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Suppression by T_{FR} cells leads to durable and selective inhibition of B cell effector function

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Follicular regulatory T cells (T_{FR} cells) inhibit follicular helper T cell (T_{FH} cell)–mediated antibody production. The mechanisms by which T_{FR} cells exert their key immunoregulatory functions are largely unknown. Here we found that T_{FR} cells induced a distinct suppressive state in T_{FH} cells and B cells, in which effector transcriptional signatures were maintained but key effector molecules and metabolic pathways were suppressed. The suppression of B cell antibody production and metabolism by T_{FR} cells was durable and persisted even in the absence of T_{FR} cells. This durable suppression was due in part to epigenetic changes. The cytokine IL-21 was able to overcome T_{FR} cell–mediated suppression and inhibited T_{FR} cells and stimulated B cells. By determining mechanisms of T_{FR} cell-mediated suppression, we have identified methods for modulating the function of T_{FR} cells and antibody production.

Humoral immunity is critical for the clearance of pathogens and is the basis for protection elicited by vaccines. An effector subset of $CD4^+$ regulatory T cells (T_{reg} cells) called 'follicular regulatory T cells' (T_{FR} cells) has been identified. T_{FR} cells migrate to the B cell follicle and inhibit antibody production[1–4]. T_{FR} cells express the chemokine receptor CXCR5 and the transcription factors Bcl6 and Foxp3 and have high surface expression of the costimulator ICOS and the co-inhibitor PD-1 (ref. 2). Follicular helper T cells (T_{FH} cells) also have high expression of CXCR5, ICOS, Bcl6 and PD-1 (but not Foxp3) but stimulate antibody responses[5]. Notably, the ratio of T_{FH} cells to T_{FR} cells can be used to functionally predict the magnitude of antibody responses in a wide range of disease states in mice and humans[2]. How T_{FR} cells modulate antibody responses is still largely unknown.

Antibody responses originate in germinal centers (GCs), highly specialized structures within the B cell follicle in which B cells become activated and differentiate to become effector B cells, plasma cells and memory B cells[6,7]. The GC reaction is a highly regulated process that depends on T_{FH} cells. T_{FH} cells interact with cognate GC B cells in a process called 'linked recognition'[8]. T_{FH} cells supply key cytokines, such as IL-4 and IL-21, as well as costimulatory molecules, such as CD40L, to the B cell[9]. These signals strongly activate the B cell, which then cycles between the light zone and dark zone of the GC during affinity maturation[6]. The GC B cell simultaneously supplies antigenic signals and costimulation to T_{FH} cells through B7-1, B7-2 and ICOSL[10,11]. The culmination of this interaction is class-switch recombination (CSR), somatic hypermutation and the differentiation of GC B cells into plasma cells that produce large quantities of high-affinity antibodies.

In contrast to T_{FH} cells, T_{FR} cells inhibit the GC reaction. The mechanisms by which T_{FR} cells exert their inhibitory effects are only beginning to be understood[2]. T_{FR} cells suppress the production of IL-21 and IL-4 by T_{FH} cells and inhibit CSR and antibody production by B cells[3,4,12,13]. CTLA-4 expressed by T_{FR} cells is an important mediator of the suppressive function of T_{FR} cells, since T_{FR} cells lacking CTLA-4 have a substantially diminished ability to suppress antibody production by B cells[14,15]. In contrast, PD-1 deficiency on T_{FR} cells results in a heightened suppressive ability[13].

Here we found that T_{FR} cells induced a distinct suppressive state in T_{FH} cells and B cells in which effector molecules and metabolic pathways were suppressed but global effector programs were maintained. We also found that IL-21 was able to overcome T_{FR} cell–mediated suppression by enhancing B cell metabolism and inhibiting T_{FR} cells. These data provide mechanistic insight into how T_{FR} cells suppress T_{FH} cells and B cells and identify ways to circumvent suppression by T_{FR} cells.

RESULTS

Early activation of B cells suppressed by T_{FR} cells

To define mechanisms by which T_{FH} cells and B cells are suppressed by T_{FR} cells, we used an *in vitro* suppression assay in which T_{FR} cells are cultured with T_{FH} cells and B cells, which leads to the suppression of both T_{FH} cell responses and B cell responses[12,14,16]. We obtained B cells ($CD19^+$), T_{FH} cells ($CD4^+ICOS^+CXCR5^+CD19^-Foxp3^-$)

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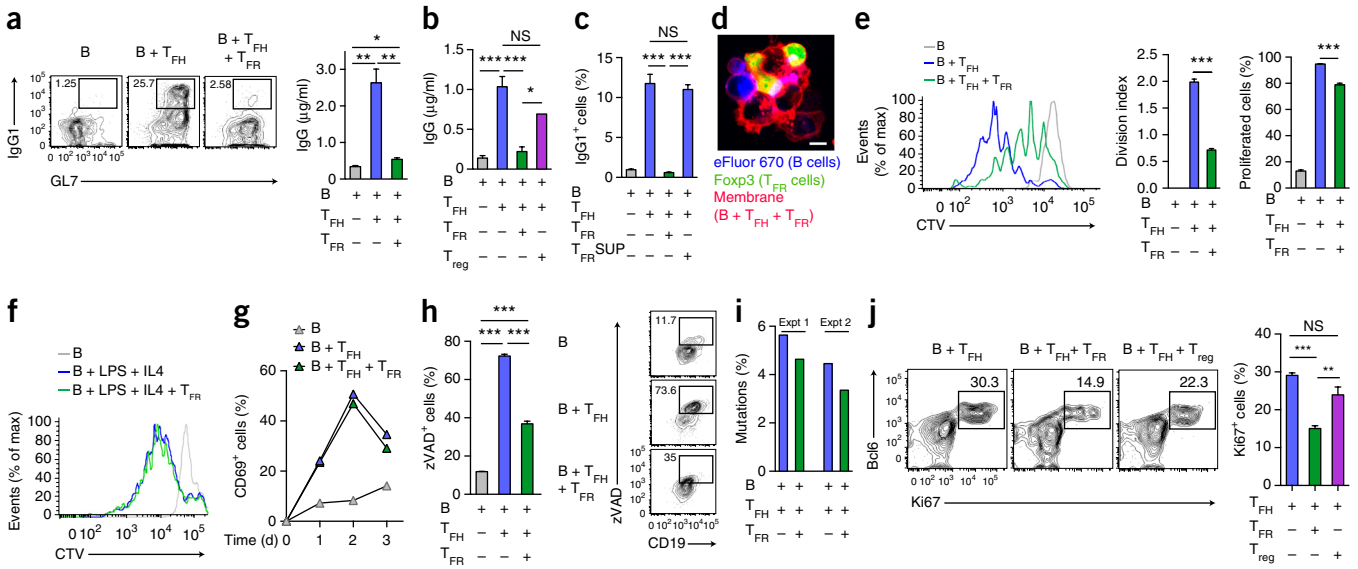

Figure 1 Suppressed B cells undergo early activation. (**a**) Flow cytometry (left) of cultures of B cells alone (B) or B cells plus T_{FH} cells with (B + T_{FH} + T_{FR}) or without (B + T_{FH}) T_{FR} cells, in the presence of anti-CD3 and anti-IgM (**Supplementary Fig. 1a**,**b**), and IgG secreted by those cultures (right). Numbers adjacent to outlined areas (left) indicate percent IgG1$^+$GL7$^+$ B cells. (**b**) Quantification of secreted antibody in cultures as in **a** and in a culture including CD4$^+$ICOS$^-$CXCR5$^-$Foxp3$^+$ T_{reg} cells (below plot). (**c**) Frequency of IgG1$^+$ B cells from cultures as in **a** and in a culture including supernatant of suppressed cultures (T_{FR} sup). (**d**) Micrograph of a culture containing B cells, T_{FH} cells and T_{FR} cells, after 4 d. Scale bar, 5 μm. (**e**) Proliferation of B cells (pre-gated as CD19$^+$IA$^+$CD4$^-$) in cultures as in **a**, measured by dilution of CellTrace Violet (CTV) at day 4. (**f**) Proliferation of B cells in cultures as in **a**, incubated for 4 d with or without lipopolysaccharide (LPS) and IL-4 and T_{FR} cells (key). (**g**) CD69 expression on B cells in cultures as in **a**. (**h**) Death of B cells in cultures as in **a**, measured by zVAD staining (indicative of cells that are undergoing death). Numbers adjacent to outlined areas (right) indicate percent zVAD$^+$CD19$^+$ (dying) B cells. (**i**) Somatic hypermutation in B cells in cultures as in **a** in the presence of NP-OVA. Expt, experiment. (**j**) Flow cytometry (left) of cultures as in **a** of cells pre-gated on T_{FH} cells (CD4$^+$Foxp3$^-$CD19$^-$IA$^-$). Numbers adjacent to outlined areas (left) indicate percent Bcl6$^+$Ki67$^+$ (cell-cycling) T_{FH} cells. NS, not significant ($P > 0.05$); $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test (**a**,**e**,**h**) or one-way analysis of variance (ANOVA) with Tukey's correction (**b**,**c**,**j**)). Data are from one experiment representative of five independent experiments with similar results (**a**,**b**,**h**,**j**; mean + s.e.m. of two to three technical replicates of cells pooled from 20 mice), three independent experiments (**c**–**g**; mean + s.e.m.) or two experiments (**i**).

and T_{FR} cells (CD4$^+$ICOS$^+$CXCR5$^+$CD19$^-$Foxp3$^+$) from *Foxp3*^{GFP} mice (which express sequence encoding green fluorescent protein (GFP) from a *Foxp3* allele) immunized with the hapten NP linked to ovalbumin (NP-OVA), then cultured the B cells and T_{FH} cells in the presence or absence of T_{FR} cells, along with antibody to the invariant signaling protein CD3 (anti-CD3) and antibody to immunoglobulin M (anti-IgM) (**Supplementary Fig. 1a**,**b**). We measured robust upregulation of expression of the GC B cell marker GL7 on B cells and CSR to IgG1 and substantial quantities of secreted IgG for B cells cultured with T_{FH} cells alone (**Fig. 1a**). When T_{FR} cells were added, CSR, GL7 expression and secretion of antibody were diminished (**Fig. 1a**). CD4$^+$CXCR5$^-$ICOS$^-$Foxp3$^+$ T_{reg} cells were not able to suppress as efficiently as T_{FR} cells did (**Fig. 1b**), as previously reported[12]. T_{FR} cells also suppressed CSR of B cells in response to specific antigen (**Supplementary Fig. 1c**). Furthermore, the suppression of CSR by T_{FR} cells required cell contact, as supernatant from T_{FR} cultures did not suppress B cells (**Fig. 1c**). Time-lapse microscopy of cultures revealed that T_{FR} cells closely interacted with both T_{FH} cells and B cells (**Fig. 1d** and **Supplementary Video 1**). These data suggested that T_{FR} cells might physically disrupt T_{FH} cell– and B cell–linked recognition during suppression[2,17]. Therefore, our culture system was a robust model for studying synchronized T_{FR} cell–mediated suppression of T_{FH} cells and B cells.

