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

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Abstract

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

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Author Contributions

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

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Introduction

Humoral immunity is critical for the clearance of pathogens and is the basis for protection elicited by vaccines. An effector subset of CD4⁺ T regulatory (T_{reg}) cells called T follicular regulatory (T_{FR}) cells has been identified. T_{FR} cells migrate to the B cell follicle and inhibit antibody production^{1, 2, 3, 4}. T_{FR} cells express the chemokine receptor CXCR5, the transcription factors Bcl6 and Foxp3, and have high surface expression of the ICOS costimulatory receptor and the PD-1 coinhibitory receptor². CXCR5, ICOS, Bcl6 and PD-1 (but not Foxp3) are also highly expressed by T follicular helper (T_{FH}) cells, which stimulate antibody responses⁵. Importantly, the ratio of T_{FH} and T_{FR} cells functionally predicts the magnitude of antibody responses in a wide range of disease states in mice and humans². 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 where 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, which depends on T_{FH} cells. T_{FH} cells interact with cognate GC B cells in a process called linked recognition⁸. T_{FH} cells supply key cytokines such as IL-4 and IL-21, as well as costimulatory molecules such as CD40L to the B cell⁹. These signals strongly activate the B cell which then cycles between the light zone and dark zone of the GC during affinity maturation⁶. 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 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. We are only beginning to understand mechanisms by which T_{FR} cells exert their inhibitory effects². T_{FR} cells suppress IL-21 and IL-4 production 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 T_{FR} cell suppressive functions, since T_{FR} cells lacking CTLA-4 have severely 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 capacity¹³.

Here we show that T_{FR} cells induce a distinctive suppressive state in T_{FH} and B cells in which effector molecules and metabolic pathways are suppressed, but global effector programs are maintained, and that IL-21 can overcome T_{FR} cell-mediated suppression by enhancing B cell metabolism and inhibiting T_{FR} cells. These data provide mechanistic insights into how T_{FR} cells suppress T_{FH} and B cells, and identify ways to circumvent T_{FR} cell suppression.

Results

B cells suppressed by T_{FR} cells undergo early activation

To define mechanisms of suppression of T_{FH} and B cells by T_{FR} cells, we utilized an *in vitro* suppression assay in which T_{FR} cells are cultured with T_{FH} and B cells, leading to suppression of both T_{FH} and B cell responses^{12, 14, 16}. T_{FR}

(CD4⁺ICOS⁺CXCR5⁺CD19⁻FoxP3⁺) cells were cultured with B (CD19⁺) and T_{FH} (CD4⁺ICOS⁺CXCR5⁺CD19⁻FoxP3⁻) cells from NP-OVA immunized *Foxp3*^{GFP} mice along with anti-CD3 and anti-IgM (Supplementary Fig. 1a–b). We measured robust upregulation of the GC B cell marker GL7 on B cells, CSR to IgG1, and substantial quantities of secreted IgG when B cells were cultured with T_{FH} cells alone (Fig. 1a). When T_{FR} cells were added, CSR, GL7 expression and secreted of antibody were diminished.

CD4⁺CXCR5⁻ICOS⁻FoxP3⁺ T_{reg} cells were not able to suppress as efficiently as T_{FR} cells (Fig. 1b), as previously reported¹². T_{FR} cells also suppressed CSR of B cells in response to specific antigen (Supplementary Fig. 1c). Furthermore, T_{FR} cell suppression of CSR required cell contact, as supernatant from T_{FR} cultures could not suppress B cells (Fig. 1c). Time-lapse microscopy of cultures revealed that T_{FR} cells closely interacted with both T_{FH} and B cells (Fig. 1d and Supplementary video 1). These data suggest that T_{FR} cells may physically disrupt T_{FH} and B cell linked recognition during suppression^{2, 17}. Therefore, our culture system is a robust model for studying synchronized T_{FR} cell-mediated suppression of T_{FH} and B cells.

We first investigated whether T_{FR} cells prevent initial B cell activation by using cell trace violet (CTV) dye dilution of B cells to assess 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 could not inhibit proliferation of B cells in co-cultures of only T_{FR} and B cells (without T_{FH} cells) and LPS + IL4 (Fig. 1f). We also analyzed CD69 expression and found that CD69 was upregulated in B cells whether or not T_{FR} cells were present (Fig. 1g). Studies with the active caspase reagent zVAD revealed that T_{FR} cells do not enhance B cell apoptosis as a means of suppression (Fig. 1h). These data demonstrate that early activation of B cells still occurs even in the presence of T_{FR} cells.

To determine if T_{FR} cells alter somatic hypermutation, we performed our *in vitro* suppression assay using NP-OVA. We found a low frequency of mutations in B cells when cultured with T_{FH} cells, and that T_{FR} cells reduced somatic hypermutation in B cells (Fig. 1i). T_{FR} cells (but not T_{reg} cells) also suppressed T_{FH} cell activation as evidenced by reduced Bcl6 and Ki67 expression (Fig. 1j). Taken together, these data demonstrate that T_{FR} cells allow initial B cell activation, but suppress downstream effector responses including CSR and antibody production.

