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Foxp3+ follicular regulatory T cells control T follicular helper cells and the germinal center response

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Abstract

Follicular helper (T_{FH}) cells provide crucial signals to germinal center B cells undergoing somatic hypermutation and selection that results in affinity maturation. Tight control of T_{FH} numbers maintains self-tolerance. We describe a population of Foxp3$^+$Blimp-1$^+$CD4$^+$ T cells constituting 10-25% of the CXCR5^{high}PD-1^{high}CD4$^+$ T cells found in germinal center after immunization. These follicular regulatory T cells (T_{FR}) share phenotypic characteristics with T_{FH} and conventional Foxp3$^+$ regulatory T cells (T_{reg}) yet are distinct from either. Similar to T_{FH} cells, T_{FR} development depends on Bcl-6, SAP, CD28 and B cells; however T_{FR} originate from thymic-derived Foxp3$^+$ precursors, not naïve or T_{FH} cells. T_{FR} are suppressive *in vitro* and limit T_{FH} and germinal center B cell numbers *in vivo*. In the absence of T_{FR}, an outgrowth of non-antigen-specific B cells in germinal centers leads to fewer antigen-specific cells. Thus, T_{reg} cells use the T_{FH} differentiation pathway to produce specialized suppressor cells that control the germinal center response.

Germinal centers are clusters of rapidly-dividing B cells formed in secondary lymphoid tissues in response to T-dependent antigens. Within germinal centers, mutation of the B cell receptor V-region genes together with subsequent selection results in the production of high affinity plasma cells and memory B cells[1]. Defective selection can result in the production of autoantibodies and a break in self-tolerance[2, 3]. Germinal center B cell selection can in part be mediated by a specialized helper T cell subset, CXCR5^{high}PD-1^{high} T follicular helper (T_{FH}) cells[4]. T_{FH} cells develop in a Bcl-6-dependent manner and provide germinal

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center B cells with survival and selection signals. Limiting the numbers of T_{FH} cells within germinal centers has been shown to be critical to prevent the emergence of autoantibodies[5,6]. Little is known about T_{FH} control; in mice, Qa-1-restricted CD8 T cells can regulate the T_{FH} compartment[7] and in humans, $CD4^+CD25^+CD69^-$ T cells with a suppressive function *in vitro* have been found in germinal centers[8, 9]. Regulatory T Cells (T_{reg}) have also been shown enter the primary B cell follicle in mice, but their phenotype, ontogeny and ability to control T_{FH} cells remain unknown[10].

T_{reg} that develop in a Foxp3-dependent manner repress the growth and function of $CD4^+$ effector T cells. Humans and mice lacking Foxp3 cannot form T_{reg} and develop fatal autoimmunity[11-15]. In order to repress T_H1-, T_H2- and T_H17- mediated immune responses, T_{reg} have been shown to co-opt selective aspects of the differentiation programs required for these T_H subsets: Tbet/Stat1, IRF-4 or Rorγt signaling respectively[16-18]. Here we show that $Foxp3^+$ T_{reg} can be diverted to become T_{FH} repressors via expression of Bcl6 and SAP-mediated interaction with B cells. The resulting follicular regulatory T cells (T_{FR}) share features of both T_{FH} and T_{reg} cells, localize to germinal centers, and regulate the size of the T_{FH} cell population and germinal centers *in vivo*.

$Foxp3^+$ follicular regulatory T cells are distinct from T_{FH} and T_{reg}

After immunization with a T-dependent antigen we observed that ~10-25% of $CD4^+CXCR5^{high}PD\text{-}1^{high}$ 'T_{FH}' cells expressed the transcriptional regulator of the T_{reg} lineage, Foxp3 (Figure 1a). These cells followed the same formation and resolution kinetics as conventional T_{FH} cells (Figure 1b). $Foxp3^+$ cells could be visualized within germinal centers identified by immunofluorescence staining of frozen spleen sections from immunized mice (Fig. 1c); 17% ± 8% of germinal center $CD3^+$ cells also expressed Foxp3.

To obtain information about the identity and function of $CD4^+CXCR5^{high}PD\text{-}1^{high}$ $Foxp3^+$ cells - designated T_{FR}, we performed microarray expression profiling on sorted populations from *Foxp3*^{GFP} mice[19] seven days after SRBC immunization. T_{reg}, T_{FH}, non-T_{FH} effector/memory cells (T_{EM}) and naïve (T_N) T cells were also included (sorting strategy is depicted in Supplementary Fig. 1). T_{FR} more closely resembled T_{reg} than T_{FH}, T_{EM} or T_N (Fig. 1d and Supplementary Table 1), with elevated expression of many T_{reg} associated genes including *Foxp3, Ctla4, Gitr, Klrg1* and *Prdm1*. Nevertheless, T_{FR} also expressed high amounts of the prototypic T_{FH} genes *Cxcr5, Pdcd1, Bcl6, Cxcl13*, and *Icos*. T_{FR} did not express the helper cytokines IL-21 or IL-4 (Fig. 1e) or the costimulatory ligand CD40L (Fig. 1f), but expressed comparably high levels of the ligand for CXCR5, CXCL13[20], as T_{FH} cells (Figure 1f). Differential expression of T_{FH} or T_{reg} associated molecules was confirmed by flow cytometry and/or real time PCR (Fig. 1g-i and Supplementary Fig. 2).

T_{FR} expressed numerous molecules characteristically expressed by T_{reg}, such as GITR and CTLA-4, but at higher levels than T_{reg}, consistent with an activated T_{reg} phenotype[21] (Figure 1g). *Gzma* expression was comparable to T_{reg} (Figure 1h) but *Gzmb,* a reported target of Bcl-6 repression[22] was barely detectable. T_{FR} also expressed high levels of *Il10* mRNA and surface ICOS protein, which are common to both T_{FH} and T_{reg} cells (Figure 1j and Supplementary Fig. 2). The elevated levels of GITR, IL-10 and ICOS on T_{FR} compared with the rest of the T_{reg} pool is consistent with an effector T_{reg} phenotype[23], which suggests T_{FR} have a regulatory function. The phenotypic features shared by T_{FH} cells and T_{FR} may account for their common germinal center localization.

