LETTERS

Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression

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The naturally occurring regulatory T cell (T_R) is the pivotal cell type that maintains self-tolerance and exerts active immune suppression. The development and function of T_R cells is controlled by Foxp3 (refs 1, 2), a lack of which results in loss of T_R cells and massive multi-organ autoimmunity in scurfy mice and *IPEX* (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) patients[3,4]. It is generally thought that, through a binary mechanism, Foxp3 expression serves as an on-and-off switch to regulate positively the physiology of T_R cells; however, emerging evidence associates decreased Foxp3 expression in T_R cells with various immune disorders[5–7]. We hypothesized that Foxp3 regulates T_R cell development and function in a dose-dependent, non-binary manner, and that decreased Foxp3 expression can cause immune disease. Here, by generating a mouse model in which endogenous *Foxp3* gene expression is attenuated in T_R cells, we show that decreased Foxp3 expression results in the development of an aggressive autoimmune syndrome similar to that of scurfy mice, but does not affect thymic development, homeostatic expansion/maintenance or transforming-growth-factor-β-induced *de novo* generation of Foxp3-expressing cells. The immune-suppressive activities of T cells with attenuated Foxp3 expression were nearly abolished *in vitro* and *in vivo*, whereas their anergic properties *in vitro* were maintained. This was accompanied by decreased expression of T_R cell 'signature genes'. Notably, T cells expressing decreased Foxp3 preferentially became T-helper 2 (T_H2)-type effectors even in a T_H1-polarizing environment. These cells instructed T_H2 differentiation of conventional T cells, which contributed to the immune diseases observed in these mice. Thus, decreased Foxp3 expression causes immune disease by subverting the suppressive function of T_R cells and converting T_R cells into effector cells; these findings are important for understanding the regulation of T_R cell function and the aetiology of various human immune diseases.

T_R cells, a central component for immune suppression, are critical for establishing self-tolerance, controlling inflammatory responses and maintaining immune homeostasis[8,9]. Foxp3, an X-chromosome-linked factor that controls T_R cell development and function[1,2], is generally thought to control positively the functions of T_R cells in a binary fashion, as Foxp3 expression is sufficient to specify immune-suppressive activities in conventional T cells[1,2,10]. Thus, current efforts are focused on associating abnormal numbers of T_R cells with immune disorders. However, the quality of T_R cells is also critical for their function[11]. We observed lowered levels of Foxp3 in intra-islet T_R cells compared with T_R cells from other peripheral lymphoid organs in diabetic NOD mice (Fig. 1a), whereas the frequencies of Foxp3-expressing T_R cells among different compartments were comparable (data not shown). However, such a specific decrease in Foxp3 expression was not observed in non-diabetes-prone C57BL/6 mice (Supplementary Fig. 1). We hypothesized that one of the quality control mechanisms for T_R cells is through tuning the expression levels of Foxp3, and that decreased Foxp3 expression can cause immune disease. To test this, we generated a mouse model, where attenuated expression of the endogenous *Foxp3* gene was achieved by a targeted gene 'knock-in' approach, allowing us to investigate the effects of decreased Foxp3 expression on T_R cell function and to provide potential mechanistic explanations for the aetiologies of certain human immune disorders. In this model, a gene cassette co-expressing luciferase and enhanced green fluorescent protein (eGFP)—the translation of which was under the control of two tandem internal ribosomal entry sites (IRES)—was inserted into the 3′-untranslated region (UTR) of the endogenous *Foxp3* locus of C57BL/6 mice to generate a Foxp3-IRES-luciferase-IRES-eGFP (*FILIG*) allele (Supplementary Fig. 2). Using a similar approach, we have previously generated a knock-in mouse model where Foxp3-expressing cells are marked by the co-expression of a monomeric red fluorescent protein (RFP)[12]. Such Foxp3-IRES-mRFP (*FIR*) mice can be used to isolate wild-type T_R cells based on RFP expression. In the following experiments, hemizygous male *FIR* mice (*FIR/Y*), heterozygous female *FIR* mice (*FIR/+*) or homozygous female *FIR* mice (*FIR/FIR*) and T cells from these mice were used as wild-type controls where appropriate.

FILIG mice were born at a mendelian ratio. Heterozygous *FILIG* female mice (*FILIG/+*) were fertile and phenotypically normal; however, hemizygous *FILIG* male mice (*FILIG/Y*) were barren and runted (Fig. 1b). Over 50% of *FILIG/Y* mice developed scaly skin (data not shown) and nearly all of them developed eyelid defects resembling blepharitis, a T_H2 disorder, at around 4 weeks of age (Supplementary Fig. 3). By 3 months of age, all the *FILIG/Y* mice succumbed to an aggressive lymphoproliferative autoimmune syndrome, manifested by enlarged spleens and lymph nodes (Fig. 1b), infiltration of lymphocytes into non-lymphoid organs, drastically increased serum levels of auto-antibodies (Supplementary Fig. 4), and activated $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. 5). Overall, *FILIG/Y* mice displayed phenotypes reminiscent of scurfy mice[13] and T-cell-specific *Foxp3* knockout mice[1]. To investigate whether transcription of the endogenous *Foxp3* gene was abolished in *FILIG* mice, we first detected luciferase expression by live imagining. In *FILIG/+* mice, cells expressing luciferase were concentrated in lymphoid organs (Fig. 1c). However, in *FILIG/Y* mice, these cells were detected in lymphoid as well as non-lymphoid organs (Fig. 1c), suggesting that the endogenous *Foxp3* gene was transcribed in *FILIG* lymphocytes, and that *FILIG* lymphocytes infiltrated non-lymphoid organs in *FILIG/Y* mice. By flow cytometry, we detected GFP expression only in $CD4^+$ T cells (Supplementary Fig. 6). Notably, among $CD4^+$ T cells, there was a higher percentage of GFP^+ cells in *FILIG/Y* mice compared with RFP^+ cells in *FIR/Y* mice, whereas a lower percentage of GFP^+ cells was detected in *FILIG/+* mice compared with RFP^+ cells in *FIR/+* mice (Fig. 1d). By intracellular staining, we detected

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Foxp3 expression in GFP$^+$ CD4$^+$ T cells from *FILIG/Y* and *FILIG/+* mice, but not in GFP$^-$ CD4$^+$ T cells from *FILIG/Y* mice (Fig. 1e). Therefore, GFP expression reflected Foxp3 expression with high fidelity in *FILIG* mice. Compared with wild-type T_R cells, GFP$^+$ cells from *FILIG* mice expressed 5–10-fold less Foxp3. Thus, although Foxp3 was expressed at decreased levels, *FILIG* mice generated Foxp3-expressing CD4$^+$ T cells that were faithfully marked by GFP expression. Compared with wild-type T_R cells, the surface expression and messenger RNA levels of signature genes for T_R cells (such as *CD25*, *Ctla4* and *Gitr*[14–16]) were decreased in GFP$^+$ CD4$^+$ T cells from *FILIG/+* mice (Fig. 1f, g). The exact mechanism by which attenuated Foxp3 expression was achieved in *FILIG* CD4$^+$ T cells is unclear. However, four AU-rich elements (ARE), the presence of which in the 3′-UTR of a gene is known to destabilize mRNA[17], were found dispersed in the luciferase complementary DNA. Therefore, reduced Foxp3 expression in *FILIG* CD4$^+$ T cells is probably due to mRNA destabilization caused by localization of the luciferase sequence in the 3′-UTR of the *Foxp3* mRNA. As a consequence of random X-chromosome inactivation, the *FILIG/+* female mice contain a mixture of Foxp3$^+$ cells: those with wild-type levels of Foxp3 expression and those having low levels due to the gene knock-in approach; this mixture of cells probably accounts for lack of overt disease in these mice.