T_{FR} cells inhibit specific genes but not effector programs

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 and T_{FH} cells, we performed RNA-seq transcriptional analysis. We cultured B and T_{FH} cells (from NP-OVA immunized *Foxp3*^{GFP} mice) with or without T_{FR} cells (from NP-OVA immunized *Actin*^{CFP} *Foxp3*^{GFP} mice) and NP-OVA. B (CD19⁺IA⁺CD4⁻) and T_{FH} (CD4⁺IA⁻CD19⁻CFP⁻) cells were sorted from the ‘activated’ culture (T_{FH} and B cells) or from the ‘suppressed’ culture (T_{FH}, B and T_{FR} cells) and transcriptional analysis was performed (Supplementary Figure 2a–b). Principal component analysis (PCA) demonstrated modest separation of activated and suppressed B cells, but not activated or suppressed T_{FH} cells (Fig. 2a). We identified 1171 differentially expressed genes (FDR-adjusted *P* value

<0.05) between activated and suppressed B cells, and 407 genes between activated and suppressed T_{FH} cells (Fig. 2b and Supplementary Figure 2c–e).

We next investigated whether T_{FR} suppression alters T_{FH} cell identity. We compiled a curated list of “T_{FH} genes” that encoded regulators of T_{FH} cell differentiation/function. The expression of many T_{FH} genes, including the essential T_{FH} transcription factors *Bcl6* and *Asc12*, were not changed during T_{FR} cell-mediated suppression (Fig. 2c). *Bcl6* and *Cxcr5* were slightly elevated in T_{FH} cells upon suppression. In contrast, *Il4* and *Il21* transcripts were markedly reduced in T_{FH} cells during T_{FR} cell suppression, consistent with previous findings¹². These data suggest that suppressed T_{FH} cells still retain their T_{FH} program, and either fail to upregulate, or actively downregulate, specific effector molecules. To confirm that suppressed T_{FH} cells still maintain a T_{FH} transcriptional program, we performed single sample gene set enrichment analysis (ssGSEA) with transcriptional signatures of T_{FH} cells¹⁸. Suppressed T_{FH} cells qualitatively retained their T_{FH}-like transcriptional signature (Fig. 2d). To determine if the suppressed state in T_{FH} cells resembles anergy, senescence or exhaustion, we compared activated and suppressed T_{FH} cells using GSEA. Activated T_{FH} cells were enriched for effector (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 (NES=-1.45, FDR=0.110) nor exhaustion signatures (NES=1.03, FDR=0.664)(Fig. 2e). Therefore, T_{FR} cell suppression of T_{FH} cells results in a unique suppressed state that does not strongly resemble anergy or exhaustion.

Next we examined whether B cells retain their effector program during suppression by T_{FR} cells. We found lower expression of *Ighg1*, *Ighg2c* and *Igha* (Fig. 2f–g). *Ighg2b* transcripts were more abundant upon suppression, however IgG2b was not increased at the protein level (data not shown). Three of the most attenuated B cell transcripts in B cells suppressed by T_{FR} cells were *Pou2af1* (encoding a transcription factor essential for GC B cell formation¹⁹), *Xbp1* (encoding a protein important for secretion of antibody²⁰) and *Aicda* (which encodes AID, the enzyme responsible for initiating CSR^{21, 22}). *Pax5*, *Bach2* and *IRF8*, genes downregulated upon differentiation to plasma cells^{23, 24}, were more highly expressed in B cells suppressed by T_{FR} cells, suggesting that T_{FR} cells may prevent differentiation of B cells to plasma cells. However, *Mxd4*, which is highly expressed in plasma cells, was upregulated in suppressed B cells²³. These data suggest that the suppressed B cell signature is more complex than inhibition of B cell subset differentiation.

Additional comparative analyses 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 enrich for exhaustion (NES=-0.69, FDR=0.994), senescence (NES=1.05, FDR= 0.561), nor anergy signatures (NES=1.27, FDR=0.837) (Fig. 2i). Together, these data indicate that B cells and T_{FH} cells maintain their transcriptional signature when suppressed by T_{FR} cells, but specific effector molecules are actively downregulated.

We next used GSEA to determine if any non-effector subset related pathways were altered in B and T_{FH} cells during suppression by T_{FR} cells. Activated B cells showed strong enrichment of gene sets for Myc targets, MTORc1 signaling, oxidative phosphorylation and glycolysis compared to suppressed B cells (Table 1). Activated T_{FH} cells also showed

enrichment of E2F targets, glycolysis and MTORc1 signaling compared to suppressed T_{FH} cells; however, this enrichment was not as strong as in B cells (Table 1). Thus, T_{FR} cell suppression leads to marked changes in pathways associated with metabolism in B and T_{FH} cells.

T_{FR} suppression alters Myc and mTOR pathways in B cells

We next tested whether Myc pathway genes were altered in B cells suppressed by T_{FR} cells, since this was one of the most attenuated gene sets in suppressed B cells (NES=2.77, FDR<0.0001), has roles in metabolism²⁵, and is essential for GC reactions^{26,27}. Most Myc target genes in B cells showed lower expression during T_{FR} cell suppression (Fig. 3a). To determine if suppressing Myc signals in B cells could recapitulate suppression of B cells by T_{FR} cells, we cultured B cells and T_{FH} cells with the Myc inhibitor 10058-F4 (F4)²⁸. F4 slightly reduced CSR, but robustly attenuated secreted IgG, to a similar extent as T_{FR} cells (Fig. 3b–c). We also tested whether Myc overexpression could result in resistance of B cells to suppression by T_{FR} cells. We cultured B cells from WT or *Igh^{Myc}* (Myc overexpressing²⁹) mice with T_{FH} and T_{FR} cells. Myc overexpression did not rescue CSR nor secreted antibody suppression (Fig. 3d–e). However, GL7 expression in B cells was partially rescued by Myc overexpression, suggesting that Myc overexpression may prevent some minor aspects of T_{FR} cell suppression of B cells.

