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

Long-lived antibody memory is mediated by the combined effects of long-lived plasma cells (PLCs) 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 potentially 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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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 mono- genic primary immunodeficiencies has identified some of the molecular requirements for memory B cell generation. Thus, mutations in B cell–intrinsic genes (CD19/CD81, CD40, IKBKCG, DOCK8, and IL2Rγ) 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 (TLR7/8/9/10), 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 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 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 (STAT3MUT) 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-activating 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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RESULTS

**STAT3**<sub>MUT</sub> CD27<sup>+</sup> B cells phenotypically resemble normal memory B cells

The population of circulating CD27<sup>+</sup> 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<sup>+</sup>. 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<sup>+</sup> B cells in STAT3<sub>MUT</sub> patients.

The size and granularity of CD27<sup>-</sup> and CD27<sup>+</sup> B cells were determined by flow cytometry. This demonstrated that CD27<sup>+</sup> B cells from both normal donors and STAT3<sub>MUT</sub> patients were significantly larger and more granular than corresponding CD27<sup>-</sup> B cells. However, these morphological features were not significantly different between cells from normal donors and STAT3<sub>MUT</sub> 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; Elyard et al., 2004; Good et al., 2009). CD24, CD80, CD95, and TACI were significantly higher on CD27<sup>+</sup> B cells from normal donors than on corresponding CD27<sup>-</sup> B cells (Fig. 1, A and C). CD86 also tended to be higher on normal CD27<sup>+</sup> versus CD27<sup>-</sup> B cells (Fig. 1, A and C). The same pattern was seen for samples from STAT3<sub>MUT</sub> patients, with CD80, CD86, CD95, and TACI being significantly higher on CD27<sup>+</sup> than on CD27<sup>-</sup> 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<sub>MUT</sub> 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<sub>MUT</sub> memory B cells (Fig. 1, B and C; and Table 1).

We also determined expression of Ig isotypes by CD27<sup>-</sup> and CD27<sup>+</sup> B cells from normal and STAT3-deficient individuals. Approximately ~90% of CD27<sup>-</sup> B cells and ~40–50% of CD27<sup>+</sup> 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<sub>MUT</sub> CD27<sup>+</sup> B cells that expressed IgG was greater than that observed for normal CD27<sup>+</sup> B cells (Table 1), the distribution of IgG subclasses within the CD27<sup>-</sup> and CD27<sup>+</sup> B cell subsets was comparable, with IgG1 being preferentially expressed by both CD27<sup>-</sup> and CD27<sup>+</sup> normal and STAT3<sub>MUT</sub> B cells (Table 1). Furthermore, we did not detect an enrichment of B1 cells, based on cells with a CD20<sup>+</sup>CD43<sup>+</sup>CD27<sup>+</sup> phenotype, in STAT3<sub>MUT</sub> 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<sup>+</sup> B cells in STAT3<sub>MUT</sub> 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

### Table 1. Characteristics of CD27<sup>-</sup> and CD27<sup>+</sup> B cells in STAT3-deficient individuals

| Parameter | CD27<sup>-</sup> B cells | CD27<sup>+</sup> B cells | CD27<sup>-</sup> B cells | CD27<sup>+</sup> B cells |
|-----------|--------------------------|--------------------------|--------------------------|--------------------------|
| % Cells   | Normal                   | STAT3<sub>MUT</sub>     | Normal                   | STAT3<sub>MUT</sub>     |
| % IgM<sup>+</sup> | 75.2 ± 2.7               | 94.9 ± 0.77              | 24.8 ± 2.7               | 5.1 ± 0.77               |
| % IgG<sup>+</sup> | 87.0 ± 2.2               | 90.0 ± 2.0               | 47 ± 3.8                 | 48 ± 3.1                 |
| % IgD<sup>+</sup> | 88.0 ± 1.9               | 93.0 ± 1.8               | 41 ± 2.2                 | 45 ± 2.0                 |
| % IgG<sub>4</sub><sup>+</sup> | 3.2 ± 0.45               | 1.6 ± 0.6<sup>a</sup>   | 25 ± 2.1                 | 32 ± 2.8<sup>a</sup>    |
| % IgG<sub>1</sub><sup>+</sup> (% total IgG<sup>+</sup> cells) | 2.3 ± 1.0 (62.1)         | 0.63 ± 0.24 (40.1)       | 11 ± 1.7 (51.4)          | 22 ± 2.8 (72.7)<sup>a</sup> |
| % IgG<sub>2</sub><sup>+</sup> (% total IgG<sup>+</sup> 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)          |
| % IgG<sub>3</sub><sup>+</sup> (% total IgG<sup>+</sup> 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)          |
| % IgG<sub>4</sub><sup>+</sup> (% total IgG<sup>+</sup> 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<sup>+</sup> | 1.2 ± 0.2                 | 0.64 ± 0.15<sup>a</sup> | 17 ± 1.2                 | 8.6 ± 1.1<sup>c</sup>   |
| % CD23<sup>+</sup> | 62.0 ± 3.9                | 86.0 ± 3.7<sup>c</sup>  | 18 ± 3.9                 | 46 ± 4.7<sup>c</sup>    |

Values represent percentage (or absolute number for CD4<sup>+</sup>CXCR5<sup>+</sup> 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<sub>MUT</sub> B cells.

