Naive and memory human B cells have distinct requirements for STAT3 activation to differentiate into antibody-secreting plasma cells

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Long-lived antibody memory is mediated by the combined effects of long-lived plasma cells (PCs) and memory B cells generated in response to T cell–dependent antigens (Ags). IL-10 and IL-21 can activate multiple signaling pathways, including STAT1, STAT3, and STAT5; ERK; PI3K/Akt, and potently promote human B cell differentiation. We previously showed that loss-of-function mutations in *STAT3*, but not *STAT1*, abrogate IL-10– and IL-21–mediated differentiation of human naive B cells into plasmablasts. We report here that, in contrast to naive B cells, STAT3-deficient memory B cells responded to these STAT3-activating cytokines, differentiating into plasmablasts and secreting high levels of IgM, IgG, and IgA, as well as Ag-specific IgG. This was associated with the induction of the molecular machinery necessary for PC formation. Mutations in *IL21R*, however, abolished IL-21–induced responses of both naive and memory human B cells and compromised memory B cell formation in vivo. These findings reveal a key role for IL-21R/STAT3 signaling in regulating human B cell function. Furthermore, our results indicate that the threshold of STAT3 activation required for differentiation is lower in memory compared with naive B cells, thereby identifying an intrinsic difference in the mechanism underlying differentiation of naive versus memory B cells.

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Abbreviations used: Ab, antibody; AD-HIES, autosomal-dominant hyper-IgE syndrome; Ag, antigen; PC, plasma cell; Tfh cell, T follicular helper cell.

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Long-lived immunological memory is mediated by the combined effects of long-lived plasma cells (PCs) and memory B cells generated in response to T-dependent antigens (Ags) and underlies the success of most currently available vaccines (Ahmed and Gray, 1996; Rajewsky, 1996; Tangye and Tarlinton, 2009; Goodnow et al., 2010). PCs reside in survival niches in bone marrow and secondary lymphoid tissues and constantly produce high titers of neutralizing antibodies (Abs; Tangye and Tarlinton, 2009; Tangye, 2011). In contrast, memory B cells recirculate throughout peripheral blood, secondary lymphoid tissues, and bone marrow. Upon reexposure to Ag, they can proliferate and differentiate into Ab-secreting plasmablasts more rapidly than naive cells, thereby replenishing the PC pool and simultaneously expanding the memory cell population (Ahmed and Gray, 1996; Rajewsky, 1996; Tangye and Tarlinton, 2009).

Analysis of gene-targeted mice and humans with monogenic primary immunodeficiencies has identified some of the molecular requirements for memory B cell generation. Thus, mutations in B cell–intrinsic genes (*CD19/CD81*, *CD40*, *IKBKG*, *DOCK8*, and *IL2RG*) or genes expressed by $CD4^+$ T helper cells (*CD40LG*, *ICOS*, and *SH2D1A* [SAP]) all result in reductions in the frequencies of memory B cells and associated deficiencies in total serum Ig levels or Ag-specific Ab (Tangye and Tarlinton, 2009; Recher et al., 2011; Jabara et al., 2012; Tangye et al., 2012). We also have some understanding of the mechanisms that enable memory B cells to respond more rapidly and vigorously than naive cells to cognate Ag. First, memory B cells are recruited into division significantly earlier and undergo more rounds of division than naive cells (Bernasconi et al., 2002; Tangye et al., 2003a,b; Macallan et al., 2005). Second, memory B cells have higher expression of cell surface receptors, TLRs (TLR7/9/10), CD21, CD27, and TACI, that could enable them to respond more efficiently to co-stimulatory signals (Tangye et al., 1998; Bernasconi et al., 2002, 2003; Darce et al., 2007; Good et al., 2009). Third, memory B cells express heightened levels of CD80 and CD86 (Liu et al., 1995; Tangye et al., 1998; Ellyard et al., 2004; Good et al., 2009), which facilitate soliciting help from T helper cells. Fourth, memory B cells express lower levels of genes that restrict the entry of naive B cells into division, limiting their activation (Good and Tangye, 2007; Horikawa et al., 2007). Lastly, distinct signaling pathways downstream of the B cell receptor expressed by naive (i.e., IgM) or memory (IgG) cells have been identified that preferentially promote responsiveness of memory cells (Martin and Goodnow, 2002; Engels et al., 2009; Davey and Pierce, 2012). However, the requirements for cytokine-mediated regulation of naive and memory B cells remain to be determined.

Human B cell differentiation is regulated by the actions of numerous cytokines, with IL-10 and IL-21, produced by T follicular helper cells (Tfh cells), being key factors in promoting proliferation, isotype switching, PC differentiation, and secretion of most Ig isotypes by not only naive B cells, but also memory B cells, including both IgM^+ and isotype-switched subsets (Banchereau et al., 1994; Arpin et al., 1997; Pène et al., 2004; Ettinger et al., 2005; Bryant et al., 2007; Avery et al., 2008a,b). Although the functions of IL-10 and IL-21 on human B cells are similar, the effects of IL-21 exceed those of IL-10 by 10–100-fold (Bryant et al., 2007). The importance of IL-21 to immune regulation has been validated by the recent identification of IL-21R–deficient humans, who exhibit infectious susceptibility to several pathogens (Kotlarz et al., 2013). The predominance of IL-21 in regulating human B cell function over IL-10 is also indicated by the fact that *IL21R* mutations result in poor Ab responses after vaccination (Kotlarz et al., 2013), whereas specific Abs are produced at normal levels in individuals with mutations in *IL10/IL10R* (Kotlarz et al., 2012). IL-10 and IL-21 activate STAT1, STAT3, STAT5, as well as MAPK/ERK and PI3K/Akt pathways (Asao et al., 2001; Zeng et al., 2007; Avery et al., 2008b, 2010; Diehl et al., 2008). Autosomal-dominant hyper-IgE syndrome (AD-HIES) is caused by heterozygous mutations in *STAT3* (Holland et al., 2007; Minegishi et al., 2007; Casanova et al., 2012). These mutations operate in a dominant-negative manner, effectively reducing the level of functional STAT3 by 75%. Loss-of-function mutations in *STAT1* also underlie several immunodeficiency states, such as those characterized by selective susceptibility to infection with environmental mycobacteria and, depending on the nature of the mutation (i.e., dominant/recessive), some viruses (Boisson-Dupuis et al., 2012; Casanova et al., 2012). By examining these patients, we previously found that functional STAT3 deficiency not only severely compromised the generation of memory (i.e., $CD27^+$) B cells in vivo, but prevented IL-10– and IL-21–mediated induction of *PRDM1* (Blimp-1 [B lymphocyte induced maturation protein-1]) and *XBP1* (*X-box binding protein 1*) in naive B cells and their subsequent differentiation to the PC lineage in vitro. However, STAT3 mutant ($STAT3_{MUT}$) naive B cells could still acquire expression of *AICDA* (*activation-induced cytidine deaminase*) and undergo IL-21–induced isotype switching in vitro. In contrast, STAT1 was dispensable for human B cell differentiation in vivo and in vitro (Avery et al., 2010).

These findings led us to investigate further the role of STATs in governing human B cell differentiation. We have now discovered that the small number of memory B cells generated in STAT3-deficient patients are unaffected by these mutations; thus, they are capable of differentiating into Ab-secreting cells in response to STAT3-actvating cytokines as efficiently as normal memory cells. These findings demonstrate that the threshold of STAT3 activation required for B cell differentiation is significantly lower in memory compared with naive cells. Consequently, limiting amounts of functional STAT3 are sufficient to mediate memory, but not naive, B cell differentiation, thereby revealing an intrinsic difference in the requirements for activating naive versus memory B cells. The memory B cell deficiency in AD-HIES patients likely contributes to impaired Ag-specific Ab responses characteristic of these individuals. Thus, by targeting the residual population of STAT3-deficient memory B cells to respond to IL-21, it may be possible to improve humoral immunity in AD-HIES.

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Table 1. Characteristics of CD27$^-$ and CD27$^+$ B cells in STAT3-deficient individuals

Parameter	CD27$^-$ B cells		CD27$^+$ B cells	
	Normal	STAT3$_{MUT}$	Normal	STAT3$_{MUT}$
% Cells	75.2 ± 2.7	94.9 ± 0.77	24.8 ± 2.7	5.1 ± 0.77
% IgM$^+$	87.0 ± 2.2	90.0 ± 2.0	47 ± 3.8	48 ± 3.1
% IgD$^+$	88.0 ± 1.9	93.0 ± 1.8	41 ± 2.2	45 ± 2.0
% IgG$^+$	3.2 ± 0.45	1.6 ± 0.6[a]	25 ± 2.1	32 ± 2.8[a]
% IgG1$^+$ (% total IgG$^+$ cells)	2.3 ± 1.0 (62.1)	0.63 ± 0.24 (40.1)	11 ± 1.7 (51.4)	22 ± 2.8 (72.7)[b]
% IgG2$^+$ (% total IgG$^+$ cells)	0.72 ± 0.14 (19.4)	0.54 ± 0.1 (34.4)	6.7 ± 2.1 (31.3)	3.9 ± 0.9 (12.9)
% IgG3$^+$ (% total IgG$^+$ cells)	0.62 ± 0.15 (16.7)	0.36 ± 0.12 (22.9)	3.6 ± 0.9 (16.8)	4.1 ± 1.1 (13.5)
% IgG4$^+$ (% total IgG$^+$ cells)	0.065 ± 0.02 (1.8)	0.04 ± 0.014 (2.5)	0.11 ± 0.04 (0.5)	0.27 ± 0.08 (0.9)
% IgA$^+$	1.2 ± 0.2	0.64 ± 0.15[a]	17 ± 1.2	8.6 ± 1.1[c]
% CD23$^+$	62.0 ± 3.9	86.0 ± 3.7[c]	18 ± 3.9	46 ± 4.7[c]

Values represent percentage (or absolute number for CD4$^+$CXCR5$^+$ T cells) of cells expressing the indicated surface molecule; each value represents the mean ± SEM; normal donors: n = 8–24; STAT3 patients: n = 9–27. P-values were determined by Student's t test, comparing normal with STAT3$_{MUT}$ B cells.
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.

