Self-reactive VH4-34–expressing IgG B cells recognize commensal bacteria

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The germline immunoglobulin (Ig) variable heavy chain 4–34 (*VH4-34*) gene segment encodes in humans intrinsically self-reactive antibodies that recognize I/i carbohydrates expressed by erythrocytes with a specific motif in their framework region 1 (FWR1). VH4-34–expressing clones are common in the naive B cell repertoire but are rarely found in IgG memory B cells from healthy individuals. In contrast, CD27$^+$IgG$^+$ B cells from patients genetically deficient for IRAK4 or MYD88, which mediate the function of Toll-like receptors (TLRs) except TLR3, contained VH4-34–expressing clones and showed decreased somatic hypermutation frequencies. In addition, *VH4-34*–encoded IgGs from IRAK4- and MYD88-deficient patients often displayed an unmutated FWR1 motif, revealing that these antibodies still recognize I/i antigens, whereas their healthy donor counterparts harbored FWR1 mutations abolishing self-reactivity. However, this paradoxical self-reactivity correlated with these *VH4-34*–encoded IgG clones binding commensal bacteria antigens. Hence, B cells expressing germline-encoded self-reactive VH4-34 antibodies may represent an innate-like B cell population specialized in the containment of commensal bacteria when gut barriers are breached.

INTRODUCTION

The clonal selection theory by Burnet and Talmage (Burnet, 1976) postulates that B cells express antibodies on their cell surface, allowing negative selection of autoreactive clones during early B cell development while permitting the activation and expansion of specific mature B cells recognizing foreign antigens. Antigen stimulation drives the maturation of naive B cells into memory B cells and plasma cells after the induction of somatic hypermutations (SHMs), followed by a series of selection steps allowing antibody maturation in germinal centers (Rajewsky, 1996; Goodnow et al., 2010; Victora and Nussenzweig, 2012). The induction of class-switch recombination in B cells permits the production of either IgG$^+$ or IgA$^+$ class-switched memory B cells that are able to react quickly to a recurrent antigenic challenge, thereby providing serological immune protection (Berkowska et al., 2011). IgA$^+$ memory B cells are mostly present in mucosa where they regulate gut microbiota homeostasis and mediate protection against invading pathogens, whereas IgG$^+$ memory B cells are generated after systemic antigenic exposure (Fagarasan et al., 2002; Boullier et al., 2009). Although IgG$^+$ and IgA$^+$ human memory B cells are produced during specific immune challenges, both populations express a higher frequency of multispecific/polyreactive and autoreactive antibodies compared with the mature naive B cells from which they originated, a feature not anticipated by the clonal selection theory (Tiller et al., 2007; Berkowska et al., 2015; Prigent et al., 2016). However, the proportions of autoreactive clones in naive and memory B cell compartments were not assessed in the same individuals, and gene variants have recently been shown to significantly increase the frequency of autoreactive B cells in the naive compartments of asymptomatic healthy donors (HDs), thereby questioning whether self-reactivity is really enriched in memory B cells (Tiller et al., 2007).

Impaired CD27$^+$IgM$^+$ memory responses have been reported in IRAK4- and MYD88-deficient patients, but the frequency and numbers of isotype-switched B cells did not appear to be affected by IRAK4 or MYD88 deficiency (Weller et al., 2012). However, the specific Ig repertoire and

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Abbreviations used: AVY, Ala-Val-Tyr; BCR, B cell receptor; CDR, complementary determining region; dsDNA, double-stranded DNA; FWR, framework region; NHS, N-glycosylation site; SHM, somatic hypermutation; SLE, systemic lupus erythematosus; TACI, transmembrane activator and CAML interactor.

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SHM frequencies of IgG^+ and IgA^+ B cells from IRAK4- and MYD88-deficient patients have not been analyzed. Because IRAK4 and MYD88 mediate most TLR functions (Picard et al., 2003; von Bernuth et al., 2008; Casanova et al., 2011) and MyD88 deficiency in mice induces dysregulated gut microbiota containment (Slack et al., 2009; Kirkland et al., 2012), we investigated antibacterial reactivity for IgG and IgA clones from IRAK4- and MYD88-deficient patients. Herein, we report altered IgG repertoire and reactivity in these patients, characterized by abnormal *VH4-34* gene usage, poor SHM frequencies, and reactivity targeting commensal bacteria. Hence, the germline-encoded self-reactive VH4-34 antibodies that also recognize I/i carbohydrates expressed on erythrocytes and B cells may have beneficial functions by cross reacting with antigens expressed by commensal bacteria that reach the circulation when gut microbiota fails to be contained.

RESULTS

Antigen selection is the major force shaping the IgG^+ and IgA^+ memory B cell compartments

We analyzed the reactivity of antibodies expressed by $CD27^+IgG^+$ (IgG^+) and $CD27^+IgA^+$ (IgA^+) conventional memory B cells from various HDs, which include individuals carrying or not carrying the *1858T PTPN22* allele, a polymorphism that results in the accumulation of autoreactive clones in the peripheral mature naive B cell compartment from which memory B cells originate (Menard et al., 2011). In healthy individuals who did not carry the *1858T PTPN22* allele, we found that the frequency of HEp-2–reactive and polyreactive clones was significantly enriched in IgG^+ B cells isolated from HDs in which they represented on average 42.1 and 23.7%, respectively, compared with 17–26% and 5–13% in mature naive B cells from the same individuals (Fig. 1, A and B; and Fig. S1, A and B). Antibodies expressed by IgA^+ B cells also displayed increased frequencies of HEp-2–reactive (36.2%) and polyreactive (19%) antibodies compared with their mature naive B cell counterparts (Fig. 1, A and B; and Fig. S1, C and D). In contrast, HDs who carry the *1858T PTPN22* allele showed similar frequencies of HEp-2–reactive clones among all B cell compartments (mature naive, 41.2%; IgG^+, 35.4%; and IgA^+, 40.4%; Fig. 1 A and Fig. S2, A and B). The lack of an increase in the frequency of autoreactive clones between the mature naive B cell stage and isotype-switched memory B cells in the presence of the *1858T PTPN22* allele was further indicated by similar frequencies of polyreactive antibodies in their various B cell compartments (Fig. 1 B and Fig. S2, C and D). These data also reveal that IgG and IgA memory B cells from HDs carrying the *PTPN22* polymorphism contain proportions of autoreactive clones that resembled those from their counterparts in noncarrier HDs. We henceforth grouped carrier and noncarrier HDs in the rest of this study. Similarly to *1858T PTPN22* allele carriers, the elevated frequency of autoreactive clones in the mature naive B cell compartment of IRAK4- and MYD88-deficient patients did not result in an increased proportion of HEp-2–reactive IgG^+ B cells (Fig. 1 A and Fig. S3 A), whereas IgA^+ B cells were significantly less self-reactive as compared with mature naive B cells (35.4 ± 3.3 vs. 52.28 ± 2.5 in mature naive; Fig. 1 A and Fig. S3 B). In addition, IRAK4- and MYD88-deficient IgG^+ and IgA^+ B cells contained proportions of polyreactive clones that were similar to those in mature naive B cells (Fig. 1 B and Fig. S3, C and D). Of note, indirect immunofluorescence assays with HEp-2 cell–coated slides revealed that antinuclear clones in IgG^+ and IgA^+ B cells were modestly increased in all individuals, but differences with mature naive B cells did not reach significance (Fig. S3 F). Altogether, although mature naive B cells can display very different proportions of self-reactive clones among subjects, we found that IgG^+ and IgA^+ B cells from HDs and patients expressed remarkably similar frequencies of HEp-2–reactive and polyreactive antibodies, suggesting that self-reactivity is associated with the development of isotype-switched memory B cells.

