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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 Gimap5sph/sph 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 Gimap5sph/sph mice. We show that CD4+ 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+ T cells are associated with the loss of Forkheadbox group O (Foxo)1, 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 to 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 (hyp/hyp) 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+ 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+ 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+ T cells undergoing LIP, including colitis. Classic studies involving the adoptive transfer of naive CD45RBhigh CD4+ 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+CD25+ regulatory T (Treg) cells were cotransferred, suggesting that the presence or absence of Treg cells is an important determinant of immunemediated sequelae induced by CD4+ T cells undergoing LIP.

Our laboratory previously described an ENU germline mutant, designated sphinx, which contained a recessive mutation in
**Gimap5 Deficiency Is Associated with Loss of Foxo Expression**

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 Gimap5sph/sph mice acquire a LIP phenotype similar to that of CD4+ T cells transferred into lymphopenic hosts (18). Around 10–12 wk of age, Gimap5sph/sph mice develop wasting disease and colitis, limiting their survival (19). Interestingly, adoptive transfer of Rag-sufficient splenocytes into Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph mice could be prevented by transferring wild-type Treg cells into 3-wk-old Gimap5sph/sph mice. Because Foxo-deficient mice exhibit many of the phenotypes observed in Gimap5sph/sph 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. Gimap5sph/sph 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-Foxo3α (2947), anti-Foxo1 (2880), anti-pFoxo1(Thr24)/pFoxo3α(Thr32) (9464), anti-Foxo3 (9472), p27, p-retinoblastoma protein (pRb) (S807, S811), p-pRb (S780), and pan-actin Abs) were purchased from Cell Signaling Technology. Purified CD3e (145-2C11) and CD28 (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 Gimap5sph/sph and C57Bl/6J mice using L3T4 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 II instrument (Roche) using the following primer pairs: Foxo1, forward, 5′-TTCTGGAATGACCTATGGAT-3′; Foxo4, forward, 5′-GGACTGCTCTACCTGTCTC-3′; Foxo3, forward, 5′-AGTGATGGTCGCTGTTG-3′, reverse, 5′-TCTGAACGCGCATGAAGC-3′; Foxo4, forward, 5′-GAGAACCTGGAGTGCGACATG-3′, reverse, 5′-GAGAACCTGGAGTGCGACATG-3′.

**Flow cytometry and T cell analyses**

To quantify T cell proliferation, MACS-purified (Miltenyi Biotec) CD4+ T cells were labeled with 5μM 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 (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 Gimap5sph/sph 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 × 104 MACS-purified CFSE-labeled CD8+ T cells or CD4+ T cells. Also included were 1 × 105 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, 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.

**Adaptive transfer and survival assays**

For adaptive transfer studies, Gimap5sph/sph mice at 25–35 d of age were injected i.v. with 3 × 106 Treg cells isolated from C57BL/6J 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 considered statistically significant if p values were <0.05.

**Results**

Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph mice (Fig. 1A). Six-week-old Gimap5sph/sph CD4+ T cells had a CD44high...
CD62L<sup>low</sup> phenotype characteristic of T cells undergoing LIP (19) and showed increased incorporation of BrdU (Fig. 1B). To assess whether the loss of CD4<sup>+</sup> T cells in the spleen and lymph nodes also was observed in GALT, we isolated LP cells from the colons of 6-wk-old Gimap<sup>5ph/sph</sup> mice and quantified the number of CD4<sup>+</sup> T cells. Similar to the spleen, reduced numbers of CD4<sup>+</sup> T cells were observed in the LP (Fig. 1C). Further analysis revealed that close to 100% of the colonic CD4<sup>+</sup> T cells were CD44<sup>high</sup>/CD62L<sup>low</sup>, resembling the LIP phenotype of the CD4<sup>+</sup> T cells in the peripheral lymphoid tissues (Fig. 1C). Together, these data suggest that CD4<sup>+</sup> 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<sup>+</sup> T cells in Gimap<sup>5ph/sph</sup> mice and their potential for contributing to the development of colitis. Our previous work indicated that 8-wk-old Gimap<sup>5ph/sph</sup> CD4<sup>+</sup> 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-κB and MAPK pathways (19). Because of the latter observation, we investigated whether CD4<sup>+</sup> 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 Gimap<sup>5ph/sph</sup> mice and incubated cells for 6 h with or without PMA/ionomycin in the presence of brefeldin. Interestingly, a higher percentage of CD4<sup>+</sup> T cells derived from Gimap<sup>5ph/sph</sup> 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 Gimap<sup>5ph/sph</sup> 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<sup>+</sup> T cells derived from Gimap<sup>5ph/sph</sup> mice become Th1/Th17 polarized and effectively produce cytokines.

