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Activation-Induced Cytidine Deaminase Expression and Activity in the Absence of Germinal Centers: Insights into Hyper-IgM Syndrome1

Masayuki Kuraoka,* Dongmei Liao,* Kaiyong Yang,* Sallie D. Allgood,† Marc C. Levesque,† Garnett Kelsoe,‡* and Yoshihiro Ueda*  

Somatic hypermutation normally occurs as a consequence of the expression of activation-induced cytidine deaminase (AID) by Ag-activated, mature B cells during T cell-dependent germinal center responses. Nonetheless, despite their inability to express CD154 and initiate GC responses, patients with type 1 hyper-IgM syndrome (HIGM1) support populations of IgM+IgD+CD27+ B cells that express mutated Ig genes. The origin of these mutated B cells is unknown; the IgM+IgD+CD27+ cells do not express AID and appear to acquire mutations independent of stringent selection by Ag. Here, we demonstrate that immature/transitional 1 B cells from the bone marrow of CD154-deficient mice express AID and acquire Ig mutations that lack the hallmarks of antigenic selection via BCR signaling. Comparable levels of AID expression was found in developmentally immature B cells recovered from murine fetal liver and from human immature/transitional 1 B cells recovered from umbilical cord blood. AID expression in human fetal liver was also robust, approaching that of human tonsil tissue and the human germinal center B cell line, Ramos. These observations led us to conclude that AID expression in developing human B cells is the origin of the mutated IgM+IgD+CD27+ B cells present in HIGM1 patients, and we propose that both mice and humans share a latent, AID-dependent pathway for the preimmune diversification of B lymphocytes that is more prominent in chicken, sheep, and rabbits. The Journal of Immunology, 2009, 183: 3237–3248.

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3 Abbreviations used in this paper: SHM, somatic hypermutation; AID, activation-induced cytidine deaminase; AMuLV, Abelson murine leukemia virus; BL/6, C57BL/6; BM, bone marrow; CGG, chicken γ-globulin; CSR, class-switch recombination; GC, germinal center; HIGM1, type-1 hyper IgM syndrome; im/T1, immature/transitional 1; MF, mature follicular; MZ, marginal zone; NIP, (4-hydroxy-5-iiodo-3-nitrophenyl)acetyl; NP, (3-hydroxy-4-nitrophenyl)acetyl; PP, Peyer’s patch; Tα, T cell-dependent; Tγ, T cell-independent; T2, transitional 2.

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general restriction of SHM to GC B cells and identification of CD27 as a marker of post-GC memory B lymphocytes (30, 31). In contrast, Weller et al. (28) have suggested that these mutated IgM* IgD* CD27* B cells are not memory cells but circulating precursors of marginal zone (MZ) B cells, noting that the mutated IgM* IgD* CD27* B cells from HIGM1 patients exhibit a highly diverse Ig repertoire and mutations that do not appear to be Ag-selected (34). Recent evidence indicates that SHM in HIGM1 patients arises independently of both Tg and T cell-independent (Tlo) humoral responses (34) and that mutated IgM* IgD* CD27* B cells may be recovered from human fetal spleen (14), observations that suggest the possibility of physiologic SHM during the development of human B-lineage cells.

Programmed AID expression during B cell development is found in a diverse collection of unrelated vertebrate species, and the primary repertoires of B cells in birds (35–37), rabbits (38, 39), and sheep (40) are significantly modified by AID-dependent SHM or gene conversion. Although the primary B cell repertoires of mice and humans are generated virtually wholly by combinatorial association, the low levels of AID observed in im/T1 B cells (10–12, 41) may represent a conserved developmental program that is active in some species (e.g., birds and sheep) and latent in others (mice and humans).

Here, we show that im/T1 B cells from the bone marrow (BM) of CD154+/− mice constitutively express AID and exhibit significant SHM in rearranged VH gene segments. These mutant B cells do not display evidence for Ag-driven clonal selection and are unlikely to represent GC B lymphocytes given the absence of serum Ab responses to Tg Ab and lack of splenic and Peyer’s patch (PP) GC in CD154+/− mice. Parallel studies of human im/T1 B cells from umbilical cord blood and fetal liver also demonstrated significant levels of AID expression at levels comparable to or above those of murine im/T1 B cells.