IRAK4 and MYD88 deficiency specifically alter the IgG^+ B cell compartment

We have previously reported that IRAK-4– and MYD88-dependent pathways are essential for the removal of developing autoreactive naive B cells and the establishment of central and peripheral B cell tolerance checkpoints (Isnardi et al., 2008). However, despite the accumulation of autoreactive B cells in their naive compartments, IRAK4- and MYD88-deficient patients do not suffer from autoimmune manifestations, likely because IRAK4- and MYD88-dependent pathways are also essential for autoreactive B cell activation (Isnardi et al., 2008). To determine whether IRAK4 and MYD88 deficiencies could impact isotype-switched memory B cell development in humans, we first examined IgH, Igκ, and Igλ light chain gene repertoires of these B cells. IRAK4- and MYD88-deficient IgG^+ B cells revealed a *VH* repertoire enriched in *VH4* gene segments (Fig. 2 A); this increase in *VH4* was mainly because of significantly enhanced frequencies of *VH4-34* gene segment usage that averaged 6.4% compared with only 1.4% in HDs as previously reported (Fig. 2 B; Tiller et al., 2007). Indeed, *VH4-34* gene–expressing clones were rarely found in the isotype-switched IgG B cells from HDs, whereas they were common in mature naive B cells (Fig. 2 B). In contrast, frequencies of VH4-34–expressing clones were similar in mature naive and IgG^+ B cells from IRAK4- and MYD88-deficient patients (Fig. 2 B). Pooled heavy-chain gene sequence analyses from IRAK4- and MYD88-deficient IgA^+ B cells revealed no consistent differences in IgH *VH*, *D*, or *J* gene usage or IgH complementary determining region 3 (CDR3) amino acid length or positive charges between patients and HDs (Fig. 2 A and Fig. S4, A–D). However, IgA^+ B cells from IRAK4- and MYD88-deficient patients were devoid of clones expressing the *VH4-34* gene (Fig. 2 B).

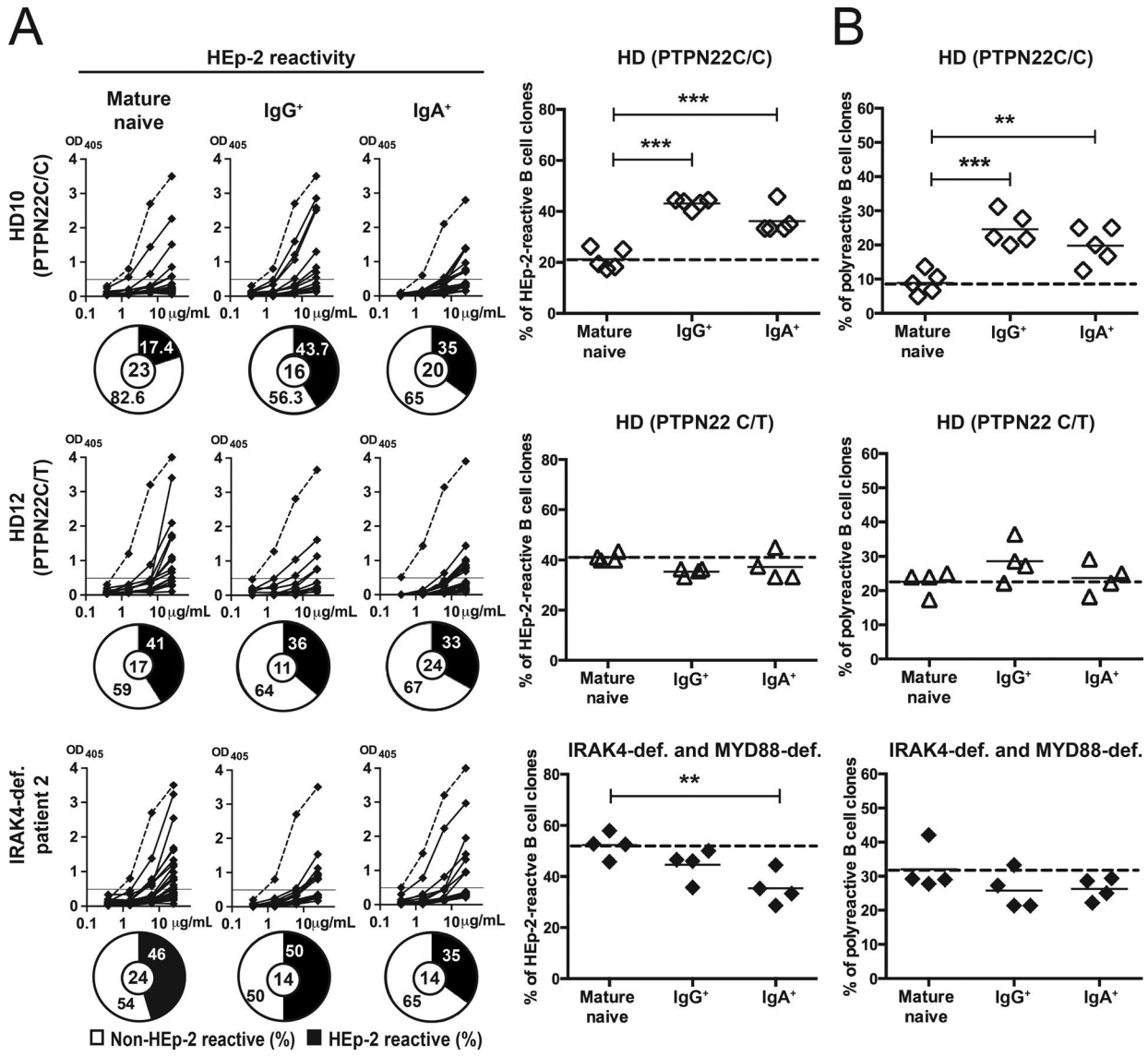

Figure 1. **Poly- and self-reactive antibodies are enriched in the IgG$^+$ and IgA$^+$ B cell compartments of HDs.** (A) Antibodies from mature naive (CD19$^+$CD10$^-$IgM$^+$CD21$^+$CD27$^-$), IgG$^+$ (CD19$^+$CD27$^+$CD21$^+$IgG$^+$), and IgA$^+$ (CD19$^+$CD27$^+$CD21$^+$IgA$^+$) B cells from five HDs that do not carry the *1858T PTPN22* risk allele (PTPN22 C/C), four HDs carrying one *1858T PTPN22* risk allele (PTPN22 C/T), and three IRAK4-deficient patients and one MYD88-deficient patient were tested by ELISA for anti–HEp-2 cell reactivity. Dotted lines show the ED38-positive control, and continuous lines show binding for each cloned recombinant antibody. Horizontal lines show cutoff OD_{405nm} for positive reactivity. For each individual, the frequency of HEp-2–reactive and nonreactive clones is summarized in pie charts, with the number of antibodies tested indicated in the center. (B) The frequencies of polyreactive clones are compared between the mature naive, IgG$^+$, and IgA$^+$ B cells compartments. Each diamond represents an individual, horizontal bars denote means, and dashed lines show the mean frequency in the mature naive B cell compartment. Statistically significant differences are indicated. **, $P \leq 0.01$; ***, $P \leq 0.001$; mixed effect logistic regression. def., deficient.