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T_{FR} and T_{FH} cells require similar differentiation cues for their formation and maintenance

As both T_{FH} cells and T_{FR} co-localize in germinal centers, we sought to determine whether T_{FR} formation was dependent on similar developmental cues. T cell priming through CD28 is one of the first signals required for T_{FH} development[24, 25]. Enumeration of T_{FH} and T_{FR} in mixed *Cd28*$^{-/-}$ CD45.2: CD45.1 *Cd28*$^{+/+}$ bone marrow chimeras immunized seven days previously with SRBC revealed a complete absence of both T_{FH} and T_{FR} cells in the absence of CD28 signaling (Fig. 2a-c). Consistent with previous reports[26, 27], CD28-deficiency moderately reduced peripheral T_{reg} numbers (Supplementary Fig. 3a).

SAP-dependent interactions of T_{FH} precursors with B cells are required for T_{FH} formation and/or maintenance[5, 28-30]. We therefore investigated whether interactions with B cells and/or SAP-mediated signals are essential for T_{FR} formation. Neither T_{FH} nor T_{FR} cells formed after SRBC immunization of B cell deficient μMT mice (Fig. 2d-f) whereas T_{reg} formed normally (Supplementary Fig. 3b). In addition, immunization of Sap-deficient (*Sh2d1a*$^{-/-}$) mice revealed that, similar to T_{FH} cells (Figure 3g, h), T_{FR} cells are dependent on SAP for their formation (Figure 3i). T_{reg} were only slightly reduced in the absence of SAP (Supplementary Fig. 3c). These data demonstrate that the developmental requirements of T_{FR} are similar to those that govern T_{FH} formation and dispensable for the generation of T_{reg}.

Coordinated Bcl-6 and Blimp-1 expression in T_{FR} cells

Bcl-6, the transcriptional regulator of the T_{FH} subset, regulates key molecules required for follicular localization and function[31-33] in a process thought to be counteracted by the transcriptional repressor Blimp-1[31]; Bcl-6 and Blimp-1 also mutually repress each other during B cell differentiation[34, 35]. We asked whether, similar to T_{FH} cells, T_{FR} cells also expressed Bcl-6 and would be devoid of Blimp-1 expression. Quantitative RT-PCR revealed that *Bcl6* was expressed in T_{FR} cells (Fig. 3a). Of note, T_{FR} co-expressed *Prdm1*, the gene encoding Blimp-1, and its expression on T_{FR} was higher than in any other CD4 T cell subset (Fig. 3a). Expression of Bcl-6 and Blimp-1 protein in Foxp3^{+} cells within germinal center identified was also confirmed by immunofluorescence staining of spleen sections from SRBC-immunized mice. All Foxp3^{+} T_{Fr} within AID^{+} germinal center expressed Bcl-6, albeit at low levels (Fig. 3b) and 75% stained positive for Blimp-1 7 days after immunization; this proportion was reduced to 50% by day 14.

Bcl-6 is required for T_{FR} formation and Blimp-1 regulates T_{FR} homeostasis

To determine whether Blimp-1 and/or Bcl-6 play a role in T_{FR} cell formation or homeostasis, we reconstituted sub-lethally irradiated CD45.1 mice with a 1:1 ratio of fetal liver cells from congenically-marked *Prdm1*$^{+/+}$ and *Prdm1*$^{gfp/gfp}$ embryos, *Bcl6*$^{-/-}$ and *Bcl6*$^{+/+}$ embryos or control *Prdm1*$^{+/+}$ and *Prdm1*$^{gfp/+}$ embryos. Eight weeks after reconstitution the mice were infected intranasally with influenza virus (HKx31), and 10 days later T_{FR} formation was assessed in the mediastinal lymph node. In contrast to published data[31], loss of Blimp-1 did not alter the proportion of T_{FH} cells but caused T_{FR} to double (Fig. 3c), suggesting that Blimp-1 limits the size of the T_{FR} population. This is consistent with a recent report showing Blimp-1 limits the numbers of effector T_{reg} through a Bcl-2-dependent mechanism[23].

Mixed *Bcl6*$^{-/-}$: *Bcl6*$^{+/+}$ chimeras confirmed previous reports that T_{FH} cells do not form in the absence of Bcl-6 (Fig. 3c, upper panel). Cells lacking Bcl-6 expression did not give rise to T_{FR} cells despite the presence of germinal centers in the mice (Fig. 3c, lower panel).

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Similar results demonstrating the requirement for Bcl-6 in splenic T_{FR} generation were obtained after SRBC immunization (Supplementary fig. 4a-c). As reported previously[33], Bcl-6 was dispensable for T_{reg} formation (Supplementary fig. 4d). Together, this suggests that Bcl-6 is essential for T_{FR} formation and Blimp-1 expression regulates the size of the T_{FR} population.

Although co-expression of Bcl-6 and Blimp-1 seems paradoxical, there are precedents in which both *Prdm1* and *Bcl6* are co-regulated; for example in both effector and memory $CD8^+$ subsets[36]. Blimp-1 has been recently shown to influence T_{reg} function inducing an effector phenotype[23,37]. It is expressed by T_{reg} at mucosal sites and by a small (8-12%) subset of splenic T_{reg}, which produce IL-10 in a Blimp-1-dependent manner[37]. Blimp-1^+ T_{reg} and T_{FR} cells also share expression of high amounts of IL-10, GITR and ICOS[23]. T_{FR} are thus likely to be the follicular counterparts of the Blimp-1^+ IL-10^+ effector T_{reg} found at mucosal surfaces.

T_{FR} cells derive from T_{reg} precursors

The observation that T_{FR} require the same cues as T_{FH} for their differentiation raised a critical question: do T_{FR} represent induced T_{reg} that arise from T_{FH} cells that switch on Foxp3 in the germinal center, or do they derive from Foxp3^+ T_{reg}? Plasticity of CD4 helper T cell subsets is well documented[38], as is the adoption of T_H transcriptional programs by T_{reg}[16-18].

