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Loss of immunological tolerance in Gimap5-deficient mice is associated with loss of Foxo in CD4+ T cells

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Abstract

Previously, we reported the abrogation of quiescence and reduced survival in lymphocytes from *Gimap5*$^{sph/sph}$ mice, an ENU germline mutant with a missense mutation in the GTPase of immunity-associated nucleotide binding protein 5 (Gimap5). These mice showed a progressive loss of peripheral lymphocyte populations and developed spontaneous colitis, resulting in early mortality. Here, we identify the molecular pathways that contribute to the onset of colitis in *Gimap5*$^{sph/sph}$ mice. We show that CD4$^+$ T cells become Th1/Th17-polarized and are critically important for the development of colitis. Concomitantly, Treg cells become reduced in frequency in the peripheral tissues and their immune-suppressive capacity becomes impaired. Most importantly, these progressive changes in CD4$^+$ T cells are associated with the loss of 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 maintain immunological tolerance.

INTRODUCTION

The family of *GTPase of immune-associated protein* (*Gimap*) genes are predominantly expressed 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 originally identified 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 previously observed 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 recently reported to localize in lysosomes, based on studies in human, mouse and rat lymphocytes(5). Genetic aberrancies in *Gimap5* have been strongly linked to reduced

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lymphocyte survival and homeostasis, but, importantly have also been associated with autoimmune diseases. In humans, polymorphisms in *GIMAP5* were associated with increased concentrations of IA2 auto-antibodies in type 1 diabetes (T1D) patients(6) and an increased risk of systemic lupus erythematosus (SLE)(7, 8). Studies using biobreeding (BB) rats— carrying a mutation (*lyp/lyp*) in *Gimap5*— show marked lymphopenia and predisposition to the development of T1D(9-11). In addition, BB 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 thymic-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 auto-immunity(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 naïve $CD45RB^{high}$ $CD4^+$ 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 co-transferred, suggesting that the presence or absence of Treg cells is an important determinant of immune-mediated sequela induced by $CD4^+$ T cells undergoing LIP.

Our laboratory previously described an ENU germline mutant, designated *sphinx*, which contained a recessive mutation in *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). Following the collapse of lymphocyte populations, $CD4^+$ T cells in $Gimap5^{sph/sph}$ mice acquire a LIP phenotype similar to $CD4^+$ T cells transferred into lymphopenic hosts(18). Around 10-12 weeks of age, $Gimap5^{sph/sph}$ mice develop wasting disease and colitis, limiting their survival(19). Interestingly, adoptive transfer of Rag-sufficient splenocytes into $Gimap5^{sph/sph}$ mice around 5 weeks of age could restore lymphocyte homeostasis and prevent colitis and wasting(19).

In this report, we show that $CD4^+$ T cells are required for development of colitis in $Gimap5^{sph/sph}$ mice. Whereas $CD4^+$ T cells exhibited impaired proliferation, they remained highly capable of producing proinflammatory cytokines, including IL-17A and IFNγ. Importantly, $CD4^+$ T cells in $Gimap5^{sph/sph}$ mice exhibited a LIP phenotype and exhibited a progressive and complete loss of full-length Foxo1, -3 and -4 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 $Gimap5^{sph/sph}$ mice could be prevented by transferring wild-type Treg cells into 3-week-old $Gimap5^{sph/sph}$ mice. Since Foxo-deficient mice exhibit many of the phenotypes observed in $Gimap5^{sph/sph}$ 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 experiments were performed according to US National Institutes of Health guidelines and were approved by the IACUC of The Cincinnati Children's Hospital. *C57BL/6J*, *Rag1*$^{-/-}$, *CD45.1* congenic and *CD90.1* congenic mice were obtained from Jackson. *Gimap5*$^{sph/sph}$ mice were generated as previously described(19) and bred in the vivarium of the Cincinnati Children's Hospital. All mice were maintained under specific pathogen-free conditions.

All antibodies used for flow cytometry were purchased from eBioscience or Biolegend. Antibodies for western blotting [anti-Foxo3a (#2497), Foxo1 (2880), pFoxo1(Thr24)/ pFox03a(Thr32) (9464), p27, p-pRB (S807,S811), p-pRb (S780) and pan-Actin antibody] were purchased from Cell Signaling. Purified CD3ε (145-2C11) and CD28 (37.51) antibodies (eBioscience) were used for T cell activation. PMA and ionomycin was obtained from Sigma.

