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*J Immunol* published online 10 May 2013
http://www.jimmunol.org/content/early/2013/05/10/jimmunol.1202711

**Supplementary Material**

[http://www.jimmunol.org/content/suppl/2013/05/10/jimmunol.1202711](http://www.jimmunol.org/content/suppl/2013/05/10/jimmunol.1202711)

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Separation of Function between Isotype Switching and Affinity Maturation In Vivo during Acute Immune Responses and Circulating Autoantibodies in UNG-Deficient Mice

Astrid Zahn,* Matthieu Daugan,* Shiva Safavi,* David Godin,* Cheolho Cheong,*†‡§ Alain Lamarre,† and Javier M. Di Noia*‡§¶

Activation-induced deaminase converts deoxycytidine to deoxyuridine at the Ig loci. Complementary pathways, initiated by the uracil-DNA glycosylase (UNG) or the mismatch repair factor MSH2/MSH6, must process the deoxyuridine to initiate class-switch recombination (CSR) and somatic hypermutation. UNG deficiency most severely reduces CSR efficiency and only modestly affects the somatic hypermutation spectrum in vitro. This would predict isotype-switching deficiency but normal affinity maturation in Ung−/− mice in vivo, but this has not been tested. Moreover, puzzling differences in the amount of circulating Ig between UNG-deficient humans and mice make it unclear to what extent MSH2/MSH6 can complement for UNG in vivo. We find that Ab affinity maturation is indeed unaffected in Ung−/− mice, even allowing IgM responses with higher than normal affinity. Ung−/− mice display normal to only moderately reduced basal levels of most circulating Ig subclasses and gut-associated IgA, which are elicited in response to chronically available environmental Ag. In contrast, their ability to produce switched Ig in response to immunization or vesicular stomatitis virus infection is strongly impaired. Our results uncover a specific need for UNG in CSR for timely Ab response against the cognate Ag by introducing amino acid changes in the IgV regions, which are then selected through their interaction with APCs and T cells. At the same time, CSR mediates isotype switching to endow the Ab response with new biological properties such as complement fixation, transport across epithelia, or opsonization ability. AID deaminates deoxycytidine in DNA, thereby producing deoxyuridine (reviewed in Refs. 4, 5). This lesion is mutagenic by creating a C-to-U/A (eventually fixed as T:A) transition mutation. However, AID cannot directly produce the full spectrum of SHM, which includes transversion mutations at C-G pairs, as well as mutations at A:T pairs (4). Similarly, CSR requires DNA double-strand break intermediates that AID cannot produce by itself (6, 7). The full spectrum of SHM and CSR requires processing of the deoxycytidine, which is initiated by either the uracil-DNA glycosylase (UNG) or the mismatch repair factor MSH2/MSH6 (7–10) (Fig. 1A). Indeed, the simultaneous absence of both UNG and MSH2/MSH6 results in SHM restricted to C-G-to-T:A mutations and the complete absence of all secondary isotypes and only IgM circulating in the serum of these mice (10, 11). UNG and MSH2/MSH6 are partially redundant for SHM and CSR, but they are not completely equivalent. In vitro CSR to IgG1 and IgG3 (i.e., measured in purified, cytokine-stimulated naive B cells from mice) is reduced by >10-fold in Ung−/− B cells, but only by ∼3-fold in Msh2/MSH6-deficient B cells (9–15). However, Msh2−/− mice produce up to 10-fold less anti-NP IgG1 titers than wild-type (wt) mice after immunization (15, 16). Furthermore, Msh2−/− and Msh6−/− mice show not only severe skewing of the SHM spectrum because of reduced SHM at A:T pairs, but also a change in the distribution of mutations and significant reduction in overall SHM accumulation (11, 13, 16, 17). The defects on Ab diversification displayed by Msh2−/− mice are therefore more severe...
than what could be predicted from the defects measured by in vitro assays. This seems to be related to imbalances in B cell populations (15, 16) and impaired germinal center B cell proliferation in vivo (12, 15, 17), which may be linked to the MSH2/MSH6 functions in cell-cycle regulation (18). Although the analysis of Msh2−/− mice highlights the limitations of deducing in vivo phenotypes from in vitro data, the fact that UNG deficiency in vitro has a much greater effect on CSR than MSH2/MSH6 deficiency (9, 10) would predict isotype switching to be severely compromised by UNG ablation in vivo. However, the actual evidence for this is scant. Ung−/− mice have very reduced IgG3 levels but display fairly normal basal serum levels of IgG1 and only modestly reduced IgA (9, 10). In contrast, the three UNG-deficient human patients who have been identified to date have very low levels of circulating switched isotype, susceptibility to recurrent infections (19, 20), and in one case, Sjögren’s disease (20, 21). However, this small cohort may be biased, as only severe pathologies would lead to their identification at immunodeficiency clinics. Moreover, preimmune serum Ig levels could be confounded by homeostatic compensation. The analysis of Ab responses to experimental immunization in ung−/− mice is limited to measuring the primary response against SRBC, which was significantly but quite modestly reduced for some isotypes (11). Thus, it is, in fact, still uncertain how much MSH2/MSH6 could compensate for UNG in catalyzing CSR in vivo after immunization or infection, and whether it may do so differently for different Ig classes.

One further reason for analyzing the immune response of Ung−/− mice is that the overall SHM load in chronically stimulated Peyer’s patches B cells is normal to slightly increased in Ung−/− mice (9–11). More importantly, the spectrum of SHM is modestly affected in Ung−/− mice, showing only a 65–95% increase in the proportion of transitions at C:G, but a normal proportion of all other types of mutations (9). This suggests that affinity maturation would be normal in these mice, although it could conceivably be influenced by secondary defects resulting from the loss of UNG in B cells. Again, this has never been tested experimentally.