The *FILIG* model allowed us to investigate further which biological functions of T_R cells are altered owing to decreased Foxp3 expression. To assess whether attenuated Foxp3 expression affected T_R cell development, the percentages of Foxp3-expressing cells (Foxp3$^+$) among CD4$^+$ single positive thymocytes from 10- and 16-day-old *FILIG/Y* and *FIR/Y* mice were determined and compared, but no difference was observed (Fig. 2a). Whether homeostatic expansion/maintenance of Foxp3$^+$ cells was affected by attenuated Foxp3 expression was addressed by transferring cell mixtures combining conventional CD4$^+$ T cells (RFP$^-$) from *FIR/FIR* mice with Foxp3$^+$ (RFP$^+$ or

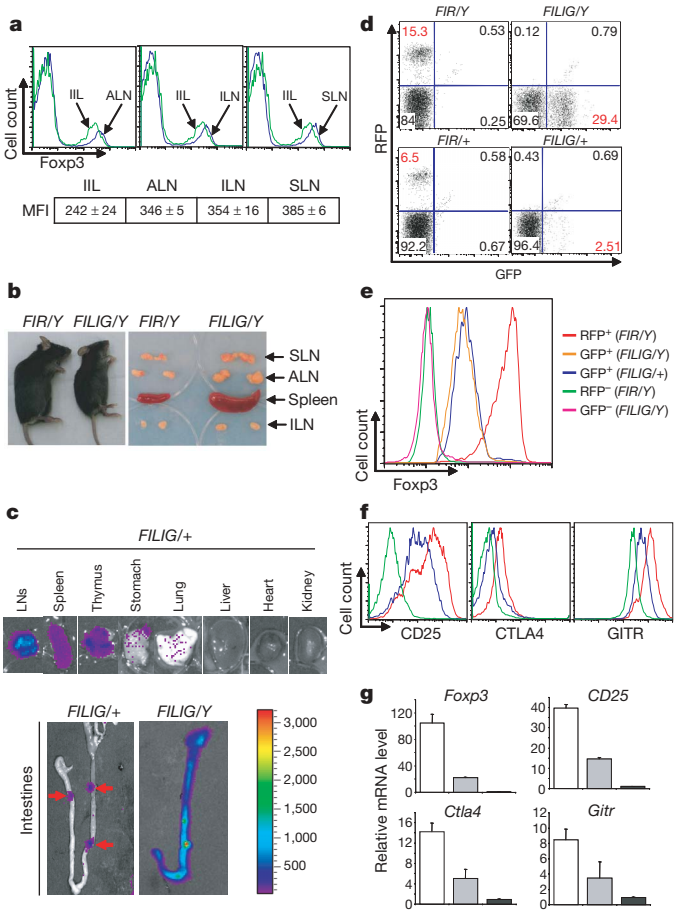

Figure 1 | Attenuated Foxp3 expression in T_R cells results in an aggressive autoimmune syndrome and altered surface properties of T_R cells. a, Flow cytometry for Foxp3 expression in CD4$^+$ T cells isolated from intra-islet lymphocytes (IIL; green line), and axillary (ALN), superficial-inguinal (ILN) and submandibular (SLN) lymph nodes (blue lines) from diabetic NOD mice. Mean fluorescence intensity (MFI) of Foxp3 staining among Foxp3$^+$ cells is indicated. Data are mean ± s.d. of two samples with one sample combining lymphocytes from three mice. **b**, Size comparison of 2-month-old *FIR/Y* and *FILIG/Y* mice (left panel); sizes of submandibular (SLN), axillary (ALN) and superficial-inguinal (ILN) lymph nodes and spleens are also shown (right panel). **c**, Localization of luciferase-expressing cells in various organs in *FILIG/+* and *FILIG/Y* mice. The colour scale with corresponding count units is shown. **d**, The percentage (highlighted in red) of GFP$^+$ cells among CD4$^+$ T cells in *FILIG/Y* and *FILIG/+* mice and of RFP$^+$ cells in *FIR/Y* and *FIR/+* control mice is shown. Results representative of at least ten experiments are shown. **e**, Flow cytometry of Foxp3 in sorted GFP$^+$ CD4$^+$ T cells from *FILIG/+* mice; GFP$^+$ and GFP$^-$ CD4$^+$ T cells from *FILIG/Y* mice; and RFP$^+$ and RFP$^-$ CD4$^+$ T cells from *FIR/Y* mice. Results representative of four experiments are shown. **f**, Surface expression of CD25, CTLA4 and GITR on GFP$^+$ CD4$^+$ T cells from *FILIG/+* mice (blue lines); RFP$^+$ CD4$^+$ T cells from *FIR/+* mice (red lines); and RFP$^-$ CD4$^+$ T cells from *FIR/FIR* mice (green lines). Results are representative of three experiments. **g**, Relative mRNA levels of *Foxp3*, *CD25*, *Ctla4* and *Gitr* in sorted GFP$^+$ CD4$^+$ T cells (grey bars) from *FILIG/+* mice, and RFP$^+$ (white bars) and RFP$^-$ (black bars) CD4$^+$ T cells from *FIR/FIR* mice. Data are mean ± s.d. of combined results from three experiments. The differences observed among samples for different genes are statistically significant as determined by the 'Statistical analysis' section of the Methods.

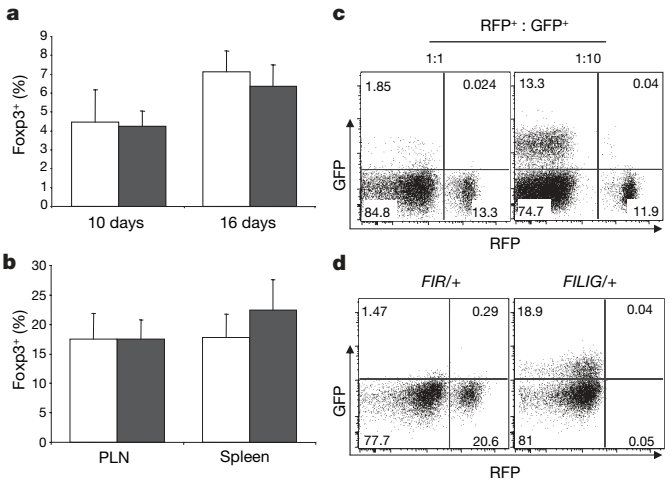

Figure 2 | Thymic development, homeostatic expansion/maintenance and TGF-β-induced *de novo* generation of T_R cells are normal when Foxp3 expression is decreased. a, The percentage of Foxp3$^+$ CD4$^+$ single positive thymocytes in 10- and 16-day-old *FIR/Y* (white bars) and *FILIG/Y* mice (black bars) is shown. Data are mean ± s.d. of results from four mice for each time point. **b**, Sorted RFP$^-$ CD4$^+$ T cells from *FIR/FIR* mice were mixed with either RFP$^+$ CD4$^+$ T cells from *FIR/+* mice (white bars) or GFP$^+$ CD4$^+$ T cells from *FILIG/+* mice (black bars) at a ratio of 2:1 and then transferred into female *Rag1*$^{-/-}$ mice. The percentage of Foxp3$^+$ (RFP$^+$ or GFP$^+$) CD4$^+$ T cells from different origins in different lymphoid organs was determined by flow cytometry 9 weeks later. PLN, peripheral lymph nodes and spleen. Data are mean ± s.d. of results from six mice of one experiment representative of two. **c**, RFP$^+$ and GFP$^+$ CD4$^+$ T cells were sorted from *FIR/+* and *FILIG/+* mice respectively, and then combined at the indicated ratios. 1×10^5 cell mixtures and 2×10^5 RFP$^-$ CD4$^+$ T cells sorted from *FIR/FIR* mice were co-transferred into *Rag1*$^{-/-}$ mice. The distribution of Foxp3$^+$ *FIR* (RFP$^+$) and Foxp3$^+$ *FILIG* (GFP$^+$) cells in the recipient mice was determined by flow cytometry 9 weeks later. Results representative of six mice are shown. **d**, CD4$^+$CD25$^-$ T cells that were negative for RFP and GFP were sorted from *FIR/+* and *FILIG/+* mice respectively, and then activated in the presence of TGF-β1. Foxp3 expression was measured by the expression of RFP and GFP. Results representative of three experiments are shown.