In order to test whether T_{FR} derive from T_{FH} cells that turn on Foxp3 expression we transferred 1×10^5 naïve cells ($CD4^+CD44^{low}CD25^-$) from CD45.1 mice expressing the 3A9 TCR transgene (TCR^{HEL}), which recognizes hen egg lysozyme (HEL) peptide presented by I-A^k, into congenic CD45.2 B10.BR mice. Seven days after immunization with HEL in alum, 6-10% of T_{FH} cells derived from the donor HEL-TCR T cells, but no donor-origin T_{FR} could be identified; all T_{FR} derived exclusively from the recipients' cells (Fig. 4a). Between 1-2% of the transferred naïve TCR^{HEL} donor population developed into inducible T_{reg} (Fig. 4b). To exclude that an idiosyncratic effect of the 3A9 transgene had precluded development of T_{FR} cells, this experiment was repeated with OT-II transgenic T cells: Again, OT-II T cells could form T_{FH} but not T_{FR} (Fig. 4c, d), suggesting that T_{FR} do not derive from T_{FH} cells.

To test whether T_{FR} derive from Foxp3^+ precursors, 1×10^6 naïve $CD4^+CD44^{low}$Foxp3^- T cells or $CD44^{int}$Foxp3^+ T_{reg} from unimmunized *Foxp3*^{GFP} mice were adoptively transferred into congenically-marked mice. Seven days after immunization with KLH in Ribi, ~1-2% of both donor-origin T_{reg} and donor-origin naïve cells had upregulated CXCR5 and PD-1 to high levels (Figure 4e). More than 90% of donor-origin $CXCR5^{high}PD\text{-}1^{high}$ T_{reg} cells retained Foxp3 expression, but none of the transferred naïve T cells that became $CXCR5^{high}PD\text{-}1^{high}$ after immunization switched on Foxp3 to become T_{FR} cells (Fig. 4e).

We then asked whether thymic T_{reg} (nT_{reg}) could become T_{FR} cells. Thymic Foxp3^+CD4^{SP} or Foxp3^-CD4^{SP} from Foxp3^{GFP} CD45.2 mice were adoptively transferred into CD45.1 mice. Seven days after SRBC immunization only Foxp3^+ CD45.2 cells had become T_{FR}; Foxp3^- CD45.2 cells had become T_{FH} but not T_{FR} (Supplementary Fig. 5). Furthermore, ~97% of T_{FR} cells expressed Helios (Supplementary Fig. 5), a transcription factor which has been reported to be expressed by thymic-derived nT_{ref} but not T_{ref} induced in the periphery[39].

In an alternative strategy to confirm that T_{FR} derive from Foxp3^+ precursors we used mice in which the gene encoding for the diphtheria toxin receptor (DTR) has been inserted in the Foxp3 locus (Foxp3^{DTR}), so that treatment with diphtheria toxin (DT) selectively ablates all

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T_{reg} within 48 hours [40]. Foxp3^{DTR} mice were immunized with SRBC and treated with either DT or saline immediately afterwards. Six days after immunization, T_{FR} cells had formed normally in mice that did not receive DT but were absent in DT-treated mice (Fig. 4f, g) demonstrating T_{FR} cannot form if Foxp3^{+} cells are absent at the time of immunization.

Within Peyer's patches, but not in the spleen, it has been demonstrated that a proportion of Foxp3^{-} T_{FR} cells derive from Foxp3^{+} precursors[41]. To investigate whether splenic T_{FR} cells stably express Foxp3, we immunized Foxp3-Cre[42] x ROSA-Stop-flox-YFP mice, in which any cell that has expressed Foxp3 is permanently marked. Seven days after SRBC immunization, the majority (97%) of YFP^{+} CD4^{+}CXCR5^{high}PD-1^{high} T_{FR} cells coexpressed Foxp3 and less than ~0.6% of CXCR5^{high}PD-1^{high} cells were Foxp3^{-}YFP^{+} (Fig. 4h), consistent with the observation that Foxp3 expression is stable in the natural T_{reg} population[43]. These data suggest that T_{FR} cells derive from Foxp3^{+} thymic T_{reg} that co-opt the T_{FH} cell differentiation program to migrate to germinal center, where they maintain Foxp3 expression.

T_{FR} cells suppress T cell proliferation *in vitro* and repress T_{FH} cells *in vivo*

Expression of Foxp3 by CD4^{+} T cells initiates a transcriptional program that confers suppressor function[11-13]. T_{FR} and T_{reg} sorted from immunized Foxp3^{GFP} mice displayed comparable suppressive ability *in vitro* (Supplementary Fig. 6a). To determine whether T_{FR} cells are suppressive *in vivo,* we sought to ablate T_{FR} cells after the germinal center response had been established. For this, DT was first administered to Foxp3^{DTR} mice 5d after SRBC immunization, when T_{FH} and T_{FR} cells have already formed, but the response has not yet reached its peak[44]. After 3 days of DT treatment – 8 days after immunization – only 1% of T_{FR} cells and 5% of T_{reg} cells were present compared with vehicle-only-treated mice (Supplementary Fig. 7), and DT-treated mice displayed a significant increase in both the proportion and total number of T_{FH} cells compared to controls (Fig. 5a). At this time point, germinal center B cell numbers were comparable between DT- and vehicle-treated groups (Figure 5b).

To confirm the *in vivo* regulatory role of T_{FR}, it is necessary to deplete them while leaving the T_{reg} and T_{FH} compartments intact. To achieve this we generated mixed bone marrow chimeras with a 1:1 ratio of either congenically marked *Sh2d1a*$^{-/-}$:Foxp3^{DTR} or *Sh2d1a*$^{+/+}$:Foxp3^{DTR} marrow. After immunization and DT treatment, CD45.2^{+} *Sh2d1a*$^{-/-}$ cells should not able to form T_{FH} or T_{FR} cells, but should form T_{reg} cells normally, whereas Foxp3^{DTR} cells should form T_{FH} cells, but lack all Foxp3^{+} cells. Thus, T_{FH} and T_{reg} populations should still be present in both chimeras, but T_{FR} cells will be selectively reduced in *Sh2d1a*$^{-/-}$: Foxp3^{DTR} mice (Supplementary Table 2).

