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Id2 and Id3 maintain the regulatory T cell pool to suppress inflammatory disease

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Abstract

Regulatory T (T_{reg}) cells suppress the development of inflammatory disease, but our knowledge of transcriptional regulators that control this function remains incomplete. Here we show that expression of *Id2* and *Id3* in T_{reg} cells was required to suppress development of fatal inflammatory disease. We found that T cell antigen receptor (TCR)-driven signaling initially decreased the abundance of *Id3*, which led to the activation of a follicular regulatory T (T_{FR}) cell-specific transcription signature. However, sustained lower abundance of *Id2* and *Id3* interfered with proper development of T_{FR} cells. Depletion of *Id2* and *Id3* expression in T_{reg} cells resulted in compromised maintenance and localization of the T_{reg} cell population. Thus, *Id2* and *Id3* enforce T_{FR} cell checkpoints and control the maintenance and homing of T_{reg} cells.

Homeostasis of the immune system requires careful control mechanisms at mucosal barriers, sites exposed to abundant foreign antigens. Immune system cells must provide protection against a broad range of invading pathogens but also ensure tolerance to self antigens and

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Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

AUTHOR CONTRIBUTIONS

M.Miy., K.M. and S.C. performed the majority of experiments. K.M. performed RNA-seq analysis. M.I. performed immunostaining of spleen and lymph nodes. N.V. contributed to the analysis of pathology. M.Mil. contributed to the analysis of lung tissue. L.-F.L. suggested key experiments and edited the manuscript. A.N.C. analyzed RNA-seq data. M.Miy. and C.M. wrote the manuscript. D.H.B. and C.M. supervised the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

innocuous non-self antigens^{1–3}. Failure of the immune system to enforce tolerance readily leads to the development of autoimmune disease and allergies, including asthma and atopic dermatitis. Allergy is characterized by the expression of T_H2 cell cytokines, high concentrations of serum immunoglobulin E (IgE) and eosinophilia^{4,5}. T_{reg} cells are prominent among the cell types that suppress spontaneous inflammation and are characterized by expression of the transcription factor Foxp3 (refs. 6–11). Absence of *Foxp3* in mice and *FOXP3* in humans rapidly results in the development of multiorgan autoimmunity, inflammatory bowel disease and allergy. T_{reg} cells develop in the thymus (tT_{reg} cells) as well as in the peripheral organs (pT_{reg} cells)^{1–3}. pT_{reg} cells act primarily to control the development of mucosal inflammation¹². T_{reg} cells are also essential in regulation of humoral immunity; loss of T_{reg} cells leads to elevated concentrations of autoantibodies, hyper-IgE syndrome, increased numbers of follicular helper T (T_{FH}) cells and spontaneous development of germinal centers (GCs)¹³. Recent studies have identified a subset of T_{reg} cells named T_{FR} cells that control GC reactions, characterized by the expression of *Cxcr5*, *Bcl6*, *Pdcd1* and *Prdm1* (refs. 14–16).

Members of the helix-loop-helix (HLH) family regulate many developmental trajectories in the thymus¹⁷. These include E proteins as well as Id proteins. E proteins function as transcriptional activators or repressors with the ability to bind specific DNA sequences termed E-box sites. Four E proteins have been identified and characterized: E12, E47, HEB and E2-2. E12 and E47 are encoded by the *Tcf3* locus and are generated by differential splicing¹⁸. HEB and E2-2 are related to the *Tcf3* gene products but diverge substantially in the N-terminal transactivation domains. DNA-binding activity of E proteins is regulated by the Id proteins^{19,20}. Four Id proteins named Id1, Id2, Id3 and Id4 contain an HLH dimerization domain but lack the basic DNA-binding region. Interactions between Id proteins and E proteins suppress DNA-binding activity of E proteins. Id2 and Id3 are particularly important in modulating the developmental progression of T lineage cells^{21–26}.

Here we found that depletion of *Id2* and *Id3* expression in T_{reg} cells resulted in the early onset of fatal T_H2 cell-mediated inflammatory disease. We found that upon TCR-mediated signaling in T_{reg} cells, expression of *Id2* and *Id3* declined, leading to higher binding activity of E proteins and induction of a T_{FR} cell-specific program of gene expression, including *Cxcr5* and *Il10*. Loss of *Id2* and *Id3* in T_{reg} cells resulted in compromised T_{reg} cell homeostasis, increased susceptibility to cell death upon stimulation and aberrant tissue localization. Taken together, we propose that Id2 and Id3 maintain the T_{reg} cell pool and act as gatekeepers to enforce multiple checkpoints during T_{FR} cell differentiation.

RESULTS

Id2 and *Id3* expression in T_{reg} cells

As a first approach to explore potential roles for *Id2* and *Id3* in T_{reg} cells, we analyzed their expression patterns using *Id2*-YFP and *Id3*-GFP reporter mice^{26,27}. We found that the thymic T_{reg} cell population can be segregated into Id2⁺Id3⁺ as well as Id2⁺Id3⁺ compartments (Fig. 1a). In maturing T_{reg} cells, which first express CD69 and then upregulate CD62L, *Id2* expression declined, leading to an increase of the Id2⁺Id3⁺ compartment (Fig. 1a). In the peripheral lymphoid organs, the majority of T_{reg} cells

consisted of $Id2^{-/-}Id3^{+/+}$ cells (Fig. 1a). To examine the dynamics of *Id2* and *Id3* expression upon stimulation, sorted T_{reg} cells carrying the *Id2*-YFP and *Id3*-GFP reporters were activated *in vitro* by exposure to anti-CD3e and anti-CD28 in the presence of non- T_{reg} cells as well as antigen-presenting cells (APCs) (Fig. 1b). The most pronounced change occurred in *Id3* expression, which declined substantially upon exposure to TCR-mediated signaling (Fig. 1b). Thus, the majority of T_{reg} cells isolated from peripheral organs expressed abundant *Id3* but lacked *Id2*, but upon *in vitro* stimulation, *Id3* expression declined in a fraction of cells, leading to $Id2^{lo}Id3^{lo}$ and $Id2^{int}Id3^{lo}$ T_{reg} cell populations.

***Id2* and *Id3* expression suppresses fatal inflammation**

To evaluate the roles of *Id2* and *Id3* in T_{reg} cell function, we crossed $Id2^{loxP/loxP}$ ($Id2^{fl/fl}$) and $Id3^{fl/fl}$ mice with *Foxp3*^{Cre} mice^{23,28,29}. The *Foxp3*^{Cre} mice used throughout this study carried an internal ribosomal entry site followed by a sequence encoding a Cre-YFP functional fusion protein located downstream of the *Foxp3* termination codon²⁹. The *Foxp3* locus is X chromosome-linked, and consequently gender-based differences in excision of the *loxP*-flanked alleles impact the population of T_{reg} cells present in these mice. Here we will refer to male mice carrying the *Foxp3*-Cre-YFP allele as *Foxp3*^{Cre} mice and to female mice as *Foxp3*^{Cre/Cre} or *Foxp3*^{Cre/+} mice. Mice deficient for *Id2* or *Id3* alone in T_{reg} cells did not show evidence of inflammatory disease (Supplementary Fig. 1 and data not shown). To examine whether *Id2* and *Id3* compensate for each other, we sorted T_{reg} cells from $Id2^{fl/fl}Foxp3^{Cre}$ mice as well as $Id3^{fl/fl}Foxp3^{Cre}$ mice. We isolated RNA from the sorted populations and examined it by real-time PCR for *Id2* and *Id3* expression. In the absence of *Id2* expression, the abundance of *Id3* increased, whereas in T_{reg} cells deficient for *Id3* expression, abundance of *Id2* increased, as compared to controls (Fig. 1c).

