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IRAK-4- and MyD88-Dependent Pathways Are Essential for the Removal of Developing Autoreactive B Cells in Humans

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SUMMARY

Most autoreactive B cells are normally counterselected during early B cell development. To determine whether Toll-like receptors (TLRs) regulate the removal of autoreactive B lymphocytes, we tested the reactivity of recombinant antibodies from single B cells isolated from patients deficient for interleukin-1 receptor-associated kinase 4 (IRAK-4), myeloid differentiation factor 88 (MyD88), and UNC-93B. Indeed, all TLRs except TLR3 require IRAK-4 and MyD88 to signal, and UNC-93B-deficient cells are unresponsive to TLR3, TLR7, TLR8, and TLR9. All patients suffered from defective central and peripheral B cell tolerance checkpoints, resulting in the accumulation of large numbers of autoreactive mature naive B cells in their blood. Hence, TLR7, TLR8, and TLR9 may prevent the recruitment of developing autoreactive B cells in healthy donors. Paradoxically, IRAK-4-, MyD88-, and UNC-93B-deficient patients did not display autoreactive antibodies in their serum or develop autoimmune diseases, suggesting that IRAK-4, MyD88, and UNC-93B pathway blockade may thwart autoimmunity in humans.

INTRODUCTION

Autoreactive B cells generated by random V(D)J immunoglobulin gene assembly are normally eliminated during their development by both central and peripheral B cell tolerance checkpoints (Wardemann et al., 2003). The mechanisms that ensure human central B cell tolerance are poorly characterized, but they are mostly controlled by intrinsic B cell factors that sense whether B cell receptors (BCRs) recognize autoantigens (Goodnow, 1996; Nemazee et al., 2000; Samuels et al., 2005a). In addition to their BCRs, B cells also express germline-encoded transmembrane receptors called Toll-like receptors (TLRs) that were originally described to bind microbial components but that are also able to recognize self-antigens (Marshak-Rothstein, 2006). Indeed, in addition to TLR1 and TLR10 complexes, whose ligands are unknown, human B cells express TLR7 and TLR9 that bind RNA and DNA, respectively, and could play a role in the removal of developing anti-nuclear antibody (ANA)-expressing B cells (Bernasconi et al., 2003; Bourke et al., 2003; Hasan et al., 2005). The regulation of the peripheral B cell tolerance checkpoint may involve other cell populations such as regulatory T (Treg) cells, whose development and function may depend on some TLR expression (Hervé et al., 2007).

To assess whether the mechanisms that preside over the removal of developing autoreactive B cells involve TLRs, we analyzed B cell tolerance checkpoints in interleukin-1 receptor-associated kinase 4 (IRAK-4)-, myeloid differentiation factor 88 (MyD88)-, and UNC-93B-deficient patients. Triggering of all TLRs except TLR3 induces the recruitment to their intracytoplasmic domain of complexes formed by the adaptor protein MyD88 and IRAK-4, which mediate signaling of these receptors (Akira and Takeda, 2004; Beutler, 2004). In addition, it has been recently reported that the endoplasmic reticulum membrane protein UNC-93B interacts with, and is required for, intracellular TLR3, TLR7, TLR8, and TLR9 trafficking (Brinkmann et al., 2007; Casrouge et al., 2006; Kim et al., 2008; Tabeta et al., 2006). Consistent with the role of TLRs in innate immunity, mice lacking

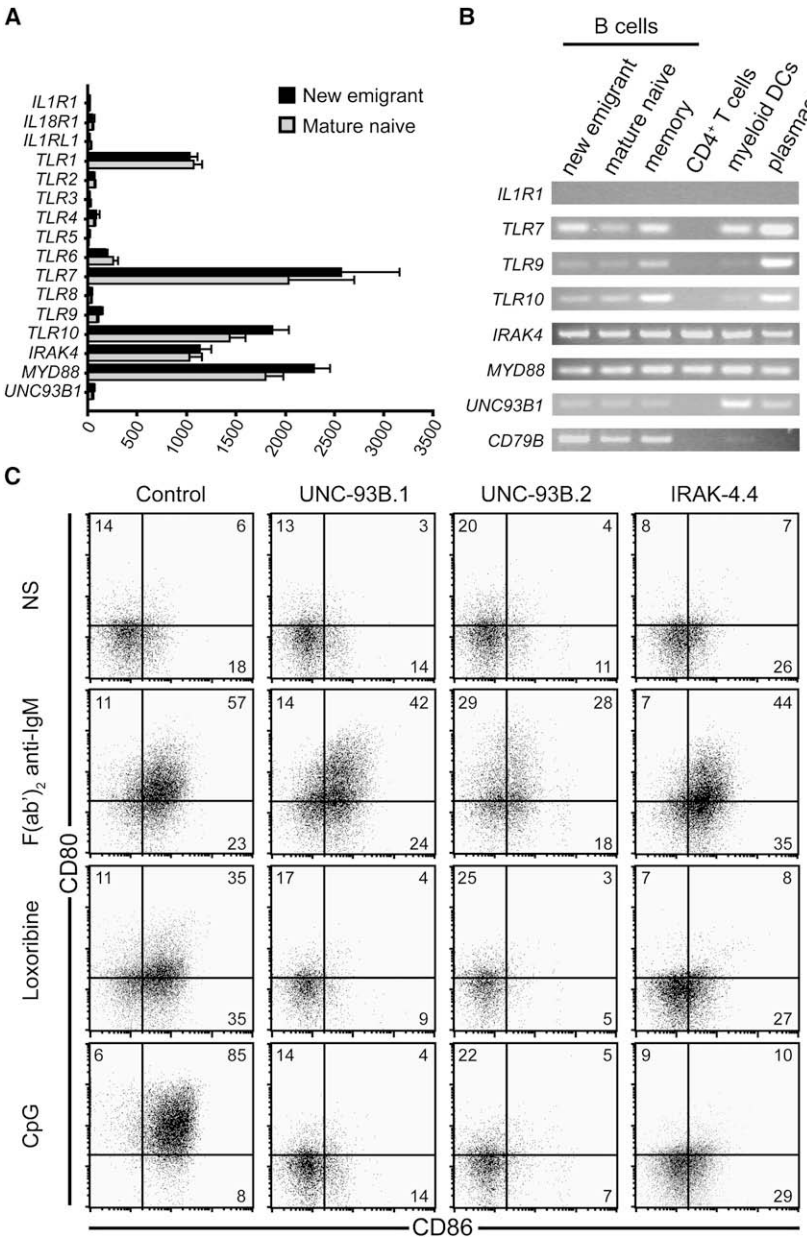

Figure 1. Human B Cell Activation by TLR7 and TLR9 Requires UNC-93B

(A) Human new emigrant and mature naive B cells do not express IL-1R family members. Histograms represent means ± standard error of the mean (SEM) of the expression of studied genes (*IL1R1*, *IL18R1*, *IL1RL1*, *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR6*, *TLR7*, *TLR8*, *TLR9*, *TLR10*, *IRAK4*, *MYD88*, and *UNC93B1*) in human new emigrant (black bars, n = 8) or mature naive (gray bars, n = 4) B cells assessed by gene-expression profiling with the Affimetrix Human Genome U133 Plus 2.0 Array.

(B) *UNC93B1* gene is expressed in human DCs and, to a lesser extent, in B cells, and it is not expressed in T cells. *IL1R*, *TLR7*, *TLR9*, *TLR10*, *IRAK4*, *MYD88*, *UNC93B1*, and *CD79B* gene expression was analyzed by RT-PCR in $CD19^+CD10^+IgM^+CD27^-$ new emigrant, $CD19^+CD10^-IgM^+CD27^-$ mature naive, and $CD19^+CD10^-CD27^+$ total memory B cells; $TCR\alpha\beta^+CD4^+CD25^-$ T cells; $CD19^-CD11c^+HLA\text{-}DR^+$ myeloid DC-enriched cells; and $CD19^-CD11c^-HLA\text{-}DR^+$ plasmacytoid DC-enriched cells from healthy controls.

