

# Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses

Rachel L. Clement<sup>1</sup>, Joe Daccache<sup>1</sup>, Mostafa T. Mohammed<sup>1</sup>, Alos Diallo<sup>2</sup>, Bruce R. Blazar<sup>3</sup>, Vijay K. Kuchroo<sup>4,5,6</sup>, Scott B. Lovitch<sup>7</sup>, Arlene H. Sharpe<sup>2,4,7</sup> and Peter T. Sage<sup>1\*</sup>

**Follicular regulatory T (T<sub>FR</sub>) cells have specialized roles in modulating follicular helper T (T<sub>FH</sub>) cell activation of B cells. However, the precise role of T<sub>FR</sub> cells in controlling antibody responses to foreign antigens and autoantigens *in vivo* is still unclear due to a lack of specific tools. A T<sub>FR</sub> cell-deleter mouse was developed that selectively deletes T<sub>FR</sub> cells, facilitating temporal studies. T<sub>FR</sub> cells were found to regulate early, but not late, germinal center (GC) responses to control antigen-specific antibody and B cell memory. Deletion of T<sub>FR</sub> cells also resulted in increased self-reactive immunoglobulin (Ig) G and IgE. The increased IgE levels led us to interrogate the role of T<sub>FR</sub> cells in house dust mite models. T<sub>FR</sub> cells were found to control T<sub>H</sub>13 cell-induced IgE. *In vivo*, loss of T<sub>FR</sub> cells increased house-dust-mite-specific IgE and lung inflammation. Thus, T<sub>FR</sub> cells control IgG and IgE responses to vaccines, allergens and autoantigens, and exert critical immunoregulatory functions before GC formation.**

T<sub>FR</sub> cells migrate to B cell follicles to stimulate antibody production by B cells in the GC reaction<sup>1</sup>. The GC reaction results in somatic hypermutation, affinity maturation and class-switch recombination, although these processes may also occur outside GCs<sup>2</sup>. T<sub>FR</sub> cells provide essential costimulation (through the inducible costimulatory molecule ICOS and CD40L) and cytokines (such as interleukin (IL)-21 and IL-4) to help promote B cell responses<sup>3,4</sup>. T<sub>FR</sub> cells possess a degree of phenotypic plasticity that can be altered by the inflammatory milieu, causing T<sub>FR</sub> cells to produce cytokines typically made by T helper (T<sub>H</sub>)1, T<sub>H</sub>2 and T<sub>H</sub>17 cells<sup>5–7</sup>. T<sub>FR</sub> cells are thought to be distinct from T<sub>H</sub>2 cells because T<sub>H</sub>2 cells can produce both IL-4 and IL-13 and express the transcription factor Gata3, but T<sub>FR</sub> cells can produce only IL-4 and do not express IL-13 or GATA3 (ref. <sup>8</sup>). Although T<sub>H</sub>2 cells can mediate IgE responses, T<sub>FR</sub> cells might also play a role. Studies have suggested that the T<sub>FR</sub> cell cytokine IL-21 is essential for IgE responses to house-dust mite (HDM) antigen, and that T<sub>FR</sub> cells may convert to T<sub>H</sub>2-like cells in the lung<sup>9,10</sup>. IgE responses are not completely dependent on GATA3 expression, suggesting that cells other than T<sub>H</sub>2 cells may promote IgE. T regulatory (T<sub>reg</sub>) cells can inhibit allergic inflammation, possibly through suppressing T<sub>H</sub>2 cells<sup>11,12</sup>.

T<sub>FR</sub> cells inhibit T<sub>FR</sub> cell-mediated B cell responses<sup>13,14</sup>. *In vitro* assays have shown that T<sub>FR</sub> cells can inhibit antibody secretion, class-switch recombination and somatic hypermutation through metabolic reprogramming and epigenetic remodeling of B cells<sup>15–17</sup>. In addition, T<sub>FR</sub> cells can suppress T<sub>FR</sub> cell production of effector cytokines such as IL-4 and IL-21 *in vitro*, while maintaining the T<sub>FR</sub> cell transcriptional program<sup>17</sup>. The role of T<sub>FR</sub> cells in controlling T<sub>FR</sub> cell-mediated B cell responses *in vivo* is less clear. Adoptive transfer studies into lymphopenic mice have shown that T<sub>FR</sub> cells inhibit antigen-specific IgG levels<sup>16,18,19</sup>. However, studies using bone

marrow chimera and/or genetic models, in which the transcription factor Bcl6 was deleted in FoxP3<sup>+</sup> cells, have suggested that T<sub>FR</sub> cells regulate non-antigen-specific B cell responses but do not substantially affect GC B cells or antigen-specific IgG levels; however, results have been inconsistent<sup>20–22</sup>. Moreover, IL-10 produced by T<sub>FR</sub> cells can promote, rather than inhibit, plasma cell formation<sup>23</sup>. One explanation for the variability between studies may be due to the models used because Bcl6 can be expressed on T<sub>reg</sub> cell subsets other than T<sub>FR</sub> cells, Bcl6 might not be completely necessary for development of all T<sub>FR</sub> cells, and compensatory effects may rescue T<sub>FR</sub> cell deletion in non-inducible systems.

To determine the precise role of T<sub>FR</sub> cells in controlling B cell responses a T<sub>FR</sub> cell-deleter mouse model was developed to inducibly delete T<sub>FR</sub> cells in intact hosts at specific time points during immune responses. It was demonstrated that T<sub>FR</sub> cells potentially regulate antigen-specific and memory IgG levels early during responses before GC formation. Using a T<sub>H</sub>2 cell-like HDM challenge model, it was found that T<sub>FR</sub> cells can regulate IL-13 production by T<sub>FR</sub> cells and control IgE responses. Deletion of T<sub>FR</sub> cells *in vivo* during HDM sensitization resulted in increased HDM-specific IgE and lung inflammation. Taken together, these data demonstrate that T<sub>FR</sub> cells are key regulators of humoral and allergic immunity by controlling early GC responses.

## Results

**Development of a specific and inducible T<sub>FR</sub> cell-deleter mouse model.** To study the role of T<sub>FR</sub> cells during immune responses *in vivo*, we created a mouse model to perturb T<sub>FR</sub> cells in an inducible manner. To achieve this, a mouse containing a *Cxcr5*<sup>IRRES-LoxP-STOP-LoxP-DTR</sup> allele knocked into the *Cxcr5* locus was generated, which was crossed to a FoxP3<sup>IRRES-CreYFP</sup> allele-containing

<sup>1</sup>Transplantation Research Center, Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. <sup>2</sup>Department of Immunology, Harvard Medical School, Boston, MA, USA. <sup>3</sup>Department of Pediatrics, Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN, USA. <sup>4</sup>Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA, USA. <sup>5</sup>Broad Institute, Cambridge, MA, USA. <sup>6</sup>Ann Romney Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA, USA. <sup>7</sup>Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA. \*e-mail: [psage@bwh.harvard.edu](mailto:psage@bwh.harvard.edu)

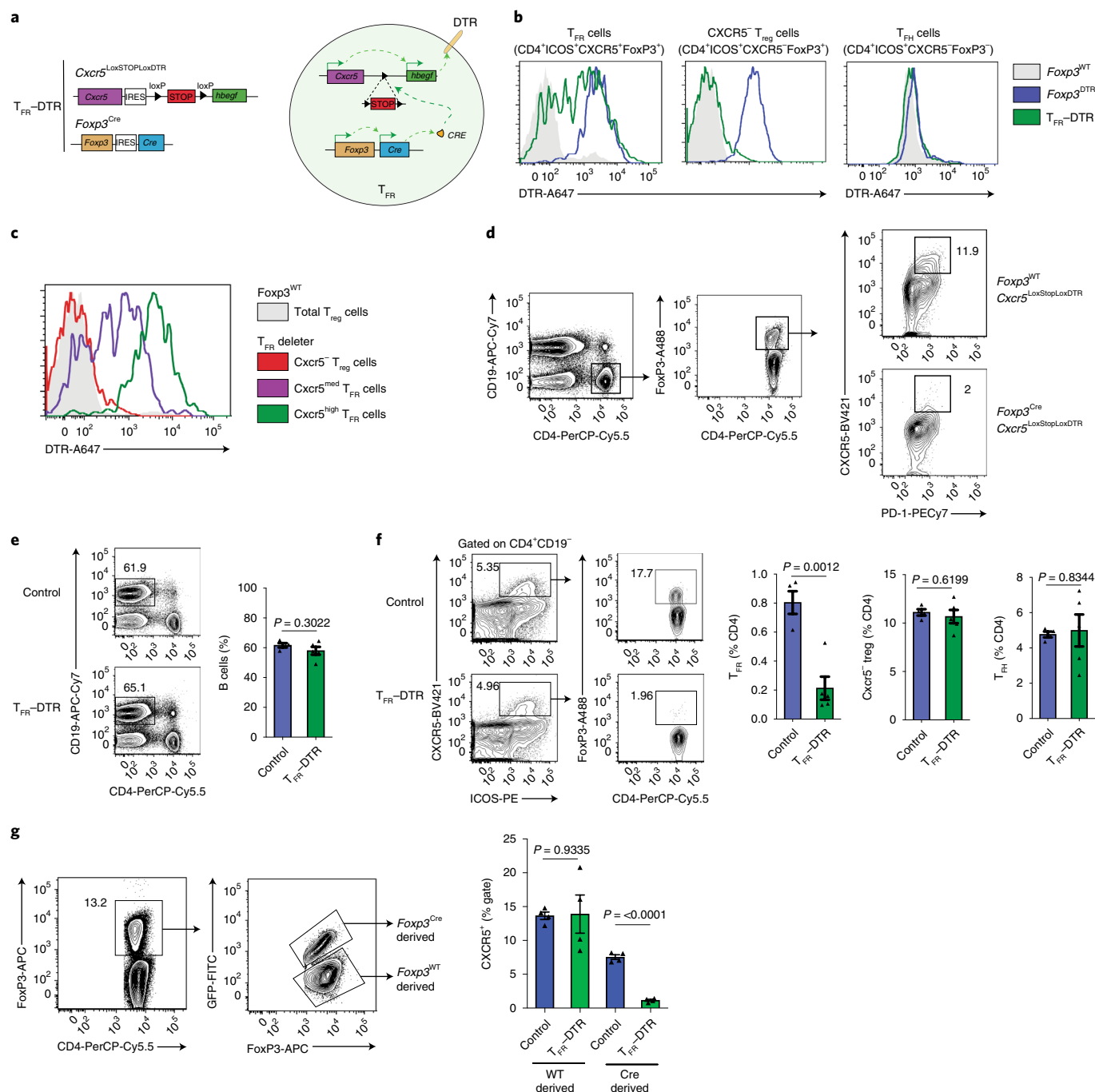


**Fig. 1 | Development of a  $T_{FR}$  cell-specific deleter model.** **a**, Schematic diagram of the  $T_{FR}$ -DTR strain. Allele details (left) and schematic of events leading to  $T_{FR}$  cell-specific DTR expression (right) are shown. **b**, DTR expression on  $T_{FR}$  cells (left), CXCR5<sup>+</sup>  $T_{reg}$  cells (middle) or  $T_{FH}$  cells (right) from control ( $Foxp3^{WT}$ ),  $Foxp3^{DTR}$  or  $T_{FR}$ -DTR mice. **c**, DTR expression on CXCR5-negative, CXCR5-medium or CXCR5-high  $T_{FR}$  cells from  $T_{FR}$ -DTR mice. **d**, Quantification of  $T_{FR}$  cells from  $T_{FR}$ -DTR ( $Foxp3^{Cre}Cxcr5^{LoxStopLoxDTT/WT}$ ) or control ( $Foxp3^{WT}Cxcr5^{LoxStopLoxDTT/WT}$ ) mice that were immunized 7 d previously and received DT 2, 4 and 6 d after immunization. **e**, Quantification of B cells from  $T_{FR}$ -DTR or control mice ( $Foxp3^{Cre}Cxcr5^{LoxStopLoxDTT/WT}$ ) that were immunized 7 d previously and received DT 2, 4 and 6 d after immunization. **f**, Quantification of  $T_{FR}$  cells, CXCR5<sup>+</sup>  $T_{reg}$  and  $T_{FH}$  cells from mice as in **e**. **g**, Quantification of  $T_{FR}$  cells (by assessing CXCR5<sup>+</sup>  $T_{reg}$  cells) from  $Foxp3^{Cre/WT}Cxcr5^{LoxStopLoxDTT/WT}$  or control ( $Foxp3^{Cre/WT}Cxcr5^{WT/WT}$ ) mice, gated on Cre-derived or WT-derived FoxP3 alleles. Column graphs represent the mean with error bars indicating s.e.m. The  $P$  value indicates a two-tailed Student's  $t$ -test. Data are from individual experiments and represent two (**b-d,g**) or four (**e,f**) independent experiments with similar results.

mouse to generate a  $Cxcr5^{IRES-LoxP-STOP-LoxP-DTR}Foxp3^{IRES-CreYFP}$  strain, referred to as the  $T_{FR}$ -DTR strain (where DTR is diphtheria toxin receptor) (Fig. 1a). In  $T_{FR}$ -DTR mice, FoxP3-expressing cells produce Cre recombinase which excises the stop cassette in the  $Cxcr5^{IRES-LoxP-STOP-LoxP-DTR}$  allele, resulting in an active  $Cxcr5^{IRES-DTR}$

allele and, hence, DTR expression under the control of  $Cxcr5$ . Therefore, only cells expressing both FoxP3 and CXCR5, such as  $T_{FR}$  cells, express DTR on the cell surface, making them susceptible to deletion with diphtheria toxin (DT). DTR expression was evaluated on  $T_{FR}$  cells and CXCR5<sup>+</sup>  $T_{reg}$  cells from wild-type (WT) FoxP3,

FoxP3–DTR (ref. <sup>24</sup>) or  $T_{FR}$ –DTR mice using an anti-DTR antibody. It was found that  $T_{FR}$  cells (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{+}$  cells) had substantial DTR expression in  $T_{FR}$ –DTR mice, albeit slightly lower compared with FoxP3–DTR mice (Fig. 1b and see Supplementary Fig. 1a,b). Importantly,  $CXCR5^{+}T_{reg}$  cells expressed DTR only in FoxP3–DTR mice and not in  $T_{FR}$ –DTR mice.  $T_{FH}$  cells (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{-}$  cells) did not express DTR, except for a very small population which are probably ex- $T_{FR}$  cells (Fig. 1b and see Supplementary Fig. 1a,b)<sup>25</sup>. DTR expression was highest on  $T_{FR}$  cells expressing high levels of the CXCR5 chemokine receptor type 5 (CXCR5), further demonstrating CXCR5-driven DTR expression (Fig. 1c).

To determine the efficiency of  $T_{FR}$  cell deletion, mice were immunized with (4-hydroxy-3-nitrophenyl)acetyl-ovalbumin (NP-OVA) and given DT. It was found that  $T_{FR}$  cells were deleted from  $T_{FR}$ –DTR but not from either  $Cxcr5^{IRES-LoxP-STOP-LoxP-DTR}FoxP3^{wt}$  or  $FoxP3^{Cre}Cxcr5^{wt/wt}$  control mice (Fig. 1d–f). Deletion of  $T_{FR}$  cells was selective in  $T_{FR}$ –DTR mice because B cells,  $T_{FH}$  cells, total CXCR5- $T_{reg}$  cells or activated Ki67<sup>+</sup>  $T_{reg}$  cells were not deleted in  $T_{FR}$ –DTR mice (Fig. 1e,f and see Supplementary Fig. 1c). Moreover, deletion of  $T_{FR}$  cells in  $T_{FR}$ –DTR mice resulted in the loss of FoxP3<sup>+</sup> cells within individual GCs (see Supplementary Fig. 1d). To determine whether deletion of  $T_{FR}$  cells was cell intrinsic,  $Cxcr5^{IRES-LoxP-STOP-LoxP-DTR}FoxP3^{Cre/wt}$  mice (in which only ~50% of the  $T_{FR}$  cells will express DTR on the surface) were immunized and given DT. DTR-expressing  $T_{FR}$  cells were deleted, but not non-DTR-expressing  $T_{FR}$  cells from the same mouse, demonstrating cell-intrinsic deletion in the  $T_{FR}$ –DTR model (Fig. 1g).