In this study, we have analyzed chronic and acute Ab responses in Ung−/− mice, which show that they are very inefficient in eliciting switched Ab in response to immunization and viral infection. In contrast, Ung−/− mice can quite efficiently produce switched Ab against chronically available Ag, as evidenced by the levels of circulating Ig subclasses and gut-associated IgA. Our results therefore suggest a previously unexplored differential importance for UNG in CSR in vivo, depending on the type of Ag exposure. Because we show that Ung−/− mice are capable of normal affinity maturation, these mice could be used as separation of function models for isotype switching and affinity maturation in the case of acute Ag challenges. Finally, we show that Ung−/− mice have circulating auto-antibodies, revealing a novel additional phenotype of these mice.

Materials and Methods

Mice

All mice were in C57BL6/J background and housed at the specific-pathogen-free facility at Institut de Recherches Cliniques de Montréal (IRCM). Ung−/− mice (22) were a kind gift from Dr. H. Krokan (Norwegian University of Science and Technology, Trondheim, Norway). Aid−/− mice (2) were a kind gift from Dr. T. Honjo (Kyoto University, Japan), and AID-GFP reporter mice (23) were a kind gift from Dr. R. Casellas (National Institutes of Health). All animal procedures were reviewed and approved by the IRCM animal protection committee following Canadian Animal Care guidelines.

Immunizations

Mice of 8–10 wk of age were immunized i.p. with NP15-CGG precipitated in alum and boosted at day 30 postimmunization as previously described (24). Blood samples were collected at day −1 (preimmune), day 11 postimmunization (primary response), and day 37 (secondary response). Immunizations with KLH were done i.p. with 100 μg KLH (Sigma) mixed with 1:1 (v/v) with Inject Alum (Thermo Scientific) and boosted at day 46 with 50 μg of the same mix. Blood samples were collected at days −1 (preimmune), 7, 14, 22, 30, and 42 (primary response), and 53, 60, 67, 77, 91, and 147 (secondary response) postimmunization.

Ig level determinations

Anti-isotype–specific Ab (BD Pharmingen or Southern Biotech) were used to capture and detect total serum IgM, IgG1, IgG3, IgG2b, and IgA, which were quantified by sandwich ELISA using biotinylated Ab followed by HRP-conjugated streptavidin (1:5000; Thermo Scientific) and developed using 2.2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate for detection as described previously (24). Standard curves for each Ig isotype (BD Pharmingen) were used to calculate serum Ig concentrations. Levels of total or high affinity anti-NP–specific Abs were determined in serum by ELISA, as described previously (24). ODs of preimmune serum wells were subtracted from the OD of the test wells. Arbitrary unit titers were calculated by multiplying the OD values of a point within the linear range of a serial 2-fold serum dilutions curve made for each mouse, by its corresponding dilution factor. OD 50% was estimated graphically using Prism 5.0 (GraphPad). Serum samples from mice were analyzed as described above by ELISA, as described previously, using plates coated with KLH (100 ng/ well). For measuring fecal IgA concentrations, fecal supernatants were prepared from freshly collected fecal pellets as described previously (25) and determined by ELISA against a standard curve. Self-reactive Abs in serum were measured by ELISA using plates coated with 100 ng/well whole-mouse NIH-3T3 fibroblast extract made in radioimmunoprecipitation assay buffer and detected using biotinylated anti-mouse Ig L chain + IgA1/2 and IgA3 L chain (1:1000; BD Pharmingen).

Ab affinity assays

Sodium thiocyanate (NaSCN) displacement ELISA was performed following published protocols (26, 27) with minor modifications. Serum samples were diluted to similar, linear range concentrations (i.e., the dilution of the serum giving an OD between 0.8 and 1 in the standard NP26-BSA or KLH ELISA) and bound to NP25-BSA– or KLH-coated plates. After extensive washing, bound Abs were treated with a range of increasing concentrations of freshly prepared NaSCN (Sigma) (0, 0.05, 0.1, 0.25, 0.5, 1, 1.25, 2, 2.5, 3, 3.5, 4, and 5 M) for 10 min at room temperature (RT). Plates were washed, blocked with 0.1% gelatin, and processed following the standard ELISA protocol. The ODs in the NaSCN-treated wells were expressed as a percentage of the OD of the untreated well (= 100%). Relative affinity values (RAV) were calculated as described previously (26). In brief, the difference between the untreated well and the well treated with 0.05 M NaSCN was multiplied by 0.05; the difference between this well and the well treated with 0.1 M NaSCN was multiplied by 0.1, and so on until the remaining fraction of Abs after 5 M NaSCN washing, which was multiplied by 6. The sum of all these products is the RAV and provides a quantitative measure of the overall affinity of the response for the Ag considered.

Neutralization assay

Quantitation of vesicular stomatitis virus (VSV)–neutralizing Abs was performed as described previously (28). In brief, 4 mice per group were i.v. infected with 2 × 106 PFU VSV Indiana, and blood samples were collected at different time points postinfection. Serially diluted sera were mixed with 50 PFU VSV and transferred on Vero monolayers to form plaques. The highest dilution of serum that reduced the plaques by 50% was taken as titer.