(C) Human B cells responses to TLR7 and TLR9 agonists depend on UNC-93B expression. Dot plots show CD80 and CD86 expression on naive $CD19^+CD27^-$ B cells from one healthy control, two UNC-93B-deficient patients, and one IRAK-4-deficient patient that were either left unstimulated (NS) or stimulated with $F(ab')_2$ anti-IgM, CpG (TLR9 agonist), or Loxoribine (TLR7 agonist) for 48 hr.

IRAK-4 or MyD88 showed severely impaired immunological responses to bacterial challenges but were resistant to a lethal dose of lipopolysaccharide (LPS) (Kawai et al., 1999; Suzuki et al., 2002; Takeuchi et al., 2000). Mice with a missense mutation in the *Unc93b1* gene encoding UNC-93B also suffer from hypersusceptibility to infection from mouse cytomegalovirus and other microbes (Tabeta et al., 2006). In humans, MyD88- and IRAK-4-deficient patients are susceptible to pyogenic Gram-positive bacterial infections because of the inability of their blood cells to produce proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in response to TLR and interleukin-1 receptor (IL-1R) ligation (Haraguchi et al., 1998; Ku et al., 2007; Medvedev et al., 2003; Picard et al., 2003). In contrast, human UNC-93B deficiency, like TLR3 deficiency, does not compromise immunity to most pathogens but elicits specific defects in clearing herpes simplex viral infections, resulting in recurrent encephalitis (Casrouge et al., 2006; Zhang et al., 2007). By studying the reactivity of recombinant antibodies from single B cells from one MyD88-, three IRAK-4-, and two UNC-93B-deficient patients, we found a high proportion of autoreactive B cells in all patients, suggesting that TLR pathways may prevent these B lymphocytes from entering the mature naive B cell compartment.

RESULTS

TLR7 and TLR9 Require UNC-93B Expression to Activate Human B Cells

IRAK-4 and MyD88 are required to mediate intracellular signaling generated upon triggering of IL-1R family members, including IL-1R1, IL-18R1, IL-1RL1 (also known as IL-33R), and most TLRs except TLR3 (Akira and Takeda, 2004; Beutler, 2004). Because the genes encoding IL-1R1, IL-18R1, and IL-1RL1 are not expressed in new emigrant and mature naive B cells, these molecules are not likely to play a direct role in the removal of developing autoreactive B cells (Figures 1A and 1B). In agreement

with previous reports, we found that *TLR1*, *TLR6*, *TLR7*, *TLR10*, *IRAK4*, *MYD88*, and, to a lower degree, *TLR9* genes were expressed when we analyzed gene-expression profiles in both new emigrant and mature naive B cells from healthy controls, but we found that *TLR2*, *TLR3*, *TLR4*, *TLR5*, and *TLR8* were not (Figure 1A) (Bernasconi et al., 2003; Bourke et al., 2003; Hasan et al., 2005). In addition, *UNC93B1* did not seem to be expressed in human B cells, whereas *Unc93b1* is highly expressed in mouse B cells (Figure 1A) (Tabeta et al., 2006). Because of the very low expression, if any, of *UNC93B1* transcripts detected by microarray analysis of gene-expression profiles, we analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) the expression of *UNC93B1* and other TLR-related genes in new emigrant, mature naive, and memory B cells, as well as in $CD4^+$ T cells and myeloid and plasmacytoid dendritic cell (DC)-enriched fractions (Figure 1B). The *CD79B* gene encodes immunoglobin β (Igβ), a component of the BCR, and its expression was used as a positive control for all B cell fractions. PCR analysis confirmed microarray gene-expression data in that *TLR7*, *TLR10*, *IRAK4*, *MYD88*, and, to a lesser extent, *TLR9* were expressed in all B cell fractions (Figure 1B). In addition, both *TLR9* and *TLR10* expression was increased in memory B cells compared to new emigrant and mature naive B cells (Figure 1B) (Bernasconi et al., 2003; Bourke et al., 2003). *UNC93B1* transcripts were weakly detected in human B cells, whereas they were more abundant in myeloid and plasmacytoid DC-enriched fractions (Figure 1B).

To determine whether UNC-93B expression was required by TLR7 and TLR9 to activate human B cells, we isolated naive $CD19^+CD27^-$ B cells from healthy controls, UNC-93B-, and IRAK-4-deficient patients and stimulated them by TLR agonists. Human naive B cells from healthy controls stimulated with $F(ab')_2$ anti-IgM, Loxoribine (TLR7 agonist), or CpG (TLR9 agonist) upregulated the expression at their cell surface of costimulation molecules CD80 and CD86 and activation markers CD25 and CD69, as well as TLR9 and TLR10 (Figure 1C, Figures S1 and S2 available online, and data not shown). Naive B cells from UNC-93B- and IRAK-4-deficient patients showed responses to $F(ab')_2$ anti-IgM stimulation similar to those of control B cells (Figure 1C and Figures S1 and S2). However, UNC-93B-deficient B cells were similar to IRAK-4-deficient B cells and failed to upregulate CD80, CD86, CD25, CD69, TLR9, and TLR10 when stimulated by Loxoribine or CpG (Figure 1C, Figures S1 and S2, and data not shown) (Ku et al., 2007). We conclude that human B cells express TLR7 and TLR9 that bind RNA- and DNA-containing antigens, respectively, and TLR10 that can associate with TLR1 and whose ligands are unknown. In addition, human B cells express IRAK-4 and MyD88 complexes and UNC93B1, which is required for TLR7 and TLR9 to function.

Defective Central B Cell Tolerance Checkpoint in IRAK-4-, MyD88-, and UNC-93B-Deficient Patients

A central B cell tolerance checkpoint removes the vast majority of developing B cells that express highly polyreactive antibodies in the bone marrow of healthy donors, and only a small fraction of weakly polyreactive clones migrate to the periphery (Wardemann et al., 2003). To determine whether this checkpoint is affected by the absence of most TLR functions, we analyzed the reactivity of antibodies cloned from single IRAK-4-, MyD88-, and UNC-93B-deficient $CD19^+CD10^+IgM^+CD27^-$ new emigrant B cells. Antibody reactivities were then compared to those of the corresponding B cell subpopulation from a healthy donor (HD10) and five previously reported controls (Figure 2) (Hervé et al., 2007; Ng et al., 2004; Tsuiji et al., 2006; Wardemann et al., 2003). We found that polyreactive new emigrant B cells were significantly increased in IRAK-4-, MyD88-, and UNC-93B-deficient patients, in which they represented 15.6%–56.1% of the clones, whereas polyreactive new emigrant B cells represented only 5.0%–8.3% in healthy controls (Figures 2A and 2B). Using enzyme-linked immunosorbent assay (ELISA) against human epithelial HEp-2 cell lysate, we further demonstrated defects in central tolerance in IRAK-4- and MyD88-deficient patients by a high frequency of HEp-2-reactive clones (Figure 2C and Figure S3). However, the frequency of HEp-2-reactive new emigrant B cells from UNC-93B-deficient patients was only slightly increased compared to that in controls, and differences failed to reach statistical significance (Figure 2C and Figure S3). Thus, the removal of developing autoreactive B cells is severely defective in the absence of IRAK-4 and MyD88 expression, whereas the absence of UNC-93B partially affects the central B cell tolerance checkpoint.

IRAK-4- and MyD88-Dependent Counterselection of ANA B Cells Does Not Require UNC-93B

Central B cell tolerance is also responsible for the removal of developing B cells expressing ANAs, as illustrated by the very low frequency of new emigrant B cells with such reactivity in healthy controls (Figure 3A) (Wardemann et al., 2003). In contrast, IRAK-4- and MyD88-deficient new emigrant B cells showed a high frequency of ANA clones, which represented 34.1%, 18.5%, and 15.4% of the new emigrant B cells in the three IRAK-4-deficient patients and 7.9% in the MyD88-deficient patient (Figure 3A). ANAs expressed by IRAK-4- and MyD88-deficient B cells could be divided into those that reacted with the condensed chromatin material in mitotic cells and those that did not (Figures 3B and 3C). Chromatin-nonreactive ANAs accounted for 10.9% and 5.3% of new emigrant B cells of IRAK-4- and MyD88-deficient patients, respectively, whereas chromatin-reactive ANAs represented 10.7% and 2.6% of the clones (Figure 3B). Autoreactive antibodies expressed by IRAK-4- and MyD88-deficient B cells showed a large diversity of speckled, nucleolar, or homogeneous anti-nuclear staining patterns (Figure 3C). Paradoxically, UNC-93B-deficient patients were able to properly counterselect ANA-expressing developing B cells, thereby suggesting that ANA B cell removal did not seem to require UNC-93B (Figures 3A and 3B).