Since we found lower expression of the mTORc1 pathway in B and T_{FH} cells during suppression by T_{FR} cells (Fig. 3f and Table 1) and the mTOR pathway promotes protein synthesis during activation and has been linked to enhancing cellular metabolism³⁰, we also investigated whether blocking the mTOR could suppress antibody production. We cultured B and T_{FH} cells with either the mTORc1 inhibitor rapamycin or T_{FR} cells. Rapamycin potently diminished CSR and antibody production similarly to 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 suppresses B cell responses similarly to T_{FR} cells.

T_{FR} cells suppress metabolism in B cells

Since our GSEA analysis revealed that the expression of genes associated with glycolysis, oxidative phosphorylation, Myc and mTOR pathways was lower in B cells suppressed by T_{FR} cells, we further analyzed metabolic alterations. Expression of a number of metabolic pathways including serine biosynthesis, purine metabolism, one-carbon metabolism, TCA cycle, subunits of the mitochondrial electron transport chain, and glycolysis in suppressed B cells was reduced (Fig. 4a and Supplementary Figures 3–4), suggesting that central metabolic and anabolic pathways are defective in B cells suppressed by T_{FR} cells.

We examined the effects of T_{FR} cell suppression on glycolysis since this pathway is essential for antibody production³¹. 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, suggesting that T_{FR} cells suppress B cell glycolysis (Fig. 4b). Suppression of Glut1 expression (and CSR) in B cells by T_{FR} cells was not due to an increase in non-dividing cells (which are low expressers of Glut1) (Fig. 4c and Supplementary Figure 5) because

comparisons of B cells that have undergone the same number of cell divisions revealed reduced Glut1 and CSR in the suppressed B cells. In addition, T_{FR} suppression of class switching and metabolism occurred prior to changes in B cell proliferation. When we analyzed B cells that had been added to activated or suppressed cultures and harvested 20 hours later (before the first cell division), B cells in suppressed cultures had lower Glut1 and IgG1 expression (Fig. 4d and Supplementary Figure 5). These studies decouple CSR and metabolism from proliferation, demonstrating T_{FR} suppression of CSR and metabolism in B cells can occur independently of changes in proliferation. T_{FR} cells (but not T_{reg} cells) also caused lower expression of Glut1 on T_{FH} cells (Fig. 4e).

We next analyzed glucose uptake in the culture supernatants. Glucose utilization was markedly decreased in cultures containing 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 markedly reduced in cultures with T_{FR} cells (Fig. 4g). We next investigated whether inhibiting glycolysis could recapitulate T_{FR} cell suppression. We cultured B and T_{FH} cells with 2-deoxyglucose (2DG), a glucose analog that inhibits glycolysis. 2DG robustly suppressed antibody production, similar to T_{FR} suppression of B cells (Fig. 4h–i). In addition, glutaminolysis was lower in the presence of T_{FR} cells and inhibition of glutaminolysis resulted in diminished antibody production (Supplementary Figure 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, folate-mediated one-carbon metabolism pathway catabolizes serine to generate one-carbon units (10-formyl THF) for de-novo purine biosynthesis. Genes encoding enzymes in one-carbon metabolism and serine biosynthetic pathway were all significantly lower in suppressed B cells (Fig. 4j and Supplementary Figure 3). Reduced expression of one-carbon metabolism genes in B cells suppressed by T_{FR} cells was not due to altered proliferation, because Shmt1 ($P=0.0167$) and Shmt2 ($P=0.0104$) (early upregulated cytosolic and mitochondrial enzymes in one-carbon metabolism, respectively³²) were attenuated before the first cell division (Fig. 4k). Inhibitors of purine metabolism could recapitulate T_{FR} cell suppression. Methotrexate (a purine synthesis inhibitor targeting Dhfr) or azathioprine (a purine analog) robustly suppressed antibody production when added to B and T_{FH} cell cultures (Fig. 4l–m). These data demonstrate that T_{FR} cells suppress multiple metabolic pathways in B cells, and inhibiting these pathways can recapitulate suppression of antibody production by T_{FR} cells.

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

Next we examined whether the effects of T_{FR} cell suppression on B cells are durable and persist in the absence of T_{FR} cells. After 3 days of culture, we sorted B cells from activated (“act B”) or suppressed B (“supp B”) conditions, and cultured these B cells with new T_{FH} cells in a secondary culture (Supplementary Figure 6). We compared B cells from these secondary cultures with B cells from primary cultures (Fig. 5a). T_{FH} cells cultured with supp B cells in the secondary cultures co-expressed much higher levels of Ki67 and Bcl6 and were more numerous compared to T_{FH} cells cultured with B and T_{FR} cells in the primary

cultures (Fig. 5a–b). Thus, B cells suppressed by T_{FR} cells are able to facilitate T_{FH} cell expansion after T_{FR} cells are no longer present.

We also analyzed IgG1 in B cells from these secondary cultures. Supp B cells cultured with T_{FR} cells were still severely defective in their ability to undergo CSR (Fig. 5c). Although most cultures contained less secreted antibody, in some cases antibody could be found in these cultures (Supplementary Figure 6), suggesting that a small population of B cells may escape suppression. Glycolysis was also defective upon restimulation of supp B cells. Glut1 expression was lower in supp B cells that were reactivated in secondary cultures with T_{FR} cells compared to activated B cells from primary cultures (Fig. 5d). Additionally, glucose uptake from suppressed B cell secondary cultures was much lower than activated primary cultures (Fig. 5e). Together, these data indicate that B cells suppressed by T_{FR} cells have defects in CSR and metabolism, which continue in the absence of continued T_{FR} cell contact.