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GFP$^+$) CD4$^+$ T cells from either *FIR*/+ or *FILIG*/+ mice into *Rag1*$^{-/-}$ mice. RFP$^+$ and GFP$^+$ CD4$^+$ T cells in the recipient mice were detected 9 weeks after transfer (Supplementary Fig. 7); the percentage of RFP$^+$ and GFP$^+$ cells among transferred CD4$^+$ T cells was comparable (Fig. 2b), suggesting that decreased Foxp3 expression did not result in intrinsic defects of the homeostatic expansion/maintenance of GFP$^+$ *FILIG* cells. However, when RFP$^+$ and GFP$^+$ CD4$^+$ T cells were co-transferred into the same hosts in the presence of conventional CD4$^+$ T cells, GFP$^+$ cells competed poorly with RFP$^+$ cells (Fig. 2c), in agreement with the observation that a lower than expected percentage of GFP$^+$ cells was found in the *FILIG*/+ mice.

The fact that CD25 expression on GFP$^+$ cells from *FILIG*/+ mice was decreased relative to that on RFP$^+$ cells from *FIR*/+ mice could account for this phenomenon, as T_R cell maintenance is dependent on interleukin-2 (IL-2) signalling[18–20]. Extra-thymic generation of Foxp3$^+$ T cells can be promoted *in vitro* by transforming growth factor-β (TGF-β)[12,21,22]. TGF-β induced *de novo* Foxp3 expression in *FILIG* CD4$^+$ T cells to a similar extent as in *FIR* CD4$^+$ T cells (Fig. 2d).

In vitro, anergy and immune-suppressive activities are two defining properties for T_R cells that are thought to go hand-in-hand[23]. Notably, upon T-cell-receptor (TCR) stimulation *in vitro*, although GFP$^+$ cells from *FILIG* mice remained anergic (Fig. 3a and

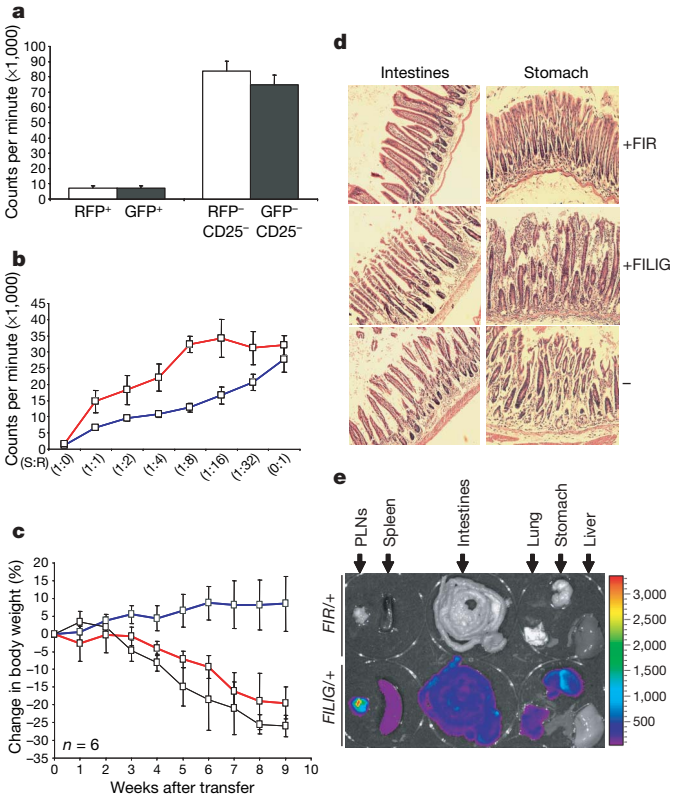

Figure 3 | Attenuation of Foxp3 expression abrogated the immune-suppressive but not hypoproliferative activities of T_R cells. **a**, RFP$^+$, GFP$^+$, RFP$^-$CD25$^-$ and GFP$^-$CD25$^-$ CD4$^+$ T cells were sorted from *FIR*/+ (white bars) and *FILIG*/+ (black bars) mice. Purified cells were activated *in vitro*, and cell proliferation was determined by a T-cell proliferation assay. Data are mean ± s.d. of triplicates done in one experiment representative of three. **b**, Suppression assay performed using sorted RFP$^+$ CD4$^+$ T cells from *FIR*/+ mice (blue line) or GFP$^+$ CD4$^+$ T cells from *FILIG*/+ mice (red line) as suppressor cells (S) and RFP$^-$ CD4$^+$ T cells sorted from *FIR/FIR* mice as responder cells (R). Data are mean ± s.d. of triplicates done in one experiment representative of three. **c**, *Rag1*$^{-/-}$ mice were transferred with RFP$^-$ CD4$^+$ T cells sorted from *FIR/FIR* mice alone (black line), or with T-cell mixtures containing one-third of RFP$^+$ CD4$^+$ T cells sorted from *FIR*/+ mice (blue line) or GFP$^+$ CD4$^+$ T cells sorted from *FILIG*/+ mice (red line) and two-thirds of RFP$^-$ CD4$^+$ T cells sorted from *FIR/FIR* mice. The percentage of body weight change in the recipient mice was determined weekly for 9 weeks after transfer. Data are mean ± s.d. of six mice from one experiment representative of two. **d**, Intestines and stomach were removed from recipient mice at the end of the experiments described in **c**. Haematoxylin-and-eosin staining was performed to detect pathological changes in mice that received conventional CD4$^+$ T cells together with RFP$^+$ CD4$^+$ T cells from *FIR*/+ (+FIR) or GFP$^+$ CD4$^+$ T cells from *FILIG*/+ (+FILIG) mice; or without Foxp3$^+$ cells (−). **e**, At the end of experiments described in **c**, localization of transferred GFP$^+$ *FILIG* cells in different organs (as indicated) was determined by live imaging. The colour scale with corresponding count units is shown. As a control, organs from a mouse that received RFP$^+$ *FIR* cells are shown.