We further analyzed the reactivity of recombinant antibodies from IRAK-4-, MyD88-, and UNC-93B-deficient patients and healthy donor controls by indirect immunofluorescence on *Crithidia luciliae* (Figures 3D and 3E). Antibody recognition of the kinetoplast of *C. luciliae*, an organelle composed of dsDNA, is the most specific assay for identifying anti-native dsDNA and is routinely used for the detection of these autoantibodies in systemic lupus erythematosus (SLE) patients. Using this assay, we found that new emigrant B cells from controls and UNC-93B-deficient patients contained no anti-dsDNA clones, confirming the proper removal of ANA B cells in these individuals (Figure 3D). In contrast, 2.6%–17.1% of antibodies expressed by IRAK-4- and MyD88-deficient new emigrant B cells bound both the

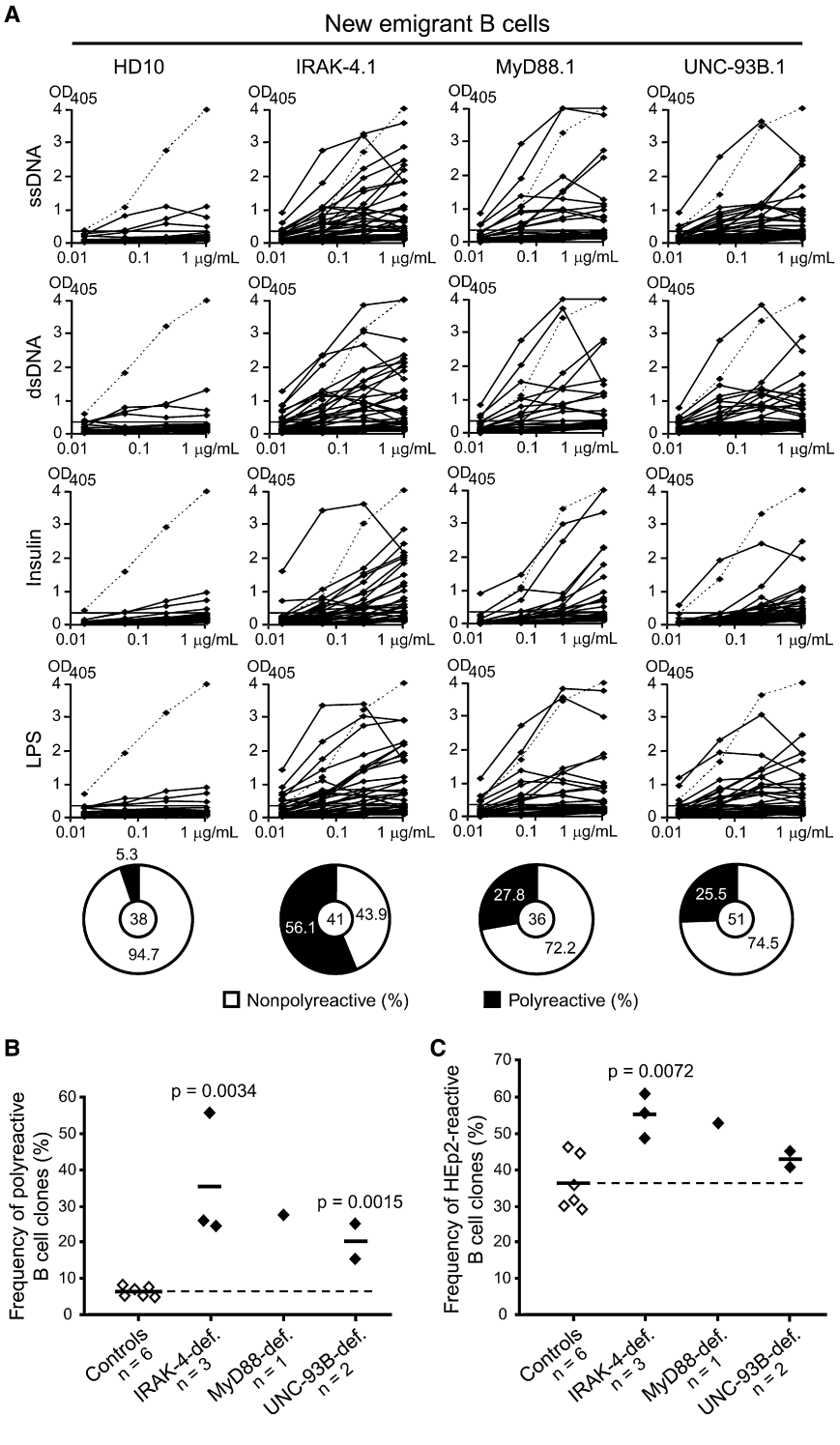

Figure 2. Defective Central B Cell Tolerance Checkpoint in IRAK-4-, MyD88-, and UNC-93B-Deficient Patients

(A) Antibodies from new emigrant B cells from controls and patients were tested by ELISA for reactivity against single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), insulin, and LPS. Antibodies are considered polyreactive when they recognize at least two and usually all four antigens. Dotted lines show ED38-positive control (Wardemann et al., 2003). Horizontal lines show cutoff OD_{405} for positive reactivity. For each individual, the frequency of polyreactive and nonpolyreactive clones is summarized in pie charts, with the number of antibodies tested indicated in the centers.

(B and C) The frequencies of polyreactive (B) and HEp-2-reactive (C) new emigrant B cells are compared between controls and patients, and statistically significant differences are indicated. Each diamond represents an individual, and the average is shown with a bar.

nucleus and the kinetoplast or only the kinetoplast of *C. luciliae* (Figures 3D and 3E and Tables S1–S14). In addition, we found that kinetoplast-nonreactive ANAs and kinetoplast-reactive antibodies display immunoglobulin heavy chain (IgH) complementarity-determining regions 3 (CDR3) that contained the highest number of positively charged amino acids (aa), such as arginines previously shown to favor anti-DNA autoreactivity (Figure 3F) (Hervé et al., 2007; Radic et al., 1993; Wardemann et al., 2003). Hence, ANA clones, including those reacting with chromatin, are enriched in the new emigrant B cell compartment when the MyD88 and IRAK-4 signaling pathway is defective, independently of UNC-93B.

Altered Receptor-Editing Regulation in Developing IRAK-4- and MyD88-Deficient B Cells

Receptor editing represents a major B cell tolerance mechanism by which developing autoreactive B cells can be silenced, especially those that express ANAs (Radic and Weigert, 1995). Secondary recombination events, first on the kappa locus and then on the lambda locus, provide attempts to edit autoreactive antibodies by substituting light chains until BCR autoreactivity is either abolished or diminished to allow B cell development to proceed (Goodnow, 1996; Nemazee et al., 2000; Radic and Weigert, 1995). As a result, upstream variable (V) gene usage combined with downstream joining (J) segments is a signature for secondary recombination events. In humans, lambda chains are more efficient at silencing autoreactive and polyreactive antibodies than are kappa chains, as illustrated by the lower frequency of autoreactive clones in new emigrant B cells expressing lambda chains in healthy controls (Figure 4A) (Wardemann et al., 2004). In contrast, lambda-chain usage in IRAK-4- and MyD88-deficient B cells did not diminish antibody autoreactivity compared to that in kappa-chain-expressing clones, which suggest defects in receptor editing in these patients (Figure 4A). In