To investigate whether the durable defects in B cell effector function and metabolism may be caused by epigenetic changes enforced by T_{FR} cells during suppression, we assessed chromatin accessibility using ATAC-sequencing (ATAC-seq). Comparisons of activated versus suppressed B cells from primary cultures revealed 2,334 genes with evidence of less accessibility in the suppressed B cell condition (Fig. 5f). Of these genes, 114 also had lower expression in 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, suggesting epigenetic modification (Fig. 5g).

To explore how these genes may be regulated, we overlaid our ATAC-seq data with the B cell regulome annotated using ChIA-pet³³ (which identifies interactions of promoters with long range enhancers). The *Aicda* locus showed less accessibility in two regions, ~8kb and ~21kb, upstream of the *Aicda* transcriptional start site (TSS) (Fig. 5h). These enhancer regions are essential for AID expression^{33, 34}. We identified 7 putative enhancer regions in the *Myc* locus that were less accessible in B cells suppressed by T_{FR} cells (Fig. 5i). We found one peak that was less accessible in the *Pou2af1* locus, which was located in an intronic region in between exons 1 and 2 (Fig. 5j). We noticed that many of the less accessible regions in suppressed B cells were not at the gene TSS, but at sites of long-range enhancers. When we quantified the distribution of all ATAC-seq regions in relation to the gene TSS, we found that less accessible regions in B cells suppressed by T_{FR} cells tended to be further away from the TSS compared to all regions (Fig. 5k). These data indicate that critical B cell function genes, but not the key metabolic enzymes, show evidence of epigenetic regulation during T_{FR} cell suppression.

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

We tested whether IL-21 can overcome T_{FR} cell suppression of B 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 attenuation of B cell proliferation and CSR by T_{FR} cells (Fig. 6a–b). IL-21

and IL-6, but not IL-4, rescued IgG secretion in cultures with T_{FR} cells (Fig. 6c). Rescue of CSR by IL-21 and IL-6 was not due to enhanced proliferation (Supplementary Figure 7).

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

To identify which B cell transcripts were rescued by IL-21, we performed RNA-seq analysis on B cells from activated (T_{FH} + B cells), suppressed (T_{FH}, B cells + T_{FR} cells), and IL-21 rescue (T_{FH}, B, T_{FR} cells + IL-21) cultures. Transcripts for IgG isotypes showed evidence of significant upregulation in IL-21 rescue cultures compared to suppressed cultures (Fig. 6h). Only 12 genes were differentially expressed in B cells from both suppressed versus IL-21 rescue cultures and suppressed versus activated cultures (FDR<0.05), and of these genes, only *Ighg1* and *Ighg2c* were rescued with IL-21 (Fig. 6i and Supplementary Figure 7). Metabolic pathways such as Myc, glycolysis and oxidative phosphorylation showed evidence of some rescue by IL-21 (Fig. 6j).

We next determined whether IL-21 rescued antibody production by acting directly on B cells by performing 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, we found no evidence that antibody responses were rescued by the addition of IL-21 (Fig. 7a). Loss of IL-21R did not abolish increases in the number of B cells, yet prevented rescue of class switch and Glut1 expression (Supplementary Figure 7). Since GL7 upregulation is a robust indicator of B cell activation, we compared GL7 upregulation in suppressed cultures with addition of IL-21. IL-21 rescued GL7 expression when WT B cells, but not when *Il21r*^{-/-} B cells, were present (Fig. 7b–c), suggesting that IL-21 signaling into B cells is essential for the rescue of B cell activation.

To test if IL-21 also affected T_{FR} cells in the cultures, we compared T_{FR} cells from cultures. T_{FR} cells expressed much less Ki67 in the presence of IL-21 and there were significantly fewer T_{FR} cells in suppressed cultures with IL-21, suggesting IL-21 inhibits cell cycling in T_{FR} cells (Fig. 7d–e). Glut1 expression was higher on T_{FR} cells in the presence of IL-21 suggesting that IL-21 can alter T_{FR} cell metabolism as well as activation (Fig. 7f). These data demonstrate that IL-21 can act on both B cells and T_{FR} cells to overcome suppression of B cells by T_{FR} cells.

Discussion

Although T_{FR} cells potentially control antibody production, the mechanisms by which T_{FR} cells exert these immunoregulatory functions are not clear. Here we demonstrate that T_{FR}

cells inhibit expression of a subset of effector genes and multiple metabolic pathways in B and T_{FH} cells. T_{FR} suppression of B cells is long-lived, and persists even when T_{FR} cells are no longer present. IL-21 can overcome T_{FR} suppression by increasing B cell metabolism, and directly inhibiting T_{FR} cells. These data demonstrate that T_{FR} cells control antibody responses by imposing a novel suppressive state in T_{FH} and B cells.

T_{FR} cells did not alter the effector program in T_{FH} cells, but potently suppressed transcripts for IL-4 and IL-21, two important effector cytokines that stimulate antibody responses. Surprisingly, genes encoding T_{FH} transcription factors such as Bcl6³⁷, Tcf1³⁸ (encoded by *Tcf7*) and Ascl2³⁹ were either unchanged or elevated in suppressed T_{FH} cells, and Blimp1 (*Prdm1*), which inhibits T_{FH} cells^{37, 40}, was lower in suppressed T_{FH} cells. Suppressed B cells had minimal changes in effector B cell genes. However, the genes that were attenuated during suppression encode proteins that have important roles in effector B cell function, including Pou2af1¹⁹ and AID. Suppressed B cells may be arrested in a state of late activation since transcripts for genes encoding late activation/GC B cell transcription factors Pax5, Bach2 and Irf8 were more highly expressed upon suppression.

