. By 4 wk of age, a reduced number of CD4+ T cells were found in the spleen, and a further decline in T cell numbers was observed in 6- and 10-wk-old Gimap5poph mice (Fig. 1A). Six-week-old Gimap5poph CD4+ T cells had a CD44hig
CD62Llow phenotype characteristic of T cells undergoing LIP (19) and showed increased incorporation of BrdU (Fig. 1B). To assess whether the loss of CD4+ T cells in the spleen and lymph nodes also was observed in GALT, we isolated LP cells from the colons of 6-wk-old Gimap5ph/sph mice and quantified the number of CD4+ T cells. Similar to the spleen, reduced numbers of CD4+ T cells were observed in the LP (Fig. 1C). Further analysis revealed that close to 100% of the colonic CD4+ T cells were CD44highCD62Llow, resembling the LIP phenotype of the CD4+ T cells in the peripheral lymphoid tissues (Fig. 1C). Together, these data suggest that CD4+ T cells are present in GALT and exhibit a LIP phenotype similar to that observed in the spleen.

We next investigated the functional capacity of CD4+ T cells in Gimap5ph/sph mice and their potential for contributing to the development of colitis. Our previous work indicated that 8-wk-old Gimap5ph/sph CD4+ T cells were unable to proliferate ex vivo after stimulation with PMA/ionomycin or anti-CD3 Ab, even though lymphocytes exhibited normal activation of NF-kB and MAPK pathways (19). Because of the latter observation, we investigated whether CD4+ T cells were capable of producing cytokines after such stimulation and, if so, were Th1, Th2, or Th17 polarized. We isolated total lymphocytes from spleens and MLNs from C57BL/6J control or Gimap5ph/sph mice and incubated cells for 6 h with or without PMA/ionomycin in the presence of brefeldin. Interestingly, a higher percentage of CD4+ T cells derived from Gimap5ph/sph spleens or MLNs produced IFN-γ, IL-17A, or both cytokines after PMA/ionomycin stimulation (Fig. 1D). Notably, T cell cytokine production was observed even in the absence of PMA/ionomycin in Gimap5ph/sph MLN cells (but not splenic leukocytes), suggesting constitutive activation of T cells in GALT in these mice. Overall, these data indicate that, despite their inability to proliferate normally ex vivo, CD4+ T cells derived from Gimap5ph/sph mice become Th1/Th17 polarized and effectively produce cytokines.

**Colitis in Gimap5ph/sph mice is driven by CD4+ T cells**

Because of their LIP phenotype and spontaneous production of IL-17A and IFN-γ, we hypothesized that MLN CD4+ T cells may support the development of colitis in Gimap5ph/sph mice. We tested this hypothesis by depleting CD4+ T cells in Gimap5ph/sph mice using weekly injections of anti-CD4 (GK1.5) Abs, starting at 3 wk of age—before the CD4+ T cells “collapse” and the subsequent intestinal inflammation normally occurs in Gimap5ph/sph mice. Importantly, GK1.5 treatment, but not isotype treatment, prevented wasting disease (Fig. 2A) and significantly decreased intestinal inflammation as determined by histology in 15-wk-old Gimap5ph/sph mice (Fig. 2B–H). These data support our hypothesis that the development of colitis in Gimap5ph/sph mice requires CD4+ T cells.

Gimap5ph/sph mice fail to maintain a Treg cell population with normal immunosuppressive function

Colitis induced by naive CD45RBhigh T cell transfer into SCID recipients does not occur when Treg cells are cotransferred.