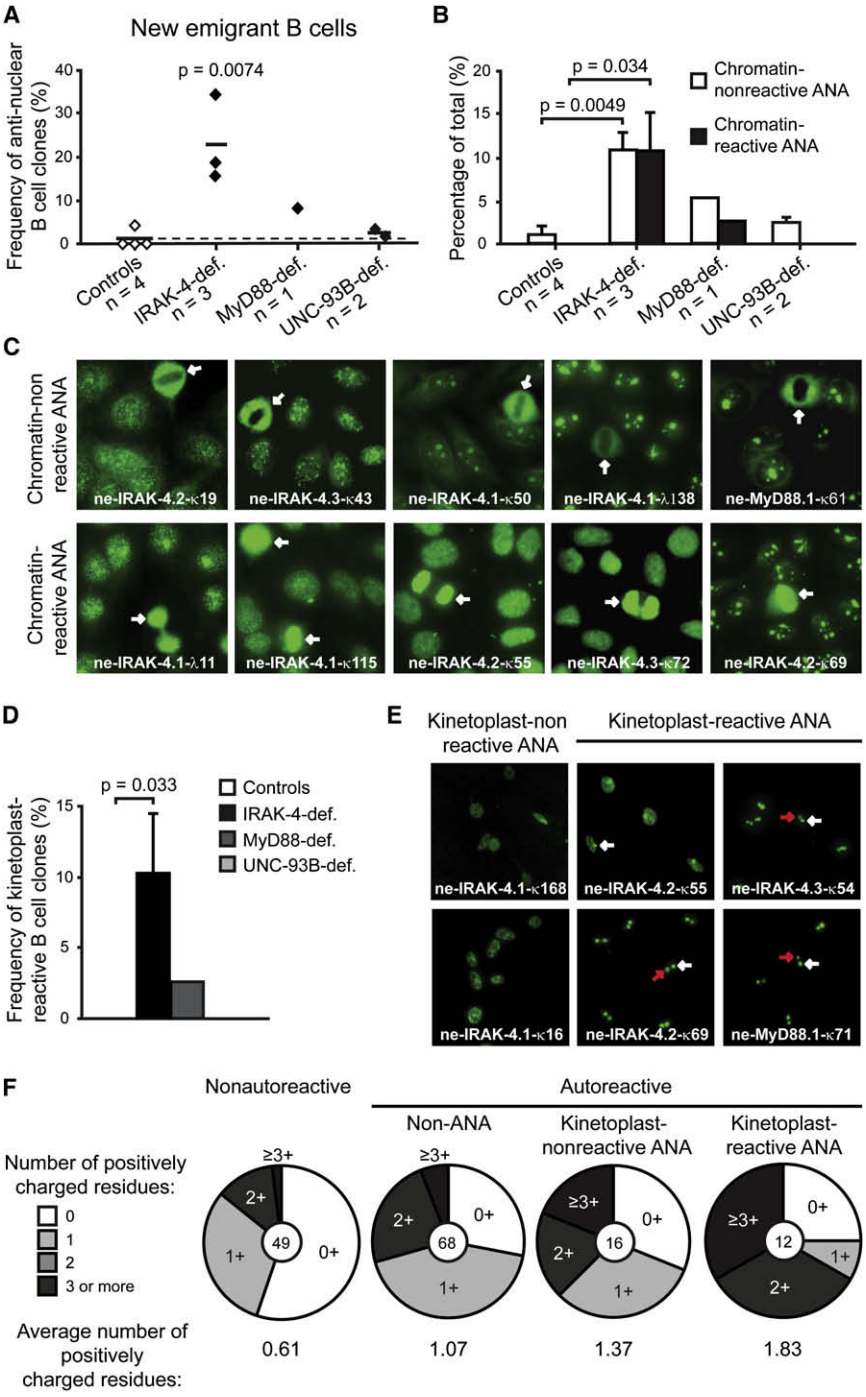

Figure 3. ANA-Expressing B Cell Removal Requires IRAK-4 and MyD88, but Not UNC-93B

(A) The frequency of anti-nuclear new emigrant B cells in IRAK-4 and MyD88-deficient patients is higher than in controls and UNC-93B-deficient patients. Each diamond represents an individual, and the average is shown with a bar.

(B) The frequencies of chromatin-nonreactive and -reactive clones in IRAK-4- and MyD88-deficient patients are higher than in controls and UNC-93B-deficient patients. Histograms represent means ± SEM of frequencies of chromatin-nonreactive (open bars) or chromatin-reactive (black bars) antibodies. Statistically significant differences between patients and controls are indicated.

(C) ANAs from IRAK-4- and MyD88-deficient new emigrant B cells show various patterns of anti-nuclear staining. White arrows indicate dividing cells that demonstrate negative (top row) or positive (bottom row) staining for the condensed chromatin material.

(D) Increased kinetoplast-reactive antibodies in new emigrant B cells from IRAK-4- (black bars) and MyD88-deficient (dark gray bars) compared to controls (open bars) and UNC-93B-deficient patients (light gray bars). Histograms represent means ± SEM of the proportion of kinetoplast-reactive clones. Statistically significant differences between patients and controls are indicated.

(E) IRAK-4- and MyD88-deficient clones express ANAs that can recognize the kinetoplast of *C. Luciliae*. White and red arrows indicate positively stained kinetoplasts and nucleus of *C. Luciliae*, respectively.

(F) Kinetoplast-reactive ANA-expressing B cells display the highest frequency of positively charged residues in their heavy-chain CDR3s. New emigrant B cells from IRAK-4- and MyD88-deficient patients were grouped into nonautoreactive, autoreactive non-ANA (HEp-2-reactive and/or polyreactive), kinetoplast-nonreactive ANA, and kinetoplast-reactive ANA clones. Pie charts show the proportion of heavy-chain CDR3s with zero, one, two, and three or more positive charges. The absolute number of clones analyzed in each B cell fraction is indicated in the center. The average number of positively charged residues per CDR3 is indicated below each fraction. The numbers of positively charged residues in immunoglobulin heavy-chain CDR3s were calculated excluding the arginin or lysin found at position 94 in most germline VHs.

UNC-93B-deficient patients, lambda-chain usage properly decreased autoreactivity, suggesting differences from IRAK-4- and MyD88-deficient patients (Figure 4A). When lambda clones were separated into nonautoreactive versus autoreactive, we found that control and IRAK-4- and MyD88-deficient nonautoreactive B cells expressed a similar Jλ gene usage (Figure 4B). In contrast, IRAK-4- and MyD88-deficient autoreactive lambda new emigrant B cells were biased toward Jλ1 gene usage, which is the most upstream of all Jλs, suggesting that there was no attempt at editing these autoreactive antibodies compared to autoreactive lambda clones in controls that preferentially used the downstream Jλ3 gene (Figure 4B). Despite the strong bias toward downstream Vλ gene usage in healthy controls, IRAK-4- and MyD88-deficient autoreactive lambda clones showed an increase in downstream Vλ gene usage, whereas there was no bias in Vλ gene usage between control and patient nonautoreactive lambda cells (Figure S4). Thus, downstream Vλ usage combined with highly increased upstream Jλ1 usage in IRAK-4- and MyD88-deficient autoreactive lambda new emigrant B cells reflect a dearth of secondary recombination on this locus when