Such chimeric mice were generated and treated with DT 1 day prior to, and 2 and 5 days after SRBC immunization. At day 8 after immunization >90% of T_{FH} cells in *Sh2d1a*$^{-/-}$:Foxp3^{DTR} chimeras derived from Foxp3^{DTR} cells compared to ~50% in the control *Sh2d1a*$^{+/+}$:Foxp3^{DTR} mice (Supplementary Fig. 8a). The size of the T_{reg} population was comparable in both groups of mice (Fig. 5c) with the majority (97%) of T_{reg} being CD45.2^{+} (Supplementary Fig. 8b). Critically, the number of T_{FR} was reduced by 5 times in *Sh2d1a*$^{-/-}$: Foxp3^{DTR} mice compared with the control chimeras (Fig. 5d, Supplementary Fig. 7c). There was also an increase in both the number and proportion of T_{FH} cells (Fig. 5e) and of germinal center B cells (Fig. 5f), indicating that T_{FR} limit the germinal center response during a T-dependent immune reaction *in vivo*.

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T_{FR} limit the outgrowth of non-antigen-specific germinal center B cells

To further characterize the role of T_{FR} during the germinal center response, T_{FR} were transiently depleted at the peak of the germinal center response. For this, Foxp3^{DTR} and control Foxp3^{WT} mice were immunized with NP-KLH in alum, and treated with DT six days later. Ten days after immunization, the fraction of germinal center B cells was not significantly different between the two groups (Fig. 6a, b) but combined T_{reg} and T_{FR} depletion led to a reduction in the proportion of germinal center B cells specific for the dominant epitope of the immunizing antigen, the hapten NP (Fig. 6a, c).

In order to determine whether this had any long-term impact on the antigen-specific antibody response, Foxp3^{DTR} mice were immunized with NP-KLH in alum and treated with either DT or saline at 7, 10 and 13 days after immunization, then boosted with NP-KLH in alum 24 days after primary immunization (Fig. 6d). High (anti-NP2) & low (anti-NP12) affinity anti-NP antibody titers were assessed by ELISA prior to and d10, 15, 10 and d28 after immunization. Titers of high and low affinity antibodies were comparable until d20, when both were reduced in DT-treated mice compared with controls (Fig 6e). Four days after secondary immunization (day 28 after primary challenge), high affinity antibodies remained lower in DT-treated mice (Fig 6e). This suggests that depletion of T_{FR} and T_{reg} during the germinal center response does not increase antigen-specific antibody production.

To test whether the reduction in the antigen-specific germinal center response was a consequence of T_{FR} depletion rather than general T_{reg} depletion, we generated *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ mixed fetal liver chimeras that selectively lack T_{FR} cells. Three groups of control chimeras were generated in parallel: *Sh2d1a*$^{+/+}$*:Foxp3*$^{+/+}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{-/-}$ and *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$. Eight weeks after reconstitution, chimeric mice were immunized with alum-precipitated NP-chicken gammaglobulin (NP-CGG). 21 days post-immunization, *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ chimeras had a reduction in T_{FR} cells compared to all control groups (Supplementary Fig. 9a and b) and an expanded T_{FH} population (Supplementary Fig. 9c). The proportion of Foxp3^{+} T_{reg} was comparable amongst all 4 groups (Supplementary Fig. 9d). Consistent with a selective defect in T_{FR} and not T_{reg}, circulating T_H1 and T_H2 cells were not expanded in *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ mice 14 days post-immunization (Supplementary Fig. 10a and b) nor in the spleen 21 days after immunization (Supplementary Fig. 10c and d). This also confirms that T_{FR} cells are specialized in the regulation of follicular responses, while other T_{reg} effectors control T_H1 and T_H2 cells.

As observed in Foxp3^{DTR} mice, the proportion and absolute number of antigen-specific (NP^{+}) germinal center B cells was reduced in *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ mice compared with control chimeras (Fig. 6f), despite formation of abundant germinal centers (Fig. 6g). NP-specific splenic memory B cells and NP-specific bone marrow plasma cells appeared reduced in *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ chimeras compared with controls 21 days after immunization (Figure 6h and i) and the differences were statistically-significant against all control groups except for the *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$ group, in which there was greater variability. At this time point there was no difference in anti-NP antibody titers between *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$ mice compared with control chimeras (Supplementary Fig. 11), probably due to a large component being of extrafollicular origin given that abundant NP^{+} plasma cells were still detectable in the spleen (Supplementary Fig. 11). Together, this suggests that T_{FR} act to limit the outgrowth of non-antigen specific clones in the germinal center.

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Discussion

We have shown here that in response to T-dependent antigens a proportion of naïve T_{reg} can turn on Bcl-6, which allows them to adopt the T_{FH} differentiation program and express the follicular homing receptor CXCR5 to localize to the germinal center. Here they exert suppressive functions on T_{FH} cells and the germinal center response. Unlike T_{FH} cells, T_{FR} cells express Blimp-1, which is required to control their numbers in the germinal center. In response to other extracellular stimuli, naïve Foxp3$^+$ cells can turn on Tbet or increase activity of IRF4 or STAT3, required for T_H1, T_H2 and T_H17 cell formation respectively[16-18]. The specialized regulatory program determined by each of these transcription factors is likely to operate via modification of the T_{reg} chemokine receptor profile in order to allow migration into an anatomical location where T_{reg} are poised to regulate specific T cell responses to prevent autoimmunity or inflammation-associated tissue damage.