RESULTS

STAT3$_{MUT}$ CD27$^+$ B cells phenotypically resemble normal memory B cells

The population of circulating CD27$^+$ memory B cells is significantly reduced in STAT3-deficient patients (n = 27) compared with normal donors (Table 1; Avery et al., 2010). In contrast, the frequency of memory B cells in STAT1-deficient individuals is comparable with normal donors (i.e., 24.4 ± 6.1%; n = 9). Although it is generally accepted that CD27 is expressed on human memory B cells (Tangye and Tarlinton, 2009), recent studies have suggested that B1 cells (Griffin et al., 2011) and some bone marrow progenitor B cells (Nilsson et al., 2005) are also CD27$^+$. Conversely, a small proportion of memory B cells lack CD27 (Tangye and Tarlinton, 2009). Thus, it was important to establish the nature of the residual population of CD27$^+$ B cells in STAT3$_{MUT}$ patients.

The size and granularity of CD27$^-$ and CD27$^+$ B cells were determined by flow cytometry. This demonstrated that CD27$^+$ B cells from both normal donors and STAT3$_{MUT}$ patients were significantly larger and more granular than corresponding CD27$^-$ B cells. However, these morphological features were not significantly different between cells from normal donors and STAT3$_{MUT}$ patients (Fig. 1, A–C).

We next examined a series of surface receptors that are differentially expressed by human naive and memory B cells (Liu et al., 1995; Tangye et al., 1998; Ellyard et al., 2004; Good et al., 2009). CD24, CD80, CD95, and TACI were significantly higher on CD27$^+$ B cells from normal donors than on corresponding CD27$^-$ B cells (Fig. 1, A and C). CD86 also tended to be higher on normal CD27$^+$ versus CD27$^-$ B cells (Fig. 1, A and C). The same pattern was seen for samples from STAT3$_{MUT}$ patients, with CD80, CD86, CD95, and TACI being significantly higher on CD27$^+$ than on CD27$^-$ B cells (Fig. 1, B and C). In contrast to these molecules, CD23 is present on normal naive B cells but is significantly down-regulated on normal memory B cells (Fig. 1, A and C; and Table 1). Interestingly, CD23 expression was dysregulated on STAT3$_{MUT}$ B cells inasmuch as its level exceeded that on normal naive B cells by >10-fold, whereas it was detected on a substantial proportion of STAT3$_{MUT}$ memory B cells (Fig. 1, B and C; and Table 1).

We also determined expression of Ig isotypes by CD27$^-$ and CD27$^+$ B cells from normal and STAT3-deficient individuals. Approximately ∼90% of CD27$^-$ B cells and ∼40–50% of CD27$^+$ B cells from normal and STAT3-deficient individuals expressed IgM and IgD, with the remaining memory B cells expressing predominantly IgG or IgA (Table 1). Although the proportion of STAT3$_{MUT}$ CD27$^+$ B cells that expressed IgG was greater than that observed for normal CD27$^+$ B cells (Table 1), the distribution of IgG subclasses within the CD27$^-$ and CD27$^+$ B cell subsets was comparable, with IgG1 being preferentially expressed by both CD27$^-$ and CD27$^+$ normal and STAT3$_{MUT}$ B cells (Table 1). Furthermore, we did not detect an enrichment of B1 cells, based on cells with a CD20$^+$CD43$^+$CD27$^+$ phenotype, in STAT3$_{MUT}$ individuals compared with healthy donors (not depicted). Collectively, these findings confirm there is a significant contraction of the memory B cell compartment in AD-HIES and provide evidence that the small population of CD27$^+$ B cells in STAT3$_{MUT}$ individuals are indeed memory B cells (Table 1).

IL-21 activates STAT1, STAT3, and STAT5 in naive and memory B cells

IL-21 has been reported to activate multiple signaling pathways in different cell types (Asao et al., 2001; Zeng et al., 2007; Diehl et al., 2008). To determine whether different subsets of human B cells used similar signaling pathways downstream of the IL-21 receptor, we examined phosphorylation of STAT proteins in normal naive, IgM memory, and

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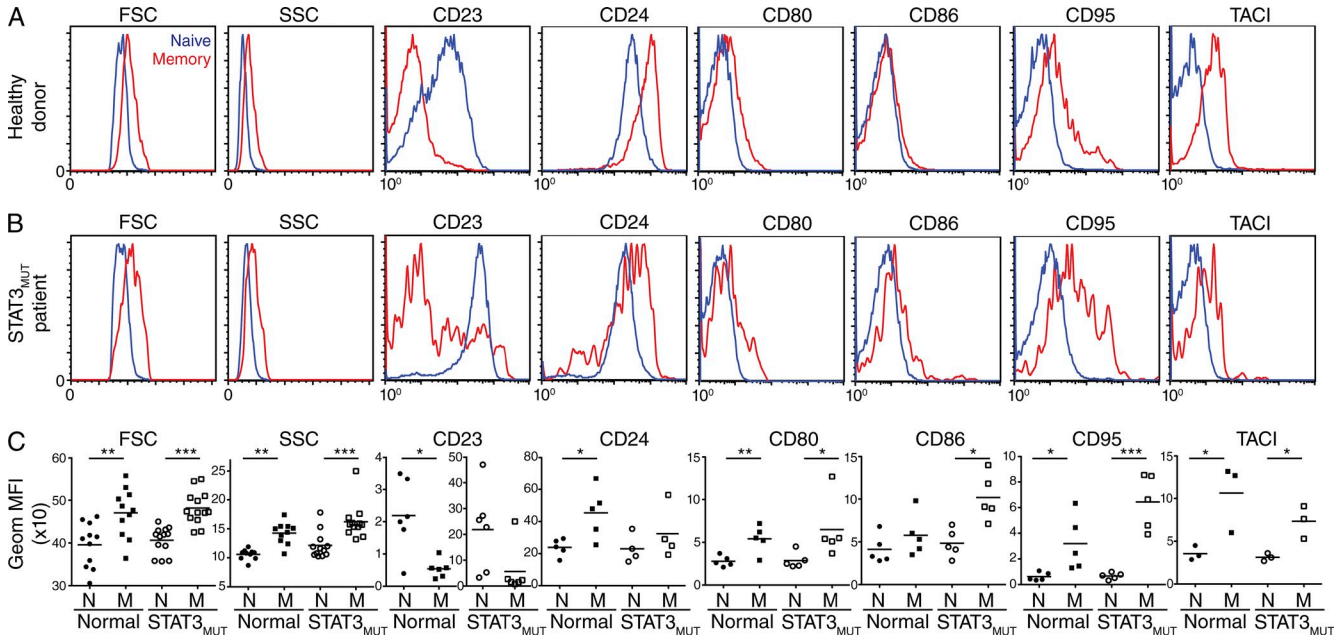

Figure 1. Morphology and phenotype of CD27⁺ B cells in $STAT3_{MUT}$ individuals resemble normal memory B cells. (A–C) PBMCs from normal donors and patients with AD-HIES caused by mutations in *STAT3* were stained with mAbs specific for CD20, CD27, and CD23, CD24, CD80, CD86, CD95, or TACI. The forward scatter (FSC) and 90° light/side scatter (SSC) and surface expression of the indicated molecules on $CD27^-$ (naive) and $CD27^+$ (memory) B cells were determined. The histograms in A and B are from a representative normal donor and patient, respectively, whereas the graphs in C depict the geometric mean fluorescence intensity (MFI) of each of the indicated cellular features for $CD27^-$ naive (N) and $CD27^+$ memory (M) B cells from 3–12 normal donors and $STAT3_{MUT}$ patients. Each value represents an individual donor or patient; the horizontal lines correspond to the mean. Because of the large difference in the level of expression of CD23 on normal versus $STAT3_{MUT}$ naive and memory B cells, individual graphs are depicted for normal and patient B cell subsets. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

isotype-switched memory B cells in response to IL-21. Phosphorylation of STAT1, STAT3, and STAT5 was greater in naive B cells than in memory B cells; however, STAT1 and STAT3 were both clearly activated by IL-21 in both IgM-expressing and Ig-switched memory B cell subsets (Fig. 2, A and B). In contrast to these STATs, IL-21 had minimal, if any, effect on phosphorylation of STAT4 or STAT6 (Fig. 2 A). Although IL-21 has been reported to activate ERK and AKT (Zeng et al., 2007), we observed no ERK or AKT phosphorylation in response to IL-21 in naive or memory B cells (Fig. 2, C and D). These results demonstrate that IL-21R signaling activates similar pathways in human naive and memory B cells, predominantly STAT1, STAT3, and STAT5.

Mutations in *STAT3* impair the response of naive, but not memory, B cells to the stimulatory effects of IL-10 and IL-21

STAT3 is required for some, but not all, aspects of naive B cell differentiation. For instance, isotype switching was intact in $STAT3_{MUT}$ B cells, as indicated by the detection of circulating IgG^+ and IgA^+ B cells in AD-HIES patients ex vivo (Table 1) and the ability of their naive B cells to up-regulate *AICDA* and switch to IgG in response to IL-21 in vitro (Avery et al., 2010). Consistent with intact *AICDA* expression in $STAT3_{MUT}$ naive B cells in vitro, somatic hypermutation was comparable in normal and $STAT3_{MUT}$ memory B cells (Avery et al., 2010). These observations lead us to question whether there were also differences between naive and memory B cells in their requirement for STAT3 function to respond to cytokines such as IL-10 and IL-21, which are well known for their abilities to induce human B cell differentiation (Banchereau et al., 1994; Arpin et al., 1997; Pène et al., 2004; Ettinger et al., 2005; Bryant et al., 2007; Avery et al., 2008a,b).