We propose that developmentally regulated AID expression in im/T1 B cells represents a phylogenetically conserved program for Ig diversification that is normally latent in mice and humans but may be revealed in the absence of CD154-dependent regulation.

Materials and Methods

Human cord blood samples

Fresh cord blood samples were harvested from healthy term placentas and were provided without identifiers for the quantification of human AID mRNA by quantitative RT-PCR. Informed consent for donation to a public cord blood bank was obtained from mothers as part of a protocol approved by the Duke University Institutional Review Board.

Mice

Female C57BL/6 (BL/6), congenic AID−/− (4), and CD154+/− (19) mice were bred and maintained under specific pathogen-free conditions at the Duke University Animal Care Facility. Mice used in experiments were 6 to 12 wk of age. All experiments involving animals were approved by the Duke University Institutional Animal Care and Use Committee.

Cell lines

The Abelson murine leukemia virus (AMuLV)-transformed cell lines SJLx86 (gift of Y. Zshuagh), C12, PD31 (42), K7G, 63-12 (43), and 220-19 (44) (gifts of M. Schlissel), or Ramos cell lines (45) (gifts of M. Schaff) were blindly subcloned; paired clones were subsequently used to quantify AID expression by quantitative RT-PCR.

Ag and immunizations

Mice were immunized i.p. with 100 μg of (3-hydroxy-5-iodo-3-nitrophenyl)acetyl (NP)-chicken γ-globulin (CGG) in alum. NP-CGG contained 10–15 μl of NP/mg CGG. Mice were killed 16 days after immunization, and BM and spleen were harvested for flow cytometry and/or histology.

Flow cytometry

Analyses of cell phenotype and cell sorting were performed by flow cytometry. Human B cells from umbilical cord blood were enriched by negative selection using the RosetteSep B cell enrichment reagent (StemCell Technologies) and labeled with fluorochrome-conjugated mAb specific for human cell surface molecules: FITC-, PE-, PerCP-, allophycocyanin-, and allophycocyanin-Cy7-. Pacific Blue-conjugated mAb specific for human IgM, IgD, CD3, CD16, CD235a, CD34, CD19, CD27 (BD Bioscience), and CD14 (Caltag Laboratories) were used to identify human T1, transitional 2 (T2), and mature follicular (MF) B cell compartments (46). Murine fetal liver samples were prepared from embryo on 16 days and 19 days of gestation. Murine pro-, im/T1, MF, MZ, and GC B cells from fetal liver, BM, PP, and spleen were identified and sorted as described (12). Labeled cells were analyzed/sorted in a FACSVerse with DiVa option (BD Bioscience) or FACSaria (BD Bioscience). Flow cytometric data were analyzed with FlowJo software (Tree Star).

Immunohistochemistry

Tissues from naive and immunized mice were embedded in TissueTek OCT compound (Sakura Finetek) and snap-frozen in 2-methylbutane cooled with liquid nitrogen. Frozen tissues were stored at −80°C. Serial 5-μm-thick cryosections were cut on a Leica CM3050 S cryostat (Leica Microsystems) and thaw-mounted onto SuperFrost Plus slides (Thermo Scientific). After air drying, sections were fixed with ice-cold acetone/methanol (1:1) at −20°C for 10 min and stored at −80°C until use. Frozen sections were rehydrated and then blocked with PBS containing 5% normal goat serum, 5% rat serum, and 100 μg/ml rat IgG (Pierce) before immuno- staining. Sections were stained with a combination of fluorochrome-conjugated mAb specific for mouse IgM, IgD, GL-7, TCRβ, and/or CD3δ in blocking buffer at 4°C overnight. After washing, stained sections were mounted in Fluoromount-G (SouthernBiotech) and examined with an Axiovert 200M microscope. Representative images were photographed and analyzed with AxioCam MRm and Axiovision AC 4.5 software (Zeiss).