Our data suggests T_{FR} cells are specialized in controlling the germinal center reaction through limiting the numbers of T_{FH} cells and inhibiting selection of non-antigen-specific B cells including those carrying self-reactive receptors. Furthermore, the ability of T_{FR} cells to ensure dominance of antigen-specific clones over the germinal center response appears essential for formation of normal numbers of long-lived plasma cells and memory B cells. Although it is likely that T_{FR} control germinal center B cells indirectly, through their ability to limit T_{FH} numbers, it is also possible that T_{FR} also negatively regulate germinal center B cells directly. This would be akin to the description of direct regulation of antigen-presenting cells by T_{reg}[45, 46] and reports that T_{reg} can directly regulate B cell function[9]. Germinal center B cells are the predominant APCs within the germinal center microenvironment, and this makes them attractive candidates for T_{FR}-mediated inhibition.

Selection of cognate germinal center B cells by T_{FH} cells is one of the key mechanisms by which germinal center tolerance is regulated[47]. Dysregulation of the T_{FH} population has been previously demonstrated to result in autoimmunity[3, 5, 7, 48], highlighting the need to tightly control positive selection in germinal centers. Understanding the mechanisms by which T_{FR} are regulated and their TCR specificity will be important for dissecting the pathogenesis of the increasing number of pathologies in which T_{FH} cells appear to play a role, including disease-associated ectopic germinal center formation seen in many autoimmune diseases[3], atherosclerosis[49], and chronic allograft rejection[50]. We therefore postulate that T_{FR} cells may represent a critical peripheral tolerance mechanism, essential for preventing germinal center-derived autoimmunity.

Supplementary Material

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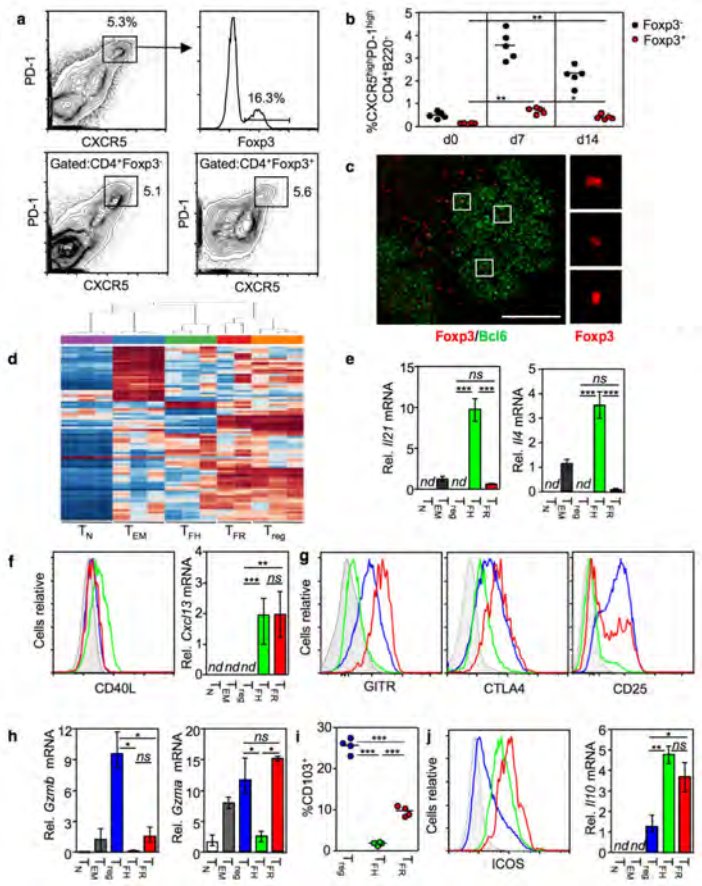

Figure 1. A proportion of CXCR5^{high}PD-1^{high}CD4^{+} cells express the transcription factor Foxp3

(a,b) After SRBC immunization Foxp3^{+} cells were identified in the CXCR5^{high}PD-1^{high}CD4^{+} 'T_{FH}' compartment, these cells follow the same kinetics as classic T_{FH} cells. **(c)** Foxp3^{+} cells (red) are present within the Bcl6^{+} germinal center area (green) following SRBC immunization. Scale bar represents 100μm. **(d)** Heat map comparing the gene expression profiles of different CD4^{+} T cell subsets from Foxp3^{GFP} mice seven days after immunization. Red: high gene expression; blue: low gene expression. The cells were sorted using the following markers and for simplicity will be referred by the abbreviations in parentheses throughout: CD4^{+}CD44^{low}Foxp3^{-} naïve (T_N) cells, CD4^{+}CD44^{high}CXCR5$^{int/low}$PD-1$^{int/low}$Foxp3^{-} effector/memory (T_{EM}) cells, CD4^{+}CD44^{int}CXCR5$^{int/low}$PD-1$^{int/low}$Foxp3^{+} regulatory T cells (T_{reg}), CD4^{+}CXCR5^{high}PD-1^{high}Foxp3^{-} T follicular helper (T_{FH}) cells and CD4^{+}CXCR5^{high}PD-1^{high}Foxp3^{+} follicular regulatory (T_{FR}) cells. **(e)** *Il21* and *Il4* mRNA measured by quantitative PCR from sorted cells using the strategy described in (d) normalized to *Gapdh*. Heights of the bars represent the mean and error bars represent the range of expression from 3 biological replicates. *nd*: gene expression not detected. **(f)** Left: Intracellular expression of CD40L as determined by flow cytometry in T_{reg} (blue), T_{FH} (green) and T_{FR} (red) cell populations; the grey histogram represents a staining control from an immunized CD40L-deficient mouse. Right: *Cxcl13 mRNA* measured by quantitative RT-PCR as described in (e). **(g)** Cell surface expression of GITR, CD25 and intracellular CTLA4 in T_{reg} (blue), T_{FH} (green) and T_{FR} (red) cell populations; grey histograms represent the isotype control. **(h)** Relative *Gzmb* and *Gzma* mRNA determined by quantitative RT-PCR as described in (e). **(i)** Percentage of CD103^{+} cells within the T_{reg}, T_{FH} & T_{FR} populations, each symbol represents one mouse. **(j)** Left: Cell surface expression of ICOS as

determined by flow cytometry in T_{reg} (blue), T_{FH} (green) and T_{FR} (red) cell populations; the grey histogram represents staining level of an isotype control. Right: *Il10* mRNA detected by quantitative RT-PCR of as described in (e). Flow cytometric and RT-PCR data are representative of at least three independent experiments. In (e)-(i): Statistical significance was determined using a one-way ANOVA analysis with Bonferroni's multiple testing correction; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