We first investigated whether T_{FR} cells prevent the initial activation of B cells by assessing dilution of the dye CellTrace Violet by B cells to measure their proliferation. Despite a decrease in the number of cell divisions, most B cells proliferated at least one cell cycle when T_{FR} cells were present (**Fig. 1e**). T_{FR} cells did not inhibit the proliferation of B cells in co-cultures of only T_{FR} cells and B cells (without T_{FH} cells) and lipopolysaccharide plus IL-4 (**Fig. 1f**). We also analyzed expression of the activation marker CD69 and found that this was upregulated in B cells whether T_{FR} cells were present or not (**Fig. 1g**). Studies with fluorescein isothiocyanate–zVAD, a fluorescent reagent that binds active caspase, revealed that T_{FR} cells did not enhance the apoptosis of B cells as a means of suppression (**Fig. 1h**). These data demonstrated that early activation of B cells still occurred even in the presence of T_{FR} cells.

To determine if T_{FR} cells alter somatic hypermutation, we performed our *in vitro* suppression assay with NP-OVA. We found a low frequency of mutations in B cells cultured with T_{FH} cells and that T_{FR} cells diminished somatic hypermutation in B cells (**Fig. 1i**). T_{FR} cells (but not T_{reg} cells) also suppressed the activation of T_{FH} cells, as indicated by reduced expression of Bcl6 and the proliferation marker Ki67 (**Fig. 1j**). Together these data demonstrated that T_{FR} cells allowed initial activation of B cells but suppressed downstream effector responses, including CSR and antibody production.

Inhibition of specific genes but not effector programs by T_{FR} cells

Our findings suggested that T_{FR} cells suppress B cells in a manner that allows B cells to receive activation signals but not perform effector functions. To further characterize suppressed B cells and T_{FH} cells, we performed RNA-sequencing (RNA-seq) transcriptional analysis. We cultured B cells and T_{FH} cells (from *Foxp3*^{GFP} mice immunized with NP-OVA) with or without T_{FR} cells (from *Actin*^{CFP}*Foxp3*^{GFP} mice (*Foxp3*^{GFP} mice that express cyan fluorescent protein (CFP) from an allele encoding β-actin) immunized with NP-OVA) and

NP-OVA. We sorted B cells ($CD19^+IA^+CD4^-$) and T_{FH} cells ($CD4^+IA^-CD19^-CFP^-$) from the 'activated' culture (T_{FH} cells and B cells) or from the 'suppressed' culture (T_{FH} cells, B cells and T_{FR} cells) and performed transcriptional analysis (**Supplementary Fig. 2a**,**b**). Principal-component analysis demonstrated modest separation of activated B cells from suppressed B cells but no separation of activated T_{FH} cells from suppressed T_{FH} cells (**Fig. 2a**). We identified 1,171 genes that were expressed differentially (false-discovery rate (FDR)-adjusted *P* value, <0.05) by activated B cells relative to their expression in suppressed B cells, and 407 that were expressed differentially by activated T_{FH} cells relative to their expression in suppressed T_{FH} cells (**Fig. 2b** and **Supplementary Fig. 2c–e**).

We next investigated whether suppression by T_{FR} cells alters T_{FH} cell identity. We compiled a curated list of 'T_{FH} cell genes' encoding regulators of the differentiation and/or function of T_{FH} cells. The expression of many such genes, including those encoding the essential T_{FH} cell transcription factors Bcl6, Ascl2 and Tcf1 (encoded by *Tcf7*), was not attenuated during T_{FR} cell–mediated suppression (**Fig. 2c**). *Prdm1* (which encodes Blimp-1, a transcription factor that inhibits T_{FH} cell differentiation) was attenuated during T_{FR} cell–mediated suppression (**Fig. 2c**). The expression of *Bcl6* and *Cxcr5* was slightly elevated in T_{FH} cells after suppression (**Fig. 2c**). In contrast, expression of *Il4* and *Il21* was markedly reduced in T_{FH} cells during T_{FR} cell–mediated suppression (**Fig. 2c**), consistent with published findings[12]. These data suggested that suppressed T_{FH} cells still retained their T_{FH} cell program and either failed to upregulate, or actively downregulated, the expression of specific effector molecules. To confirm that the suppressed T_{FH} cells still maintained a T_{FH} cell transcriptional program, we performed single-sample gene-set–enrichment analysis (GSEA) with transcriptional signatures of T_{FH} cells[18]. Suppressed T_{FH} cells qualitatively retained their T_{FH} cell–like transcriptional signature (**Fig. 2d**). To determine if the

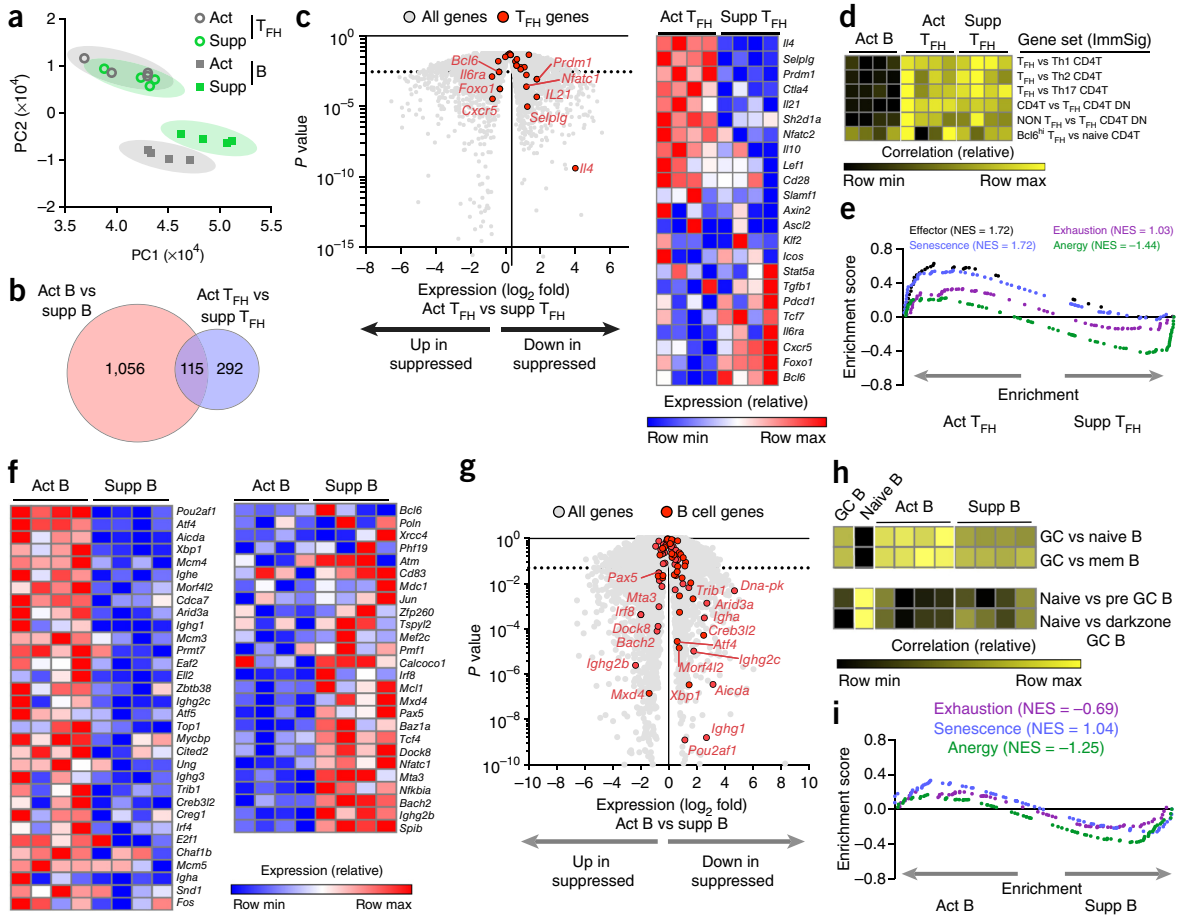

Figure 2 Suppressed T_{FH} cells and B cells retain transcriptional programs except for inhibition of genes encoding specific effector molecules. (**a**) Principal-component analysis of B cells and T_{FH} cells obtained from NP-OVA–immunized *Foxp3*^{GFP} mice and cultured for 4 d alone (activated (Act)) or with T_{FR} cells (suppressed (Supp)) from *Foxp3*^{GFP}*Actin*^{CFP} mice in the presence of NP-OVA, followed by sorting of B cells and T_{FH} cells. PC1 and PC2, principal components 1 and 2. (**b**) Comparisons of genes expressed differentially (FDR-adjusted *P* value, <0.05) in activated B cells versus suppressed B cells (left) or activated T_{FH} cells versus suppressed T_{FH} cells (right) or in both (middle). (**c**) Expression of all genes and 'T_{FH} cell genes' (key) in activated T_{FH} cells versus that in suppressed T_{FH} cells in culture, plotted against *P* values (left), and expression of 'T_{FH} cell genes' (right margin) in activated and suppressed T_{FH} cells (above plots) (right). (**d**) Single-sample GSEA showing correlation of results for activated B cells, activated T_{FH} cells and suppressed T_{FH} cells (above plot) to those of ImmSig gene sets (right margin; GEO accession codes, GSE11924, GSE16697, GSE21380 and GSE24574)[18]. (**e**) GSEA of effector T cells, senescent cells[45], exhaustion signatures[46,47] or anergy signatures (GEO accession codes, GSE2323) in activated T_{FH} cells and suppressed T_{FH} cells. (**f**) Expression of the 'B cell genes' (right margins) most upregulated or downregulated in activated and suppressed B cells. (**g**) Expression of all genes and 'B cell genes' (key) in activated B cells versus that in suppressed B cells, plotted against *P* values. (**h**) Single-sample GSEA (presented as in **d**) showing the correlation of results obtained for activated or suppressed B cells to ImmSig gene sets (GEO accession codes, GSE12366 and GSE12845). (**i**) GSEA (presented as in **e**) of gene sets for effector T cells, senescent T cells, exhaustion or anergy in activated versus suppressed B cells. Data are pooled from four experiments (each with one biological replicate of cells pooled from 20 mice).