Next, we generated $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ as well as $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}$ mice. $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}$ mice were born with Mendelian frequencies and were healthy up to 4 weeks of age. However, 6–8-week-old mice with no sex bias developed dermatitis with swelling and fur loss across the eyelids, and the majority of $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}$ mice died within ten weeks (Fig. 1d and Supplementary Fig. 2a). Neither $Id2^{fl/+}Id3^{fl/fl}Foxp3^{Cre}$ nor $Id2^{fl/fl}Id3^{fl/+}Foxp3^{Cre}$ mice displayed early morbidity (data not shown). Approximately one-third of $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}$ mice showed intense dermatitis with scaling and fur loss throughout the body but not in the skin of the tail (data not shown). Similar to the case with human atopic dermatitis, $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ mice displayed dermatitis as evidenced by compulsive scratching of the face and back (**Supplementary Video 1**).

$Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}$ mice also showed splenomegaly and lymphadenopathy of subcutaneous lymph nodes accompanied by increasing cell numbers, whereas the size of mesenteric lymph nodes (MLNs) was comparable to that of littermate control mice (Supplementary Fig. 2a,b). As the *Foxp3* locus is located on the X chromosome, half of the T_{reg} cells in heterozygous female mice that carry one copy of *Foxp3*^{Cre} express normal amounts of *Id2* and *Id3* (ref. 30). We found that heterozygous female $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}$ mice lacked dermatitis across the eyelids, lacked detectable

splenomegaly and lymphadenopathy, and had normal percentages of naive CD4⁺ and CD8⁺ T cells (Supplementary Fig. 2c,d and data not shown). Histological analysis of tissue samples from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice revealed leukocytic infiltrates in the lung, esophagus and eyelids, with an associated reactive hyperplasia of the adjacent mucosal epithelial cells (Fig. 2a). The inflammatory infiltrates in the eyelid were localized to the subepithelial area and there was no inflammation in the lachrymal gland (Supplementary Fig. 3a). The inflammatory infiltrates in the lungs were peribronchial and perivascular, accompanied by production of mucus in the bronchial mucosal goblet cells (Fig. 2b). Many of the peribronchial and perivascular infiltrates of inflammatory cells in the lungs of *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice displayed expression of major basic protein (MBP), indicative of eosinophils, accompanied by infiltrating B cells and T cells (Fig. 2b and Supplementary Fig. 3b). In a similar fashion, inflammatory infiltrates across subepithelial regions of eyelids and the esophagus of *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* as well as *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice were composed primarily of eosinophils (Fig. 2c). We also found considerable eosinophilic infiltration in the hilar lymph nodes as well in the pancreas but not across the pancreatic islands (Supplementary Fig. 3c,d). We did not observe esophagitis in *Foxp3* mutant mice, which indicated that pathologies associated with depletion of *Id2* and *Id3* expression differ from that observed in mice lacking *Foxp3* (Supplementary Fig. 3e). Notably, the larger bronchioles were filled with denuded epithelial cells and cell debris in dying *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice (Supplementary Fig. 3a).

In a minor fraction of mice (<20%), other organs such as liver, kidney, stomach, pancreas and colon displayed low levels of inflammation (data not shown). Notably, three of five *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice that survived beyond 12 weeks of age showed evidence of focal pneumonia, characterized by areas in the lung that were filled with eosinophilic foamy macrophages, and had scarring and increased deposition of collagen surrounding the bronchioles (Supplementary Fig. 4). We also observed mild to moderate colitis with an increase in numbers of mucosal MBP-expressing eosinophils (Supplementary Fig. 4).

Consistent with the pathology in the lung, we found an increase in the total number of eosinophils and lymphocytes in the bronchial alveolar lavage (BAL) fluid and markedly elevated numbers of eosinophils, neutrophils, T cells and B cells in the lung derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice (Fig. 3a,b). The increase in eosinophil cellularity in the lung was closely associated with elevated numbers of type 2 innate lymphoid cells (ILC2) (Fig. 3b)^{31–33}. In addition, we observed substantially increased numbers of eosinophils, basophils and neutrophils in the lymph nodes and spleen derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice as compared to littermate control mice (Fig. 3c). These observations indicate that the absence of *Id2* and *Id3* expression in T_{reg} cells leads to spontaneous T_H2 cell-mediated fatal airway inflammation in the lungs as well as inflammation of the eyelids, the skin and the esophagus.

***Id2* and *Id3* suppress T_H2-mediated inflammation**

To determine how depletion of *Id2* and *Id3* expression in T_{reg} cells leads to inflammatory disease, we examined the lymphoid organs. We noticed increased numbers of T cells and B

cells in the spleen and subcutaneous LNs, in which we observed lower percentages of CD62L^{hi}CD44^{lo} naive CD4⁺ and CD8⁺ T cells as well as a significant increase in the percentages of T_{FH} cells, GC B cells and IgG1 class-switched B cells (Fig. 4a,b and data not shown). We observed a substantial increase in T_H2 cell-mediated production of cytokines, such as interleukin 4 (IL-4), IL-5 and IL-13 in the lymph nodes and spleen derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice (Fig. 4c). Consistent with the markedly increased numbers of T_{FH} cells and GC B cells, and the augmented expression of T_H2 cell cytokines, 6–8-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice exhibited a spontaneous increase in IgM, IgG1 and IgE serum concentrations but not in IgG2b, IgG3 and IgA (Fig. 4d). Titers of anti-nuclear antibodies in serum isolated from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice, however, were comparable to those in the littermate control (data not shown). These data indicate that expression of *Id2* and *Id3* in T_{reg} cells is required to suppress spontaneous GC development and T_H2 cell-mediated inflammatory disease.

***Id2* and *Id3* modulate CXCR5 and Foxp3 expression**

The T_{reg} population can be segregated into distinct functional subsets, including effector T_{reg} (eT_{reg}) cells³⁴. Previously, low *Id3* expression has been observed in the Blimp-1⁺ eT_{reg} cell population³⁵. Consistent with these observations, we found that a fraction of ICOS⁺ eT_{reg} cells displayed low *Id3* expression and *Id2^{lo}Id3^{lo}* T_{reg} cells expressed more CXCR5 (Fig. 5a). In line with this, T_{reg} cells that lacked *Id2* and *Id3* expression exhibited increased expression of CXCR5, PD-1, ICOS, CXCR3, CXCR4 and CTLA-4 but decreased CD62L expression as compared to *Id*-sufficient T_{reg} cells (Fig. 5b and Supplementary Fig. 5a–c)³⁶. T_{reg} cells derived from healthy heterozygous female *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice also displayed increased expression of CXCR5 and PD-1, but expression of CXCR3, CXCR4, CD62L and CTLA-4 was comparable to that in wild-type T_{reg} cells (Fig. 5c,d and Supplementary Fig. 5a–c). *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice displayed significantly reduced expression of Foxp3 protein (Fig. 5e). We observed this downregulation of Foxp3 even in heterozygous female *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice, although abundance of *Foxp3* transcript in T_{reg} cells isolated from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice did not differ from that for *Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}* mice (Supplementary Fig. 5d,e). These data indicate that loss of *Id2* and *Id3* expression in T_{reg} cells leads to decreased abundance of Foxp3 and increased proportions of CXCR5⁺PD-1⁺ T_{reg} cells.