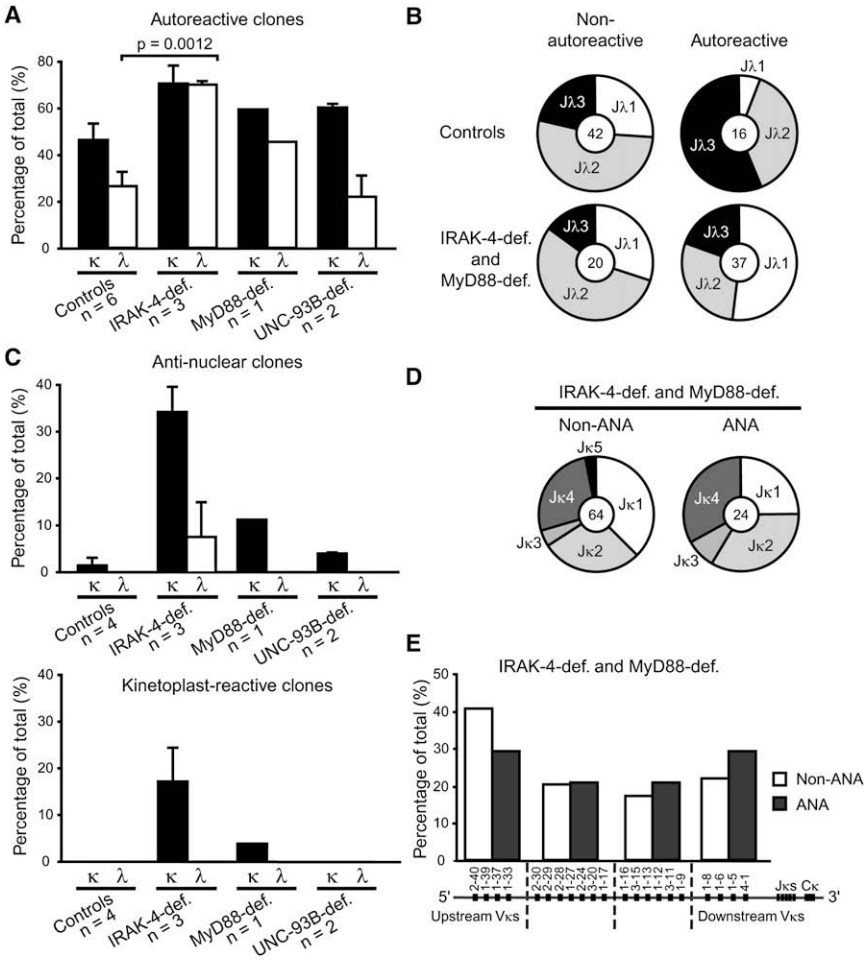

Figure 4. Defective Receptor-Editing Induction in IRAK-4- and MyD88-Deficient Developing Autoreactive B Cells

(A) New emigrant B cells from IRAK-4- and MyD88-deficient patients have an increased proportion of autoreactive lambda clones compared to those in controls and UNC-93B-deficient patients. Histograms represent means ± SEM of the proportion of κ (black bars) and λ (open bars) clones that are autoreactive (polyreactive and HEp-2-reactive combined).

(B) Autoreactive IRAK-4- and MyD88-deficient new emigrant B cells display a lambda repertoire suggesting defective receptor editing. Pie charts show the proportion of the different Jλ genes in nonautoreactive and autoreactive new emigrant B cells from healthy controls (top panel) or IRAK-4- and MyD88-deficient patients (bottom panel), with the number of analyzed sequences indicated in the centers.

(C) Most ANA and all kinetoplast-reactive B cells from IRAK-4 and MyD88-deficient patients express kappa chains. Histograms represent means ± SEM of proportion of κ (black bars) and λ (open bars) clones that are anti-nuclear (top panel) and kinetoplast-reactive (bottom panel).

(D) The ANA expressed by IRAK-4- and MyD88-deficient new emigrant B cells do not show any bias toward downstream Jκ3,4,5 usage compared to the non-ANA clones. Pie charts show the proportion of the different Jκ genes, with the number of analyzed sequences indicated in the centers.

(E) The ANA expressed in IRAK-4- and MyD88-deficient new emigrant B cells do not show any bias toward upstream Vκ gene usage compared to the non-ANA clones. The Vκ locus is shown below, clustered into four clans of Vκ gene segments. Histograms represent the proportion of genes of each Vκ group for ANA (black bars) and non-ANA (open bars) expressed by new emigrant B cells from IRAK-4- and MyD88-deficient patients.

functional IRAK-4 and MyD88 complexes could not be expressed.

The analysis of ANA-expressing new emigrant B cells that escaped central tolerance in IRAK-4- and MyD88-deficient patients revealed that most ANA and all kinetoplast-reactive clones utilized kappa chains, demonstrating that editing using lambda chains was not induced in these B cells (Figure 4C). In addition, IRAK-4- and MyD88-deficient new emigrant B cells that express ANAs did not show any evidence of secondary recombination on their kappa locus (Figures 4D and 4E). There was no bias toward downstream Jκ3,4,5 gene usage in IRAK-4- and MyD88-deficient ANA clones, nor any bias toward upstream Vκ gene usage in ANA-expressing B cells (Figures 4D and 4E). We conclude that kappa and lambda light-chain receptor editing is not efficiently induced to thwart autoreactivity and silence autoreactive and ANA-expressing B cell precursors in the absence of proper IRAK-4 and MyD88 signaling.

Defective Peripheral B Cell Tolerance Checkpoint in IRAK-4-, MyD88-, and UNC-93B-Deficient Patients

We previously demonstrated that a peripheral B cell tolerance checkpoint further counterselects autoreactive new emigrant B cells before they enter the $CD19^{+}CD10^{-}IgM^{+}CD27^{-}$ mature naive B cell compartment (Wardemann et al., 2003). In healthy donors, HEp-2-reactive mature naive B lymphocytes represent 16.7%–23.3% of the clones (Figures 5A and 5B) (Wardemann et al., 2003). In contrast, we found that the frequency of HEp-2-reactive mature naive B cells was substantially increased and reached 45.8%–65.4% in IRAK-4-, MyD88-, and UNC-93B-deficient patients (Figures 5A and 5B). The mature naive B cell compartment of all patients was also enriched in polyreactive clones compared to healthy controls (Figure 5C and Figure S5). In addition, the proportion of mature naive B cells expressing ANAs reacting or not with the chromatin was slightly elevated in IRAK-4-, MyD88-, and UNC-93B-deficient patients when compared to that in healthy donors (Figures 6A and 6B). Correlating with the presence of chromatin-reactive antibodies, kinetoplast-reactive B cells were also identified in the mature naive B cell compartment of IRAK-4-, MyD88-, and UNC-93B-deficient patients (Figure 6C). Little is known about the cells and molecules that regulate human peripheral B cell tolerance, but elevated B cell-activating factor (BAFF) concentrations may prevent the removal of autoreactive B cells, probably by increasing their survival in the periphery (Hervé et al., 2007; Lesley et al., 2004;

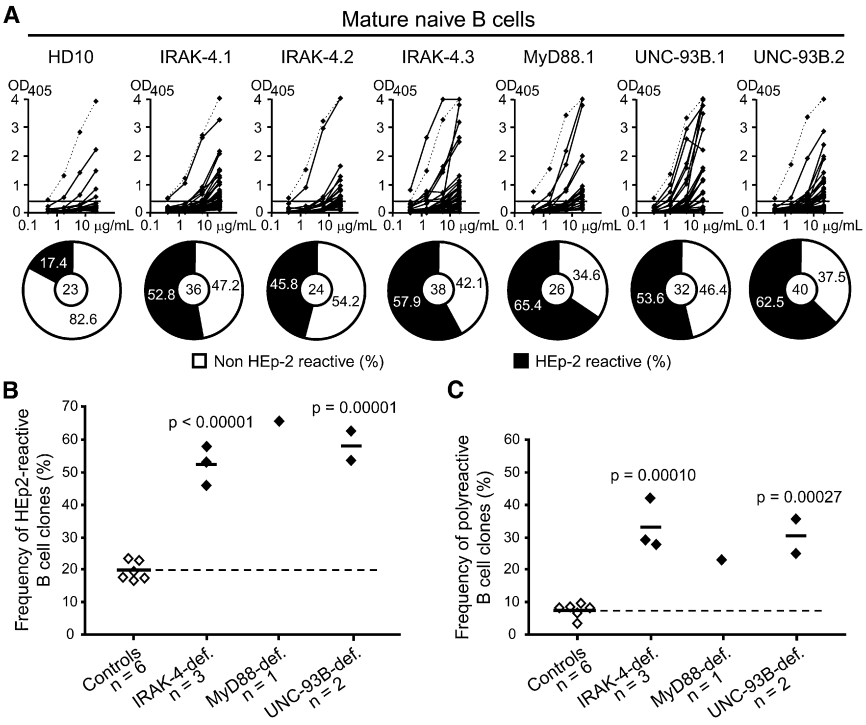

Figure 5. Defective Peripheral B Cell Tolerance Checkpoint in IRAK-4-, MyD88-, and UNC-93B-Deficient Patients

(A) Increased frequency of HEp-2-reactive antibodies in IRAK-4-, MyD88-, and UNC-93B-deficient mature naive B cells. Antibodies from mature naive B cells from one control (HD10), three IRAK-4-deficient, one MyD88-deficient, and two UNC-93B-deficient patients were tested in ELISA for reactivity with HEp-2 cell lysate. Dotted lines show ED38-positive control (Meffre et al., 2004; Wardemann et al., 2003). Horizontal lines show cutoff OD_{405} for positive reactivity. For each individual, the frequency of HEp-2-reactive (in black) and non-HEp-2-reactive (in white) clones is summarized in pie charts with the number of antibodies tested indicated in the centers.