Naive and memory B cells isolated from normal donors or $STAT3_{MUT}$ or $STAT1_{MUT}$ patients were cultured with CD40L alone or together with IL-21, and Ig secretion was determined after 10–12 d. Because of the limited numbers of memory cells recovered from $STAT3_{MUT}$ patients, we could only culture ~5,000 sorted B cells/well. Under these conditions, IL-21 potently promoted secretion of IgM and induced production of IgG and IgA by normal and $STAT1_{MUT}$ naive B cells (Fig. 3 A and Table 2). IL-21 substantially increased production of IgM, IgG, and IgA by CD40L-stimulated memory B cells from normal donors and STAT1-deficient individuals; however, the response of $STAT1_{MUT}$ memory B cells was significantly less (approximately fourfold) than that of normal memory cells (Fig. 3 B and Table 2). Ig secretion by $STAT3_{MUT}$ naive B cells in response to IL-21 was ~30-fold less than normal naive cells (Fig. 3 A and Table 2; $P < 0.001$). In distinct contrast, Ig secretion by IL-21–stimulated $STAT3_{MUT}$ memory B cells (either total memory cells [Fig. 3 B and Table 2] or IgM^+ and switched subsets [not depicted]) was largely comparable with normal memory B cells,

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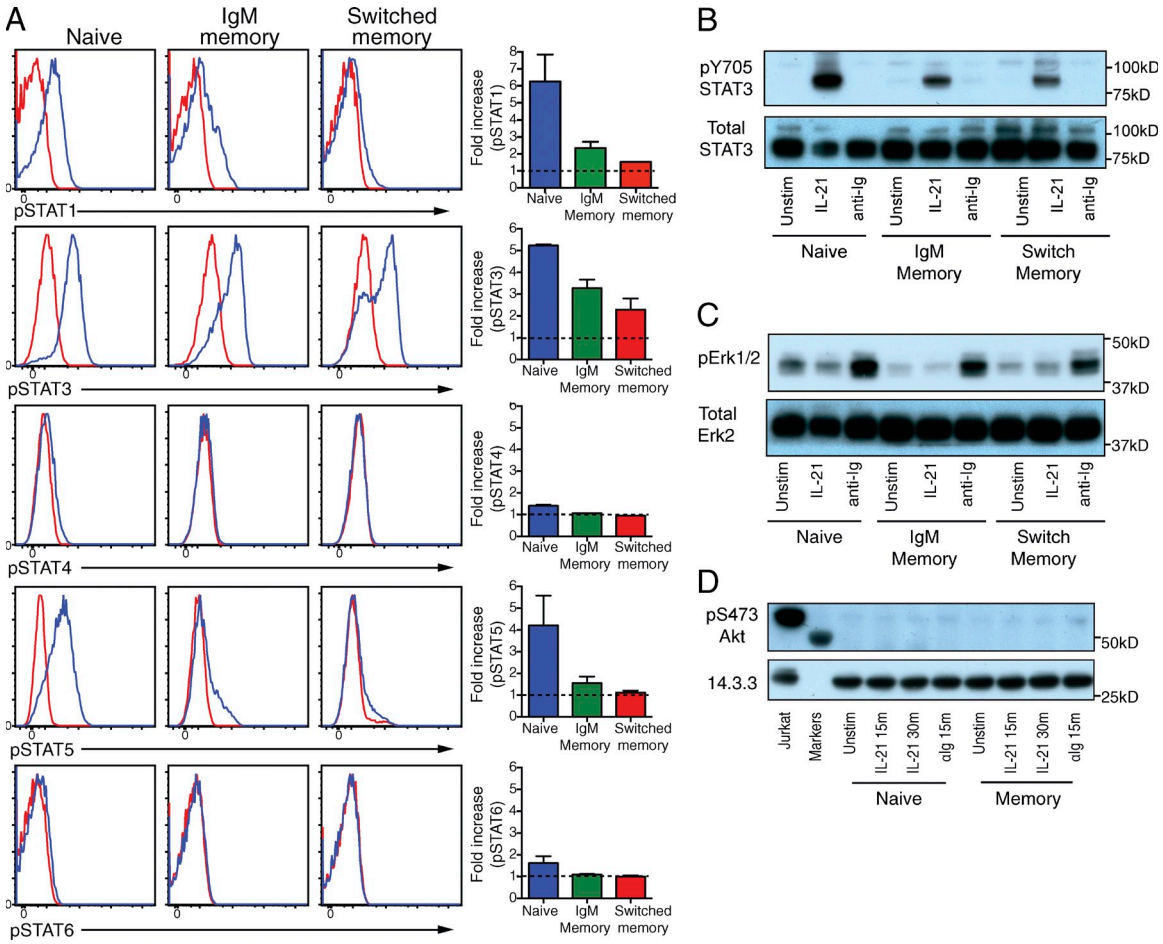

Figure 2. IL-21 induces activation of STAT1, STAT3, and STAT5 in human naive and memory B cells. Human naive, IgM memory and isotype-switched memory, or total memory, B cells were sort-purified from normal donor spleens. (A) These B cell subsets were cultured for ~18 h with anti-Ig, rested, and then cultured in the absence (red histograms) or presence (blue histograms) of IL-21 for 30 min. Phosphorylation of STAT1, STAT3, STAT4, STAT5, and STAT6 was determined by intracellular staining. Histograms on the left show representative staining in naive and memory B cells. Right panels plot increase in mean fluorescence intensity of pSTATs in naive, IgM memory, and isotype-switched memory B cells cultured with IL-21; response of unstimulated cells were normalized to a value of 1.0. These values represent the mean ± SEM of two independent experiments using B cells from different donor spleens. Identical results were obtained when the B cell subsets were prestimulated with CD40L/anti-Ig. (B–D) Human B cell subsets were cultured for ~18 h with anti-Ig, rested, and then left unstimulated or stimulated with IL-21 or anti-Ig for 15–30 min. Cells lysates were prepared and subjected to SDS-PAGE and Western blotting to detect phosphorylated or total STAT3 (B), phosphorylated or total ERK (C), or phosphorylated AKT or 14.3.3 as a loading control (D). B–D are representative of three to four similar experiments.

with the only significant difference (less than twofold) being noted for the levels of IgM secreted by normal versus STAT-3_{MUT} memory B cells (Table 2). Furthermore, both IgM memory (i.e., IgM^+CD27^+) and switched (IgM/D^-CD27^+) memory cells from STAT3-deficient patients could respond to the stimulatory effects of IL-10 (not depicted).

The inability of $STAT3_{MUT}$ naive B cells to respond to IL-21 did not reflect a general impairment in differentiation because these cells produced IgM after stimulation with CD40L plus CpG (Fig. 3 C) and up-regulated expression of *AICDA* (Fig. 3 E) and Ig ε germline transcripts, a precursor to producing mature IgE (Geha et al., 2003), in response to CD40L/IL-4 (Fig. 3 F) as efficiently as normal naive B cells. Consistent with the intact response of $STAT3_{MUT}$ memory B cells to CD40L together with IL-10 or IL-21 (Fig. 3 B, Table 2, and not depicted), these cells also exhibited normal responses to stimulation with CD40L/CpG (Fig. 3 D) or CD40L/IL-4 (Fig. 3, E and F). Because many facets of lymphocyte differentiation are linked to cell division (Hodgkin et al., 1996; Deenick et al., 1999; Tangye et al., 2003a,b; Avery et al., 2005, 2008a), we also assessed the proliferative potential of $STAT3_{MUT}$ naive and memory B cells. IL-21 enhanced proliferation of both normal and $STAT3_{MUT}$ naive B cells over that induced by CD40L alone, yet the response of $STAT3_{MUT}$ naive B cells was approximately threefold less than that of normal B cells (Fig. 3 G). Consistent with the differential dependency on STAT3 function for IL-21–induced Ig secretion by naive versus memory B cells, $STAT3_{MUT}$ memory

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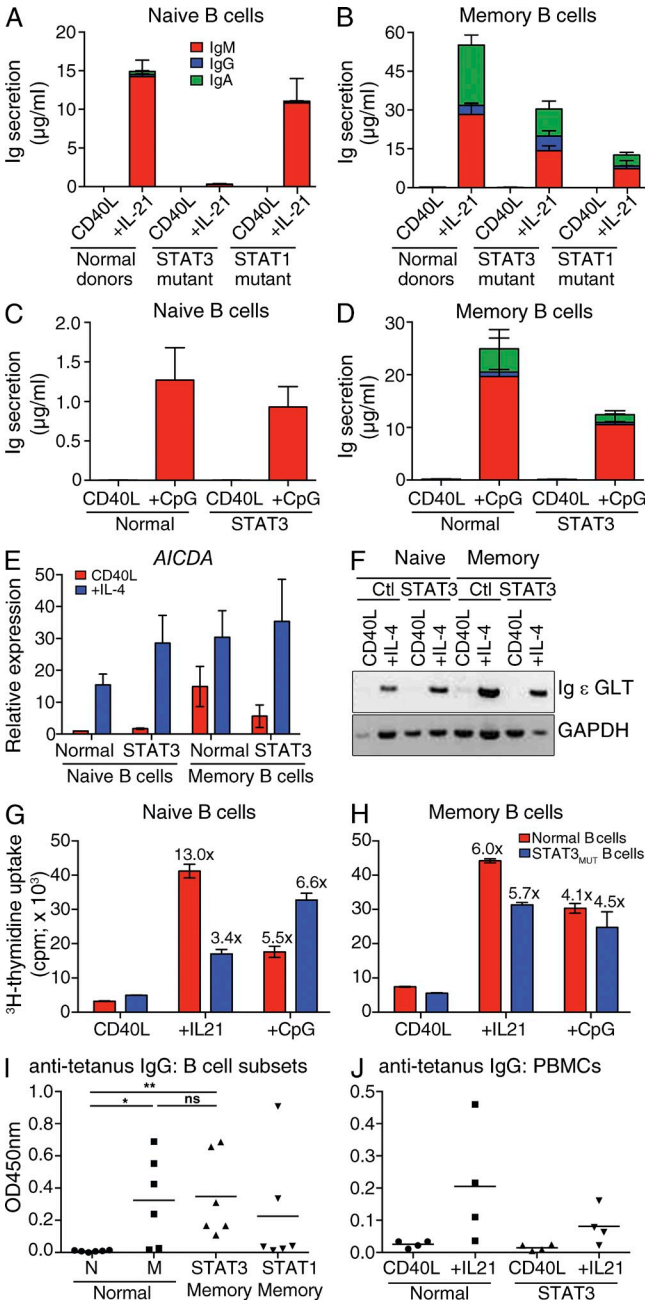