ELISA

Serum Ab to (4-hydroxy-5-iodo-3-nitrophenyl)acetetyl (NP) from naive and immunized mice were determined by ELISA (6, 47). Briefly, 96-well plates were coated with 3 μg/ml NIP-, BSA, NIP25-BSA, or BSA in 0.1 M carbonate buffer (pH 9.0) at 4°C overnight and blocked with 1% BSA in PBS. After washing with PBS containing 0.1% Tween 20, serially diluted samples or mAb standards were added to each well and incubated at room temperature for 2 h. For the dissociation of pentameric IgM into monomers, we treated serum samples and mAb standards with 2-ME as described (6, 47). After washing, HRP-conjugated goat anti-mouse IgM or HRP-conjugated goat anti-mouse IgG (both from SouthernBiotec) was added and incubated at room temperature for 1 h. HRP activity was visualized using a TMB peroxidase kit (Bio-Rad Laboratories), and optical densities were determined at 450 nm. The NP/NIP mAb B1–8μ (48) and H33L-y1 (22) Ab were used as ELISA standards.

Amplification of VμDμJμ rearrangements

Genomic DNA was isolated by phenol-chloroform extraction from sorted B cell subsets from BL/6, CD154+/−, and AID−/− mice. Specific VμDJμ rearrangements were amplified by a nested PCR by Pfu polymerase (Stratagene) (49) and forward primers specific for the V3 subgroup of Vμ1 gene segments and a reverse primer specific for Jμ2 (1) (supplemental Table S1). After an initial 20 cycles of amplification, a second round of 20 cycles was performed using internal primers (1). Amplified VDJ products were ligated into PBS II SK+ plasmid (Stratagene) and cloned by bacterial transformation (1). Cloned VDJ inserts were sequenced in an Applied Biosystems automated DNA sequencer and analyzed by IMGT/V-QUEST (http://imgt.cines.fr) and NCBI BLAST search software. All sequence data are available at GenBank (www.ncbi.nlm.nih.gov/GenBank, accession nos. GQ162485–GQ162778).

RT-PCR

Expression of murine and human AID mRNA was quantified by quantitative RT-PCR (12). Briefly, total RNA was extracted from various B cell populations, subcloned Ramos cells, and AMuLV transfectants. Total RNA libraries of human adult tonsil (Biochain) and fetal liver (Biochain/Stratagene) were purchased; cDNA from mouse and human tissues was prepared by standard methods (12, 50). PCR primers used in this study are listed in supplemental Table S1. AID cDNA was amplified using a nested PCR amplification with primers specific for the AID open reading frame (ORF) and cloned by bacterial transformation. Cloned AID inserts were sequenced in an Applied Biosystems automated DNA sequencer and analyzed by IMGT/V-QUEST (http://imgt.cines.fr) and NCBI BLAST search software. All sequence data are available at GenBank (www.ncbi.nlm.nih.gov/GenBank, accession nos. GQ162485–GQ162778).

4 The online version of this article contains supplemental material.
PCR method (12). The relative expression levels of AID message to Igβ message were calculated by the comparative threshold cycle method (12).

Statistical analysis
Statistical significance of data was determined by Mann-Whitney’s U test (for mutation frequencies) or Student’s t test (for serum Ab titers and AID mRNA levels). A p value of <0.05 was defined as significant.

Results
No Ab or GC responses in immunized CD154−/− mice
To determine the capacity, if any, of CD154−/− mice to respond to Td Ag, we immunized CD154−/− mice and congeneric BL/6 controls with NP-CGG in alum (1, 49) and quantified serum IgM and IgG Ab (6, 47); total and high-affinity Ab were differentiated by binding to NIP5-BSA and NIP25-BSA (6, 47). Additionally, treatment of serum samples with 2-ME to dissociate IgM Ab (6, 47) allowed us to minimize low-affinity, avidity-dependent binding by IgM (6, 47).