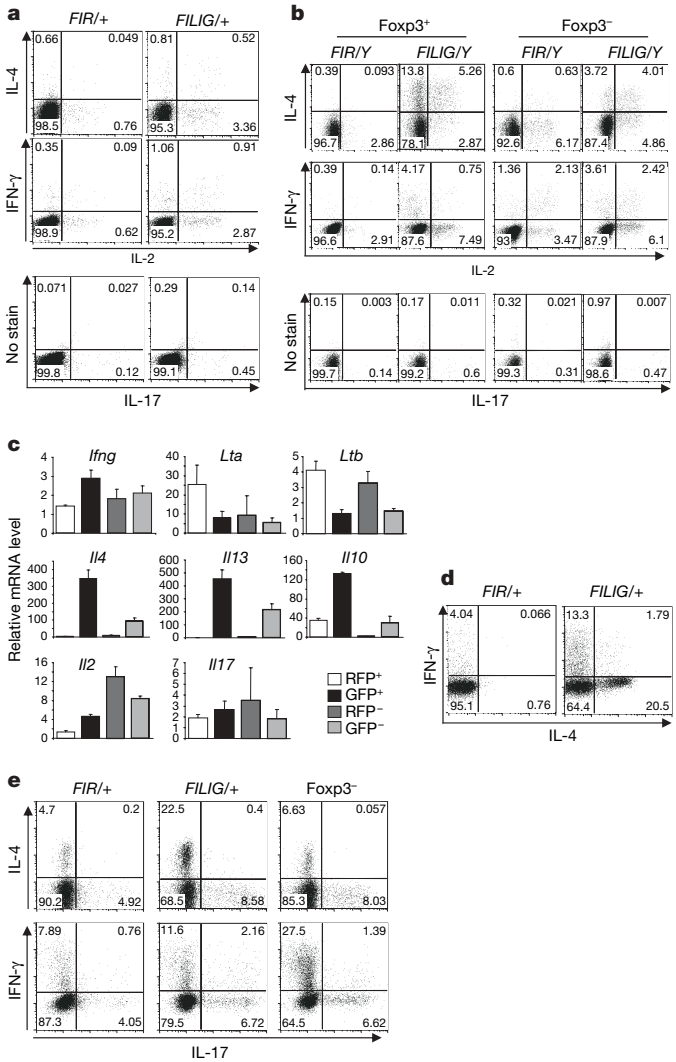

Figure 4 | T_R cells converted into T_H2 effector cells owing to decreased Foxp3 expression. **a**, **b**, The production of IL-2, IL-4, IL-17 and IFN-γ in RFP$^+$ CD4$^+$ T cells sorted from *FIR*/+ mice and in GFP$^+$ CD4$^+$ T cells sorted from *FILIG*/+ mice (**a**), or in RFP$^+$ and RFP$^-$ CD4$^+$ T cells from *FIR/Y* mice and GFP$^+$ and GFP$^-$ CD4$^+$ T cells from *FILIG/Y* mice (**b**) is shown. **c**, Relative mRNA levels of *Ifng*, *Lta* (lymphotoxin A), *Ltb* (lymphotoxin B), *Il4*, *Il13*, *Il10*, *Il2* and *Il17* among RFP$^+$, RFP$^-$, GFP$^+$ and GFP$^-$ CD4$^+$ T cells isolated from male *FIR/Y* and *FILIG/Y* mice are shown. Data are mean ± s.d. from two experiments. **d**, At the end of the experiments described in Fig. 3c, The expression of IL-4 and IFN-γ was examined in RFP$^+$ *FIR*/+ and GFP$^+$ *FILIG*/+ cells that were recovered from recipient mice. **e**, At the end of the experiments described in Fig. 2c (right panel), the expression of IL-4, IL-17 and IFN-γ was determined in co-existing RFP$^+$ *FIR*/+ cells and GFP$^+$ *FILIG*/+ cells as well as in RFP$^-$GFP$^-$ *FIR/FIR* cells. All results are representative of at least three experiments unless stated otherwise.

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Supplementary Fig. 8), their immune-suppressive activities were greatly impaired (Fig. 3b). Thus, anergy and immune suppression are two separable properties of T_R cells that are affected differentially by Foxp3 expression level. The immune-suppressive activities of Foxp3$^+$ CD4$^+$ T cells from *FILIG* mice were also abolished *in vivo*, because, unlike wild-type T_R cells, co-transferred GFP$^+$ cells from *FILIG/+* mice did not prevent conventional CD4$^+$ T-cell-elicited weight loss (Fig. 3c) or the immune pathologies in the intestines and stomach (Fig. 3d) of *Rag1*$^{-/-}$ recipient mice. This was not due to defective migration of transferred cells. In fact, adoptively transferred GFP$^+$ *FILIG* cells are found in lymphoid as well as non-lymphoid organs (Fig. 3e).

Loss of the suppressive activity of T_R cells could be sufficient to cause an aggressive autoimmune syndrome in *FILIG/Y* mice and the wasting disease observed in the aforementioned transfer model. Nevertheless, we further investigated whether Foxp3$^+$ *FILIG* cells developed effector functions that could contribute to any of these immune disorders. On the basis of cytokine production profiles, three types of effector T cells, T_H1, T_H2 and T_H17, have been described[24,25]. Although T_R cells bear self-reactive TCRs[26,27], GFP$^+$ cells from healthy *FILIG/+* mice did not exhibit an effector cell phenotype (Fig. 4a), suggesting that they did not spontaneously activate and convert into effector cells when substantial numbers of wild-type T_R cells were present. Notably, however, a large portion of GFP$^+$ cells from diseased *FILIG/Y* mice produced IL-4, whereas the percentage of cells expressing IL-2, interferon-γ (IFN-γ) or IL-17 was only modestly increased compared to wild-type cells (Fig. 4b). The percentage of IL-4-producing cells also preferentially increased in Foxp3$^-$ (GFP$^-$) CD4$^+$ T cells from *FILIG/Y* mice, consistent with the T_H2 disorder observed in these mice. Cytokine mRNA levels were also determined and agreed with aforementioned results (Fig. 4c). In addition, compared with wild-type T_R cells, the percentage of IL-4-producing GFP$^+$ *FILIG* cells showed an approximately 30-fold increase upon adoptive transfer, whereas that of IFN-γ-producing GFP$^+$ *FILIG* cells showed only an approximately 3-fold increase (Fig. 4d). In some experiments, we also noticed that the percentage of IL-4- but not IFN-γ-producing cells was substantially increased even among transferred wild-type Foxp3$^+$ *FIR* cells that expressed lower levels of Foxp3 (Supplementary Fig. 9), suggesting that IL-4 production can be induced in wild-type T_R cells expressing low levels of Foxp3 *in vivo*. To compare Foxp3$^+$ *FIR* and *FILIG* CD4$^+$ T cells directly in the same physiological environment, CD4$^+$ T cells that were RFP$^+$ from *FIR/+* mice, GFP$^+$ from healthy *FILIG/+* mice, and RFP$^-$ from *FIR/FIR* mice, were mixed at a ratio of 1:10:20 and then transferred into *Rag1*$^{-/-}$ hosts. At the time of transfer, none of these cells was producing substantial amounts of effector cytokines (Fig. 4a and data not shown). Recipient mice developed wasting disease 9 weeks after transfer (data not shown), possibly owing to the fact that insufficient numbers of wild-type T_R cells were transferred. Compared with coexisting RFP$^+$ wild-type T_R cells, the fraction of IL-4-producing cells among GFP$^+$ *FILIG* cells increased greatly, whereas that of IFN-γ- or IL-17-producing cells did not change substantially, although coexisting Foxp3$^-$ cells produced large quantities of IFN-γ, thereby providing a T_H1-polarizing condition (Fig. 4e). Moreover, GFP$^+$ *FILIG* cells potently induced T_H2 differentiation of conventional CD4$^+$ T cells *in vitro* and *in vivo*, potentially through IL-4. Intriguingly, IL-4 production by GFP$^+$ *FILIG* cells was not affected by the T_H1-polarizing environments imposed by co-cultured cells (Supplementary Fig. 10).

Decreased Foxp3 expression is associated with human immune disorders[5–7]. Our genetic evidence convincingly shows that decreased Foxp3 expression can cause defective suppressive function of T_R cells and their conversion into effector cells, which contribute to rather than inhibit immune diseases. This provides an important mechanistic explanation for the aetiology of immunopathology in our mouse model and potentially various human immune diseases.

METHODS

Mice and adoptive transfer assays. *FIR*, *FILIG*, *NOD*, C57BL/6 and *Rag1*$^{-/-}$ (C57BL/6 background) mice were kept under specific pathogen-free conditions in the animal care facility at Yale University. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Yale University. For adoptive transfer assays, conventional (RFP$^-$) CD4$^+$ T cells from *FIR/FIR* mice, RFP$^+$ CD4$^+$ T cells from *FIR/+* mice or GFP$^+$ CD4$^+$ T cells from *FILIG/+* mice were sorted by fluorescence-activated cell sorting (FACS). Sorted cells were either transferred alone or mixed at different ratios as elaborated in the text or figure legends. A total of 3×10^5 cells were transferred into female *Rag1*$^{-/-}$ mice via retro-orbital injection. Mice were weighed every week thereafter and killed 9–10 weeks after transfer.