**$T_{FR}$  cells potently regulate early GC formation.** Previous data suggest that  $T_{FR}$  cells can be limited by cytokines produced in GCs<sup>15</sup>. Moreover,  $T_{FR}$  cells seem to be less frequent in large, developed GCs (data not shown)<sup>26</sup>. These findings suggest that  $T_{FR}$  cells might regulate B cell responses most potently before mature GCs form. To assess the role of  $T_{FR}$  cells before GC initiation,  $T_{FR}$ –DTR or control ( $Foxp3^{Cre}Cxcr5^{wt/wt}$ ) mice were immunized with NP-OVA and  $T_{FR}$  cells were deleted on days 5–9 with administration of DT, and B cell responses at day 21 were assessed.  $T_{FR}$  cells were largely attenuated, even 12 d after the last DT injection (Fig. 2a). There were minor, but notable, increases in the frequency of  $T_{FH}$  cells compared with control mice. The  $CD19^{+}GL7^{+}FAS^{+}$  GC B cell frequency was approximately twofold higher in  $T_{FR}$ –DTR mice compared with control mice, demonstrating that  $T_{FR}$  cells potently regulate initial GC formation (Fig. 2b). In addition, naive B cells, gated as  $CD38^{+}IgG1^{-}$  B cells, were slightly attenuated in  $T_{FR}$ –DTR mice.  $CD138^{+}$  plasma cells,  $IgG1^{+}$  class-switched B cells and  $IgG1^{+}CD38^{+}$  memory-like B cells were also increased in  $T_{FR}$ –DTR mice, suggesting that  $T_{FR}$  cells regulate many arms of B cell effector responses (Fig. 2b,c).  $T_{FR}$  cells have previously been shown to cause metabolic reprogramming, including inhibition of glycolysis, in B cells<sup>15</sup>. It was found that Glut1 expression was higher in B cells from  $T_{FR}$  cell-deleted compared with control mice (Fig. 2d). These results are consistent with a mechanism in which  $T_{FR}$  cells inhibit metabolism in B cells in vivo.

To determine whether  $T_{FR}$  cells regulate antigen-specific antibody production by regulating early GC responses, total and NP-specific antibody levels were assessed in  $T_{FR}$ –DTR mice. There was a twofold increase in total IgG and a ~2.5-fold increase in NP-specific IgG, demonstrating that  $T_{FR}$  cells regulate antigen-specific antibody responses (Fig. 2e and see Supplementary Fig. 2a). Moderate increases in total IgA and substantial increases in total IgE were also found in  $T_{FR}$  cell-deleted mice (Fig. 2e). The latter result was unexpected because  $T_{FR}$ –DTR mice are on a C57bl/6 background. These results were not due to preferential deletion of a  $T_{FR}$  cell subset because the small number of  $T_{FR}$  cells remaining in  $T_{FR}$ –DTR mice phenotypically resemble  $T_{FR}$  cells from control mice (see Supplementary Fig. 2b). Increased Ki67 expression was found in the  $T_{FR}$  cells remaining in  $T_{FR}$ –DTR mice, probably due

to a compensatory mechanism to overcome  $T_{FR}$  cell deficiency (see Supplementary Fig. 2b).

Next, it was determined whether  $T_{FR}$  cells can regulate B cell responses after GCs have already formed.  $T_{FR}$ –DTR or control ( $Foxp3^{Cre}Cxcr5^{wt/wt}$ ) mice were immunized with NP-OVA and given DT on days 10–14 to delete  $T_{FR}$  cells. A reduction in  $T_{FR}$  cells was found in the  $T_{FR}$ –DTR mice, and slight increases in the frequency of  $T_{FH}$  cells, polarizing the follicular T cell subset toward  $T_{FH}$  cells (Fig. 2f). However, when GC B cells were assessed, no differences were found between  $T_{FR}$ –DTR and control mice (Fig. 2g). Likewise, no increases in total or NP-specific IgG were found, although plasma cells were slightly elevated, as were levels of IgA and IgE (Fig. 2g,h and see Supplementary Fig. 2c). The few  $T_{FR}$  cells remaining in  $T_{FR}$ –DTR mice had a similar phenotype to control mice except for elevated Ki67 (see Supplementary Fig. 2d). The lack of increased antigen-specific antibodies when  $T_{FR}$  cells were deleted post-GC formation was due to the stage of GC, and not the total duration of  $T_{FR}$  cell deletion, because pre-GC deletion strategies have a phenotype as early as day 5 after  $T_{FR}$  cell deletion, and deletion of  $T_{FR}$  cells after GC formation does not result in a phenotype, even at day 26 (see Supplementary Fig. 2e,f). These data demonstrate that  $T_{FR}$  cells can regulate GC B cell development and antigen-specific antibody responses early, before GC formation, and have less regulatory control after GCs have been initiated.

**$T_{FR}$  cells regulate autoreactive IgG and IgE antibodies.** Next, we assessed whether  $T_{FR}$  cells can regulate autoreactive antibodies.  $T_{FR}$  cells were deleted in  $T_{FR}$ –DTR mice before GC formation (at days 5–9), and the sera analyzed at day 21 with autoantigen protein arrays. Autoreactive IgG was increased for a third of the autoantigens in  $T_{FR}$ –DTR mice compared with control mice using a stringent cutoff (Mann–Whitney *U*-test,  $P < 0.01$ ) (Fig. 3a). In most cases, control mice had antibodies that recognize autoantigens, but levels were higher in the  $T_{FR}$ –DTR mice. However, in one example, there was a substantial signal for anti-histone H1 autoantibodies in  $T_{FR}$ –DTR mice, but no detectable signal in control mice (Fig. 3a). These data demonstrate that  $T_{FR}$  cells can regulate levels and formation of autoreactive IgG antibodies.

Next, it was determined whether any of the substantial amounts of IgE in the  $T_{FR}$  cell-deleted mice were specific for autoantigens. Such autoreactive antibodies could be pathogenic, because patients with systemic lupus erythematosus (SLE) can generate autoreactive IgE responses, and autoreactive IgE can exacerbate disease in mouse models of lupus<sup>27,28</sup>. Fifteen autoantigens were recognized to a higher degree by IgE in  $T_{FR}$ –DTR compared with control mice (Mann–Whitney *U*-test,  $P < 0.01$ ) (Fig. 3b). These autoantigens include La/SSB and Ro-52/SSA, which are targets for autoreactive IgE responses in SLE patients; anti-SSB and -SSA IgE may be indicators of immune complex-mediated disease<sup>28</sup>. In addition, complement had higher IgE autoantibody scores in  $T_{FR}$ –DTR compared with control mice. When a less stringent cutoff of  $P < 0.05$  was used, anti- $\beta_2$ -microglobulin and anti-GP2 IgE were present in  $T_{FR}$ –DTR mice, but not in control mice (data not shown). Using the stringent  $P < 0.01$  cutoff, there was evidence of increases in IgG and IgE targeting the same eight autoantigens, including Ro-52/SSA, MPO, CENP-B, PL-7, TTG and M2 (Fig. 3c,d). Some of these IgG and IgE autoantibodies, such as anti-Ro-52/SSA, are increased in SLE patients.

Next, it was determined whether  $T_{FR}$  cells can regulate initial activation and class-switch recombination of autoreactive B cells. A  $T_{FH}$  cell-mediated, antigen-specific, autoreactive B cell, class-switch recombination assay was developed. Myelin oligodendrocyte glycoprotein (MOG) was chosen as the model autoantigen because MOG immunization generates functional  $T_{FH}$  and  $T_{FR}$  cells<sup>18,29</sup>, MOG-specific B cells cause a Devic-like disease in experimental autoimmune encephalomyelitis models<sup>30,31</sup> and B cell depletion has a large

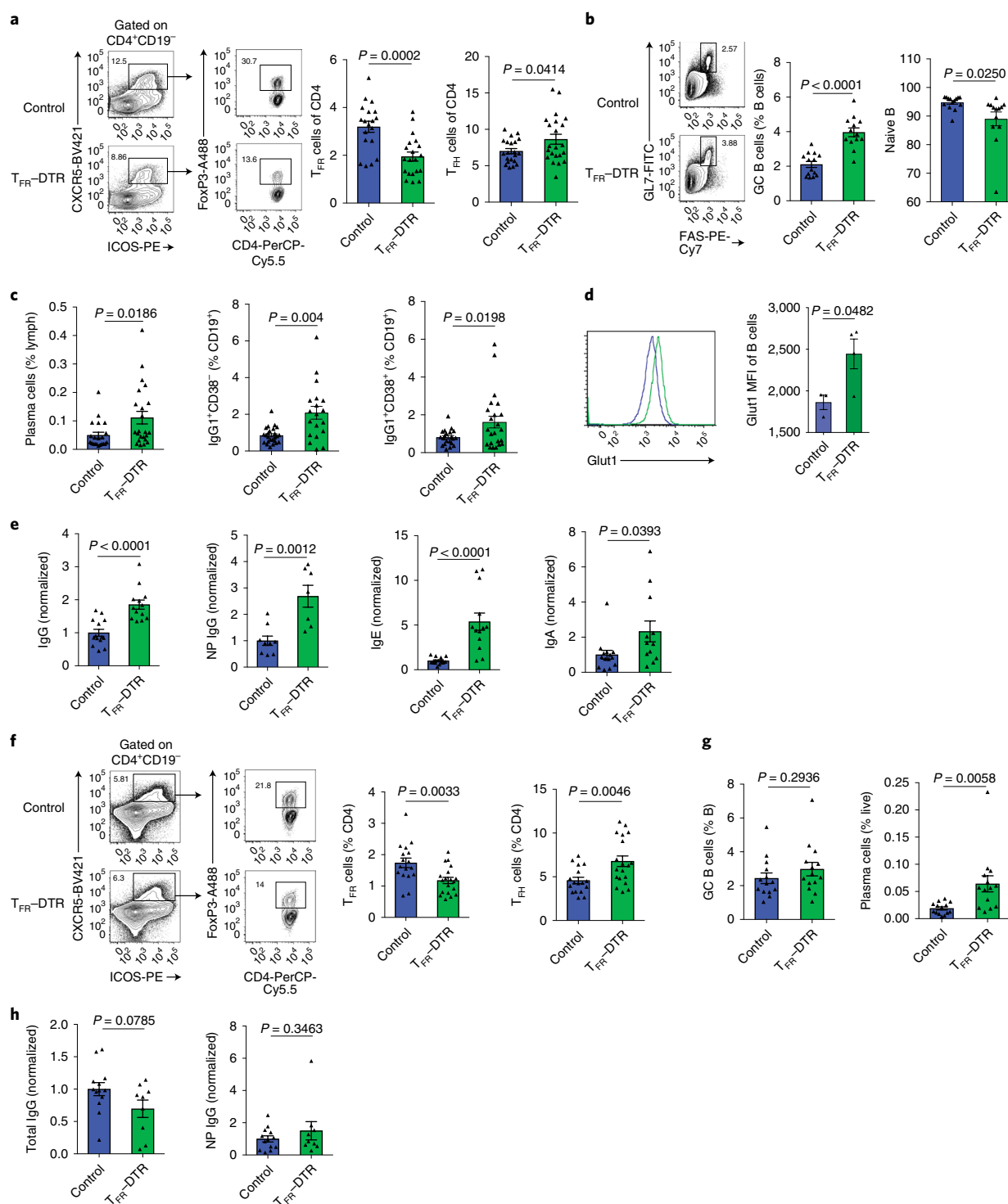


**Fig. 2 | T<sub>FR</sub> cells potentially regulate early GC formation.** **a**, Quantification of T<sub>FR</sub> (gated as CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>+</sup>) and T<sub>H</sub> (gated as CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>+</sup>) cells from dLNs of T<sub>FR</sub>-DTR (*Foxp3<sup>Cre</sup>Cxcr5<sup>LoxStopLoxDTTR/WT</sup>*) or control (*Foxp3<sup>Cre</sup>Cxcr5<sup>WT/WT</sup>*) mice 21 d after immunization. DT was administered on days 5, 7 and 9 to delete T<sub>FR</sub> cells before GC initiation. **b**, Quantification of GC B cells (gated as CD19<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>) and naive B cells (gated as CD38<sup>+</sup>IgG1<sup>+</sup>) from dLNs at day 21 after immunization as in **a**. **c**, Quantification of plasma cells (gated as CD138<sup>+</sup>), class-switched B cells (gated as CD19<sup>+</sup>IgG1<sup>+</sup>CD38<sup>+</sup>) and memory-like B cells (gated as CD19<sup>+</sup>IgG1<sup>+</sup>CD38<sup>+</sup>) at day 21 after immunization as in **a**. **d**, Glut1 expression on B cells from mice as in **a**. Representative histogram is shown on the left and quantification on the right. MFI, mean fluorescence intensity. **e**, Quantification of total IgG (far left), NP-specific IgG (middle left), total IgE (middle right) and total IgA (far right) analyzed from serum of mice as in **a**. **f**, Quantification of T<sub>FR</sub> (gated as CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>+</sup>) and T<sub>H</sub> (gated as CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>+</sup>) cells from dLNs of T<sub>FR</sub>-DTR (*Foxp3<sup>Cre</sup>Cxcr5<sup>LoxStopLoxDTTR/WT</sup>*) or control (*Foxp3<sup>Cre</sup>Cxcr5<sup>WT/WT</sup>*) mice at day 21 after immunization. DT was administered on days 10, 12 and 14 to delete T<sub>FR</sub> cells after GC formation. **g**, Quantification of GC B cells (gated as CD19<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>) and plasma cells (CD138<sup>+</sup>) from dLNs at day 21 after immunization as in **f**. **h**, Quantification of total IgG (left) and NP-specific IgG (right). Column graphs represent the mean with error bars indicating s.e.m. The *P* value indicates a two-tailed Student's *t*-test. Data are either combined results from four (**a–c, e**) or three (**f–h**) independent experiments, or from an individual experiment that represents two independent experiments (**d**).

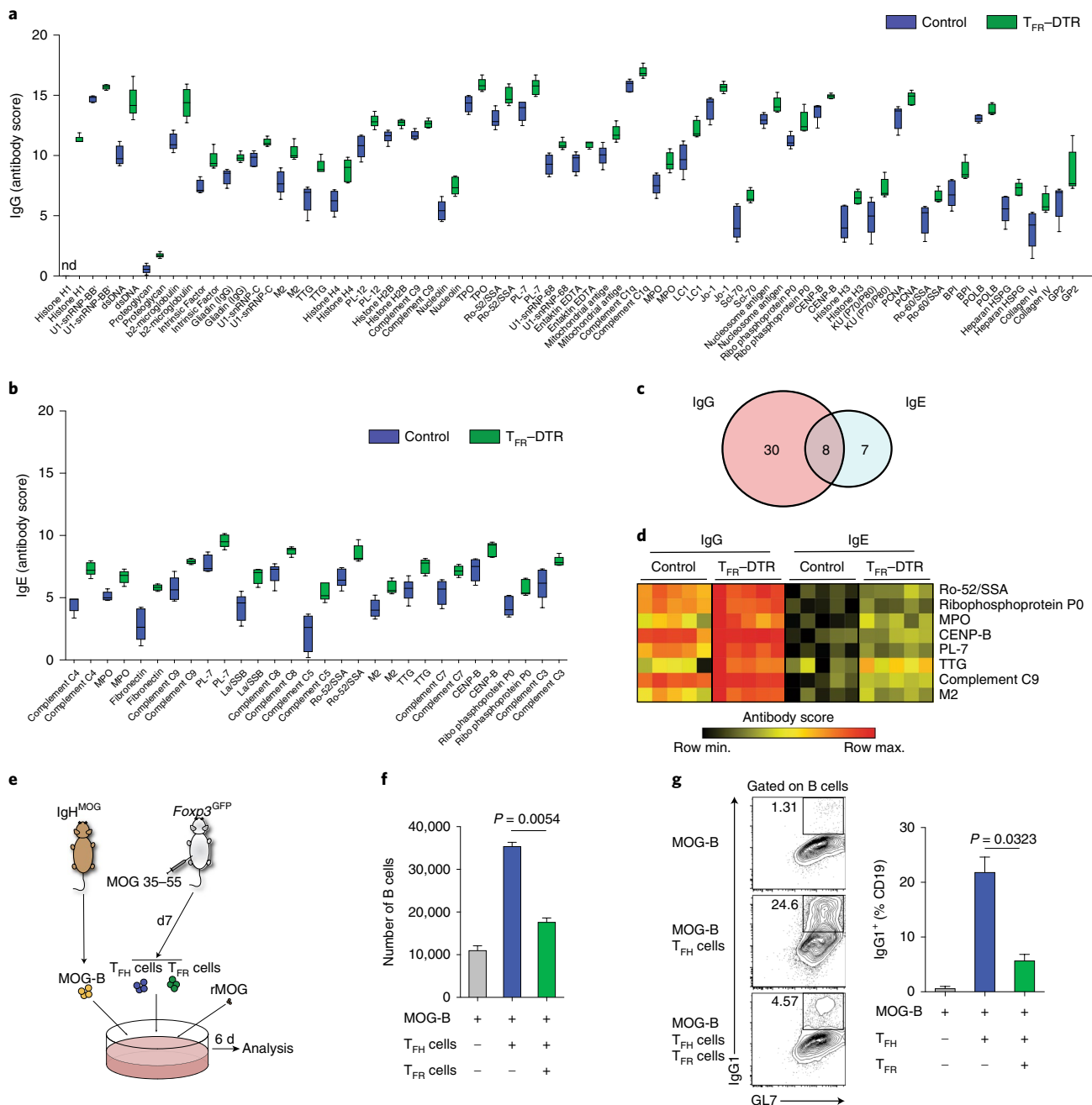


**Fig. 3 |  $T_{FR}$  cells control autoreactive IgG and IgE during foreign antibody responses.** **a**, Quantification of IgG scores for indicated autoantigens from  $T_{FR}$ -DTR ( $Foxp3^{Cre} Cxcr5^{LoxStopLox/DTR/WT}$ ) or control ( $Foxp3^{Cre} Cxcr5^{WT/WT}$ ) mice that were immunized with NP-OVA and given DT to delete  $T_{FR}$  cells before GC initiation, as in Fig. 2a. Serum was collected at day 21 after immunization. Autoantigens that showed a significant difference between control and  $T_{FR}$ -DTR mice (38 out of 123,  $P < 0.01$ , Mann-Whitney  $U$ -test) are shown. **b**, Quantification of IgE scores for indicated autoantigens as in **a**. Autoantigens that showed a significant difference between control and  $T_{FR}$ -DTR mice (15 out of 123,  $P < 0.01$ , Mann-Whitney  $U$ -test) are shown. **c**, Venn diagram showing overlap of differentially expressed autoantibodies for IgG and IgE isotypes in  $T_{FR}$ -DTR mice. **d**, Heatmap showing IgG and IgE autoantibody scores for the eight overlapping autoantigens in  $T_{FR}$ -DTR compared with control mice. **e**, Schematic of in vitro, autoreactive, B cell suppression assay.  $T_{FH}$  and  $T_{FR}$  cells from MOG<sub>35-55</sub>-immunized mice were cultured with IgH<sup>MOG</sup> B cells in the presence of rMOG for 6 d. **f**, Relative count of B cells from suppression assays as in **e**. **g**, Quantification of class switching to IgG1 in suppression assays as in **e**. Representative plots (left) and quantification (right) are shown. Graphs are box-and-whisker plots with horizontal lines indicating the mean and bars indicating the range of values (**a**, **b**). Column graphs represent the mean with error bars indicating s.e.m. (**f**, **g**). Data are from an individual experiment with five mice per group (**a**, **b**), or are replicate suppression assays from an individual experiment and represent three independent experiments (**e**–**g**). The  $P$  value indicates a two-tailed Student's  $t$ -test.