Real time PCR

CD4^{+}T cells were isolated from spleen and lymph nodes of 4 week-old and 7 week-old *Gimap5*$^{sph/sph}$ and C57BL/6 mice using L3T4 MicroBeads (Miltenyi Biotech). RNA isolation was done with RNeasy Micro Kit (Qiagen) and reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were amplified with Lightcycler 480 SYBR Green I Master (Roche) and quantified by Roche Light Cycler 480 II instrument using the following primer pairs: Foxo1: Fwd: TTCGGAATGACCTCATGGATG Rev TGGACTGCTCCTCAGTTCCTG Foxo3: Fwd; AGTGGATGGTGCGCTGTGT, Rev: TCTGAACGCGCATGAAGCFoxo4: Fwd: GAGAACCTGGAGTGCGACATG, Rev: TGTGTTGCCACCAAT

Flow cytometry and T cell analyses

To quantify T cell proliferation, MACS (Miltenyi Biotech)-purified CD4^{+} T cells were labeled with CFSE (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(50ng/mL)/ionomycin(1μg/mL). After 3 days of incubation, proliferation was measured by analyzing CFSE dilution using flow cytometry. To assess the capacity of T cells to produce cytokines, MACS (Miltenyi Biotech) purified CD4^{+} T cells were incubated for 6 hours with/without PMA(10ng/mL)/ ionomycin(10μg/mL) and subsequently fixed and analyzed for intracellular IL-17A, IL-4 or IFNγ production using flow cytometry. To measure surface markers *ex vivo*, CD4^{+} T cells from spleen, MLN or lamina propria were isolated and stained with fluorochrome-labeled antibodies 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 a 24-hr interval, wildtype or *Gimap5*$^{sph/sph}$ 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 hours after the last injection using flow cytometry.

In vitro Treg cell suppressor assays

The Treg cell suppressor assay was performed under conditions previously described(21, 22). Briefly, spleens were isolated and Treg cells were MACS purified using the

$CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi). Subsequently, Treg cells were harvested and co-cultured at indicated ratios with 5×10^4 MACS purified CFSE-labeled $CD8^+$ T cells or $CD4^+$ T cells. Also included were 1×10^5 T cell-depleted, γ-irradiated (1,500 rad) splenocytes as bystander cells and 0.5 μg/mL soluble CD3 antibody. CFSE dilution was assessed by flow cytometry after three days of co-culture.

Histology

Colon tissue was collected and immediately fixed in 10% buffered formalin solution overnight, followed by routine paraffin embedding. Hematoxylin and eosin staining was performed on 4 μm sections from the paraffin-embedded tissue blocks for conventional light microscopy analysis. Histological scoring was performed as described before (23) Briefly, scoring parameters included quantitation 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 from 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; and 3, severe. Total disease score was expressed as the mean of all combined scores per genotype.

Adoptive transfer and survival assays

For adoptive transfer studies, *Gimap5*$^{sph/sph}$ mice at 25-35 days of age were injected *i.v.* with 3×10^5 Treg cells isolated from C57BL/6J mice using a Treg cell isolation kit (Miltenyi). Purity was confirmed by Foxp3 staining using flow cytometry and cells were >90% $Foxp3^+$. Mice were monitored and weighed every week following cell transfer.

Statistical analysis

Data were analyzed using GraphPad Prism4® software (GraphPad Software, San Diego, CA). Unless indicated otherwise, statistical significance of the differences among groups was determined from the mean and standard deviation 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

Gimap5$^{sph/sph}$ $CD4^+$ T cells from MLN are Th1/17-polarized

In previous work, we determined that NK, NKT, $CD8^+$, $CD4^+$ and B lymphocyte survival are impaired in *Gimap5*$^{sph/sph}$ mice. In addition, they developed spontaneous colitis that required the presence of microbiota and survived poorly, with most mice succumbing by 150 days of age(19). As several mouse models have linked impaired lymphocyte function with colitis development, we further explored the contribution of lymphocytes to the immunopathology observed in *Gimap5*$^{sph/sph}$ mice. Firstly, we investigated the survival and functional capacity of $CD4^+$ T cells at different ages. By 4 weeks 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-week old *Gimap5*$^{sph/sph}$ mice (Figure 1A). Six-week-old *Gimap5*$^{sph/sph}$ $CD4^+$ T cells had a $CD44^{high}CD62L^{low}$ phenotype characteristic of T cells undergoing LIP(19) and showed increased incorporation of BrdU (Figure 1B). To assess whether the loss of $CD4^+$ T cells in the spleen and lymph nodes was also observed in gut-associated lymphoid tissue, we isolated lamina propria (LP) cells from the colons of 6-week-old *Gimap5*$^{sph/sph}$ mice and quantified the number of $CD4^+$ T cells. Similar to the spleen, reduced numbers of $CD4^+$ T cells were observed in the LP (Figure 1C). Further analysis revealed that close to 100% of the colonic $CD4^+$ T cells were $CD44^{high}CD62L^{low}$, resembling the LIP phenotype of the $CD4^+$ T cells in the peripheral lymphoid tissues (Figure

1C). Together these data suggest that $CD4^+$ T cells are present in the GALT and exhibit a LIP phenotype similar to what is observed in the spleen.