To determine whether IRAK4 and MYD88 deficiency affect the acquisition of SHM in isotype-switched memory B cells, we compared mutation numbers in Ig genes cloned from IgG$^+$ and IgA$^+$ B cells from IRAK4- and MYD88-deficient patients to those from HDs. Indeed, TLRs and transmembrane activator and CAML interactor (TACI), which bind IRAK4/MYD88 complexes, can induce B cells to express activation-induced cytidine deaminase, the enzyme that catalyzes class-switch recombination and SHM (He et al., 2004, 2010; Pone et al., 2012). We found that *VH*, *Vκ*, and *Vλ* genes expressed by IRAK4- and MYD88-deficient IgG$^+$ B cells harbored decreased averages of mutations, especially in their heavy chain genes (*VH*: 18.31 ± 0.83 vs. 14.04 ± 1.3; P = 0.0018; Fig. 2 C). SHM frequencies in IgA$^+$ B cells from patients were also significantly decreased compared with those in HD counterparts (*VH*: 17.57 ± 0.5 vs. 14.3 ± 0.93, P =

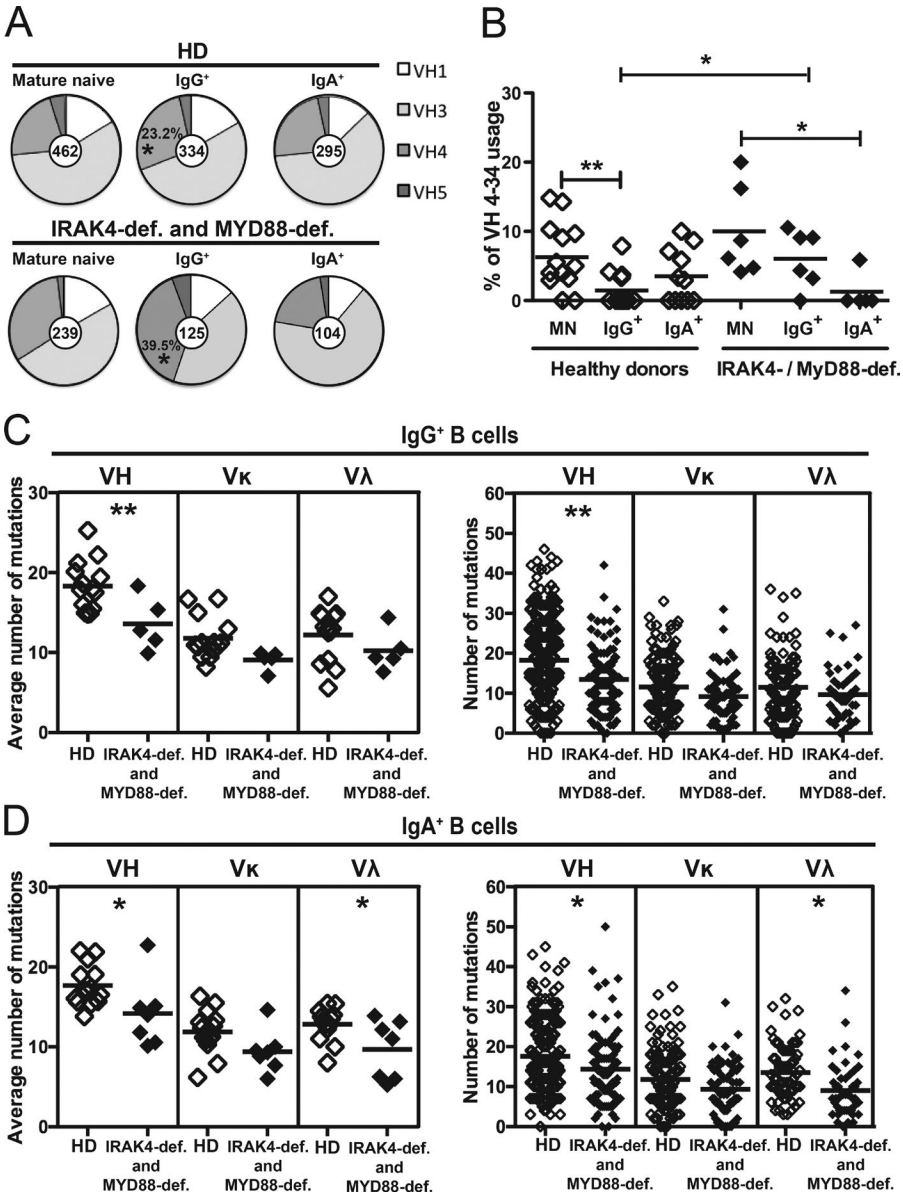

Figure 2. **IRAK4- and MyD88-deficient IgG$^+$ B cells display an unusual IgH repertoire.** (A) *VH* gene usage frequencies in mature naive, IgA$^+$, and IgG$^+$ B cells are represented for 11 HDs, 4 IRAK4-deficient patients, and 1 MYD88-deficient patient. (B) The increase in *VH4* genes and *VH4-34* gene usage in IRAK4- and MYD88-deficient memory B cells is further analyzed. Each diamond represents an individual. The mean is shown with a bar. Mixed-effect logistic regression was used. (C and D) The mean number of mutations per individual (left) and the number of mutation by B cell clone (right) in *VH*, *Vκ*, and *Vλ* genes in antibodies from IgG$^+$ (C) and IgA$^+$ (D) memory B cells from nine HDs, four IRAK4-deficient patients, and one MYD88-deficient patient are represented (numbers for IgG clones: HD [H:334; κ:227; λ: 96] and patients [H:125; κ:61; λ: 35]; numbers for IgA clones: HD [H:295; κ:160; λ:71] and patients [H:104; κ:52; λ:41]). Statistically significant differences are indicated. Mixed-model repeated-measures analysis was used. *, $P \leq 0.05$; **, $P \leq 0.01$. def., deficient; MN, mature naive.

0.014;Vλ: 13.55 ± 0.8 vs. 9.9 ± 1, P = 0.016; Fig. 2 D). Of note, SHM frequencies were similar between IgG$^+$ and IgA$^+$ B cells in either HDs or IRAK4- and MYD88-deficient patients, and no correlation was found with ages of subjects (Fig. 2, C and D; and the Patients and HD controls section of Materials and methods). Decreased SHM frequencies in IRAK4- and MYD88-deficient IgG$^+$ and IgA$^+$ B cells were not associated with alterations in the ratio of *V* gene replacement (R) to silent (S) mutations in framework regions (FWRs) or CDRs, suggesting normal affinity maturation processes in the absence of functional IRAK4 or MYD88 (Fig. S4 E). We conclude that IRAK4 and MYD88 deficiencies impact the induction but not the selection of SHM in both IgG$^+$ and IgA$^+$ B cells and that IRAK4- and MYD88-deficient IgG$^+$ B cells are enriched in clones expressing *VH4-34*–encoded antibodies.