Recent studies have indicated that T_{reg} cells generated in the thymus (tT_{reg} cells) abundantly express Helios or Neuropilin-1 (Nrp1)^{37,38}. We found that the proportions of Helios⁺ T_{reg} cells were comparable between 4-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice versus control mice but reduced in 8–9-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* as compared to control mice. These data indicate that *Id2* and *Id3* maintain the Helios⁺ T_{reg} cell pool (Fig. 5f). Recent observations also have indicated a critical role for Nrp1 expression in maintaining stability of T_{reg} cells in inflammatory sites³⁹. We found that Nrp1 expression was substantially reduced in T_{reg} cells derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice or *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice (Fig. 5f and Supplementary Fig. 5f). These data indicate that the expression of a subset of genes associated with T_{reg} cell function, including CXCR5, PD-1, Foxp3 and Nrp1, are directly affected by loss of *Id2* and *Id3* expression.

***Id2* and *Id3* expression affects T_{reg} cell localization**

Data described above indicate that loss of *Id2* and *Id3* expression leads to the abnormal proportions of T_{reg} cells that express CXCR5. To explore the possibility that aberrant expression of CXCR5 affects localization and/or homing of T_{reg} cells, we examined the localization of T_{reg} cells in spleens from control or *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice. Increased numbers of Foxp3⁺ cells were localized to the B cell area in the spleens derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice versus control mice (Supplementary Fig. 6). T_{reg} cells depleted for *Id2* and *Id3* expression also appeared to home more deeply into the B cell follicles across LNs (Supplementary Fig. 6). To examine how depletion of *Id2* and *Id3* expression affects T_{reg} localization across the lung tissues, we analyzed T_{reg} cells derived from 4-week-old and 7–8-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice. Whereas the Foxp3⁺ pool was not significantly affected in the lungs derived from healthy 4-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice, the percentage of Foxp3 T_{reg} cells localized across the lungs in 7–8-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice was significantly decreased (Supplementary Fig. 7a). To compare the abilities of T_{reg} cells deficient for *Id2* and *Id3* expression to home to the lymphoid organs versus lung tissues, we transferred T_{reg} cells isolated from heterozygous female *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice into wild-type CD45.1 recipient mice. We found that the homing potential of T_{reg} cells depleted for *Id2* and *Id3* expression was reduced in the lung, as compared to the lymph nodes or spleen (Supplementary Fig. 7b).

Roles of *Id2* and *Id3* in T_{reg}-mediated immunosuppression

To determine whether the development of fatal T_H2 cell-mediated inflammation upon depletion of *Id2* and *Id3* expression in T_{reg} cells was caused by loss of suppressive functions of T_{reg} cells, we examined the *in vitro* suppressive function of T_{reg} cells sorted from 4-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* or *Foxp3^{Cre}* mice. We found that T_{reg} cells depleted for *Id2* and *Id3* expression displayed normal *in vitro* immunosuppressive function (Supplementary Fig. 8).

To examine whether T_{reg} cells depleted for *Id2* and *Id3* expression have the ability to suppress T cell-mediated inflammation *in vivo*, we injected sorted CD4⁺CD25[−]CD45RB^{hi} T cells (T_{conv} cells) derived from CD45.1 mice into *Rag1^{−/−}* recipient mice with or without purified T_{reg} cells isolated from 4-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* or *Foxp3^{Cre}* mice (Fig. 6a). As expected, *Rag1^{−/−}* recipient mice transferred with T_{conv} cells alone showed severe colitis accompanied by body-weight loss (Fig. 6a,b). Cotransfer of T_{reg} cells isolated from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice resulted in partially impaired suppressive activity (Fig. 6a,b). As expected, we found a large fraction of CD45.1[−]Foxp3⁺ T_{reg} cells in the MLNs of recipient mice but fewer *Id*-deficient T_{reg} cells in the MLNs of recipient mice (Fig. 6c). These findings are consistent with the notion that expression of *Id2* and *Id3* is required to maintain the T_{reg} pool. Overall these data indicate that T_{reg} cells depleted for *Id2* and *Id3* expression have the ability to suppress, albeit partially, T cell-mediated inflammation in a transfer colitis model.

***Id2* and *Id3* maintain T_{reg} cell homeostatic potential**

To assess directly the roles of *Id2* and *Id3* under noninflammatory conditions, we analyzed heterozygous female *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice carrying the IRES-YFP reporter as described above. As inactivation of the X chromosome can occur on either allele, 50% of the T_{reg} cells in heterozygous female *Foxp3^{Cre}-YFP/+* mice expressed the *Foxp3^{Cre}-YFP* marker, whereas the rest of the T_{reg} cell population lacked expression of *Foxp3^{Cre}-YFP*. This setting allows for a direct functional comparison of T_{reg} cells that are deficient for *Id2* and *Id3* expression versus wild-type cells under noninflammatory conditions. In the thymi derived from female *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice, the YFP⁺/CD25⁺ ratio of the CD4⁺ T cell population was comparable to that of *Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}* mice, whereas the YFP⁺/CD25⁺ ratio in the spleen and LNs was substantially reduced (Fig. 7a). Similarly, the fraction of YFP⁺ cells as compared to the proportion of Foxp3⁺ cells was substantially lower in spleen and LNs, indicating that the Id-sufficient Foxp3⁺ population outcompeted the Id-deficient population (Fig. 7a). We did not detect obvious differences in CD25, Annexin-V or Ki67 expression in *Id2*- and *Id3*-deficient YFP⁺ T_{reg} cells derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* versus *Foxp3^{Cre/+}* mice (data not shown). Notably, we found that when cultured in the presence of anti-CD3 and anti-CD28, the fraction of T_{reg} cells depleted for *Id2* and *Id3* expression (YFP⁺ cells) among Foxp3⁺ populations declined progressively over time and was accompanied by elevated numbers of apoptotic cells and decreased numbers of Ki67⁺ cells at 24 h (Fig. 7b). These observations indicate that in the absence of *Id2* and *Id3*, T_{reg} cells are more susceptible to cell death upon TCR stimulation. Collectively, these data indicate that *Id2* and *Id3* expression is required to maintain the peripheral T_{reg} cell pool.