We next investigated the functional capacity of $CD4^+$ T cells in *Gimap5*$^{sph/sph}$ mice, and their potential for contributing to the development of colitis. Our previous work indicated that 8-week-old *Gimap5*$^{sph/sph}$ $CD4^+$ T cells were unable to proliferate *ex vivo* following stimulation with PMA/ionomycin or αCD3, even though lymphocytes exhibited normal activation of NF-κB and MAP kinase 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 spleen and MLNs from C57BL/6J control or *Gimap5*$^{sph/sph}$ mice and incubated cells for 6 hours with or without PMA/ionomycin in the presence of brefeldin. Interestingly, a higher percentage of $CD4^+$ T cells derived from *Gimap5*$^{sph/sph}$ spleen or MLNs produced IFNγ or IL-17A, or both cytokines following PMA/ionomycin stimulation (Figure 1D). Notably, T cell cytokine production was observed even in the absence of PMA/ionomycin in *Gimap5*$^{sph/sph}$ MLN cells (but not splenic leukocytes), suggesting constitutive activation of T cells in gut lymphoid tissue in these mice. Overall, these data indicate that, despite their inability to proliferate normally *ex vivo*, $CD4^+$ T cells derived from *Gimap5*$^{sph/sph}$ mice become Th1/17 polarized and effectively produce cytokines.

Colitis in Gimap5$^{sph/sph}$ mice is driven by CD4$^+$ T cells

Because of their LIP phenotype and spontaneous production of IL-17A and IFNγ, we hypothesized that MLN $CD4^+$ T cells may support the development of colitis in *Gimap5*$^{sph/sph}$ mice. We tested this hypothesis by depleting $CD4^+$ T cells in *Gimap5*$^{sph/sph}$ mice using weekly injections of anti-CD4 (GK1.5) antibodies, starting at 3 weeks of age—before the $CD4^+$ T cells "collapse" and the subsequent intestinal inflammation normally occurs in *Gimap5*$^{sph/sph}$ mice. Importantly, GK1.5 treatment, but not isotype treatment, prevented wasting disease (Figure 2A) and significantly decreased intestinal inflammation as determined by histology in 15-week-old *Gimap5*$^{sph/sph}$ mice (Figure 2B-H). These data support our hypothesis that the development of colitis in *Gimap5*$^{sph/sph}$ mice requires $CD4^+$ T cells.

Gimap5$^{sph/sph}$ mice fail to maintain a Treg cell population with normal immunosuppressive function

Colitis induced by naïve $CD45RB^{high}$ T cell transfer into SCID recipients does not occur when Treg cells are co-transferred. Therefore, even though Treg cell development in the thymus of *Gimap5*$^{sph/sph}$ 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 $Foxp3^+$ Treg cells in *Gimap5*$^{sph/sph}$ mice. Although relatively normal numbers of $Foxp3^+$ Treg cells were observed in 3-week-old mice (Supplemental figure 2A), Treg cells became significantly reduced in the MLNs of 6- to 8-week old mice, both as a percentage within the $CD4^+$ T cell compartment as well as in total numbers of cells (Figure 3A,B). In the spleen, the number of Treg cells were reduced but the percentage of $Foxp3^+$ $CD4^+$ T cells within the CD4 T cell population remained similar to the percentage observed in wildtype mice (Figure 3A,B). To assess their functional capacity, we purified Treg cells from 4- or 6-week-old C57BL/6J or *Gimap5*$^{sph/sph}$ spleens, and co-cultured Treg cells with CFSE-labeled wild-type $CD8^+$ T cells that were stimulated with soluble anti-CD3-antibodies. Treg cells from 4-week-old *Gimap5*$^{sph/sph}$ mice showed a slight, but significant reduction in their ability to suppress $CD8^+$ T cell proliferation *in vitro*, whereas Treg cells isolated from 6-week-old *Gimap5*$^{sph/sph}$ mice were incapable of suppressing $CD8^+$ T cell proliferation (Figure 3C). Similar results were obtained for suppression of wildtype $CD4^+$ T

cell proliferation (Supplemental Figure 1A). These findings suggest that both Treg cell survival and functional capacity become impaired as *Gimap5*$^{sph/sph}$ mice age.