VH4-34–encoded antibodies expressed by IRAK4- and MYD88-deficient IgG$^+$ B cells preferentially bind antigens from commensal bacteria

We further analyzed the reactivity of peripheral IgG$^+$ and IgA$^+$ B cells by testing the ability of their antibodies to bind lysates from specific bacteria and flagellin, which is specifically recognized by TLR5, an IRAK4/MYD88-dependent TLR (Hayashi et al., 2001). Bacteria lysates included potentially pathogenic *Escherichia coli* and *Staphylococcus aureus*, as well as commensal bacteria *Enterococcus faecalis*, *Enterobacter cloacae*, *Bacteroides fragilis*, and *Clostridium difficile*. IgG$^+$ B cells from HDs and IRAK4- and MYD88-deficient patients displayed similar antibody reactivity toward flagellin and lysates from *E. coli* or *S. aureus* (Fig. 3 A). In contrast, we found an increase in commensal bacteria–reactive antibodies

expressed by IRAK4- and MYD88-deficient IgG^+ B cells compared with HD counterparts. Indeed, the percentages of *E. faecalis*–, *E. cloacae*–, *B. fragilis*–, and *C. difficile*–reactive IgG^+ B cells in HDs ranged between 13.6 and 14.4%, whereas reactivity means toward the same bacteria strains represented 25.4–28.8% in IgG^+ B cells from IRAK4- and MYD88-deficient patients (Fig. 3 A, $P = 0.02$). In contrast, bacteria reactivity of antibodies expressed by $CD27^+IgA^+$ B cells were similar between HDs and patients, suggesting that IRAK4 and MYD88 deficiency may not alter the specificity of this compartment, potentially because anticommensal reactivity appeared enriched in scarce circulating IgA^+ B cells that do not express CD27 (Fig. S5 A; Berkowska et al., 2015). Antibacteria reactivity was mostly restricted to polyreactive clones, whereas IgG^+ B cells from IRAK4- and MYD88-deficient patients were especially enriched in anticommensal-specific antibodies that did not bind *E. coli* and *S. aureus* lysates (Fig. 3 B and Fig. S5 B). In addition, four out of six *VH4-34*–encoded antibodies expressed by IgG^+ B cells from IRAK4- and MYD88-deficient patients recognized antigens from commensal bacteria, whereas none of the three *VH4-34*–encoded IgG antibodies isolated from HDs bound any of the four tested commensal bacteria strains (Fig. 3 A). This bias for IgG antibodies using the *VH4-34* gene in IRAK4 and MYD88 deficiency to react with components from commensal bacteria was significant when compared with IgG antibodies encoded by other *VH* genes ($P = 0.014$). In contrast, frequencies of *E. coli*– or *S. aureus*–reactive IgG^+ B cells from IRAK4- and MYD88-deficient patients was not different between *VH4-34*– versus other *VH*-encoded antibodies, further supporting the specificity of VH4-34–expressing clones to recognize commensal bacteria in these patients (Fig. 3 C). Moreover, $VH4\text{-}34^+IgG^+$ B cells do not appear to be the result of abnormal switching toward IgG instead of IgA because none of the four *VH4-34*–encoded antibodies cloned from IgA^+ B cells from HDs recognized commensal bacteria lysate (Fig. S5 C). Hence, human IRAK4 and MYD88 deficiencies result in the emergence of anticommensal bacteria–reactive $VH4\text{-}34^+IgG^+$ B cells that may reflect the occurrence of systemic immune responses against gut microbiota that fail to be contained in these patients (Slack et al., 2009).

VH4-34 self-reactive motif is preserved in IgG^+ B cells from IRAK4- and MYD88-deficient patients

Igs encoded by the *VH4-34* gene are intrinsically autoreactive and bind conserved I/i carbohydrate self-epitope expressed at the surface of red blood cells and other cell types (Pascual et al., 1991; Grillot-Courvalin et al., 1992; Parr et al., 1994). The Ala-Val-Tyr (AVY) motif in VH4-34 FWR1 is responsible for I/i binding that is independent of IgH CDR3 or associated light chain; therefore, mutations in the AVY motif abrogate self-reactivity (Potter et al., 2002; Thorpe et al., 2008; Reed et al., 2016). Another unusual characteristic of *VH4-34*–encoded antibodies is the presence of an Asn-X-Ser N-glycosylation site (NHS) in the CDR2 region that allows modulation of antibody avidity to the cognate antigen (Sabouri et al., 2014). To determine how SHM alters these two VH4-34 motifs, we analyzed IgG and IgA sequences from HDs and IRAK4- and MYD88-deficient patients using NCBI IGBLAST software and aligned mutated *VH4-34* sequences with the germline *VH4-34*01* sequence (Fig. 4 A). Additional *VH4-34* sequences from 11 single-sorted IgG^+ and 3 IgA^+ B cells that were previously isolated from other HDs were included in this study (Tiller et al., 2007). We found that 11 out of 17 *VH4-34*–encoded antibodies expressed by IgG^+ B cells, and eight out of nine IgA^+ B cells from HDs harbored mutations in their AVY motif, thereby abrogating self-reactivity (Fig. 4 B and Fig. S5 D; Reed et al., 2016). In contrast, *VH4-34*–encoded antibodies expressed by IRAK4- and MYD88-deficient IgG^+ B cells often displayed unmutated AVY sequences (five out of eight), preserving carbohydrate recognition and potentially self-reactivity (Fig. 4, A and B). In addition, SHM often abolished the NHS motif, thereby removing CDR2 glycosylation in VH4-34–expressing IgG (12 out of 17) and IgA (six out of nine) clones from HDs (Fig. 4 B and Fig. S5 D). In contrast, this glycosylation site was preserved in 62.5% (five out of eight) of VH4-34 IgG sequences isolated from IRAK4- and MYD88-deficient patients (Fig. 4 B and Fig. S5 D). Hence, SHM normally abrogates AVY and NHS motifs during immune responses involving VH4-34 clones in HDs, whereas antigenic selection in IRAK4- and MYD88-deficient patients preserves *VH4-34* germline–encoded AVY and NHS sequences, suggesting that carbohydrate recognition mediated by the AVY motif may play an important role in systemic responses targeting commensal bacteria.

Anticommensal reactivity is enriched in *VH4-34*–encoded IgG clones with unmutated AVY and NHS motifs

To further assess whether *VH4-34*–encoded IgG clones often recognize commensal bacteria, we analyzed the reactivity of $9G4^+CD27^+IgG^+$ B cells that express *VH4-34*–encoded antibodies isolated from two HDs and two IRAK4-deficient patients. Indeed, the 9G4 monoclonal antibody recognizes a FWR1-encoded *VH4-34*–specific epitope, which therefore allows the identification and isolation of rare $VH4\text{-}34^+IgG^+$ B cells in HDs and patients (Mockridge et al., 2004; Zheng et al., 2004). In agreement with our sequencing results, we found increased frequencies of $9G4^+$ B cells in the IgG^+ compartment of IRAK4-deficient patients, whereas $9G4^+IgG^+$ B cells were scarce in HDs as previously reported (Fig. 5, A and B; Pugh-Bernard et al., 2001). SHM analyses of Ig genes cloned from $9G4^+IgG^+$ B cells isolated from IRAK4-deficient patients showed a decrease in the overall number of mutations in Ig genes (Fig. 5 C). However, most VH4-34 clones from both HDs and patients displayed an unmutated AVY motif, which differed with VH4-34 sequences obtained from total IgG^+ B cells (Figs. 5 D and 4 B). This bias, which likely results from 9G4 recognition of its germline-encoded FWR1 epitope near or overlapping with the AVY sequence, prevented