**FIGURE 1.** Phenotypic characterization of CD4+ T cells in Gimap5ph/sph mice. A and B, Splenic CD4+ T cell population collapse around 6 wk of age in Gimap5ph/sph mice (A), at which time they exhibit a LIP phenotype with increased BrdU uptake (B). C, The number of CD4+ T cells and the percentage of CD44highCD62Llow CD4+ T cells in LP of 6-wk-old wild-type and Gimap5ph/sph mice. D, Ex vivo cytokine production by CD4+ T cells isolated from wild-type or Gimap5ph/sph spleens and MLNs, left unstimulated or after stimulation with PMA/ionomycin (100 ng/ml) for 6 h (mean values ± SEM; n ≥ 4 mice per genotype from two independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001.
Therefore, even though Treg cell development in the thymus of Gimap5<sup>sh/sph</sup> mice appeared to occur normally (19), we considered that Treg cell function may be impaired in the peripheral tissues of these lymphopenic mice and contribute to the development of colitis. Thus, we examined the presence and immunosuppressive capacity of Foxp3<sup>+</sup> Treg cells in Gimap5<sup>sh/sph</sup> mice. Although relatively normal numbers of Foxp3<sup>+</sup> Treg cells were observed in 3-wk-old mice (Supplemental Fig. 2A), Treg cells became significantly reduced in the MLNs of 6- to 8-wk-old mice, both as a percentage within the CD4<sup>+</sup> T cell compartment and as a total number of cells (Fig. 3A, 3B). In the spleen, the number of Treg cells was reduced, but the percentage of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells remained similar to the percentage observed in wild-type mice (Fig. 3A, 3B). To assess their functional capacity, we purified Treg cells from 4- or 6-wk-old C57BL/6J or Gimap5<sup>sh/sph</sup> spleens and cocultured Treg cells with CFSE-labeled wild-type CD8<sup>+</sup> T cells that were stimulated with soluble anti-CD3 Abs. Treg cells from 4-wk-old Gimap5<sup>sh/sph</sup> mice showed a slight but significant reduction in their ability to suppress CD8<sup>+</sup> T cell proliferation in vitro, whereas Treg cells isolated from 6-wk-old Gimap5<sup>sh/sph</sup> mice were incapable of suppressing CD8<sup>+</sup> T cell proliferation (Fig. 3C). Similar results were obtained for the suppression of wild-type CD4<sup>+</sup> T cell proliferation (Supplemental Fig. 1A). These findings suggest that both Treg cell survival and functional capacity become impaired as Gimap5<sup>sh/sph</sup> mice age.

We next questioned whether reduced peripheral Treg cell accumulation in Gimap5<sup>sh/sph</sup> mice resulted from a cell-intrinsic phenomenon. We injected CD4<sup>+</sup> splenocytes from 3-wk-old wild-type and/or Gimap5<sup>sh/sph</sup> mice into Rag-deficient recipients, as either a mixture or alone, and quantified the presence of Foxp3<sup>+</sup> Treg cells 5 wk after injection (Supplemental Fig. 2A).

Whereas no differences in the percentage of Treg cells within the CD4<sup>+</sup> T cell compartment were observed at the time of injection, after 5 wk the Gimap5<sup>sh/sph</sup> Treg population was lost, regardless of whether wild-type cells were cotransferred or not (Fig. 3D). Overall, these data indicate that cell-intrinsic expression of Gimap5 is required to allow normal Treg cell survival.

Gimap5<sup>sh/sph</sup> CD4<sup>+</sup> T cells exhibit progressive loss of Foxo1, Foxo3, and Foxo4 expression