We next questioned whether reduced peripheral Treg cell accumulation in *Gimap5*$^{sph/sph}$ mice resulted from a cell-intrinsic phenomenon. We injected CD4^{+} splenocytes from 3 week-old wildtype and/or *Gimap5*$^{sph/sph}$ mice into Rag-deficient recipients, either as a mixture, or alone, and quantified the presence of Foxp3^{+} Treg cells 5 weeks after injection (Supplemental Figure 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 weeks the *Gimap5*$^{sph/sph}$ Treg population was lost, regardless of whether wild-type cells were cotransferred or not (Figure 3D). Overall, these data indicate that cell-intrinsic expression of Gimap5 is required to allow normal Treg cell survival.

Gimap5$^{sph/sph}$CD4^{+} T cells exhibit progressive loss of Foxo1, -3 and -4 expression

Our data indicate that the *Gimap5*$^{sph/sph}$ CD4^{+} T cell population collapses around 5 weeks 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 Forkheadbox group O (Foxo) transcription factors. The family of Foxo transcription factors contain 4 members of which three (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 the regulation of life span(27). Foxo1 expression is critical for maintaining naïve T cell quiescence. Foxo1-deficient CD4^{+} T cells exhibit a CD44^{high}CD62L^{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 expression of Foxo1, Foxo3 and Foxo4 in *Gimap5*$^{sph/sph}$ CD4^{+} T cells. Strikingly, immunoblot analysis of CD4^{+} T from 6-week-old *Gimap5*$^{sph/sph}$ mice revealed a near absence of full-length Foxo1, -3a and -4 protein (Figure 4A). At the mRNA level, a reduction in Foxo1 but not Foxo3 and 4 could be observed in CD4^{+} T cells isolated from *Gimap5*$^{sph/sph}$ compared to wildtype mice (Figure 4B), suggesting that regulation of Foxo3 and Foxo4 protein expression occurred at the post-transcriptional level. Since many of the T cell-specific phenotypes observed in *Gimap5*$^{sph/sph}$ occur after 4 weeks 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 weeks, somewhat reduced after 4 weeks, and almost absent after 6 to 10 weeks of age (Figure 4C and supplementary Figure 1A). Concordant with the loss of Foxo expression, we detected reductions in the abundance of the cyclin-dependent kinase (Cdk) inhibitor, p27^{kip1}, a downstream target of Foxo proteins and an important regulator of cell cycle entry (Figure 4C)(32, 33). As p27^{kip1} inhibits Cdk4, we measured Cdk4 activity and detected increased phosphorylation of its substrate, retinoblastoma protein (pRb), in *Gimap5*$^{sph/sph}$ cells (Figure 4C). Due to the progressive nature of this phenotype, we considered that lymphocytes isolated from young *Gimap5*$^{sph/sph}$ mice with intact Foxo expression might respond normally to mitogenic stimuli. Indeed, CD4^{+} T cells isolated from 4 week-old, but not 8-week-old, *Gimap5*$^{sph/sph}$ mice were able to proliferate after TCR stimulation (Supplemental Figure 3A and(19)). Finally, we assessed whether the loss of Foxo expression was also observed in regulatory T cells from *Gimap5*$^{sph/sph}$ mice. Indeed, Foxo1 and Foxo3 expression was mostly absent in CD4^{+}CD25^{+} T cells from 6-week-old *Gimap5*$^{sph/sph}$ mice (Figure 4D).

Interestingly, the progressive loss of Foxo expression appeared to correlate with a progressive increase in the number of CD4^{+} T cells undergoing LIP (CD44^{hi}, CD62L^{lo}) in 4- to 10-week old *Gimap5*$^{sph/sph}$ mice as previously reported(19). Subsequent analysis of CD44^{lo}, CD62L^{hi} and CD44^{hi}, CD62L^{lo} CD4^{+} T cells from 5-week-old *Gimap5*$^{sph/sph}$ and

wildtype mice revealed loss of FoxO expression specifically in $CD4^+$ T cells undergoing LIP but not naïve $CD4^+$ T cells in *Gimap5*$^{sph/sph}$ mice (Figure 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*$^{sph/sph}$ lymphocytes, the impaired cell cycle control and proliferative capacity in such lymphocytes, and the reduced Treg cell survival and function in *Gimap5*$^{sph/sph}$ mice.