Figure 3. STAT3-deficient memory B cells differentiate into Ab-secreting cells in response to IL-21. (A–D) Naive ($CD20^+CD10^-CD27^-IgG^-$) and memory ($CD20^+CD10^-CD27^+$) B cells were sort-purified from normal donors (A and B, $n = 16$; C and D, $n = 7$), $STAT3_{MUT}$ patients (A and B, $n = 8$; C and D, $n = 7$), or $STAT1_{MUT}$ patients ($n = 6$), and then cultured with CD40L alone or together with IL-21 (A and B) or CpG (C and D). The levels of secreted IgM, IgG, and IgA were determined by ELISA after 10–12 d. The columns represent the mean ± SEM of experiments performed using naive B cells from 7–16 normal donors, 7–8 $STAT3_{MUT}$ patients, or 6 $STAT1_{MUT}$ patients. (E and F) Naive and memory B cells were sort-purified from normal donors or $STAT3_{MUT}$ patients and then cultured with CD40L alone or together with IL-4. Expression of *AICDA* (E) and Ig ε germline transcript (GLT; F) was determined by qPCR and PCR, respectively. The graphs in E represent the mean ± SEM of three experiments using B cells from different donors or patients. The gel depicted in F is representative of experiments performed using B cells from two to three different donors or patients. (G and H) Naive and memory B cells were sort-purified from a single normal donor or $STAT3_{MUT}$ patient and then cultured with CD40L alone or together with IL-21 or CpG. Proliferation was assessed after 5 d by determining incorporation of [^{3}H]thymidine during the last 18 h of culture. The graphs are the mean ± SEM of replicate cultures of naive or memory B cells from one normal donor or one $STAT3_{MUT}$ patient. The annotated values indicate the fold increase in proliferation of normal or $STAT3_{MUT}$ naive or memory B cells cultured with CD40L/IL-21 or CD40L/CpG over that induced by CD40L alone. (I and J) Normal naive (N) or normal, $STAT3_{MUT}$, or $STAT1_{MUT}$ memory (M) B cells ($n = 6$; I) or total PBMCs from normal donors or $STAT3_{MUT}$ patients ($n = 4$; J) were cultured with CD40L and IL-21 for 10–12 d. The levels of antitetanus IgG in culture supernatants were determined by ELISA using immobilized tetanus toxoid as solid phase Ag. Each symbol represents the response of B cells from an individual control or patient; the horizontal bars represent means. ns, no significant; *, $P < 0.05$; **, $P < 0.01$.

B cells proliferated to a similar extent as normal memory B cells in response to IL-21 (Fig. 3 H). Not surprisingly, CD40L/CpG induced comparable proliferation in STAT3-sufficient and -deficient naive and memory B cells (Fig. 3, G and H). Thus, impaired proliferation of naive $STAT3_{MUT}$ B cells to IL-21 correlates with poor differentiation of these cells to plasmablasts under these culture conditions. However, naive $STAT3_{MUT}$ B cells do undergo some proliferation to IL-21 (Fig. 3 G; Avery et al., 2010), indicating that the block in differentiation is not simply caused by *STAT3* mutations abrogating cell division.

The memory cell pool in STAT3-deficient individuals contains Ag-specific B cells

Although the total levels of Ig produced by $STAT3_{MUT}$ memory B cells in response to STAT3 cytokines were normal, it was unknown whether these B cells could contribute to an Ag-specific Ab response. To address this, we quantified the relative amounts of antitetanus IgG produced by B cells from normal donors or $STAT3_{MUT}$ or $STAT1_{MUT}$ patients after in vitro culture with CD40L/IL-21. As expected, the levels of antitetanus IgG produced by normal naive B cells were very low/undetectable, whereas memory cells from most normal donors produced significantly higher amounts of tetanus-specific IgG (Fig. 3 I). Importantly, $STAT3_{MUT}$ memory B cells from all patients tested produced significantly higher amounts of tetanus-specific IgG than normal naive B cells (Fig. 3 I). Memory B cells from some $STAT1_{MUT}$ patients exhibited a lower response than others, but in general this exceeded that of normal naive B cells and, on average, approximated that of normal and $STAT3_{MUT}$ memory B cells (Fig. 3 I). We also assessed production of antitetanus IgG in cultures of total PBMCs from normal donors and $STAT3_{MUT}$ individuals that had been stimulated with CD40L and IL-21. On average, normal PBMCs produced approximately threefold higher levels of antitetanus IgG than did $STAT3_{MUT}$ PBMCs (Fig. 3 J). However, there are several caveats to screening PBMCs, rather than purified B cells, for the production of Ag-specific Ab.

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Table 2. Ig secretion by IL-21–stimulated normal, $STAT3_{MUT}$, and $STAT1_{MUT}$ naive and memory B cells

Cell type and culture	Ig secretion								
	IgM			IgG			IgA		
	Normal	$STAT3_{MUT}$	$STAT1_{MUT}$	Normal	$STAT3_{MUT}$	$STAT1_{MUT}$	Normal	$STAT3_{MUT}$	$STAT1_{MUT}$
	ng/ml	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*
Naive B cells									
CD40L	2.0 ± 1.0	5.0 ± 2.4[a]	<0. 1[a]	<1	<1[a]	<1[a]	<1	<1[a]	<1[a]
+IL-21	14,640 ± 2,021	444 ± 177[d]	10,801 ± 3,183[a]	267 ± 80	7.3 ± 2.7[b]	86 ± 40[a]	365 ± 106	4.6 ± 2.6[b]	174 ± 40[a]
Memory B cells									
CD40L	166 ± 51	100 ± 25[a]	2.5 ± 2.5[a]	6.1 ± 1.4	11.8 ± 4.7[a]	0.2 ± 0.14[a]	21.3 ± 6.1	26.2 ± 10.6[a]	<1[a]
+IL-21	29,389 ± 4,107	15,214 ± 1,905[b]	7,396 ± 3,097[d]	4,202 ± 1,122	5,651 ± 1,743[a]	953 ± 195[a]	25,208 ± 4,134	13,330 ± 4,027[a]	4,265 ± 989[c]

Naive and memory B cells were sort-purified from normal healthy donors ($n = 17$), $STAT3_{MUT}$ patients ($n = 8$), or $STAT1_{MUT}$ patients ($n = 6$) and then cultured with CD40L alone or together with IL-21. The levels of secreted IgM, IgG, and IgA were determined by ELISA after 10–12 d. The values represent the mean ± SEM from the indicated number of donors/patients and correspond to the data depicted in Fig. 3 (A and B). Statistical analyses were performed using one-way ANOVA; differences are indicated for normal donors compared with $STAT3_{MUT}$ or $STAT1_{MUT}$ B cells.

[a]Not significant.
[b]$P < 0.01$.
[c]$P < 0.001$.
[d]$P < 0.0001$.

First, there is substantial variability in the frequencies of B cells within the population of all PBMCs, as well as in the proportion of B cells that are memory cells. Indeed, there is a strong correlation between the frequency of memory B cells and production of antitetanus IgG in vitro (not depicted). Second, the addition of CD40L to cultures of PBMCs will activate myeloid cells (monocytes, macrophages, and DCs) to secrete molecules such as BAFF and APRIL (Litinskiy et al., 2002; Craxton et al., 2003), which can preferentially promote differentiation and Ig secretion by human memory B cells presumably in a STAT3-independent manner (Avery et al., 2003). Even taking these into account, it is clear that STAT3-deficient individuals are capable of generating Ag-specific Ab responses and that these Ag-specific cells reside within the residual subset of $CD27^+$ B cells. This further substantiates that these cells are indeed memory B cells and are likely to contribute to Ag-specific Ab responses in vivo.

STAT1 deficiency does not affect the early differentiation of memory B cells into Ig-secreting plasmablasts

The accumulated levels of Ig secreted by $STAT1_{MUT}$ memory B cells were 2.5–5-fold less than those by normal memory cells (Fig. 3 B and Table 2). To determine whether this reflected a quantitative defect in generating Ab-secreting cells from $STAT1_{MUT}$ memory B cells, we performed kinetic analyses of plasmablast formation and Ig secretion by normal and $STAT1_{MUT}$ naive and memory B cells that had been stimulated with CD40L alone or together with IL-21. CD40L alone resulted in <0.5% of normal and $STAT1_{MUT}$ naive B cells and ~1–3% of memory B cells acquiring a $CD38^{hi}CD27^{hi}$ phenotype, which corresponds to plasmablasts (Fig. 4, A, B, D, and E; Avery et al., 2005). Addition of IL-21 had minimal effect on naive B cell differentiation, in terms of the frequency of plasmablasts and Ig secretion, after 4 d of culture (Fig. 4, A–C); however, a substantial proportion of memory B cells had differentiated to become Ig-secreting plasmablasts at this time (Fig. 4, D–F). The rate of plasmablast formation from naive and memory B cells increased after 5.5 d and tended to plateau or decline at later times (7 d; Fig. 4, B and E). Co-incident with this was a dramatic increase in Ig secretion by both naive and memory B cells between 4 and 5.5 d of in vitro culture (Fig. 4, C and F). The rate of formation of plasmablasts and Ig secretion by IL-21–stimulated naive and memory B cells was not affected by *STAT1* mutations (Fig. 4). Collectively, these results suggested that $STAT1_{MUT}$ memory B cells could initially generate normal numbers of functional plasmablasts. However, in contrast to this normal rate of differentiation of $STAT1_{MUT}$ memory B cells between days 4 and 7 of culture (Fig. 4, D–F), Ig secretion by these cells after 11 d of culture was consistently less than that by normal memory B cells (Fig. 3 B and Table 2). Thus, STAT1 may play a role in sustaining Ig secretion by differentiated memory B cells.