therapeutic benefit in multiple sclerosis<sup>32</sup>. For this assay,  $T_{FH}$  and  $T_{FR}$  cells were sorted from MOG<sub>35-55</sub>-immunized  $Foxp3^{GFP}$  mice and cultured with B cells isolated from naive IgH<sup>MOG</sup> mice in the presence

of recombinant MOG (rMOG) (Fig. 3e).  $T_{FH}$  cells could stimulate IgH<sup>MOG</sup> B cells to expand and undergo class-switch recombination (Fig. 3f,g). Importantly,  $T_{FR}$  cells could potentially suppress IgH<sup>MOG</sup> B

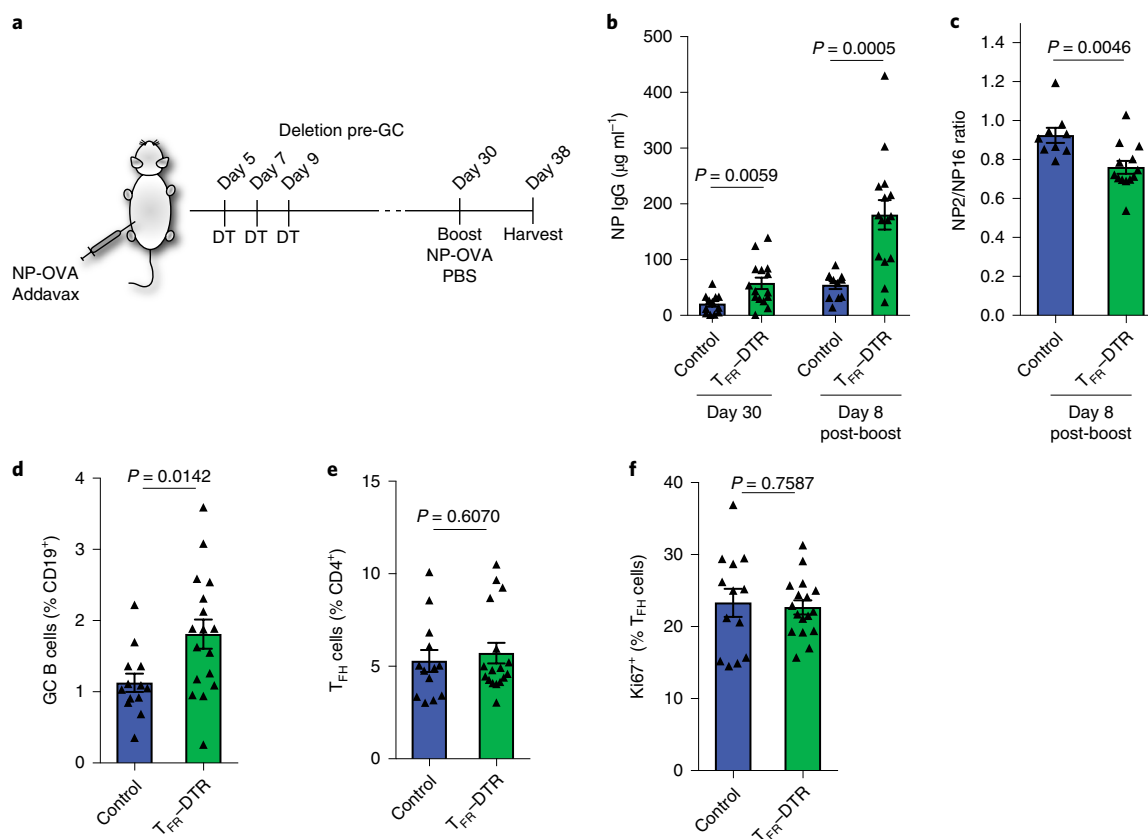


**Fig. 4 |  $T_{FR}$  cells regulate antibody memory responses.** **a**, Schematic of  $T_{FR}$  cell deletion to assess memory responses.  $T_{FR}$ -DTR or control mice were immunized with NP-OVA in Addavax and DT was given on days 5, 7 and 9 to delete  $T_{FR}$  cells before GC formation. Mice received a boost of NP-OVA without adjuvant at day 30. Mice were harvested on day 38. **b**, Analysis of NP-specific IgG levels before and after NP-OVA boost as in **a**. **c**, Quantification of the NP2/NP16 ratio in experiments as in **a**. **d**, Quantification of GC B cells (gated as  $CD19^{+}GL7^{+}FAS^{+}$ ) from dLNs of mice at day 38 as in **a**. **e**, Quantification of  $T_{FH}$  (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{-}CD19^{-}$ ) cells at day 38 as in **a**. **f**, Quantification of Ki67 expression in  $T_{FH}$  cells gated as in **e**. Column graphs represent the mean with error bars indicating s.e.m. The *P* value indicates a two-tailed Student's *t*-test. Data represent combined data from three independent experiments.

cell expansion and class-switch recombination. These data demonstrate that  $T_{FR}$  cells can regulate initial autoreactive B cell activation and class-switch recombination.

**$T_{FR}$  cells regulate antigen-specific antibody during memory responses.** Next, we determined whether  $T_{FR}$  cells regulate memory B cell responses. To do this,  $T_{FR}$ -DTR or control (*Foxp3<sup>Cre</sup>Cxcr5<sup>wt/wt</sup>*) mice were immunized with NP-OVA containing a mild adjuvant, MF59-like Addavax, and given DT from day 5 to day 9. At day 30, mice were boosted intraperitoneally with NP-OVA that did not contain adjuvant (Fig. 4a). It was found that NP-specific antibody responses were ~2.8 times higher before, and ~3.3-fold higher after, the boost in  $T_{FR}$ -DTR compared with control mice (Fig. 4b). These data suggest that antigen-specific memory responses are substantially increased when  $T_{FR}$  cells are deleted before GC initiation, suggesting, in turn, that  $T_{FR}$  cells control B cell memory to limit antibody responses. Next, it was determined whether the increased antigen-specific antibody had altered affinity because it was hypothesized that  $T_{FR}$  cells can set thresholds on B cell responses. ELISAs were performed with low and high ratios of NP and the NP2/NP16 ratio was calculated to approximate the affinity of NP-specific antibody. After a boost, antibody had a lower NP2/NP16 ratio in  $T_{FR}$ -DTR compared with control mice, suggesting lower affinity (Fig. 4c and see Supplementary Fig. 3a). Notable changes in the NP2/NP16 ratio were not found in the non-boosted experiments (see Supplementary Fig. 3b,c).

Memory B cell responses probably require secondary GCs to produce high-affinity antibody<sup>33</sup>. Therefore, the study next analyzed GC responses in  $T_{FR}$ -DTR mice in which  $T_{FR}$  cells were deleted and boosted with NP-OVA. Increases in GC B cells were found in  $T_{FR}$ -DTR compared with control mice, suggesting augmented secondary GCs after rechallenge (Fig. 4d). However, it is important to note that this assay cannot distinguish GC B cells that form from memory B cells or naive B cells. In contrast to GC B cells, there were no significant increases in  $T_{FH}$  cell frequency or expression of Ki67 in  $T_{FR}$ -DTR mice after rechallenge (Fig. 4e,f). Taken together, these data indicate that  $T_{FR}$  cells restrain the quantity, but promote the affinity, of antigen-specific antibody during memory responses by regulating early GCs.

#### HDM antigen generates distinct populations of $T_{FH}$ and $T_{FR}$ cells.

The finding that  $T_{FR}$  cell deletion results in increased IgE levels in mice immunized with NP-OVA suggested that  $T_{FR}$  cells may be able to regulate  $T_H2$ -like responses. Therefore, it was next determined whether HDM exposure, a  $T_H2$ -like response, generated  $T_{FH}$  and  $T_{FR}$  cells. C57bl/6 mice were challenged with HDM intranasally every 2 d and mediastinal lymph nodes were assessed on day 7. Unimmunized mice had a very small  $ICOS^{+}CXCR5^{+}CD4^{+}$  population made up of ~60%  $T_{FR}$  cells, a ratio that is typical in basal states<sup>13</sup> (Fig. 5a). In comparison, HDM-exposed mice developed a substantial population of  $ICOS^{+}CXCR5^{+}CD4^{+}$  cells in which  $T_{FR}$  cells were only ~30%. Moreover, a subpopulation of  $T_{FH}$  and  $T_{FR}$  cells was

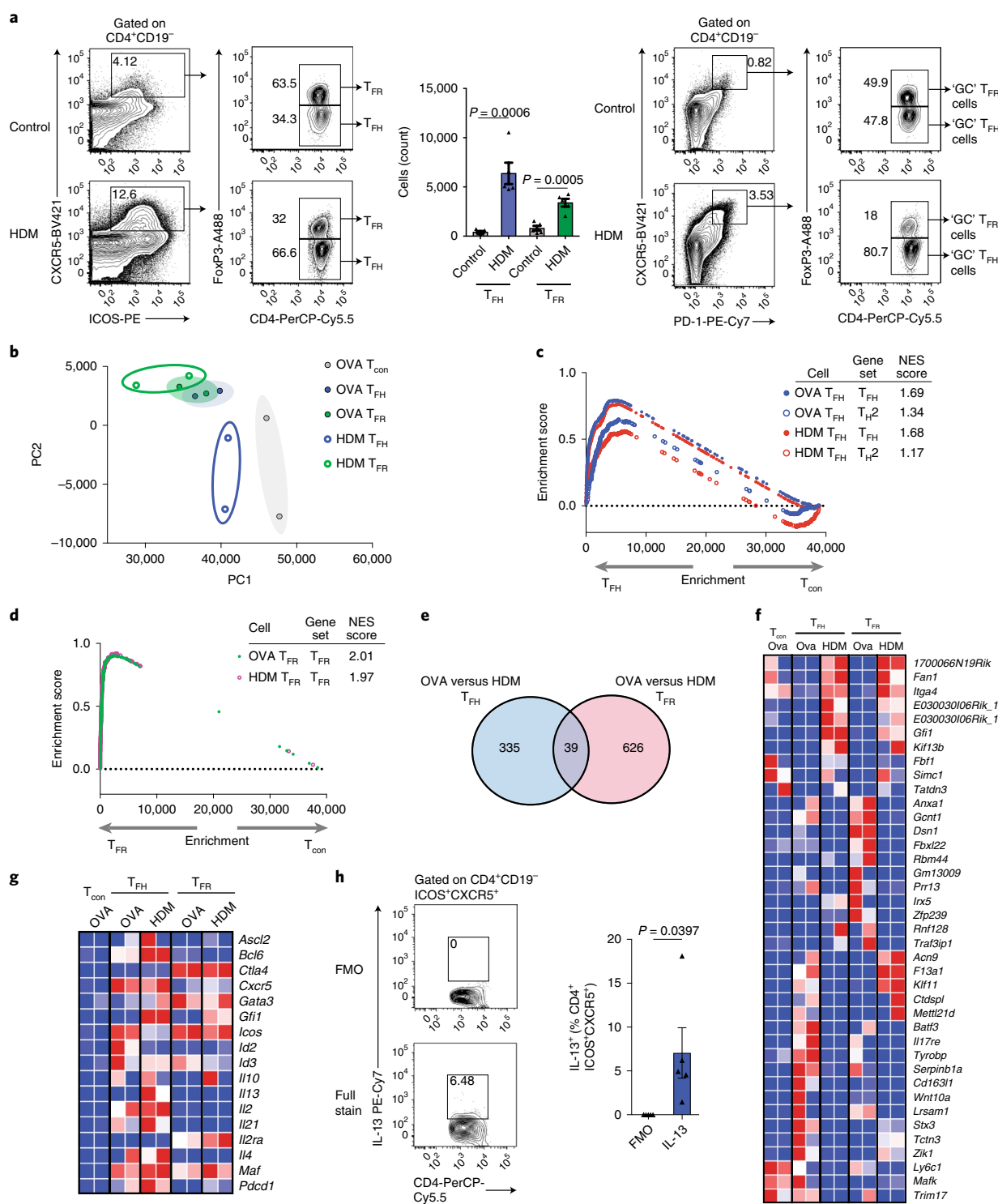


**Fig. 5 | HDM antigen generates distinct populations of T<sub>FR</sub> and T<sub>FR</sub> cells.** **a**, Quantification of T<sub>FR</sub> and T<sub>FR</sub> cells in response to HDM challenge. WT mice were either challenged or not challenged (control) with HDM intranasally on days 0, 2, 4 and 6. The dLNs were harvested on day 7. The gating strategy to identify T<sub>FR</sub> and T<sub>FR</sub> cells (left), total numbers of T<sub>FR</sub> and T<sub>FR</sub> cells (middle), and gating strategy for 'GC' T<sub>FR</sub> and T<sub>FR</sub> cells (right) is shown. **b**, PCA showing the relationship between transcriptional profiles of T<sub>FR</sub> (CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>-</sup>) and T<sub>FR</sub> (CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>-</sup>) cells generated in response to NP-OVA (subcutaneous) or HDM (intranasal) challenge in Foxp3<sup>GFP</sup> mice. PC, principal component. **c**, GSEA comparing T<sub>FR</sub> cells generated in response to NP-OVA or HDM for T<sub>FR</sub> or T<sub>H2</sub> signatures (GSE14308). NES, normalized enrichment score. **d**, GSEA comparing T<sub>FR</sub> cells generated in response to NP-OVA or HDM for T<sub>FR</sub> signatures. **e**, Venn diagram demonstrating the overlap of differentially expressed genes ( $P < 0.05$ ) between NP-OVA and HDM components for T<sub>FR</sub> and T<sub>FR</sub> cells. **f**, Heatmap showing the 39 common differentially expressed genes in T<sub>FR</sub> and T<sub>FR</sub> cells in NP-OVA versus HDM challenge as in **e**. **g**, Heatmap of common follicular T cell and T<sub>H2</sub> genes in T<sub>FR</sub> and T<sub>FR</sub> cells generated in response to NP-OVA or HDM challenge. **h**, IL-13 production by HDM T<sub>FR</sub> cells. Intracellular staining was performed on HDM-treated mice as in **a**. FMO, stain without anti-IL-13 antibody. Column graphs represent the mean with error bars indicating s.e.m. The  $P$  value indicates a two-tailed Student's  $t$ -test. Data either represent three independent experiments (**a,h**) or are combined data from two independent experiments (**b-g**).

found, which assumed a GC-like phenotype, suggesting proper  $T_{FH}$  and  $T_{FR}$  cell effector differentiation.

To determine whether after HDM exposure  $T_{FH}$  and  $T_{FR}$  cells transcriptionally resemble  $T_{FH}$  and  $T_{FR}$  cells, transcriptional analysis was performed. *Foxp3*<sup>RES-GFP</sup> mice were immunized with NP-OVA (emulsified in Freund's complete adjuvant) subcutaneously on day 0 or HDM intranasally on days 0, 2, 4 and 6, and  $T_{FH}$  (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{-}CD19^{-}$ ),  $T_{FR}$  (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{+}CD19^{-}$ ) or T conventional ( $T_{con}$ , gated as  $CD4^{+}ICOS^{-}CXCR5^{-}FoxP3^{-}CD19^{-}$ ) cells were sorted on day 7 and RNA sequencing (RNA-seq) transcriptional analysis was performed. Using principal component analysis (PCA), most follicular T cell populations clustered separately from  $T_{con}$  cells; however, HDM  $T_{FH}$  cells clustered closer to  $T_{con}$  cells than other cells (Fig. 5b). Next, it was determined whether  $T_{FH}$  cells from HDM-challenged mice transcriptionally resemble  $T_{FH}$  cells or whether they take on a  $T_{H2}$ -like phenotype. OVA- and HDM-specific  $T_{FH}$  cells had a similar enrichment for  $T_{FH}$  genes and HDM  $T_{FH}$  cells did not have enrichment for  $T_{H2}$  genes (Fig. 5c). Similarly,  $T_{FR}$  cells from both OVA and HDM challenges had strong enrichment for  $T_{FR}$  genes. (Fig. 5d).