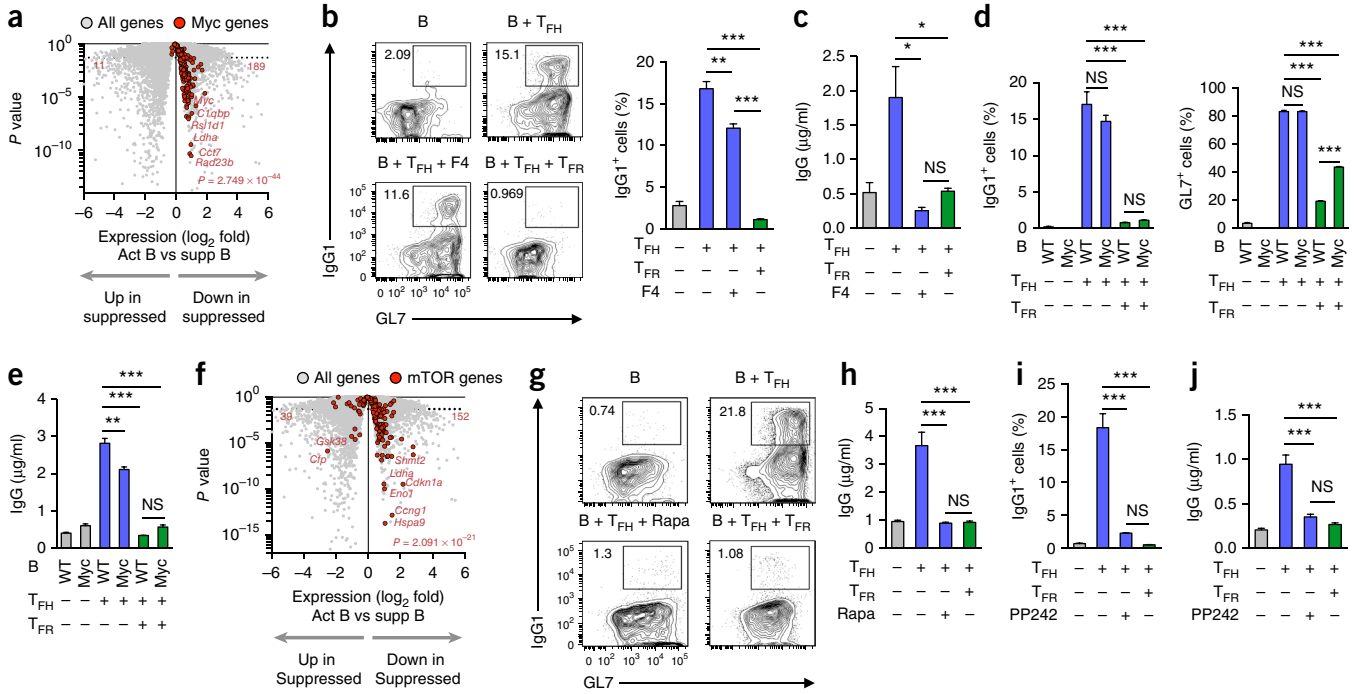

Figure 3 Inhibition of the Myc and mTOR pathways suppresses B cell effector function. (**a**) RNA-seq data (from **Fig. 2**) of all genes or genes encoding Myc targets (GSEA gene set 'Hallmark_MYC_TARGETS_V1') (key) in activated B cells versus that of suppressed B cells, plotted against *P* values. (**b**) Flow cytometry of cells from cultures of various combinations (above plots) of B cells, T_{FH} cells, and T_{FR} cells (as in **Fig. 1a**) in the presence (+ F4) or absence of the Myc inhibitor 10058-F4 (numbers in plots (left), as in **Fig. 1a**). (**c**) Concentration of IgG in cultures as in **b**. (**d**) Frequency of $IgG1^+$ cells (left) or $GL7^+$ cells (right) among B cells in cultures containing various combinations (below plot) of B cells expressing wild-type Myc (WT) or overexpressing Myc (Myc), T_{FH} cells and T_{FR} cells. (**e**) Concentration of IgG in supernatants of cultures as in **d**. (**f**) RNA-seq data (from **Fig. 2**) of all genes (gray) or genes encoding products involved in mTOR signaling (GSEA gene set 'Hallmark_MTORC1_SIGNALING') (key) in activated B cells versus that in suppressed B cells, plotted against *P* values. (**g**) Flow cytometry of cells from cultures of various combinations (above plots) of B cells, T_{FH} cells, and T_{FR} cells (as in **Fig. 1a**) in the presence (+ Rapa) or absence of the mTOR inhibitor rapamycin (numbers in plots, as in **Fig. 1a**). (**h**) Concentration of IgG in supernatants of cultures as in **g**. (**i**) Frequency of $IgG1^+$ B cells (gated as in **Fig.1a**) among cultures as in **b** incubated in the presence (+) or absence (–) of the mTOR inhibitor PP242 (below plots). (**j**) Concentration of IgG in supernatants of cultures as in **i**. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way ANOVA with Tukey's correction (**b**–**e**,**h**–**j**) or χ^2 test (**a**,**f**)). Data are from four experiments (**a**,**f**) or one experiment representative of three independent experiments with similar results (**b**–**e**,**g**–**j**; mean + s.e.m. of three technical replicates with cells pooled from 20 mice).

suppressed state in T_{FH} cells resembles anergy, senescence or exhaustion, we used GSEA to compare activated T_{FH} cells with suppressed T_{FH} cells. Activated T_{FH} cells showed enrichment for the expression of effector signatures (normalized enrichment score (NES) = 1.72; FDR < 0.001) and senescence signatures (NES = 1.72, FDR < 0.001), but suppressed T_{FH} cells did not show substantial enrichment for anergy signatures (NES = −1.45; FDR = 0.110) or exhaustion signatures (NES = 1.03; FDR = 0.664) (**Fig. 2e**). Therefore, the suppression of T_{FH} cells by T_{FR} cells resulted in a unique suppressed state that did not strongly resemble anergy or exhaustion.

Next we investigated whether B cells retained their effector program during suppression by T_{FR} cells. We found lower expression of *Ighg1*, *Ighg2c* and *Igha* by B cells after suppression by T_{FR} cells (**Fig. 2f**,**g**). *Ighg2b* transcripts were more abundant after such suppression (**Fig. 2f**,**g**); however, IgG2b was not increased at the protein level (data not shown). Three of the 'B cell genes' (which encode products involved in B cell function) most attenuated in B cells suppressed by T_{FR} cells were *Pou2af1* (which encodes a transcription factor essential for GC B cell formation[19]), *Xbp1* (which encodes a transcription factor important for the secretion of antibody[20]) and *Aicda* (which encodes the cytidine deaminase AID, the enzyme responsible for initiating CSR[21,22]) (**Fig. 2f**,**g**). *Pax5*, *Bach2* and *Irf8*, genes downregulated after B cells differentiate into plasma cells[23,24], had higher expression in B cells after suppression by T_{FR} cells (**Fig. 2f**,**g**), which suggested that T_{FR} cells might prevent such differentiation. However, *Mxd4*, which has high expression in plasma cells, was upregulated in suppressed B cells[23] (**Fig. 2f**,**g**). These data suggested that the gene-expression signature of suppressed B cells was more complex than inhibition of B cell subset differentiation.

Additional comparative analysis of activated and suppressed B cells revealed that suppressed B cells still retained their GC B cell signature (**Fig. 2h**). Moreover, suppressed B cells did not show enrichment for exhaustion signatures (NES = −0.69; FDR = 0.994), senescence signatures (NES = 1.05; FDR = 0.561) or anergy signatures (NES = 1.27; FDR = 0.837) (**Fig. 2i**). Together these data indicated that B cells and T_{FH} cells maintained their transcriptional signature when suppressed by T_{FR} cells but that the expression of specific effector molecules was actively downregulated.

We next used GSEA to determine if any non–effector-subset-related pathways were altered in B cells and T_{FH} cells during suppression by T_{FR} cells. Activated B cells showed substantial enrichment for the expression of sets of genes that are targets of the oncoprotein Myc or that encode products involved in signaling via the metabolic checkpoint kinase complex MTORc1, oxidative phosphorylation and glycolysis, relative to the expression of these genes in suppressed B cells (**Supplementary Table 1**). Activated T_{FH} cells also showed enrichment for the expression of genes that are targets of the transcription factor E2F or that encode products involved in

glycolysis and signaling via MTORc1, relative to the expression of these genes in suppressed T_{FH} cells; however, this enrichment was not as strong as that in B cells (**Supplementary Table 1**). Thus, suppression by T_{FR} cells led to substantial changes in pathways associated with metabolism in B cells and T_{FH} cells.

Suppression by T_{FR} cells alters B cell Myc and mTOR pathways

We next investigated whether the Myc pathway was altered in B cells suppressed by T_{FR} cells, since genes that are targets of Myc were one of the gene sets whose expression was most attenuated in suppressed B cells (NES = 2.77; FDR<0.0001), and Myc has roles in metabolism[25] and is essential for GC reactions[26,27]. In B cells, most genes that are targets of Myc showed lower expression during suppression by T_{FR} cells (**Fig. 3a**). To determine if suppressing Myc signaling in B cells could recapitulate the suppression of B cells by T_{FR} cells, we cultured B cells and T_{FH} cells with the Myc inhibitor 10058-F4 (ref. 28). 10058-F4 slightly reduced CSR but robustly attenuated the secretion of IgG, to a similar extent to that achieved with T_{FR} cells (**Fig. 3b**,**c**). We also investigated whether overexpression of Myc would result in the resistance of B cells to suppression by T_{FR} cells. We obtained B cells