The most substantial change in suppressed B cells was lower expression of multiple metabolic pathways, including glycolysis, glutaminolysis, one-carbon metabolism, serine biosynthesis, purine biosynthesis, as well as their upstream mediators, Myc and mTOR. It is unclear if T_{FR} cells prevent their upregulation or actively downregulate, these pathways. Our findings are consistent with previous work showing that B cell activation induces glycolysis in B cells, which may 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 differentiation⁴², but our work indicates that these pathways also may be important for T_{FH} cell effector functions.

Our data suggest that T_{FR} cells may suppress T_{FH} and B cells by interrupting bidirectional costimulation and linked recognition during T-B immune synapse formation^{2, 17}. We hypothesize that interactions of T_{FR} cells with T_{FH} and B cells may allow sufficient activation signals to maintain T_{FH} and B cell transcriptional programs, but not to support effector functions.

Suppression of B cells by T_{FR} cells results in a durable suppressive state in which there are prolonged defects in CSR and glycolysis. Interestingly, suppressed B cells that were reactivated could stimulate T_{FH} cells. Therefore, the suppressed B cell state uncouples two major effector functions of B cells: T cell activation and CSR. The durability of T_{FR} cell suppression led us to hypothesize that T_{FR} cells can inhibit B cells through epigenetic modification. *Aicda*, *Myc* and *Pou2af2* showed considerable chromatin inaccessibility in suppressed B cells. We hypothesize that epigenetic modification of these three genes may result in prolonged defects in B cell function.

IL-21 is an important cytokine produced by T_{FH} cells which stimulates antibody production in GC. IL-21, but not the related cytokine IL-4, can overcome T_{FR} cell-mediated suppression by stimulating B cell metabolism and function. Increased glucose metabolism was necessary for IL-21 rescue of antibody production, demonstrating the link between metabolism and B

cell effector functions. IL-21 also can inhibit T_{FR} cells, consistent with previous studies showing that IL-21 can inhibit other types of T_{reg} cells^{35, 36}. IL-21 enhanced Glut1 expression on T_{FR} cells, suggesting that IL-21 may alter T_{FR} cell metabolism and thereby reduce suppressive capacity as has been observed in PTEN deficient T_{reg} cells which possess enhanced glycolytic activity and decreased capacity to suppress B cell responses^{43, 44}. Therefore, IL-21 plays multifaceted roles in rendering B and T_{FH} cells insensitive to T_{FR} mediated suppression.

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

Methods

Mice

WT C57Bl/6J and Igh-Myc mice were purchased from Jackson Laboratories. *FoxP3^{ΔRES-GFP}* (*FoxP3^{GFP}*) on the C57Bl/6 background have been published previously⁴⁸. *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 previously¹². 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.

Immunizations

Mice were immunized with 100μg NP-OVA (Biosearch Technologies) emulsified in H37RA CFA s.c. in the mouse flanks as previously described^{12, 16}. Mice were sacrificed 7 days 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 1mM EDTA. T_{FH} 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 we have 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 WT 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 assays

In vitro suppression assays were performed as described previously^{12, 14, 16}. Briefly, *FoxP3*^{GFP} reporter mice were immunized with NP-OVA and 7 days later dLN were harvested and CD19⁺ B cells and CD4⁺CXCR5⁺ICOS⁺FoxP3⁻CD19⁻ T_{FH} cells were purified by cell sorting. B 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. 5×10⁴ B cells, 3×10⁴ T_{FH} cells and/or 1.5×10⁴ T_{FR} cells were plated in 96-well plates along with 2μg/ml anti-CD3 (BioXcell) and 5μg/ml anti-IgM (Jackson ImmunoResearch). For some experiments, 20μg/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 cell trace violet (Thermo Scientific). Cultures were harvested after 2–6 days as described. 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 resorting 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 days of culture with NP-OVA and were gated as CD19⁺IA⁺CD4⁻ cells. For Igh sequencing, B cells were harvested after 6 days of culture with NP-OVA and were gated as CD19⁺IA⁺CD4⁻ cells. For reculturing of suppressed cells, suppressed cultures (which contained anti-CD3/IgM) were harvested after 3 days and B cells were sorted as CD19⁺IA⁺CD4⁻. 2.5×10⁴ suppressed B cells (or freshly isolated B cells) were then cultured with freshly isolated 3×10⁴ T_{FR} cells, prepared as above, in the presence of anti-CD3/IgM. In some studies, small molecule compounds were added to cultures: Myc inhibitor 10058-F4 (100μM), mTOR inhibitors rapamycin (10nM) and PP242 (200nM), methotrexate (1μM), azathioprine (50μM) (all from Tocris Bioscience), glycolysis inhibitor 2-deoxyglucose (2DG; 500μM) and glutaminolysis inhibitor BPTES (8μM) (both from Sigma). In some experiments recombinant IL-21, IL-4 or IL-6 (Peprotech) were added to wells (at 60–90ng/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 library preparations were performed (Chevrier, N. *manuscript in preparation*). Briefly, RNA

was isolated using MyOne Silane Dynabeads (Thermo Fisher Scientific). RNA was fragmented, and barcoded using 8bp 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 Sequencing reactions were sequenced on an Illumina HiSeq 2000 or Illumina NextSeq sequencer (Illumina) according to manufacturer's instructions, sequencing 50bp reads. Analysis was performed using the CLC Genomics Workbench version 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.