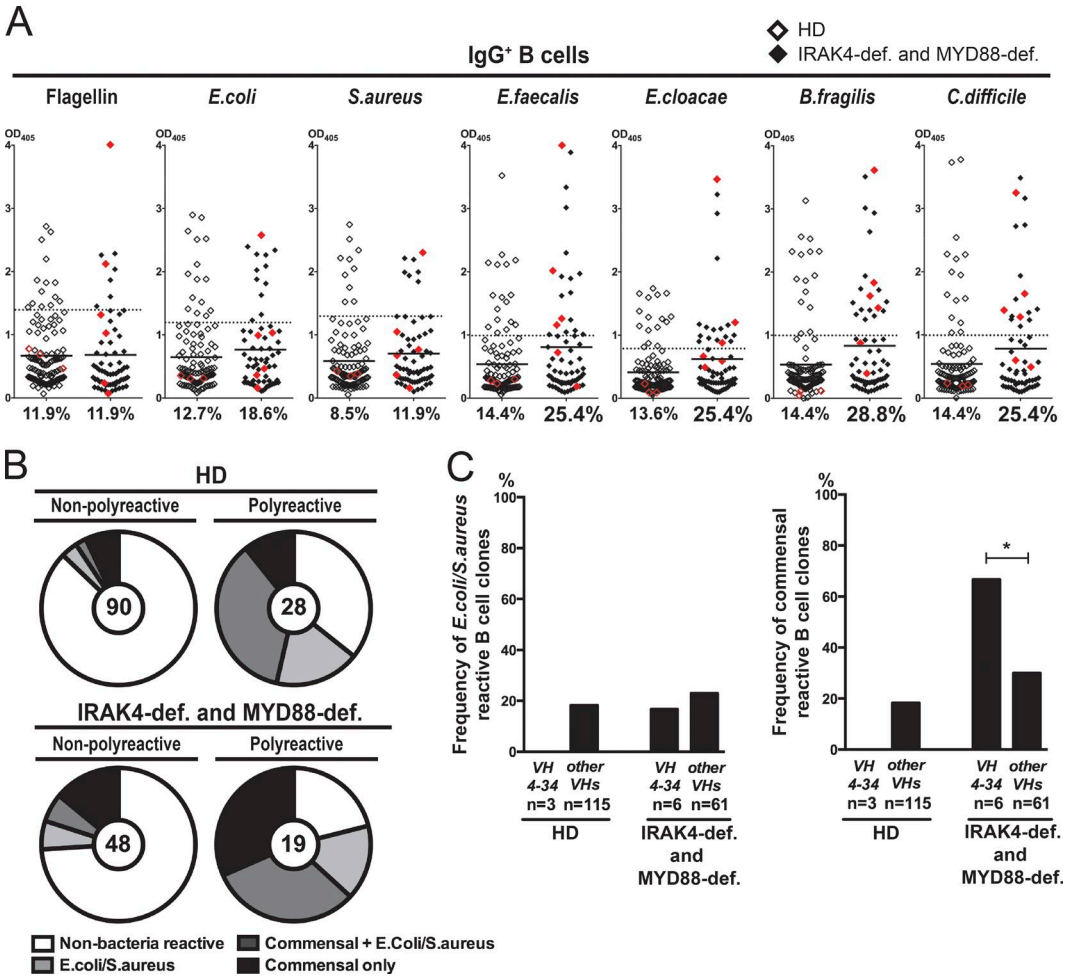

Figure 3. **Increased anticommensal bacteria reactivity in IRAK4- and MYD88-deficient VH4-34–expressing IgG+ B cells.** (A) Recombinant antibodies cloned from 118 IgG+ B cells isolated from HDs and from 59 IgG+ B cells of IRAK4- and MYD88-deficient patients were tested by ELISA for reactivity against flagellin, *E. coli*, *S. aureus*, *E. faecalis*, *E. cloacae*, *B. fragilis*, and *C. difficile*. Each diamond represents a single antibody. VH4-34–expressing clones are displayed in red. The frequency of reactive clones is indicated for each bacterial strain. Antibodies with binding above the dotted line were considered flagellin or bacteria reactive. (B) Pie charts representing bacteria reactivity among the nonpolyreactive and polyreactive IgG+ B cells in HDs and IRAK4- and MYD88-deficient patients. (C) Frequencies of *E. coli*– or *S. aureus*–reactive (left) and commensal bacteria–reactive (*E. faecalis*, *E. cloacae*, *B. fragilis*, or *C. difficile* reactive) clones (right) among *VH4-34*–encoded IgG antibodies versus IgG using other VHs from HDs and IRAK4- and MYD88-deficient memory B cells. *VH4-34*–encoded IgG from IRAK4- and MYD88-deficient patients preferentially bind antigens from commensal bacteria. *, P = 0.014; mixed-effect logistic regression. def., deficient.

further assessments of the impact of AVY mutation on the recognition of commensal bacteria. However, significant differences in the mutational status of the NHS motif were found between HD and patient VH4-34 sequences, thereby revealing that the removal of the glycosylation in VH4-34 CDR2 is a common feature associated with immune responses in HDs (Fig. 5 D, P = 0.04). Reactivity studies revealed anticommensal bacteria associated with antibodies expressed by 9G4+IgG+ B cells with unmutated AVY motif (Fig. 5 E). Indeed, the frequencies of anticommensal 9G4+IgG+ B cells in both HDs and IRAK4-deficient patients were 36.8 and 36.8%, respectively, whereas such reactivity is only found in ∼20% of non–VH4-34 IgG+ B cells in HDs (Figs. 5 E and 3 C). Moreover, anticommensal reactivity was significantly enriched in VH4-34 IgG clones from HDs that displayed an unmutated NHS motif (Fig. 5 F, P = 0.0001). We conclude that, unmutated, the AVY motif conferring self-reactivity, combined with CDR2 glycosylation resulting from the intact NHS sequence, favors anticommensal bacteria reactivity to *VH4-34*–encoded antibodies.

DISCUSSION

The present study has indirectly evaluated the strength of antigen selection in shaping the switched memory B cell compartment generated from naive B cell repertoires that contain either low or elevated frequencies of autoreactive