The serum of naive BL/6 and CD154−/− mice contained undetectable levels of IgM reactive with NIP5, but ~300 μg/ml NIP25 binding IgM (Fig. 1A). This binding, however, was abolished completely by exposure to 2-ME (Fig. 1A), indicating avidity-dependent binding and demonstrating that neither naive knockout or control mice express detectable levels of NIP-specific serum IgM Ab. Low levels (20–30 μg/ml) of IgG reactive to NIP25 could not be detected in either group (Fig. 1B).

Immunization of BL/6 mice with NP-CGG/alum elicited significant IgM responses. At 16 days after immunization, serum IgM (1348 ± 426 μg/ml) reactive with NIP25 was increased ~4-fold over naive controls (p = 0.0031), and in contrast to naive mice,
>50% of this IgM Ab retained NIP 25-binding after reduction with 2-ME (Fig. 1A).

NP-CGG, however, did not induce Ag-specific IgM in CD154−/− mice. Although immunization resulted in a >3-fold increase (1063 ± 269 μg/ml) of NIP 25-binding IgM (p = 0.0016), this activity was completely abolished by 2-ME (Fig. 1A). We conclude that immunization did not elicit specific IgM production in CD154−/− mice but increased serum IgM levels nonspecifically. Indeed, CD154−/− mice injected with adjuvant alone also exhibited similar increases in serum IgM levels (data not shown).

**FIGURE 2.** Absence of GC in CD154−/− mice. GC formations in the spleen and PP of naive and immunized BL/6 and CD154−/− mice were assessed by flow cytometry and immunofluorescence microscopy. Mice were immunized with NP-CGG/alum i.p. and cells and tissues were harvested 16 days after immunization. A–D, Splenocytes from naive (A and C) and immunized (B and D) animals were labeled with mAb specific for B220 and GL-7. M and N, PP cells from naive animals were labeled with mAb specific for B220 and GL-7. Numbers represent percentages of B220highGL-7high GC B cells and B220intGL-7low developing B cells in propidium iodide− live cells. Serial frozen sections of naive (E, I, G, and K) and immunized (F, J, H, and L) spleen or PP (O–R) were labeled with a combination of mAb specific for IgD (green; FITC) and GL-7 (red; PE) or TCRβ (blue, PE) as indicated. Original magnification, ×100. Data shown are representative of at least three independent experiments.

Immunization elicited robust IgG responses in BL/6 mice; by 16 days postimmunization (18), NIP 25-binding serum IgG levels increased 1000-fold above naive controls (p = 0.0008) (Fig. 1B). In contrast, NIP-specific IgG did not increase in the sera of immunized CD154−/− mice (Fig. 1B).

The lack of serum Ab in immunized CD154−/− mice was correlated with the lack of splenic GC responses (Fig. 2). Naive BL/6 mice contained few (0.23 ± 0.1%) B220highGL-7high GC B cells (22) (Fig. 2A), but by day 16 after immunization, their frequency increased ≥4-fold (0.93 ± 0.18%; Fig. 2B). In contrast, only rare (<0.1%) splenic B cells with the GC phenotype could be detected.
in naive CD154−/− mice, and their frequency was not increased by immunization (Fig. 2, C and D).

Immunohistochemical labeling with mAb for the GL-7 Ag and IgD revealed typical GL-7−IgD− splenic GC (51, 52) in immunized BL/6 mice (Fig. 2F), whereas no GL-7−IgD− B cell clusters were present in the spleens of naive and immunized CD154−/− mice (Fig. 2, G and H). Thus, immunization of CD154−/− mice with NP-CGG/alum neither elicits Ag-specific serum Ab or splenic GC responses.

No PP GC in CD154−/− mice

Immunization with Tγ type II Ag can elicit “abortive GC” in CD154−/− mice (53) that have been linked to low frequencies of Ig SHM in some (54), but not all (55), studies. To determine whether Tγ GC might provide an alternative source for SHM in CD154−/− mice, we determined whether PP GC were present in CD154−/− mice. PP samples the Tγ and Tγ Ag of the intestinal flora to trigger humoral responses (56, 57), and the PP GC constitutively present in normal mice may be suppressed by antibiotics that reduce the gut flora (56, 57). PP GC, therefore, provide a significant test of the capacity of Tγ GC in CD154−/− mice.