B cell purification and in vitro CSR

Splenic resting B cells were isolated as previously described (29) and cultured at 0.5 × 106 cells/ml for 3–4 d in RPMI 1640 medium (Wisent, Saint-Bruno, QC, Canada) supplemented with 50 μM 2-ME and 10% FBS (Wisent) in the presence of cytokines. Saturating switching conditions used 25 μg/ml LPS (Sigma-Aldrich) and 50 ng/ml marine IL-4 (PeproTech) for IgG1, and 5 μg/ml LPS and 0.5 ng/ml recombinant human TGF-β1 (R&D Systems) for IgG2b. Nonlimiting conditions were 5 μg/ml LPS and 5 ng/ml IL-4 for 1.5 × 105 cells/ml for IgG1, 1 μg/ml LPS and 0.05 ng/ml TGF-β1 for 2.5 × 105 cells/ml for IgG2b, and 5 μg/ml LPS and 3 ng/ml mouse anti-IgD-dextran (Fina BioSolutions) for 1.5 × 105 cells/ml for IgG3. Surface Ig was determined by flow cytometry as described previously (29) using a FACS Calibur apparatus (BD Biosciences) and analyzed using FlowJo (Tree Star). Retroviral delivery into mouse primary B cells has been described previously (29).
VH analysis

IgM* AID* germlinal center B cells were identified by staining splenocytes from NP-CGG immunized mice at 7 d postimmunization (d.p.i.) with anti-IgM-PE (BD Pharmingen) and GFP expression, and FACS-sorted into RNA extraction buffers. RNA was extracted using RNeasy mini kit (Qiagen) and cDNA produced using Protoscript kit (Biolas). The suitability of AID-GFP expression in these mice to identify germinatal center B cells has been established (23). PCRs were performed on cDNA using oligonucleotides OJ794 5'-CTTCTTCTGGACAGCAACAG-3' , priming at the leader region of VH186.2 and OJ795 5'-CCAATCTTTTACAGACAGG-3' priming at the IgM Cα1 for 35 cycles of 95°C 20 s + 55°C 10 s + 70°C 9 s using KOD DNA polymerase. Products were cloned into pGEM-T Easy (Promega) and sequenced at Macrogen (Seoul, Korea). Sequences were analyzed using Sequencer, and the V, D, and J usage and mutations were scored by comparing each sequence with the germline sequences at the IMGT server (http://www.imgt.org). The JH and DH gene fragment names provided are according to the IMGT data.

Leukocyte population analysis

Splenocytes were harvested and stained through a 70-μm cell strainer. For dendritic cell extraction, the mice were previously extracted by collagenase treatment. Single-cell splenocyte suspensions in RPMI 1640 10% FBS were centrifugated at 400 × g for 10 min at RT, resuspended in 1 ml erythrocytes lysis buffer (Sigma) for 5 min at RT, washed, and resuspended in PBS BSA 1%. Aliquots of 5 × 10⁷ cells were stained for 15 min using combinations of the following Abs: anti-B220 Biotin, anti-CD21 PerCP, anti-CD23 PE (all BD Pharmingen), anti-IgM PE, anti-IgD FITC (1:100), anti-CD3 PE (1:50), and PNA FITC (1:200), mounted overnight in Mounting medium (Invitrogen). Dead cells were excluded by staining with 10 μg/ml propidium iodide. Data were acquired in a FACSCalibur (BD Biosciences) and analyzed using FlowJo (Tree Star). Cellularity was estimated from the total lymphocyte count after extraction, and the proportion of the different lymphocyte subpopulations was determined as described earlier. Heparinized whole blood from 1- to 3-month-old mice was analyzed for cellular and biochemical parameters using an Advia 120 hematology system (Siemens).

Histology and immunohistochemistry

Tissues were fixed with 4% formaldehyde overnight at 4°C and embedded in paraffin. Sections were stained with H&E. IHC was performed on 5-μm sections of paraffin-embedded tissues, deparaffinized, rehydrated, and subjected to Ag retrieval. After blocking endogenous peroxidase activity with 3% hydrogen peroxide, sections were incubated overnight with rat anti-IgM-Alexa 488, rat anti-CD21 FITC, rat anti-CD23 PE (all BD Pharmingen 1:200). After blocking endogenous peroxidase activity with 3% H₂O₂, sections were incubated overnight with rat anti-CD8 allophycocyanin (all BD Pharmingen), anti-CD21 FITC, anti-CD23 PE (all BD Pharmingen). Single-cell splenocyte suspensions in RPMI 10% FBS were resuspended in PBS BSA 1%. Aliquots of 5 × 10⁷ cells were stained for 15 min at RT, washed, and resuspended in PBS BSA 1%. Aliquots of 5 × 10⁷ cells were stained for 15 min using combinations of the following Abs: anti-B220 Biotin, anti-CD21 PerCP, anti-CD23 PE (all BD Pharmingen), anti-IgM PE, anti-IgD FITC (1:100), anti-CD3 PE (1:50), and PNA FITC (1:200), mounted overnight in Mounting medium (Invitrogen). Dead cells were excluded by staining with 10 μg/ml propidium iodide. Data were acquired in a FACSCalibur (BD Biosciences) and analyzed using FlowJo (Tree Star). Cellularity was estimated from the total lymphocyte count after extraction, and the proportion of the different lymphocyte subpopulations was determined as described earlier. Heparinized whole blood from 1- to 3-month-old mice was analyzed for cellular and biochemical parameters using an Advia 120 hematology system (Siemens).

Statistical analysis

Unpaired two-tailed Student t test was used throughout unless indicated otherwise using Prism 6.0 (GraphPad Software). The p values <0.05 were considered significant.