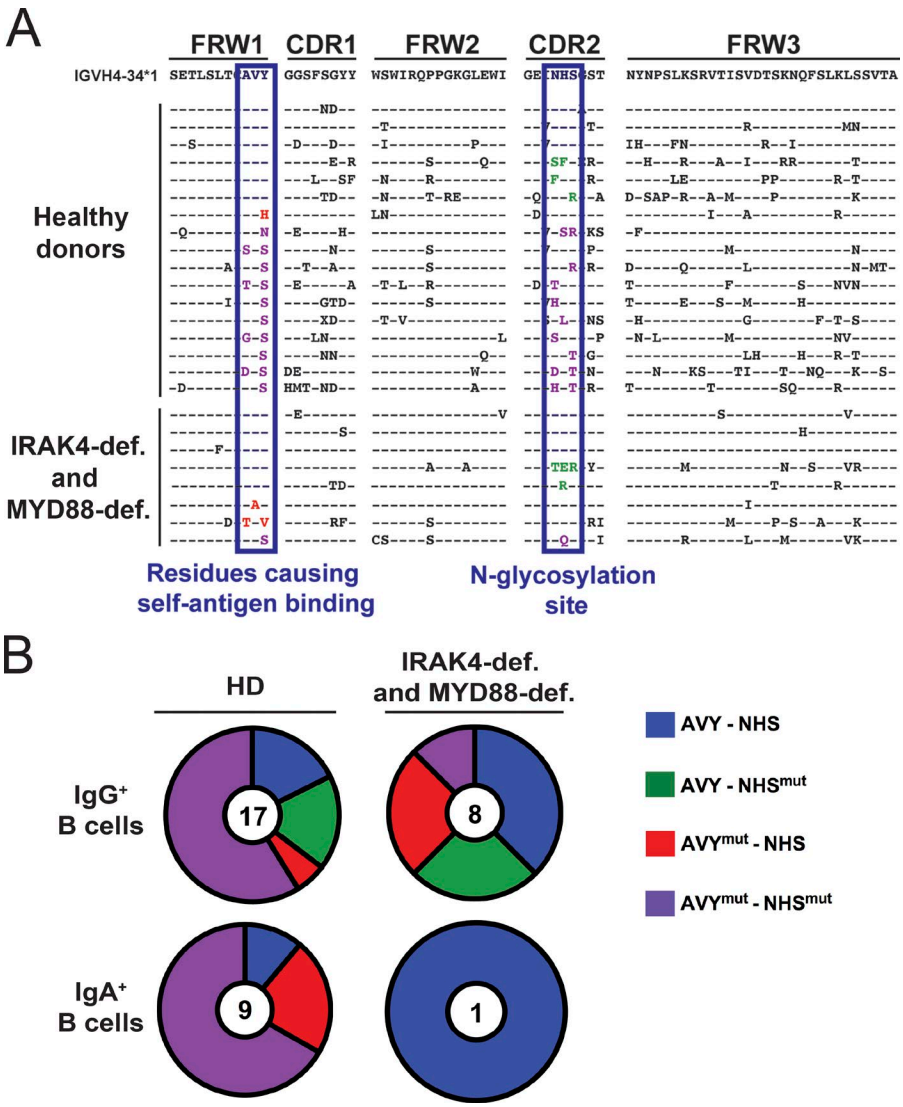

Figure 4. **VH4-34$^+$ IgG$^+$ memory B cells isolated from IRAK4- and MYD88-deficient patients display unusual mutation profiles.** (A) VH4-34 amino acid sequence alignment for IgG$^+$ memory B cells from HDs and IRAK4- and MYD88-deficient patients. The germline *IGHV4-34*01* amino acid sequence is shown at the top. Identity to germline is denoted by a dash, and substituted residues are in black. In blue are the germline AVY and NHS sequences in FWR1 and CDR2. Mutations in only NHS or only AVY are shown in green and red, respectively, and mutations in clones mutated for both AVY and NHS are represented in purple. (B) Pie charts representing proportions of clones mutated in the AVY and/or the NHS VH4-34 sequences from IgG$^+$ B cells of HDs and IRAK4- and MYD88-deficient patients use the same color code as in A. def., deficient.

clones in *1858T PTPN22* noncarrier and carrier HD and IRAK4- and MYD88-deficient patients. We show that the amount of self-reactivity in isotype-switched memory B cells is independent of the frequency of the autoreactive naive B cells from which they originate and that IRAK4- and MYD88-deficient patients show evidence of systemic anti-commensal responses correlating with an increase in *VH4-34*–encoded self-reactive clones.

Both IgG$^+$ and IgA$^+$ conventional memory B cells in HDs who do not carry the *1858T PTPN22* allele contained elevated frequencies of polyreactive and autoreactive clones compared with mature naive B cells from the same individuals. These results confirm previous studies showing increased autoreactivity in the IgG$^+$ and IgA$^+$ memory B cell compartments for which comparisons were made with mature naive B cells from different control subjects (Tiller et al., 2007; Berkowska et al., 2015). However, we found that the proportion of autoreactive clones does not increase in the memory B cells from subjects who contained increased frequencies of self-reactive mature naive B cells associated with the presence of *1858T PTPN22* polymorphism or biallelic mutations in the *IRAK4* or *MYD88* gene (Isnardi et al., 2008; Menard et al., 2011). This suggests that antigen selection favors specific clones independently of their initial autoreactive frequencies, resulting in the accumulation in all individuals of a similar proportion of self-reactive clones in their circulating IgG$^+$ and IgA$^+$ conventional B cells.

Why is polyreactivity/self-reactivity favored in isotype-switched memory B cells, whereas this feature is counterselected during early development of naive B cells? Cross-reactivity allows antibodies to bind and neutralize bacteria or viruses of related strains. For instance, antibody responses generated during flu season may potentially be protective for a related virus strain in the next season (Wrammert et al., 2011). Multispecificity for anti-gut commensal IgA that is enriched in CD27$^-$IgA$^+$ B cells may allow antibodies to recognize related molecular variants of common bacteria strains (Berkowska et al., 2015). Similarly, we also found

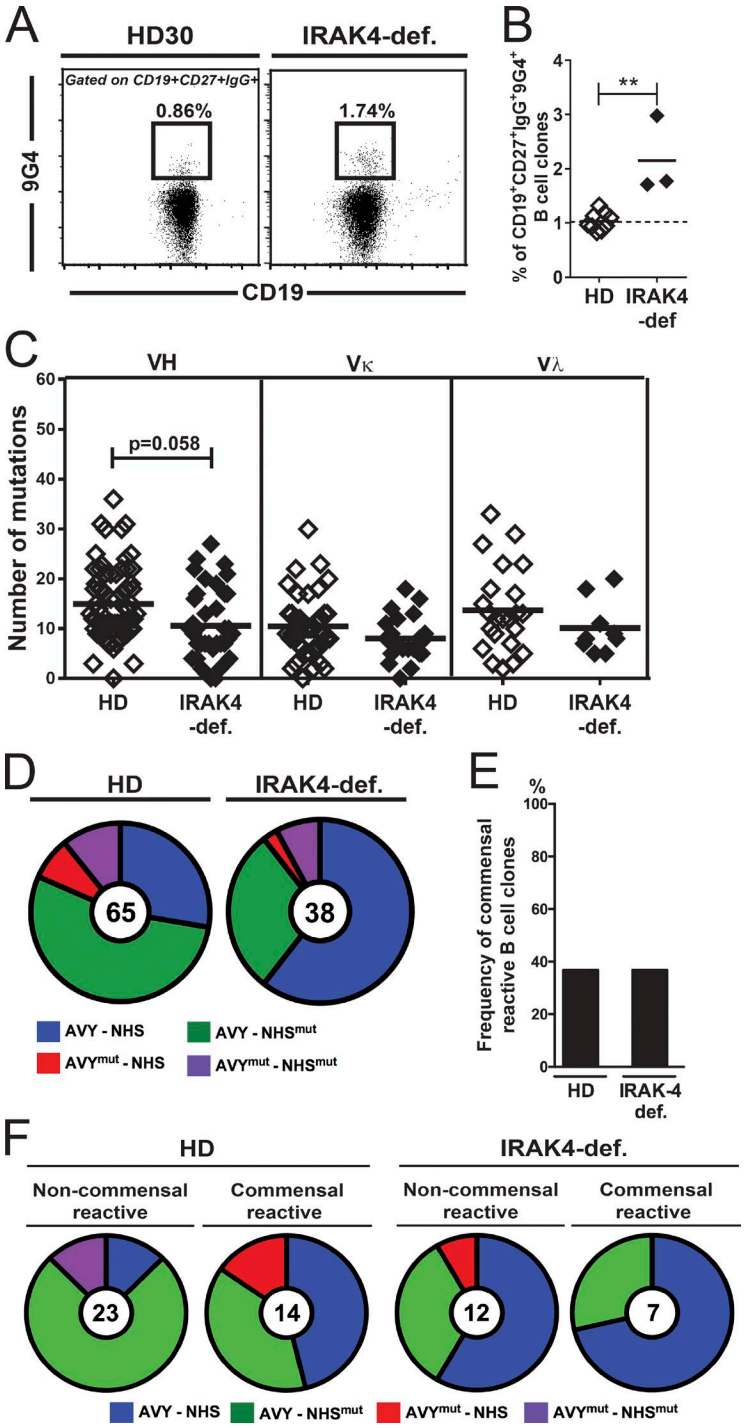

Figure 5. **Anticommensal 9G4$^+$IgG$^+$ B cells are expanded in IRAK4-deficient patients.** (A) Dot plots represent staining for 9G4 recognizing *VH4-34*–encoded heavy chains and CD19 on gated CD19$^+$CD27$^+$IgG$^+$ memory B cells from a representative healthy control and an IRAK4-deficient patient. (B) The frequencies of CD19$^+$CD27$^+$IgG$^+$9G4$^+$ in 11 healthy controls and 3 IRAK4-deficient patients. (C) The number of mutations in *VH*, *Vκ*, and *Vλ* genes in antibodies from 9G4$^+$IgG$^+$ memory B cells from five HDs (H:65; κ:35; λ: 23) and two IRAK4-deficient patients (H:38; κ:23; λ: 9). (D) Pie charts representing proportions of 9G4$^+$IgG$^+$ memory B cell clones mutated in the AVY and/or the NHS sequences in HDs and IRAK4-deficient patients. (E) Frequencies of commensal bacteria–reactive (*E. cloacae*, *E. faecalis*, *B. fragilis*, or *C. difficile* reactive) antibodies cloned from 37 single 9G4$^+$IgG$^+$ B cells from five HDs and 19 single 9G4$^+$IgG$^+$ B cells from two IRAK4-deficient patients. (F) Pie charts representing proportions of clones mutated in the AVY and the NHS sequences among noncommensal and commensal-reactive 9G4$^+$IgG$^+$ B cells from HDs and IRAK4- and MYD88-deficient patients. Statistically significant differences are indicated. **, $P \leq 0.01$; Mann-Whitney *U* tests. def., deficient.