Commitment of memory B cells to the PC lineage is unaffected by mutations in *STAT1* or *STAT3*

The differentiation of human and mouse B cells into PCs is regulated by the coordinated actions of several transcription factors. PAX5 is down-regulated in activated B cells, thereby relieving PAX5-mediated repression of Blimp-1, resulting in Blimp-1 expression. Although Blimp-1 is not required for initial commitment to the PC lineage, it is indispensable for the generation of terminally differentiated PCs. Other transcription factors, XBP-1 and IRF4 (interferon-induced regulatory

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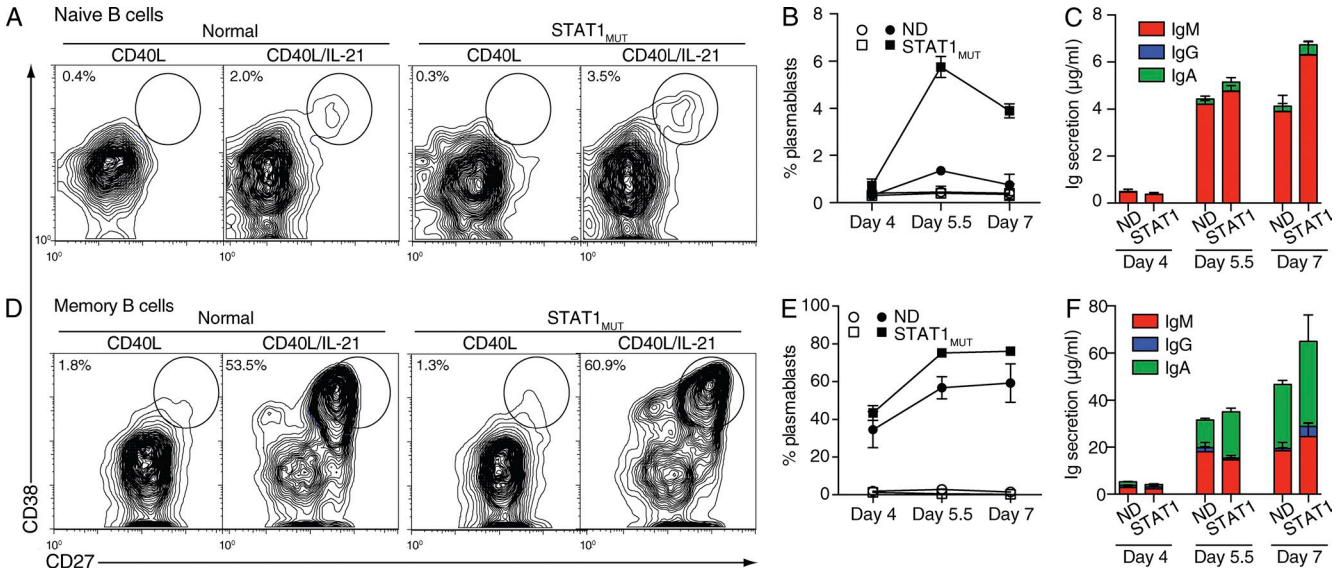

Figure 4. *STAT1* mutations do not affect the generation of plasmablasts but impair sustained Ig secretion. (A–F) Naive (A–C) and memory (D–F) B cells were sort-purified from normal donors or $STAT1_{MUT}$ patients and then cultured (25×10^3/well/100 µl) with CD40L alone (open symbols in B and E) or together with IL-21 (closed symbols in B and E). The generation of plasmablasts, defined as cells acquiring a $CD38^{hi}CD27^{hi}$ phenotype (A, B, D, and E), as well as secretion of IgM, IgG, and IgA (C and F), was determined after 4, 5.5, and 7 d. The contour plots (A and D) are representative of plasmablasts detected after 5 d of culture. The graphs depicting Ig secretion are from cultures of CD40L/IL-21–stimulated B cells. The values represent the mean (±SEM for B, C, E, and F) of experiments using cells from two normal donors and two $STAT1_{MUT}$ patients. Similar results were obtained in a second independent experiment.

factor-4), are also involved in PC differentiation (Nutt et al., 2011). A key mechanism by which IL-21 mediates differentiation of naive B cells into PCs is by modulating expression of these transcription factors. Thus, naive B cells lose PAX5 and acquire Blimp-1 and XBP-1 in response to IL-21 in vitro (Ettinger et al., 2005; Bryant et al., 2007).

Our finding that $STAT3_{MUT}$ memory, but not naive, B cells were capable of secreting near-normal levels of Ig in response to IL-21 (Fig. 3 and Table 2) led us to investigate transcriptional changes in normal and $STAT3_{MUT}$ naive and memory B cells after stimulation with IL-21. We also examined $STAT1_{MUT}$ naive and memory B cells as the latter had some defects in secreting normal levels of Ig (Fig. 3 and Table 2). Addition of IL-21 to cultures of B cells from normal donors resulted in the down-regulation of *PAX5* and up-regulation of *PRDM1* and *XBP1* in naive and memory B cells (Fig. 5, A and B). *PRDM1* and *XBP1* were substantially higher, and PAX5 much lower, in memory versus naive cells (Fig. 5, A and B). Furthermore, although IL-21 had no detectable effect on *IRF4* expression in normal naive B cells (Fig. 5 A), it induced an approximately threefold increase in *IRF4* in normal memory B cells (Fig. 5 B). These differences likely contribute to memory B cells secreting 10–20-fold more Ig than naive cells (Figs. 3 and 4 and Table 2).

$STAT1_{MUT}$ naive and memory B cells modulated expression of *PAX5*, *PRDM1*, *XBP1*, and *IRF4* in a manner indistinguishable from normal B cells (Fig. 5, A and B), consistent with normal Ig secretion during short-term cultures (Fig. 4, C and F). However, $STAT3_{MUT}$ B cells revealed marked differences in the behavior of naive and memory cells. Although naive $STAT3_{MUT}$ B cells down-regulated *PAX5* in response to IL-21, they failed to up-regulate *PRDM1* and *XBP1* (Fig. 5 A). In stark contrast, IL-21–mediated induction of *PRDM1*, *XBP1*, and *IRF4* in $STAT3_{MUT}$ memory B cells was intact (Fig. 5 B), mirroring the ability of these cells to secrete large amounts of Ig in response to IL-21 (Fig. 3 B). Induction of *AICDA* in $STAT3_{MUT}$ naive B cells by IL-21 was comparable with normal and $STAT1_{MUT}$ naive B cells (Fig. 5 A), further demonstrating that $STAT3_{MUT}$ naive B cells can respond to IL-21 under the culture conditions used here. In contrast to naive B cells, IL-21 reduced *AICDA* expression in memory B cells from all individuals compared with stimulation with CD40L alone (Fig. 5 B). This is probably a result of memory B cells expressing much higher levels of *PRDM1* (Fig. 5 B), which directly represses *AICDA* (Nutt et al., 2011). Thus, differentiation of naive and memory B cells into Ab-secreting cells, as determined both at the cellular and molecular level, exhibit distinct sensitivity to mutations in *STAT3*.

Memory cells exhibit greater sensitivity to the stimulatory effects of STAT3-activating cytokines IL-21 and IL-10

One possible explanation for this differential susceptibility to mutations in *STAT3* would be that in memory B cells IL-21 activates an alternate STAT3-independent signaling pathway. However, we observed little activation of pathways other than STAT1 and STAT3 in memory B cells (Fig. 2). An alternative possibility was that memory B cells expressed higher levels of STAT3 or were enriched for expression of the wild-type

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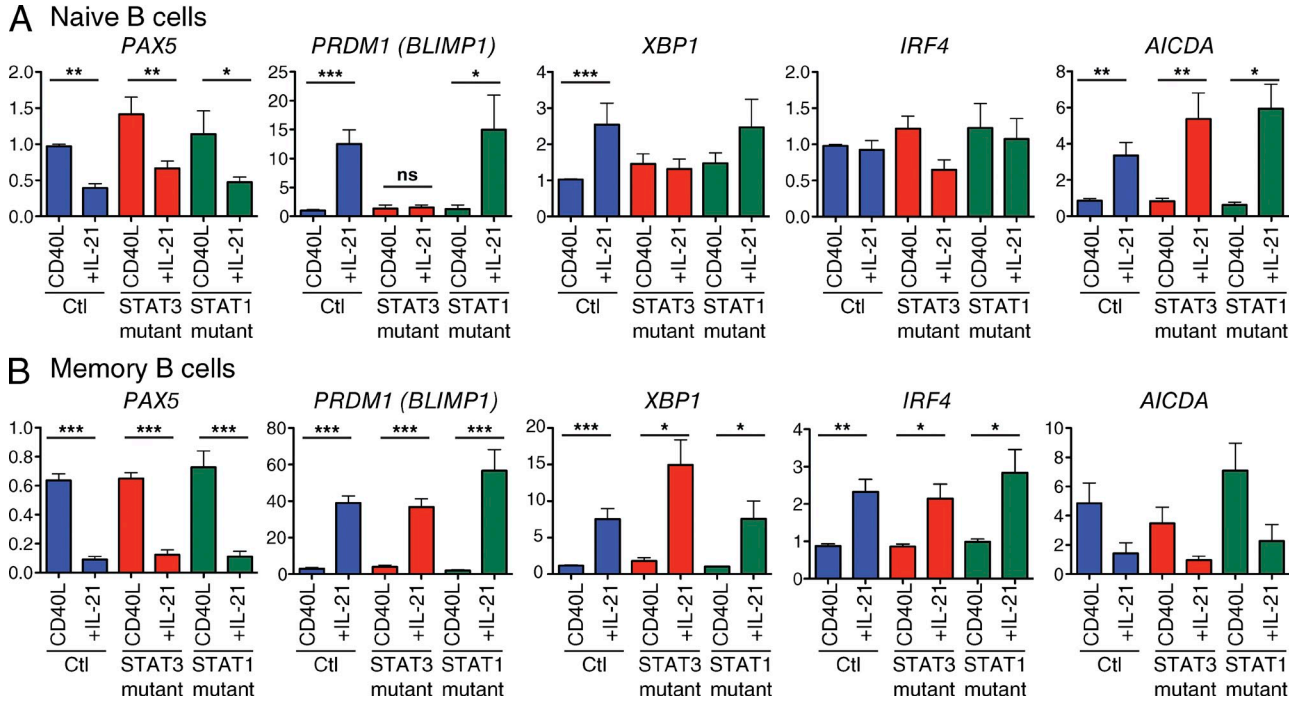