Results

Intact affinity maturation of IgG1 in UNG-deficient mice

The initial impetus of this work was to experimentally determine to what extent UNG deficiency could provide separation of function between affinity maturation and isotype switching in vivo. We first analyzed affinity maturation of the Ab response against the hapten NP after immunization with NP-CGG, which is dominated by IgG1. Although anti-NP IgG1 titers were severely reduced in Ung⁻/⁻ mice compared with wt (see later), the immunization protocol still elicited sufficient NP-specific IgG1 to measure their affinity. We analyzed affinity maturation by using the ELISA-based NaSCN washing method (27) (see Materials and Methods). This detailed analysis showed that the overall profile of the anti-NP IgG1 affinities produced by UNG-deficient mice was comparable with and showed a shift toward higher affinity Ab from the primary to the secondary response of similar magnitude than wt mice (Fig. 1B). There was a small reduction in the abundance of anti-NP IgG1 in the secondary response of Ung⁻/⁻ mice (Fig. 1B). This is very likely explained by the clonally restricted nature of the anti-NP response, which uses VH186.2 and Vα1 (30), plus the fact that high affinity for NP is achieved by the hallmark W33L substitution in VH186.2 (31). Changing Trp33, encoded by TGG in VH186.2, to Leu can be achieved by a single-point mutation to TGG or else it needs two independent mutations. TGG to TTG is a transversion mutation at a C-G pair, which is the only type of mutation specifically and significantly affected by UNG deficiency (7, 9) (see Supplemental Fig. 1). Nevertheless, this minor difference did not reduce the overall affinity of the response. This was demonstrated by calculating the RAVs (see Materials and Methods), which, on average, were not different between Ung⁻/⁻ and wt mice (Fig. 1C).

To exclude that there was a small general defect in affinity maturation in Ung⁻/⁻ mice, we repeated the analysis of affinity maturation by immunizing the mice with the more complex Ag KHL. This large and heterogeneous Ag elicits a complex Ab response that allows measuring the affinity of a mixture of specificities against a much larger number of epitopes, which unlike NP cannot be biased by the requirement for one specific mutation. Again, in this case, the predominant response was IgG1 (data not shown), and the Ab affinity profile of the primary and secondary responses was identical between Ung⁻/⁻ and wt mice (Fig. 1B). That the overall affinity of the response was also the same in Ung⁻/⁻ and wt was reflected by their similar average RAV (Fig. 1C). We conclude that affinity maturation of the IgG1 response is unaffected by UNG deficiency.

Improved affinity maturation of IgM in UNG-deficient mice

We then asked whether IgM could undergo affinity maturation in Ung⁻/⁻ mice. This was the case, as secondary Ab responses in Ung⁻/⁻ mice contained significantly more high-affinity Ag-specific IgM than wt controls after NP and KLH immunizations (Fig. 2A). The RAV values confirmed the higher overall affinity of anti-NP and anti-KHL IgM in Ung⁻/⁻ (Fig. 2B). This is most likely due to the impairment in CSR caused by UNG deficiency (see later), leading to persistence of IgM⁺ cells in the germinal center reaction to accumulate SHM, which can be selected before isotype switching has occurred. We could confirm that the spleen of Ung⁻/⁻ mice contained more IgM⁺ AID* cells after immunization than the controls by using an AID-GFP reporter mouse that allows identifying AID⁺ germinal center cells in response to immunization (23). Indeed, the ratio of IgM⁺ to IgM⁻ mice was reflected by their similar average RAV (Fig. 1C). We conclude that affinity maturation of the IgG1 response is unaffected by UNG deficiency.
detecting polyclonal responses but, as they shared VH186.2, it allowed comparing the mutation load of similar sequences without the strong bias that sorting for NP binders would have introduced through selection of high-affinity clones. Sequence analysis demonstrated that IgM+ B cells from \textit{Ung}^{2/2} mice accumulated mutations to at least the same, and perhaps higher, levels than the same population from control mice, and that the mutations were similarly distributed over the VH186.2 (Fig. 2E, 2F). We conclude that \textit{Ung}^{2/2} mice maturate the affinity of Ag-specific IgM responses above the usual level achieved by wt mice, through the accumulation of SHM at the VH of IgM+ B cells.

Basal IgG serum levels in \textit{Ung}^{2/2} mice are not indicative of their ability to respond to acute immunization

To determine whether isotype switching in vivo behaved as predicted by the importance of UNG in in vitro CSR assays (9, 10), we analyzed the levels of preimmune Ig subclasses compared with Ag-specific Ab titers after immunizations in the same mice. The preimmune serum of \textit{Ung}^{2/2} mice showed the expected hyper-IgM phenotype (9, 10, 19), with 2.6-fold higher circulating IgM than wt littermate controls, but variable effects on circulating switched isotypes. Compared with wt littermates, IgG1 levels were normal in \textit{Ung}^{2/2} mice, IgG2b and IgA were only ∼3-fold lower, whereas IgG3 was reduced 10-fold (Fig. 3A). These differences between Ig subclasses could not be explained by differences in maternal Ig as older mice showed similar differences (9, 10, and data not shown), nor by the half-life of the isotypes, which are all similar (6–8 d for IgG1 and IgG3, 4–6 d for IgG2b) (32). They do not reflect either isotype-specific differences in the intrinsic efficiency with which the MSH2/MSH6 pathway triggers CSR, because in vitro CSR assays using purified \textit{Ung}^{2/2} B cells show that switching to all isotypes are similarly and drastically impaired, as previously reported for IgG1 and IgG3 (9, 10), and shown in this article for IgG2b stimulated in vitro (Fig. 3B). Thus, the requirement for UNG in catalyzing CSR to generate circulating switched Ig classes is variable depending on the isotype. We then compared this with the requirement for UNG in catalyzing CSR in the same mice in response to acute immunizations. To this end, we immunized mice with NP-CGG and analyzed anti-NP titers at the primary and secondary responses. Anti-NP IgG3 was reduced 10-fold in \textit{Ung}^{2/2} mice (Fig. 3C), consistent with the reduction in basal IgG3 accumulation in serum. However,
anti-NP responses are dominated by IgG1, an isotype present in normal levels in the Ung−/− mice preimmune sera. Despite this, the elicitation of total and high-affinity anti-NP–specific IgG1 was reduced by ∼15-fold in the primary and secondary responses (Fig. 3D). This decrease was compensated for in Ung−/− mice by an increase in anti-NP–specific IgM at all times (Fig. 3E). Interestingly, despite UNG haploinsufficient mice showing no defect in serum Ig accumulation or anti-NP IgG1 production (Fig. 3A, 3D), they still had increased anti-NP IgM titers in the primary response (Fig. 3E). The latter result and the altered ratio of IgM+ germinal center B cells (Fig. 2C) suggested that slower switching kinetics in vivo might be behind the impaired response to immunization in Ung−/− mice. To test this, we assayed the primary and secondary IgG1 response to KLH at various time points. Anti-KLH IgG1 Ab titers increased with slower kinetics in Ung−/− than in wt mice (Fig. 3F). Nonetheless, anti-KLH IgG1 levels never reached those of wt controls, even after booster immunization (Fig. 3F). We conclude that UNG plays a fundamental role in allowing efficient isotype switching in response to acute immunization, even for isotypes like IgG1, which the same mice produce as natural Abs to normal levels. The defect is due to both delayed switching and overall reduced efficiency.