To enumerate PP GC B cells, we determined the frequencies of GL-7highCD93−B220low cells by flow cytometry. In naive BL/6 mice, a substantial fraction (5.25 ± 0.78%; Fig. 2M) of PP B cells expressed the phenotype of GC B lymphocytes, whereas no or few (0.30 ± 0.23%; Fig. 2N) GC B cells were present in the PP of CD154−/− mice. Although the PP of BL/6 mice contained prominent IgD−GL-7+ GC (Fig. 2O), GC-like structures could not be demonstrated in the PP of CD154−/− mice (Fig. 2P).

The absence of GC in CD154−/− mice was not due to any generalized disorganization of lymphoid architecture in the spleen or PP. Characteristic T cell zones and B cell follicles were present in both BL/6 (Fig. 2, I, J, and Q) and CD154−/− (Fig. 2, K, L, and R) mice. We conclude that neither Tγ nor Tγ GC are formed in CD154−/− mice under physiologic conditions.

Immunochemical increases the numbers of developing B cells in the spleens of CD154−/− mice

Whereas immunization with NP-CGG/alum did not induce GC responses in CD154−/− mice (Fig. 2, D, H, and L), immunization comparably mobilized developing (GL-7highCD93−B220low) B cells from the BM to the spleen in both BL/6 and CD154−/− animals (58) (Fig. 2, B and D). In naive BL/6 mice, GL-7highCD93−B220low cells comprised 0.2% of splenocytes (Fig. 2A), but immunization increased their frequency 7-fold by day 16 (Fig. 2B). Similarly, GL-7high CD93−B220low cells represented 0.1% of splenocytes in naive CD154−/− mice (Fig. 2C); immunization increased their frequency 6-fold (Fig. 2D).

To locate CD93+ B cells in naive and immunized BL/6 and CD154−/− mice, we labeled histologic sections of spleen with fluorochrome-tagged mAb specific for CD93, IgM, and TCRβ (Fig. 3). Whereas all sections (naive, Fig. 3, A and D) contained populations of spindle-shaped CD93+ endothelial cells (59, 60), immunization obviously increased the numbers of CD93+ B lymphocytes (compare Fig. 3A and 3B), consistent with our flow cytometric analyses (Fig. 2, A and B). This increase in CD93+ splenic lymphocytes was most obvious in the splenic bridging channels (61) (Fig. 3, B and C) where plasmacytes

FIGURE 3. Immunization with alum adjuvant mobilizes CD93+ cells from the BM and localizes them in splenic bridging channels. Mice were immunized with NP-CGG/alum i.p. and 16 days after immunization, the location of mobilized CD93+ BM cells was determined by immunofluorescence microscopy. Frozen spleen sections from naive (A and D) and immunized (B, C, E, and F) BL/6 (A–C) or CD154−/− (D–F) mice were labeled with mAb specific for IgM (blue; Alexa Fluor 350), TCRβ (green; FITC), and CD93 (red; PE). Arrows indicate CD93+ lymphocyte-like cells. Bridging channel areas indicated by rectangles in panels B and E are shown at higher magnification in panels C and F, respectively. Original magnification, ×100. Data shown are typical of at least two independent experiments.
expressing intracellular IgM were also present (62, 63) (Fig. 3C). Immunization comparably increased the numbers of CD39 + splenic lymphocytes in the bridging channels of CD154−/− mice (Fig. 3, D and E) where they accumulated along with IgM + plasmacytes (Fig. 3F). We note that SHM in autoreactive plasmablasts present in splenic bridging channels has been reported (9).

In the BM of BL/6 and CD154−/− mice, the patterns of GL-7, IgM, CD21, and CD23 expression on developing (CD93−) and recirculating mature (CD93+) B cells are identical (supplemental Fig. S1A). After immunization, CD93+ B220 + splenic emigrants in BL/6 mice comprised similar numbers of IgM + and IgM − cells (12, 64) (supplemental Fig. S1B), whereas >80% of CD93+ B220 + splenocytes in immunized CD154−/− mice expressed IgM (supplemental Fig. S1B). The spleens of immunized CD154−/− mice, therefore, contain fewer (IgM + CD93+) pro-/pre-B cells compared with BL/6 controls; inflammatory mobilization of BM B-lineage cells in CD154−/− may preferentially target im/T1 B cells.

