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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 (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.

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Abbreviations used in this article: Cdk, cyclin-dependent kinase; Foxo, Forkheadbox group O; Gimap, GTPase of immunity-associated protein; iTreg, inducible regulatory T; LIP, lymphopenia-induced proliferation; LP, lamina propria; MLN, mesenteric lymph node; p53, p53-mutated lymphoma; p<sub>0.05</sub>, p<sub>0.05</sub> to p<sub>0.01</sub> protein (group 0); T, CD4<sup>+</sup> T cells; Treg, regulatory T.

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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 Biologend. Abs for western blotting [anti-Foxo3a (2497), anti-Foxo1 (2880), anti-pFoxo1(Thr24)/pFoxo3a(Thr32) (9464), anti-Foxo4 (9472), p27, or Biolegend. Abs for Western blotting [anti-Foxo3a (2497), anti-Foxo1 (2880), anti-pFoxo1(Thr24)/pFoxo3a(Thr32) (9464), anti-Foxo4 (9472)] were purchased from Cell Signaling Technology. Purified CD3 ε(145-281) and CD4 (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, Gimap5sph/sph mice at 25–35 d of age were injected i.v. with 3 × 105 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 profile, CD8α−CD25−Foxp3−, and were capable of producing proinflammatory cytokines, including IL-2, IL-6, IL-17, and IFN-γ. 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 × 10^5 MACS-purified CFSE-labeled CD8+ T cells or CD4+ T cells. Also included were 1 × 10^5 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 culture.

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.
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 colonos of 6-wk-old Gimap5sph/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 Gimap5sph/sph mice and their potential for contributing to the development of colitis. Our previous work indicated that 8-wk-old Gimap5sph/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 Gimap5sph/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 Gimap5sph/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 Gimap5sph/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 Gimap5sph/sph mice become Th1/Th17 polarized and effectively produce cytokines.

Colitis in Gimap5sph/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 Gimap5sph/sph mice. We tested this hypothesis by depleting CD4+ T cells in Gimap5sph/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 Gimap5sph/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 Gimap5sph/sph mice (Fig. 2B–H). These data support our hypothesis that the development of colitis in Gimap5sph/sph mice requires CD4+ T cells.

Gimap5sph/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.
Gimap5sph/sph mouse anti-CD4 (GK1.5) or isotype control Abs i.p. weekly, beginning at 6 wk of age (Fig. 3B). At the time of injection, 50% of the CD4+ T cells compartment were observed at the time of injection, after 5 wk the Gimap5sph/sph 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.

Gimap5sph/sph CD4+ T cells exhibit progressive loss of Foxo1, Foxo3, and Foxo4 expression

Our data indicate that the Gimap5sph/sph 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 CD44highCD62Llow 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 Gimap5sph/sph CD4+ T cells. Strikingly, immunoblot analysis of CD4+ T cells from 6-wk-old Gimap5sph/sph mice revealed a near absence of full-length Foxo1, Foxo3a, and Foxo4 expression 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 Gimap5sph/sph mice occur after 4 wk of age, we next quantified the temporal progression of changes in Foxo expression in lymphocytes. Immunoblot analyses revealed that Foxo1 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 Foxo1 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 Gimap5sph/sph cells (Fig. 4C). Due to the progressive nature of this phenotype, we considered the

Therefore, even though Treg cell development in the thymus of Gimap5sph/sph 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 Foxo3 Treg cells in Gimap5sph/sph mice. Although relatively normal numbers of Foxo3+ 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 Foxo3+ CD4+ T cells within the CD4+ T cell population remained similar to the percentage observed in wild-type mice (Fig. 3A, 3B). To
Gimap5sph/sph

Prevention of colitis in Gimap5sph/sph old onset of lymphopenia in these data link the loss of full-length Foxo expression with 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- to 6-wk-old Gimap5sph/sph mice were able to proliferate after TCR stimulation (Supplemental Fig. 3A) (19). Finally, we measured by CFSE dilution after 72 h of incubation in vitro. The percentage of congeneric (CD45.1) and Gimap5sph/sph Foxp3+ CD4+ T cells in spleens and MLNs 25 wk after transfer in Rag1−/− recipient mice. The Gimap5sph/sph but not wild-type Treg cell population was lost in Rag1−/− mice injected with a mixture of wild-type and Gimap5sph/sph CD4+ T cells. Data represents mean values ± SEM (n = 4 mice per genotype from two independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 3. Reductions in the numbers and function of peripheral Foxp3+ Treg cells in Gimap5sph/sph mice precede the onset of colitis. A and B, Flow cytometric analysis of 6-wk-old Gimap5sph/sph mice reveals a reduced percentage of Foxp3+ cells within the CD4+ T cell compartment (A) and a reduced absolute number of Treg cells (B). C, Treg cells isolated from 4- or 6-wk-old Gimap5sph/sph mice have a reduced capacity to suppress the proliferation of anti-CD28/anti-CD3-activated, cocultured C57BL/6J CD8+ T cells, as measured by CFSE dilution after 72 h of incubation in vitro. D, The percentage of congenic (CD45.1) and Gimap5sph/sph Foxp3+ CD4+ T cells in spleens and MLNs 25 wk after transfer in Rag1−/− recipient mice. The Gimap5sph/sph but not wild-type Treg cell population was lost in Rag1−/− mice injected with a mixture of wild-type and Gimap5sph/sph CD4+ T cells. Data represents mean values ± SEM (n = 4 mice per genotype from two independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001.