**Colitis in Gimap<sup>5ph/sph</sup> mice is driven by CD4<sup>+</sup> T cells**

Because of their LIP phenotype and spontaneous production of IL-17A and IFN-γ, we hypothesized that MLN CD4<sup>+</sup> T cells may support the development of colitis in Gimap<sup>5ph/sph</sup> mice. We tested this hypothesis by depleting CD4<sup>+</sup> T cells in Gimap<sup>5ph/sph</sup> mice using weekly injections of anti-CD4 (GK1.5) Abs, starting at 3 wk of age—before the CD4<sup>+</sup> T cells “collapse” and the subsequent intestinal inflammation normally occurs in Gimap<sup>5ph/sph</sup> 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 Gimap<sup>5ph/sph</sup> mice (Fig. 2B–H). These data support our hypothesis that the development of colitis in Gimap<sup>5ph/sph</sup> mice requires CD4<sup>+</sup> T cells.

Gimap<sup>5ph/sph</sup> mice fail to maintain a Treg cell population with normal immunosuppressive function

Colitis induced by naive CD45RB<sup>high</sup> T cell transfer into SCID recipients does not occur when Treg cells are cotransferred.
Gimap5 DEFICIENCY IS ASSOCIATED WITH LOSS OF Foxo EXPRESSION

Therefore, even though Treg cell development in the thymus of Gimap5phpk 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+ Treg cells in Gimap5phpk mice. Although relatively normal numbers of Foxp3+ 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+ 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+ CD4+ T cells within the CD4 T cell population 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 Gimap5phpk spleens and cocultured Treg cells with CFSE-labeled wild-type CD8+ T cells that were stimulated with soluble anti-CD3 Abs. Treg cells from 4-wk-old Gimap5phpk mice showed a slight but significant reduction in their ability to suppress CD8+ T cell proliferation in vitro, whereas Treg cells isolated from 6-wk-old Gimap5phpk mice were incapable of suppressing CD8+ T cell proliferation (Fig. 3C). Similar results were obtained for the suppression of wild-type CD4+ T cell proliferation (Supplemental Fig. 1A). These findings suggest that both Treg cell survival and functional capacity become impaired as Gimap5phpk mice age.

We next questioned whether reduced peripheral Treg cell accumulation in Gimap5phpk mice resulted from a cell-intrinsic phenomenon. We injected CD4+ splenocytes from 3-wk-old wild-type and/or Gimap5phpk mice into Rag-deficient recipients, as either a mixture or alone, and quantified the presence of Foxp3+ Treg cells 5 wk after injection (Supplemental Fig. 2A). Whereas no differences in the percentage of Treg cells within the CD4+ T cell compartment were observed at the time of injection, after 5 wk the Gimap5phpk 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.

Gimap5phpk CD4+ T cells exhibit progressive loss of Foxo1, Foxo3, and Foxo4 expression