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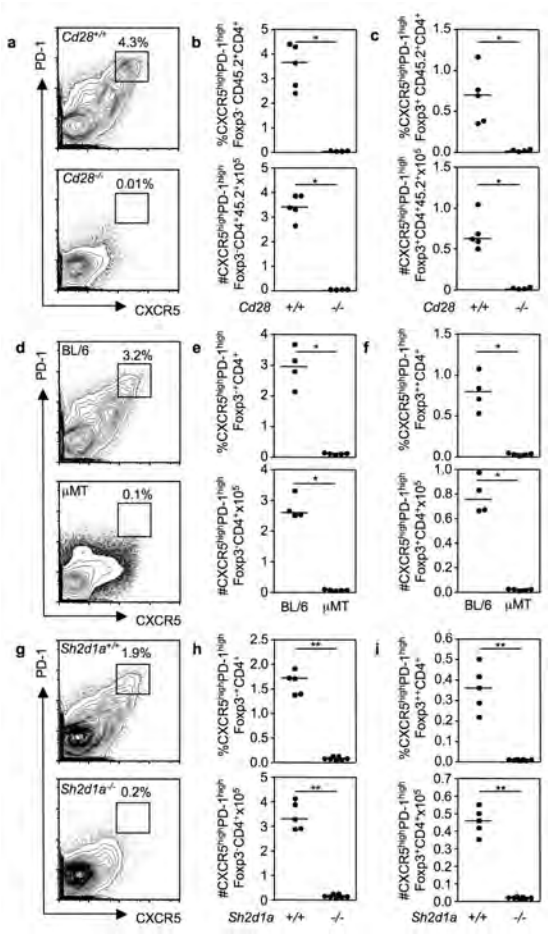

Figure 2. T_{FR} require the same differentiation cues as T_{FH} cells for their development

Flow cytometric contour plots **(a, d, g)** and dot plots of T_{FH} **(b, e, h)** and T_{FR} **(c, f, i)** cells in the groups of mice described below, seven days after SRBC immunization. **(a-c)** Mixed bone marrow chimeras generated by sub-lethally irradiating *Rag2*$^{-/-}$ mice and reconstituting their immune system with a 1:1 ratio of bone marrow cells from CD45.1 *Cd28*$^{+/+}$ and CD45.2 *Cd28*$^{-/-}$ mice or control CD45.1 *Cd28*$^{+/+}$ and CD45.2 *Cd28*$^{+/+}$. **(d-f)** C57BL/6 (BL/6) and B-cell deficient μMT mice. (g-i) *Sh2d1a*$^{+/+}$ and *Sh2d1a*$^{-/-}$ mice. Each symbol represents one mouse and horizontal bars represent median values. Figures represent one of 3 independent experiments with similar results. Statistical significance was determined using a Mann-Whitney Test: *P<0.05, **P<0.01.

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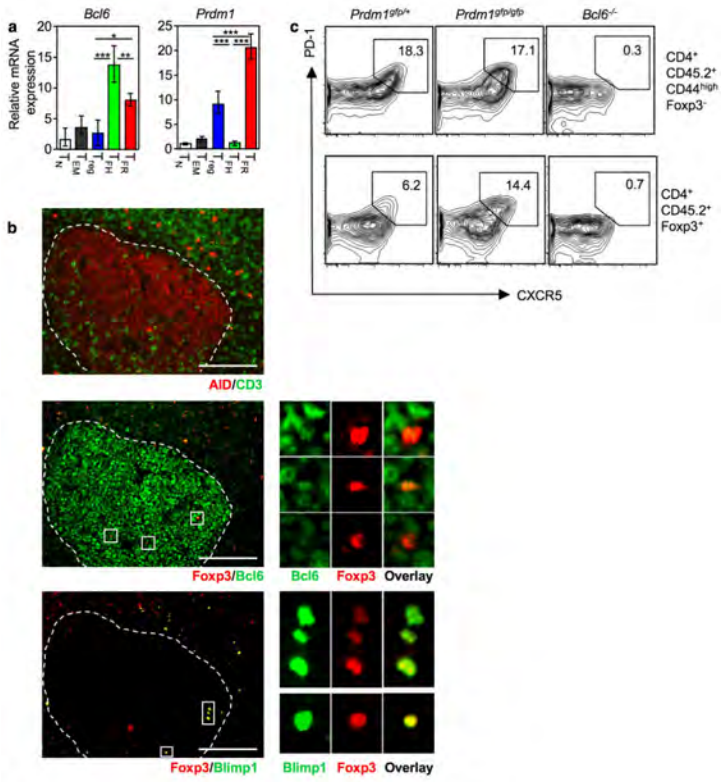

Figure 3. T_{FR} cells express Bcl-6 and Blimp-1

(a) *Bcl6* and *Prdm1* mRNA normalized to *Gapdh* determined by quantitative RT-PCR from sorted cells using the strategy described in Fig. 1d and Supplementary Fig. 1. Heights of the bars represent the mean and error bars represent the range of expression from 3 biological replicates. Statistical significance was determined using a one-way ANOVA analysis with Bonferroni's multiple testing correction; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Bar graphs are representative of 3 experiments. **(b)** Immunofluorescence of frozen spleen sections from mice immunized seven days previously with SRBC. The germinal center is demarcated by the white dotted line in the three consecutive sections. Upper panel: AID (red) and CD3 (green); middle panel: Foxp3 (red) and Bcl-6 (green); lower panel: Foxp3 (red) and Blimp1 (green). Scale bar represents 100μm. **(c)** Flow cytometric contour plots of T_{FH} (upper panels) & T_{FR} (lower panels) formation in the draining (mediastinal) lymph node ten days after intranasal influenza infection of mixed fetal liver chimeras reconstituted with a 1:1 ratio of fetal liver cells from E14.5 CD45.2 *Prdm1*$^{gfp/gfp}$: CD45.1 *Prdm1*$^{+/+}$ embryos, E14.5 CD45.2 *Bcl6*$^{-/-}$: CD45.1 *Bcl6*$^{+/+}$ embryos or control E14.5 CD45.2 *Prdm1*$^{gfp/+}$: CD45.1 *Prdm1*$^{+/+}$ embryos.