Prevention of colitis in Gimap5$^{sph/sph}$ mice by the adoptive transfer of wildtype Treg cells

Our previous data show that colitis can be prevented in *Gimap5*$^{sph/sph}$ 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 *Gimap5*$^{sph/sph}$ mice, we next examined whether adoptively transferred wild-type Treg cells could prevent the development of colitis. *Gimap5*$^{sph/sph}$ recipients of 3×10^5 wild-type $CD4^+CD25^+$ T cells showed prolonged survival, delayed wasting disease (Figure 5A) and, importantly, did not develop colitis (Figure 5B-D). Characterization of lymphocyte populations 25 weeks after transfer of CD45.1 congenically marked $CD4^+CD25^+$ Treg cells revealed that Treg cell reconstitution of the spleen of *Gimap5*$^{sph/sph}$ mice achieved ~50% of the level observed in wild-type C57BL/6J mice (Figure 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 *Gimap5*$^{sph/sph}$–derived (Figure 5E). Functional analysis of isolated $CD4^+$ T cell from spleen and MLN of 15-week-old treated *Gimap5*$^{sph/sph}$ mice revealed no background cytokine production and a similar activation as observed for wildtype $CD4^+$ T cells following stimulation with PMA/Ionomycin (supplementary Figure 2D). Around 25 weeks 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 Figure 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-deficienty. Together, these data link impaired Treg cell survival and function to the development of colitis in *Gimap5*$^{sph/sph}$ mice. In addition, they reveal that the *Gimap5*$^{sph/sph}$ 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 *Gimap5*$^{sph/sph}$ mice, we observed severe and spontaneous inflammation in the gut(19)—an environment where homeostasis critically depends upon maintaining tolerance to exogenous antigens and bacterial stimuli. Similar to *Gimap5*$^{sph/sph}$ mice, 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 T helper cells towards 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. Here, we explored the pathways that contribute to the loss of tolerance observed in *Gimap5*$^{sph/sph}$ mice. We show that the development of colitis in *Gimap5*$^{sph/sph}$ mice is critically dependent on $CD4^+$ T cells. Around 8 weeks of age, $CD4^+$ T cells lose their capacity to proliferate *ex vivo*, yet they remain capable of producing proinflammatory cytokines, including IL-17A and IFNγ, and contain a population of IL-17^+IFNγ^+ Th1/17 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 directly associated with a progressive loss of protein expression of Foxo1, -3 and -4—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*$^{sph/sph}$ mice(28-31). For example, *Foxo*-deficient CD4$^+$ T cells have impaired proliferative capacity and adopt CD44^{high}CD62L^{low} LIP or memory-like phenotype[28, 30]. In addition, reduced Treg cell numbers and function were observed in mice lacking Foxo1 or both Foxo1 and -3 in T cells. Similar to *Gimap5*$^{sph/sph}$ mice, mice lacking Foxo1 and -3 in T cells develop spontaneous colitis, and furthermore purified *Foxo1*$^{-/-}$*Foxo3*$^{-/-}$ Treg cells were unable to prevent colitis in *Rag1*$^{-/-}$ mice when coinjected with naïve wildtype 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 auto-immunity(29). In *Gimap5*$^{sph/sph}$ mice we found no evidence of autoreactive B cells(19), but it is important to note that, similar to CD4$^+$ T cells, *Gimap5*$^{sph/sph}$ B cells progressively lost Foxo expression (Supplementary Figure 3B) and were unable to proliferate following stimulation with IgM(19). Thus, B cell expansion and differentiation may be severely hampered in *Gimap5*$^{sph/sph}$ mice, preventing the development of auto-reactive antibody 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 TGFβ signaling pathway by interacting with SMAD proteins, ultimately fine tuning the TGFβ-induced transcriptional program(38, 39). This pathway is also critical for the development of inducible Treg (iTreg) cells(40), which develop extra-thymically and have been suggested by many studies to comprise an important population of regulatory T cells in the gut(21, 41-43). Indeed, the loss of Treg cells within the CD4$^+$ T cell compartment is most evident in the MLN of *Gimap5*$^{sph/sph}$ mice (Figure 3), suggesting the iTregs 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 *Gimap5*$^{sph/sph}$ mice, an absence of Foxo expression was observed in all 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 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 that we considered is that the loss of Foxo expression may drive a secondary phenotype resulting from the constitutive proliferation cues associated with LIP, something that may be driven by self-antigens or antigens derived from the microbiota. While we cannot exclude 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 Figure 3C), suggesting that LIP alone is insufficient to cause 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 previously been reported to occur via ubiquitination and proteosomal degradation(45). Moreover, 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 inversely correlated with increased expression of S-phase kinase-associate protein-2 (Skp2)—an E3 ubiquitin ligase that targets numerous cell cycle proteins. Foxo degradation could be reversed following down-regulation of Skp2 via shRNAs, and therefore increased Skp2 expression could pose a potential mechanism by which loss of Foxo-expression in *Gimap5*$^{sph/sph}$ T cells occurs. Alternatively, the localization of *Gimap5* in the lysosomal compartment(5) and the presumed scaffolding function of Gimap family members(4) suggests 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 Gimap5- deficient mice, perhaps supporting the hypothesis of a deregulated lysosomal compartment. Although the link between 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 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 SLE, T1D and colitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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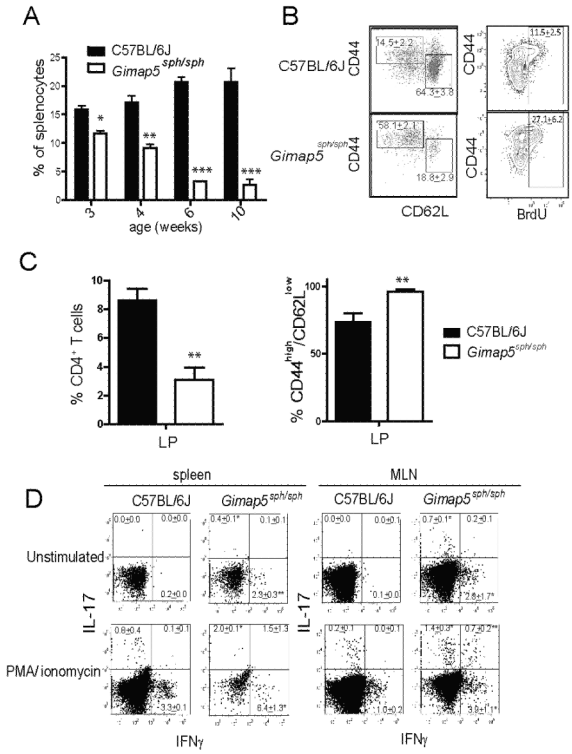