**FIGURE 2.** Affinity maturation and SHM of IgM in Ung−/− mice. (A) Affinity of the NP-specific or KLH-specific IgM secondary response was measured by NaSCN displacement affinity ELISA in Ung−/− versus wt control littersmates, and the eluted and bound fraction of Ag-specific IgM are plotted for each as in Fig. 1B. Statistically significant differences are indicated by an asterisk above the corresponding data point (p < 0.05, unpaired two-tailed Student t test). (B) RAVs of the overall IgM secondary response against NP and KLH were calculated for individual mice and plotted. Means, indicated by horizontal lines, are significantly different by unpaired two-tailed Student t test at p < 0.05. (C) Flow cytometry profile of splenic leukocytes from NP-CGG–immunized AID-GFP control or AID-GFP Ung−/− mice, stained with anti–IgM-PE at 7 d.p.i. The GFP signal identifies germinal center AID+ cells (23). The ratio of IgM+ to IgM− cells for this population is indicated below each plot. (D) Scheme of the PCR performed on cDNA obtained from sorted IgM+ AID+ B cells as in (C) to amplify VH186.2 IgM. The number in the center of the pie charts (each summarizing data from two mice) indicates the total number of sequences analyzed. Each slice represents the proportion of sequences using each indicated JH or DH fragment. (E) The number of mutations found in each individual VH186.2 IgM sequence (excluding the VDJ junction) is plotted for each mouse analyzed. (F) The distribution and frequency per position of all mutations scored in (E) are plotted along the VH186.2 region analyzed for AID-GFP control (top) and AID-GFP Ung−/− mice (bottom).
FIGURE 3. Normal serum levels but impaired Ag-specific IgG1 production in \textit{Ung}^{-/-} mice. (A) Serum Ig concentrations in 8- to 10-wk-old unimmunized \textit{Ung}^{-/-}, \textit{Ung}^{+/+}, and wt (Ctrl) littermates were determined by isotype-specific ELISA. As in all subsequent panels, each symbol represents an individual mouse and horizontal lines indicate median values. Ig concentrations of age-matched \textit{Ad}^{-/-} mice were included as background control. The \(p\) values were calculated by unpaired \(t\) test. (B) Naive resting B cells from \textit{Ung}^{-/-} and wt age-matched mice (3–6 mo old) were cultured with LPS+IL-4 or LPS+TGF-\(\beta\)1 for 4 d to induce switching to IgG1 or IgG2\(b\), respectively. Representative flow cytometry profiles are shown next to the mean proportion \(6\) SEM for two mice. (C) NP-specific IgG3 titers in serum of 8- to 10-wk-old \textit{Ung}^{-/-} and littermate control mice immunized with NP15-CGG and boosted at 30 d.p.i. were determined by ELISA at day 37 d.p.i (secondary [2\textsuperscript{ry}] response) by using plates coated with NP26-BSA or NP4-BSA to capture total or high-affinity Ab, respectively. Each symbol represents an individual mouse and means are indicated. (D) Total and high-affinity serum NP-specific IgG1 titers were determined by ELISA in \textit{Ung}^{-/-} and littermate controls immunized as in (C), at the primary (1\textsuperscript{ry}; 1 d.p.i.) and 2\textsuperscript{ry} (37 d.p.i.) responses. Littermate or age-matched \textit{Ung}^{+/+} and \textit{Ad}^{-/-} mice were included where indicated. (E) Total and high-affinity NP-specific IgM measured as described earlier for the same mice as in (D). (F) \textit{Ung}^{-/-} and wt littermate mice were immunized with 100 \(\mu\)g KLH, and serum samples were collected at 7, 14, 22, 30, and 42 d.p.i. (1\textsuperscript{ry} response). Mice were boosted with 50 \(\mu\)g KLH at 46 d.p.i., and sera were prepared at 53, 60, 67, 77, 91, and 147 d.p.i. (2\textsuperscript{ry} response). KLH-specific IgG1 was measured by ELISA, and the mean concentration \(6\) SD from three mice per genotype is plotted at each time point. Values for \textit{Ung}^{-/-} were significantly reduced compared with wt at each time point except at 147 d.p.i (\(p < 0.01\), unpaired two-tailed \(t\) test).
very significantly reduced considering that the NP and KLH immunization protocols use saturating amounts of Ag, which probably dissipates larger defects. To measure the ability of Ung⁻/⁻ mice to elicit isotype switching in vivo in a more physiologically relevant situation, we used VSV infection, a classical model for analyzing Ab responses to acute viral infection (33). Because SHM is not required for VSV neutralization (34, 35), the accumulation of neutralizing IgG in the serum of infected mice over time is a good indicator for CSR ability in vivo that is not conditioned by the contribution of affinity maturation. VSV-infected Ung⁻/⁻ mice showed dramatically impaired production of anti-VSV neutralizing IgG, as measured by their capacity to inhibit cell lysis in monolayers. The neutralizing IgG titer of Ung⁻/⁻ mice was 96-fold lower than wt mice (Fig. 4). The total neutralizing IgG titer was not different from wt mice because Ung-deficient mice compensated the reduction in IgG with IgM. This was confirmed by the fact that the AID-deficient mice were able to achieve similar levels of VSV-neutralizing Ab with IgM only, confirming the fact that SHM is dispensable (Fig. 4). We did not find any significant reduction in splenic NK cells in Ung⁻/⁻ mice that might have contributed to this difference (Supplemental Fig. 2). We conclude that Ung⁻/⁻ B cells are very inefficient in producing switched Abs upon acute antigenic challenge.