Generation of *FILIG* mice. *FILIG* knock-in mice were generated according to the protocols described for generating *FIR* mice[12]. A gene cassette encoding IRES-luciferase-IRES-eGFP instead of IRES-RFP was inserted into an *Ssp1* site. The floxed neomycin cassette was deleted *in vitro* by transfecting Cre-expressing plasmid into Bruce-4 embryonic stem cells originating from C57BL/6 mice.

Live imaging and histology. For live imaging analysis, mice were injected intraperitoneally with 3 mg luciferin (Xenogen) per mouse and then killed. Different lymphoid and non-lymphoid organs were surgically removed. Luciferase-expressing organs were visualized by IVIS Imaging System (Xenogen) as per the manufacturer's protocols. For histology analysis, organs from mice were removed and fixed in S.T.F. fixatives (Streck) for 24 h. Preparation of the slides, sectioning and haematoxylin-and-eosin staining were performed by the Yale histology and pathology laboratory.

Statistical analysis. Data from at least three sets of samples were used for statistical analysis. Mean ± s.d. are shown. Statistical significance was calculated by Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Supplementary Methods

Mice and adoptive transfer assays

FIR, *FILIG*, *NOD*, C57BL/6 and *Rag1*$^{-/-}$ (C57BL/6 background) mice were kept under specific pathogen-free conditions in the animal care facility at Yale University. All mouse experiments were approved by Institutional Animal Care and Use Committee of Yale University. For adoptive transfer assays, conventional (RFP^{-}) CD4 T cells from *FIR/FIR* mice, RFP^{+} CD4 T cells from *FIR*/+ or GFP^{+} CD4 T cells from *FILIG*/+ mice were sorted by FACS. Sorted cells were either transferred alone or were mixed at different ratios as elaborated in the texts or figure legends. A total of 3x10^{5} cells in a 200μl sterile 1x PBS were transferred into female *Rag1*$^{-/-}$ mice via retro-orbital injection. Mice were weighed every week thereafter and euthanized 9-10 weeks post transfer.

Generation of *FILIG* mice

FILIG knock-in mice were generated according to the protocols described for generating *FIR* mice[12]. A gene cassette encoding IRES-Luciferase-IRES-eGFP instead of IRES-RFP was inserted into an Ssp1 site. The floxed Neo cassette was deleted *in vitro* by transfecting Cre-expressing plasmid into Bruce-4 ES cells originated from C57BL/6 mice.

Live imagining and histology

For live imaging analysis, mice were injected intra-peritoneally with 3 mg luciferin (Xenogen) per mouse and then enthanized. Different lymphoid and non-lymphoid organs were surgically removed. Luciferase expressing organs were visualized by IVIS Imaging

System (Xenogen) per manufacturer's protocols. For histology analysis, organs from mice were removed and fixed in S.T.F. fixatives (Streck) for 24 hours. The preparation of the slides, sectioning and Hematoxylin-and-Eosin (H&E) staining were performed by the Yale histology and pathology laboratory.

Flow cytometry

Lymphocytes were isolated from the thymus, spleen, and lymph nodes from 1-10 week old mice. To isolate intra-islet lymphocytes, islets were prepared by Yale DERC facility. Islets from 3-4 mice were pooled and the lymphocytes were harvested via standard procedures. Except for Foxp3 (eBioscience), antibodies for FACS analysis were purchased from BD Pharmingen. Cells were stained per manufacturer's protocols. Foxp3, IL-2, IL-4, IFN-γ and IL-17 production was analyzed by intracellular staining per manufacturer's protocols. Stained cells were analyzed on a FACSCalibur or LSRII station (Becton Dickinson), or sorted on a MoFlow cell sorter (Dako Cytomation) or VantageSE station (Becton Dickinson).

T cell culture, activation, proliferation and suppression assays

T cells were cultured in Bruff's medium supplemented with 10% FBS and 1% penicillin/streptomycin. T cells were activated with plate-bound 5 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28 for long term culturing or with 100 ng/ml of PMA and 1 μM of ionomycin for 4-6 hours to stimulate cytokine production. Recombinant hTGF-β1 (R&D systems) was added to the culture at 4 ng/ml where stated. For T cell proliferation assay and suppression assay, $2x10^4$ purified responder T cells were combined with FACS purified suppressor T cells at different ratios in the presence of $8x10^4$ irradiated CD4 and CD8 depleted antigen presenting cells. The T cell mixture was stimulated with soluble

anti-CD3 (1μg/ml) and anti-CD28 (1ug/ml) antibodies for 72 hours and then 1μCi of ^{3}H-thymidine was added for 8 hours, T cell proliferation was determined by counting incorporated ^{3}H using a β-counter (PerkinElmer).

Quantitative RT-PCR

RNA was extracted using Trizol reagent and cDNA was synthesized by Superscript II reverse-transcriptase per manufacturer's protocols (Invitrogen). Quantitative PCR was performed on the ABI 7500 Real-time PCR system using primer/probe sets purchased from ABI. The relative levels of mRNA were determined by the following method. For each sample, per cell mRNA level of the gene of interest was determined by normalizing experimentally determined mRNA level of the gene of interest to internal control HPRT mRNA level. The sample with the lowest per cell mRNA level of the gene of interest was identified. Per cell mRNA level of the gene of interest of such sample was arbitrarily set as 1. The relative fold differences between per cell mRNA level of the gene of interest of this sample and other samples were calculated and presented as "relative mRNA level".

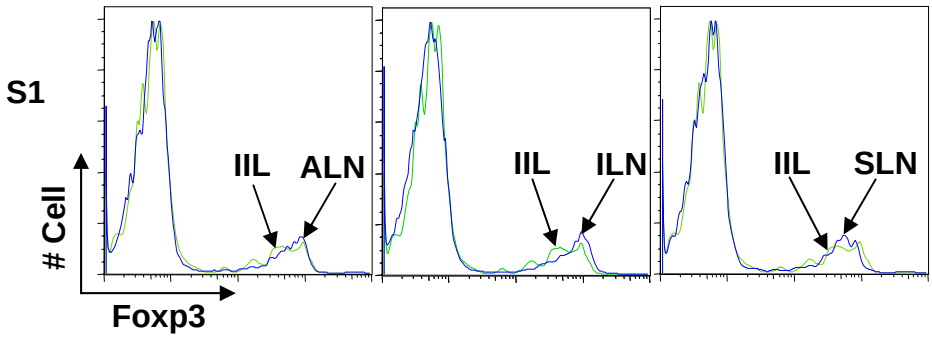
Enzyme-Linked Immunosorbent Assay (ELISA)

Peripheral blood was drawn from mice via tail bleeding. Serum was collected. The quantity of auto-antibodies against nuclear antigens (ANA) and double-stranded DNA (dsDNA) presented in the serum were determined by ELISA per manufactures protocols (Alpha Diagnostic International).