AID expression by CD154−/− im/T1 B cells

We (12) and Imanishi-Kari and colleagues (10, 11) have demonstrated levels of AID expression in im/T1 B cells from BL/6 mice sufficient to support limited CSR and Igk SHM. Although AID expression by im/T1 B cells occurs in the absence of T lymphocytes or CD154 (10–12), it is unclear whether SHM is equally independent of CD40 signals triggered by T cells or other CD154 + cell compartments (65–69). To compare AID expression in B-lineage cells from CD154−/− and normal mice, we quantified AID message in pro-/pre-B, im/T1, MF, and MZ B cells from naive and immunized BL/6 and CD154−/− mice (12). AID message in GC B cells from BL/6 mice and im/T1 and MF B cells from congenic AID−/− mice served as positive and negative comparators, respectively (3, 4) (Fig. 4). Additionally, we compared AID expression by im/t1 B cells to that of AMuLV-transformed B cell lines (42–44).

GC B cells from immunized BL/6 mice expressed the highest levels (~500 × 10−6 relative to Igβ) of AID transcript; reciprocally, AID message in MF B cells from BL/6 and CD154−/− mice or im/T1 and MF B cells from AID−/− mice (4) was undetectable (Fig. 4). Consistent with our earlier study (12), pro-/pre-B cells from BL/6 and CD154−/− mice contained low, but significant quantities (~0.6% of GC B cells; Fig. 4) of AID message. In both strains, AID message levels rose 5- to 7-fold in im/T1 B cells (p ≤ 0.02; Fig. 4).

Expression of AID by im/T1 B cells, 2–4% of GC B cells, was not quantitatively different (p = 0.71) from that of six AMuLV-transformed pre-B cell lines (Fig. 4). Indeed, AID expression by BL/6 and CD154−/− im/T1 B cells (range, 6.0–37.8 × 10−6, relative to Igβ) and AID + AMuLV-transformants (range, 1.8–19.3 × 10−6, relative to Igβ) overlapped (Fig. 4).

SHM in CD154−/− im/T1 B cells

To determine whether AID expression in developing B cells is sufficient to support IgH SHM, we amplified genomic VDJ rearrangements from highly purified (>95%) populations of pro-/pre-, im/T1 B cells from the BM and MF B cells from the spleens of naïve BL/6, CD154−/−, and AID−/− mice using Pfu polymerase (49, 70). Additionally, GC B cells were recovered from immunized BL/6 mice 16 days after immunization with NP-CGG/alum. Amplificands comprised 11 related V H gene segments (V3 subgroup) rearranged to J H2 (71); VDJ products were cloned, sequenced (49, 70), and V H mutations were identified by comparison to germline (1, 70). Mutation frequencies were estimated from unique VDJ rearrangements (n = 22–59) as defined by CDR3 sequence, and matched B cell subsets from congenic AID−/− mice (4) established a baseline for mutation frequencies (Table I). Rare mutations (≤1/V H gene segment) recovered from AID−/− B cells indicated methodological errors of ~2.2 × 10−4 mutations per base pair sequenced (range, 1.3–4.5 × 10−7/bp; Table I).

No excess V H mutations were observed in VDJ fragments recovered from any pro-/pre-B or MF B cell population (3.1–3.9 × 10−4 mutations/bp; p = 0.43–0.68), whether from BL/6 or CD154−/− mice (Table I). In contrast, numerous V H mutations were present in GC B cells (161 × 10−4 mutations/bp; p < 0.001) from immunized BL/6 animals (Table I).

Significantly elevated numbers of V H mutations were, however, recovered from im/T1 B cells from CD154−/− mice (16.3 × 10−4, p < 0.001). Indeed, the V H mutation frequency in this im/T1 B cell cohort is 10% of that observed in GC B cells from immunized BL/6 mice and demonstrates a capacity for developmental SHM in the absence of CD40-CD154 interaction.