Moderately reduced gut-associated IgA in Ung⁻/⁻ mice

Contrasting with the strong impairment in isotype switching after immunization or infection, preimmune switched Ig was not drastically reduced in Ung⁻/⁻ mice sera (except for IgG3). Because preimmune Ig most likely results from continual exposure to multiple environmental Ags, we extended the characterization of Ab responses to chronic antigenic exposure in Ung⁻/⁻ mice. To this end, we analyzed the production of gut-associated IgA, which is constantly produced in response to stimulation by the gut flora. We controlled for interindividual and environmental variations by comparing fecal IgA between littermates housed within the same cage. Ung⁻/⁻ mice 4–14 mo old showed only a modest ~2-fold decrease in fecal IgA compared with wt littermates, with all Ung⁻/⁻ mice having significantly more IgA than age-matched, AID-deficient mice (Fig. 5A). The number of IgA⁺ cells in Peyer’s patches from Ung-deficient mice was reduced by 3.7-fold compared with wt (Fig. 5B), but there were no changes in the number or cellularity of Peyer’s patches (Fig. 5C), suggesting that the defect was most likely due to decreased efficiency of CSR in situ (36). In any case, similar to what is observed for basal serum IgG1 and IgG2b accumulation, there is only a moderate effect on the chronic accumulation of gut-associated IgA. We conclude that UNG plays a much less important role in producing switched Ig in response to chronic than acute antigenic stimuli.

UNG is not limiting for CSR

The persistence of higher than normal anti-NP IgM levels in Ung haploinsufficient mice despite producing normal anti-NP IgG1 levels (Fig. 3E) also raised the question of whether UNG could be limiting for CSR. AID haploinsufficient mice show reduced preimmune serum Ig accumulation (37, 38). However, Ung⁻/⁻ mice did not show any differences compared with wt for any Ig subclass (Fig. 3A). Moreover, the efficiency of Ung⁻/⁻ B cells to switch to IgG1, IgG2b, and IgG3 in vitro was identical to wt, in contrast with the ~50% reduction shown by AID haploinsufficient B cells we used as controls (Fig. 6A). Finally, overexpressing AID in AID⁻/⁻ Ung⁻/⁻ B cells only produced CSR levels to IgG1 that were ~70% of those achieved by the same strategy in AID⁻/⁻ B cells (Fig. 6B), despite AID levels achieved being ~3-fold above the normal endogenous AID (Fig. 6C). We know that in these conditions, AID is not limiting for switching to IgG1 even at endogenous levels (data not shown). Our results in vitro and in vivo indicate that UNG is not rate limiting for CSR. Interestingly, published experiments using the same retroviral delivery strategy and assay as us showed that overexpressed AID in AID⁻/⁻ Msh2⁻/⁻ B cells produced IgG1 CSR levels that were ~50% of those it produced in AID⁻/⁻ B cells (39), raising the possibility that MSH2/MSH6 could be rate limiting.

Normal lymphocyte populations and germinal center architecture in Ung⁻/⁻ mice

To rule out defects in the Ung⁻/⁻ mice lymphoid tissues that might contribute to the phenotypes we observed, we analyzed lymphocyte populations and tissue architecture of 8- to 10-wk-old Ung⁻/⁻ mice. Nonimmunized and immunized mice (i.e., after acute antigenic stimulus) showed similar results. Ung⁻/⁻ mice showed a trend for having slightly larger spleen (Fig. 7A). However, there were no statistically significant differences versus the controls in either their proportion or absolute cell numbers for any splenic or thymic lymphocyte subset (Fig. 7B–F, and data not shown). Importantly, histological analysis of spleens and germinal center architecture from naive or immunized mice showed no differences between Ung⁻/⁻ and wt mice (Fig. 7G). Interestingly, Ung⁻/⁻ mice did not display the large germinal centers characteristic of AID-deficient mice (2, 3) (Fig. 7G, also visible from the proportion of AID⁺ cells after immunization in Fig. 2C), which is
consistent with the phenotype of UNG-deficient patients, who have lymphadenopathies but not giant germinal centers (19, 20). The major populations of dendritic cells, monocytes, and granulocytes were also found to be within normal ranges (Fig. 7H, Supplemental Fig. 3). As previously reported (40), we could find follicular hyperplasia in some older Ung−/− mice (>1 y), although their lymphocyte populations, as well as cellular and biochemical blood parameters, were otherwise normal (Supplemental Fig. 3). We conclude that the differences observed in isotype switching cannot be explained by any major defects in the lymphoid organ composition or germinal center organization in the Ung−/− mice.

**Increased levels of circulating autoantibodies in Ung−/− mice**

AID deficiency is associated with autoimmunity (41–43), and one of the UNG-deficient patients experienced development of Sjögren’s disease (20, 21), a disease characterized by the presence of autoantibodies. We therefore asked whether Ung−/− mice would show autoimmune manifestations. We tested for the presence of self-reactive Abs in Ung−/− mice by measuring the levels of circulating total Ig that reacted against mouse Ag. We found that Ung−/− mice had increased levels of autoantibodies compared with littermate wt controls (Fig. 8A). Splenic Treg populations were similar in the Ung−/− and control mice (Fig. 8B). The levels of autoantibodies in Ung−/− mice were similar to those detected in age- and sex-matched Aid−/− mice, recently described to have a defect in B cell tolerance (44). This novel phenotype of Ung−/− mice suggests some role of UNG in preventing autoimmunity.