Figure 5. Induction of the PC transcriptional program is intact in IL-21–stimulated STAT3$_{MUT}$ memory B cells. (A and B) Naive ($CD20^+CD10^-CD27^-IgG^-$; A) and memory ($CD20^+CD10^-CD27^+$; B) B cells were sort-purified from normal donor controls (Ctl; $n = 10$ [or 7 for *AICDA*]), STAT3$_{MUT}$ patients ($n = 5$), or STAT1$_{MUT}$ patients ($n = 4$) and then cultured with CD40L alone or together with IL-21 (+IL-21) for 5 d. Expression of *PAX5*, *PRDM1*, *XBP1*, *IRF4*, and *AICDA* was determined by qPCR. The columns represent the mean ± SEM of experiments performed using naive B cells from 7–10 normal donors, 5 STAT3$_{MUT}$ patients, or 4 STAT1$_{MUT}$ patients. Levels of expression are relative to the amount of *GAPDH*. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

STAT3 allele compared with naive B cells. Yet we found that naive and memory B cells from normal donors or AD-HIES patients expressed comparable levels of STAT3 both ex vivo and after in vitro culture (not depicted) and that the mutant allele still accounted for ∼50% of total STAT3 that was expressed by memory B cells (not depicted). A final explanation for the differential effects of STAT3 deficiency on the function of naive versus memory B cells derives from the heterozygous nature of the *STAT3* mutations and the fact that STATs exert their effect as dimers. This predicts that the mutant allele acts in a dominant-negative manner, thereby inhibiting the function of up to 75% of STAT3 dimers and leaving only 25% intact (Holland et al., 2007; Minegishi et al., 2007). Thus, the differential sensitivity of naive and memory B cells to *STAT3* mutations may reflect an increased responsiveness of memory cells to STAT3 action such that the residual wild-type STAT3 dimers in STAT3$_{MUT}$ memory, but not naive, B cells are sufficient to render these cells responsive to the effects of STAT3-activating cytokines. To test whether there are differences in the threshold of activation of naive and memory B cells, these B cell subsets were purified from normal donor spleens and cultured with CD40L and increasing concentrations of IL-21 or IL-10, and induction of expression of *PRDM1* and *XBP1* was determined after 4 d. The concentrations of IL-21 (Fig. 6 A, left) and IL-10 (Fig. 6 B, left) required to induce *PRDM1* in naive B cells (i.e., 10 ng/ml IL-21 and 100 U/ml IL-10) were at least five times higher than those required for induction in memory B cells (i.e., 2 ng/ml IL-21 and 20 U/ml IL-10). Furthermore, these cytokines induced *PRDM1* in memory B cells at levels that exceeded those in naive B cells by two- to five-fold (Fig. 6, A and B, left). Induction of *XBP1* followed a similar pattern, with expression being detected in memory B cells at much reduced cytokine concentrations than in naive B cells and memory B cells expressing substantially more *XBP1* than naive B cells (Fig. 6, A and B, right). Collectively, these results demonstrate that memory B cells have greater sensitivity to the stimulatory effects of these STAT3-activating cytokines, especially when present at limiting concentrations. Thus, it is likely that the small percentage of wild-type STAT3 dimers that can form in STAT3$_{MUT}$ memory B cells are sufficient to integrate signals provided by IL-10 and IL-21 to facilitate the differentiation of memory B cells into Ig-secreting plasmablasts.

Loss-of-function mutations in *IL21R* abolishes responses of naive and memory B cells to IL-21

To establish that the differences in responses of naive and memory STAT3$_{MUT}$ B cells to IL-21 involved direct signaling through the IL-21R, rather than interactions between IL-21 and a putative alternate receptor that may be expressed only on memory B cells and functions independently of

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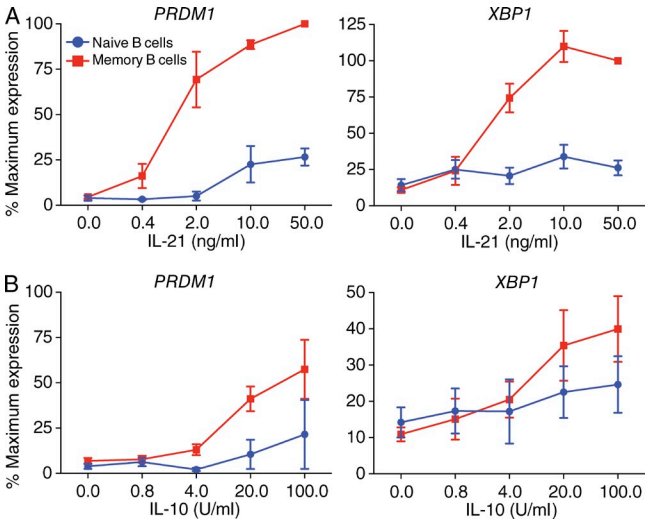

Figure 6. Memory B cells exhibit greater sensitivity to the differentiation-inducing effects of STAT3-activating cytokines IL-21 and IL-10. (A and B) Naive and memory B cells were sort-purified from normal donor spleens and then cultured with CD40L alone or with increasing concentrations of IL-21 (A) or IL-10 (B). After 4 d, expression of *PRDM1* and *XBP1* was determined by qPCR. The values are presented as the percentage of the maximum response, defined as the levels of expression induced in memory B cells by the highest dose of IL-21 tested (50 ng/ml). The data represent the mean ± SEM of experiments using naive and memory B cells from three different normal donor spleens.

STAT3, we examined the B cell compartment in recently identified individuals with loss-of-function mutations in *IL21R* (Kotlarz et al., 2013). Phenotypic analysis of three individuals revealed a marked deficiency in memory B cells that was comparable with that observed for $STAT3_{MUT}$ patients (Fig. 7 A and Table 1). Furthermore, in contrast to healthy donors, ∼95% of IL-$21R_{MUT}$ B cells were IgM^+IgD^+, revealing a deficiency in Ig isotype–switched cells (Fig. 7 B). We also quantified the proportion of memory B cells that expressed specific Ig isotypes and found that although ∼50% of memory B cells from normal donors had lost expression of IgD and IgM, >90% of IL-$21R_{MUT}$ memory B cells remained IgD^+ (Fig. 7 C and not depicted). Consistent with this, <5% of IL-$21R_{MUT}$ memory B cells had undergone switching to express IgG or IgA, whereas the memory B cell pool of normal donors is comprised of ∼25% IgG^+ and ∼20% IgA^+ cells (Fig. 7 C). Thus, IL-21 signaling is indispensable for the generation of not only a normal pool of memory B cells but also the generation of isotype-switched effector B cells within the memory cell subset.

When naive B cells were isolated from normal donors and IL-$21R_{MUT}$ individuals and cultured in vitro, only B cells from normal donors responded to IL-21, as revealed by the generation of $CD38^{hi}CD27^{hi}$ plasmablasts (Fig. 7 D), secretion of high levels of IgM, IgG, and IgA (Fig. 7 E), and down-regulation of *PAX5* while concomitantly acquiring *AICDA*, *PRDM1*, and *XBP1* expression (Fig. 7 F). All of these readouts of naive B cell differentiation were abolished by loss-of-function mutations in *IL21R* (Fig. 7, D–F). Not surprisingly, CD40L-stimulated IL-$21R_{MUT}$ memory B cells also failed to respond to the stimulatory effects of IL-21 (Fig. 7 E). IL-$21R_{MUT}$ naive B cells, though, are intrinsically functional, as indicated by intact responses to IL-4 and IL-10 with respect to induction of *AICDA* (IL-4 and IL-10) and *XBP1* (IL-4) and reduction in *PAX5* (IL-10; Fig. 7 F). These findings demonstrate the specificity of the IL-21 used in our experiments and reveal that both naive and memory B cells absolutely require a functional IL-21R for their response to IL-21.

DISCUSSION

Naive and memory B cells play distinct roles during humoral immune responses. Thus, naive B cells activated after primary encounter with foreign Ag initially produce Ag-specific IgM and eventually yield B cells that produce IgG or IgA. In contrast, memory cells respond much more efficiently upon subsequent exposure to such Ags, rapidly differentiating into Ab-secreting cells to produce substantially higher levels of protective Ig than naive cells (Ahmed and Gray, 1996; Rajewsky, 1996; Tangye and Tarlinton, 2009). This increased efficacy of memory B cell activation is one mechanism underlying long-term protective immunological memory.

B cell differentiation into plasmablasts is regulated by the integration of signals provided by Ag, T cell help (CD40L), and cytokines. Signal transduction pathways activated by these ligands converge to activate key transcriptional regulators, such as Blimp-1, that mediate the commitment of activated B cells to a PC fate (Nutt et al., 2011). Cytokines important for human B cell differentiation include IL-10 and IL-21, which induce isotype switching, PC generation, and Ab secretion from activated naive and memory B cells (Banchereau et al., 1994; Arpin et al., 1997; Pène et al., 2004; Ettinger et al., 2005; Bryant et al., 2007; Avery et al., 2008a). A common feature of these cytokines is their ability to activate similar signaling intermediates, such as STAT1 and STAT3. Remarkably, the effects of IL-10 or IL-21 on human naive B cells are abolished by heterozygous *STAT3* mutations, yet are unaffected by mutations in *STAT1* (Figs. 3–5; Avery et al., 2010). These defects likely explain defective Ab responses and reduced numbers of memory B cells in AD-HIES (Leung et al., 1988; Sheerin and Buckley, 1991; Avery et al., 2010) and, conversely, intact humoral responses in STAT1-deficient patients (Boisson-Dupuis et al., 2012). Interestingly, mutations in *IL21R* recapitulated the impaired Ab responses to vaccines (Kotlarz et al., 2013) and memory B cell deficit (Fig. 7) observed in AD-HIES, despite the ability of IL-21R–deficient B cells to respond to other growth and differentiation-inducing cytokines such as IL-4 or IL-10. In contrast, B cell responses in vivo appear intact in IL-10/IL-10R–deficient individuals (Kotlarz et al., 2012). Thus, STAT3, downstream of IL-21R, clearly plays a central role in establishing long-lived Ab-mediated immunity.