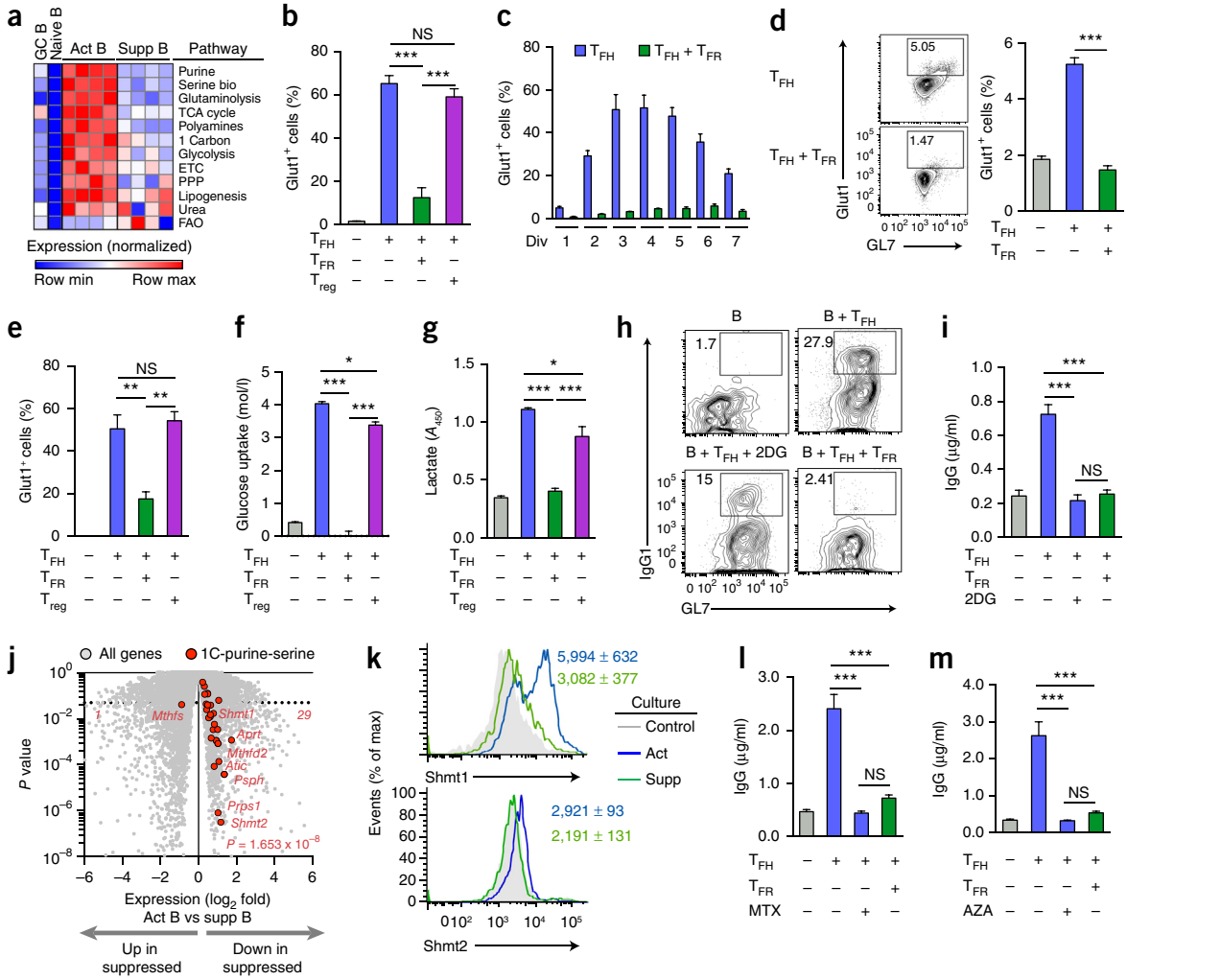

Figure 4 T_{FR} cells inhibit multiple metabolic pathways in B cells. (**a**) Expression of genes (RNA-seq data from **Fig. 2**) encoding products involved in various metabolic pathways (right margin), in GC B cells and activated or suppressed B cells, normalized to that of naive B cells (**Supplementary Fig. 4**). (**b**) Frequency of Glut1$^+$ cells among B cells from activated cultures (B cells plus T_{FH} cells), T_{FR} cell–suppressed cultures (B cells plus T_{FH} cells plus T_{FR} cells), or T_{reg} cell–suppressed cultures (B cells plus T_{FH} cells plus T_{reg} cells) (below plot). (**c**) Frequency of Glut1$^+$ cells among B cells cultured with T_{FH} cells only or with T_{FH} cells plus T_{FR} cells (key), gated to indicate CellTrace Violet division peaks (horizontal axis). (**d**) Flow cytometry of B cells added to cultures of T_{FH} cells or T_{FH} cells plus T_{FR} cells (left margin) at day 3 and assessed 20 h later (left), and frequency of Glut1$^+$ cells among those B cells (right). Numbers in outlined areas (left) indicate percent Glut1$^+$ B cells. (**e**) Frequency of Glut1$^+$ cells among T_{FH} cells in cultures as in **b**. (**f**) Glucose uptake by cultures as in **b**, presented as the concentration of glucose in supernatants. (**g**) Lactate production by cultures as in **b**, presented as the abundance of lactate in supernatants (absorbance at 450 nm (A_{450})). (**h**) Flow cytometry of cultures as in **b** in the presence (+ 2DG) or absence of 2DG (numbers in plots, as in **Fig. 1a**). (**i**) Concentration of IgG in supernatants of cultures as in **h**. (**j**) Expression (RNA-seq data from **Fig. 2**) of all genes or genes encoding enzymes involved in one-carbon, serine or purine metabolism (key) in activated B cells versus that in suppressed B cells, plotted against *P* values. (**k**) Expression of Shmt1 (top) and Shmt2 (bottom) in B cells added to cell-free cultures (Control) or to activated or suppressed cultures (key) at day 3 and assessed 20 h later. Numbers in plots indicate mean fluorescent intensity (mean ± s.e.m.) of Shmt1 ($P = 0.0167$) or Shmt2 ($P = 0.0104$) (colors match key). (**l**,**m**) Concentration of IgG in supernatants of cultures as in **i** in the presence or absence of methotrexate (MTX) (**l**) or azathioprine (AZA) (**m**). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test (**d**), one-way ANOVA with Tukey's correction (**b**,**e**–**i**,**l**,**m**) or χ^2 test (**j**)). Data are from four experiments (**a**,**j**) or one experiment representative of three independent experiments with similar results (**b**–**i**,**k**–**m**; mean + s.e.m. of three technical replicates with cells pooled from 20 mice).

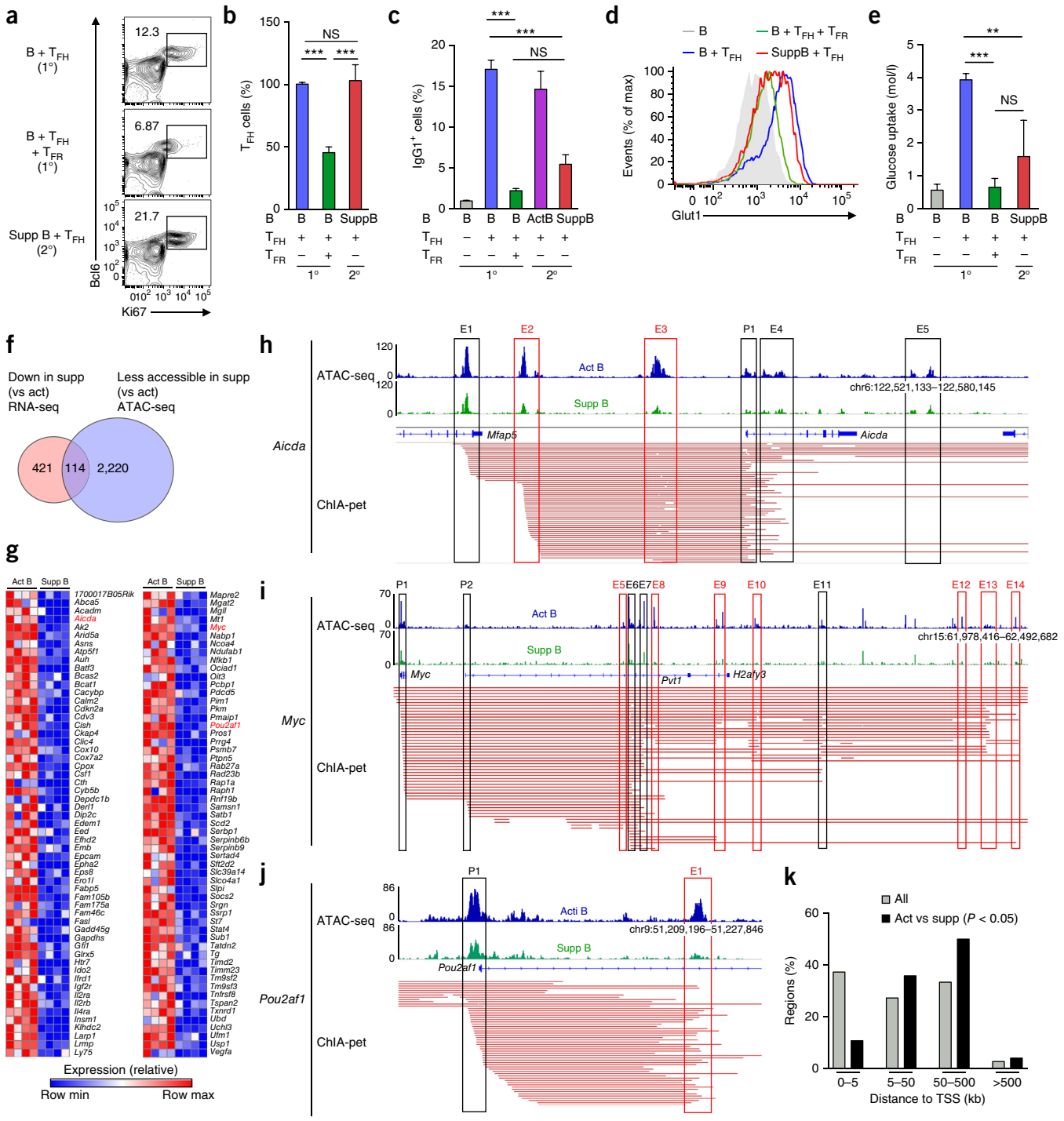

Figure 5 Suppression by T_{FR} cells results in sustained inhibition and epigenetic changes in B cells. (**a**) Flow cytometry of T_{FH} cells from primary (1°) cultures of B cells and T_{FH} cells only (activated; top) or those cells together with T_{FR} cells (suppressed; middle) incubated for 3 d, and in reactivation cultures (2°) generated by the culture of new T_{FH} cells with B cells sorted from the activated culture (ActB; not shown) or suppressed culture (SuppB; bottom) (numbers adjacent to outlined areas as in **Fig. 1j**). (**b**) Frequency of T_{FH} cells in cultures as in **a**. (**c**) Frequency of $IgG1^+GL7^+$ B cells from cultures as in **a**. (**d**) Glut1 expression in B cells from cultures as in **a**. (**e**) Glucose uptake in cultures as in **a**, measured in supernatants. (**f**) Genes with lower expression in B cells after suppression relative to that in activated B cells (Down in Supp vs Act (left); RNA-seq data as in **Fig. 2**) or loci with evidence of chromatin inaccessibility in the corresponding comparison (right; by ATAC-seq), and the overlap of those two gene sets (middle). (**g**) RNA-seq analysis of transcripts with lower expression in suppressed B cells that also showed less accessibility by ATAC-seq analysis (red denotes genes shown in more detail in the panels that follow). (**h–j**) ATAC-seq peaks and ChIA-pet annotated B cell regulome gene tracks[33] for *Aicda* (**h**), *Myc* (**i**) and *Pou2af1* (**j**); red boxes indicate significant downregulation ($P < 0.05$). (**k**) Distance of ATAC-seq peaks from TSSs for all peaks or peaks less accessible in suppressed B cells (key). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way ANOVA with Tukey's correction). Data are pooled from ten independent experiments, each with cells pooled from 20 mice (**a–e**; mean + s.e.m. in **b**,**c**,**e**), or are representative of two independent experiments (**f–k**).

from wild-type mice or *Igh*^{Myc} mice (which overexpress Myc on B cells[29]) and cultured them with T_{FH} and T_{FR} cells. Overexpression of Myc did not restore CSR or antibody secretion (**Fig. 3d**,**e**). However, GL7 expression in B cells was partially restored by Myc overexpression (**Fig. 3d**), which suggested that overexpression of Myc might prevent some minor aspects of the suppression of B cells by T_{FR} cells.

Since we found lower expression of components of the mTORc1 pathway in B cells and T_{FH} cells during suppression by T_{FR} cells (**Fig. 3f** and **Supplementary Table 1**) and the mTOR pathway promotes protein synthesis during activation and has been linked to the enhancement of cellular metabolism[30], we also investigated whether blocking mTOR would suppress antibody production. We cultured B cells and T_{FH} cells with either the mTORc1 inhibitor rapamycin or T_{FR} cells. Rapamycin potently diminished CSR and antibody production to a degree similar to that achieved with T_{FR} cells (**Fig. 3g**,**h**). The mTORc1-mTORc2 inhibitor PP242 similarly reduced CSR and antibody production (**Fig. 3i**,**j**). Therefore, inhibiting the mTOR pathway suppressed B cell responses to a degree similar to that achieved with T_{FR} cells.

Suppression of B cell metabolism by T_{FR} cells

Since our GSEA revealed that the expression of genes encoding products associated with the glycolysis, oxidative-phosphorylation, and the Myc and mTOR pathways was lower in B cells suppressed by T_{FR} cells, we further analyzed metabolic alterations in such cells. The expression of genes encoding products involved in various metabolic pathways, including serine biosynthesis, purine metabolism, one-carbon metabolism, the tricarboxylic acid cycle and glycolysis, as well as subunits of the mitochondrial electron-transport chain, was lower in suppressed B cells than in activated B cells (**Fig. 4a** and **Supplementary Figs. 3** and **4**). This suggested that central metabolic and anabolic pathways were defective in B cells suppressed by T_{FR} cells.