(B and C) The frequency of (B) HEp-2-reactive and (C) polyreactive (tested against ssDNA, dsDNA, insulin, and LPS) clones in mature naive B cells of IRAK-4-, MyD88-, and UNC-93B-deficient patients is higher than in controls. Each diamond represents an individual, and the average is shown with a bar. Statistically significant differences between patients and controls are indicated.

Mackay et al., 1999; Thien et al., 2004). However, BAFF does not account for the defective peripheral B cell tolerance checkpoint in IRAK-4-, MyD88-, and UNC-93B-deficient patients because they all showed normal BAFF amounts in their serum (Figure S6). We conclude that the peripheral B cell tolerance checkpoint requires IRAK-4, MyD88, and UNC-93B to be functional.

Human Autoreactive B Cells Fail to Secrete Autoantibodies in the Absence of IRAK-4, MyD88, and UNC-93B

Despite the accumulation of autoreactive B cells expressing ANAs and polyreactive antibodies in their blood, IRAK-4-, MyD88-, and UNC-93B-deficient patients suffer from bacterial and viral infections, respectively, and not from autoimmune diseases (Casanova and Abel, 2007; Casrouge et al., 2006; von Bernuth et al., 2008). To investigate the role of IRAK-4-, MyD88-, and UNC93-B-dependent TLRs on the activation of autoreactive B cells through the secretion of autoreactive antibodies, we tested nine IRAK-4-, three MyD88-, and three UNC-93B-deficient patients for the presence of autoantibodies in their serum. We found that these patients displayed titers of ANAs and polyreactive IgG antibodies in their serum that were similar to those found in healthy controls (Figure 7 and Figure S7). Thus, autoreactive B cells in the blood of IRAK-4-, MyD88-, and UNC-93B-deficient patients do not secrete ANAs and other polyreactive antibodies when TLR signaling and functions are altered in the absence of IRAK-4, MyD88, or UNC-93B expression.

DISCUSSION

We report herein that IRAK-4-, MyD88-, and UNC-93B-deficient patients showed defects in the establishment of B cell tolerance. Indeed, IRAK-4-, MyD88-, and UNC-93B-dependent pathways prevent the accumulation of large numbers of autoreactive B cells in the periphery of healthy donors. Developing autoreactive B cells are normally removed at two different checkpoints, first in the bone marrow and then in the periphery. The central B cell tolerance checkpoint seems to be mainly controlled by intrinsic B cell molecules that regulate BCR signaling induced by binding to autoantigens at the immature B cell stage (Goodnow, 1996; Nemazee et al., 2000; Samuels et al., 2005a). Alterations of BCR signaling thresholds, such as in B cells from X-linked agammaglobulinemia patients who carry mutations in their *BTK* gene, result in defective central B cell tolerance checkpoint and in the release of autoreactive B cells in the periphery (Ng et al., 2004). In contrast, the peripheral B cell tolerance checkpoint seems to rely on extrinsic molecular and cellular regulators, such as BAFF concentration and Treg cells (Hervé et al., 2007). How does the absence of IRAK-4, MyD88, and UNC-93B affect mechanisms that normally mediate these counterselection steps?

We demonstrated that signaling through IRAK-4 and MyD88 complexes plays a major role in the establishment of central B cell tolerance in the bone marrow by counterselecting developing autoreactive B cells, including ANA-expressing clones. Because *IL1R1*, *IL18R1*, and *IL1RL1* genes are not expressed in control B cells, defects in the central B cell tolerance checkpoint in IRAK-4 and MyD88-deficient patients are likely to result from the inability of TLRs to signal in the absence of functional IRAK-4 and MyD88 complexes. TLR7 and TLR9, which bind RNA- and DNA-containing antigens, respectively, were obvious candidates for the removal of ANA-expressing clones. UNC-93B has been reported to be required for these TLRs to reach endolysosomes, where they bind ligands and initiate signaling (Kim et al., 2008; Tabeta et al., 2006). In agreement with this observation, we found that B cells from UNC-93B-deficient patients showed impaired responses to TLR7 and TLR9 triggering. The apparent removal

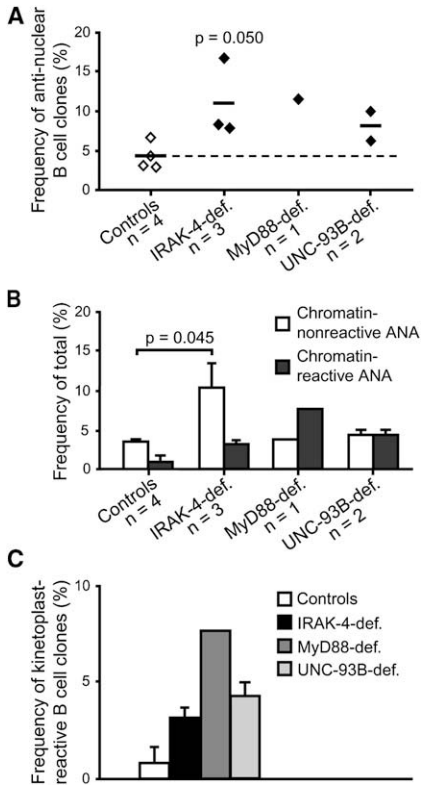

Figure 6. Identification of ANA-Expressing Mature Naive B Cells in IRAK-4-, MyD88-, and UNC-93B-Deficient Patients

(A) The frequency of anti-nuclear mature naive B cells in IRAK-4, MyD88-, and UNC-93B-deficient patients is higher than in controls. Each diamond represents an individual, and the average is shown with a bar.

(B and C) Chromatin-nonreactive and -reactive (B) and kinetoplast-reactive (C) clones are enriched in the mature naive B cell compartment of IRAK-4-, MyD88-, and UNC-93B-deficient patients compared to those in controls. Histograms represent means ± SEM of proportion of (B) chromatin-nonreactive (open bars) and chromatin-reactive (black bars) clones and (C) kinetoplast-reactive clones.

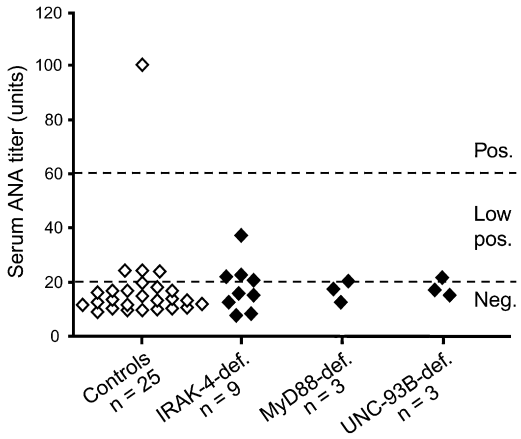

Figure 7. Lack of Serum ANAs in IRAK-4-, MyD88-, and UNC-93B-Deficient Patients

The presence of IgG ANAs was assessed by ELISA in the serum of healthy controls and IRAK-4-, MyD88-, and UNC-93B-deficient patients. Each diamond represents an individual. Dashed lines indicate threshold between negative, low-positive, and positive individuals for serum IgG ANAs.