Although  $T_{FH}$  and  $T_{FR}$  cells from HDM-challenged mice had intact transcriptional programs, differentially expressed genes were found between these cells and their OVA challenge counterparts. There were 374 genes differentially expressed ( $P < 0.05$ ) between OVA and HDM  $T_{FH}$  cells, and 665 genes differentially expressed ( $P < 0.05$ ) between OVA and HDM  $T_{FR}$  cells, with 39 genes being differentially expressed in both  $T_{FH}$  and  $T_{FR}$  cells (Fig. 5e). When these 39 genes were assessed in more detail, a subset of genes was found expressed in HDM  $T_{FH}$  and  $T_{FR}$  cells, but not OVA populations, such as *Gfi1* ( $T_{FH}$  cells:  $P = 0.0130$ ;  $T_{FR}$  cells:  $P = 0.0424$ ), which has a role in stabilizing  $T_{H2}$  cells<sup>34</sup> (Fig. 5f). Genes commonly expressed in  $T_{FH}$  and  $T_{FR}$  cells were also evaluated. Some genes, such as *Icos* and *Id2*, seemed to be expressed less in HDM  $T_{FH}$  cells compared with OVA  $T_{FH}$  cells, although only *Id2* was statistically significant ( $P = 0.0400$ ) (Fig. 5g). A low, but positive, transcript for *Il13* was found in HDM  $T_{FH}$  cells that was not present in OVA  $T_{FH}$  cells. In addition, HDM  $T_{FH}$  cells expressed more *Gata3*. To assess whether a subset of  $T_{FH}$  cells could produce IL-13, intracellular cytokine staining was performed and a small proportion of  $T_{FH}$  cells was found that produced IL-13 (Fig. 5h). Taken together, these data demonstrate that  $T_{FH}$  and  $T_{FR}$  cells from HDM challenge have  $T_{FH}$  and  $T_{FR}$  transcriptional programs, but also some distinct transcriptional characteristics, such as an IL-13 transcript in HDM  $T_{FH}$  cells. As HDM  $T_{FH}$  cells have an intact  $T_{FH}$ , but not a  $T_{H2}$ , program yet express IL-13, these cells are referred to as ' $T_{FH13}$ -like' cells.

**$T_{FR}$  cells regulate  $T_{FH13}$  cell-mediated IgE responses to HDM in vitro.** As it was found that  $T_{FH13}$ -like cells from HDM-challenged mice expressed IL-13, and  $T_{FR}$  cell-deleted mice had elevated levels of autoreactive IgE, it was hypothesized that  $T_{FR}$  cells may regulate IL-13 and IgE responses in the context of  $T_{H2}$ -like allergic responses. To test this hypothesis, an in vitro suppression assay was developed in which  $T_{FH13}$ -like cells mediate class switching of B cells to IgE in response to HDM antigen. HDM was given intranasally to *Foxp3*<sup>RES-GFP</sup> mice every 2d and, on day 7, total B,  $T_{FH}$  (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{-}CD19^{-}$ ) and  $T_{FR}$  (gated as  $CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{+}CD19^{-}$ ) cells were sorted from mediastinal lymph nodes. Cells were cultured together along with HDM for 6d (Fig. 6a).  $T_{FR}$  cells inhibited  $T_{FH}$  cell proliferation and were still present at the end of the culture (Fig. 6b). It was found that cultures containing  $T_{FH13}$  and B cells contained large amounts of  $T_{H2}$ -like cytokines, including IL-5, IL-13 and IL-4, all of which were suppressed by the addition of  $T_{FR}$  cells (Fig. 6c,d). In addition,  $T_{FR}$  cells suppressed the frequency of  $T_{FH}$  cells (gated as  $CD4^{+}FoxP3^{-}$  cells) expressing IL-13 protein (Fig. 6e).  $T_{FH13}$  cells stimulated B cells to

undergo class switching to IgG1 and, to a lesser extent, IgE (Fig. 6f). Importantly, addition of  $T_{FR}$  cells resulted in near-complete reduction in IgE<sup>+</sup> B cells and a substantial reduction in IgG1<sup>+</sup> B cells (Fig. 6f,g). No evidence was found of class switching to IgE when HDM was omitted from the wells, or when similar cultures were performed using cells from NP-OVA immunization (see Supplementary Fig. 4; data not shown). Although HDM  $T_{FR}$  cells suppressed class switching of NP-OVA B cells to IgG1, NP-OVA  $T_{FR}$  cells may suppress  $T_{FH13}$  cell-mediated IgE class switching of B cells less potentially than HDM  $T_{FR}$  cells (see Supplementary Fig. 4).

To determine whether  $T_{FR}$  cells prevent class switching to IgE, suppress already class-switched IgE<sup>+</sup> B cells, or both, levels of GL7, a GC B cell-expressed molecule that is attenuated on B cells during  $T_{FR}$  suppression, were assessed. It was found that IgE<sup>+</sup> B cells had lower expression of GL7 in the presence of  $T_{FR}$  cells, suggesting that IgE<sup>+</sup> class-switched B cells are less activated (Fig. 6h). Protein levels of IgE and IgG1 were also analyzed within switched B cells. Both IgE- and IgG1-expressing B cells had lower expression of IgE and IgG1, respectively, if  $T_{FR}$  cells were present, although this did not reach statistical significance for IgE (Fig. 6i). To determine whether  $T_{FH13}$  cell cytokines were essential for full IgE responses, IL-13- or IL-4-blocking antibodies were added to cultures and the levels of IgE assessed.  $T_{FH}$  cells stimulated large amounts of IgE secretion, which was strongly attenuated when IL-13- or IL-4-blocking antibodies were added, demonstrating that cytokines produced by  $T_{FH13}$  cells stimulate IgE (Fig. 6j). Importantly, IgE and IgG production was substantially suppressed by the presence of  $T_{FR}$  cells (Fig. 6j). Taken together, these data demonstrate that  $T_{FR}$  cells can suppress  $T_{FH}$  cell-mediated IL-13 and IgE responses in vitro.

**$T_{FR}$  cells regulate antigen-specific IgE responses in vivo.** Next the role of  $T_{FR}$  cells was assessed in allergic immunity in vivo. For this, a HDM sensitization and challenge model was used that results in antigen-specific IgE responses and IL-13-dependent eosinophilic lung inflammation<sup>10,35</sup>.  $T_{FR}^{-DTR}$  or control (*Foxp3*<sup>Cre</sup>*Cxcr5*<sup>wt/wt</sup>) mice were sensitized with HDM and given DT to delete  $T_{FR}$  cells. On day 7 mice with HDM were challenged (Fig. 7a). Robust deletion of  $T_{FR}$  cells was found in  $T_{FR}^{-DTR}$  mice as a percentage of both total  $CXCR5^{+}CD4^{+}$  cells and total  $CD4^{+}$  cells (Fig. 7b). No evidence was found of deletion of activated  $T_{reg}$  cells in  $T_{FR}^{-DTR}$  mice (see Supplementary Fig. 5a). Deletion of  $T_{FR}$  cells did not result in altered frequencies of  $T_{FH}$  cells, GC B cells, IgG1<sup>+</sup> B cells, IgE<sup>+</sup> B cells or plasma cells (Fig. 7c). In addition, deletion of  $T_{FR}$  cells did not alter the relative class switching to IgG or IgE in GC B cells (Fig. 7d). However, when plasma cells were assessed, it was found that deletion of  $T_{FR}$  cells resulted in small increases in IgE plasma cells (Fig. 7e). Moreover,  $T_{FR}^{-DTR}$  mice had substantially higher levels of total and HDM-specific IgE compared with control mice (Fig. 7f). These data demonstrate that  $T_{FR}$  cells can control HDM-specific IgE responses in vivo.

To determine whether deletion of  $T_{FR}$  cells results in altered lung inflammation, bronchoalveolar lavage fluid was analyzed and increases in eosinophils found (Fig. 7g). Histological analysis of lungs showed increased inflammation consisting of cell infiltration to the airway/vessel walls and alveolar parenchyma in  $T_{FR}$  cell-deleted mice compared with control mice (Fig. 7h). It was found that the immune cell infiltrate in the lungs of  $T_{FR}$  cell-deleted mice was positive for Gr1 or SiglecF, suggesting the presence of granulocytes and eosinophils, respectively (Fig. 7i and see Supplementary Fig. 5b). Taken together, these data demonstrate that  $T_{FR}$  cells regulate HDM-specific IgE responses in vivo and control immune cell infiltrate during HDM sensitization and challenge.

## Discussion

The precise role of  $T_{FR}$  cells in modulating B cell responses has been elusive due to the lack of specific mouse models to study  $T_{FR}$  cells.

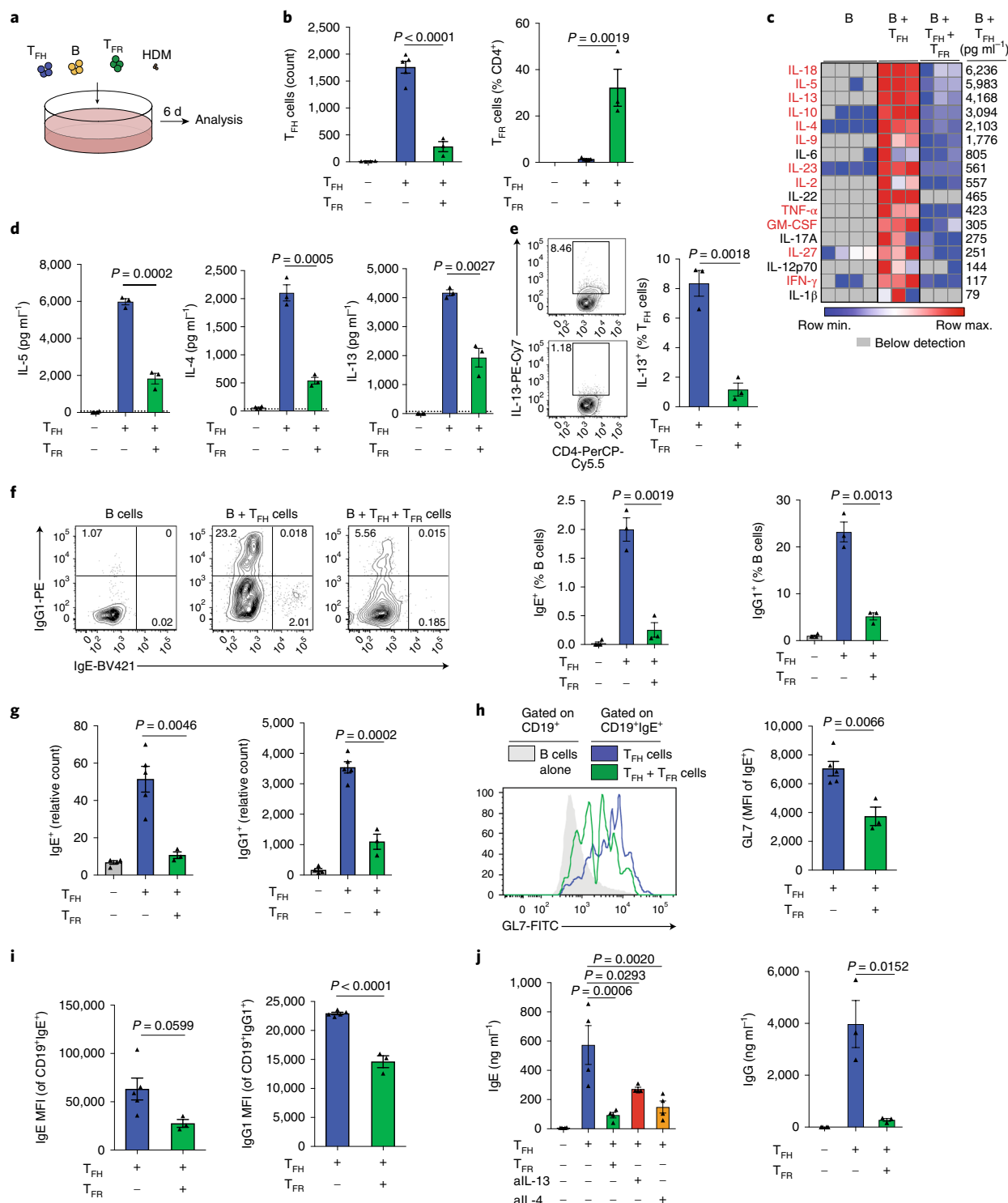


**Fig. 6 |  $T_{FR}$  cells regulate  $T_{FH}$ 13 cell-mediated IgE responses in vitro.** **a**, Schematic of experimental design for an in vitro HDM suppression assay. Total B,  $T_{FH}$  and  $T_{FR}$  cells were purified from the dLNs of mice that received HDM on day 0, 2, 4 and 6, and were added to culture wells along with HDM for 6 d. **b**, Quantification of total  $T_{FH}$  cells (left) and the percentage of  $T_{FR}$  cells (FoxP3<sup>+</sup> of CD4<sup>+</sup>IA<sup>+</sup>CD19<sup>-</sup> cells) (right) from cultures as in **a**. **c**, Quantification of cytokines in culture supernatants from cultures as in **a**. Cytokines listed in red have levels notably lower in cultures containing  $T_{FR}$  cells compared with cultures without  $T_{FR}$  cells. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; TNF, tumor necrosis factor. **d**, Column graphs of IL-5, IL-4 and IL-13 from the data in **c**. **e**, Intracellular cytokine staining of IL-13 in  $T_{FH}$  cells from cultures as in **a**. **f**, Analysis of class switching to IgG1 and IgE in cultures as in **a**. Representative gating (left, pregated on CD19<sup>+</sup>IA<sup>+</sup>CD4<sup>-</sup> cells), IgE<sup>+</sup> B cell quantification (middle) and IgG1<sup>+</sup> B cell quantification (right) are shown. **g**, Counts of total IgE<sup>+</sup> (left) and IgG1<sup>+</sup> (right) B cells expressed as relative counts from experiments as in **a**. **h**, Expression of GL7 on IgE<sup>+</sup> B cells from indicated cultures as in **a**. **i**, Expression of IgE on IgE<sup>+</sup> B cells (left) and IgG1 on IgG1<sup>+</sup> B cells are shown from cultures as in **a**. MFI, mean fluorescence intensity. **j**, Levels of IgE (left) and IgG (right) in culture supernatants from cultures as in **a**. Anti-IL-13 (aIL-13) or anti-IL-4 (aIL-4) was added to the indicated wells. Column graphs represent the mean with error bars indicating s.e.m. The *P* value indicates a two-tailed Student's *t*-test (**b–i**; right) or one-way analysis of variance with Tukey's correction (**j**; left). Data are from individual experiments and represent three independent experiments.