that polyreactive antibodies expressed by CD27$^+$IgG$^+$ and CD27$^+$IgA$^+$ B cells were enriched in antibacteria clones. In addition, polyreactivity can increase binding to pathogens expressing antigens at very low density. It has been reported that polyreactivity enhances binding of anti-gp120 antibodies that recognize one of the 10–15 gp120 molecules expressed on the surface of HIV virions (Mouquet et al., 2010). However, it is unclear whether polyreactivity/self-reactivity is selected during B cell affinity maturation in germinal centers or later, thereby promoting the survival of long-term memory B cells. Indeed, plasma cells rarely express polyreactive or self-reactive antibodies, suggesting that autoreactive features are not favored during B cell affinity maturation processes (Benckert et al., 2011; Scheid et al., 2011). As B cell receptor (BCR) expression is essential for B cell survival (Kraus et al., 2004), we would like to propose that the few polyreactive B cell

clones expressing antibodies specific for cognate antigens may preferentially accumulate in the long-term isotype-switched memory B cell compartment because BCRs from these polyreactive memory B cells will likely generate survival signals because of chronic low BCR triggering after cognate antigens have been cleared. In line with this hypothesis is the dearth of autoreactive clones in IgG-expressing plasma cells that do not express BCRs on their cell surface and therefore do not rely on BCR signals to survive (Scheid et al., 2011). Additional studies on the evolution of polyreactivity/autoreactivity during the development of isotype-switched memory B cells are warranted to further investigate whether self-reactivity plays a role in the maintenance of these B cells.

The IRAK4/MYD88 pathway is essential for the generation of $CD27^+IgM^+$ memory B cells that are proposed to mediate anti-carbohydrate responses and provide protection against Gram-positive encapsulated bacteria (Weller et al., 2012; Maglione et al., 2014). However, the generation of isotype-switched IgG^+ and IgA^+ B cells and their proportion in polyreactive/autoreactive clones seem unaffected in these patients, suggesting that the maintenance of isotype-switched memory B cells and the selection of self-reactive antibodies in these compartments does not rely on TLR/TACI/IL-1R family members, as their function requires IRAK-4 and MYD88 (Weller et al., 2012). However, both IgG^+ and IgA^+ B cells from IRAK4- and MYD88-deficient patients displayed decreased SHM frequencies compared with HD counterparts, revealing that TLR/TACI/IL-1R play a role for the proper accumulation of SHM. Decreased SHM could be a consequence of defective TLR/TACI function in B cells, which results in reduced expression of activation-induced cytidine deaminase, the enzyme that catalyzes SHM (He et al., 2010; Kasturi et al., 2011; Pone et al., 2012; Romberg et al., 2013; Soni et al., 2014). In addition, altered TLR function in other antigen-presenting cells may also affect B and T cell activation and germinal center responses as shown in mouse models (Hou et al., 2011).

The absence of functional IRAK4 or MYD88 resulted in the emergence of clones expressing antibodies encoded by the *VH4-34* gene in IgG^+ but not in IgA^+ B cells. What are the origins of VH4-34–expressing $CD27^+IgG^+$ B cells in IRAK4- and MYD88-deficient patients? *VH4-34* gene usage is rare in $CD27^+IgG^+$ B cells from HDs because the unmutated *VH4-34* gene encodes self-reactive antibodies recognizing I/i antigens because of the AVY motif in its FWR1 (Pascual et al., 1991; Grillot-Courvalin et al., 1992; Parr et al., 1994; Pugh-Bernard et al., 2001; Potter et al., 2002; Thorpe et al., 2008; Sabouri et al., 2014; Young et al., 2015; Reed et al., 2016). In addition, human VH4-34–expressing B cells have been reported to be anergic, a property that does not allow naive B cells to be recruited into B cell follicles (Ekland et al., 2004; Cappione et al., 2005; Sabouri et al., 2014). In agreement with previous studies, we found that the majority of the rare VH4-34–expressing IgG^+ B cells from HDs acquired mutations that abolish the AVY motif and thereby eliminate self-reactivity, suggesting that anergy may be overcome by $VH4\text{-}34^+$ B cells (Sabouri et al., 2014; Reed et al., 2016). The antigens recognized by VH4-34–expressing IgG^+ B cells from HDs remain to be identified to determine whether tolerance is really broken. In contrast, the majority of VH4-34–expressing IgG^+ B cells from IRAK4- and MYD88-deficient patients display an unmutated AVY motif, revealing that these clones are still self-reactive (Young et al., 2015). However, these B cells acquired SHMs preferentially targeting CDRs, suggesting proper antigenic selection like in conventional memory B cells (Berek et al., 1991). Hence, the absence of functional IRAK4 and MYD88 may lead to a break in B cell tolerance as indicated by the recruitment of autoreactive VH4-34 B cells. However, we found that most VH4-34–expressing IgG^+ B cells with an unmutated AVY motif often combined with the unmutated NHS in CDR2-recognized antigens from commensal bacteria. Therefore, it appears that the paradoxical observation of autoreactive $VH4\text{-}34^+$ clones in the IgG^+ B cell compartment may in fact represent systemic immune responses targeting foreign antigens that may cross react with the I/i antigen recognized by VH4-34 antibodies. A more detailed analysis of anticommensal reactivity will be needed to confirm our ELISA results and identify the commensal bacteria antigens recognized by IgG^+ memory B cells in IRAK4- and MYD88- deficient patients. VH4-34–expressing $CD27^+IgG^+$ B cells from IRAK4- and MYD88-deficient patients are not likely caused by dysregulated switching processes in the gut toward IgG instead of IgA isotype because $CD27^+IgA^+$ B cells from healthy controls do not favor *VH4-34* gene usage with anticommensal reactivity. In fact, anticommensal bacteria reactivity in the IgA^+ memory compartment was found to be enriched in unconventional $CD27^-IgA^+$ B cells, but it did not appear to correlate with *VH4-34* usage, perhaps because VH4-34–expressing B cells are anergic and cannot be recruited in mucosal tissues (Berkowska et al., 2015). Thus, the VH4-34 self-reactive sequence may have been selected in the human genome because it spontaneously produces antibodies that may neutralize uncontained gut microbiota. In line with this hypothesis, $MyD88^{-/-}$ mice display abnormal IgG production toward commensal bacteria (Slack et al., 2009). In addition, mice with B cell–specific *MyD88* deletion succumb to lethal dissemination of commensal bacteria during DSS-induced colitis, suggesting that MyD88 expression in B cells plays an essential role in preventing commensal bacteria dissemination (Kirkland et al., 2012). Similarly, IRAK4- and MYD88-deficient patients may have issues in maintaining gut homeostasis. Reactivity toward *C. difficile* in IgG^+ B cells from IRAK4- and MYD88-deficient patients may also be explained by their prophylactic antibiotic treatment, which has been reported to induce *C. difficile* infection (Settle and Wilcox, 1996). We conclude that VH4-34–expressing IgG^+ B cells represent systemic immune responses against commensal bacteria in patients with IRAK4/MYD88 deficiency.