Statistical analysis

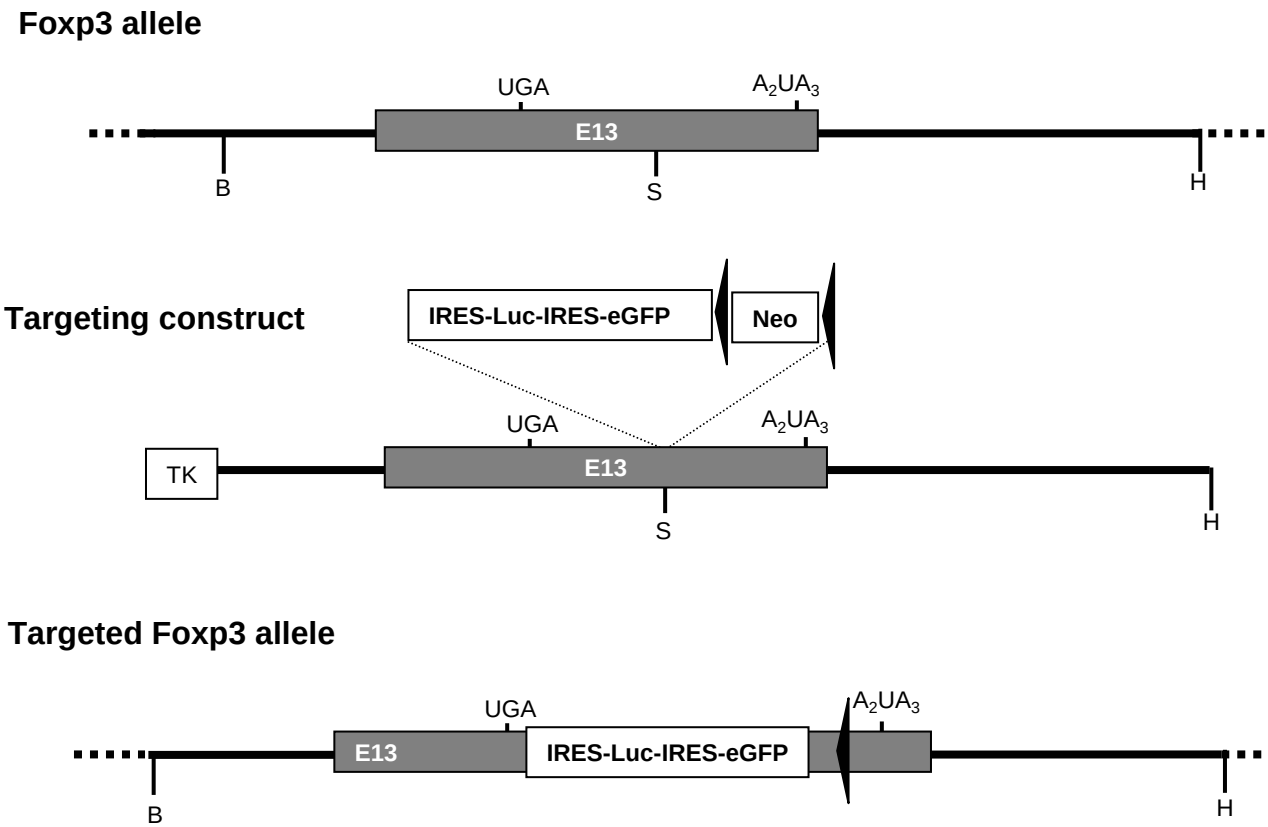
Data from at least three sets of samples were used for statistical analysis. Mean ± s.d. were shown. Statistical significance was calculated by Student's *t*-test. A *P* value of less than 0.05 was considered significant.

Supplementary Figures



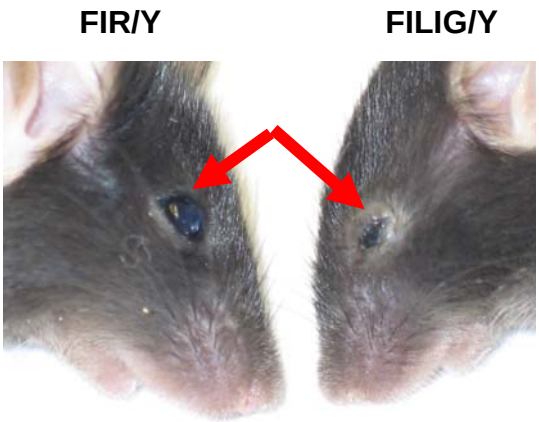
S1. Similar levels of Foxp3 expression were detected in Treg cells from islets and peripheral lymph-nodes in C57BL/6 mice. Foxp3 expression levels were determined and compared in CD4 T cells (ILN) and submendubullar (SLN) lymph-nodes (isolated from intra-islet lymphocytes (IIL) (green line), axillary (ALN), superficial-inguinal blue lines) from C57BL/6 mice by intracellular staining. Result shown is one representative experiment of two, where lymphocytes from 4 mice were pooled for each experiment.

S2



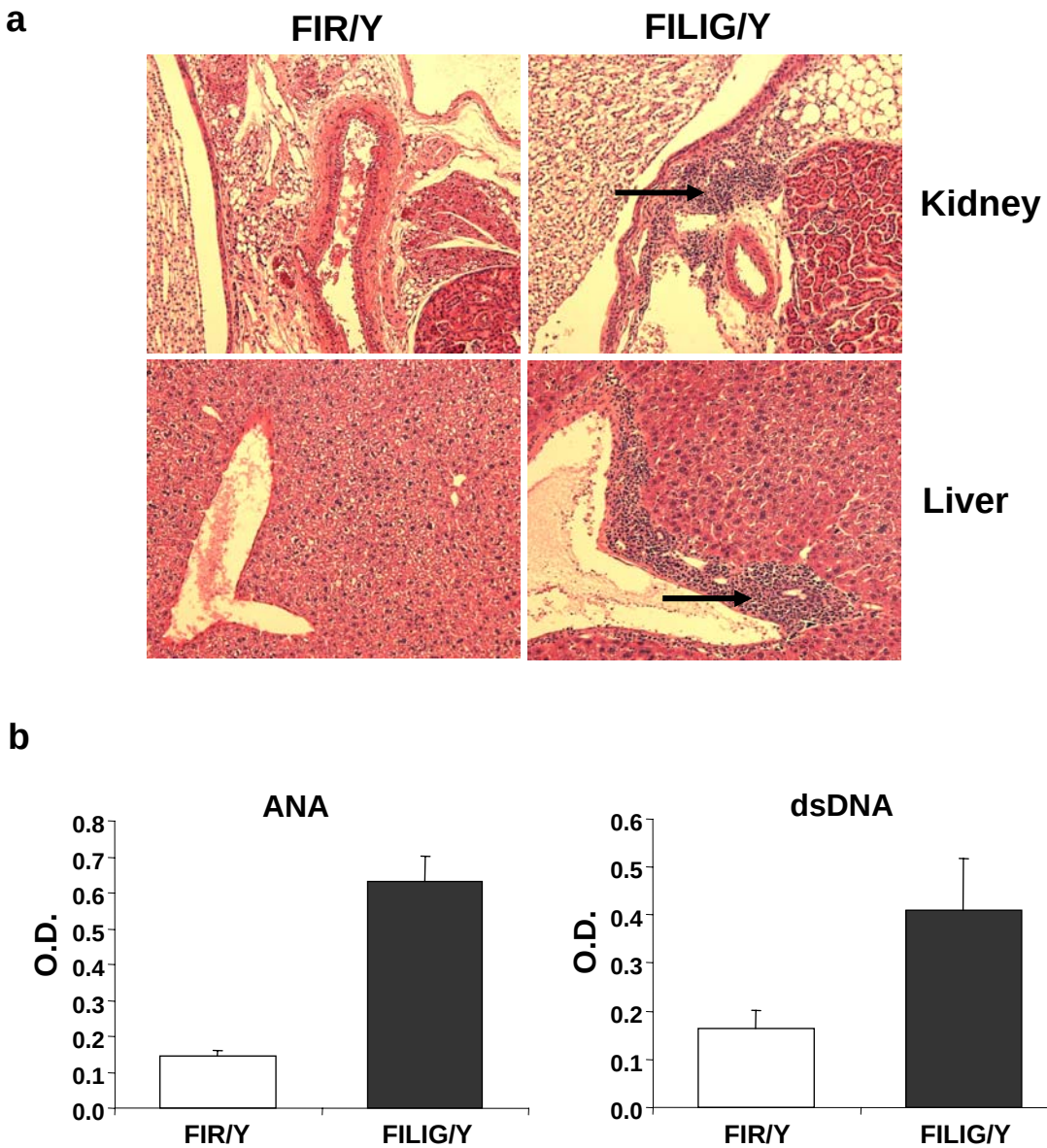
S2. Generation of *FILIG* knock-in mice. Maps for mouse *Foxp3* locus, targeting DNA construct, and the targeted Foxp3 locus are shown. An 11 kb mouse genomic DNA including Exon13 of the *Foxp3* gene was excised using BstZ17I (B) and HpaI (H) (top panel) and cloned into the pEasy-Flox vector adjacent to the Thymindine Kinase (TK) selection marker. A cassette containing IRES-luciferase-IRES-eGFP (IRES-Luc-IRES-eGFP) and LoxP-flanked Neomycin (Neo) selection marker was inserted into an SspI (S) site between the translation stop codon (UGA) and the polyadenylation signal (A_2UA_3) of the *Foxp3* gene (middle panel). The Neo gene was removed *in vitro* by transfecting C57BL/6 ES cells (Bruce-4) with a Cre recombinase expressing construct. ES cells bearing the correctly targeted (lower panel) *Foxp3* allele were used to create chimeras and germline-transmitted C57BL/6 mice.