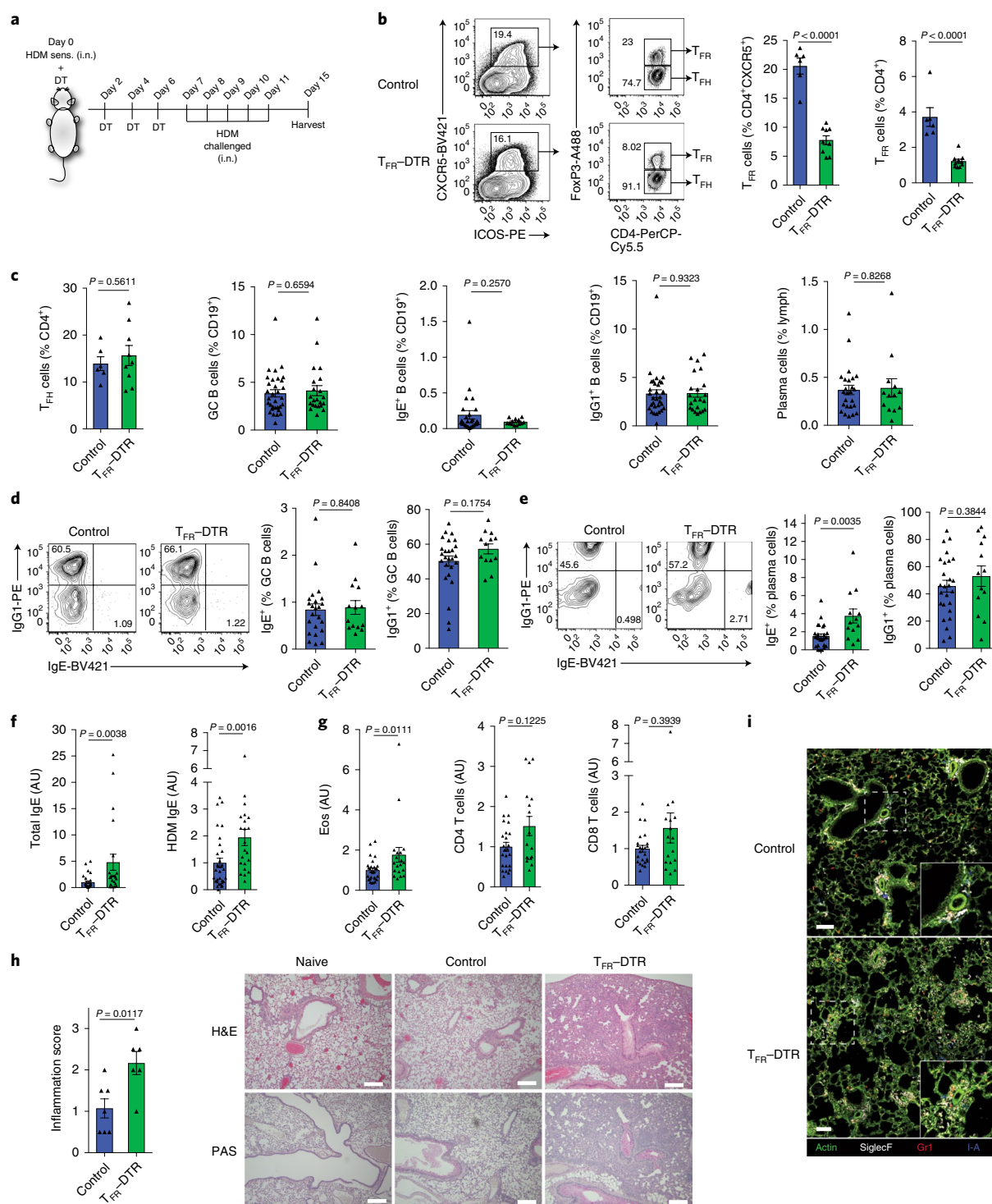


**Fig. 7 |  $T_{FR}$  cells regulate HDM-specific IgE responses in vivo.** **a**, Schematic of HDM sensitization-and-challenge model to induce lung inflammation.  $T_{FR}$ -DTR (*Foxp3<sup>Cre</sup>Cxcr5<sup>LoxStopLox</sup>DTR/WT*) or control (*Foxp3<sup>Cre</sup>Cxcr5<sup>WT/WT</sup>*) mice received HDM sensitization (sens.) intranasally (i.n.) at day 0, followed by DT administration at days 0, 2, 4 and 6. Mice were challenged with HDM on days 7–11 and harvested on day 15. **b**, Analysis of  $T_{FR}$  cells from dLNs of HDM-challenged mice as in **a**. Representative gating (left) and quantification (right) are shown. **c**, Quantification of  $T_{FH}$ , GC B cells (CD19<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>), total IgE<sup>+</sup> B cells (CD19<sup>+</sup>IgE<sup>+</sup>), total IgG1<sup>+</sup> B cells (CD19<sup>+</sup>IgG1<sup>+</sup>) and total plasma cells (CD138<sup>+</sup>) from dLNs of HDM-challenged mice as in **a**. **d**, Quantification of IgG1 and IgE expression in GC B cells (CD19<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>). **e**, Quantification of IgG1 and IgE expression in plasma cells (CD138<sup>+</sup>). **f**, Quantification of total IgE or HDM-specific IgE from HDM-challenged mice as in **a** ( $n = 30$ , control;  $n = 22$ ,  $T_{FR}$ -DTR). AU, arbitrary units. **g**, Quantification of eosinophils (Eos, left), CD4 T cells (middle) or CD8 T cells (right) from the bronchoalveolar lavage fluid of mice as in **a** ( $n = 24$ , control;  $n = 17$ ,  $T_{FR}$ -DTR). **h**, Lung inflammation scores (left) and representative images of hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) staining of lung samples. Scale bars, 500  $\mu$ m. **i**, Immunofluorescence micrographs of lungs stained for actin, SiglecF, Gr1 and I-A. Scale bars, 100  $\mu$ m. Column graphs represent the mean with error bars indicating s.e.m. The  $P$  value indicates a two-tailed Student's  $t$ -test (**b–e**) or a Mann-Whitney  $U$ -test (**f–h**). Data are from individual experiments and represent three independent experiments (**b,c** left), are combined data from four independent experiments (**c** right, **d–g**) or are from one experiment (**h,i**).

In the present study, a new  $T_{FR}$ -DTR mouse strain was developed to study  $T_{FR}$  cells at distinct stages of immune responses. It was found that  $T_{FR}$  cells potentially regulate foreign and self-reactive IgG responses, especially before initial GC development. Surprisingly, a population of IL-13-expressing,  $T_{FH13}$ -like cells was found during HDM challenge, and  $T_{FR}$  cells were found to potentially regulate these cells to control IL-13- and HDM-specific IgE responses. Taken together, these data indicate that  $T_{FR}$  cells have a dynamic role in controlling many types of B cell responses to foreign and self-reactive antigens, particularly before initial GC formation.

Although  $T_{FR}$  cells can be found inside and outside of GCs, there has been a debate in the field as to where and when  $T_{FR}$  cells regulate B cells.  $T_{FR}$  cells are less frequently found in GCs compared with the B cell follicle, and cytokines produced in high levels in GCs, such as IL-21, can inhibit  $T_{FR}$  cells<sup>15,26</sup>. Based on these observations, it has been suggested that  $T_{FR}$  cells do not substantially regulate B cell responses within GCs<sup>15</sup>. However,  $T_{FR}$  cells can regulate GC B cells in vitro<sup>17</sup>. In the present study, it has been demonstrated that  $T_{FR}$  cells potentially regulate antibody responses before, but not after, GC formation. Although the experiments in this study cannot eliminate the possibility of  $T_{FR}$  cells having roles within very early GCs, the lack of a phenotype in mature GCs suggests that  $T_{FR}$  cells regulate GC development. However, it is important to note that  $T_{FR}$  cells may have more subtle roles in GCs such as facilitating GC resolution and promoting immune homeostasis.

It was also found that  $T_{FR}$  cells regulate memory B cell responses. It was proposed that they do this by preventing GC formation where some memory B cells originate. However, it is possible that non-GC memory B cells may also be regulated by  $T_{FR}$  cells in the B cell follicle. As reactivation of memory B cells may require secondary GCs<sup>33</sup>,  $T_{FR}$  cells probably have two roles in modulating B cell memory: regulation of memory B cell formation and regulation of secondary GCs during memory B cell reactivation. In addition it was found that  $T_{FR}$  cells promote antibody affinity during memory, suggesting that  $T_{FR}$  cells can modulate not only the quantity of antigen-specific antibody, but also the quality of the antibody. Previous studies found that deletion of Bcl6 in all  $T_{reg}$  cell subsets from birth resulted in increased autoreactive antibodies, but these antibodies develop only after months<sup>22,36</sup>. It was found that deletion of  $T_{FR}$  cells resulted in increases in a variety of autoreactive IgG antibodies and, surprisingly, autoreactive IgE antibodies. Autoreactive IgE has been found in autoimmune diseases such as SLE and has been suggested to enhance autoimmune pathology<sup>27,28</sup>; it has also been found in models of epithelial damage<sup>37</sup>.

The role of  $T_{FH}$  and  $T_{FR}$  cells in  $T_H2$ -like immunity has been unclear. Although  $T_{FH}$  cells are not thought to make IL-13,  $T_{FH}$  cells have been implicated in controlling IgE responses to HDM<sup>9,10</sup>. Likewise, attenuated percentages of  $T_{FR}$  cells correlate with worse allergy in patients<sup>38</sup>. Evidence was found of a small frequency of IL-13-producing  $T_{FH}$  cells in vivo after HDM administration. Using an in vitro HDM IgE assay, it was shown that these  $T_{FH}$  cells could produce large amounts of IL-13, IL-5 and IL-4, and potentially stimulate B cell IgE responses. These  $T_{FH}$  cells have been referred as  $T_{FH13}$ -like cells to distinguish them from previously described  $T_{FH2}$  cells. Both IL-13 and IgE responses were potentially suppressed by  $T_{FR}$  cells. Interestingly, deletion of  $T_{FR}$  cells in vivo during HDM sensitization/challenge resulted in higher levels of antigen-specific IgE and increased lung inflammation. High-affinity IgE responses occur preferentially through sequential switching of IgG1 to IgE<sup>39,40</sup>. As there was such a profound increase in IgE serum levels and alterations in IgE<sup>+</sup>, but not IgG1<sup>+</sup>, plasma cells, these data suggest that  $T_{FR}$  cells probably limit IgE plasma cell responses rather than sequential switching. However, more in-depth studies are necessary to fully determine the role of  $T_{FR}$  cells in sequential switching. Taken together, these results demonstrate that  $T_{FH}$  cells can have roles in  $T_H2$ -like responses to HDM and that these responses are controlled

by  $T_{FR}$  cells. Therefore,  $T_{FR}$  cells are likely to have roles in regulating allergic inflammation and immunity to helminth infections, and modulation of  $T_{FR}$  cells may help to control these responses.

## Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at <https://doi.org/10.1038/s41590-019-0472-4>.

Received: 27 December 2018; Accepted: 21 July 2019;

Published online: 2 September 2019

## References

- Crotty, S. Follicular helper CD4 T cells (TFH). *Annu. Rev. Immunol.* **29**, 621–663 (2011).
- Victora, G. D. & Nussenzweig, M. C. Germinal centers. *Annu. Rev. Immunol.* **30**, 429–457 (2012).
- Crotty, S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* **41**, 529–542 (2014).
- Vinuesa, C. G., Linterman, M. A., Yu, D. & MacLennan, I. C. Follicular helper T cells. *Annu. Rev. Immunol.* **34**, 335–368 (2016).
- Cannons, J. L., Lu, K. T. & Schwartzberg, P. L. T follicular helper cell diversity and plasticity. *Trends Immunol.* **34**, 200–207 (2013).
- Weinstein, J. S. et al. TFH cells progressively differentiate to regulate the germinal center response. *Nat. Immunol.* **17**, 1197–1205 (2016).
- Luthje, K. et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat. Immunol.* **13**, 491–498 (2012).
- Liang, H. E. et al. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat. Immunol.* **13**, 58–66 (2011).
- Ballesteros-Tato, A. et al. T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. *Immunity* **44**, 259–273 (2016).
- Coquet, J. M. et al. Interleukin-21-producing CD4<sup>+</sup> T cells promote type 2 immunity to house dust mites. *Immunity* **43**, 318–330 (2015).
- Noval Rivas, M. & Chatila, T. A. Regulatory T cells in allergic diseases. *J. Allergy Clin. Immunol.* **138**, 639–652 (2016).
- Curto de Lafaille, M. A. et al. Adaptive Foxp3<sup>+</sup> regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* **29**, 114–126 (2008).
- Sage, P. T. & Sharpe, A. H. T follicular regulatory cells. *Immunol. Rev.* **271**, 246–259 (2016).
- Maceiras, A. R., Fonseca, V. R., Agua-Doce, A. & Graca, L. T follicular regulatory cells in mice and men. *Immunology* **152**, 25–35 (2017).
- Sage, P. T. et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat. Immunol.* **17**, 1436–1446 (2016).
- Sage, P. T., Paterson, A. M., Lovitch, S. B. & Sharpe, A. H. The coinhibitory receptor ctla-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* **41**, 1026–1039 (2014).
- Sage, P. T., Alvarez, D., Godec, J., von Andrian, U. H. & Sharpe, A. H. Circulating T follicular regulatory and helper cells have memory-like properties. *J. Clin. Invest.* **124**, 5191–5204 (2014).
- Sage, P. T., Francisco, L. M., Carman, C. V. & Sharpe, A. H. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat. Immunol.* **14**, 152–161 (2013).
- Wollenberg, I. et al. Regulation of the germinal center reaction by Foxp3<sup>+</sup> follicular regulatory T cells. *J. Immunol.* **187**, 4553–4560 (2011).
- Wu, H. et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur. J. Immunol.* **46**, 1152–1161 (2016).
- Linterman, M. A. et al. Foxp3<sup>+</sup> follicular regulatory T cells control the germinal center response. *Nat. Med.* **17**, 975–982 (2011).
- Fu, W. et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J. Exp. Med.* **215**, 815–825 (2018).
- Laidlaw, B. J. et al. Interleukin-10 from CD4<sup>+</sup> follicular regulatory T cells promotes the germinal center response. *Sci. Immunol.* **2**, ea4767 (2017).
- Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* **8**, 191–197 (2007).
- Hou, S. et al. FoxP3 and Ezh2 regulate TFR cell suppressive function and transcriptional program. *J. Exp. Med.* **216**, 605–620 (2019).
- Sayin, I. et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J. Exp. Med.* **215**, 1531–1542 (2018).
- Dema, B. et al. Immunoglobulin E plays an immunoregulatory role in lupus. *J. Exp. Med.* **211**, 2159–2168 (2014).

28. Dema, B. et al. Autoreactive IgE is prevalent in systemic lupus erythematosus and is associated with increased disease activity and nephritis. *PLoS ONE* **9**, e90424 (2014).
29. Sage, P. T. et al. Dendritic cell PD-L1 limits autoimmunity and follicular T cell differentiation and function. *J. Immunol.* **200**, 2592–2602 (2018).
30. Bettelli, E., Baeten, D., Jager, A., Sobel, R. A. & Kuchroo, V. K. Myelin oligodendrocyte glycoprotein-specific T and B cells cooperate to induce a Devic-like disease in mice. *J. Clin. Invest.* **116**, 2393–2402 (2006).
31. Krishnamoorthy, G., Lassmann, H., Wekerle, H. & Holz, A. Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation. *J. Clin. Invest.* **116**, 2385–2392 (2006).
32. Mulero, P., Midaglia, L. & Montalban, X. Ocrelizumab: a new milestone in multiple sclerosis therapy. *Ther. Adv. Neurol. Disord.* **11**, 1756286418773025 (2018).
33. McHeyzer-Williams, L. J., Milpied, P. J., Okitsu, S. L. & McHeyzer-Williams, M. G. Class-switched memory B cells remodel BCRs within secondary germinal centers. *Nat. Immunol.* **16**, 296–305 (2015).
34. Zhu, J., Jankovic, D., Grinberg, A., Guo, L. & Paul, W. E. Gfi-1 plays an important role in IL-2-mediated Th2 cell expansion. *Proc. Natl Acad. Sci. USA* **103**, 18214–18219 (2006).
35. Tomlinson, K. L., Davies, G. C., Sutton, D. J. & Palframan, R. T. Neutralisation of interleukin-13 in mice prevents airway pathology caused by chronic exposure to house dust mite. *PLoS ONE* **5**, e13136 (2010).
36. Botta, D. et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat. Immunol.* **18**, 1249–1260 (2017).
37. Crawford, G. et al. Epithelial damage and tissue gammadelta T cells promote a unique tumor-protective IgE response. *Nat. Immunol.* **19**, 859–870 (2018).
38. Yao, Y. et al. Allergen immunotherapy improves defective follicular regulatory T cells in patients with allergic rhinitis. *J. Allergy Clin. Immunol.* **144**, 118–128 (2019).
39. He, J. S. et al. IgG1 memory B cells keep the memory of IgE responses. *Nat. Commun.* **8**, 641 (2017).
40. Xiong, H., Dolpady, J., Wabl, M., Curotto de Lafaille, M. A. & Lafaille, J. J. Sequential class switching is required for the generation of high affinity IgE antibodies. *J. Exp. Med.* **209**, 353–364 (2012).

## Acknowledgements

We would like to thank T. Chatila, R. Anthony, D. Wesemann and M. Carroll for helpful discussions, the MICRON imaging core for help with microscopy and H. Wekerle and S. Zamvil for reagents. This work was supported by the US National Institutes of Health through grant nos. K22AI132937 (P.T.S.), P01AI056299 (A.H.S.), R37AI34495 (B.R.B.) and R01HL11879 (B.R.B.), and the Evergrande Center for Immunologic Diseases.

## Author contributions

R.L.C., J.D., M.T.M. and P.T.S. performed the experiments. R.L.C., A.D., S.B.L. and P.T.S. analyzed the data. B.R.B., V.K.K. and A.H.S. provided key technical help and reagents. P.T.S. conceived of the project and wrote the manuscript. All the authors edited the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary information** is available for this paper at <https://doi.org/10.1038/s41590-019-0472-4>.

**Reprints and permissions information** is available at [www.nature.com/reprints](http://www.nature.com/reprints).

**Correspondence and requests for materials** should be addressed to P.T.S.

**Peer review information:** Z. Fehervari was the primary editor on this article, and managed its editorial process and peer review in collaboration with the rest of the editorial team.