**Discussion**

The conversion of AID-generated uracils into DNA double-strand breaks, which is necessary for CSR, requires the recognition of the
uracil by either UNG or MSH2/MSH6, which therefore provide alternative pathways for CSR (10, 11). The relative intrinsic efficiency of each pathway has been well established in vitro, with UNG being quantitatively more important than MSH2/MSH6 for CSR ex vivo (9–15). However, extrapolating this result to in vivo might not be straightforward, as exemplified by the Msh2−/− mice.

**FIGURE 7.** Normal splenic leukocyte populations in Ung−/− mice. (A) Spleens were collected from immunized mice and weighted. As in subsequent panels, each symbol represents an individual mouse, and horizontal lines indicate median values. (B) Proportion of splenic B (CD3− B220+) and T (CD3+ B220−) cells were calculated from flow cytometry profiles of nonimmunized mice (two pairs) and NP-CGG–immunized mice euthanized after primary (four pairs) or secondary (three pairs) immunization. (C) Absolute numbers of splenocytes, B cells, and T cells were calculated for immunized mice euthanized after primary (two pairs) or secondary (three pairs) immunization. (D) Proportion of marginal zone (MZ; CD21+ CD23−), follicular (Fo; CD21+ CD23+), newly formed (NF; CD21− CD23−), mature (IgMhigh IgDhigh) B cells were calculated from flow cytometry profiles of B220+ splenic cells stained with a combination of either anti-CD21FITC and anti-CD23PE or anti-IgDFITC and anti-IgMPE for the same mice as in (B). (E) Proportion of CD4+ and CD8+ splenic T cells calculated from flow cytometry profiles of two pairs of nonimmunized and two pairs of immunized mice euthanized 11 d.p.i. (F) Absolute number of splenic B cell subsets calculated for immunized mice euthanized after the primary (two pairs) or secondary (three pairs) responses. (G) Representative histological analysis of splenic follicles and germinal centers. Spleens were collected at day 11 postimmunization from 8-wk-old mice, and paraffin-embedded sections were stained with H&E by IHC using rat anti-B220 followed by anti-rat IgGHRP (B220) or cryosections stained with PNAFITC, anti-CD3PE, and anti-B220allophycocyanin (PNA CD3 B220). Original magnification ×2.5 (left, middle), ×20 (right). (H) Proportion of splenic dendritic cells (DC) defined as MHC II+ CD11c+ and monocytes defined as Ly6G− Ly6C+ CD11b− (see Supplemental Fig. 2) by flow cytometry from four pairs of immunized mice at 8 d.p.i. There were no statistically significant differences by unpaired two-tailed Student t test in this figure.
Ab RESPONSES IN THE ABSENCE OF UNG

(12, 14–17). The more severe than expected Ab diversification phenotype in Msh2−/− mice is likely related to MSH2 roles in cell-cycle checkpoints that presumably affect the viability of germinal center B cells in vivo (15, 17), which does not seem to be an issue with UNG as previous results (9, 22) and our results indicate. Still, the presumably strong CSR deficiency of Ung−/− mice in vivo was not obvious from the available data. The hyper-IgM, low IgG levels and susceptibility to infections of the three human patients identified with UNG deficiency (19, 20) seemed to suggest this. In contrast, UNG-deficient mice showed very low serum IgG3 but normal levels of IgG1 and only modestly reduced IgA (9, 10). We confirmed this and additionally found only <3-fold reduction for IgG2b. One possible explanation for this apparent discrepancy between human and mouse is that the patients’ phenotype reflected the sampling bias inherent to clinical identification, as the very reduced number of patients may suggest (see later). Alternatively, there could be a difference in the mechanism between the species (19). For instance, the uracil DNA glycosylase SMUG1 is more abundant in mice than in humans (45), and could partly compensate for UNG in producing serum Ig. However, given SMUG1’s inability to potentiate CSR in Ung−/− Msh2−/− or Ung−/− Msh6−/− mice (10, 11), this seems unlikely.

Alternatively, MSH2/MSH6 could have a more prominent backup role for CSR in mice than in humans. However, we find severe defects in isotype switching in vivo in Ung−/− mice, indicating that MSH2/MSH6 is not such an efficient backup, although this depends on the type of antigenic exposure.

Previous work comparing the UNG and MSH2/MSH6 pathways for CSR in vivo has not analyzed immunization in detail or infection (10, 11). In this article, we document a striking difference in the ability to produce preimmune and Ag-specific IgG1 in Ung−/− mice. Despite having normal preimmune circulating IgG1, Ung−/− mice display an ∼15-fold defect in producing Ag-specific IgG1 in response to immunizations. Because we used saturating amounts of Ag and adjuvant, this most likely underestimates what happens when Ag is limiting, as would be the case with natural infections. Indeed, the ability of VSV-infected Ung−/− mice to produce neutralizing anti-VSV IgG is reduced by 100-fold compared with wt mice. So, why is it that Ung−/− mice produce normal or substantial levels of preimmune IgG1, IgG2b, and IgA? “Natural” switched Ig must be generated in response to environmental Ab and nonpathogenic microbe to which these mice are chronically exposed in our specific pathogen-free facility. In this situation, even infrequent CSR events could be selected by persistent Ag availability, further facilitated by the fact that the switched B cells are able to undergo normal affinity maturation, as we show. This could result from the generation of long-lived plasma cells and homeostatic compensation of serum switched Ig levels (46). This seems to work best for IgG1 and fairly well for IgG2b and IgA, but not at all for IgG3. The reason behind these differences is unclear, but the in vitro CSR assays exclude the possibility of intrinsic differences in the efficiency of the mechanism, so it is probably related to the nature of the Ag and B cell subsets involved in each response (46). In any case, supporting the notion that Ung−/− mice produce fairly normal levels of switched Ig when Ag is chronically available, we find that fecal IgA, which is elicited in response to commensal gut flora, is only modestly diminished. We did not directly address the pathway mediating the substantial production of switched Ab against chronic Ag in Ung−/− mice. However, because the combined absence of UNG and MSH2 or MSH6 leads to the virtual absence of any other Ab isotypes than IgM in circulation and complete lack of in vitro CSR (10, 11), isotype switching in Ung−/− mice most likely proceeds via MSH2/MSH6. This suggests that MSH2/MSH6 is not sufficient to allow an efficient Ab response upon acute antigenic challenge. In this situation, Ung seems to have a critical role in ensuring the appropriate kinetics of CSR in vivo. Supporting this inference, we observe reduced kinetics in the response to KLH (Fig. 3F) and a drastic alteration of the IgM+ to IgM− switch in Ung−/− mice (Fig. 2C). Thus, our results reveal a critical need for UNG for a timely and efficient T cell–dependent switched Ab response to intentional immunization and infection, and a less important role in the buildup of natural Ab levels elicited by chronic Ag exposure.