S3



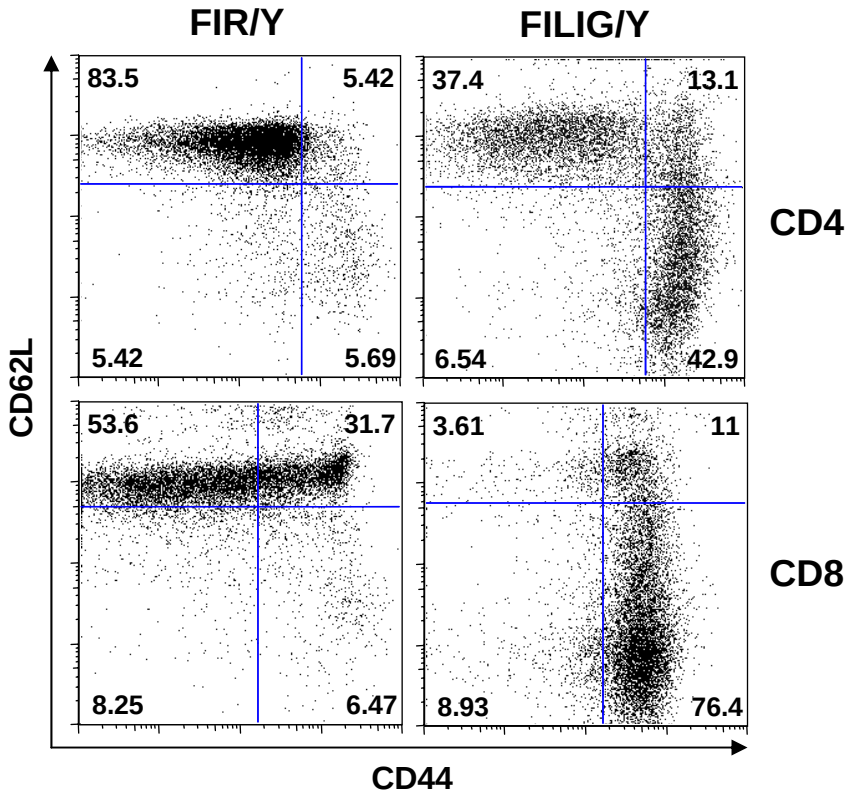
S3. Eyelid defects resembling blepharitis developed in *FILIG/Y* mice. The gross appearance of eyelid areas of two-month old *FIR/Y* and *FILIG/Y* mice was shown.

S4



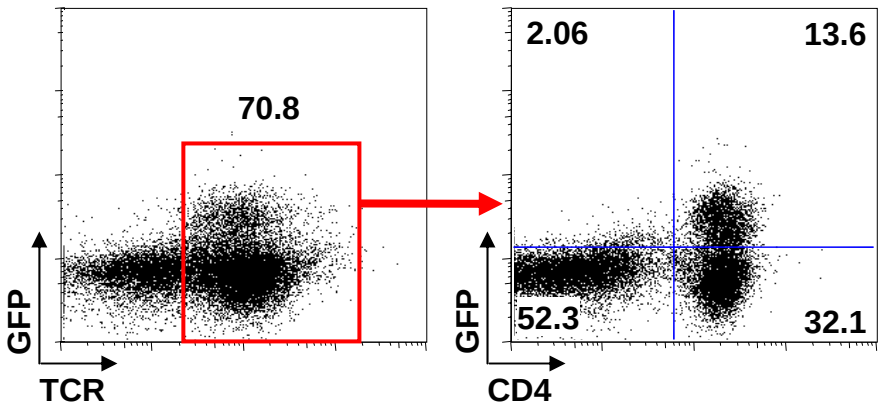
S4. Aggressive autoimmunity developed in *FILIG/Y* mice. a, H&E staining performed on the tissue sections from kidneys and livers from *FIR/Y* and *FILIG/Y* mice. Aggregation of infiltrated lymphocytes were indicated by arrows. **b,** ELISA analysis of antibodies against nuclear antigen (ANA) and double-stranded DNA (dsDNA) in the serum from *FIR/Y* and *FILIG/Y* mice. Optical density (O.D.) values are presented. Data are mean value ± s.d. of the results from 4 mice. The differences observed are statistically significant as determined by the 'Statistical analysis' descried in 'Methods' section.

S5



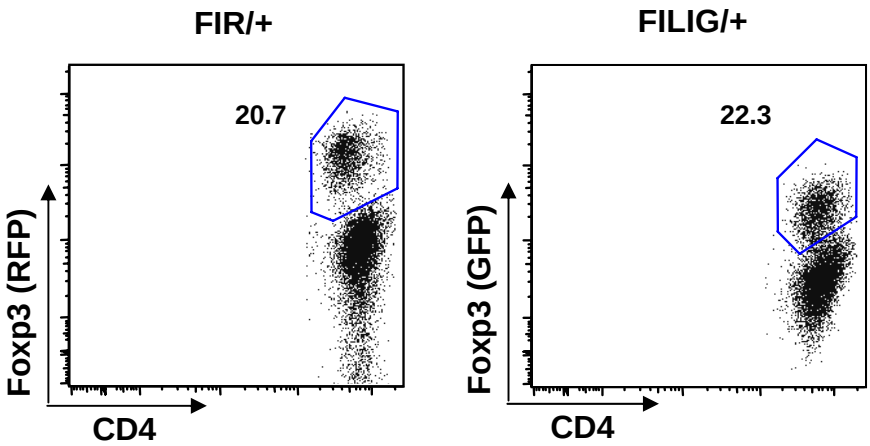
S5. CD4 and CD8 T cells in *FILIG/Y* mice displayed an activated phenotype. Flow cytometry analysis of CD62L and CD44 expression on the CD4 and CD8 T cells isolated from peripheral lymph-nodes in *FIR/Y* and *FILIG/Y* mice.

S6



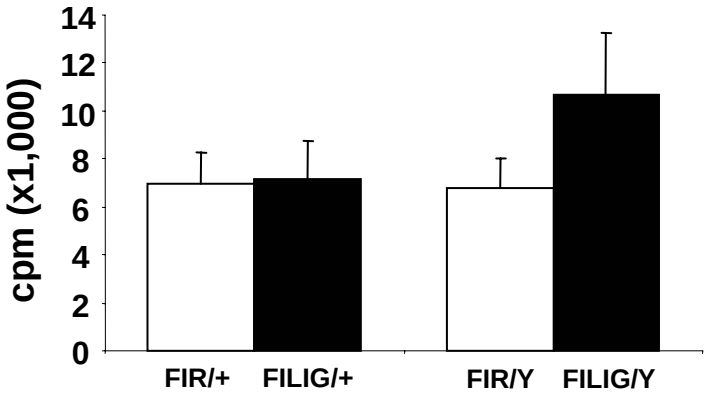
S6: GFP expressing cells were only detected in CD4 T cells, but not in CD8 T cells or non-T cells in *FILIG* mice. Flow-cytometry analysis of GFP expressing cells among lymphocytes isolated from peripheral lymph-nodes in *FILIG/Y* mice.