<sup>a</sup>P < 0.05.

<sup>b</sup>P < 0.01.

<sup>c</sup>P < 0.001.
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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$^{\text{MUT}}$ or STAT1$^{\text{MUT}}$ 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$^{\text{MUT}}$ patients, we could only culture $\sim 5,000$ 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$^{\text{MUT}}$ naive B cells (Fig. 3A and Table 2). IL-21 substantially increased production of IgM, IgG, and IgA by CD40L-stimulated memory B cells from normal donors and STAT3$^{\text{MUT}}$ 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$^{\text{MUT}}$ naive and memory B cells, individual graphs are depicted for normal and patient B cell subsets. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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-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$^{\text{MUT}}$ 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$^{\text{MUT}}$ naive B cells in vitro, somatic hypermutation was comparable in normal and STAT3$^{\text{MUT}}$ 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 humna 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$^{\text{MUT}}$ or STAT1$^{\text{MUT}}$ 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$^{\text{MUT}}$ patients, we could only culture $\sim 5,000$ 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$^{\text{MUT}}$ naive B cells (Fig. 3A and Table 2). IL-21 substantially increased production of IgM, IgG, and IgA by CD40L-stimulated memory B cells from normal donors and STAT1$^{\text{MUT}}$ individuals; however, the response of STAT1$^{\text{MUT}}$ memory B cells was significantly less (approximately fourfold) than that of normal memory cells (Fig. 3B and Table 2). Ig secretion by STAT3$^{\text{MUT}}$ naive B cells in response to IL-21 was $\sim 30$-fold less than normal naive cells (Fig. 3A and Table 2; $P < 0.001$). In distinct contrast, Ig secretion by IL-21–stimulated STAT3$^{\text{MUT}}$ memory B cells (either total memory cells [Fig. 3B and Table 2] or IgM$^+$ and switched subsets [not depicted]) was largely comparable with normal memory B cells.
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\(^{\text{MUT}}\) naive and memory B cells. IL-21 enhanced proliferation of both normal and STAT3\(^{\text{MUT}}\) naive B cells over that induced by CD40L alone, yet the response of STAT3\(^{\text{MUT}}\) 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\(^{\text{MUT}}\) 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\(^{\text{MUT}}\) naive and memory B cells. IL–21 enhanced proliferation of both normal and STAT3\(^{\text{MUT}}\) naive B cells over that induced by CD40L alone, yet the response of STAT3\(^{\text{MUT}}\) 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\(^{\text{MUT}}\) memory

with the only significant difference (less than twofold) being noted for the levels of IgM secreted by normal versus STAT3\(^{\text{MUT}}\) 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\(^{\text{MUT}}\) 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\(\varepsilon\) 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\(^{\text{MUT}}\) 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\(^{\text{MUT}}\) naive and memory B cells. IL–21 enhanced proliferation of both normal and STAT3\(^{\text{MUT}}\) naive B cells over that induced by CD40L alone, yet the response of STAT3\(^{\text{MUT}}\) 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\(^{\text{MUT}}\) memory

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 was 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.
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 STAT3MUT B cells to IL-21 correlates with poor differentiation of these cells to plasmablasts under these culture conditions. However, naive STAT3MUT 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 STAT3MUT 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 STAT3MUT or STAT1MUT 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, STAT3MUT 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 STAT1MUT 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 STAT3MUT memory B cells (Fig. 3 I). We also assessed production of antitetanus IgG in cultures of total PBMCs from normal donors and STAT3MUT individuals that had been stimulated with CD40L and IL-21. On average, normal PBMCs produced approximately threefold higher levels of antitetanus IgG than did STAT3MUT PBMCs (Fig. 3 J). However, there are several caveats to screening PBMCs, rather than purified B cells, for the production of Ag-specific Ab.
Table 2. Ig secretion by IL-21–stimulated normal, STAT3MUT, and STAT1MUT naive and memory B cells

| Cell type and culture | IgM | IgG | IgA |
|-----------------------|-----|-----|-----|
|                       | ng/ml | ng/ml | ng/ml | ng/ml | ng/ml | ng/ml |
| Naive B cells          |       |       |       |       |       |       |
| CD40L                 | 2.0 ± 1.0 | 5.0 ± 2.4<sup>a</sup> | <1<sup>0.1<sup>a</sup> | 267 ± 80 | 7.3 ± 2.7<sup>a</sup> | 86 ± 40<sup>a</sup> |
| +IL-21                | 14,640 ± 2,021 | 444 ± 177<sup>d</sup> | 10,801 ± 3,183<sup>a</sup> | 365 ± 106 | 4.6 ± 2.6<sup>a</sup> | 174 ± 40<sup>a</sup> |
| Memory B cells         |       |       |       |       |       |       |
| CD40L                 | 166 ± 51 | 100 ± 25<sup>a</sup> | 2.5 ± 2.5<sup>a</sup> | 6.1 ± 1.4 | 11.8 ± 4.7<sup>a</sup> | 0.2 ± 0.14<sup>a</sup> |
| +IL-21                | 29,389 ± 4,107 | 15,214 ± 1,905<sup>a</sup> | 7,396 ± 3,097<sup>d</sup> | 4,202 ± 1,122 | 5,651 ± 1,743<sup>a</sup> | 953 ± 195<sup>a</sup> |
| Naive and memory B cells were sorted from normal healthy donors (n = 17), STAT3MUT patients (n = 8), or STAT1MUT 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 STAT3MUT or STAT1MUT B cells.