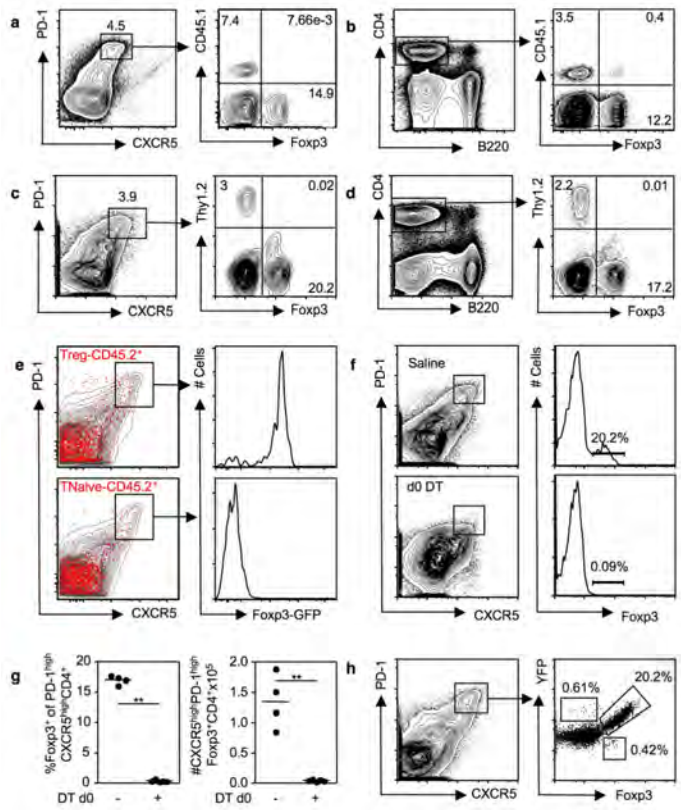

Figure 4. T_{FR} derive from Foxp3$^+$ precursors

Flow cytometric contour plots of splenic CD4$^+$CXCR5^{high}PD-1^{high} cells **(a)** or CD4$^+$ cells **(b)** seven days after 1×10^5 transferred transgenic TCR^{3A9} HEL-specific CD45.1 T cells were adoptively transferred into congenically distinct CD45.2 B10.Br mice and immunized with HEL in alum. Flow cytometric contour plots of splenic CD4$^+$CXCR5^{high}PD-1^{high} cells **(c)** or CD4$^+$ cells **(d)** seven days after adoptive transfer of 1×10^5 OT-II OVA-specific Thy1.2 T cells into congenically distinct Thy1.1 C57BL6 mice and immunization with OVA in alum. **(e)** Flow cytometric contour plots of splenic CD4$^+$ T cells from CD45.1 C57BL/6 mice seven days after adoptive transfer of 1×10^6 sorted naïve CD4$^+$CD44^{int}Foxp3$^+$ T_{reg} (top panel) or CD4$^+$CD44^{low}Foxp3$^-$ naïve T cells (lower panel) from unimmunized CD45.2 Foxp3^{GFP} mice and KLH in Ribi immunization. Transferred CD45.2 cells are shown in red, the endogenous CD45.1 cells are represented by the grey contour plots. Histograms showing Foxp3-GFP expression in transferred CD45.2$^+$CD4$^+$CXCR5^{high}PD-1^{high} cells. **(f)** Contour plots of splenic CD4$^+$ T cells and quantification of T_{FR} cells **(g)** from Foxp3^{DTR} mice six days after SRBC immunization and administration of either 0.9% saline (top panel) or DT (lower panel). Histograms show Foxp3$^+$ cells within the CD4$^+$CXCR5^{high}PD-1^{high} compartment. **(h)** Flow cytometric contour plots of splenic CD4$^+$ cells from Foxp3-cre x ROSA-Stop-flox-YFP mice immunized seven days previously with SRBC (left panel). Enumeration of the proportion of CD4$^+$CXCR5^{high}PD-1^{high} cells that expressed YFP and/or Foxp3 (right panel). Each symbol represents one mouse and horizontal bars represent median values. Figures are representative of 2-4 independent experiments.

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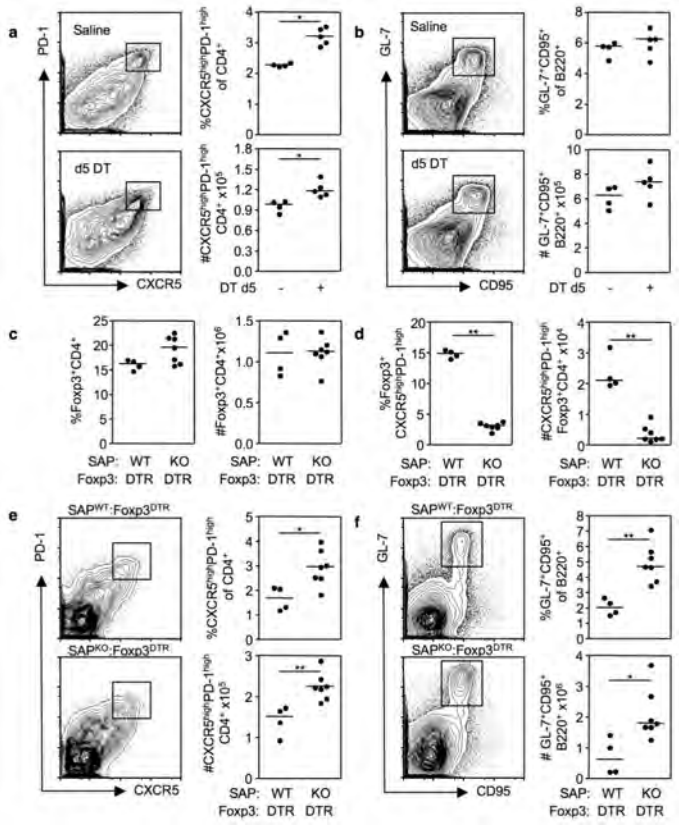