Figure 1. Phenotypic characterization of CD4$^+$ T cells in *Gimap5*$^{sph/sph}$ mice

(**A**) Splenic CD4$^+$ T cell population collapse around 6 weeks of age in *Gimap5*$^{sph/sph}$ mice, at which time they exhibit a LIP phenotype with increased BrdU uptake (B). (C) The number of CD4$^+$ T cells and the percentage of CD44^{high}CD62L^{low} CD4$^+$ T cells in lamina propria of 6-week-old wildtype and *Gimap5*$^{sph/sph}$ mice. (D) *Ex vivo* cytokine production by CD4$^+$ T cells isolated from wildtype or *Gimap5*$^{sph/sph}$ spleen and mesenteric lymph node (MLN), left unstimulated or following stimulation with PMA/ionomycin (100ng/ml) for six hours (mean values ± SEM; n ≥ 4 mice per genotype from 2 independent experiments). (*P<0.05, **P<0.01, ***P<0.001)

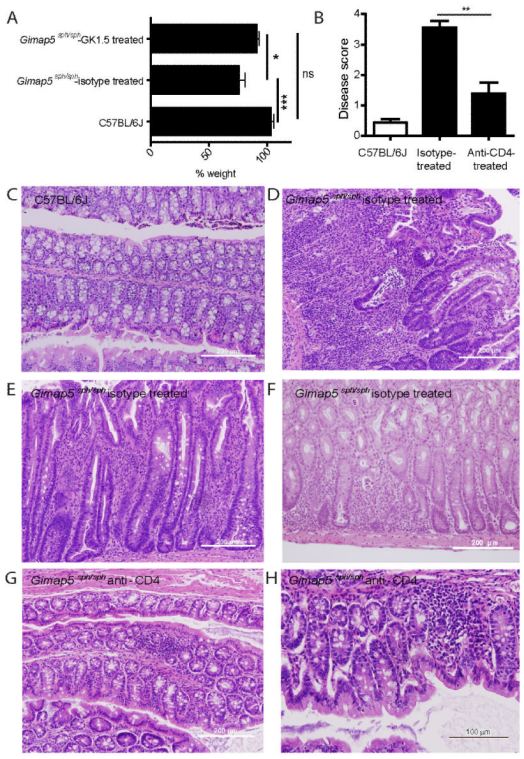

Figure 2. Depletion of CD4^{+} T cells prevents wasting disease and colitis in *Gimap5$^{sph/sph}$* mice