ATAC-seq

50,000 sorted cells per biological replicate were washed once in cold PBS and lysed in 50µL cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% IGEPAL CA-630). Lysed nuclei were incubated in Tn5 transposition reaction mix as described⁴⁹ 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 utilizing the basal extension method⁵⁰. 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 cells³³.

Microscopy

In vitro suppression assays were performed as above except B cells were pre-labeled with eFluor670 proliferation dye (eBioscience) and cells were cultured in cytek imaging chambers coated with celltak (VWR). After 4 days of culture, cell mask orange (Thermo Fisher Scientific) was added to the imaging chambers to detect membrane of B, T_{FH} and T_{FR} cells. Samples were imaged on a Nikon spinning disk confocal microscope using a 43x objective and standard lasers/filters. Z-stacks were converted to projections using ImageJ software. B cells were identified as blue (eFluor670) and red (cell mask orange), T_{FR} cells were identified as green (*FoxP3*^{IRE5-GFP}) and red (cell mask orange), and Tfh cells were identified as red (cell mask orange). Movie shown was representative of imaging studies that were repeated in three different 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).

Gene Set Enrichment Analysis (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) utilizing Student's two-tailed unpaired t test or 1-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 Chi² test. Samples were not randomized or blinded, 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 3–4 technical replicate stimulation/suppression assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Suppressed B cells undergo early activation

(a) Quantification of IgG1⁺GL7⁺ B cells (left) and secreted antibody (right) from cultures of B and T_{FH} cells with or without T_{FR} cells in the presence of anti-CD3/IgM (Supplementary Figure 1a–b). (b) Quantification of secreted antibody as in (a) with the addition of a culture with CD4⁺ICOS⁺CXCR5⁺FoxP3⁺ T_{reg} cells. (c) Quantification of IgG1⁺GL7⁺ B cells from assays performed as in (a) with addition of supernatant from suppressed cultures (T_{FR} sup'). (d) Micrograph of culture containing B, T_{FH} and T_{FR} cells after 4 days. Scale bar = 5μm. (e) Proliferation of B cells measured by (cell trace violet) CTV dilution at day 4. B cells are

pregated on CD19⁺IA⁺CD4⁻. **(f)** Proliferation of B cells measured in which B cells were cultured with LPS and IL-4 and T_{FR} cells for 4 days. **(g)** CD69 expression on B cells from suppression assays as in (a). **(h)** Cell death in B cells measured by zVAD staining. **(i)** Somatic hypermutation in B cells during suppression assays as in (a) with the addition of NP-OVA. **(j)** Expression of Bcl6 and Ki67 in T_{FH} cells from suppression assays as in (a). Cells are pregated on CD4⁺FoxP3⁻CD19⁻IA⁻. *p<0.05, **p<0.01, ***p<0.001 (Student's t-test (**a,e,h**) or 1-way ANOVA with Tukey's correction (**b,c,j**)). Data are from one experiment (technical replicate suppression assays utilizing cells from 20 pooled mice) and are representative of 5 independent experiments with similar results (**a–b, h, j**) or 3 independent experiments (**c–g**; mean±s.e.m.).

Figure 2. Suppressed TFH and B cells retain transcriptional programs except for inhibition of specific effector genes

(a) Principal component analysis of activated and suppressed B and TFH cells. B and TFH cells, from immunized *FoxP3^{GFP}* mice, were cultured alone ("Activated") or with TFH cells ("Suppressed") from *FoxP3^{GFP} Actin^{CFP}* mice, in the presence of NP-OVA. After 4 days B and TFH cells were sorted. (b) Venn diagram of differentially expressed (FDR adjusted p < 0.05) genes in activated versus suppressed B and TFH cells. (c) (Left) Volcano plot of all genes (gray) or "TFH genes" (red) in TFH cells from cultures. (Right) Heat map showing TFH genes. (d) Single sample GSEA showing correlation of activated B, activated TFH and

suppressed T_{FH} cells to ImmSig gene sets (GSE11924, GSE16697, GSE21380, GSE24574)¹⁸. **(e)** GSEA enrichment plot for effector T cells, senescent cells⁴⁵, exhaustion^{46, 47} or anergy (GSE2323) signatures in activated versus suppressed T_{FH} cells. **(f)** Heat map of B cell genes in activated or suppressed B cells (only most upregulated or downregulated are shown). **(g)** Volcano plot showing data from all genes (gray) or B cell function genes (red) in activated versus suppressed B cells. **(h)** Single sample GSEA showing correlation of activated or suppressed B cells to ImmSig gene sets (GSE12366, GSE12845). **(i)** GSEA enrichment plot for gene sets for effector T cells, senescent T cells, exhaustion or anergy in activated versus suppressed B cells. Data are from 4 biological replicates on different experimental days, each containing cells from 20 pooled mice.