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2019

## Methods

**Mice.** Studies of *Foxp3*<sup>IRE5-GFP</sup> mice on the C57Bl/6 background have been published previously<sup>41</sup>. *Foxp3*<sup>IRE5-CreYFP</sup> and *Foxp3*<sup>DTR</sup> mice on the C57Bl/6 background were from Jackson Laboratories. IgH<sup>MOG</sup> mice were a kind gift from Hartmut Wekerle<sup>42</sup>. *Cxcr5*<sup>IRE5-LoxP-STOP-LoxP-DTR</sup> knockin mice were generated by constructing a targeting vector in which an IRES-Frt-PGKNeo-Frt-LoxP-STOP-LoxP-hbfgf sequence was placed directly downstream of the stop sequence of *Cxcr5*. The targeting vector was introduced into C57Bl/6 embryonic stem cells by electroporation, and the resulting neomycin-resistant embryonic stem cells were screened for homologous recombination. Positive clones were microinjected into albino-B6 blastocysts and implanted into pseudopregnant female mice to generate chimeras. Germline transmission was achieved and mice were bred to FlpE-carrying mice to remove the NEO cassette<sup>39</sup>. The resulting mice were then bred to *Foxp3*<sup>IRE5-CreYFP</sup> mice to generate the T<sub>FR</sub>-DTR colony. Mouse progeny were routinely screened for leakiness of the *Foxp3*<sup>IRE5-CreYFP</sup> allele by flow cytometry. All mice were used according to Brigham and Women's Hospital and Harvard Medical School Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

**Immunization.** Mice were immunized with 100 µg NP-OVA (Biosearch Technologies) or 100 µg MOG<sub>35-55</sub> (UCLA Biopolymer Facility) emulsified in H37RA Freund's complete adjuvant subcutaneously in the mouse flanks as previously described<sup>15,43</sup>, unless otherwise noted. For memory studies, mice were immunized with 100 µg NP-OVA mixed with Addavax MF59-like adjuvant (Invivogen) subcutaneously in one flank. Then, 30 d later mice received a boost of 100 µg NP-OVA in PBS intraperitoneally. For allergy studies, mice were sensitized with 10 µg HDM (Greer Labs) in PBS intranasally and then challenged with 10 µg HDM in PBS intranasally on days 7, 8, 9, 10 and 11, followed by harvesting at day 15. In some cases mice received 1 µg DT in PBS intraperitoneally to delete T<sub>FR</sub> cells at indicated timepoints.

**Antibodies.** The following antibodies were used for surface staining at 4 °C for 30 min: anti-CD4 (Biolegend, 1:200, RM4-5), anti-ICOS (Biolegend, 1:200, 15F9), anti-CD19 (Biolegend, 1:200, 6D5), anti-PD-1 (1:200, RMP1-30), anti-CXCR5 biotin (BD Biosciences, 1:100, 2G8), anti-GL7 (BD Biosciences, 1:200, GL-7), anti-HB-EGF/DTR (R&D Systems, 1:200, AF-259-NA), anti-CD38 (Biolegend, 1:200, 90), anti-CD138 (Biolegend, 1:200, 281-2), anti-IA (Biolegend, 1:200, M5/114.15.2), anti-SiglecF (BD Biosciences, 1:200, E50-2440), anti-CD8a (Biolegend, 1:200, 53-6.7), anti-CD11c (Biolegend, 1:200, N418) and anti-CD11b (Biolegend, 1:200, M1/70). For CXCR5 detection, streptavidin-BV421 (Biolegend, 1:400, 405225) was used at 4 °C. In some cases, anti-IgE (BD Biosciences, 1:200, R35-72) was included to block IgE bound to cell surfaces. For intracellular staining, samples were fixed with the Foxp3 Fix/Perm buffer set according to the manufacturer's instructions (eBioscience). Samples were then intracellularly stained with anti-IgG1 (BD Biosciences, 1:200, A85-1), anti-IgE (BD Biosciences, 1:200, R35-72), anti-FoxP3 (eBiosciences, 1:200, FJK-16S), anti-Ki67 (BD Biosciences, 1:100, B56) or anti-Glut1 (Abcam, 1:200, EPR3915). In some cases, a donkey anti-goat A647 secondary antibody was used (Invitrogen, 1:400, A-21447). See the Reporting Summary for more details.

**Sorting.** The draining lymph nodes (dLNs) were passed through 70-µm filters and resuspended in PBS supplemented with 1% fetal bovine serum and 1 mM ethylenediaminetetraacetic acid. CD4<sup>+</sup> cells were enriched by magnetic positive selection (Miltenyi Biotec). CD4<sup>+</sup>-enriched cells were then stained and sorted on an Aria II cell sorter (70-µm nozzle) using optimal purity settings. T<sub>FR</sub> cells were gated as CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>-</sup> and T<sub>FR</sub> cells were gated as CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>-</sup>. B cells were isolated by flow-through from CD4<sup>+</sup> selection, which was then positively selected using CD19 beads (Miltenyi Biotec).

**Suppression assays.** In vitro stimulation/suppression assays were performed by culturing 5 × 10<sup>4</sup> B cells, 3 × 10<sup>4</sup> T<sub>FR</sub> cells and 1.5 × 10<sup>4</sup> T<sub>FR</sub> cells (isolated from dLNs of HDM-, NP-OVA- or MOG<sub>35-55</sub>-challenged mice) along with 20 µg ml<sup>-1</sup> of HDM, NP-OVA or rMOG in 96-well culture plates for 6 d. In some cases 20 µg ml<sup>-1</sup> of anti-IL-4 (Biolegend, 11B11) or anti-IL-13 (R&D Systems, MAB413) was added. Cells and supernatants were collected for flow cytometric and ELISA.

**ELISA.** For total IgG and IgE levels in serum, Maxisorp (Nunc) plates were coated with anti-mouse Ig (SouthernBiotech) or anti-IgE (BD Biosciences, R35-72), respectively, and the serum was incubated. Alkaline phosphatase secondary reagents (SouthernBiotech) were added, followed by phosphatase

substrate (Sigma), and plates were read on a plate reader (Spectramax). For NP-specific antibody ELISAs, Maxisorp plates were coated with NP-BSA (Biosearch Technologies), followed by the secondary reagents above. Anti-HDM IgE kits (Chondrex) were used for HDM-specific antibody quantification.

**Autoantigen arrays.** Autoantibody reactivities against a panel of autoantigens were measured using an autoantigen microarray platform developed by the University of Texas Southwestern Medical Center. Genepix Pro v.6.0 software was used to analyze IgG or IgE fluorescence. The antibody score was generated through the following equation: log<sub>2</sub>(net fluorescence intensity × signal-to-noise ratio + 1). The Mann-Whitney *U*-test was used to determine statistical significance.

**RNA-seq.** RNA-seq was performed as described previously<sup>15</sup>. Briefly, RNA was isolated using MyOne Silane Dynabeads (Thermo Fisher Scientific). RNA was fragmented, and barcoded using 8-basepair barcodes together with standard Illumina adaptors. Primers were removed using Agencourt AMPure XP bead cleanup (Beckman Coulter/Agencourt) and samples were amplified with 14 PCR cycles. Libraries were gel purified and quantified using a Qubit high-sensitivity DNA kit (Invitrogen) and library quality was confirmed using TapeStation high-sensitivity DNA tapes (Agilent Technologies). RNA-seq reactions were sequenced on an Illumina NextSeq sequencer (Illumina) according to the manufacturer's instructions, sequencing 50-basepair reads. Analysis was performed using the CLC Genomics Workbench v.8.0.1 RNA-seq analysis software package (Qiagen). Briefly, reads were aligned (mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8) to the mouse genome and differential expression analysis was performed (total count filter cutoff = 5.0). Results were normalized to reads per million. Gene-e (Broad Institute) was used to generate heatmaps. The datasets generated during the current study are available on the Gene Expression Omnibus (GSE134153) and are available from the corresponding author on reasonable request.

**Gene set enrichment analysis.** RNA-seq data were compared with T<sub>FR</sub> signatures<sup>25</sup>, T<sub>FR</sub> signatures<sup>25</sup> or T<sub>H</sub>2 signatures (GSE14308) using default settings in gene set enrichment analysis (GSEA) software (Broad Institute).

**Histology.** Paraffin-embedded lung sections were stained with hematoxylin and eosin or periodic acid-Schiff. Images were taken on an Olympus BX41 microscope and Olympus DP26 camera and images obtained using Olympus CellSens v.1.1 software. Lung inflammation was scored as: no inflammation: 0; perivascular/peribronchial inflammation without infiltration of airway/vessel walls: 1; infiltration of airway/vessel walls without extension into alveolar parenchyma: 2; destruction of alveolar parenchyma: 3.

**Statistics.** Most statistical tests were performed using Prism v.6.0 (GraphPad) using the two-tailed, unpaired, Student's *t*-test for normalized data, or the Mann-Whitney *U*-test for non-normal data, as indicated. Statistics for RNA-seq were performed using CLC Genomics Workbench (Qiagen). Statistics for gene set enrichment was performed at the GSEA (Broad Institute). All measurements were taken from distinct samples, except for memory experiments in which the same mice were bled before and after boost.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

The data that support the findings of this study are available from the corresponding author upon request. Transcriptomic data have been deposited in the Gene Expression Omnibus with the accession code GSE134153.

## References

- Bettelli, E. et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238 (2006).
- Litzenburger, T. et al. B lymphocytes producing demyelinating autoantibodies: development and function in gene-targeted transgenic mice. *J. Exp. Med.* **188**, 169–180 (1998).
- Sage, P. T. & Sharpe, A. H. In vitro assay to sensitively measure TFR suppressive capacity and TFR stimulation of B cell responses. *Methods Mol. Biol.* **1291**, 151–160 (2015).

## Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

### Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- |                                     |                                                                                                                                                                                                                                                                                                |
|-------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| n/a                                 | Confirmed                                                                                                                                                                                                                                                                                      |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The exact sample size ( $n$ ) for each experimental group/condition, given as a discrete number and unit of measurement                                                                                                                                    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly                                                                                                                                    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The statistical test(s) used AND whether they are one- or two-sided<br><i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>                                                               |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of all covariates tested                                                                                                                                                                                                                     |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons                                                                                                                                        |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. $F$ , $t$ , $r$ ) with confidence intervals, effect sizes, degrees of freedom and $P$ value noted<br><i>Give <math>P</math> values as exact values whenever suitable.</i>                            |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings                                                                                                                                                                      |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes                                                                                                                                                |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Estimates of effect sizes (e.g. Cohen's $d$ , Pearson's $r$ ), indicating how they were calculated                                                                                                                                                                    |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

### Software and code

Policy information about [availability of computer code](#)

Data collection

BD FACSDIVA software, Softmax Pro, Illumina NextSeq Software Suite, Olympus CellSens v1.1, Nikon Elements Acquisition Software AR 5.02, Genepix Pro v6.0

Data analysis

FlowJo v9, Graphpad Prism v7, Softmax Pro, CLC Genomics v9, ImageJ v. 2.0.0, GSEA v3.0

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

### Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNAseq has been deposited in GEO under accession number GSE134153

### Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- ☒ Life sciences      ☐ Behavioural & social sciences      ☐ Ecological, evolutionary & environmental sciences

# Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

|                 |                                                                                                                                                                                                                                                           |
|-----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sample size     | No sample size calculations were performed. Sample sizes were chosen based on empirical data, previous publications and availability of mice.                                                                                                             |
| Data exclusions | Analysis of mice included QC steps during flow cytometric analysis, samples which had inconsistencies in genotype confirmation or showed evidence of leakiness of alleles (pre-established to be a B cell frequency below 40%) were not analyzed further. |
| Replication     | All experiments were performed multiple independent times with separate mice. Littermates were used for experimental and control mice within experiments. All attempts at replication were successful, no experiment could not be replicated.             |
| Randomization   | Randomization is not relevant, mendelian genetics determined experimental and control groups. All controls were from littermates.                                                                                                                         |
| Blinding        | Histology was scored in a blind manner. Other experiments were not performed blinded because the same analysis gates and cutoffs were used for all samples regardless of group.                                                                           |

# Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

## Materials & experimental systems

## Methods

| n/a                                 | Involved in the study                                           |
|-------------------------------------|-----------------------------------------------------------------|
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Antibodies                  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Eukaryotic cell lines                  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology                          |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Animals and other organisms |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Human research participants            |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data                          |

| n/a                                 | Involved in the study                              |
|-------------------------------------|----------------------------------------------------|
| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Flow cytometry |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging    |

## Antibodies

|                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|-----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Antibodies used | Antibodies. The following antibodies were used for surface staining at 4°C for 30 minutes: anti-CD4 (Biolegend, 1:200, RM4-5, 100540, Lot# B261856), anti-ICOS (Biolegend, 1:200, 15F9, 107706, Lot# B259751), anti-CD19 (Biolegend, 1:200, 6D5, 115530, Lot# B276004), anti-PD-1 (Biolegend, 1:200, RMP1-30, 109110, Lot# B63885), anti-CXCR5 biotin (BD Biosciences, 1:100, 2G8, 551960, Lot# 8285670), anti-GL7 (BD Biosciences, 1:200, GL-7, 553666, Lot# 6021880), anti-FAS (BD Biosciences, 1:200, Jo2, 557653, Lot# 9039631), anti-HB-EGF/DTR (R&D Systems, 1:200, AF-259-NA, AF-259-NA, Lot# PX1017051), anti-CD38 (Biolegend, 1:200, 90, 102720, Lot# B240225), anti-CD138 (Biolegend, 1:200, 281-2, 142506, Lot# B268275), anti-IA (Biolegend, 1:200, M5/114.15.2, 107620, Lot# B252427), anti-SiglecF (BD Biosciences, 1:200, E50-2440, 565527, Lot# 7244817), anti-CD8a (Biolegend, 1:200, 53-6.7, 100714, Lot# B247008), anti-CD11c (Biolegend, 1:200, N418, 117310, Lot# B262130), anti-CD11b (Biolegend, 1:200, M1/70, 101208, Lot# B253922). For further CXCR5 detection, streptavidin-BV421 (Biolegend, 1:400, 405225, Lot# B274677) was used at 4°C. In some cases, anti-IgE (BD Biosciences, 1:200, R35-72, 553413, Lot# 3081842) was included to block IgE bound to cell surfaces. For intracellular staining, samples were fixed with the Foxp3 Fix/Perm buffer set according to the manufacturer's instructions (eBioscience). Samples were then intracellularly stained with anti-IgG1 (BD Biosciences, 1:200, A85-1, 550083, Lot# 9087965), anti-IgE (BD Biosciences, 1:200, R35-72, 564207, Lot# 7349503), anti-FoxP3 (eBiosciences, 1:200, FJK-16S, 20201221, Lot# 2011698), or anti-Ki67 (BD Biosciences, 1:100, B56, 561126, Lot# 8194601), anti-Glut1 (abcam, EPR3915, ab195020, Lot# GR310910-3), anti-IL-13 (eBioscience, 1:200, eBio13A, 2020-01-31, Lot# 4323739). In some cases, a donkey anti-goat A647 secondary was used (Invitrogen, 1:400, A-21447, Lot# 872645). For blocking desired cytokines in vitro, the following antibodies were added: anti-IL-4 (Biolegend, 11B11, 504121, Lot# B242087), and anti-IL-13 (R&D Systems, 38213, MAB413, Lot# AEQ1218031). For immunofluorescence the following antibodies were used: anti-actin, anti-Grl (Biolegend, RB6-8C5, 108408, Lot# B220797), anti-SiglecF (BD Biosciences, E502440, 562680, Lot# 8008651), and anti-IA as previously shown. |
| Validation      | All antibodies used were commercially available, passed manufacturer's quality controls, have been validated for indicated applications, and have been previously published using specific applications.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |

## Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

|                    |                                                                                                                     |
|--------------------|---------------------------------------------------------------------------------------------------------------------|
| Laboratory animals | Foxp3IRES-GFP, Foxp3IRES-CreYFP, Foxp3DTR, IgHMOG, Cxcr5IRES-LoxP-STOP-LoxP-DTR mice on the C57bl/6 background were |
|--------------------|---------------------------------------------------------------------------------------------------------------------|

|                         |                                                                                                                                                                                   |
|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Laboratory animals      | used. All mice were 6-8 weeks at the start of experiments. Males or females were used for individual experiments, but repeats included experiments with both male or female mice. |
| Wild animals            | This study does not include wild animals.                                                                                                                                         |
| Field-collected samples | This study does not include field collected samples.                                                                                                                              |
| Ethics oversight        | Brigham and Women's Hospital and Harvard Medical School Institutional Animal Care and Use Committees                                                                              |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Flow Cytometry

### Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

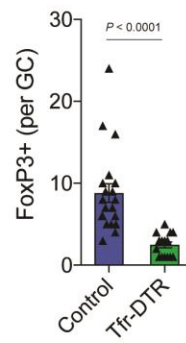
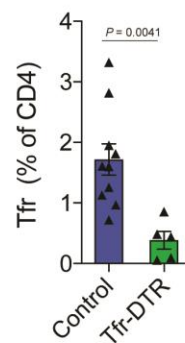
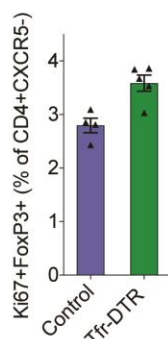
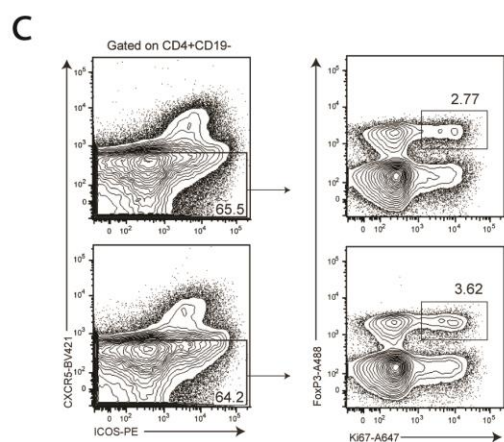
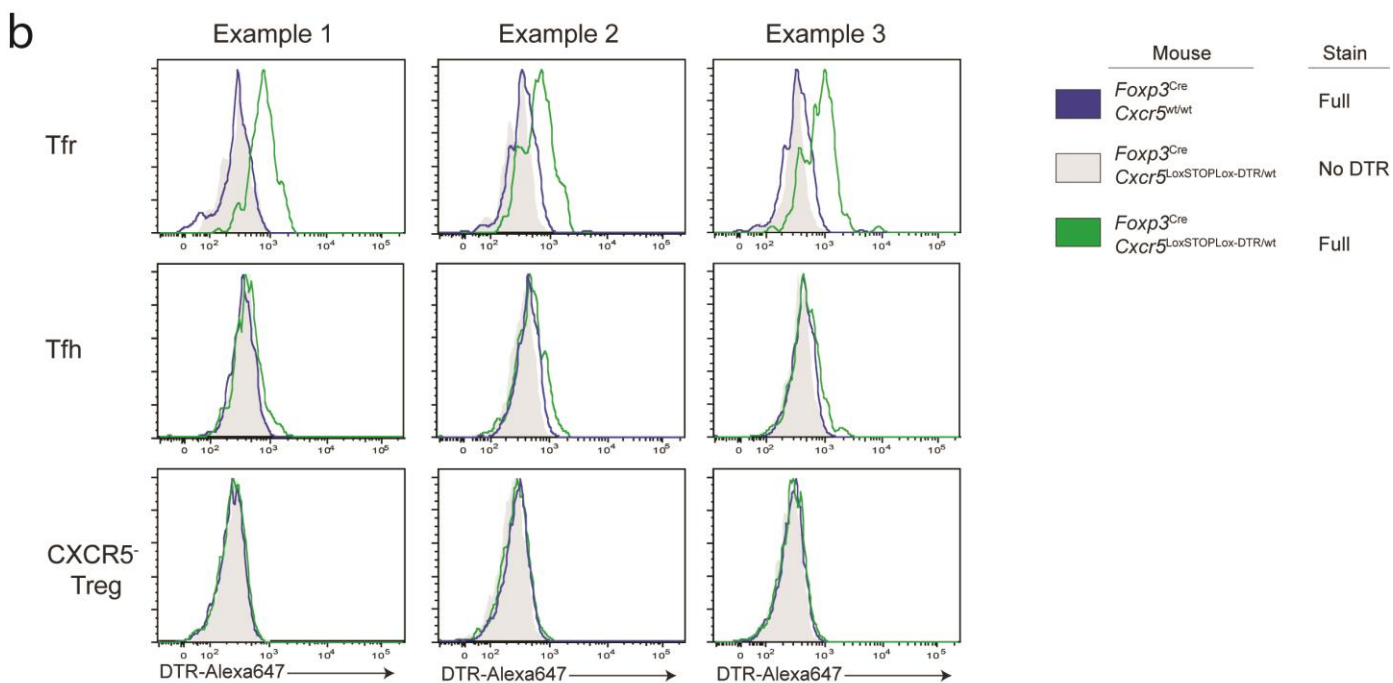
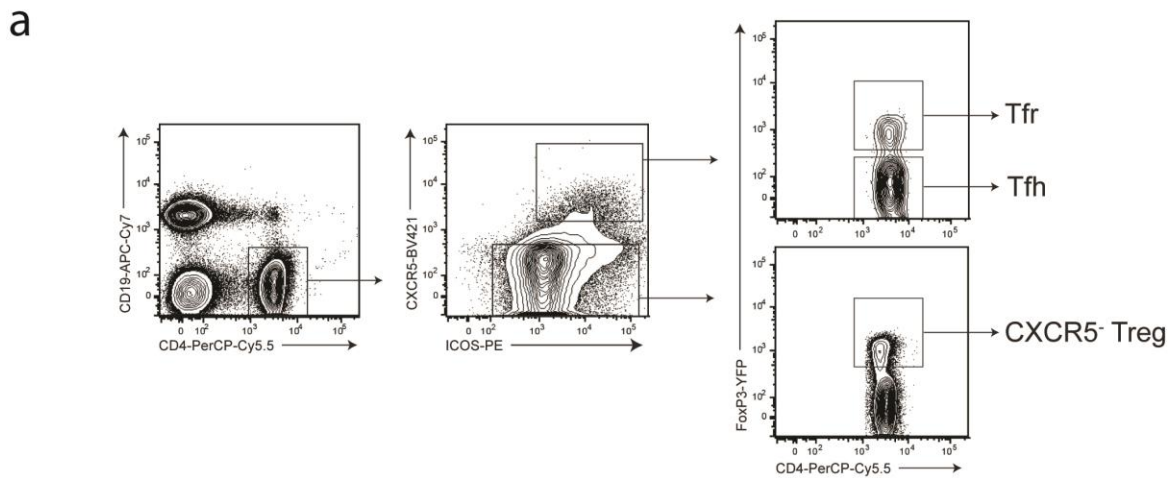
|                                                                                                                                                           |                                                                                                                              |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------|
| Sample preparation                                                                                                                                        | Draining lymph nodes were passed through 70micron filters and resuspended in PBS supplemented with 1% FBS with 1mM EDTA.     |
| Instrument                                                                                                                                                | BD LSRII, BD FACS Symphony, BD FACS Canto, BD ARIA II                                                                        |
| Software                                                                                                                                                  | BD FACS DIVA (collection), FlowJo (Analysis)                                                                                 |
| Cell population abundance                                                                                                                                 | Post sort analysis was done by re-running collection tubes on cell sorter.                                                   |
| Gating strategy                                                                                                                                           | All samples were pregated on SSA-SSH singlets, FSC-SSA for lymphocyte population. Gating strategies are included in figures. |
| <input checked="" type="checkbox"/> Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. |                                                                                                                              |

In the format provided by the authors and unedited.

# Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses

Rachel L. Clement<sup>1</sup> , Joe Daccache<sup>1</sup>, Mostafa T. Mohammed<sup>1</sup>, Alos Diallo<sup>2</sup>, Bruce R. Blazar<sup>3</sup>, Vijay K. Kuchroo<sup>4,5,6</sup>, Scott B. Lovitch<sup>7</sup> , Arlene H. Sharpe<sup>2,4,7</sup>  and Peter T. Sage<sup>1\*</sup> 

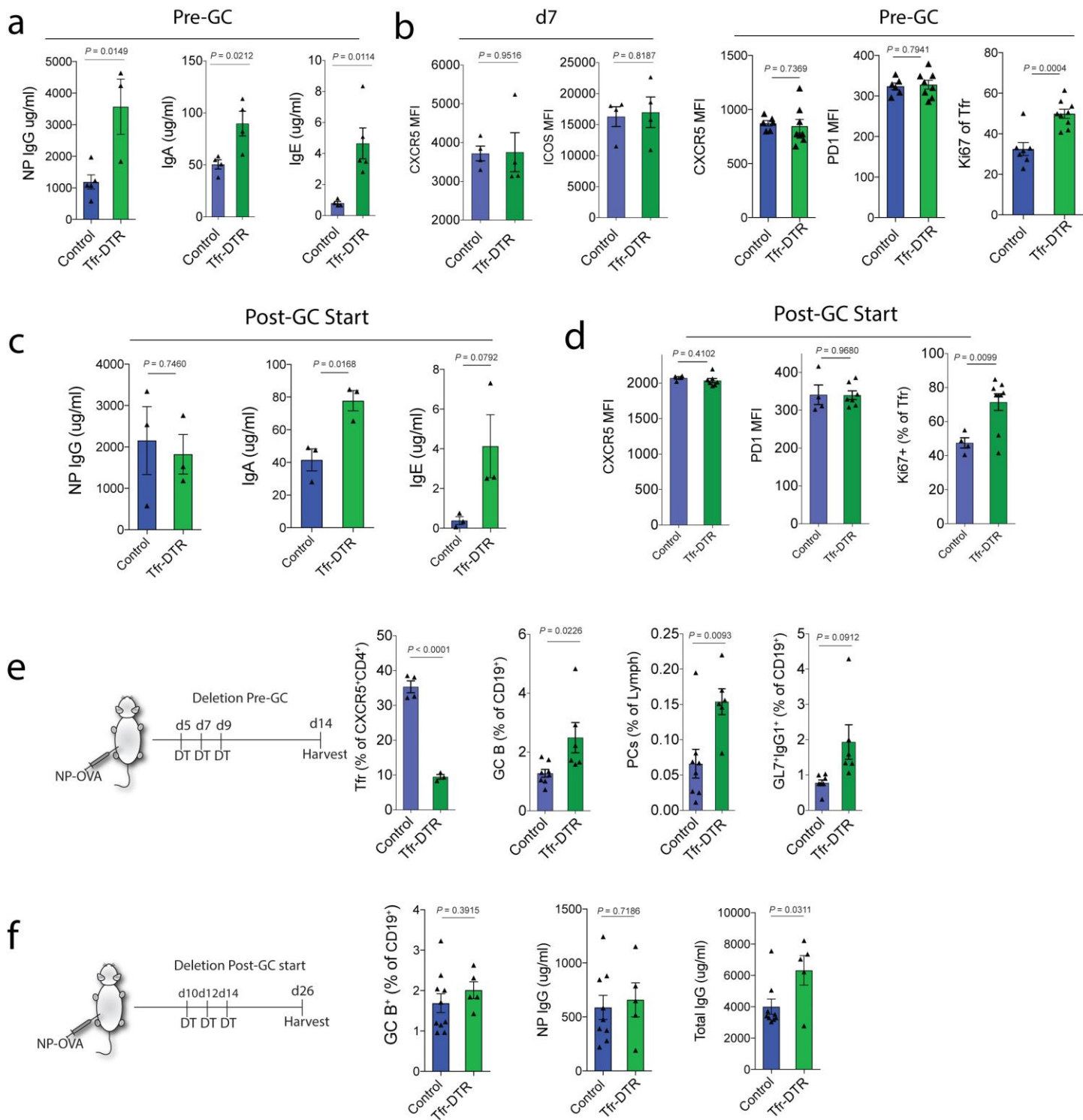
<sup>1</sup>Transplantation Research Center, Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. <sup>2</sup>Department of Immunology, Harvard Medical School, Boston, MA, USA. <sup>3</sup>Department of Pediatrics, Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN, USA. <sup>4</sup>Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA, USA. <sup>5</sup>Broad Institute, Cambridge, MA, USA. <sup>6</sup>Ann Romney Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA, USA. <sup>7</sup>Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA. \*e-mail: [psage@bwh.harvard.edu](mailto:psage@bwh.harvard.edu)



### Supplementary Figure 1

Characterization of the Tfr-DTR strain

- a) Gating strategy to identify Tfh, Tfr and CXCR5<sup>-</sup> T<sub>reg</sub> cells in draining lymph nodes of mice immunized with NP-OVA 7 days previously.
- b) DTR expression on Tfh, Tfr and CXCR5<sup>-</sup> T<sub>reg</sub> cells gated as in (a) in indicated mouse strains. Full stain = all antibodies used along with secondary reagent for DTR staining. No DTR = all antibodies except anti-DTR primary was not added.
- c) Quantification of activated T<sub>reg</sub> cells in Tfr-DTR mice. Control or Tfr-DTR mice were immunized and given DT to delete Tfr cells as in Fig. 1f. CXCR5-Ki67<sup>+</sup>FoxP3<sup>+</sup> activated T<sub>reg</sub> cells were quantified. Representative gating (left) and analysis (right) are shown.
- d) Quantification of Tfr cells by flow cytometry (left) and quantification of FoxP3<sup>+</sup> cells within individual GCs by microscopy (right) in Tfr-DTR mice as in (c).
- Column graphs represent the mean with error bars indicating standard error. P value indicates two-tailed student's T test. Data are from an individual experiment and are representative of two experimental repeats (a-c), or are combined data from two experiments (d).

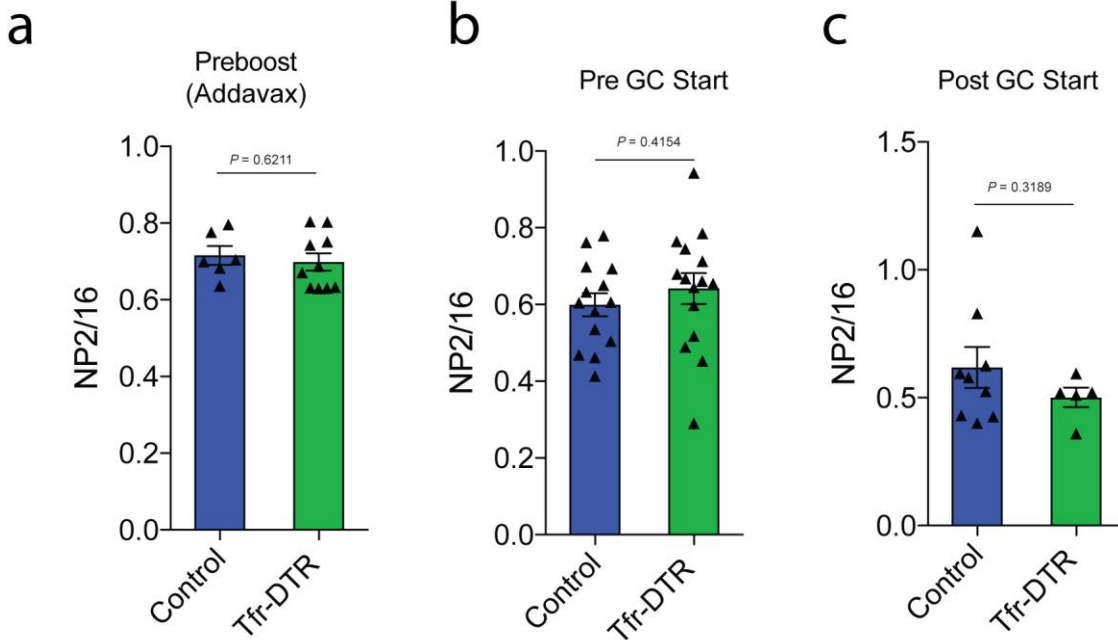


**Supplementary Figure 2**

Tfr cells regulate early GC responses

- a) Antibody responses in Tfr-DTR mice with pre-GC Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA and received DT on days 5,7 and 9. Serum was collected on day 21 and NP-specific IgG (left), total IgA (middle) and total IgE (right) were measured.
- b) Phenotype of Tfr cells in Tfr-DTR mice with early (left) or pre-GC (right) start Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA and received DT on days 2,4,6 (left) or 5,7 and 9 (right). Expression of CXCR5, ICOS, PD-1 or Ki67 was measured on day 7 (left) or day 21 (right).
- c) Assessment of B cell responses in Tfr-DTR mice with post-GC Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA and received DT on days 10,12 and 14. Serum was collected on day 21 and NP-specific IgG (left), total IgA (middle) and total IgE (right) were measured.
- d) Expression of CXCR5, ICOS and Ki67 on Tfr cells in control or Tfr-DTR mice in post-GC deletion experiments. *FoxP3<sup>Cre</sup> Cxcr5<sup>wt</sup>* control or Tfr-DTR mice which were immunized with NP-OVA, given DT on days 10,12, and 14 and draining lymph nodes harvested on day 21.
- e) Evaluation of B cell responses early during pre-GC Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA and received DT on days 5,7 and 9. Draining lymph nodes were collected on day 14 for the indicated analysis. Tfr (CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>FoxP3<sup>+</sup>CD19<sup>-</sup>) cells, GC B (CD19<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>) cells, Plasma cells (CD138<sup>+</sup>) and class switched B cells (CD19<sup>+</sup>GL7<sup>+</sup>IgG1<sup>+</sup>) cells were quantified.
- f) Evaluation of B cell responses late during post-GC Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA and received DT on days 10,12 and 14. Draining lymph nodes and serum was collected on day 26 for analysis. GC B (CD19<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>) cells from lymph nodes, NP specific IgG from serum, and total IgG from serum were quantified.

Column graphs represent the mean with error bars indicating standard error. P value indicates two-tailed student's T test. Data are from an individual experiments and are representative of two experimental repeats.