Male *Gimap5$^{sph/sph}$* mice were given 200μg/mouse anti-CD4 (GK1.5) or isotype control antibodies i.p. weekly, beginning at 3 weeks of age. (A) Weights of 10-week-old wild-type, isotype-treated or GK1.5 treated *Gimap5$^{sph/sph}$* mice. (B) CD4-depletion significantly reduces colitis in *Gimap5$^{sph/sph}$* mice Data represents histological scoring as described in the material and methods. (C-H) At fifteen weeks of age, mice were sacrificed and the colon was analyzed for signs of intestinal inflammation following H&E staining (C, wildtype untreated; D-F, *Gimap5$^{sph/sph}$*-isotype treated; G-H, *Gimap5$^{sph/sph}$* GK1.5-treated). Data represent mean weight percentage (wild type mice =100%) ± SEM from at least 3 animals per group and histology is representative of 3 analyzed mice per group. (*P<0.05, **P<0.01, ***P<0.001)

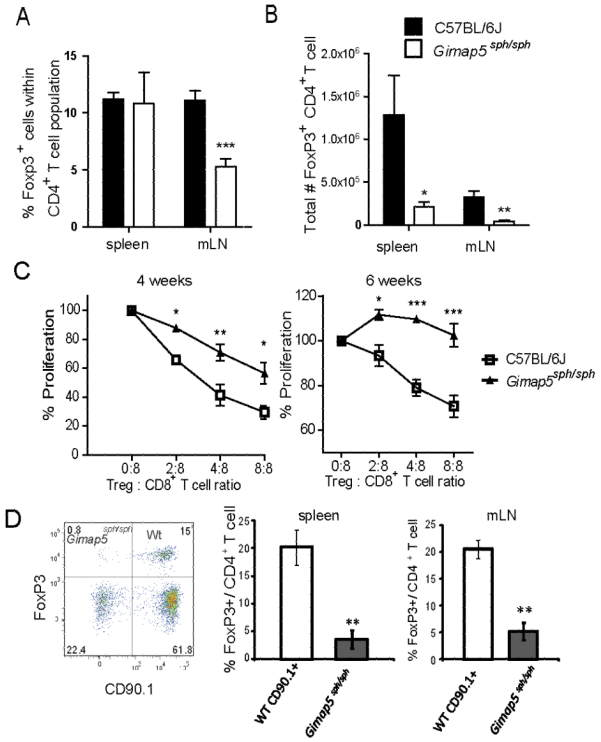

Figure 3. Reductions in the numbers and function of peripheral Foxp3$^+$ Treg cells in *Gimap5$^{sph/sph}$* mice precedes the onset of colitis

Flow cytometric analysis of 6 week-old *Gimap5$^{sph/sph}$* mice reveals a reduced percentage of Foxp3$^+$ cells within the CD4$^+$ T cell compartment (**A**) and a reduced absolute number of Treg cells and (**B**). (**C**) Treg cells isolated from 4- or 6-week-old *Gimap5$^{sph/sph}$* mice have a reduced capacity to suppress the proliferation of αCD28/αCD3-activated, cocultured C57BL/6J CD8$^+$ T cells, as measured by CFSE dilution after 72 hours incubation *in vitro*. (**D**) The percentage of congenic (CD45.1) and *Gimap5$^{sph/sph}$* Foxp3$^+$ CD4$^+$ T cells in spleen and MLN 25-weeks after transfer in *Rag1$^{-/-}$* recipient mice. The *Gimap5$^{sph/sph}$* but not wildtype Treg cell population was lost in *Rag1$^{-/-}$* mice injected with a mixture of wild-type and *Gimap5$^{sph/sph}$* CD4$^+$ T cells. Data represents mean values ± SEM; n ≥ 4 mice per genotype from 2 independent experiments (*P<0.05, **P<0.01, ***P<0.001).

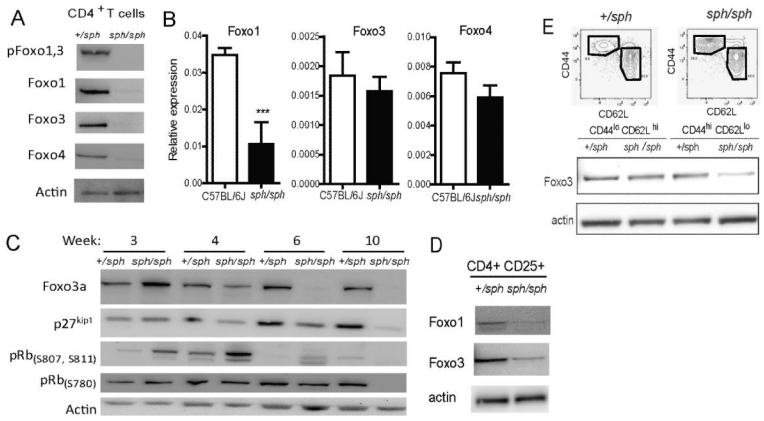

Figure 4. CD4$^+$ T cells in *Gimap5*$^{sph/sph}$ mice progressively lose full-length Foxo expression in lymphocytes