Figure 3. Inhibition of MYC and mTOR pathways suppress B cell effector functions

(a) Volcano plot showing RNA-seq data from Figure 2 with all genes (gray) or Myc pathway (Hallmark_MYC_TARGETS_V1) genes (red) in activated versus suppressed B cells. (b) Quantification of IgG1⁺GL7⁺ B cells from suppression assays performed as in Figure 1 with the addition of the Myc inhibitor 10058-F4 (F4). (c) IgG antibody concentration in culture supernatants from suppression assays in (b). (d) IgG1⁺GL7⁺ (left) or GL7 expression (right) in B cells from suppression assays in which control (WT) or Myc overexpressing (Myc) B cells were cultured with T_{FH} alone or with T_{FR} cells. (e) IgG concentration in culture supernatants from assays as in (c). (f) Volcano plot showing RNA-seq data from Figure 2 with all genes (gray) or mTOR signaling (Hallmark_MTORC1_SIGNALING) genes (red) in activated versus suppressed B cells. (g) Quantification of IgG1⁺GL7⁺ B cells from suppression assays performed as in Figure 1 with the addition of the mTOR inhibitor rapamycin (Rapa). (h) IgG concentration in culture supernatants from suppression assays in (b). (i) Quantification of IgG1⁺GL7⁺ B cells from suppression assays performed as in (b) with the addition of the mTOR inhibitor PP242. (j) IgG concentration in culture supernatants from suppression assays in (i). *p<0.05, **p<0.01, ***p<0.001 (using 1-way

ANOVA with Tukey's correction (**b,c,d,e,h,i,j**) or Chi² test (**a,f**). Data are from one experiment (technical replicate suppression assays utilizing cells from 20 pooled mice) and are representative of 3 independent experiments with similar results (**b–e, g–j** mean±s.e.m.).

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Figure 4. T_{FR} cells inhibit multiple metabolic pathways in B cells

(a) Heat map of metabolic pathways using RNA-seq data from Fig. 2 (normalized to naive B cells) (Supplementary Figure 4). (b) Glut1 expression in B cells from activated (B + T_{FH}), T_{FR}-suppressed (B + T_{FH} + T_{FR}), or T_{reg}-suppressed (B + T_{FH} + T_{reg}) cultures. (c) Glut1 expression in B cells, gated to indicate CTV division peaks. (d) Glut1 expression in B cells added to activated or suppressed day 3 cultures and harvested 20 hours later. (e) Glut1 expression in T_{FH} cells. (f) Glucose uptake in culture supernatants as in (b). (g) Lactate production in culture supernatants from cultures as in (b). (h–i) IgG⁺GL7⁺ B cells (left) and IgG concentration (right) in supernatants from cultures as in (b) to which 2DG was added. (j) Volcano plot of activated versus suppressed B cells showing all genes (gray) or genes encoding enzymes in one-carbon/serine/purine metabolism (red) using RNA-seq data from Fig. 2. (k) Shmt1 and Shmt2 expression in B cells added to activated or suppressed day 3 cultures and harvested 20 hours later. (l) IgG concentration in supernatants as in (c) with the addition of MTX. (m) IgG concentration in supernatants as in (c) with the addition of

AZA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t-test (**d**), 1-way ANOVA with Tukey's correction (**b**, **e-i**, **l-m**), or χ^2 test (**j**). Data are from one experiment (technical replicate suppression assays utilizing cells from 20 pooled mice) and are representative of 3 independent experiments with similar results (**b-i**, **k-m** mean $\pm$ s.e.m.).

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suppression from RNA-seq data (as in Fig. 2) and gene loci showing evidence of chromatin inaccessibility by ATAC-seq. **(g)** RNA-seq data for gene transcripts expressed less in suppressed B cells by RNA-seq and which also showed less accessibility by ATAC-seq analysis. **(h–j)** ATAC-seq peaks and ChIA-pet annotated B cell regulome gene track³³ for *Aicda*, *Myc* and *Pou2af1*. Red boxes indicate statistical downregulation ($P < 0.05$). **(k)** Distance of ATAC-seq peaks from gene transcriptional start sites (TSS) for all peaks or peaks less accessible in suppressed B cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (1-way ANOVA with Tukey's correction **(a–e)**). Data are pooled data from 10 independent experiments, each of which included cells from 20 pooled mice (**a–e** mean $\pm$ s.e.m.), or are representative of two independent experiments (**f–k**).

Figure 6. IL-21 can overcome T_{FR} -mediated suppression of B cell metabolism and antibody production

(a) (Left) B cell proliferation in B cells from cultures of T_{FH} cells alone, T_{FH} and T_{FR} cells, or T_{FH} and T_{FR} cells with IL-21. (Right) Quantification of proliferation index. (b) IgG1⁺GL7⁺ B cells from cultures as in (a). (c) IgG concentration in culture supernatants from cultures as in (a). IL-4 (left) or IL-6 (right) was added. (d) Glut1 expression in B cells from cultures as in (a). (e) Glucose uptake from culture supernatants as in (a). (f) Lactate production in culture supernatants as in (a). (g) IgG concentration in culture supernatants as in (a), to which 2DG was added. (h) Volcano plot showing all genes (grey) or B cell function genes (red) from sorted B cells in Suppressed + IL-21 versus Suppressed cultures. Cultures were performed as in (a) except NP-OVA was used instead of anti-CD3/IgM. (i) Venn diagram of all genes differentially expressed (FDR adjusted p < 0.05) for RNA-seq analysis as in (h). (j) Collapsed ssGSEA correlation plots for Activated, Suppressed or Suppressed +

IL-21 B cell RNA-seq data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using 1 way ANOVA with Tukey's correction (**a–g**). Data are from one experiment (technical replicate suppression assays utilizing cells from 20 pooled mice) are representative of 3 independent experiments with similar results (**a–g**, mean $\pm$ s.e.m.), or are combined 3 biological replicates (**h–j**).