Our data indicate that the Gimap5<sup>sh/sph</sup> CD4<sup>+</sup> T cell population collapses around 5 wk of age and that the remaining CD4<sup>+</sup> T cells undergo LIP thereafter. At the same time, they fail to maintain a functional Treg cell population. Interestingly, these T cell phenotypes show striking similarities with those seen in mice with T cells deficient in the family of Foxo transcription factors. The family of Foxo transcription factors contains four members, three of which (Foxo1, Foxo3, and Foxo4) have overlapping patterns of expression and transcriptional activities (24–26). They play an essential role in the regulation of cell cycle progression, apoptosis, glucose metabolism, and life span (27). Foxo1 expression is critical for maintaining naive T cell quiescence. Foxo1-deficient CD4<sup>+</sup> T cells exhibit a CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> low LIP or effector memory phenotype (28–30). In addition, Foxo expression has been reported to be essential for Treg cell development and function (29, 31). We therefore analyzed the expression of Foxo1, Foxo3, and Foxo4 in Gimap5<sup>sh/sph</sup> CD4<sup>+</sup> T cells. Strikingly, immunoblot analysis of CD4<sup>+</sup> T cells from 6-wk-old Gimap5<sup>sh/sph</sup> mice revealed a near absence of full-length Foxo1, Foxo3a, and Foxo4 protein (Fig. 4A). At the mRNA level, a reduction in Foxo1 but not Foxo3 and Foxo4 could be observed in CD4<sup>+</sup> T cells isolated from Gimap5<sup>sh/sph</sup> mice compared with those isolated from wild-type mice (Fig. 4B), suggesting that regulation of Foxo3 and Foxo4 protein expression occurred at the posttranscriptional level. Because many of the T cell-specific phenotypes observed in Gimap5<sup>sh/sph</sup> mice occur after 4 wk of age, we next quantified the temporal progression of changes in Foxo expression in lymphocytes. Immunoblot analyses revealed that Foxo expression was normal at 3 wk, somewhat reduced after 4 wk, and almost absent after 6–10 wk of age (Fig. 4C, Supplemental Fig. 1A). Concordant with the loss of Foxo expression, we detected reductions in the abundance of the cyclin-dependent kinase (Cdk) inhibitor p27<sup>Kip1</sup>, a downstream target of Foxo proteins and an important regulator of cell cycle entry (Fig. 4C) (32, 33). Because p27<sup>Kip1</sup> inhibits Cdk4, we measured Cdk4 activity and detected increased phosphorylation of its substrate, pRb, in Gimap5<sup>sh/sph</sup> cells (Fig. 4C). Due to the progressive nature of this phenotype, we considered the
onset of lymphopenia in these data link the loss of full-length Foxo expression with the possibility that T cell activation. Overall, Treg cells from Gimap5sph/sph mice did not 8-wk-old, Gimap5sph/sph assessed whether the loss of Foxo expression also was observed in TCR stimulation (Supplemental Fig. 3A) (19). Finally, we characterized whether the loss of Foxo expression also was observed in Treg cells from Gimap5sph/sph mice. Indeed, Foxo1 and Foxo3 expression was mostly absent in CD4+CD25+ T cells from 6-wk-old Gimap5sph/sph mice. Interestingly, colitis could be prevented by the transfer of CD45.1 congenically marked CD4+CD25+ Treg cells (17). Notably, the Foxp3+CD4+ Treg cell population constituted 40% of the overall CD4+ T cell population and was entirely congenic, whereas the Foxp3+CD4+ Treg cell population was predominantly Gimap5sph/sph-derived (Fig. 5E). Functional analysis of isolated CD4+ T cells from spleens and MLNs of 15-wk-old treated Gimap5sph/sph mice revealed no background cytokine production and activation similar to that observed for wild-type CD4+ T cells after stimulation with PMA/ionomycin (Supplemental Fig. 2D). Around 25 wk of age, Treg cell-recipient mice still developed wasting disease. Necropsy at this time revealed severe inflammation in the lung and infiltration of macrophages in a number of mice (Supplemental Fig. 2C). Interestingly, colitis could be prevented by the transfer of Il10−/− splenocytes (data not shown), suggesting that IL-10–independent regulatory pathways are more perturbed by Gimap5 deficiency. Together, these data link impaired Treg cell survival and function to the development of colitis in Gimap5sph/sph mice. In addition, they reveal that the possibility that lymphocytes isolated from young Gimap5sph/sph mice with intact Foxo expression might respond normally to mitogenic stimuli. Indeed, CD4+ T cells isolated from 4-wk-old, but not 8-wk-old, Gimap5sph/sph mice were able to proliferate after TCR stimulation (Supplemental Fig. 3A) (19). Finally, we assessed whether the loss of Foxo expression also was observed in Treg cells from Gimap5sph/sph mice. Indeed, Foxo1 and Foxo3 expression was mostly absent in CD4+CD25+ T cells from 6-wk-old Gimap5sph/sph mice (Fig. 4D).

Interestingly, the progressive loss of Foxo expression appeared to correlate with a progressive increase in the number of CD4+ T cells undergoing LIP (CD44hiCD62Llo) in 4- to 10-wk-old Gimap5sph/sph mice as reported previously (19). Subsequent analysis of CD44hiCD62Llo and CD44hiCD62Lhi CD4+ T cells from 5-wk-old Gimap5sph/sph and wild-type mice revealed the loss of Foxo expression specifically in CD4+ T cells undergoing LIP but not in naïve CD4+ T cells in Gimap5sph/sph mice (Fig. 4E), suggesting that the loss of Foxo expression follows T cell activation. Overall, these data link the loss of full-length Foxo expression with the onset of lymphopenia in Gimap5sph/sph lymphocytes, impaired cell cycle control and proliferative capacity in such lymphocytes, and reduced Treg cell survival and function in Gimap5sph/sph mice.