Increased frequency of $VH4\text{-}34^+IgG^+$ B cells that contained less SHMs were previously reported in the memory

compartment of patients with systemic lupus erythematosus (SLE; Anolik and Sanz, 2004). Clonally related *VH4-34*–expressing plasma cells were also reported to be expanded during lupus flares (Tipton et al., 2015). We would like to speculate that SLE flares may be triggered by systemic responses toward commensal bacteria. In humans, monocyte transcriptome analysis in SLE patients revealed a signature mark of chronic endotoxin exposure that could indicate a breach in the gut barrier, allowing the escape of commensal antigens into the periphery (Shi et al., 2014). In this regard, a study also described intestinal dysbiosis in SLE patients characterized by an increased frequency of the Bacteroides bacteria phylum in the gut (Hevia et al., 2014). Interestingly, we found that *VH4-34*–encoded IgG^+ B cells recognized *B. fragilis* that belongs to this phylum, further supporting altered microbiota as a potential origin for the appearance and expansion of these B cells in the periphery of SLE patients. It remains to be determined whether a more specific commensal bacteria strain is responsible for VH4-34 responses during SLE flares or whether various species or strains may account for these dysregulated systemic IgG responses (Tipton et al., 2015).

In summary, our data highlight the versatile function of autoreactive VH4-34–expressing B cells and their activation, which is likely associated with a failure to contain gut commensal bacteria in IRAK4- and MYD88-deficient patients. The commensal bacteria inducing systemic responses and the specific bacterial antigen responsible for *VH4-34*–encoded IgG antibody production remain to be determined.

MATERIALS AND METHODS

Patients and HD controls

The five IRAK4-deficient and the MYD88-deficient patient have been described previously (Isnardi et al., 2008), with the exception of IRAK4-deficient patient 4 who is a 19-yr-old Caucasian female with compound heterozygote IRAK4 gene mutations (877C>T, Q293X, and a null allele), patient 5 who is a 35-yr-old Caucasian female with compound heterozygote mutations (877C>T, Q293X, and a 620–621 delAC), and patients 6 who is a 22-yr-old Caucasian female with compound heterozygote mutations (877C>T, Q293X T, and a null allele). HDs carrying or not carrying the *1858T PTPN22* risk allele were described previously (Menard et al., 2011). Overall, the ages of healthy controls range from 5 to 37 yr old and match the diverse ages of the patients enrolled in the study. All samples were collected after patients signed informed consent in accordance with protocols reviewed by the institutional review board of Yale University.

Single-cell sorting

Post-Ficoll mononuclear cells from HDs and IRAK4- and MYD88-deficient patients were enriched for B cells by magnetic separation with CD20 microbeads (Miltenyi Biotec) and stained with anti–human CD19–Pacific blue, anti–human CD27-PerCP Cy5.5, anti–human CD10–PE-Cy7, anti–human CD21-APC, anti–9G4-FITC (IGM Biosciences), anti–human IgG-PE (BioLegend), or anti–human IgA-PE (SouthernBiotech) before purification. Single $CD19^+CD27^+CD21^+IgG^+$, $CD19^+CD27^+CD21^+IgA^+$, or $CD19^+CD27^+CD21^+IgG^+9G4^+$ memory B cells were sorted on a FACSAria flow cytometer (BD) into 96-well PCR plates and immediately frozen on dry ice.

cDNA synthesis, Ig gene amplification, antibody production, and purification

RNA from single cells was reverse-transcribed in the original 96-well plate in 12.5-µl reactions containing 100 U of Superscript II reverse transcriptase (Gibco) for 45 min at 42°C. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as previously described (Wardemann et al., 2003; Tiller et al., 2008), except for the *IGHA*-specific primer (5′-CTTTCGCTC CAGGTCACACTGAG-3′) and *IGHG*-specific primer (5′-GTTCGGGGAAGTAGTCCTTGAC-3′) that were used in the first PCR reaction. H. Wardemann (German Cancer Research Center, Heidelberg, Germany) provided mutated *VH4-34* sequences amplified from single $CD27^+IgG^+$ B cells from HDs previously studied (Tiller et al., 2007).

ELISAs and immunofluorescence assays

Antibody reactivity analysis was performed as described previously with the highly polyreactive ED38 antibody as a positive control for HEp-2 reactivity and polyreactivity (Wardemann et al., 2003). Polyreactivity was determined by the binding of antibodies to three individual antigens of unrelated structure (double-stranded DNA [dsDNA], insulin, and LPS) using three specific ELISAs with plates coated with each single antigen. Antibodies were considered polyreactive when they bound all three antigens. ELISA plates for bacteria reactivity testing were coated with purified flagellin from *Bacillus subtilis* (InvivoGen) or sonicated lysates from cultured *E. cloacae* (13047; ATCC), *E. faecalis* (29212; ATCC), *E. coli*, or *S. aureus* or were obtained by multiple cycles of freezing and thawing lysates from *Bacteroides fragilis* (2528; ATCC) and *C. difficile* (9689; ATCC) at the concentration of 1 ng/µl. For indirect immunofluorescence assays, HEp-2 cell–coated slides (Bion Enterprises Ltd.) were incubated in a moist chamber at room temperature with purified recombinant antibodies at 50–100 µg/ml according to the manufacturer's instructions. FITC-conjugated goat anti–human IgG was used as a detection reagent.

Statistical analysis

Statistical analyses were performed using mixed effect logistic regression on binary outcomes (i.e., binomial distributed data) by including a random effects/covariance structure to account for correlations and different denominators to compare rate of various reactivities between different types of naive and memory B cells from subjects and patients. Mixed model repeated-measure analysis was used to compare the mutational status of Ig heavy- and light-chain genes. Differ-

ences between groups of research subjects for the presence of $CD19^+CD27^+IgG^+9G4^+$ B cells were analyzed for statistical significance with unpaired two-tailed Student's *t* tests. A p-value ≤ 0.05 was considered significant after simple Bonferroni correction for multiple comparisons.

Online supplemental material

Figs. S1 and S2 show the frequencies of HEp2-reactive and polyreactive IgG^+ and IgA^+ B cells from HDs that do not or do carry the *1858T PTPN22* risk allele, respectively. Fig. S3 shows the frequencies of HEp2-reactive and polyreactive IgG^+ and IgA^+ B cells from IRAK4- and MYD88-deficient patients. Fig. S4 shows D and JH gene segment usage and IgHCDR3 length of memory B cells from HDs and IRAK4- and MYD88-deficient patients. Fig. S5 shows bacteria reactivity of IRAK4- and MYD88-deficient IgA^+ B cells.

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Author contributions: E. Meffre initiated the collaboration with G. Uzel, C. Picard, A. Puel, and J.-L. Casanova, who provided blood samples from IRAK4- and MYD88-deficient patients. J.-N. Schickel and E. Meffre designed the experiments. J.-N. Schickel, S. Glauzy, Y.-S. Ng, N. Chamberlain, C. Massad, N. Katz, and I. Isnardi performed the experiments. J.-N. Schickel and E. Meffre wrote the manuscript. All authors reviewed the manuscript and provided scientific input.

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