of ANA-expressing B cells in UNC-93B-deficient patients seems, however, to argue against a role of TLR7 and TLR9 in this process. Nevertheless, UNC-93B-deficient patients displayed an increased frequency of polyreactive new emigrant B cells, suggesting that TLR7 and TLR9 play a partial role in the establishment of central B cell tolerance. In addition to TLR7 and TLR9, human B cells express the UNC-93B-independent TLR10, either as homodimers or as heterodimers with TLR1 (Hasan et al., 2005). TLR10 complexes do not have any known ligands and may be responsible for the counterselection of B cells expressing ANAs. Alternatively, other IRAK-4 and MyD88-dependent receptors yet to be identified may be responsible for the removal of ANA-expressing B cell clones in the bone marrow. During their development, B cells may encounter nuclear self-antigens in blebs expressed at the surface of bone marrow cells dying by apoptosis or from the enucleated material from millions of red blood cells (Cocca et al., 2002). The binding of DNA- and RNA-containing antigens to ANAs and other receptors may therefore generate synergistic signals that may mediate the silencing of these clones. Interestingly, we found that TLR10 and, to some extent, TLR9 cell-surface expression were upregulated after either BCR triggering or BCR and TLR9 cotriggering. Thus, developing autoreactive B cells stimulated by self-antigens express increased cell-surface TLR expression that may cosignal with BCRs and induce tolerance mechanisms. In the absence of functional IRAK-4 and MyD88 expression, a lack of intracellular signaling may result in altered removal of ANA clones. In agreement with this hypothesis, we found that kappa and lambda light-chain receptor editing was not properly induced in IRAK-4- and MyD88-deficient B cell precursors, which resulted in the appearance of a population of kappa-expressing anti-nuclear B cells. Hence, the TLR and BCR coengagement paradigm may apply not only to B cell activation and proliferation, but also to the selection of developing B cells in the bone marrow.

All IRAK-4-, MyD88-, and UNC-93B-deficient patients displayed a similar defective peripheral B cell tolerance checkpoint, resulting in the accumulation of large numbers of autoreactive B cells in the blood of these patients. Because TLR3, TLR7, TLR8, and TLR9 are the only receptors that depend on UNC-93B to function, and because TLR3 signaling does not require IRAK-4 and MyD88 complexes, it is likely that TLR7, TLR8, and TLR9 are responsible for the removal of autoreactive B cells in the periphery. Little is known about the cells and molecules that regulate human peripheral B cell tolerance or about how TLR7, TLR8, and TLR9 could be involved. In agreement with findings from mouse models, our recent data suggest that elevated BAFF concentrations may prevent the removal of autoreactive B cells, probably by increasing their survival in the periphery (Hervé et al., 2007; Lesley et al., 2004; Mackay et al., 1999; Thien et al., 2004). However, BAFF does not account for the defective peripheral B cell tolerance checkpoint in IRAK-4-, MyD88-, and UNC-93B-deficient patients because they all showed normal BAFF amounts in their serum. In addition to BAFF, Treg cells may also control the elimination of autoreactive B cells in the periphery (Hervé et al., 2007). Indeed, the decreased numbers of Treg cells in CD40L- and major histocompatibility complex

class II-deficient patients, who display a specific increase of autoreactive B cells in the mature naive B cell compartment, but not in the new emigrant B cell compartment, suggest that these T cells may mediate peripheral B cell tolerance (Hervé et al., 2007). A similar hypothesis was previously proposed with transgenic mouse models, in which $CD4^+$ T cells and the receptor-ligand pairs CD40-CD40L and Fas-FasL have been shown to play an important role in removing tolerant hen-egg-lysozyme-binding B cells (Rathmell et al., 1995; Rathmell et al., 1996). A direct involvement of TLR7, TLR8, and TLR9 on human Treg cell development and functions is difficult to assess because TLR stimulation can either increase or decrease the regulatory properties of these T cells (Conroy et al., 2008). However, the roles of TLR7 and TLR9 on DCs have been well characterized, especially in plasmacytoid DCs (pDCs), which mostly express these TLRs (Kadowaki et al., 2001), Interestingly, pDCs have been reported to play an important role in the development of Treg cells when primed by TLR agonists (Ito et al., 2007; Moseman et al., 2004; Ouabed et al., 2008; Watanabe et al., 2005). We would therefore like to propose that in the absence of TLR7 and TLR9 signaling in IRAK-4, MyD88-, and UNC-93B-deficient patients, pDCs may not be able to induce the development of Treg cells specific for a whole set of self-antigens. Moreover, DC-Treg cell interactions may also be affected because IRAK-4-deficient DCs show lower CD40, CD80, and CD86 expression after stimulation by a TLR agonist (Ku et al., 2007). Altogether, a failure to properly educate Treg cells may generate an incomplete Treg cell repertoire unable to counterselect some autoreactive B cells in the periphery. In addition, the inability of IRAK-4-, MyD88-, and UNC-93B-deficient monocytes and DCs to properly secrete cytokines such as IFNs and TNF-α in response to TLR stimulation may also impact on the peripheral B cell tolerance checkpoint (Ku et al., 2007). In conclusion, altered Treg cell and/or DC functions in IRAK-4-, MyD88-, and UNC-93B-deficient patients may lead to the defective peripheral B cell tolerance checkpoint observed in these patients.

Despite the large number of peripheral autoreactive B cells in IRAK-4-, MyD88-, and UNC-93B-deficient patients, these individuals suffer from bacterial or viral infections and not from autoimmune diseases. We found that these patients did not display ANAs or show any increase in polyreactive antibodies in their serum. Thus, the autoreactive and polyreactive B cells in their blood are not activated and do not secrete these antibodies when TLR signaling is altered. In agreement with this observation, many reports using mouse models demonstrated that TLR7 and TLR9 play an essential role in activating autoreactive B cells by transducing costimulatory signals to BCRs that bind DNA- and RNA-containing immune complexes (Baccala et al., 2007; Marshak-Rothstein, 2006). In addition, mouse models further support a role for MyD88 and TLR7 in the development of autoimmunity (Berland et al., 2006; Christensen et al., 2006; Ehlers et al., 2006; Pisitkun et al., 2006). The TLR7 pathway was therefore proposed to represent a therapeutic target in diseases like SLE (Berland et al., 2006; Christensen et al., 2006; Pisitkun et al., 2006). Our data show that IRAK-4-, MyD88-, and UNC-93B-deficient patients present frequencies of autoreactive mature naive B cells similar to those from patients suffering from rheumatoid arthritis and SLE (Samuels et al., 2005b; Yurasov et al., 2005). However, in contrast to these patients, IRAK-4-, MyD88-, and UNC-93B-deficient patients did not develop autoimmune diseases, further suggesting that blocking TLR pathways in humans may thwart the development of autoimmunity.

In conclusion, the accumulation of autoreactive B cells in the mature naive B cell compartment of IRAK-4-, MyD88-, and UNC-93B-deficient patients suggests that pathways mediated by IRAK-4, MyD88, and UNC-93B play an important role in counterselecting developing autoreactive B cells and preventing their maturation into long-lived mature naive B cells in healthy individuals.