***Id2* and *Id3* regulate T_{FR} cell-specific transcription signatures**

As a first approach to identify critical target genes affected by the depletion of *Id2* and *Id3* in T_{reg} cells, we isolated by cell sorting YFP⁺ T_{reg} cells from subcutaneous LNs derived from 3-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* or *Id2^{fl/+}Id3^{fl/+}Foxp3^{Cre}* mice. 792 genes were differentially expressed (greater than twofold; fragments per kilobase of exons per million fragments mapped; FPKM) in YFP⁺ T_{reg} cells derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice when compared to those in *Id2^{fl/+}Id3^{fl/+}Foxp3^{Cre}* mice (Fig. 8a). Hierarchical clustering showed that a large fraction of transcripts that were differentially expressed were associated with genes encoding for metabolic process, transcription, T cell lymphocyte activation, cytokine/chemokine inflammation, cell cycle and cell death by Gene Ontology (GO) analysis (Supplementary Fig. 9). Prominent among the putative targets were *Cxcr5*, *Ikzf3*, *Cebpa*, *Hif1a*, *Pparg*, *Il10*, *Il10ra*, *Ccr2*, *Nrp1* and *Ccr6* (Fig. 8a–c and Supplementary Fig. 10a). However, expression of genes encoding transcriptional regulators, such as *Tbx21*, *Irf4*, *Gata3*, *Stat3*, *Prdm1* and *Sh2d1a*, was not affected (data not shown). Similarly, cytokine genes including *Il2*, *Il4*, *Il5*, *Il13*, *Ifng*, *Il17* and *Il21* were not modulated upon depletion of *Id2* and *Id3* expression in the T_{reg} cell pool (data not shown). Similar to the case in T_{FR} cells, we found elevated expression of *Gzma*, *Il10* and *Ctla4*. On the other hand, expression of *Bcl6* and *Prdm1* was not altered¹⁵ (Fig. 8a,b and Supplementary Fig. 10a).

To identify target genes directly affected by depletion of *Id2* and *Id3* expression rather than genes affected by inflammatory disease, we isolated RNA from YFP⁺ T_{reg} cells from

heterozygous female *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* and *Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}* mice and examined it by real-time PCR. We found that ablation of *Id2* and *Id3* expression in the T_{reg} cell pool in healthy mice affected *Cxcr5*, *Ikzf3*, *Hif1a*, *Myb*, *Apoe*, *Il10*, *Il10ra*, *Ccr2*, *E2f2*, *Nrp1* and *Il18r1* transcript abundance (Fig. 8c). In contrast, *Ikzf4*, *Ccr6*, *Cebpa*, *Pparg1* and *Slpr1* transcript abundance was not altered in T_{reg} cells depleted of *Id2* and *Id3* (data not shown). To determine whether the differences in transcript abundance directly relate to transcription factor occupancy across putative enhancer elements, we inspected previous genome-wide studies for E2A occupancy as well as deposition of monomethylation of histone H3K4 (H3K4me1) in CD4⁺CD8⁺ double-negative (DN3) thymocytes, pro-B cells and T cell lymphomas^{26,40}. Notably, E2A-bound sites were closely associated with putative enhancer regions across the *Cxcr5*, *IL10*, *Hif1a*, *Ikzf3*, *Myb*, *Il10ra*, *Nrp1*, *E2f2* and *Bmp7* loci, which indicated that this ensemble of loci is directly regulated by E proteins (Fig. 8d and Supplementary Fig. 10b). These data indicate that a subset of genes associated with T_{FR} cells, including *Il10* and *Cxcr5*, but not *Bcl6* and *Prdm1*, are directly regulated by the expression of *Id2* and *Id3* in T_{reg} cells to orchestrate the developmental progression of the T_{FR} cell population (Supplementary Fig. 10c).

DISCUSSION

Here we demonstrated that expression of the antagonist HLH proteins, *Id2* and *Id3*, in T_{reg} cells is required for suppression of early onset inflammation at mucosal barriers. How does depletion of *Id2* and *Id3* expression in T_{reg} cells lead to the development of severe inflammation? Our data indicate that expression of *Id2* and *Id3* is essential to maintain the T_{reg} cell population. In 3–4-week-old mice that lacked expression of *Id2* and *Id3*, we observed T_{reg} cells in near wild-type numbers. However, in 7–8-week-old mice, the T_{reg} cell population depleted for expression of *Id2* and *Id3* was severely reduced across the lung tissues. Homing of T_{reg} cells to the lung in transfer experiments was also compromised in the absence of *Id2* and *Id3* expression. Finally, we found that activated T_{reg} cells depleted for expression of *Id2* and *Id3* displayed decreased viability and impaired proliferation upon stimulation. Collectively, these data indicate that *Id2* and *Id3* coordinate the survival, expansion and homing of T_{reg} cells under inflammatory conditions.

In addition to the roles of *Id2* and *Id3* in suppressing T_{H2} cell-mediated inflammation, our observations also point to a role for *Id2* and *Id3* in T_{FR} cell development. How may *Id2* and *Id3* regulate T_{FR} differentiation? We suggest that *Id2* and *Id3* expression enforces distinct T_{FR} checkpoints. Upon TCR-mediated signaling, *Id2* and *Id3* levels readily decline permitting increased E-protein binding activity and the induction of a T_{FR}-specific transcription signature, including the activation of *Cxcr5* and IL-10 expression. We propose that abundance of *Id2* and/or *Id3* is elevated and required again in differentiating T_{FR} cells to indirectly modulate Bcl-6 and Blimp-1 protein abundance, which ultimately leads to the development of a mature T_{FR} cell population.

There are interesting parallels between the roles of E and Id proteins in development of T_{FR} cells and T_{FH} cells. Differentiation of T_{FH} cells is characterized by distinct developmental stages. First, upon activation, effector T_{FH} cells induce the expression of CXCR5 to migrate to the border that separates the T cell areas from the B cell follicles. Followed by ICOS-

ICOSL interaction with B cells, T_{FH} cells continue to differentiate, acquire Bcl-6 expression and migrate to GCs^{41,42}. Two critical factors control the early developmental progression of naive CD4⁺ T cells toward the effector T_{FH} cell type: *Id3* and a heterodimeric complex involving the basic HLH protein Ascl2 and E proteins to activate the expression of CXCR5 and other critical target genes^{26,43,44}. Based on these observations, we suggest that a common genetic circuitry that involves the E-Id protein module controls the homing of both T_{FR} cells and T_{FH} cells.

In sum, higher *Id2* and *Id3* expression enforces the early T_{FR} cell checkpoint, whereas at intermediate developmental stages the abundance of E protein versus Id protein controls developmental progression toward a fully mature T_{FR} cell fate. We note this property of Id proteins and E proteins is not unique to T_{reg} cells. Previous studies have demonstrated critical roles for E and Id proteins acting as gatekeepers at the pre-TCR, TCR and B cell antigen receptor checkpoints^{21,23,45–47}. During the developmental progression of the CD8⁺ and T_{FH} cell lineages, the Id proteins also control key developmental transitions^{27,48,49}. Each of these checkpoints involves the regulation of Id protein expression by antigen receptor-mediated signaling. Thus, a picture is now emerging in which antigen receptors directly connect to the E protein and Id protein machinery to orchestrate the developmental transitions of cells that comprise the adaptive immune system under healthy as well as inflammatory conditions.

ONLINE METHODS

Mice

C57BL/6, *Id3*^{fl/fl}, *Id2*^{fl/fl} *Id3*^{-/-}, *Foxp3*^{Cre-YFP} and *Id3*^{GFP/+} *Id2*^{YFP/+} mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California, San Diego.