Figure 5. T_{FR} regulate the size of the T_{FH} population

Flow cytometric contour plots and graphs of T_{FH} cells **(a)** and germinal center B cells **(b)** from the spleens of Foxp3^{DTR} mice immunized eight days previously (d0) with SRBC. Five days after immunization the mice were treated with either DT or saline. **(c-f)** Analysis of mixed bone marrow chimeras generated by sub-lethally irradiating *Rag2*$^{-/-}$ mice and reconstituting their immune system with either a 1:1 ratio of *Sh2d1a*$^{-/-}$ CD45.2 : Foxp3^{DTR} CD45.1 bone marrow or control *Sh2d1a*$^{+/+}$ CD45.2 : Foxp3^{DTR} CD45.1 bone marrow. Eight weeks after reconstitution chimeric mice were immunized with SRBC and treated with 50μg/Kg of DT on one day prior to immunization and d2 and d5 thereafter. Splenocytes were analyzed on d8 for the proportion and total number of CD4$^+$CXCR5^{high}PD-1^{high}Foxp3$^+$ T_{FR} cells **(c)**, CD4$^+$Foxp3$^+$ T_{reg} **(d)**, CD4$^+$CXCR5^{high}PD-1^{high} T_{FH} cells **(e)** and B220$^+$ GL-7^{high}CD95^{high} germinal center B cells **(f)**. Each symbol represents one mouse and horizontal bars represent median values. Statistical significance was determined using a Mann-Whitney Test: **P*<0.05, ***P*<0.01.

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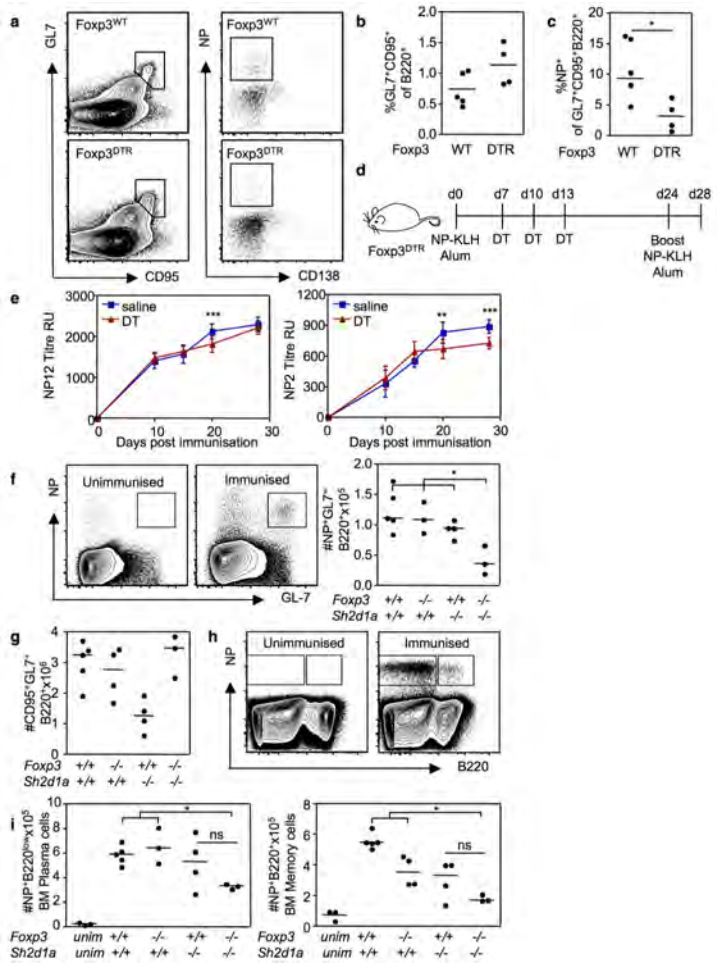

Figure 6. T_{FR} restrict the outgrowth of non-antigen specific clones in the germinal center

Flow cytometric contour plots **(a)** and graphs **(b)** of total GL-7$^+$CD95$^+$ germinal center B cells and **(c)** NP$^+$ germinal center B cells ten days after immunization of Foxp3^{WT} and Foxp3^{DTR} mice that have been treated with DT 6 days after NP-KLH immunization. Statistical analyses performed using Mann Whitney U-test. Experimental outline **(d)** of immunization and DT or saline treatment scheme of Foxp3^{DTR} mice (n=8 per group) to examine the antigen specific immunoglobulin response over time, mice were bled prior to, and d10, d15, d20 and d28 after primary immunization. Mice were given a booster immunization 24 days after the primary immunization. **(e)** ELISA analysis of NP12 and NP2 antibodies in the experiment outlined in (d). Error bars represent the standard error of the mean from eight individual mice from one experiment, representative of two experiments. Statistical analyses in (e) were performed using a two-way ANOVA with Bonferroni post test to compare differences at each time point. Graphs and flow cytometric contour plots of NP$^+$ germinal center B cells **(f)**, total GL-7$^+$CD95$^+$ germinal center B cells **(g)** and NP$^+$ bone marrow plasma and memory cells **(h, i)** 21 days after NP-CGG immunization of chimeric mice generated by reconstituting *Rag2*$^{-/-}$ mice with a 1:1 mix of *Sh2d1a*$^{-/-}$*:Foxp3*$^{-/-}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{+/+}$, *Sh2d1a*$^{+/+}$*:Foxp3*$^{-/-}$ and *Sh2d1a*$^{-/-}$*:Foxp3*$^{+/+}$ fetal liver. Statistical analyses in (f, g, h and i) were performed using a one-way ANOVA with Bonferroni post test correction. Each symbol represents one mouse and horizontal bars represent median values. **P*<0.05, ***P*<0.01, ****P*<0.001.