We assessed the effects of suppression by T_{FR} cells on glycolysis, since this pathway is essential for antibody production[31]. We first compared expression of the glucose transporter Glut1 in B cells suppressed by T_{FR} cells. T_{FR} cells (but not T_{reg} cells) suppressed Glut1 expression in B cells (**Fig. 4b**), which suggested that the T_{FR} cells suppressed B cell glycolysis. The suppression of Glut1 expression (and CSR) in B cells by T_{FR} cells was not due to an increase in the abundance of non-dividing cells (which have low expression of Glut1), because comparison of B cells that had undergone the same number of cell divisions revealed diminished Glut1 expression and CSR in the suppressed B cells (**Fig. 4c** and **Supplementary Fig. 5**). In addition, the suppression of CSR and metabolism by T_{FR} cells occurred before the changes in B cell proliferation; when we analyzed B cells that had been added to activated or suppressed cultures and harvested 20 h later (before the first cell division), B cells in suppressed cultures had lower expression of Glut1 and IgG1 than that of B cells in activated cultures (**Fig. 4d** and **Supplementary Fig. 5**). These studies indicated a decoupling of CSR and metabolism from proliferation and demonstrated that the suppression of CSR and metabolism in B cells by T_{FR} cells could occur independently of changes in proliferation. T_{FR} cells (but not T_{reg} cells) also caused lower expression of Glut1 in T_{FH} cells (**Fig. 4e**).

We next analyzed glucose uptake, as measured by glucose in the culture supernatants. Glucose use was much lower in cultures containing T_{FR} cells than in cultures without T_{FR} cells (**Fig. 4f**). We also measured lactate production, since a large fraction of glucose-derived carbon is secreted from cells in the form of lactate. Lactate production was also much lower in cultures with T_{FR} cells than in cultures without T_{FR} cells (**Fig. 4g**). We next investigated whether inhibiting glycolysis could recapitulate the effects of suppression by T_{FR} cells. We cultured B cells and T_{FH} cells with 2-deoxyglucose (2DG), a glucose analog that inhibits glycolysis. 2DG robustly suppressed antibody production, similar to results obtained by the suppression of B cells by T_{FR} cells (**Fig. 4h**,**i**). In addition, glutaminolysis was lower in the presence of T_{FR} cells than in their absence, and inhibition of glutaminolysis resulted in diminished antibody production (**Supplementary Fig. 5**).

Since one-carbon, serine and purine metabolism were attenuated during T_{FR} cell–mediated suppression, we assessed these pathways in more detail. In proliferating cells, the folate-mediated one-carbon metabolism pathway catabolizes serine to generate one-carbon units (10-formyl tetrahydrofolate) for *de novo* purine biosynthesis. The expression of all genes encoding enzymes involved in one-carbon metabolism and the serine-biosynthetic pathway was significantly lower in suppressed B cells than in activated B cells (**Fig. 4j** and **Supplementary Fig. 3**). Reduced expression of genes encoding products involved in one-carbon metabolism in B cells suppressed by T_{FR} cells was not due to altered proliferation, because the expression of Shmt1 and Shmt2 (a cytosolic enzyme and mitochondrial enzyme, respectively, in one-carbon metabolism that are upregulated within hours of lymphocyte activation[32]) were attenuated before the first cell division (**Fig. 4k**). Next we sought to determine whether inhibitors of purine metabolism could recapitulate suppression by T_{FR} cells. Methotrexate (an inhibitor that targets Dhfr, an enzyme essential for purine biosynthesis) or azathioprine (a purine analog) robustly suppressed antibody production when added to cultures of B cells and T_{FH} cells (**Fig. 4l**,**m**). These data demonstrated that the T_{FR} cells suppressed multiple metabolic pathways in B cells and that inhibiting these pathways was able to recapitulate the suppression of antibody production by T_{FR} cells.

T_{FR} cell suppression of B cells results in epigenetic changes

Next we investigated whether the effects of the suppression of B cells by T_{FR} cells were durable and persisted in the absence of T_{FR} cells. After 3 d of culture, we sorted B cells from activated or suppressed B cell conditions and cultured those B cells with new T_{FH} cells in a secondary culture (**Supplementary Fig. 6**). We compared B cells from those secondary cultures with B cells from primary cultures (**Fig. 5a**). T_{FH} cells cultured with suppressed B cells in the secondary cultures had much higher co-expression of Ki67 and Bcl6 than that of T_{FH} cells cultured with B cells and T_{FR} cells in the primary cultures and were more numerous than the T_{FH} cells in those primary cultures (**Fig. 5a**,**b**). Thus, B cells suppressed by T_{FR} cells were able to facilitate T_{FH} cell population expansion after T_{FR} cells were no longer present.

We also analyzed IgG1 in B cells from those secondary cultures. Suppressed B cells cultured with T_{FH} cells were still severely defective in their ability to undergo CSR (**Fig. 5c**). Although most cultures of suppressed B cells that were reactivated in secondary culture with T_{FH} cells contained less secreted antibody than that of activated primary cultures, in some cases antibody could be found in these cultures (**Supplementary Fig. 6**), which suggested that a small population of B cells might have escaped suppression. Glycolysis was also defective after restimulation of the suppressed B cells; Glut1 expression was lower in suppressed B cells that were reactivated in secondary culture with T_{FH} cells than in activated B cells from primary cultures (**Fig. 5d**). Additionally, the uptake of glucose was much lower by suppressed B cell secondary cultures than by activated primary cultures (**Fig. 5e**). Together these data indicated that the B cells suppressed by T_{FR} cells had defects in CSR and metabolism that continued in the absence of continued contact with T_{FR} cells.

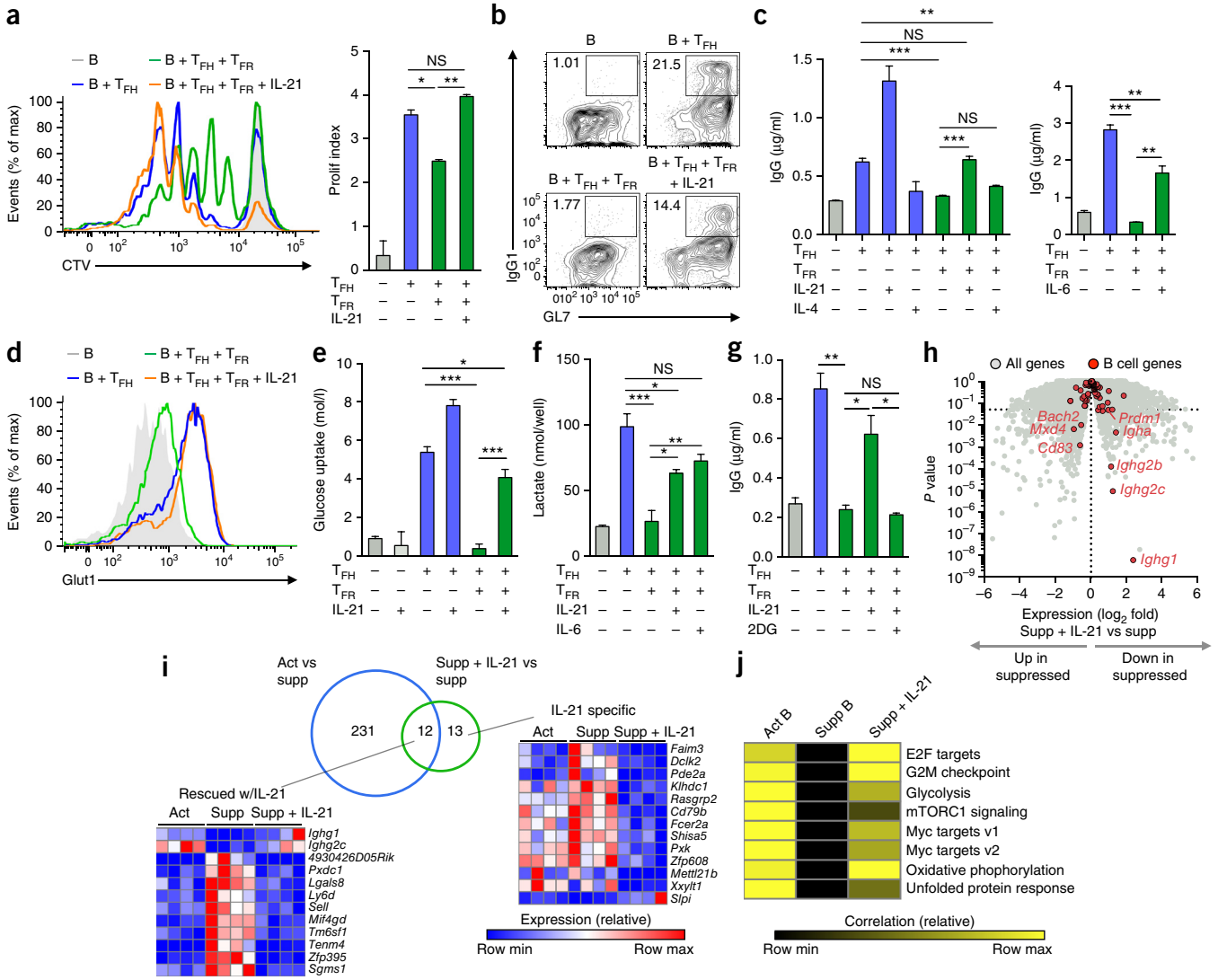

Figure 6 IL-21 can overcome T_{FR} cell–mediated suppression of B cell metabolism and antibody production. (**a**) Proliferation of B cells in cultures of B cells alone or with T_{FH} cells alone, T_{FH} cells and T_{FR} cells, or T_{FH} cells and T_{FR} cells with IL-21 (key), incubated with anti-CD3 and anti-IgM (left), and proliferation (Prolif) index of those cultures (right). (**b**) Flow cytometry of B cells from cultures as in **a** (numbers in plots, as in **Fig. 1a**). (**c**) Concentration of IgG in supernatants of cultures as in **a** with the addition of IL-4 (left) or IL-6 (right) to some cultures (below plots). (**d**) Glut1 expression in B cells from cultures as in **a**. (**e**) Glucose uptake by cultures as in **a** (presented as in **Fig. 4f**). (**f**) Lactate production by cultures as in **c**, measured in supernatants. (**g**) Concentration of IgG in supernatants of cultures as in **a**, with the addition of 2DG to some cultures (below plot). (**h**) Expression of all genes or 'B cell genes' (key) in B cells sorted from suppressed cultures (B cells plus T_{FH} cells plus T_{FR} cells) as in **a** (but with NP-OVA instead of anti-CD3 and IgM) containing IL-21 (Supp + IL-21), relative to that in B cells sorted from similar suppressed cultures without IL-21 (Supp), plotted against *P* values. (**i**) All genes expressed differentially (FDR-adjusted *P* value, <0.05) in activated B cells versus suppressd B cells (left) or in comparisons as in **h** (right) or both (middle). (**j**) Collapsed single-sample GSEA correlation plots for RNA-seq data (FDR < 0.05) of activated B cells (Act), suppressed B cells (Supp) or suppressed B cells from cultures containing IL-21 (Supp + IL-21). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way ANOVA with Tukey's correction). Data are from one experiment representative of three independent experiments with similar results (**a**–**d**,**g**; mean + s.e.m. of three technical replicates of cells pooled from 20 mice), are pooled from three experiments (**e**, mean + s.e.m.) each with two technical replicates, or are from four experiments, each with one biological replicate with cells pooled from 20 mice (**h**–**j**).