In contrast to CD154−/− mice, we found that the number of V H mutations in im/T1 B cells from naïve BL/6 mice (5.1 × 10−4/bp) was modestly, but not significantly (p = 0.23), elevated above AID−/− controls (2.2 × 10−4/bp; Table I).

SHM in GC B cells exhibits a characteristic bias for transition mutations; transitions typically constitute some 50–60% of AID-driven V(D)J mutations (72). In our samples, transitions accounted for 48% of V H mutations in CD154−/− im/T1 B cells and 61% of
the mutations recovered from GC B cells (supplemental Fig. S2). Mutation at RGYW/WRCY motifs is also a hallmark of AID-mediated SHM (73) and constitutes ~30% of V_H mutations in human B cells (74, 75). The distribution of V_H mutations in im/T1 B cells from CD154^{-/−} mice revealed a preference (24%) for these “hotspots” similar to that of GC B cells (33%) and consistent with AID-dependent SHM (Table I) (73).

**V_H mutations in im/T1 B cells are unselected by Ag**

Replacement (R) mutations are strongly linked to affinity-driven selection (70, 76), and ~75% (70/95) of the V_H mutations recovered from GC B cells were R substitutions (Table I). In contrast, R mutations constituted 52% (11/10) of the V_H mutations recovered from the im/T1 B cells of CD154^{-/−} mice (Table I). Additionally, the distribution of V_H mutation sites differed substantially between GC B cells and im/T1 B cells. Mutations in GC B cells were frequent (35% of total) in CDR1 and CDR2, whereas CDR mutations in im/T1 B cells constituted ≤5% of total mutations (data not shown). Reduced frequencies of R and CDR1/2 mutations in IM/T1 B cells indicate a lack of stringent Ag-driven selection.

V_H gene usage in mutated im/T1 B cells differed significantly from that of GC B cells elicited by NP-CGG. GC B cell populations were dominated by rearrangements of the V186.2 (50%) and C1H4 (29%) gene segments (supplemental Fig. S3); these frequencies underestimate Ag selection in GC, as we excluded clonally related sequences from our analysis (1, 70). Instead, V_H gene usage by BM im/T1 B cells from CD154^{-/−} mice was diverse (supplemental Fig. S3). The V_H mutations recovered in CD154^{-/−} im/T1 B cells were distributed uniformly among the various V_H amplicands (data not shown).

**AID expression in murine fetal liver**

In both mice and humans, the fetal liver supports lymphopoiesis and, as gestation proceeds, contains increasingly mature B-lineage cells (77, 78). The fetus is normally isolated from infection and exposure to exogenous Ag (79), and any AID expression in the murine fetal liver is therefore unlikely to be induced by extrinsic factors. To determine whether murine B-lineage cells express AID, we amplified AID message from fetal liver B cells recovered at 16 and 19 days of gestation (E16 and E19; Fig. 5).

As reported (80, 81), only pro-/pre-B cells were present in E16 fetal liver, but pro-/pre-B and im/T1 B cells could be recovered from E19 samples. Some 2% of E16 fetal liver cells were B220^{+}/CD93^{-} (2.4 ± 0.6%) and, of these, all (99.8 ± 0.1%) expressed the IgM^{−}/IgD^{−} phenotype (supplemental Fig. S4). In E19 samples, B220^{+}/CD93^{-} cells comprised ~20% of fetal liver.

**FIGURE 5.** AID expression in developing B-lineage cells from murine fetal liver. AID mRNA expression in pro-/pre-B (B220^{+}/CD93^{-}/IgM^{−}/IgD^{−}) and im/T1 B (B220^{+}/CD93^{-}/IgM^{−}/IgD^{−}) cell compartments from murine fetal liver samples (16 days and/or 19 days of gestation; supplemental Fig. S4) were measured by quantitative RT-PCR. AID mRNA levels are shown as relative values to IgB mRNA levels (mean ± SD) in each sample (n = 2–4).