Our study revealed that *STAT3* mutations do not affect memory B cell function, as $STAT3_{MUT}$ memory cells underwent the molecular and cellular changes required for plasmablast

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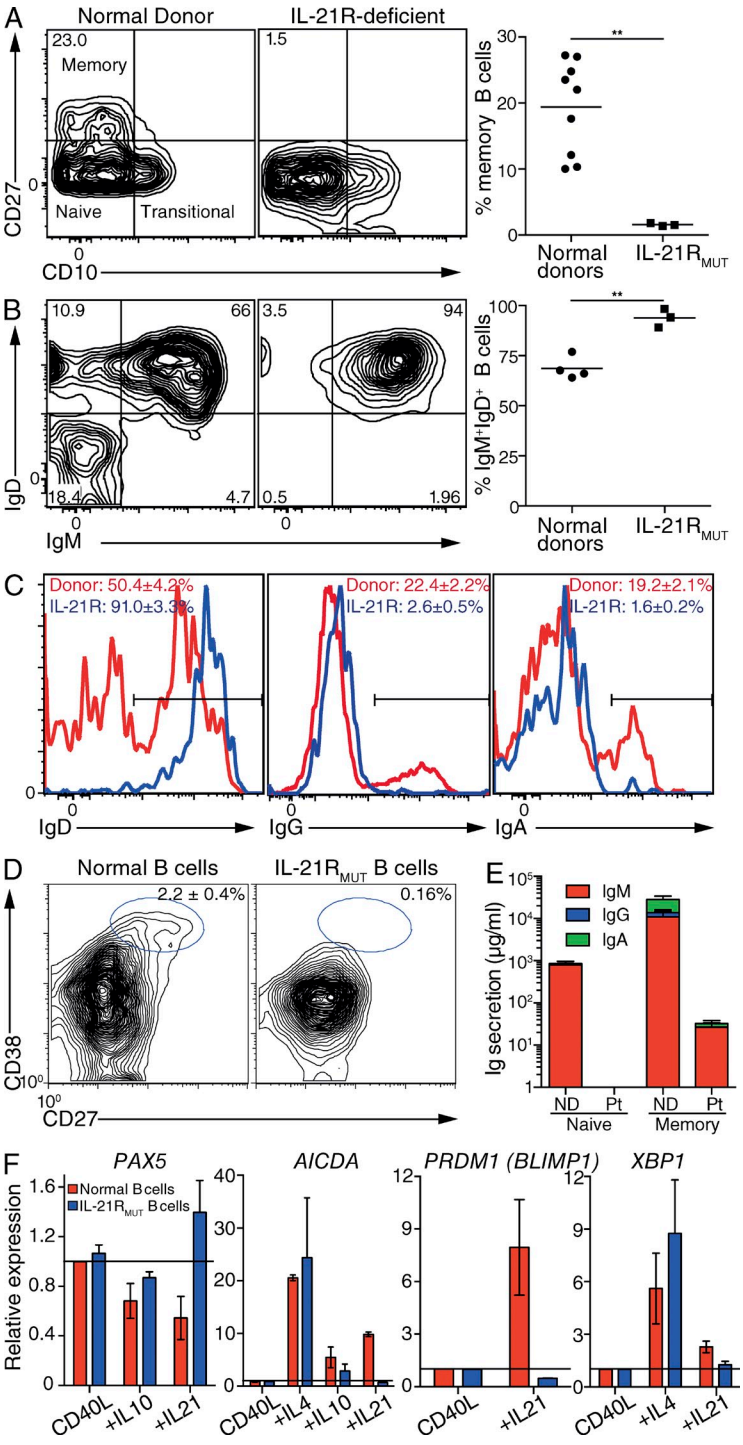

Figure 7. Loss-of-function mutation in *IL21R* abolishes B cell responses to IL-21. (A and B) PBMCs from age-matched normal donors and three patients with loss-of-function mutations in *IL21R* were labeled with mAb against CD20, CD10, CD27, IgM, IgD, IgG, and IgA. (A) Memory B cells were quantified based on the frequency of $CD20^+$ B cells that were $CD10^-CD27^+$. (B and C) The percentages of total B cells in normal donors or IL-21R–deficient patients that coexpressed IgM and IgD (B) and of memory (i.e., $CD27^+$) B cells from normal donors and IL-$21R_{MUT}$ patients that expressed IgD, IgG, or IgA (C) were determined. **, $P < 0.01$. Each symbol in A and B represents an individual normal donor or IL-$21R_{MUT}$ patient; the horizontal bars represent means. The values in C represent the mean percentage ± SEM of memory B cells from four normal donors or three IL-$21R_{MUT}$ patients that express IgD, IgG, or IgA. (D–F) Naive or memory B cells sort-purified from normal donors or IL-21R1$_{MUT}$ patients were cultured with CD40L alone or CD40L/IL-21 (D and E) or CD40L/IL-4, CD40L/IL-10, or CD40L/IL-21 (F). After 5 d, the percentage of plasmablasts (i.e., $CD38^{hi}CD27^{hi}$) generated (D) and expression of *PAX5*, *AICDA*, *PRDM1*, and *XBP1* by cultured naive B cells (F) were determined by flow cytometry or qPCR. The values in D represent the mean (±SEM) percentage of naive B cells that acquired a plasmablast phenotype in response to CD40L/IL-21 in experiments using naive B cells from three different normal donors or one IL-$21R_{MUT}$ patient. In the absence of IL-21 <0.5% plasmablasts were detected in these cultures. (E) The levels of secreted IgM, IgG, and IgA by naive and memory B cells from normal donors (ND) or IL-$21R_{MUT}$ patients (Pt) in response to stimulation with CD40L/IL-21 were determined by ELISA after 10 d. The amounts of Ig secreted by IL$21R_{MUT}$ memory B cells in response to CD40L/IL-21 did not differ from those induced by CD40L alone (not depicted). These data are from one of three experiments using naive B cells from five different healthy controls or two unrelated IL-$21R_{MUT}$ patients. The values in F represent the mean ± SEM from experiments using two to five different healthy controls or of three experiments using cells from two unrelated IL-$21R_{MUT}$ patients. The horizontal black lines on the graphs in F indicate a value of 1.0, which corresponds to the relative level of expression of the indicated gene in CD40L-stimulated normal naive B cells.

differentiation in response to IL-10 or IL-21. Importantly, although memory cells are numerically deficient in AD-HIES patients, they produced normal levels of Ag-specific IgG in vitro on a per cell basis, inferring that $STAT3_{MUT}$ memory B cells would be functional in vivo. This explains the variability in impairment in humoral immunity in AD-HIES patients (Sheerin and Buckley, 1991; Avery et al., 2010). There are several explanations for the normal response of $STAT3_{MUT}$ memory B cells to STAT3 cytokines. First, these cytokines may activate signaling pathways in memory B cells distinct from naive cells, thereby allowing memory cells to respond independently of STAT3, unlike naive B cells. This is unlikely as we detected comparable activation of STATs, yet little activation of Erk and Akt in IL-21–stimulated naive and memory B cells. Second, STAT3 may be differentially expressed by naive and memory B cells; however, this was also

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found to not be the case. Third, because $STAT1_{MUT}$ B cells secreted less Ig in vitro over time, STAT1 may contribute to the function of activated memory B cells. Even if correct, the reduction in Ig secretion by $STAT1_{MUT}$ memory B cells is probably not physiologically significant as these patients have intact humoral immunity (Boisson-Dupuis et al., 2012). This may reflect the intact early differentiation of $STAT1_{MUT}$ naive and memory B cells in response to IL-10 and IL-21 in vitro, the generation of normal numbers of memory B cells in vivo, and the ability of $STAT1_{MUT}$ memory B cells to produce sufficient quantities of specific Abs after reexposure to immunizing Ags or infectious pathogens. These findings suggest that STAT1 plays only a minor, if any, role in inducing and maintaining humoral immunity. A final possibility is that memory B cells require less activated STAT3 to respond to specific cytokines than do naive B cells. Thus, the residual amounts of functional STAT3 in $STAT3_{MUT}$ B cells are sufficient to mediate plasmablast differentiation induced by IL-10 and IL-21 in memory, but not naive, B cells. This is supported by our finding that IL-10 and IL-21 induced expression of the key PC transcription factors Blimp-1 and XBP-1 in memory B cells at concentrations that had no effect on gene expression in corresponding naive B cells (Fig. 6). This, therefore, is our favored model, which is also consistent with memory B cells having a lower threshold for activation than naive B cells (Yefenof et al., 1986; Poudrier and Owens, 1994), which underlies their rapid response on subsequent encounters with specific Ag (Ahmed and Gray, 1996; Rajewsky, 1996; Tangye and Tarlinton, 2009; Goodnow et al., 2010).