Prevention of colitis in Gimap5sph/sph mice by the adoptive transfer of wild-type Treg cells

Our previous data show that colitis can be prevented in Gimap5sph/sph mice through adoptive transfer of normal, but not Rag-deficient, splenocytes (19), indicating that a lymphocyte population is responsible for the rescue. Given the impaired Treg cell survival and function observed in Gimap5sph/sph mice, we next examined whether adoptively transferred wild-type Treg cells could prevent the development of colitis. Gimap5sph/sph recipients of 3 × 10^5 wild-type CD4+CD25+ T cells showed prolonged survival and delayed wasting disease (Fig. 5A) and, importantly, did not develop colitis (Fig. 5B–D). Characterization of lymphocyte populations 25 wk after transfer of CD45.1 congenically marked CD4+CD25+ Treg cells revealed that Treg cell reconstitution of the spleens of Gimap5sph/sph mice achieved ~50% of the level observed in wild-type C57BL/6J mice (Fig. 5E). Notably, the Foxp3+CD4+ Treg cell population constituted 40% of the overall CD4+ T cell population and was entirely congenic, whereas the Foxp3+CD4+ Treg cell population was predominantly Gimap5sph/sph-derived (Fig. 5E). Functional analysis of isolated CD4+ T cells from spleens and MLNs of 15-wk-old treated Gimap5sph/sph mice revealed no background cytokine production and activation similar to that observed for wild-type CD4+ T cells after stimulation with PMA/ionomycin (Supplemental Fig. 2D). Around 25 wk of age, Treg cell-recipient mice still developed wasting disease. Necropsy at this time revealed severe inflammation in the lung and infiltration of macrophages in a number of mice (Supplemental Fig. 2C). Interestingly, colitis could be prevented by the transfer of Il10−/− splenocytes (data not shown), suggesting that IL-10–independent regulatory pathways are more perturbed by Gimap5 deficiency. Together, these data link impaired Treg cell survival and function to the development of colitis in Gimap5sph/sph mice. In addition, they reveal that the...
**FIGURE 4.** CD4+ T cells in *Gimap5sph/sph* progressively lose full-length Foxo expression in lymphocytes. *A*, Immunoblot analysis of phosphorylated Foxo1/3, total Foxo1, Foxo3, and Foxo4 in total splenocytes from 6-wk-old mice. *B*, Foxo1, Foxo3, and Foxo4 mRNA abundance in CD4+ T cells isolated from wild-type or *Gimap5sph/sph* spleens, as measured by quantitative real-time PCR. *C*, Loss of Foxo expression in *Gimap5sph/sph* lymphocytes correlates with decreased p27kip1 expression and increased phosphorylation of pRb. *D*, Foxo protein expression in CD4+CD25+ T cells isolated from 6-wk-old wild-type or *Gimap5sph/sph* spleens (sph/sph, homozygote; +/sph, heterozygote). *E*, Foxo3 expression in CD44hiCD62Llo and CD44hiCD62Lhi CD4+ T cells isolated from 5-wk-old homozygote *sphinx* mice and heterozygote littermate controls. Data represent mean values ± SEM, and blots are representative of three independent experiments (*n* = 3). ***p < 0.001.

*Gimap5sph/sph* environment is capable of supporting a functional Treg cell population.