Flow cytometry

Single-cell suspensions from thymus, lymph nodes and spleen were stained with the following: FITC-, PE-, APC-, APC-Cy7-, Pacific Blue-, Alexa Fluor 700-, Alexa Fluor 780-, PerCP-Cy5.5-, PE-Cy7- or biotin-labeled monoclonal antibodies were purchased from BD PharMingen including CD11c (HL3), CD44 (IM7), CXCR5 (2G8), CD95 (Jo-2), GL-7 (GL7), SiglecF (E50-2440), IL-5 (TRFK5), CD8 (53-6.7), CD19 (ID3), CD38 (90), CD62L (MEL-14), CD44 (IM7), B220 (RA3-6B2), Mac1 (M1/70), Gr1 (RB6-8C5), Nk1.1 (PK136), Ter119 (TER119), TCR β (H57), TCR $\gamma\delta$ (GL3), PD-1 (J43), ICOS (7E.17G9), CXCR4 (2B11), IL-4 (11B11), IL-13 (eBio13A), IL-17 (eBio17B7), IFN- γ (XMG1.2), FOXP3 (FJK-16s) and TBR2 (Dan11mag) were purchased from eBioscience. Fc ϵ R1 (MAR-1), CD3e (2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD25 (PC61), CD122 (TMb1), CTLA4 (UC10-4B9), CCR6 (29-2L17), Helios (22F6) and PD-1 (RMP1-30) were obtained from BioLegend. Anti-Nrp1 (AF566) was purchased from R&D Systems. Biotinylated antibodies were labeled with streptavidin-conjugated Qdot-665 or Qdot-605 (Invitrogen). Clone 2.4 G2 anti-CD32:CD16 (eBioscience) was used to block Fc receptors. Dead cells were removed from analysis and sorting by staining with propidium iodide (PI) (Sigma-Aldrich). Isolation of lung single cells was performed as described⁵¹. Mouse T_{reg}

cell staining kit (eBioscience) was used for intracellular Foxp3, CTLA4 and Helios staining. Samples were collected on a LSRII (BD Biosciences) and were analyzed with FlowJo software (Tree Star). Cells were sorted on a FACS Aria (BD). For intracellular staining of IFN- γ , IL-4, IL-5, IL-13 and IL-17, isolated cells from spleen and lymph nodes were treated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (4 h) and Golgi stop (2 h). After culture, cells were stained with anti-CD4, CD8, TCR β and CD3e. Intracellular staining was performed with the BD Biosciences Cytotfix/Cytopermkit.

RNA sequencing

Total RNA was isolated using the RNeasy-Mini Kit (Qiagen) and was processed for deep sequencing as described previously with slight modifications. Briefly, mRNA was purified from total RNA using Dynabeads mRNA purification kit (Invitrogen). The mRNA was then subjected to first-strand cDNA synthesis using a combination of random hexamers and oligo(dT). Second-strand synthesis was performed using dUTP instead of dTTP. The double-stranded cDNA was sonicated using S220 Focused-ultrasonicator (Covaris). Sonicated cDNA was next ligated to adaptors and size-selected. The size-selected cDNA was treated with uracil-N-glycosylase and then multiplex-amplified by PCR. cDNA libraries were sequenced with a HiSeq 2000 sequencer (Illumina). Sequence reads from each cDNA library were analyzed with Galaxy (<http://galaxyproject.org/>). They were mapped to mouse genome build NCBI37 mm9 using TopHat. The expression values were obtained using Cufflink and Cuffdiff. GO analyses were performed using Homer (<http://homer.salk.edu/homer/index.html>). Visualization files were generated using Homer and read pile-ups visualized using the University of California Santa Cruz Genome Browser.

Histology and immunostaining

Tissues were fixed in 4% paraformaldehyde (Electron Microscopy Sciences). Fixed tissues were embedded in paraffin and sliced, followed by hematoxylin and eosin (H&E) staining, periodic acid Schiff (PAS) staining and MBP staining. For MBP immunostaining, we used a monoclonal antibody directed against MBP⁵¹. For quantification of MBP⁺ or PAS⁺ cells, we chose five bronchioles and count positive cells in one mouse. For Foxp3 staining, spleen and lymph nodes were embedded in optimal cutting temperature (OCT) compound and snap-frozen. 5- μ m serial sections were stained with anti-CD45R (B220; supernatant isolated from the medium of cultured hybridomas) or anti-Foxp3 (FJK-16s). Secondary antibody used was donkey anti-rat IgG Alexa Fluor 488 (Molecular Probes). The images were captured by Olympus Provis AX80 microscopy. The digital images were acquired using RETIGA EXi (Nippon Rooper). Image process was done by Photoshop (Adobe). IBD scores were calculated as described⁵⁰.

Enzyme-linked immunosorbent assay

Serum IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were done as described⁵². For IgE, ELISA was performed using Mouse IgE Standard Set Module (BioLegend).

RNA isolation and reverse transcriptase PCR

First, cDNA was obtained from purified YFP⁺ or CD25⁺ T_{reg} cells from *Id2*^{+/+}*Id3*^{+/+}*Foxp3*^{Cre/+} or *Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre/+} mice. Total RNA was isolated from sorted cells with RNeasy kit (Quagen), followed by reverse transcription with the SuperScript III RT-PCR system (Invitrogen) and quantitative real-time reverse transcription (RT)-PCR.

In vitro and *in vivo* suppression and *in vitro* stimulation

For *in vitro* suppression assay, YFP⁺ T_{reg} cells and responder CD4⁺CD25⁻ T cells were purified by sorting. 2×10^4 Responder CD4⁺ T cells were cocultured with T_{reg} cells at indicated ratios and were stimulated with soluble anti-CD3 antibody (1 µg/ml) in the presence of T cell-depleted splenocytes treated with mitomycin C (1×10^5) in 96-well plates for 72 h. Cells were pulsed with [³H]thymidine deoxyribose (1 µCi/well) for the last 12 h. *In vivo* suppression assay was performed as described⁵³. For *in vitro* stimulation, total lymphocytes were cultured *in vitro* and stimulated with anti-CD3ε (0.5 µg/ml) plus anti-CD28 (1 µg/ml) antibodies in the presence of human IL-2 (100 U/ml) as described³⁰.

Statistical analysis

P values were calculated with the two-tailed Student's *t*-test for two-group comparison as applicable with Microsoft Excel software. The statistical significance level was 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* 2012; 30:531–564. [PubMed: 22224781]
2. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* 2008; 133:775–787. [PubMed: 18510923]
3. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat. Rev. Immunol.* 2008; 8:523–532. [PubMed: 18566595]
4. Locksley RM. Asthma and allergic inflammation. *Cell.* 2010; 140:777–783. [PubMed: 20303868]
5. Rothenberg ME, Hogan SP. The eosinophil. *Annu. Rev. Immunol.* 2006; 24:147–174. [PubMed: 16551246]
6. Sakaguchi S, et al. Immunologic self-tolerance maintained by activated T cells expressing IL2-receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 1995; 155:1151–1164. [PubMed: 7636184]