(**A**) Immunoblot analysis of phosphorylated Foxo1/3, total Foxo1, Foxo3 and Foxo4 in total splenocytes from 6-week-old mice. (**B**), Foxo1-, 3- and 4-mRNA abundance in CD4$^+$ T cells isolated from wildtype or *Gimap5*$^{sph/sph}$ spleens, as measured by qRT-PCR. (**C**) Loss of Foxo expression in *Gimap5*$^{sph/sph}$ lymphocytes correlates with decreased p27^{kip1} expression and increased phosphorylation of Retinoblastoma (Rb). (**D**) Foxo protein expression in CD4$^+$CD25$^+$ T cells isolated from 6 week-old wildtype or *Gimap5*$^{sph/sph}$ spleens. (*sph/sph*, homozygote; *+/sph* = heterozygote). (**E**) Foxo3 expression in CD44^{hi}, CD62L^{lo} and CD44^{lo} and CD62L^{hi} CD4$^+$ T cells isolated from 5-week-old homozygote *sphinx* mice and heterozygote littermate controls. Data represents mean values + SEM and blots are representative blots of three independent experiments (n = 3).

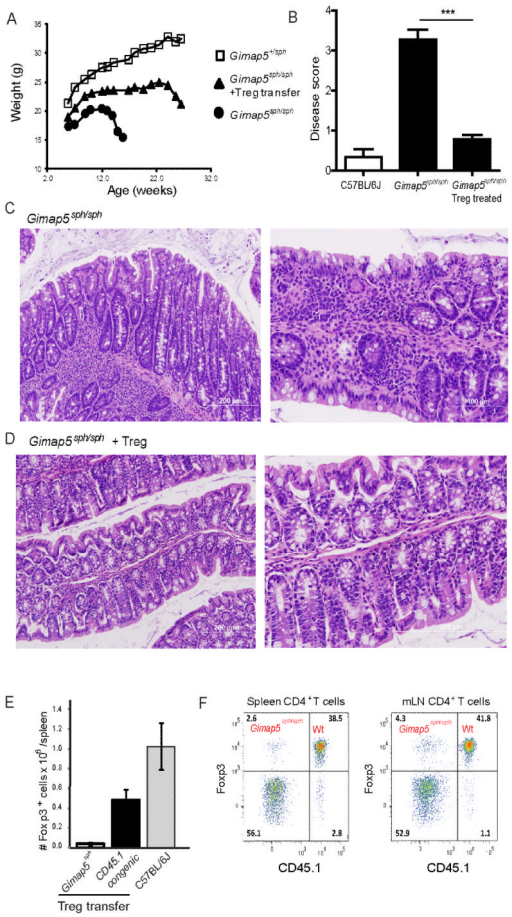

Figure 5. Colitis in *Gimap5*$^{sph/sph}$ mice can be prevented by the adoptive transfer of wild-type Treg cells

(**A**) Three- to four-week old *Gimap5*$^{sph/sph}$ mice were injected with 3 × 10^5 $CD25^+CD4^+$ splenocytes *iv*, isolated from wild-type mice. Recipient mice were weighed for up to 25 weeks and compared to untreated heterozygote and homozygote *Gimap5*$^{sph/sph}$ mice. (**B**) *Gimap5*$^{sph/sph}$ mice treated with wildtype Treg cells were protected from colitis development as determined by histological scoring (**C,D**) H&E stained colon sections from untreated 12-week-old *Gimap5*$^{sph/sph}$ (**C**) and 25-week-old Treg cell-recipient *Gimap5*$^{sph/sph}$ mice (**D**). (**E,F**) The total number (**E**) and percentage (**F**) of *Gimap5*$^{sph/sph}$ ($CD45.1^-$) and congenic ($CD45^+$) $Foxp3^+$ Treg cells in spleen and MLN of 25-week-old in $CD4^+CD25^+$ Treg cell-recipient *Gimap5*$^{sph/sph}$ mice. In C, the numbers of $Foxp3^+$ cells in wild-type C57BL/6J mice are presented as a comparison. All studies were performed with n ≥ 4 mice per genotype from 2 independent experiments and data is represented as mean values ± SEM (*** P<0.001)