These findings also provide important insights into the hierarchy by which cytokines operate to induce B cell differentiation. Thus, although IL-21R–deficient naive B cells could respond to IL-4 and IL-10 in vitro to induce key events required for Ig isotype switching (*AICDA* expression) and PC generation (*PRDM1* and *XBP1*), responses to these and other cytokines such as BAFF and APRIL (Banchereau et al., 1994; Litinskiy et al., 2002; Avery et al., 2003; Craxton et al., 2003) are insufficient in vivo to compensate for a complete absence of IL-21/IL-21R signaling. This is indicated by B cells in IL-21R–deficient individuals expressing only IgM and IgD, with essentially no isotype-switched cells being detected (Fig. 7). Thus, despite IL-4 and IL-10 inducing *AICDA* expression in IL-21R–deficient B cells, a primary signal via the IL-21R appears to be the critical and rate-limiting step for B cells to undergo isotype switching, after which cytokines such as IL-4, IL-10, BAFF, and APRIL can cooperate to enhance IL-21–induced switching and B cell differentiation (Litinskiy et al., 2002; Avery et al., 2003, 2008a; Craxton et al., 2003). This is reminiscent of the immunological phenotype of X-linked or JAK3-deficient SCID patients who have undergone stem cell transplant but retain autologous (i.e., *IL2RG* or *JAK3* mutant) B cells, inasmuch that these patients have significant reductions in memory B cells, isotype-switched B cells, and serum IgM and lack serum IgG and IgA (Recher et al., 2011), thereby highlighting the requirement for intact signaling through γc/JAK3 downstream of IL-4R and IL-21R for B cell differentiation and effector function. Interestingly though, populations of IgG^+ and IgA^+ cells were detectable within the memory B cell subset of STAT3-deficient individuals, despite the reduction in total memory B cells in these patients. Because IL-21 could induce CD40L-activated $STAT3_{MUT}$ naive B cells to express *AICDA*, but not *PRDM1*, it is likely that the level of STAT3 required to mediate class switching in naive B cells is significantly less that that required for plasmablast formation. Thus, although IL-21R is indispensable for class switching in vivo, the residual amount of functional STAT3 in $STAT3_{MUT}$ naive B cells is sufficient to mediate IL-21–induced class switching in vivo. These findings demonstrate that within the same cell type (i.e., naive B cells) the thresholds of activation of STAT3 required for different biological processes (i.e., class switching versus plasmablast generation) are distinct, thereby providing a rational explanation for (a) intact class switch recombination but defective plasmablast formation by STAT3-deficient naive B cells to IL-21 and (b) phenotypic differences between memory B cells in patients with mutations in *STAT3* or *IL21R*.

As a key attribute of memory B cells is their ability to respond more rapidly than naive B cells, a question that arises is why $STAT3_{MUT}$ memory B cells do not increase in frequency over time to improve humoral immunity in AD-HIES. Because $STAT3_{MUT}$ memory B cells exhibit normal responses to IL-21 in vitro, this would suggest that availability of, or access to, stimulatory cytokines in vivo is limiting. We have reported that the proportions (Ma et al., 2012; Mazerolles et al., 2013) and absolute numbers (101 ± 7 cells/ml in normal donors vs. 51.6 ± 10 cells/ml peripheral blood in STAT3 deficiency) of circulating $CD4^+CXCR5^+$ T cells, which like Tfh cells present in secondary lymphoid tissues are enriched for IL-21–producing cells (Chevalier et al., 2011), are reduced in AD-HIES patients. Furthermore, $STAT3_{MUT}$ $CD4^+$ T cells are impaired in their ability to generate Tfh-like cells in vitro, thereby compromising IL-21–mediated help for B cell differentiation (Ma et al., 2012). These observations are consistent with a scenario whereby $STAT3_{MUT}$ memory B cells, despite their intact ability to respond to IL-21, are constrained in doing so in vivo because of diminished production of IL-21 by STAT3-deficient $CD4^+$ T cells.

The deficit in memory B cells observed in $STAT3_{MUT}$ patients is comparable with that in other immune-deficient individuals, such as patients with mutations in *SH2D1A* (XLP; Ma et al., 2005, 2006), *CD40LG* (hyper-IgM syndrome; Notarangelo et al., 2006) or *ICOS* (common variable immunodeficiency; Warnatz et al., 2006), or transplanted X-linked/JAK3-deficient SCID patients who retain autologous B cells (Recher et al., 2011). Although all of these latter conditions are characterized by reductions in serum Ig levels (Ma et al., 2005; Notarangelo et al., 2006; Warnatz et al., 2006; Recher et al., 2011), serum levels of IgM, IgG, and IgA are normal in STAT3-deficient patients despite a generalized impairment in the ability to elicit sustained Ag-specific Ab

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responses (Leung et al., 1988; Sheerin and Buckley, 1991; Avery et al., 2010). Our results provide a potential explanation for the apparent discrepancy between reduced memory B cells but normal serum Ig levels in STAT3 deficiency, inasmuch that the residual memory B cells can respond to B cell differentiating cytokines and thus contribute to the pool of serum Ig. The corollary of this is that the small population of memory B cells in XLP, hyper-IgM syndrome, ICOS deficiency, and posttransplant SCID are unable to access appropriate $CD4^+$ T cell–derived signals (i.e., SAP-dependent interactions; CD40L; ICOS; IL-2/IL-4/IL-21) and thus are limited in their ability to contribute to humoral immunity. An extension of our findings is that because we could detect functional Ag-specific $STAT3_{MUT}$ memory B cells, directed targeting of these cells with IL-21–mediated signals may improve humoral immunity in AD-HIES patients.

MATERIALS AND METHODS

Human blood and tissue samples. Buffy coats and spleens from healthy donors were provided by the Australian Red Cross Blood Service. Peripheral blood was also collected from patients with loss-of-function mutations in *STAT1* ($STAT1_{MUT}$; Dupuis et al., 2001; Chapgier et al., 2006, 2009; Sampaio et al., 2012; Hirata et al., 2013; Ives et al., 2013), *STAT3* ($STAT3_{MUT}$; Ma et al., 2008, 2012; Avery et al., 2010) or *IL21R* ($IL\text{-}21R_{MUT}$; Kotlarz et al., 2013; Ives et al., 2013; Table S1). Approval for this study was obtained from the human research ethics committees of the St. Vincent's Hospital and Sydney South West Area Health Service (Australia), the Rockefeller University Institutional Review Board (New York), and the National Institute of Allergy and Infectious Diseases Intramural Institutional Review Board (Bethesda, MD).

Lymphocyte phenotyping and isolation. PBMCs were incubated with mAb to CD20 and CD27 and an isotype control or mAb specific for CD23, CD24, CD80, CD86, CD95, TACI, IgM, IgD, IgG, IgG1, IgG2, IgG3, IgG4, and IgA, and expression of these molecules on $CD20^+CD27^-$ (naive) and $CD20^+CD27^+$ (memory) B cells was determined by flow cytometry (Ma et al., 2006). Naive and memory B cells were purified by labeling either PBMCs or total B cells with mAb against CD10, CD20, and CD27 and sorting $CD10^-CD20^+CD27^-$ (naive) and $CD10^-CD20^+CD27^+$ (memory) cells (FACSAria; BD; (Avery et al., 2008b, 2010). Splenic B cells were labeled with mAbs against CD20, CD27, IgG, and IgA, and subsets of either naive ($CD20^+CD27^-$) and total memory B cells ($CD20^+CD27^+$) or IgM memory ($CD20^+CD27^-IgG^-IgA^-$) and isotype-switched ($CD20^+CD27^-IgG^+IgA^+$) memory B cells were collected (FACSAria; Tangye et al., 2003a; Bryant et al., 2007; Good et al., 2009). The purity of the recovered populations was typically >98%. To enumerate circulating Tfh-like cells, PBMCs were labeled with mAbs against CD3, CD4, and CXCR5, and the absolute number of $CD4^+CXCR5^+$ T cells was then determined.

In vitro activation of naive and memory B cells. Naive and memory B cells isolated from normal donors or $STAT3_{MUT}$, $STAT1_{MUT}$, or $IL\text{-}21R_{MUT}$ patients were cultured ($\sim$5–10 $\times$ 10^3/200 µl/well for proliferation, Ig secretion, and qPCR and $\sim$4 $\times$ 10^4/400 µl/well for phenotyping; BD) with CD40L alone or together with 100 U/ml IL-4, 100 U/ml IL-10, 50 ng/ml IL-21 (PeproTech), or 1 µg/ml CpG 2006 (Sigma-Aldrich). Expression of *STAT3*, *PAX5*, *PRDM1*, *XBP1*, *IRF4*, and *AICDA* was determined after 5 d by real-time PCR and standardized to *GAPDH* (Avery et al., 2010). Expression of Ig ε germline transcripts were determined by PCR as described previously (Avery et al., 2008b). Differentiation of B cells to plasmablasts was assessed by determining the frequency of cells acquiring a $CD38^{hi}CD27^{hi}$ phenotype during in vitro culture (Avery et al., 2005). B cell proliferation was determined by assessing the incorporation of [^{3}H]thymidine (1 µCi/ml per well; ICN Biomedicals) during the final 18 h of a 5-d culture (Good et al., 2006). Ig secretion was determined by ELISA after 4–12 d of culture (Bryant et al., 2007). Relative levels of antitetanus IgG in culture supernatants were determined by ELISA using plates precoated with tetanus toxoid (Sigma-Aldrich) and then detecting bound IgG (Avery et al., 2010).

Analysis of intracellular signaling. Naive and memory splenic B cells were cultured with $F(ab')_2$ fragments of goat anti–human Ig (Jackson ImmunoResearch Laboratories, Inc.) for $\sim$18 h, washed, and then recultured with media alone, 100 ng/ml IL-21, or $F(ab')_2$ anti-Ig for 15–30 min. Cells were then fixed, permeabilized, labeled with anti–phospho-STAT1, STAT3, STAT4, STAT5, and STAT6 mAb (Avery et al., 2008b, 2010) and analyzed by flow cytometry. Alternatively, cells were lysed and Western blotting was performed using rabbit polyclonal anti-STAT3 (C-20), anti-ERK2 (C-14), and anti-14.3.3 (K-19; Santa Cruz Biotechnology, Inc.); and anti-STAT3 pY705 (3E2), anti-AKT pS473 (587F11), and anti-ERK1/2 pT202/Y204 (Cell Signaling Technology).

Statistical analysis. Significant differences between datasets were determined using either the unpaired Student's *t* test when comparing two variables or ANOVA for more than two variables (Prism; GraphPad Software).

Online supplemental material. Table S1 provides details of the patients analyzed in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130323/DC1.

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