**Discussion**

Genetic aberrancies in *Gimap5* have been linked to lymphopenia and the loss of immunological tolerance (6, 7, 9, 10). Although we found no evidence of autoimmune responses in *Gimap5sph/sph* mice, we observed severe and spontaneous inflammation in the gut (19)—an environment where homeostasis critically depends upon maintaining tolerance to exogenous Ags and bacterial stimuli. Similar to *Gimap5sph/sph*, mice, the loss of immunological tolerance in the lyp/lyp rat has been associated with reduced Treg cell survival and function as well as the polarization of Th cells toward a Th17 pattern of differentiation (34, 35). However, the molecular pathways underlying the loss of immunological tolerance in rat and mouse models of *Gimap5* deficiency have remained elusive. In this study, we explored the pathways that contribute to the loss of tolerance observed in *Gimap5sph/sph* mice. We show that the development of colitis in *Gimap5sph/sph* mice is critically dependent on CD4+ T cells. Around 8 wk of age, CD4+ T cells lose their capacity to proliferate ex vivo, yet they remain capable of producing proinflammatory cytokines, including IL-17A and IFN-γ, and contain a population of IL-17A+IFN-γ+ Th1/Th17 cells that have been associated with IL-23 signaling and more severe colitis (36). At the same time, Treg cell numbers and function decline. Most importantly, we found that these phenotypes are associated directly with a progressive loss of protein expression of Foxo1, Foxo3, and Foxo4—important transcription factors that regulate both quiescence and survival of lymphocytes (37). Mice with T cell-specific deletions of Foxo1 and/or Foxo3 mimic many of the immunological and pathological phenotypes observed in *Gimap5sph/sph* mice (28–31). For example, Foxo-deficient CD4+ T cells have impaired proliferative capacity and adopt CD44highCD62Llow LIP or memory-like phenotypes (28, 30). In addition, reduced Treg cell numbers and function were observed in mice lacking Foxo1 or both Foxo1 and Foxo3 in T cells. Similar to *Gimap5sph/sph* mice, mice lacking Foxo1 and Foxo3 in T cells develop spontaneous colitis, and furthermore purified Foxo1−/−Foxo3−/− Treg cells were unable to prevent colitis in Rag1−/− mice when coinfected with naive wild-type CD4+ T cells (31). Interestingly, the impaired Treg cell development and function in T cell-specific Foxo1-deficient mice caused exaggerated T follicular helper cell accumulation, which contributed to B cell-mediated autoimmunity (29). In *Gimap5sph/sph* mice, we found no evidence of autoreactive B cells (19), but it is important to note that, similar to CD4+ T cells, *Gimap5sph/sph* B cells progressively lost Foxo expression (Supplemental Fig. 3B) and were unable to proliferate after stimulation with IgM (19). Thus, B cell expansion and differentiation may be hampered severely in *Gimap5sph/sph* mice, preventing the development of autoreactive Ab responses.

The mechanisms by which Foxo transcription factors control Treg cell development, homeostasis, and function have been studied in some detail. Foxo proteins have been shown to serve as coactivators downstream of the TGF-β signaling pathway by interacting with SMAD proteins, ultimately fine-tuning the TGF-β-induced transcriptional program (38, 39). This pathway is also critical for the development of inducible Treg (iTreg) cells (40), which develop extrathymically and have been suggested by many studies to comprise an important population of Treg cells in the gut (21, 41–43). Indeed, the loss of Treg cells within the CD4+ T cell compartment is most evident in the MLNs of *Gimap5sph/sph* mice (Fig. 3), suggesting that the iTreg cells in particular are impaired. In addition, Foxo1 and Foxo3 can cooperatively control the differentiation of Foxp3+ Treg cells through the regulation of a number of Treg cell-associated genes, including Foxp3 itself (31). Furthermore, conditional deletion of Foxo1 in T cells
resulted in reduced surface expression of CTLA-4 and CD25 in Foxp3\(^+\)CD4\(^+\) T cells (29). Analysis of the \(\text{Ctla-4} \) gene showed that the promoter region contained a conserved Foxo binding site 193 bp upstream of the transcription start site (44). Thus, the impaired function of Foxp3\(^+\) Treg cells is likely the result of an incomplete transcriptional program in the absence of Foxo expression. In summary, the loss of Foxo expression affects multiple pathways that regulate Treg cell development, homeostasis, and function as well as the generation of iTreg cells.