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 (Litinskaya 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<sup>+</sup> 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 STAT1MUT memory B cells were 2.5–5-fold less than those by normal memory B cells (Fig. 3 B and Table 2). To determine whether this reflected a quantitative defect in generating Ab-secreting cells from STAT1MUT memory B cells, we performed kinetic analyses of plasmablast formation and Ig secretion by normal and STAT1MUT 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 STAT1MUT naive B cells and ~1–3% of memory B cells acquiring a CD38<sup>hi</sup>CD27<sup>hi</sup> 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<sup>a</sup> mutations (Fig. 4). Collectively, these results suggested that STAT1MUT memory B cells could initially generate normal numbers of functional plasmablasts. However, in contrast to this normal rate of differentiation of STAT1MUT 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
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 STAT3MUT 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 STAT3MUT naive and memory B cells after stimulation with IL–21. We also examined STAT1MUT 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).

STAT1MUT 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, STAT3MUT B cells revealed marked differences in the behavior of naive and memory cells. Although naive STAT3MUT 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 STAT3MUT 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 STAT3MUT naive B cells by IL–21 was comparable with normal and STAT1MUT naive B cells (Fig. 5 A), further demonstrating that STAT3MUT 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
Further, 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.

**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 **STAT3**s 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
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*<sub>MUT</sub> patients (Fig. 7 A and Table 1). Furthermore, in contrast to healthy donors, ~95% of *IL21R*<sub>MUT</sub> B cells were IgM<sup>+</sup>IgD<sup>-</sup>, 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 *IL21R*<sub>MUT</sub> memory B cells remained IgD<sup>+</sup> (Fig. 7 C and not depicted). Consistent with this, ~5% of *IL21R*<sub>MUT</sub> 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<sup>+</sup> and ~20% IgA<sup>+</sup> 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 *IL21R*<sub>MUT</sub> individuals and cultured in vitro, only B cells from normal donors responded to IL-21, as revealed by the generation of CD38<sup>hi</sup>CD27<sup>hi</sup> 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<sub>MUT</sub> memory B cells also failed to respond to the stimulatory effects of IL-21 (Fig. 7 E). IL-21R<sub>MUT</sub> 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; Ettenger 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-21–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*<sub>MUT</sub> memory cells underwent the molecular and cellular changes required for plasmablast
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 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\textsubscript{MUT} memory B cells would be functional in vivo. This explains the variation 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\textsubscript{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
found to not be the case. Third, because STAT1\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsuperscript{+} and IgA\textsuperscript{+} 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\textsubscript{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\textsubscript{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\textsubscript{MUT} memory B cells do not increase in frequency over time to improve humoral immunity in AD-HIES. Because STAT3\textsubscript{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\textsuperscript{+}CXCR5\textsuperscript{+} 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\textsubscript{MUT} CD4\textsuperscript{+} 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\textsubscript{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\textsuperscript{+} T cells.

The deficit in memory B cells observed in STAT3\textsubscript{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
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 STAT3 (STAT3±MUT; Dupuis et al., 2001; Chapgier et al., 2006, 2009; Sampaio et al., 2012; Hirata et al., 2013; Ives et al., 2013), STAT4 (STAT4±MUT; Ma et al., 2008, 2012; Avery et al., 2010) or IL21R (IL–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, TAC1, 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±CD27 (naive) and CD10±CD27+ (memory) cells (FACSAnA; BD). By (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 (FACSAnA; Tangye et al., 2003a; Bryant et al., 2007; Good et al., 2009). The purity of the recovered populations was typically $>98\%$. To enumerate circulating Th–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, STAT4±MUT, or IL–21R±MUT patients were cultured (5–10 $\times$ 104/200 µl/well for proliferation, Ig secretion, and qPCR; and 4 $\times$ 104/400 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, BXP1, IRF4, and AICDA was determined after 5 d by real–time PCR and standardized to GAPDH (Avery et al., 2010). Expression of Ig (g) 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±CD22± phenotype during in vitro culture (Avery et al., 2005). B cell proliferation was determined by assessing the incorporation of [3H]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 anti–tetanus 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 Immuno–Research Laboratories, Inc.) for ~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–I4.3.3 (K–19; Santa Cruz Biotechnology, Inc.); and anti–STAT3 pY705 (3E2), anti–AKT pS473 (S87F11), 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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