S7



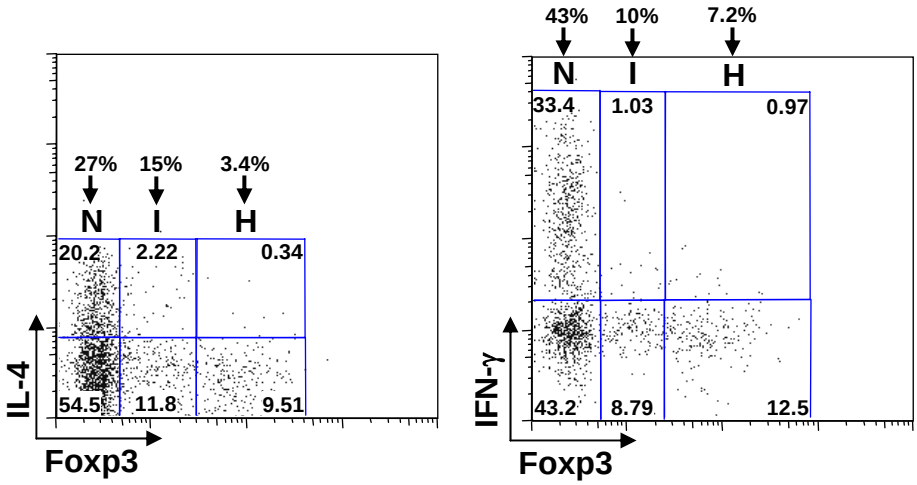
S7. Homeostatic expansion/maintenance of Foxp3 expressing *FIR* and *FILIG* cells in *Rag*$^{-/-}$ recipient mice. Sorted RFP^{-} CD4 T cells from *FIR/FIR* mice were mixed with sorted RFP^{+} cells from *FIR/+* or GFP^{+} cells from *FILIG/+* mice at a 2:1 ratio. A total of 3x10^{5} cells were transferred into female *Rag*$^{-/-}$ mice. The RFP^{+} and GFP^{+} CD4 T cells in peripheral lymph-nodes were detected by flow-cytometry and the percentages of these cells among CD4 T cells were determined 9 weeks later.

S8



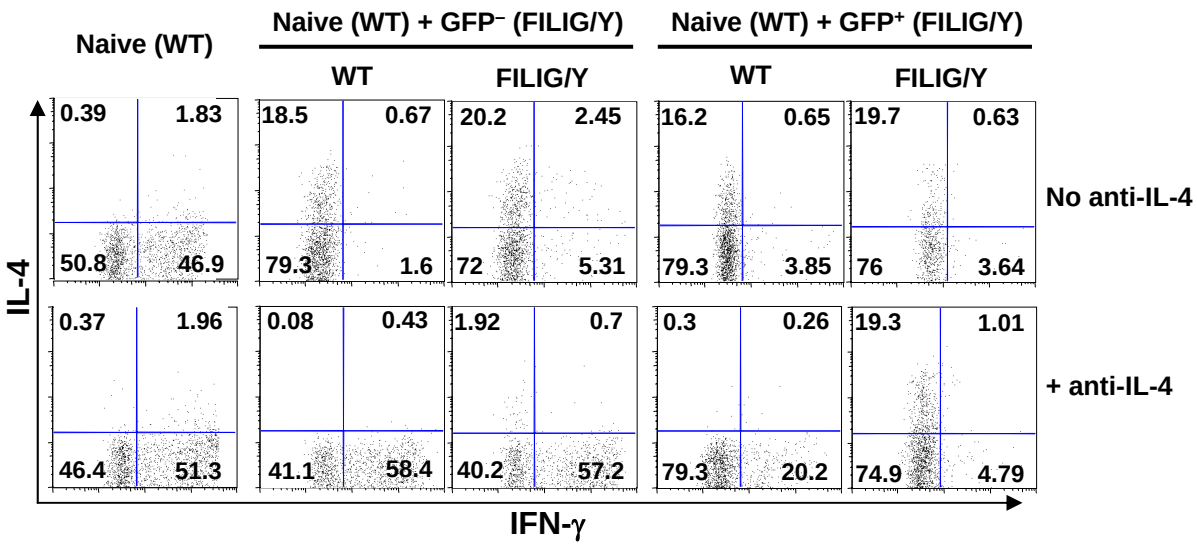
S8. Foxp3$^+$ *FILIG* cells are hypo-proliferative *in vitro*. RFP$^+$ (white bars) and GFP$^+$ (black bars) CD4 T cells were sorted from *FIR/+*, *FILIG/+*, *FIR/Y* and *FILIG/Y* mice (as indicated). Purified cells were activated *in vitro*, and cell proliferation was determined by a T cell proliferation assay. Data are mean value ± s.d. of triplicates done in one experiment representative of three.

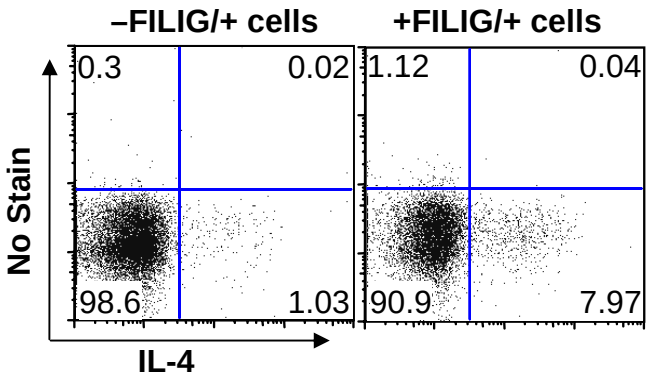
S9



S9: IL-4 but not IFN-γ were preferentially produced by Treg cells with lower Foxp3 expression. Sorted RFP^{-} CD4 T cells from *FIR/FIR* mice were mixed with RFP^{+} CD4 T cells from *FIR/+* mice at a 2:1 ratio. A total of 3x10^{5} cells were transferred into female *Rag*$^{-/-}$ mice. Nine weeks post transfer, CD4 T cells recovered from recipient mice were re-stimulated and then stained intracellularly for IL-4, IFN-γ and Foxp3. Cells did not express (N) or expressed intermediate (I) or high (H) levels of Foxp3 were indicated. The percentages of cytokine producing cells among each of the three populations were shown.

S10





S10: GFP⁺ *FILIG* cells promote Th2 differentiation of conventional T cells *in vitro* and *in vivo*. a, Naive ($CD25^{-}CD62L^{high}CD44^{low}$) CD4 T cells ($2x10^5$) sorted from CD45.1 wild-type (WT) mice were either stimulated alone (Naive) or with $0.5x10^5$ GFP^{-} or GFP^{+} cells isolated from CD45.2 *FILIG/Y* mice in the absence (no anti-IL-4) or presence (+anti-IL-4) of 10μg/ml IL-4 neutralizing antibodies. Four days post stimulation, IL-4 production in $CD45.1^{+}$ (WT) and $CD45.2^{+}$ (FILIG/Y) CD4 T cells were assayed. **b,** *Rag*$^{-/-}$ mice were transferred with total of $3x10^5$ $Foxp3^{-}$ (RFP^{-}) CD4 T cells sorted from *FIR/FIR* mice alone (–*FILIG*/+ cells) or with a mixture of $2x10^5$ $Foxp3^{-}$ CD4 T cells and $1x10^5$ GFP^{+} *FILIG*/+ cells (+*FILIG*/+ cells). Nine weeks after transfer, IL-4 production in $Foxp3^{-}$ CD4 T cells recovered from recipient mice were assessed.