In Gimap5\(^{ sph/sph} \) mice, the absence of Foxo expression was observed in all of the lymphocyte populations examined, including peripheral Foxp3\(^+\) Treg cells, conventional Foxp3\(^-\)CD4\(^+\) T cells, and B cells. Although conventional Foxp3\(^-\)CD4\(^+\) T cells lack Foxo expression, our experiments reveal that colitis can be prevented by treatment with competent wild-type Treg cells, suggesting that colitogenic CD4\(^+\) T cells remain capable of being regulated when they lack Gimap5. Although we link the sphinx mutation in Gimap5 to the progressive loss of Foxo expression in lymphocytes, it is unclear to what extent these genes directly interact with each other. Given the progressive nature of the loss of Foxo expression, it is unlikely that Gimap5 directly interacts with Foxo proteins. One possibility we considered is that the loss of Foxo expression may drive a secondary phenotype resulting from constitutive proliferation cues associated with LIP, something that may be driven by self-antigens or Ags derived from the microbiota. Although we cannot exclude the possibility that LIP may contribute to the loss of Foxo expression in Gimap5\(^{ sph/sph} \) CD4\(^+\)RB45\(^{ high} \) T cells, wild-type CD4\(^+\)RB45\(^{ high} \) T cells transferred into
a lymphopenic host retained normal levels of Foxo expression (Supplemental Fig. 3C), suggesting that LIP alone is insufficient to cause the loss of Foxo expression. Our data show that Gimap5 deficiency affects Foxo3 and Foxo4 expression at the protein level, not at the mRNA level. Regulation of Foxo proteins has been reported previously to occur via ubiquitination and proteasomal degradation (45). Moreover, the loss of Foxo1 expression has been observed in mouse lymphomas, which served as a mechanism to remove the tumor suppressor activity of Foxo1 (46). Foxo degradation was correlated inversely with increased expression of S-phase kinase-associated protein-2 (Skp2)—an E3 ubiquitin ligase that targets numerous cell cycle proteins. Foxo degradation was reversed after the downregulation of Skp2 via short hairpin RNAs, and therefore, increased Skp2 expression could provide a potential mechanism by which the loss of Foxo expression in Gimap5sp/sph T cells occurs. Alternatively, the localization of Gimap5 in the lysosomal compartment (5) and the presumed scaffolding function of Gimap family members (4) suggest that Gimap5 may be necessary for optimal lysosomal function. Lysosomes are essential for the catabolic turnover of intra- and extracellular macromolecules but also can release lysosomal enzymes (such as cathepsins) that can initiate programmed cell death once in the cytosol (47). Intriguingly, lymphocytes containing large numbers of cytotoxic granules, such as CD8+ T cells and NK cells, do not survive in Foxo5-deficient mice, perhaps supporting the hypothesis of a deregulated lysosomal compartment. Although the link between the loss of Gimap5 and Foxo protein expression remains to be established in human cells, understanding the molecular pathways that lead to the degradation of Foxo proteins could provide important therapeutic targets, not only in the context of tumor growth, but potentially in the context of autoimmune or chronic inflammatory disorders.

Our data provide evidence that Gimap5 is essential for maintaining lymphocyte quiescence and immunological tolerance. In the absence of functional Gimap5, Foxo expression in lymphocytes is progressively lost, with the loss of Foxo3 and Foxo4 most likely involving a proteolytic mechanism. This progressive loss of Foxo expression is associated with concomitant decline in Treg numbers and function, which ultimately leads to the loss of immunological tolerance in the gut. Thus, not only do we establish a critical link between Gimap5 and Foxo protein levels, we also provide evidence for a novel regulatory mechanism controlling Foxo protein expression that may be involved in the development of immune-mediated diseases such as systemic lupus erythematosus, T1D, and colitis.

Disclosures
The authors have no financial conflicts of interest.

References
1. Nitta, T., M. Narren, T. Seike, A. Goji, I. Ogashashi, T. Miyazaki, T. Ohta, M. Kanno, and Y. Takahama. 2006. IAN family critically regulates survival and function, which ultimately leads to the loss of immunological disorders. Our data provide evidence that Gimap5 is essential for maintaining lymphocyte quiescence and immunological tolerance. In the absence of functional Gimap5, Foxo expression in lymphocytes is progressively lost, with the loss of Foxo3 and Foxo4 most likely involving a proteolytic mechanism. This progressive loss of Foxo expression is associated with concomitant decline in Treg numbers and function, which ultimately leads to the loss of immunological tolerance in the gut. Thus, not only do we establish a critical link between Gimap5 and Foxo protein levels, we also provide evidence for a novel regulatory mechanism controlling Foxo protein expression that may be involved in the development of immune-mediated diseases such as systemic lupus erythematosus, T1D, and colitis.