To investigate whether the durable defects in the effector function and metabolism of B cells might have been caused by epigenetic changes enforced by T_{FR} cells during suppression, we assessed chromatin accessibility by assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq). Comparison of activated B cells versus suppressed B cells, both from primary cultures, revealed 2,334 genes with evidence of less accessibility in the suppressed B cell condition (**Fig. 5f**). Of those genes, 114 also had lower expression by RNA-seq analysis (**Fig. 5g**). Genes encoding B cell functional proteins and upstream regulators of metabolism, such as *Aicda*, *Myc* and *Pou2af1*, showed evidence of chromatin inaccessibility in the suppressed condition (**Fig. 5g**), suggestive of epigenetic modification.

To explore how the genes identified above might be regulated, we overlaid our ATAC-seq data with the 'B cell regulome' (a collection of confirmed interactions of promoters with long-range enhancers in B cells) defined by ChIA-pet techniques[33]. The *Aicda* locus showed less accessibility in suppressed B cells than in activated B cells in two regions, one ~8 kilobases (kb) and another ~21 kb upstream of the *Aicda* transcriptional start site (TSS) (**Fig. 5h**). These enhancer

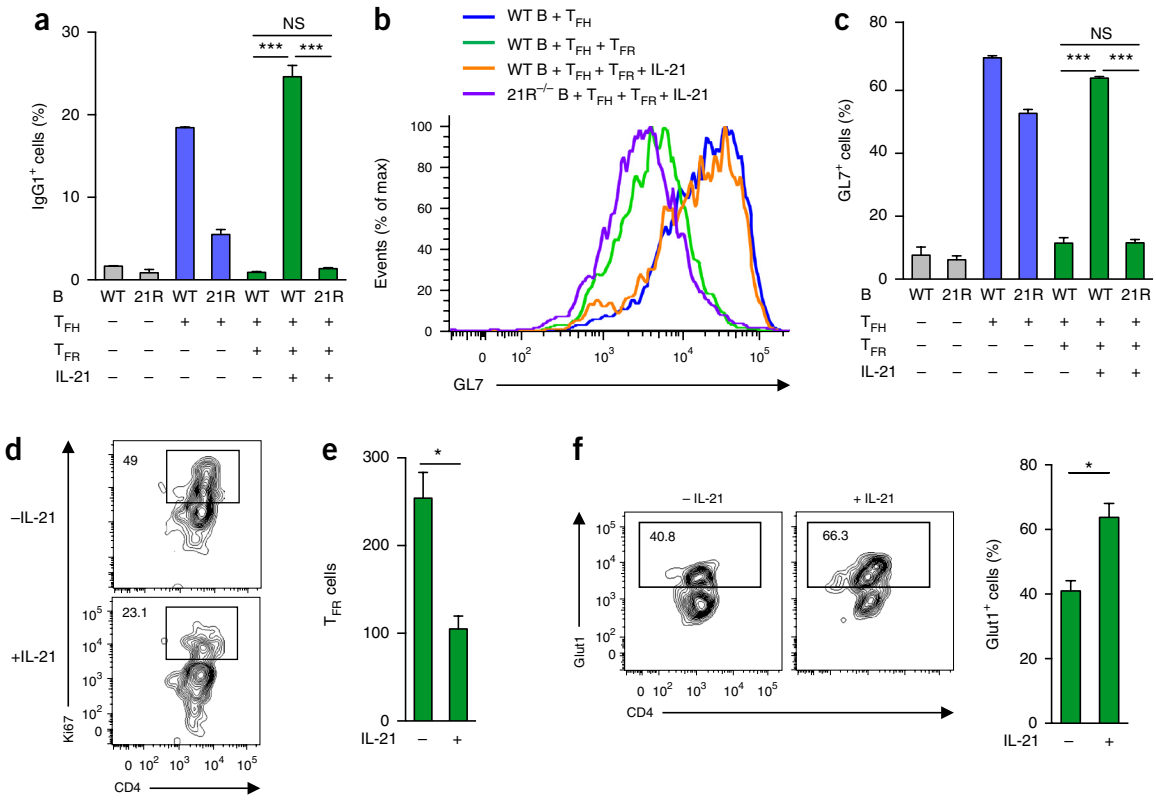

Figure 7 IL-21 directly stimulates B cells and inhibits T_{FR} cells. (**a**) Frequency of IgG1$^+$GL7$^+$ B cells in cultures (pre-gated as CD19$^+$IA$^+$CD4$^-$) of wild-type B cells (WT) or *Il21r*$^{-/-}$ B cells (21R) cultured with T_{FH} cells alone, T_{FH} and T_{FR} cells, or T_{FH} cells and T_{FR} cells plus IL-21 (below plot). (**b**) GL7 expression in B cells from cultures as in **a**. (**c**) Frequency of GL7$^+$ cells among B cells from cultures as in **a**. (**d**) Flow cytometry of T_{FR} cells from cultures (pre-gated as CD4$^+$Foxp3$^+$ cells) of B cells, T_{FH} cells and T_{FR} cells without IL-21 (–IL-21) or with IL-21 (+IL-21). Numbers adjacent to outlined areas indicate percent Ki67$^+$CD4$^+$ T_{FR} (activated) cells (gated as CD4$^+$Foxp3$^+$). (**e**) T_{FR} cells remaining at the end of culture as in **d**. (**f**) Glut1 expression in T_{FR} cells from cultures as in **d** (left), and frequency of Glut$^+$ T_{FR} cells from cultures (right). Numbers adjacent to outlined areas (left) indicate percent Glut$^+$ T_{FR} cells. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way ANOVA with Tukey's correction (**a**-**c**) or Student's *t*-test (**e**,**f**)). Data are from two independent experiments (**a**–**c**, mean + s.e.m. of three technical replicates with cells pooled from 20 mice) or one experiment representative of three independent experiments with similar results (**d**–**f**; mean + s.e.m. of three technical replicates with cells pooled from 20 mice).

regions are essential for AID expression[33,34]. We identified seven putative enhancer regions in the *Myc* locus that were less accessible in B cells suppressed by T_{FR} cells than in activated B cells (**Fig. 5i**). We found one peak that was less accessible in the *Pou2af1* locus that was located in an intronic region in between exon 1 and exon 2 (**Fig. 5j**). We noticed that many of the less-accessible regions in suppressed B cells were not at the TSS but were at sites of long-range enhancers (**Fig. 5h**–**j**). When we quantified the distribution of all ATAC-seq regions relative to the location of the TSS, we found that less-accessible regions in B cells suppressed by T_{FR} cells tended to be further away from the TSS than were all regions identified by ATAC-seq (**Fig. 5k**). These data indicated that genes encoding products critical for B cell function, but not those encoding key metabolic enzymes, showed evidence of epigenetic regulation during suppression by T_{FR} cells.

IL-21 can overcome T_{FR} cell–mediated suppression of B cells

We investigated whether IL-21 was able to overcome the suppression of B cells by T_{FR} cells because IL-21 is essential in the GC reaction, is suppressed by T_{FR} cells, can coordinate lipid and glucose metabolism in fat tissue and can inhibit T_{reg} cells[35,36]. The addition of IL-21 to suppression cultures 'rescued' the attenuation of B cell proliferation and CSR by T_{FR} cells (**Fig. 6a**,**b**). IL-21 and IL-6, but not IL-4, restored the secretion of IgG by cultures with T_{FR} cells (**Fig. 6c**). The restoration of CSR by IL-21 and IL-6 was not due to enhanced proliferation (**Supplementary Fig. 7**).

We reasoned that if IL-21 were able to restore antibody production, it would also 'rescue' defects in metabolism during suppression by T_{FR} cells, since our studies showed that metabolism and B cell function were interconnected. Glut1 expression was restored in both B cells and T_{FH} cells when IL-21 was added to cultures containing T_{FR} cells (**Fig. 6d** and data not shown). Moreover, IL-21 restored glucose uptake (**Fig. 6e**), and both IL-21 and IL-6 increased lactate production in cultures with T_{FR} cells (**Fig. 6f**). The 'rescue' by IL-21 required glycolysis, as 2DG completely prevented the restoration of antibody production by IL-21 in suppressed cultures (**Fig. 6g**). Therefore, IL-21 rendered B cells resistant to T_{FR} cell–mediated suppression at least in part by enhancing glycolysis.

To identify which B cell transcripts were 'rescued' by IL-21, we performed RNA-seq analysis of B cells from activated cultures (T_{FH} cells and B cells), suppressed cultures (T_{FH} cells, B cells and T_{FR} cells) and 'IL-21 rescue' cultures (T_{FH} cells, B cells and T_{FR} cells plus IL-21). Transcripts encoding IgG isotypes showed evidence of significant upregulation in 'IL-21 rescue' cultures relative to their expression in suppressed cultures (**Fig. 6h**). Only 12 genes were expressed differentially in B cells from suppressed cultures versus those from 'IL-21 rescue' cultures and also in B cells from suppressed cultures versus those from activated cultures, and of these genes, only *Ighg1* and

Ighg2c were 'rescued' with IL-21 (**Fig. 6i** and **Supplementary Fig. 7**). Metabolic pathways such as Myc, glycolysis and oxidative phosphorylation showed evidence of some restoration by IL-21 (**Fig. 6j**).

We next determined whether IL-21 restored antibody production by acting directly on B cells. For this, we performed suppression assays using B cells lacking the receptor for IL-21 (*Il21r*$^{-/-}$). Although baseline antibody responses were lower in cultures of *Il21r*$^{-/-}$ B cells with T_{FH} cells than in those of *Il21r*$^{+/+}$ B cells with T_{FH} cells, we found no evidence that antibody responses were restored in suppressed cultures by the addition of IL-21 (**Fig. 7a**). Loss of IL-21R did not abolish the increase in the number of B cells observed in suppressed cultures after the addition of IL-21 but did prevent the restoration of CSR and Glut1 expression (**Supplementary Fig. 7**). Since upregulation of GL7 is a robust indicator of B cell activation, we compared its expression in suppressed cultures containing either *Il21r*$^{+/+}$ B cells or *Il21r*$^{-/-}$ B cells and supplemented with IL-21. IL-21 restored GL7 expression when *Il21r*$^{+/+}$ B cells were present but not when *Il21r*$^{-/-}$ B cells were present (**Fig. 7b**,**c**), which suggested that signaling through IL-21 into the B cells was essential for the restoration of B cell activation.

To investigate if IL-21 also affected T_{FR} cells in the cultures, we compared T_{FR} cells from the various cultures noted above. T_{FR} cells expressed much less Ki67 in the presence of IL-21 than in its absence, and there were significantly fewer T_{FR} cells in suppressed cultures with IL-21 than in those without it (**Fig. 7d**,**e**); this suggested that IL-21 inhibited the cell cycling of T_{FR} cells. Glut1 expression was higher in T_{FR} cells in the presence of IL-21 than in its absence (**Fig. 7f**), which suggested that IL-21 was able alter the metabolism of T_{FR} cells as well as their activation. These data demonstrated that IL-21 was able to act on both B cells and T_{FR} cells to overcome the suppression of B cells by T_{FR} cells.