Our data indicate that the Gimap5phpk CD4+ T cell population collapses around 5 wk of age and that the remaining CD4+ 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+ T cells exhibit a CD44highCD62low 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 Gimap5phpk CD4+ T cells. Strikingly, immunoblot analysis of CD4+ T cells from 6-wk-old Gimap5phpk 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+ T cells isolated from Gimap5phpk 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 Gimap5phpk 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). Concurrent with the loss of Foxo expression, we detected reductions in the abundance of the cyclin-dependent kinase (Cdk) inhibitor p27kip1, a downstream target of Foxo proteins and an important regulator of cell cycle entry (Fig. 4C) (32, 33). Because p27kip1 inhibits Cdk4, we measured Cdk4 activity and detected increased phosphorylation of its substrate, pRb, in Gimap5phpk cells (Fig. 4C). Due to the progressive nature of this phenotype, we considered the
posibility that lymphocytes isolated from young Gimap5<sup>sph/sph</sup> mice with intact Foxo expression might respond normally to mitogenic stimuli. Indeed, CD4<sup>+</sup> T cells isolated from 4- to 10-wk-old Gimap5<sup>sph/sph</sup> 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 Gimap5<sup>sph/sph</sup> mice. Indeed, Foxo1 and Foxo3 expression was mostly absent in CD4<sup>+</sup>CD25<sup>+</sup> T cells from 6-wk-old Gimap5<sup>sph/sph</sup> mice. Interestingly, the progressive loss of Foxo expression appeared to correlate with a progressive increase in the number of CD4<sup>+</sup> T cells undergoing LIP (CD4<sup>+</sup>CD62L<sup>hi</sup>) in 4- to 10-wk-old Gimap5<sup>sph/sph</sup> mice as reported previously (19). Subsequent analysis of CD4<sup>+</sup>CD62L<sup>hi</sup> and CD4<sup>+</sup>CD62L<sup>lo</sup> CD4<sup>+</sup> T cells from 5-wk-old Gimap5<sup>sph/sph</sup> and wild-type mice revealed the loss of Foxo expression specifically in CD4<sup>+</sup> T cells undergoing LIP but not in naive CD4<sup>+</sup> T cells in Gimap5<sup>sph/sph</sup> 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 Gimap5<sup>sph/sph</sup> lymphocytes, impaired cell cycle control and proliferative capacity in such lymphocytes, and reduced Treg cell survival and function in Gimap5<sup>sph/sph</sup> mice.

Prevention of colitis in Gimap5<sup>sph/sph</sup> mice by the adoptive transfer of wild-type Treg cells

Our previous data show that colitis can be prevented in Gimap5<sup>sph/sph</sup> 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 Gimap5<sup>sph/sph</sup> mice, we next examined whether adoptively transferred wild-type Treg cells could prevent the development of colitis. Gimap5<sup>sph/sph</sup> recipients of 3 × 10<sup>5</sup> wild-type CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> Treg cells revealed that Treg cell reconstitution of the spleens of Gimap5<sup>sph/sph</sup> mice achieved ∼50% of the level observed in wild-type C57BL/6J mice (Fig. 5E). Notably, the Foxp3<sup>+</sup>CD4<sup>+</sup> T cell population constituted 40% of the overall CD4<sup>+</sup> T cell population and was entirely congenic, whereas the Foxp3<sup>-</sup>CD4<sup>+</sup> population was predominantly Gimap5<sup>sph/sph</sup>-derived (Fig. 5E). Functional analysis of isolated CD4<sup>+</sup> T cells from spleens and MLNs of 15-wk-old treated Gimap5<sup>sph/sph</sup> mice revealed no background cytokine production and activation similar to that observed for wild-type CD4<sup>+</sup> 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 the 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<sup>−/−</sup> 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 Gimap5<sup>sph/sph</sup> mice. In addition, they reveal that the

![Image](http://www.jimmunol.org/DownloadedFrom)
**Gimap5** has been linked to lymphopenia and the loss of immunological tolerance (6, 7, 9, 10). Although we found no evidence of autoimmune responses in rat models of Gimap5 deficiency have remained elusive. In this study, we explored the pathways that contribute to the loss of tolerance observed in rat mice. We show that the development of colitis in **Gimap5** 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 **Gimap5** mice (28–31). For example, Foxo-deficient CD4+ T cells have impaired proliferative capacity and adopt CD44hi CD62Llo 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 **Gimap5** 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 **Gimap5** mice, we found no evidence of autoreactive B cells (19), but it is important to note that, similar to CD4+ T cells, **Gimap5** B cells progressively lost Foxo expression (Supplemental Fig. 3) and were unable to proliferate after stimulation with IgM (19). Thus, B cell expansion and differentiation may be hampered severely in **Gimap5** 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 **Gimap5** 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