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Supplementary figure legends

Supplementary figure 1. Progressive loss immunosuppressive function in Gimap5^{sph/sph} mice is associated with a loss of Foxo expression in CD4^{+} T cells. (A) Reduced capacity of Treg cells isolated from 3- or 6-week-old Gimap5^{sph/sph} mice to suppress proliferation of C57BL/6J CD4^{+} T cells stimulated with αCD28/αCD3-activated, as measured by CFSE dilution after 72 hours incubation in vitro. (B) Immunoblot analysis of total Foxo1, Foxo3 and Foxo4 in CD4^{+} T cells from 3 or 6-week-old mice. Data represents mean values ± SEM (n > 3 mice per genotype); blots are representative of three independent experiments (n = 3).

Supplementary figure 2. (A) Transfer of a mixture of purified congenic wildtype and Gimap5^{sph/sph} CD4^{+} T cells into 6 week-old Rag1^{-/-} recipient mice. Mice were analyzed 5 weeks after transfer. The percentage of Foxp3^{+} Treg cells in wildtype and Gimap5^{sph/sph} CD4^{+} T cells at the time of injection is shown. (B) Rescue studies in which 3x10^{5} CD25^{+} CD4^{+} cells isolated from congenic (CD45.1) C57BL/6J mice are injected into 4 week-old Gimap5^{sph/sph} mice. Analysis was performed after 25 weeks. (C) Inflammatory lung infiltrate as observed in a number of Treg “rescued” Gimap5^{sph/sph} mice. (D) Ex vivo cytokine production by CD4^{+} T cells isolated from 15-week-old wildtype or Treg-treated Gimap5^{sph/sph} spleen and mesenteric lymph node (MLN), left unstimulated or following stimulation with PMA/ionomycin (100ng/ml) for six hours. (Data represents mean values ± SEM; n ≥ 3 mice per genotype from 3 independent experiments).

Supplementary Figure 3. (A) Proliferation of CD4^{+} T cells isolated from 4 or 8 week-old wildtype C57BL/6J or Gimap5^{sph/sph} mice. Cells were stimulated with αCD3 and proliferation was measured by CFSE-dilution after 3 days of incubation. (B) Progressive loss of Foxo3 expression in B cells isolated from Gimap5^{sph/sph} but not wildtype cells. (C) Foxo3 expression in CD4^{+} T cells isolated from wildtype spleen (control) or following the transfer of wt CD4^{+} RB45^{high} CD4^{+} T cells in Rag^{-/-} recipient mice. The latter were analyzed 5 weeks after transfer. Data represents mean values ± SEM; n > 4 mice per genotype from 2 independent experiments; blots are representative blots of B cells pooled from three mice.
Supplemental Figure 1

A

4 week

6 week

% proliferation

Treg : CD4+ T cell ratio

C57BL/6J

Gimap5sph/sph

* *

**

B

3 weeks

+/sph

sph/sph

6 weeks

+/sph

sph/sph

Foxo1

Foxo3

Actin

Treg : CD4+ T cell ratio

% proliferation
Supplemental Figure 2

A

3 week-old Gimap5sph/sph CD90.1 B6

CD4+ T cells

Adoptive Transfer i.v.

RAG2-/-

5 x 10^5

Analyze after 5 weeks

Normal Treg frequency at 3 weeks

B

CD45.1 B6

3 x 10^5 CD25+ CD4+ T cells

Adoptive Transfer

Gimap5sph/sph

4-week old

Analyze after 23 weeks

C

Gimap5sph/sph + Treg C57BL/6

Lung

D

spleen

MLN

C57BL/6J

Gimap5sph/sph

Unstimulated

PMA/ionomycin

IL17

IFN

CONTROL

C57BL/6J

Gimap5sph/sph

Unstimulated

PMA/ionomycin

IL17

IFN

CONTROL

C57BL/6J

Gimap5sph/sph

Unstimulated

PMA/ionomycin

IL17

IFN

CONTROL
Supplemental Figure 3

A

![Graph showing the number of cells for C57BL/6J and Gimap5^sph/sph mice under different conditions.](image)

B

<table>
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<th>3</th>
<th>4</th>
<th>6</th>
<th>10</th>
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<tr>
<td>wt</td>
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![Western blot showing total Foxo3a and actin expression.](image)

C

![Western blot showing Foxo3a and actin expression for CD45Rb^high transfer.](image)