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- to 6-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 CD44hiCD62Llo 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 naive 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+ 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. 2E). 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
In this study, we explored the pathways that contribute to the loss of immunological tolerance in rat T cells, identifying a Th17 pattern of differentiation (34, 35). However, the molecular correlates with decreased p27kip1 expression and increased phosphorylation of pRb. Foxo transcription factors have been shown to serve as direct with a progressive loss of protein expression of Foxo1, Foxo3, and Foxo4 in total splenocytes from 5-wk-old mice. 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 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 mice 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

**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-17+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 CD44high CD62Llow 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.
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...
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 Gimap5-deficient 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 catalytic 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, lysosomal enzymes (such as cathepsins) that can initiate programmed cell death once in the cytosol (47). Intriguingly, lysosomal enzymes (such as cathepsins) that can initiate programmed cell death once in the cytosol (47).

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, TID, and colitis.

Disclosures

The authors have no financial conflicts of interest.

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Supplementary figure legends

**Supplementary figure 1.** Progressive loss immunosuppressive function in *Gimap5<sup>sph/sph</sup>* mice is associated with a loss of Foxo expression in CD4<sup>+</sup> T cells. (A) Reduced capacity of Treg cells isolated from 3- or 6-week-old *Gimap5<sup>sph/sph</sup>* mice to suppress proliferation of C57BL/6J CD4<sup>+</sup> 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<sup>+</sup> 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<sup>sph/sph</sup>* CD4<sup>+</sup> T cells into 6 week-old Rag1<sup>−/−</sup> recipient mice. Mice were analyzed 5 weeks after transfer. The percentage of Foxp3<sup>+</sup> Treg cells in wildtype and *Gimap5<sup>sph/sph</sup>* CD4<sup>+</sup> T cells at the time of injection is shown. (B) Rescue studies in which 3x10<sup>5</sup> CD25<sup>+</sup>CD4<sup>+</sup> cells isolated from congenic (CD45.1) C57BL/6J mice are injected into 4 week-old *Gimap5<sup>sph/sph</sup>* mice. Analysis was performed after 25 weeks. (C) Inflammatory lung infiltrate as observed in a number of Treg “rescued” *Gimap5<sup>sph/sph</sup>* mice. (D) Ex vivo cytokine production by CD4<sup>+</sup> T cells isolated from 15-week-old wildtype or Treg-treated *Gimap5<sup>sph/sph</sup>* 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<sup>+</sup> T cells isolated from 4 or 8 week-old wildtype C57BL/6J or *Gimap5<sup>sph/sph</sup>* 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<sup>sph/sph</sup>* but not wildtype cells. (C) Foxo3 expression in CD4<sup>T</sup> cells isolated from wildtype spleen (control) or following the transfer of wt CD4<sup>+</sup> RB45<sup>high</sup> CD4<sup>+</sup> T cells in Rag<sup>−/−</sup> 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

% proliferation

Treg : CD4+ T cell ratio

B

3 weeks

6 weeks

+/sph sph/sph

+/sph sph/sph

Foxo1

Foxo3

Actin

C57BL/6J

Gimap5sph/sph
Supplemental Figure 2

A

CD90.1 B6

3 week-old Gimap5sph/sph

CD4+ T cells

Adoptive Transfer i.v.

5 x 10^5

RAG2-/-

Analyse after 5 weeks

Normal Treg frequency at 3 weeks

CD90.1 B6

Gimap5sph/sph

FoxP3

CD4

CD4

B

CD45.1 B6

3 x 10^5 CD25+ CD4+ T cells

Adoptive Transfer

Gimap5sph/sph

4-week old

Analyse after 23 weeks

C

Gimap5sph/sph + Treg

C57BL/6

Lung

D

spleen

MLN

Unstimulated

PMA/ionomycin

IL17

IFNγ