7. Bennett CL, et al. The immune dysregulation, polyendocrinopathy, X-linked syndrome (IPEX) is caused by mutations of Foxp3. *Nat. Genet.* 2001; 27:20–21. [PubMed: 11137993]
8. Wildin RS, et al. X-linked neonatal diabetes, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 2001; 27:18–20. [PubMed: 11137992]
9. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003; 299:1057–1061. [PubMed: 12522256]
10. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 2003; 4:330–336. [PubMed: 12612578]
11. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 2003; 4:337–342. [PubMed: 12612581]
12. Josefowicz SZ, et al. Extratymically generated regulatory T cells control mucosal Th2 inflammation. *Nature.* 2012; 482:395–399. [PubMed: 22318520]
13. Wing JB, Sakaguchi S. Foxp3⁺ T_{reg} cells in humoral immunity. *Int. Immunol.* 2014; 26:61–69. [PubMed: 24324208]
14. Chung Y, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat. Med.* 2011; 17:983–988. [PubMed: 21785430]
15. Linterman MA, et al. Foxp3 follicular regulatory T cells control the germinal center response. *Nat. Med.* 2011; 17:975–982. [PubMed: 21785433]
16. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat. Immunol.* 2013; 14:152–161. [PubMed: 23242415]
17. Murre C. Helix-loop-helix proteins and lymphocyte development. *Nat. Immunol.* 2005; 6:1079–1086. [PubMed: 16239924]
18. Murre C, McCaw PS, Baltimore D. A new DNA binding and dimerization motif. *Cell.* 1989; 56:777–783. [PubMed: 2493990]
19. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell.* 1990; 61:49–59. [PubMed: 2156629]
20. Yokota Y, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix protein Id2. *Nature.* 1999; 397:702–706. [PubMed: 10067894]
21. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. *Immunity.* 2000; 12:17–26. [PubMed: 10661402]
22. Verykokakis M, Boos MD, Bendelac A, Kee BL. SAP protein-dependent natural killer T-like cells regulate the development of CD8(+) T cells with innate lymphocyte characteristics. *Immunity.* 2010; 33:203–215. [PubMed: 20674402]
23. Jones-Mason ME, et al. E protein transcription factors are required for the development of CD4(+) lineage T cells. *Immunity.* 2012; 36:348–361. [PubMed: 22425249]
24. Li H, Dai M, Zhuang YA. T cell intrinsic role of Id3 in a mouse model for primary Sjogren's syndrome. *Immunity.* 2004; 21:551–560. [PubMed: 15485632]
25. Maruyama T, et al. Control of the differentiation of regulatory T cells and T_H17 cells by the DNA-binding inhibitor Id3. *Nat. Immunol.* 2011; 12:86–95. [PubMed: 21131965]
26. Miyazaki M, et al. The opposing roles of the transcription factor E2A and its antagonist Id3 that orchestrate and enforce the naive fate of T cells. *Nat. Immunol.* 2011; 12:992–1001. [PubMed: 21857655]
27. Yang CY, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8⁺ T cell subsets. *Nat. Immunol.* 2011; 12:1221–1229. [PubMed: 22057289]
28. Niola F, et al. Id proteins synchronize stemness and anchorage to the niche of neural stem cells. *Nat. Cell Biol.* 2012; 14:477–487. [PubMed: 22522171]
29. Rubtsov YP, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity.* 2008; 28:546–558. [PubMed: 18387831]
30. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J. Exp. Med.* 2008; 205:1993–2004. [PubMed: 18725526]
31. Nussbaum JC, et al. Type 2 Innate lymphoid cells control eosinophil homeostasis. *Nature.* 2013; 502:245–248. [PubMed: 24037376]

32. Monticelli LA, Sonnenberg GF, Artis D. Innate lymphoid cells: critical regulators of allergic inflammation and tissue repair in the lung. *Curr. Opin. Immunol.* 2012; 24:284–289. [PubMed: 22521139]
33. Chang YJ, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat. Immunol.* 2011; 12:631–638. [PubMed: 21623379]
34. Cretney E, Kallies A, Nutt SL. Differentiation and function of Foxp3+ effector regulatory T cells. *Trends Immunol.* 2013; 34:74–80. [PubMed: 23219401]
35. Cretney E, et al. The transcription factors, Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat. Immunol.* 2011; 12:304–311. [PubMed: 21378976]
36. Smigiel KS, et al. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J. Exp. Med.* 2014; 211:121–136. [PubMed: 24378538]
37. Thornton AM, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J. Immunol.* 2010; 184:3433–3441. [PubMed: 20181882]
38. Weiss JM, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ Treg cells. *J. Exp. Med.* 2012; 209:1723–1742. [PubMed: 22966001]
39. Delgoffe GM, et al. Stability of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature.* 2013; 501:252–256. [PubMed: 23913274]
40. Lin YC, et al. A global network of transcription factors, involving E2A, EBF1, and Foxo1, that orchestrates B cell fate. *Nat. Immunol.* 2010; 11:635–643. [PubMed: 20543837]
41. Xu H, et al. Follicular T-helper cell recruitment governed by bystanding B cells and ICOS-driven motility. *Nature.* 2013; 496:523–527. [PubMed: 23619696]
42. Choi YS, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity.* 2011; 34:932–946. [PubMed: 21636296]
43. Liu X, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature.* 2014; 507:513–518. [PubMed: 24463518]
44. Murre C, et al. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell.* 1989; 58:537–544. [PubMed: 2503252]
45. Bain G, et al. Thymocyte maturation is regulated by the activity of the helix-loop-helix protein, E47. *J. Exp. Med.* 1999; 190:1605–1616. [PubMed: 10587351]
46. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early thymocyte development is regulated by modulation of E2A protein activity. *J. Exp. Med.* 2001; 194:733–745. [PubMed: 11560990]
47. Quong M, et al. Receptor editing and marginal zone B cell development are regulated by the helix-loop-helix protein, E2A. *J. Exp. Med.* 2004; 199:1101–1112. [PubMed: 15078898]
48. Masson F, et al. Id2-mediated inhibition of E2A represses memory CD8+ T cell differentiation. *J. Immunol.* 2013; 190:4585–4594. [PubMed: 23536629]
49. Knell J, et al. Id2 influences differentiation of killer cell lectin-like receptor G1(hi) short-lived CD8+ effector T cells. *J. Immunol.* 2013; 190:1501–1509. [PubMed: 23325888]
50. Burich A, et al. *Helicobacter*-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2001; 281:G764–G778. [PubMed: 11518689]
51. Suzukawa M, et al. Sialyltransferase ST3Gal-III regulates SiglecF ligand formation and eosinophilic lung inflammation in mice. *J. Immunol.* 2013; 190:5939–5948. [PubMed: 23677475]
52. Smith KGC, et al. bcl-2 transgene expression Inhibits apoptosis in the germinal center and reveals differences in the selection of memory B cells and bone marrow antibody-forming cells. *J. Exp. Med.* 2000; 191:475–484. [PubMed: 10662793]
53. Miyazaki K, et al. The role of the basic helix-loop-helix transcription factor Dec1 in the regulatory T cells. *J. Immunol.* 2010; 185:7330–7339. [PubMed: 21057086]

Figure 1.