EXPERIMENTAL PROCEDURES

Patients and Healthy Donor Controls

The four IRAK-4-deficient patients belong to four unrelated families. They all suffered from recurrent pyogenic bacterial infections but were healthy when blood samples were obtained. They had no history of related autoimmune disorders. Patient 1 is a 23-year-old woman who was born from unrelated parents (Kuhns et al., 1997; Medvedev et al., 2003). She suffers from two different mutations on her *IRAK4* gene alleles. The first is a cytidine-to-thymidine mutation at nucleotide 877, resulting in a stop codon instead of the wild-type glutamine in the IRAK-4 kinase domain (Medvedev et al., 2003). The second mutation is a two-nucleotide deletion at nucleotides 620–621 that results in a frameshift creating a stop codon 36–39 nucleotides downstream, also in the kinase domain of IRAK-4 (Medvedev et al., 2003). Patient 2 is a 10-year-old girl who was born to consanguineous parents (Day et al., 2004; Haraguchi et al., 1998). She displays the same cytidine-to-thymidine mutation from patient 1 on both of her *IRAK4* gene alleles (Picard et al., 2003). Patient 3 is an 8-year-old boy who suffers from a homozygous deletion of the noncoding exon 1 of the *IRAK4* gene reported as P15 in Ku et al., 2007. Patient 4 is a 16-month-old boy. He suffers from two different mutations on his *IRAK4* gene alleles. The first mutation is the same cytidine-to-thymidine mutation displayed by patients 1 and 2. The second mutation is a deletion in exon 5. The MyD88-deficient patient is a 2-year-old Turkish girl born from nonconsanguineous parents (von Bernuth et al., 2008). This patient displays compound heterozygous mutations in *MYD88*: L93P in exon 1 that encodes the death domain of MyD88 and R196C in exon 3 that is contained in the Toll-IL-1 receptor (TIR) domain of the protein (von Bernuth et al., 2008). Both UNC-93B-deficient patients were teenagers born from first-cousin parents (Casrouge et al., 2006). UNC-93B-deficient patient 1 is homozygous for a four-nucleotide deletion at position 1034–1037 in exon 8, whereas patient 2 is homozygous for a single-nucleotide substitution in exon 6 that prevents the splicing of this exon (Casrouge et al., 2006). Healthy donors include a 36-year-old Caucasian male (HD10) and five previously reported controls (Hervé et al., 2007; Ng et al., 2004; Tsuiji et al., 2006; Wardemann et al., 2003). Overall, the ages of healthy controls range from 5 years old to 37 years old and match the diverse ages of the patients enrolled in the study (Hervé et al., 2007; Ng et al., 2004; Tsuiji et al., 2006; Wardemann et al., 2003). All samples were collected after patients signed informed consent in accordance with protocols reviewed by the institutional review board. Informed consent as approved by the institutional review board was obtained from the custodians of subjects younger than 18 years, and assent was signed by children and adolescents older than 8 years as well.

Cell Sorting

Peripheral B cells were purified from the blood of patients and control donors by negative selection with RosetteSep™ procedure (StemCell Technologies). Enriched B cells were stained with fluorescein isothiocyanate (FITC) anti-human CD27, phycoerythrin (PE) anti-human CD10, anti-human IgM biotin, and allophycocyanin (APC) anti-human CD19. Biotinylated antibodies were revealed with streptavidin-PECy7. Single $CD19^+CD10^+IgM^+CD27^-$ new emigrant and $CD19^+CD10^-IgM^+CD27^-$ peripheral mature naive B cells from patients and control donors were sorted on a FACSVantage (Becton Deckinson) into 96-well PCR plates containing 4 μl of lysis solution (0.5 × PBS containing 10 mM DTT, 8 U RNAsin [Promega], and 0.4 U 5′-3′ RNase Inhibitor [Eppendorf]) and immediately frozen on dry ice. All samples were stored at

−70°C. B cell subpopulations as well as T cell and DC fractions were batch sorted with antibodies described above and anti-TCRαβ-FITC, anti-CD25-PE, anti-CD4-PECy7, anti-CD11c-PE, and anti-HLA-DR-APC. All antibodies were purchased from PharMingen, Becton Dickinson.

B Cell Activation

Naive B cells were enriched with the Human Naive B Cell Isolation Kit II (Miltenyi). $CD19^{+}CD27^{-}$ naive B cells from control donors and patients were plated at 150,000–200,000 cells per well in a 96-well plate in RPMI 10% serum and 20 μg/mL polyclonal $F(ab)'_2$ rabbit anti-human IgM (Jackson Immunoresearch), 1 μg/mL CpG (Invivogen), or 0.5 μM Loxoribine (Invivogen) for 48 hr. Flow-cytometry analysis was performed with anti-CD86-FITC, anti-CD25-FITC, anti-CD10-APC (Biolegend), anti-CD80-PE, anti-CD69-PE, anti-CD19-PECy7 (PharMingen, Beckton Dickinson), anti-TLR10-FITC, and anti-TLR-9-PE (Imgenex).

RNA and RT-PCRs

Total RNA was extracted from 10^5 purified cells with Absolutely RNA microprep kit (Stratagene). RNA was reverse transcribed in 20 μl with Superscript II (GIBCO BRL). For RT-PCR reactions, 2 μl of cDNA was amplified for 32 cycles of 30 s at 94°C, 30 s at 60°C (*TLR7*) or at 63°C (*IL1R*, *TLR9*, *TLR10*, *IRAK4*, *MYD88*, *UNC93B1*, and *CD79b*), and 30 s at 72°C, with a final 7 min extension at 72°C with Taq DNA polymerase (Roche) and the following primers: IL1R1 sense 5′CATAGAGGGAACTATACTTGTCATG3′; IL1R1 antisense 5′CATTAGC TGGGCTCACAATCACAG3′; TLR7 sense 5′TTTGGAAGAAGACTAAAAATGGT G3′; TLR7 antisense 5′GAACATCCAGAGTGACATCACAG3′; TLR9 sense 5′CC GCCAGACCCTCTGGAGAAG3′; TLR9 antisense 5′ACTTCAGGAACAGCCAG TTGCAG3′; TLR10 sense 5′CCCAAGAACAACTCAAGAGAAATG3′; TLR10 antisense 5′TGCTTTTGCCAGGGTCAAAGTAG3′; IRAK4 sense 5′CATATGTG CGCTGCCTCAATGTTG3′; IRAK4 antisense 5′CCAGTCAAACAGTAATTCAGA AGTG3′; MYD88 sense 5′GACGACGTGCTGCTGGAGCTG3′; MYD88 antisense 5′GATGAAGGCATCGAAACGCTCAG3′; UNC93B1 sense 5′GCGAGGT GAAGTATGGCAACATG3′; and UNC93B1 antisense 5′GGCGTAGATGCCCA CAGCGAG3′. CD79b primers were previously described (Meffre et al., 1998). RT-PCR products were analyzed on 2% agarose gels.

Microarray Analysis of Gene-Expression Profiles

RNA was extracted from 10^5–3.10^5 batch-sorted new emigrant and mature naive B cells isolated from donors with the Absolutely RNA microprep kit (Stratagene). 100–200 ng of RNA was obtained per sample, and the quality of the purified RNA was assessed by the Bioanalyzer from Agilent. With the Ovation biotin system kit from Nugen, 30–50 ng of RNA was amplified and labeled for producing cDNA. Labeled cDNA was hybridized on chips containing the whole human genome (Human Genome U133 2.0 from Affymetrix).

cDNA, RT-PCR, and 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 RT (GIBCO BRL) for 45 min at 37°C. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, and antibody expression and purification were as described (Meffre et al., 2004; Wardemann et al., 2003). Immunoglobulin sequences were analyzed by Ig BLAST comparison with GenBank. Heavy-chain CDR3 was defined as the interval between the conserved cysteine at position 92 in the V_H framework 3 and the conserved tryptophan at position 103 in J_H segments.

ELISAs and Immunofluorescence Assays

Antibody concentrations, reactivity against specific antigens, and indirect immunofluorescence were as described (Meffre et al., 2004; Wardemann et al., 2003). Highly polyreactive ED38 was used as positive control in HEp-2-reactivity and polyreactivity ELISAs (Meffre et al., 2004; Wardemann et al., 2003). Antibodies were considered polyreactive when they recognized at least two and usually all of the four analyzed antigens, which include ssDNA, dsDNA, insulin, and LPS. Serum ANA titers of total IgG from patients and healthy controls were determined with INOVA QUANTA Lite ANA ELISA according to the manufacturer's instructions.

For indirect immunofluorescence assays, HEp-2 cell coated slides (Bion Enterprises) and *Crithidia luciliae* coated slides (Antibodies Inc.) were incubated in a moist chamber at room temperature with purified recombinant antibodies at 50–100 μg/mL and 100–200 μg/mL, respectively. FITC-conjugated goat anti-human IgG was used as detection reagent.

Statistics

Differences were analyzed for statistical significance with unpaired Student's t tests with SigmaPlot software (Systat). A p value of less than 0.05 was considered significant.

ACCESSION NUMBERS

The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession number GSE13300 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13300).

SUPPLEMENTAL DATA

Supplemental Data include seven figures and fourteen tables and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00465-2.

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