DISCUSSION

Although T_{FR} cells potently control antibody production, the mechanisms by which T_{FR} cells exert these immunoregulatory functions are not clear. Here we demonstrated that T_{FR} cells inhibited the expression of a subset of genes encoding effector molecules and components of multiple metabolic pathways in B cells and T_{FH} cells. The suppression of B cells by T_{FR} cells was long-lived and persisted even when T_{FR} cells were no longer present. IL-21 was able to overcome the suppressive effect of T_{FR} cells by increasing B cell metabolism and by directly inhibiting T_{FR} cells. These data demonstrate that T_{FR} cells control antibody responses by imposing a previously undefined suppressive state in T_{FH} cells and B cells.

T_{FR} cells did not alter the effector program in T_{FH} cells but potently suppressed transcripts encoding IL-4 and IL-21, two important effector cytokines that stimulate antibody responses. Unexpectedly, the expression of genes encoding T_{FH} cell transcription factors, such as Bcl6 (ref. 37), Tcf1 (ref. 38) and Ascl2 (ref. 39), was either unchanged or elevated in suppressed T_{FH} cells relative to that in activated T_{FH} cells, and expression of the gene encoding Blimp1, which inhibits T_{FH} cells[37,40], was lower in suppressed T_{FH} cells than in activated T_{FH} cells. Suppressed B cells had minimal changes in the expression of genes encoding effector B cell molecules. However, the genes that were attenuated during suppression encode proteins with important roles in effector B cell function, including Pou2af1 (ref. 19) and AID. The suppressed B cells might have been arrested in a state of late activation, since genes encoding the late-activation and GC B cell transcription factors Pax5, Bach2 and Irf8 had higher expression after suppression.

The most substantial change in suppressed B cells was lower expression of genes encoding products involved in multiple metabolic pathways, including glycolysis, glutaminolysis, one-carbon metabolism, serine biosynthesis and purine biosynthesis, as well as their upstream mediators Myc and mTOR. It is unclear if T_{FR} cells prevented their upregulation or actively downregulated genes encoding components of these pathways. Our findings are consistent with published work showing that the activation of B cells induces glycolysis in B cells, which might aid in antibody production[31,41]. Glycolysis and the mTOR pathway were also inhibited in T_{FH} cells suppressed by T_{FR} cells. Glycolysis and the mTOR pathway can inhibit T_{FH} cell differentiation[42], but our work indicated that these pathways also might be important for the effector function of T_{FH} cells.

Our data suggested that T_{FR} cells might suppress T_{FH} cells and B cells by interrupting bidirectional costimulation and linked recognition during the formation of T cell–B cell immunological synapses[2,17]. We hypothesize that interactions of T_{FR} cells with T_{FH} cells and B cells might allow sufficient activation signals to maintain T_{FH} cell and B cell transcriptional programs but not to support effector function.

The suppression of B cells by T_{FR} cells resulted in a durable suppressive state in which there were prolonged defects in CSR and glycolysis. Interestingly, suppressed B cells that were reactivated were still able to stimulate T_{FH} cells. Therefore, the suppressed B cell state uncoupled two main effector functions of B cells: activation of T_{FH} cells and CSR. The durability of the suppression achieved by T_{FR} cells led us to hypothesize that T_{FR} cells can inhibit B cells through epigenetic modification. *Aicda*, *Myc* and *Pou2af1* showed considerable chromatin inaccessibility in suppressed B cells. We hypothesize that epigenetic modification of these three genes might result in prolonged defects in B cell function.

IL-21 is an important cytokine produced by T_{FH} cells that stimulates antibody production in GCs. IL-21, but not the related cytokine IL-4, overcame T_{FR} cell–mediated suppression by stimulating B cell metabolism and function. Increased glucose metabolism was necessary for the restoration of B cell antibody production by IL-21, which demonstrated a link between metabolism and B cell effector function. IL-21 was also able to inhibit T_{FR} cells, consistent with published studies showing that IL-21 can inhibit other types of T_{reg} cells[35,36]. IL-21 enhanced Glut1 expression on T_{FR} cells, which suggested that IL-21 might alter T_{FR} cell metabolism and thereby reduce suppressive capacity, as has been observed in T_{reg} cells deficient in the inositol phosphatase PTEN, which have enhanced glycolytic activity and diminished ability to suppress B cell responses[43,44]. Therefore, IL-21 has multifaceted roles in rendering B cells and T_{FH} cells insensitive to T_{FR} cell–mediated suppression.

In summary, our data have demonstrated that T_{FR} cells induce a unique suppressed transcriptional state in T_{FH} cells and B cells and that IL-21 can overcome this suppressive state. Understanding the mechanisms that regulate antibody production has the potential to identify new strategies for enhancing beneficial antibody responses and limiting pathogenic antibody responses.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: RNA-seq and ATAC-seq data, GSE82003.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.T.S. performed all experiments and analyzed data; N.R.-H. performed metabolic-flux analysis; N.R.-H. and M.H. provided technical help on metabolic pathways; V.R.J. provided technical help on RNA-seq experiments; D.R.S. and W.N.H. prepared ATAC-seq samples and provided technical assistance on ATAC-seq analysis; S.M. provided technical help; W.S. and V.K.K. provided *Il21r*$^{-/-}$ mice and technical help; N.C. provided the RNA-seq library-preparation protocol and provided technical help; and P.T.S. and A.H.S. conceived of the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Linterman, M.A. *et al.* Foxp3^{+} follicular regulatory T cells control the germinal center response. *Nat. Med.* **17**, 975–982 (2011).
2. Sage, P.T. & Sharpe, A.H. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol.* **36**, 410–418 (2015).
3. Chung, Y. *et al.* Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat. Med.* **17**, 983–988 (2011).
4. Wollenberg, I. *et al.* Regulation of the germinal center reaction by Foxp3^{+} follicular regulatory T cells. *J. Immunol.* **187**, 4553–4560 (2011).
5. Crotty, S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* **41**, 529–542 (2014).
6. De Silva, N.S. & Klein, U. Dynamics of B cells in germinal centres. *Nat. Rev. Immunol.* **15**, 137–148 (2015).
7. McHeyzer-Williams, M., Okitsu, S., Wang, N. & McHeyzer-Williams, L. Molecular programming of B cell memory. *Nat. Rev. Immunol.* **12**, 24–34 (2011).
8. Crotty, S. A brief history of T cell help to B cells. *Nat. Rev. Immunol.* **15**, 185–189 (2015).
9. Crotty, S. Follicular helper CD4 T cells (TFH). *Annu. Rev. Immunol.* **29**, 621–663 (2011).
10. Stone, E.L. *et al.* ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* **42**, 239–251 (2015).
11. Choi, Y.S. *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* **34**, 932–946 (2011).
12. 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).
13. 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).
14. 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).
15. Wing, J.B., Ise, W., Kurosaki, T. & Sakaguchi, S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* **41**, 1013–1025 (2014).
16. Sage, P.T. & Sharpe, A.H. In vitro assay to sensitively measure T(FR) suppressive capacity and T(FH) stimulation of B cell responses. *Methods Mol. Biol.* **1291**, 151–160 (2015).
17. Sage, P.T. & Sharpe, A.H. T follicular regulatory cells. *Immunol. Rev.* **271**, 246–259 (2016).
18. Godec, J. *et al.* Compendium of immune signatures identifies conserved and species-specific biology in response to inflammation. *Immunity* **44**, 194–206 (2016).
19. Schubart, D.B., Rolink, A., Kosco-Vilbois, M.H., Botteri, F. & Matthias, P. B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal centre formation. *Nature* **383**, 538–542 (1996).
20. Shaffer, A.L. *et al.* XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* **21**, 81–93 (2004).
21. Muramatsu, M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553–563 (2000).
22. Revy, P. *et al.* Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102**, 565–575 (2000).
23. Shi, W. *et al.* Transcriptional profiling of mouse B cell terminal differentiation defines a signature for antibody-secreting plasma cells. *Nat. Immunol.* **16**, 663–673 (2015).
24. Qian, J. *et al.* B cell super-enhancers and regulatory clusters recruit AID tumorigenic activity. *Cell* **159**, 1524–1537 (2014).
25. Stine, Z.E., Walton, Z.E., Altman, B.J., Hsieh, A.L. & Dang, C.V. MYC, Metabolism, and cancer. *Cancer Discov.* **5**, 1024–1039 (2015).
26. Dominguez-Sola, D. *et al.* The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. *Nat. Immunol.* **13**, 1083–1091 (2012).
27. Calado, D.P. *et al.* The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. *Nat. Immunol.* **13**, 1092–1100 (2012).
28. Wang, J. *et al.* Evaluation of the antitumor effects of c-Myc-Max heterodimerization inhibitor 100258-F4 in ovarian cancer cells. *J. Transl. Med.* **12**, 226 (2014).
29. Adams, J.M. *et al.* The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318**, 533–538 (1985).
30. Powell, J.D., Pollizzi, K.N., Heikamp, E.B. & Horton, M.R. Regulation of immune responses by mTOR. *Annu. Rev. Immunol.* **30**, 39–68 (2012).
31. Caro-Maldonado, A. *et al.* Metabolic reprogramming is required for antibody production that is suppressed in anergic but exaggerated in chronically BAFF-exposed B cells. *J. Immunol.* **192**, 3626–3636 (2014).
32. Ron-Harel, N. *et al.* Mitochondrial biogenesis and proteome remodeling promote one-carbon metabolism for T cell activation. *Cell Metab.* **24**, 104–117 (2016).
33. Kieffer-Kwon, K.R. *et al.* Interactome maps of mouse gene regulatory domains reveal basic principles of transcriptional regulation. *Cell* **155**, 1507–1520 (2013).
34. Huong, T. *et al.* In vivo analysis of Aicda gene regulation: a critical balance between upstream enhancers and intronic silencers governs appropriate expression. *PLoS One* **8**, e61433 (2013).
35. Fabrizi, M. *et al.* IL-21 is a major negative regulator of IRF4-dependent lipolysis affecting Tregs in adipose tissue and systemic insulin sensitivity. *Diabetes* **63**, 2086–2096 (2014).
36. Peluso, I. *et al.* IL-21 counteracts the regulatory T cell-mediated suppression of human CD4^{+} T lymphocytes. *J. Immunol.* **178**, 732–739 (2007).
37. Johnston, R.J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* **325**, 1006–1010 (2009).
38. Choi, Y.S. *et al.* LEF-1 and TCF-1 orchestrate T_{FH} differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat. Immunol.* **16**, 980–990 (2015).
39. Liu, X. *et al.* Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* **507**, 513–518 (2014).
40. Oestreich, K.J., Mohn, S.E. & Weinmann, A.S. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a T_{FH}-like gene profile. *Nat. Immunol.* **13**, 405–411 (2012).
41. Kunisawa, J. *et al.* Mode of bioenergetic metabolism during B cell differentiation in the intestine determines the distinct requirement for vitamin B1. *Cell Rep.* **13**, 122–131 (2015).
42. Ray, J.P. *et al.* The interleukin-2-mTORc1 kinase axis defines the signaling, differentiation, and metabolism of T felper 1 and follicular B helper T cells. *Immunity* **43**, 690–702 (2015).
43. Shrestha, S. *et al.* Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat. Immunol.* **16**, 178–187 (2015).
44. Huynh, A. *et al.* Control of PI(3) kinase in T_{reg} cells maintains homeostasis and lineage stability. *Nat. Immunol.* **16**, 188–196 (2015).
45. Fridman, A.L. & Tainsky, M.A. Critical pathways in cellular senescence and immortalization revealed by gene expression profiling. *Oncogene* **27**, 5975–5987 (2008).
46. McKinney, E.F., Lee, J.C., Jayne, D.R., Lyons, P.A. & Smith, K.G. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature* **523**, 612–616 (2015).
47. Wherry, E.J. *et al.* Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* **27**, 670–684 (2007).