Ablation of *Id2* and *Id3* expression in T_{reg} cells leads to the early onset of fatal inflammatory disease. **(a)** Flow cytometric analysis of CD69 versus CD62L expression gated on the CD4⁺CD25⁺ T_{reg} cell population derived from the thymus (CD4⁺CD25⁺CD8⁻TCRβ^{hi} (left). CD4SP, CD4⁺CD8⁻. GFP versus YFP expression, gated on CD69⁺CD62L⁻ or CD69⁻CD62L⁺ T_{reg} cells derived from the thymus (middle). GFP versus YFP expression in CD4⁺CD25⁺ T_{reg} cells isolated from the spleens (Spl) of control and *Id2*^{YFP/+}*Id3*^{GFP/+} mice (right). Numbers in quadrants indicate percent cells in each compartment. **(b)** Flow

cytometric analysis of Foxp3 expression on sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells (left; number indicates the percentage of Foxp3⁺ cells gated on the CD4⁺CD25⁺ T cells); sorted CD4⁺CD25⁺ T_{reg} cells from *Id2*^{YFP/+}*Id3*^{GFP/+} mouse (CD45.2) were cocultured with CD4⁺CD25⁻ T cells (CD45.1) and stimulated with anti-CD3ε plus anti-CD28 in the presence of T cell-depleted splenocytes (APCs). Flow cytometric analysis of GFP versus YFP expression gated on CD45.2⁺CD45.1⁻CD4⁺TCRβ⁺CD25⁺ cells for the indicated time points (bottom). Percentage of *Id3*-GFP⁺*Id2*-YFP⁻ cells and *Id3*-GFP⁺*Id2*-YFP⁺ cells (middle). (c) Quantitative real-time PCR analysis of *Id2* and *Id3* transcript levels in sorted CD4⁺TCRβ⁺CD25⁺YFP⁺ T_{reg} cells derived from the lymph nodes of *Id2*^{+/+}*Id3*^{+/+}*Foxp3*^{Cre} (Ctrl), *Id2*^{fl/fl}*Foxp3*^{Cre} or *Id3*^{fl/fl}*Foxp3*^{Cre} mice. (d) Survival plot of *Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre} and *Id2*^{fl/fl}*Id3*^{fl/-}*Foxp3*^{Cre/Cre}*Id2*^{fl/fl}*Foxp3*^{Cre/Cre}*Id3*^{fl/fl}*Foxp3*^{Cre/Cre}*Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre/+} and littermate control (Ctrl) mice, monitored during a 25-week period. Data are representative of two experiments (a,b; error bars in b, s.d.; *n* = 3 technical replicates), one experiment (c; error bars, s.d.; *n* = 3 technical replicates) and one experiment (d; *n* = 11 independent biological replicates per group).

Figure 2.

Spontaneous inflammation in the lung, eyelid, skin and esophagus in mice depleted for the expression of *Id2* and *Id3* in T_{reg} cells. **(a)** Hematoxylin and eosin (H&E) staining of the lung, esophagus and eyelid derived from control (Ctrl; female $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}$) and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ mice. Original magnification: $\times 200$ (lung), $\times 200$ (esophagus) and $\times 50$ (eyelid). **(b)** Periodic acid Schiff (PAS) staining and MBP immunostaining of lungs of indicated genotypes (top; arrows indicate muous-producing goblet cell (PAS staining) and MBP-positive eosinophil). Original magnification: $\times 400$ (PAS) and $\times 200$ (MBP). Quantification of PAS-stained epithelial cells per bronchus or MBP-positive eosinophils in peribronchial area (bottom). **(c)** MBP staining of eyelid and esophagus. Original magnification: $\times 200$ (eyelid) and $\times 200$ (esophagus). Data are representative of six experiments **(a)**, two experiments **(b)**; mean $\pm$ s.d. ($n = 4$ biological replicates) and two experiments **(c)**. * $P < 0.05$, ** $P < 0.01$ (two-tailed unpaired Student's *t* test).

Figure 3.

T_H2 cell-mediated inflammation in the lungs of mice depleted for *Id2* and *Id3* expression in T_{reg} cells. **(a)** May-Giemsa staining images of cells (left) and cell numbers (right) in BAL fluid derived from 6–8-week-old control (Ctrl) or *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice (left). Green, red and blue arrowheads indicate macrophages (MF), lymphocytes (Lym) and eosinophils (Eos), respectively; Neu, neutrophil. Insets show magnification of boxed areas. Original magnification, $\times 40$ ($\times 120$ in insets). **(b)** Numbers of hematopoietic cells (CD45⁺), macrophages (MF), eosinophils (Eos), basophils (Bas), neutrophils (Neu), mast cells (Mas), T cells (T) and B cells (B) isolated from the left lobes of the lungs derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice (left). Numbers of type 2 innate lymphoid cells (ILC2; CD45⁺LineageCD25⁺IL7R⁺Sca1^{hi}Thy1.2^{hi}) isolated from the left lobes of the lungs derived from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice (right). **(c)** Flow cytometric analysis of CD19 versus TCR β expression of cells derived from the LNs isolated from 6–8-week-old control (*Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}*) or *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* mice. Adjacent panels indicate CD11c versus SiglecF, Fc ϵ r1 versus SiglecF and Mac-1 versus Gr-1 expression, gated on the TCR β ⁺CD19⁺ compartment derived from 6–8-week-old control (*Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}*) or *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* lymphocytes isolated from subcutaneous LNs. Numbers in plots indicate percent CD19⁺TCR β ⁺, CD11c⁺SiglecF⁺, Fc ϵ r1⁺SiglecF⁺ and Mac-1⁺Gr-1⁺ cells. Right, percentages of eosinophils (SiglecF⁺CD11c⁺), basophils (Baso; Fc ϵ r1^{hi}SiglecF⁺) and neutrophils (Neut; Mac-1^{hi}Gr-1^{hi}) in subcutaneous LN cells and splenocytes. Data are representative of four experiments (**a,c**; mean $\pm$ s.d.; in **a**, $n = 8$ independent biological replicates; in **b**, control $n = 5$, *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}* $n = 4$ independent biological replicates; in **c**, control $n = 6$,

Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre} $n = 9$ independent biological replicates). * $P < 0.05$, ** $P < 0.01$ (two-tailed unpaired Student's t test).

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Figure 4. Spontaneous germinal center formation and T_H2 cell-mediated inflammation in mice depleted for the expression of *Id2* and *Id3* in T_{reg} cells. **(a)** $\alpha\beta$ T cell ($\alpha\beta$ T) and B cell numbers for spleen (Spl) and subcutaneous LN (sLN) isolated from 8-week-old littermate control and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ mice. **(b)** Flow cytometric analysis of splenocytes derived from 8-week-old littermate control ($Id2^{fl/+}Id3^{fl/+}Foxp3^{Cre}$) and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ mice, showing expression of CD44 versus CD62L and CXCR5 versus PD-1, gated on the CD4⁺TCR[3⁺YFP-CD25⁻ population (left). Frequency of germinal center B cells in 8-week-old littermate control ($Id2^{fl/+}Id3^{fl/+}Foxp3^{Cre}$) and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ mice (middle). Numbers in quadrants and gates indicate percent cells in each compartment. Frequencies of T_{FH} (CXCR5^{hi}PD-1^{hi}) cells in the CD4⁺ T cell compartment and GC B (Fas^{hi}GL7^{hi}) cells (GCB) in the B cell compartment (right). **(c)** Cytokine expression in CD4⁺ T cell derived from littermate control ($Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}$) and $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/Cre}$ mice (left) Percentages of CD4⁺ T cells producing IL-4, L-5, IL-13, IFN- γ and IL-17 in 7–9-week-old mice for the indicated genotypes (right). **(d)**

Enzyme-linked immunosorbent assay (ELISA) of IgM, IgG1, IgG2a, IgG2b, IgG3, IgA and IgE in serum derived from 6–9-week-old mice for the indicated genotypes. Data are representative of four experiments (**a**; mean $\pm$ s.d.; $n = 8$ biological replicates), | three independent experiments (**b**; mean $\pm$ s.d.; control $n = 4$, $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ $n = 5$ biological replicates), two experiments (c; mean $\pm$ s.d.; $n = 4$ biological replicates), one experiment (**d**; mean $\pm$ s.d.; control $n = 8$, $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}$ $n = 7$ biological replicates) $*P < 0.05$, $**P < 0.01$ (two-tailed unpaired Student's t test).