SHM and CSR are identically initiated by AID-mediated DNA deamination (2, 3) and therefore difficult to separate. The only model achieving this used a knock-in approach to introduce a single amino acid change in Aicda. This AIDG23S shows near to normal CSR ability in vitro (47) and allows for normal levels of circulating Ig isotypes and gut-associated IgA (48). In contrast, AIDG23S results in very reduced SHM, which allowed us to demonstrate that the lack of SHM critically compromised the immune control of commensal bacteria in the gut (48). The Ung−/− mouse phenotype we report, showing a differential need for UNG depending on the type of antigenic challenge, raises the possibility that AIDG23S might not provide such a clear separation between

![FIGURE 8. Increased serum autoantibodies in Ung−/− mice.](http://www.jimmunol.org/)

(A) Total Abs reacting against whole-cell extracts of murine NIH-3T3 fibroblasts in ELISA plates were detected by biotinylated anti-Igκ+κ followed by streptavidin-HRP. Mice were 2–18 mo old, either littermates or age- and sex-matched. A similar number of male and female pairs are included. Each symbol represents an individual mouse. Horizontal lines indicate median values. Age-matched Aid−/− mice were included as positive controls. (B) Same analysis as in (A) but plotting only littermate mice pairs (joined by lines). Data include three female and three male 2-mo-old pairs and two male pairs 12 mo old. (C) Proportion of Tregs among splenic T cells for four mice of each genotype, calculated by flow cytometry analysis of CD4+ CD3+ Foxp3+ cells, as shown on the left. The p values were calculated by unpaired (A, C) or paired (B) two-tailed t test.
SHM and CSR in the case of acute antigenic challenges. In fact, AIDG23S mice show an important defect (50–80% reduced) in the production of Ag-specific switched isotypes after acute immunization (48). A complementary model to AIDG23S, using AID specifically lacking CSR, may not be possible because these AID variants seem to have dominant negative effects (49). Ung−/− mice could provide such a model, as they display normal ability for affinity maturation as shown for the residual IgG1, but also for the most abundant Ag-specific IgM response. This model will nevertheless be limited to provide separation of function between isotype switching and affinity maturation when Ag is acutely provided and not saturating. This could still be useful in infection models as a way to disentangle the contributions of SHM and CSR to the response. For instance, AID-deficient mice can mount a neutralizing response against influenza but still show significant morbidity compared with wt mice (50). This implies that unmutated IgM is enough to neutralize the virus, as we observe for VSV, but SHM and/or CSR are important to reduce morbidity during influenza infection, which could be studied in the Ung−/− mice.

The paucity of UNG deficiency among hyper-IgM patients (~1%), compared with the much higher abundance of AID deficiency (~15%) (51), is puzzling. Both are autosomal recessive defects that affect similarly sized, nonessential genes (3, 19) for which one could expect similar frequency in the incidence of AICDA and UNG mutations in the human population. Thus, it is possible that the three UNG-deficient patients reported have unusually severe phenotypes, mimicking AID deficiency to an extent that made them clinically conspicuous. Indeed, the only difference between Ung-deficient mice and humans seems to be in the levels of switched Ig in serum, which could be explained by sampling bias. Our results would suggest that other UNG-deficient patients might have higher levels of some natural Ig classes. These switched Ig but also IgM could undergo normal affinity maturation and contribute to immunity by traditional means, but also by engaging intracellular receptors (52), which might be sufficient to make these patients appear fairly normal in the modern sanitary conditions.

Finally, we find that Ung−/− mice have circulating autoantibodies to the same extent as Aicda−/− mice. The molecular or cellular basis for this novel phenotype of UNG deficiency, and whether the C57BL/6 background has any incidence, is unknown. It is suggestive that the presence of circulating autoantibodies in AID-deficient mice and patients was recently found to be a consequence of a defect in B cell tolerance (43, 44). The mechanism by which AID contributes to B cell tolerance is unknown, but our results suggest the possibility that UNG can be part of this pathway and warrants further investigation.

Acknowledgments
We are grateful to Dr. H. Krokan and G. Slupphaug for providing the Ung−/− mice; Dr. R. Casellas for AID-GFP mice; Anne-Marie Patenaude for the AID knock out mice. Dr. R. Casellas for AID-GFP mice; Anne-Marie Patenaude for the We are grateful to Dr. H. Krokan and G. Slupphaug for providing the Ung−/− mice; Dr. R. Casellas for AID-GFP mice; Anne-Marie Patenaude for the

Disclosures
The authors have no financial conflicts of interest.

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