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**FIGURE 4.** CD4+ T cells in **Gimap5** mice 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 **Gimap5** splenocytes, as measured by quantitative real-time PCR. C, Loss of Foxo expression in **Gimap5** 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 **Gimap5** splenocytes (sph/sph, homozygote; +/sph, heterozygote). E, Foxo3 expression in CD4+CD62Lhi and CD4+CD62Llo CD4+ T cells isolated from 5-wk-old homozygote sph/sph mice and heterozygote littermate controls. Data represent mean values ± SEM, and blots are representative of three independent experiments (n = 3). ***p < 0.001.

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**Discussion**

 Genetic aberrancies in **Gimap5** have been associated with IL-23 signaling and more severe colitis (34, 35). However, the molecular pathways underlying the loss of immunological tolerance in rat have been linked to lymphopenia and the loss of immunological tolerance (6, 7, 9, 10). Although we found no evidence of autoimmune responses in **Gimap5** 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 **Gimap5** mice, the loss of immunological tolerance in the lyp/lyp rat has been associated with reduced Treg cell lymphopenia and lower CD4+ T cell numbers 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 **Gimap5** mice. We show that the development of colitis in **Gimap5** 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 **Gimap5** mice (28–31). For example, Foxo-deficient CD4+ T cells have impaired proliferative capacity and adopt CD44hi CD62Llo 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 **Gimap5** 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 **Gimap5** mice, we found no evidence of autoreactive B cells (19), but it is important to note that, similar to CD4+ T cells, **Gimap5** B cells progressively lost Foxo expression (Supplemental Fig. 3) and were unable to proliferate after stimulation with IgM (19). Thus, B cell expansion and differentiation may be hampered severely in **Gimap5** 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 **Gimap5** 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 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 Gimap5sph/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 Gimap5sph/sph CD4+ RB45high T cells, wild-type CD4+RB45high T cells transferred into

FIGURE 5. Colitis in Gimap5sph/sph mice can be prevented by adoptive transfer of wild-type Treg cells. A, Three- to 4-wk-old Gimap5sph/sph mice were injected i.v. with $3 \times 10^5$ CD25+CD4+ splenocytes isolated from wild-type mice. Recipient mice were weighed for up to 25 wk and compared with untreated heterozygote and homozygote Gimap5sph/sph mice. B, Gimap5sph/sph mice treated with wild-type Treg cells were protected from colitis development as determined by histological scoring. C and D, H&E-stained colon sections from untreated 12-wk-old Gimap5sph/sph (C) and 25-wk-old Treg cell-recipient Gimap5sph/sph mice (D). Scale bars, 200 μm (C, left panel, D), 100 μm (C, right panel). E and F, The total number (E) and percentage (F) of Gimap5sph/sph (CD45.1+) and congenic (CD45.2+) Foxp3+ Treg cells in spleens and MLNs of 25-wk-old in CD4+CD25+ Treg cell-recipient Gimap5sph/sph mice. In C, the numbers of Foxp3+ cells in wild-type C57BL/6J mice are presented as a comparison. All of the studies were performed with n ≥ 4 mice per genotype from two independent experiments, and data are represented as mean values ± SEM. *** p < 0.001.

The Journal of Immunology 7
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 could be 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 Gimap5sph/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, lysophycines containing large numbers of cytotoxic granules, such as CD8+ T cells and NK cells, do not survive in Gimap5-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 disorders such as systemic lupus erythematosus, T1D, and colitis.

Disclosures

The authors have no financial conflicts of interest.

References

1. Nitta, T., M. Narrene, T. Seike, A. Goji, I. Ogashishi, T. Miyazaki, T. Ohta, M. Kanno, and Y. Takahama. 2006. IAN family critically regulates survival 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 disorders such as systemic lupus erythematosus, T1D, and colitis.

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