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Figure 7. IL-21 directly stimulates B cells and inhibits T_{FR} cells

(a) Quantitation of IgG1⁺GL7⁺ B cells from cultures of WT or *Il21r^{-/-}* B cells cultured with T_{FH} cells alone, T_{FH} and T_{FR} cells or T_{FH}, T_{FR} cells and IL-21. Cells are pregated on CD19⁺IA⁺CD4⁻. (b–c) GL7 expression in B cells from cultures as in (a). (d) Analysis of Ki67 in T_{FR} cells from cultures as in (a). Cells are pregated on CD4⁺FoxP3⁺ cells. (e) Numbers of T_{FR} cells remaining cultures as in (a). (f) Glut1 expression in T_{FR} cells from cultures as in (a) *p<0.05, **p<0.01, ***p<0.001 (1-way ANOVA with Tukey's correction (a–c)) or (Student's t-test (e,f)). Data are from one experiment (technical replicate suppression assays utilizing cells from 20 pooled mice) are representative of 3 independent experiments with similar results (a–f, mean±s.e.m.), or 2 independent experiments (a–c).

Table 1Summary of GSEA analysis from activated or suppressed T_{FH} and B cells

Enriched in Act B vs. Supp B				
Collection	Gene Set	Size	NES	FDR q-val
Hall (50)	HALLMARK_MYC_TARGETS_V1	196	2.7671254	<0.0001
	HALLMARK_MYC_TARGETS_V2	58	2.3868976	<0.0001
	HALLMARK_MTORC1_SIGNALING	192	2.346688	<0.0001
	HALLMARK_E2F_TARGETS	193	2.2503338	<0.0001
	HALLMARK_OXIDATIVE_PHOSPHORYLATION	191	2.1517582	<0.0001
	HALLMARK_UNFOLDED_PROTEIN_RESPONSE	111	2.1171036	<0.0001
	HALLMARK_GLYCOLYSIS	192	1.8493669	0.00031
	HALLMARK_G2M_CHECKPOINT	196	1.6099535	0.00383
	HALLMARK_DNA_REPAIR	138	1.505335	0.01441
	HALLMARK_FATTY_ACID_METABOLISM	147	1.500412	0.01398
GO (1011)	NUCLEOLUS	110	2.0671701	0.00094
	MITOCHONDRION	319	2.06659	0.00047
	MITOCHONDRIAL_PART	138	2.0225444	0.00129
	RIBONUCLEOPROTEIN_COMPLEX	142	1.9865174	0.00264
	RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY	81	1.9715921	0.00249
	PROTEASOME_COMPLEX	22	1.9145986	0.00636
	RNA_SPLICING	89	1.9071435	0.00599
	TRANSLATION_INITIATION_FACTOR_ACTIVITY	21	1.8895354	0.00679
	RNA_PROCESSING	166	1.883764	0.00656
	RIBOSOME_BIOGENESIS_AND_ASSEMBLY	18	1.8799101	0.00648
	ISOMERASE_ACTIVITY	35	1.8725868	0.00701
	NUCLEOLAR_PART	18	1.8698697	0.00651
	MITOCHONDRIAL_MATRIX	45	1.8656288	0.00660
	MITOCHONDRIAL_LUMEN	45	1.8638681	0.00626
TF (773)	V\$MYC_Q2	169	1.7301639	0.02973
	SGCGSSAAA_V\$E2F1DP2_01	153	1.6884224	0.02908
	V\$E2F1_Q3	219	1.683651	0.01995
	V\$E2F_Q6	214	1.6622303	0.02027
	V\$E2F4DP1_01	216	1.6378138	0.02317
	V\$E2F1_Q6	214	1.6301547	0.02114
	V\$MYCMAX_01	230	1.6162086	0.02279

Enriched in Act Tfh vs. Supp Tfh				
Hall (50)	HALLMARK_E2F_TARGETS	193	1.9549149	<0.0001
	HALLMARK_HYPOXIA	193	1.8284913	0.001524492
	HALLMARK_G2M_CHECKPOINT	196	1.7426534	0.003196406
	HALLMARK_GLYCOLYSIS	192	1.7223264	0.00311881

HALLMARK_MTORC1_SIGNALING	192	1.709111	0.002761714
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Enriched in Supp B vs. Act B				
Collection	Gene Set	Size	NES	FDR q-val
Hall (50)	HALLMARK_INTERFERON_ALPHA_RESPONSE	89	-2.015375	<0.0001
	HALLMARK_ALLOGRAFT_REJECTION	183	-1.6821588	0.00684
	HALLMARK_INTERFERON_GAMMA_RESPONSE	185	-1.6755509	0.00456
GO (1011)	G_PROTEIN_SIGNALING_COUPLED_TO_CAMP_NUCLEOTIDE_SECOND_MESSENGER	60	-2.095274	0.00208
	NEUROPEPTIDE_BINDING	21	-1.9949162	0.01007
	DEFENSE_RESPONSE_TO_BACTERIUM	20	-1.9927827	0.00672
	G_PROTEIN_SIGNALING_ADENYLATE_CYCLASE_ACTIVATING_PATHWAY	22	-1.9915489	0.00504
	CAMP_MEDIATED_SIGNALING	61	-1.9635229	0.00764
	NEUROPEPTIDE_RECEPTOR_ACTIVITY	20	-1.9402733	0.00793
	DETECTION_OF_EXTERNAL_STIMULUS	22	-1.923486	0.00904
	RESPONSE_TO_BACTERIUM	24	-1.9086182	0.00922
	ADENYLATE_CYCLASE_ACTIVATION	17	-1.908182	0.00820