Figure 5.

Depletion of *Id2* and *Id3* expression in T_{reg} cells modulates the expression of CXCR5, Foxp3 and Nrp1. (a) Flow cytometric analysis of *Id3*-GFP versus ICOS, gated on the CD4⁺CD25⁺ T_{reg} cell compartment derived from from *Id3*^{GFP/+} splenocytes (left). Numbers in quadrants indicate percent cells in each compartment. Expression of CXCR5 in the T_{reg} compartments as characterized by the expression of *Id2* and *Id3* (right). (b) Flow cytometric analysis of T_{reg} cells (Foxp3-YFP⁺CD25⁺CD4⁺) derived from LNs isolated from 8-week-old control (*Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre}) and *Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre} mice for indicated cell surface markers. Numbers in plots indicate percent Foxp3-YFP⁺CD25⁺, CXCR5⁺PD-1⁺ and CXCR5⁺PD-1⁻ cells. Spl, spleen. (c) CXCR5 expression patterns for YFP⁺T_{reg}, CD25⁺YFP⁻T_{reg} and CD25⁻non-T_{reg} CD4⁺CD8⁻ thymocytes derived from heterozygous female *Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre/+} mice (left). CXCR5 expression derived from

Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+} mice (bottom). MFI, mean fluorescence intensity. **(d)** CXCR5 and PD-1 expression in T_{reg} cells derived from the thymus and LNs isolated from control *Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}* and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice. Numbers in plots indicate percent CXCR5⁺PD-1⁺ and CXCR5⁺PD-1⁻ cells. **(e)** Flow cytometric analysis for Foxp3 and CTLA-4 expression in CD4 T cells derived from littermate control and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice. Numbers in plots indicate percent Foxp3⁺ cells. **(f)** Helios and CD25 expression (top) and Nrp1 (bottom) in Foxp3⁺ or Foxp3⁺Helios⁺ T_{reg} cells derived from 8-week-old control and *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* LNs and thymi. Numbers in plots indicate percent Helios⁺ cells. Percentages of Helios⁺ cells in Foxp3⁺ T_{reg} cells isolated from control, 4-week-old or 8–9-week-old *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice (right). Data are representative of two experiments **(a)**, three independent experiments **(b)**; mean ± s.d.; control *n* = 3, *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* *n* = 5 biological replicates), two experiments **(c)**; mean ± s.d.; *n* = 3 biological replicates), three experiments **(d)**, three (Foxp3) and five (CTLA-4) experiments **(e)**; mean ± s.d.; Foxp3 *n* = 3, CTLA-4 control *n* = 7, *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* *n* = 9 biological replicates), and three experiments **(f)**; mean ± s.d.; *n* = 4 biological replicates). **P* < 0.05, ***P* < 0.01 (two-tailed unpaired Student's *t* test).

Figure 6.

Impaired homeostasis of T_{reg} cells deleted for *Id2* and *Id3* expression after transfer into *Rag1*^{-/-} mice. **(a)** Body weight in indicated populations of mice after CD4⁺CD25⁻CD45RB^{hi} T cell (T_{conv}) from CD45.1 mice were intravenously injected into *Rag1*^{-/-} recipient mice with or without CD4⁺CD25⁺Foxp3-YFP⁺ T cell (T_{reg}) from 4-week-old *Id2*^{fl/fl}*Id3*^{fl/fl}*Foxp3*^{Cre} or wild-type *Foxp3*^{Cre} mice (CD45.2) ($n = 4$ each group). WT, wild type. **(b)** H&E staining of large intestine (left). Blue and green arrowheads indicate crypt abscess and infiltration of inflammatory cells in the submucosal muscle layer, respectively. IBD score⁵⁰ in large intestine on day 44 after transfer (right). Original magnification, $\times 100$ **(c)** Flow cytometric analysis of Foxp3 and CD45.1 expression gated on CD4⁺TCR β ⁺ cells in mesenteric LNs (left) and analysis of CD45.1Foxp3⁺ cells (right). Data are representative of one experiment (**a–c**; error bars, s.d.; $n = 4$ independent biological replicates). * $P < 0.05$, ** $P < 0.01$ (two-tailed unpaired Student's *t* test).

from heterozygous female $Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}$ or $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}$ mice cultured and stimulated with anti-CD3 ϵ plus anti-CD28 antibodies in the presence of human IL-2 (top left). Numbers indicate the fraction of YFP $^{+}Foxp3^{+}$ and YFP $^{-}Foxp3^{+}$ cells. Percentages of Ki67 $^{+}$ cells in the YFP $^{+}$ T $_{reg}$ cell populations analyzed 24 h after stimulation for the indicated genotypes (top right). Ratios of YFP $^{+}$ cells for the indicated time points as compared to the zero time point (bottom left). Percentages of annexin-V $^{+}$ cells gated on the YFP $^{+}$ T $_{reg}$ cell population (right). Data are representative of three independent experiments (**a**; mean $\pm$ s.d.; $Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}n = 5$, $Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}n = 7$ biological replicates) and one experiment (**b**; mean $\pm$ s.d.; $n = 3$ technical replicates). * $P < 0.05$, ** $P < 0.01$ (two-tailed unpaired Student's t test).

Figure 8.

Unique patterns of gene expression in T_{reg} cells depleted for *Id2* and *Id3*. **(a)** RNA-seq analysis of mRNA expression in sorted T_{reg} cells isolated from *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* versus *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* mice (log₂ FPKM values for RefSeq genes). Points represent genes upregulated in *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* T_{reg} cells > twofold (red; $P < 0.05$ (calculated by Cuffdiff)), genes downregulated in *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre}* T_{reg} cells > twofold (blue; $P < 0.05$) and not significantly changed (gray) as compared to control cells. Selected genes are labeled. **(b)** RNA-seq analysis at *Cxcr5*, *Ccr2*, *Il10*, *ApoE*, *Bcl6* and *Prdm1* loci, presented in reads per million reads aligned (RPM). Arrows indicate transcription start site and direction of transcription. **(c)** Quantitative real-time PCR analysis of genes in sorted Foxp3-YFP⁺ T_{reg} cells derived from LNs isolated from heterozygous female *Id2^{+/+}Id3^{+/+}Foxp3^{Cre/+}* or *Id2^{fl/fl}Id3^{fl/fl}Foxp3^{Cre/+}* mice, presented normalized by the abundance of *Hprt1* transcript. **(d)** E2A occupancy (E2A binding) and deposition of H3K4me1 across the *Cxcr5* and *Il10* loci in DN3, A12 and proB cells. Numbers in plots indicate total tags observed. DN3 cells; *Rag2^{-/-}* thymocytes, A12 cells; *E2a^{-/-}* E47-reconstituted T cell line, pro-B cells; *Rag2^{-/-}* bone marrow B cells. Arrows indicate transcriptional start site and direction of transcription. Data are representative of three experiments (**a,b**), one experiment (**c**; mean $\pm$ s.d.; $n = 3$ technical replicates) and one experiment (**d**).