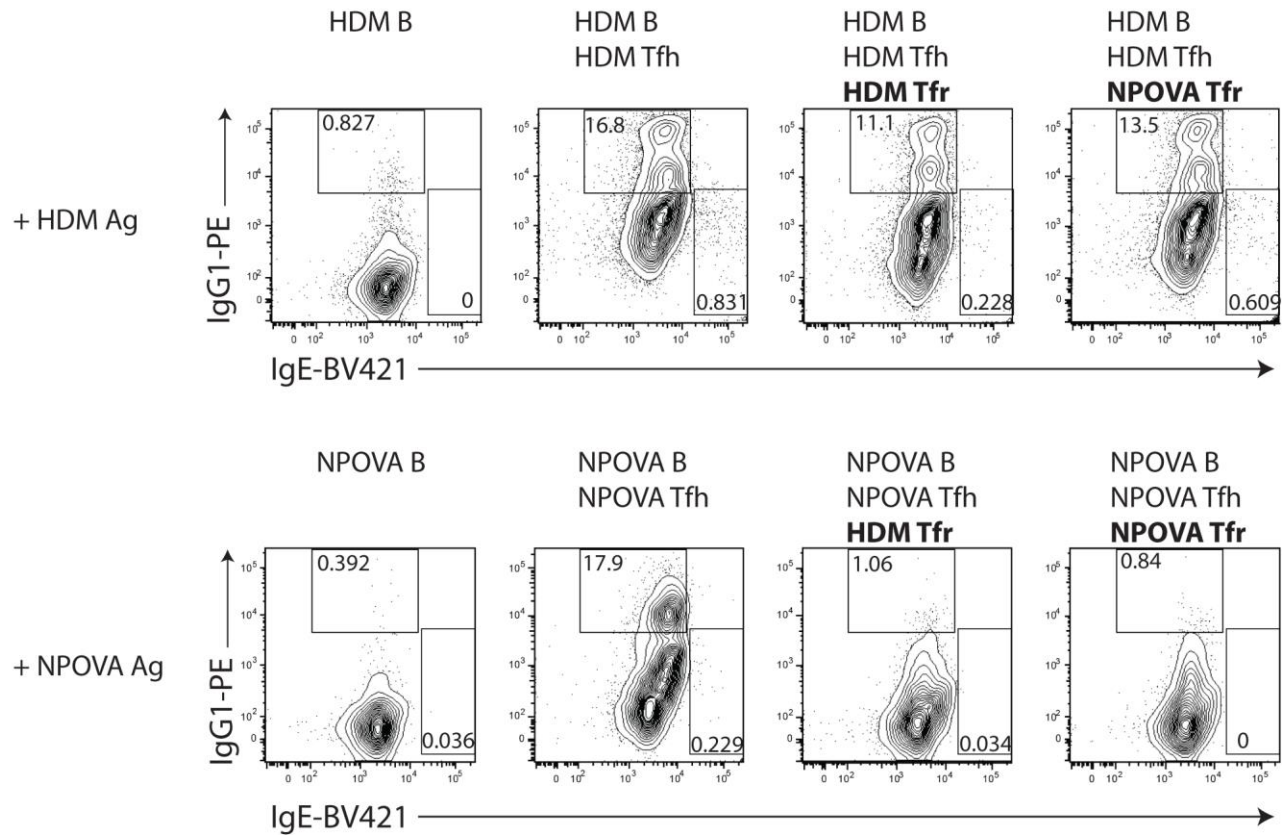
### Supplementary Figure 3

Additional analysis of memory responses in Tfr-DTR mice

- a) NP2/16 ratio in Tfr-DTR mice with pre-GC Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA/MF59-Addavax and received DT on days 5,7 and 9. Serum was collected on day 30 (pre-boost).
- b) NP2/16 ratio in Tfr-DTR mice with pre-GC Tfr deletion strategies. Tfr-DTR mice were immunized with NP-OVA and received DT on days 5,7 and 9. Serum was collected on day 21. Explain boost
- c) NP2/16 ratio in Tfr-DTR mice with Tfr deletion post-GC formation. Tfr-DTR mice were immunized with NP-OVA and received DT on days 10,12 and 14. Serum was collected on day 21.

Column graphs represent the mean with error bars indicating standard error. P value indicates two-tailed student's T test. Data are from 3 combined experiments.

a

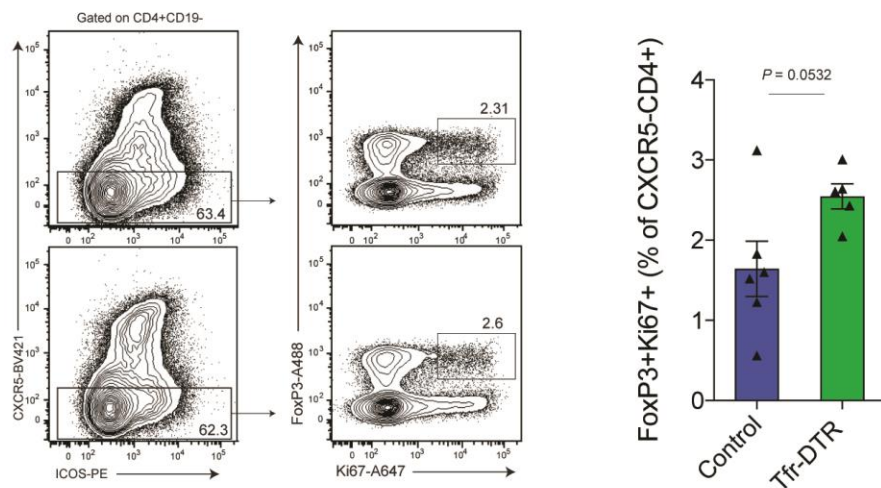


#### Supplementary Figure 4

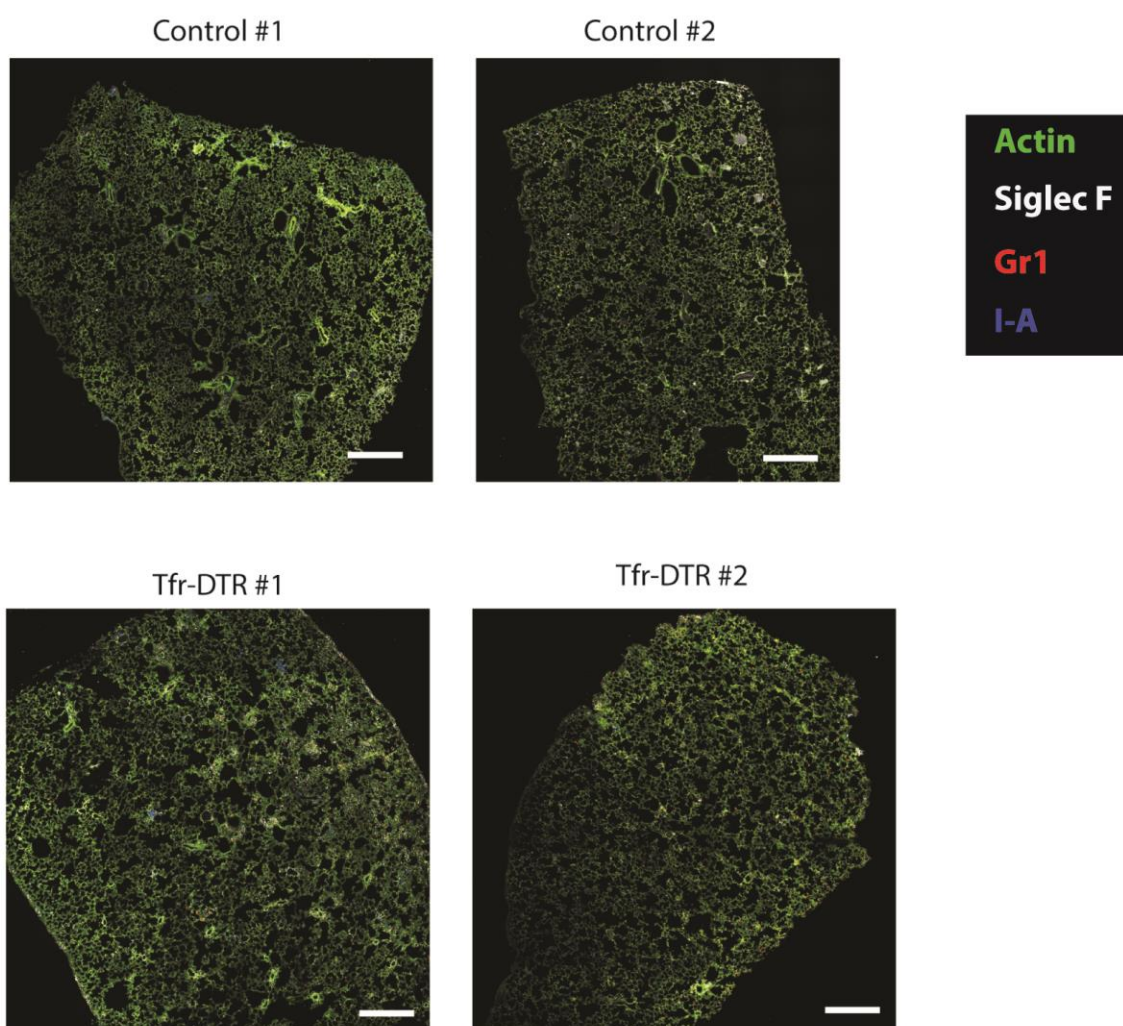
Tfr cells suppress Tfh13-mediated class switching

a) FoxP3-GFP mice were immunized with NP-OVA subcutaneously or HDM intranasally according to *materials and methods*. 7 days later draining lymph nodes were harvested and B, Tfh and Tfr cells were isolated. Indicated populations were cultured together with the indicated antigen for 6 days. Plots are pregated on CD19<sup>+</sup>CD4<sup>-</sup>. Data are from an individual experiment and are representative of two experimental repeats.

a



b



### Supplementary Figure 5

Additional analysis of allergic inflammation in Tfr-DTR mice

a) Quantification of Ki67<sup>+</sup> CXCR5- activated T<sub>reg</sub> cells in Tfr-DTR mice as in Fig. 7a-b. Representative gating (left) and representative gating (right) are shown.

b) Representative micrographs of lungs from *FoxP3*<sup>Cre</sup> *Cxcr5*<sup>wt</sup> control or Tfr-DTR mice stained for Actin (green), Siglec F (white), Gr1 (red) or I-A (blue) as in Fig. 7a. Scale bars indicate 500 microns.

Column graphs represent the mean with error bars indicating standard error. P value indicates two-tailed student's T test. Data are from an individual experiment and are representative of three experimental repeats (a), or are from one experiment (b).

## Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

### Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- |                                     |                                                                                                                                                                                                                                                                                                |
|-------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| n/a                                 | Confirmed                                                                                                                                                                                                                                                                                      |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The exact sample size ( $n$ ) for each experimental group/condition, given as a discrete number and unit of measurement                                                                                                                                    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly                                                                                                                                    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The statistical test(s) used AND whether they are one- or two-sided<br><i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>                                                               |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of all covariates tested                                                                                                                                                                                                                     |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons                                                                                                                                        |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. $F$ , $t$ , $r$ ) with confidence intervals, effect sizes, degrees of freedom and $P$ value noted<br><i>Give <math>P</math> values as exact values whenever suitable.</i>                            |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings                                                                                                                                                                      |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes                                                                                                                                                |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Estimates of effect sizes (e.g. Cohen's $d$ , Pearson's $r$ ), indicating how they were calculated                                                                                                                                                                    |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

### Software and code

Policy information about [availability of computer code](#)

Data collection

BD FACSDIVA software, Softmax Pro, Illumina NextSeq Software Suite, Olympus CellSens v1.1, Nikon Elements Acquisition Software AR 5.02, Genepix Pro v6.0

Data analysis

FlowJo v9, Graphpad Prism v7, Softmax Pro, CLC Genomics v9, ImageJ v. 2.0.0, GSEA v3.0

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

### Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNAseq has been deposited in GEO under accession number GSE134153

### Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- ☒ Life sciences      ☐ Behavioural & social sciences      ☐ Ecological, evolutionary & environmental sciences

# Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

|                 |                                                                                                                                                                                                                                                           |
|-----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sample size     | No sample size calculations were performed. Sample sizes were chosen based on empirical data, previous publications and availability of mice.                                                                                                             |
| Data exclusions | Analysis of mice included QC steps during flow cytometric analysis, samples which had inconsistencies in genotype confirmation or showed evidence of leakiness of alleles (pre-established to be a B cell frequency below 40%) were not analyzed further. |
| Replication     | All experiments were performed multiple independent times with separate mice. Littermates were used for experimental and control mice within experiments. All attempts at replication were successful, no experiment could not be replicated.             |
| Randomization   | Randomization is not relevant, mendelian genetics determined experimental and control groups. All controls were from littermates.                                                                                                                         |
| Blinding        | Histology was scored in a blind manner. Other experiments were not performed blinded because the same analysis gates and cutoffs were used for all samples regardless of group.                                                                           |

# Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

## Materials & experimental systems

## Methods

| n/a                                 | Involved in the study                                           |
|-------------------------------------|-----------------------------------------------------------------|
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Antibodies                  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Eukaryotic cell lines                  |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology                          |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Animals and other organisms |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Human research participants            |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data                          |

| n/a                                 | Involved in the study                              |
|-------------------------------------|----------------------------------------------------|
| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Flow cytometry |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging    |

## Antibodies

|                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|-----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Antibodies used | Antibodies. The following antibodies were used for surface staining at 4°C for 30 minutes: anti-CD4 (Biolegend, 1:200, RM4-5, 100540, Lot# B261856), anti-ICOS (Biolegend, 1:200, 15F9, 107706, Lot# B259751), anti-CD19 (Biolegend, 1:200, 6D5, 115530, Lot# B276004), anti-PD-1 (Biolegend, 1:200, RMP1-30, 109110, Lot# B63885), anti-CXCR5 biotin (BD Biosciences, 1:100, 2G8, 551960, Lot# 8285670), anti-GL7 (BD Biosciences, 1:200, GL-7, 553666, Lot# 6021880), anti-FAS (BD Biosciences, 1:200, Jo2, 557653, Lot# 9039631), anti-HB-EGF/DTR (R&D Systems, 1:200, AF-259-NA, AF-259-NA, Lot# PX1017051), anti-CD38 (Biolegend, 1:200, 90, 102720, Lot# B240225), anti-CD138 (Biolegend, 1:200, 281-2, 142506, Lot# B268275), anti-IA (Biolegend, 1:200, M5/114.15.2, 107620, Lot# B252427), anti-SiglecF (BD Biosciences, 1:200, E50-2440, 565527, Lot# 7244817), anti-CD8a (Biolegend, 1:200, 53-6.7, 100714, Lot# B247008), anti-CD11c (Biolegend, 1:200, N418, 117310, Lot# B262130), anti-CD11b (Biolegend, 1:200, M1/70, 101208, Lot# B253922). For further CXCR5 detection, streptavidin-BV421 (Biolegend, 1:400, 405225, Lot# B274677) was used at 4°C. In some cases, anti-IgE (BD Biosciences, 1:200, R35-72, 553413, Lot# 3081842) was included to block IgE bound to cell surfaces. For intracellular staining, samples were fixed with the Foxp3 Fix/Perm buffer set according to the manufacturer's instructions (eBioscience). Samples were then intracellularly stained with anti-IgG1 (BD Biosciences, 1:200, A85-1, 550083, Lot# 9087965), anti-IgE (BD Biosciences, 1:200, R35-72, 564207, Lot# 7349503), anti-FoxP3 (eBiosciences, 1:200, FJK-16S, 20201221, Lot# 2011698), or anti-Ki67 (BD Biosciences, 1:100, B56, 561126, Lot# 8194601), anti-Glut1 (abcam, EPR3915, ab195020, Lot# GR310910-3), anti-IL-13 (eBioscience, 1:200, eBio13A, 2020-01-31, Lot# 4323739). In some cases, a donkey anti-goat A647 secondary was used (Invitrogen, 1:400, A-21447, Lot# 872645). For blocking desired cytokines in vitro, the following antibodies were added: anti-IL-4 (Biolegend, 11B11, 504121, Lot# B242087), and anti-IL-13 (R&D Systems, 38213, MAB413, Lot# AEQ1218031). For immunofluorescence the following antibodies were used: anti-actin, anti-Grl (Biolegend, RB6-8C5, 108408, Lot# B220797), anti-SiglecF (BD Biosciences, E502440, 562680, Lot# 8008651), and anti-IA as previously shown. |
| Validation      | All antibodies used were commercially available, passed manufacturer's quality controls, have been validated for indicated applications, and have been previously published using specific applications.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |

## Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

|                    |                                                                                                                     |
|--------------------|---------------------------------------------------------------------------------------------------------------------|
| Laboratory animals | Foxp3IRES-GFP, Foxp3IRES-CreYFP, Foxp3DTR, IgHMOG, Cxcr5IRES-LoxP-STOP-LoxP-DTR mice on the C57bl/6 background were |
|--------------------|---------------------------------------------------------------------------------------------------------------------|

|                         |                                                                                                                                                                                   |
|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Laboratory animals      | used. All mice were 6-8 weeks at the start of experiments. Males or females were used for individual experiments, but repeats included experiments with both male or female mice. |
| Wild animals            | This study does not include wild animals.                                                                                                                                         |
| Field-collected samples | This study does not include field collected samples.                                                                                                                              |
| Ethics oversight        | Brigham and Women's Hospital and Harvard Medical School Institutional Animal Care and Use Committees                                                                              |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Flow Cytometry

### Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

|                           |                                                                                                                              |
|---------------------------|------------------------------------------------------------------------------------------------------------------------------|
| Sample preparation        | Draining lymph nodes were passed through 70micron filters and resuspended in PBS supplemented with 1% FBS with 1mM EDTA.     |
| Instrument                | BD LSRII, BD FACS Symphony, BD FACS Canto, BD ARIA II                                                                        |
| Software                  | BD FACS DIVA (collection), FlowJo (Analysis)                                                                                 |
| Cell population abundance | Post sort analysis was done by re-running collection tubes on cell sorter.                                                   |
| Gating strategy           | All samples were pregated on SSA-SSH singlets, FSC-SSA for lymphocyte population. Gating strategies are included in figures. |

- ☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.