ONLINE METHODS

Mice. Wild-type C57Bl/6J and Igh-Myc mice were purchased from Jackson Laboratories. *Foxp3*$^{IRES-GFP}$ (*Foxp3*^{GFP}) on the C57Bl/6 background have been published previously[48]. *Il21r*$^{-/-}$ mice on the C57Bl/6 background were from the Kuchroo Lab. *Actin*^{CFP}*Foxp3*^{GFP} mice on the C57Bl/6 background have been published previously[12]. All mice were between 6 and 8 weeks of age at the time of experiments and were housed in a SPF facility. Each individual experiment contained one sex of mice, but replicates were performed with males or females. All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Health Guidelines.

Immunization. Mice were immunized with 100 μg NP-OVA (Biosearch Technologies) emulsified in H37RA CFA s.c. in the mouse flanks as previously described[12,16]. Mice were sacrificed 7 d later and inguinal lymph nodes were harvested.

Antibodies. The following antibodies were used for surface staining at 4 °C: anti-CD4 (BioLegend, 1:200, RM4-5)[12], anti-ICOS (BioLegend, 1:200, 15F9)[12], anti-CD19 (BioLegend, 1:200, 6D5)[12], anti-CXCR5 biotin (BD Biosciences, 1:100, 2G8)[12], GL7 (BD Biosciences, 1:200, GL-7)[12], CD69 (BioLegend, 1:200, H1.2F3)[12], and anti-IA (BioLegend, 1:200, M5/114.15.2)[12]. For further CXCR5 detection, streptavidin-BV421 (BioLegend, 1:400, 405225) was used at 4 °C. 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)[12], anti-Foxp3 (eBiosciences, 1:200, FJK-16S)[12], anti-Ki67 (BD Biosciences, 1:100, B56)[12], anti-Glut1 (Abcam, 1:200, EPR3915)[31], anti-Shmt2 (Abcam, 1:200, ab64417,)[32] or anti-Shmt1 (Novus Biologicals, 1:200, NBP2-32173,)[32] at 4 °C. In some cases, a donkey anti-rabbit BV421 secondary was used (BioLegend, 1:400, 406410).

Sorting. Single-cell suspensions were diluted in PBS supplemented with 1% FBS with 1 mM EDTA. T_{FH} cells and T_{FR} cells were isolated by enriching with CD4$^+$ cells by magnetic positive selection (Miltenyi Biotec). CD4$^+$ enriched cells were then stained and sorted as follows: T_{FH} (CD4$^+$ICOS$^+$CXCR5$^+$Foxp3$^-$CD19$^-$), T_{FR} (CD4$^+$ICOS$^+$CXCR5$^+$Foxp3$^+$CD19$^-$). In some cases, an alternative gating strategy was used; T_{FH} (CD4$^+$ICOS$^+$CXCR5$^+$GITR$^-$CD19$^-$), T_{FR} (CD4$^+$ICOS$^+$CXCR5$^+$GITR$^+$CD19$^-$) as previously described[12,13]. B cells were isolated from flow-through from CD4$^+$ selection, which was then positively selected using CD19 beads (Miltenyi Biotec). For wild-type versus Igh-Myc B cells, B cells were enriched with magnetic selection and then sorted as CD19$^+$GL7$^-$ cells to rule out effects of spontaneous activation in Igh-Myc mice.

Suppression assay. *In vitro* suppression assays were performed as described previously[12,14,16]. *Foxp3*^{GFP} reporter mice were immunized with NP-OVA, and 7 d later, dLN were harvested and CD19$^+$ B cells and CD4$^+$CXCR5$^+$ICOS$^+$Foxp3$^-$CD19$^-$ T_{FH} cells were purified by cell sorting. B cells and T_{FH} cells were cultured alone (activated) or with CD4$^+$ICOS$^+$CXCR5$^+$CD19$^-$Foxp3$^+$ T_{FR} cells (suppressed) sorted from NP-OVA–immunized *Foxp3*^{GFP} or *Foxp3*^{GFP} *Actin*^{CFP} mice. 5×10^4 B cells, 3×10^4 T_{FH} cells and/or 1.5×10^4 T_{FR} cells were plated in 96-well plates along with 2 μg/ml anti-CD3 (2c11, BioXcell) and 5 μg/ml anti-IgM (115-006-020, Jackson Immunoresearch). For some experiments, 20 μg/ml NP-OVA was added to wells instead of anti-CD3 and anti-IgM. For some studies, B cells were pre-labeled with the proliferation dye CellTrace Violet (Thermo Scientific). Cultures were harvested 6 d later unless specified otherwise. For analysis, B cells were gated as CD19$^+$IA$^+$CD4$^-$ cells, T_{FH} cells were gated as CD4$^+$IA$^-$CD19$^-$Foxp3$^-$ cells, and T_{FR} cells were gated as CD4$^+$IA$^-$CD19$^-$Foxp3$^+$ cells. For re-sorting for RNA-seq analysis of NP-OVA–containing cultures, B cells were gated as CD19$^+$IA$^+$CD4$^-$CFP$^-$ and T_{FH} cells were gated as CD4$^+$IA$^-$CD19$^-$CFP$^-$. For ATAC-seq analysis, B cells were harvested after 4 d of culture with NP-OVA and were gated as CD19$^+$IA$^+$CD4$^-$ cells. For *Igh* sequencing, B cells were harvested after 6 d of culture with NP-OVA and were gated as CD19$^+$IA$^+$CD4$^-$ cells. For re-culture of suppressed cells, suppressed cultures (which contained anti-CD3 and IgM) were harvested after 3 d and B cells were sorted as CD19$^+$IA$^+$CD4$^-$. 2.5×10^4 suppressed B cells (or freshly isolated B cells) were then cultured with freshly isolated 3×10^4 T_{FH} cells, prepared as above, in the presence of anti-CD3 and IgM. In some studies, small-molecule compounds were added to cultures: Myc inhibitor 10058-F4 (100 μM), mTOR inhibitors rapamycin (10 nM) and PP242 (200 nM), methotrexate (1 μM), azathioprine (50 μM) (all from Tocris Bioscience), the glycolysis inhibitor 2-deoxyglucose (2DG; 500 μM) and the glutaminolysis inhibitor BPTES (8 μM) (both from Sigma). In some experiments, recombinant IL-21, IL-4 or IL-6 (Peprotech) were added to wells (at 60–90 ng/ml).

RNA-seq. Samples were sorted as described above, and each replicate indicates a biological replicate that was prepared using different sets of mice on different experimental days. RNA-seq libraries were prepared (N.C. data not shown). RNA was isolated using MyOne Silane Dynabeads (Thermo Fisher Scientific). RNA was fragmented and then was bar-coded using 8-bp barcodes in conjunction 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 HiSeq 2000 or Illumina NextSeq sequencer (Illumina) according to the manufacturer's instructions, sequencing 50-bp reads. Analysis was performed using the CLC Genomics Workbench version 8.0.1 RNA-seq analysis software package (Qiagen). 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 heat maps.

ATAC-seq. 5×10^4 sorted cells per biological replicate were washed once in cold PBS and lysed in 50 μl cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1% IGEPAL CA-630). Lysed nuclei were incubated in Tn5 transposition reaction mix as described[49] and purified using MinElute Reaction Cleanup kit (Qiagen). ATAC-seq fragments were size-selected for fragments between 115 and 600 bp using Pippin Prep 2% Agarose Gel Cassettes and the Pippin Prep DNA Size Selection System (Sage Science). Post size-selection, ATAC libraries were amplified and Nextera sequencing primers ligated using Polymerase Chain Reaction (PCR). Finally, PCR primers were removed using Agencourt AMPure XP bead cleanup (Beckman Coulter/Agencourt) and library quality was verified using a Tapestation machine. Samples were sequenced on an Illumina NextSeq sequencer. Peak calling and analysis was performed using CLC Genomics Workbench 8.0.1 ChipSeq analysis software (Qiagen) by combining all samples (maximum *P* value for peak calling = 0.05). Gene-peak associations were determined using the GREAT software package using the basal extension method[50]. Visualization was performed using Integrated Genomics Viewer (Broad Institute) using the mm10 mouse genome build. Gene tracks were annotated with previously published ChIA-pet gene-enhancer associations in B cells[33].

Microscopy. *In vitro* suppression assays were performed as above except B cells were pre-labeled with eFluor 670 proliferation dye (eBioscience) and cells were cultured in cytek imaging chambers coated with celltak (VWR). After 4 d of culture, CellMask Orange (Thermo Fisher Scientific) was added to the imaging chambers to detect membrane of B cells, T_{FH} cells and T_{FR} cells. Samples were imaged on a Nikon spinning-=disk confocal microscope using a 40× objective and standard lasers and filters. The *z*-stacks were converted to projections using ImageJ software. B cells were identified as blue (eFluor 670) and red (CellMask Orange), T_{FR} cells were identified as green (*Foxp3*$^{IRES-GFP}$) and red (CellMask Orange), and T_{FH} cells were identified as red (CellMask Orange). **Supplementary Video 1** is representative of imaging studies repeated for three independent biological replicates.

IgH sequencing. B cells were sorted from suppression assays as above, and IgH sequencing was performed using the Adaptive Biotechnologies ImmunoSeq mouse IgH sequencing platform (Adaptive Biotechnologies).

GSEA. For GSEA analysis, RNA-seq data were converted to human nomenclature and compared to GSEA mSigDatabases including Hallmarks, C3 and C5 collections using standard settings (Broad Institute). For enrichment plots of specific gene sets, pathways were analyzed along with 20 randomized gene sets to ensure specificity. Single sample GSEA was performed in GenePattern using indicated gene sets (Broad Institute).

ELISA. ELISA measurements of total IgG from culture supernatants were performed as described previously[12,16].

Statistics. Most statistical tests were performed using Prism 6.0 (GraphPad) using Student's two-tailed unpaired *t*-test or one-way ANOVA with Tukey's correction as specified assuming Gaussian distribution. Statistics for RNA-seq and ATAC-seq were performed using CLC Genomics Workbench (Qiagen). Statistics for gene-set enrichment was performed in GSEA (Broad Institute). Statistics for Volcano plots were performed in Microsoft excel using a χ^2 test. Samples were not randomized and investigators were not blinded to sample identity, and there was no exclusion of data. Sample sizes were chosen as the minimum number of mice (typically 20) to sort enough cells to perform three to four technical replicate stimulation or suppression assays.

48. Bettelli, E. *et al*. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238 (2006).
49. Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y. & Greenleaf, W.J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat. Methods* **10**, 1213–1218 (2013).
50. McLean, C.Y. *et al*. GREAT improves functional interpretation of cis-regulatory regions. *Nat. Biotechnol.* **28**, 495–501 (2010).

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