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**Table I. SHM in im/T1 B cells from CD154^{-/−} mice**

<table>
<thead>
<tr>
<th>B Cell Type</th>
<th>Nos. VDJ Rearrangements (in frame)</th>
<th>Basepairs Sequenced</th>
<th>Nos. Mutations</th>
<th>Nos. Mutated Rearrangements (%) total</th>
<th>Mutation Frequency (×10^{-5})</th>
<th>ΔR/ΔS&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BL/6</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SPL GC</td>
<td>22 (19)</td>
<td>5.9 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>95</td>
<td>20 (91%)</td>
<td>161***</td>
<td>31/95 (33%)</td>
</tr>
<tr>
<td>SPL MF</td>
<td>31 (25)</td>
<td>8.3 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>3 (10%)</td>
<td>3.6</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>BM pro-/pre-</td>
<td>24 (19)</td>
<td>6.4 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2</td>
<td>2 (8%)</td>
<td>3.1</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>BM im/T1</td>
<td>22 (20)</td>
<td>5.9 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>3 (14%)</td>
<td>5.1</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td><strong>CD154^{-/−}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPL MF</td>
<td>29 (25)</td>
<td>7.8 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>3 (10%)</td>
<td>3.9</td>
<td>2/3 (67%)</td>
</tr>
<tr>
<td>BM pro-/pre-</td>
<td>34 (27)</td>
<td>9.1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>3 (9%)</td>
<td>3.3</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>BM im/T1</td>
<td>48 (41)</td>
<td>12.9 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>21</td>
<td>13 (27%)</td>
<td>16.3***</td>
<td>5/21 (24%)</td>
</tr>
<tr>
<td><strong>AID^{-/−}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM pro-/pre-</td>
<td>25 (18)</td>
<td>6.7 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3</td>
<td>3 (12%)</td>
<td>4.5</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>BM im/T1</td>
<td>59 (49)</td>
<td>15.8 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2</td>
<td>2 (3%)</td>
<td>1.3</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>Total AID^{-/−}</td>
<td>84 (67)</td>
<td>22.5 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5</td>
<td>5 (6%)</td>
<td>2.2</td>
<td>1/5 (20%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> V_H(V3)DJ_H2 rearrangements from B cell subsets were cloned into bacteria and sequenced.

<sup>b</sup> Numbers of mutation in RGYW/WRCY motifs (Δhotspot), total numbers of mutations (Δtotal), and percentage of Δhotspot in Δtotal are shown.

<sup>c</sup> Numbers of replacement mutations (ΔR) and silent mutations (ΔS) are shown.

<sup>d</sup> ***p < 0.001; mutation frequency of B cell subset is significantly different from the mutation frequency of total AID^{-/−} B cells calculated by Mann-Whitney’s U test.
Human AID expression levels were highest in tonsil (1.1 × 10^{-2} relative to Igβ), with Ramos cells expressing about half as much AID message (0.5 × 10^{-2} relative to Igβ; Fig. 6). Consistent with AID expression by murine B cells (Fig. 4) (12), human im/T1 B cells expressed low but significant levels of AID message (3.6% of tonsil; Fig. 6), while AID was undetectable in T2 and MF B cells (Fig. 6). AID expression in im/T1 B cells accounted for virtually all AID message present in unsorted cord blood cells (Fig. 6), and no AID message was detected in the CD19+ compartments of cord blood (data not shown). By this relative measure, AID message levels in human cord blood im/T1 B cells are comparable to those of murine im/T1 B cells capable of CSR (11, 12) and SHM (Table I) (10, 11).

AID mRNA in two, independent fetal liver libraries (20 wk gestation, both) was substantially more abundant than in cord blood samples (Fig. 6). Remarkably, AID expression levels (3.0 × 10^{-3} and 6.0 × 10^{-3} relative to Igβ) in human fetal liver equaled that of Ramos cells (Fig. 6). The shared patterns of AID expression in mouse and human B-lineage cells suggest a conserved developmental program.

**Discussion**

HIGM1 patients support populations of IgM+/IgD+ CD27+ B cells carrying mutated V(D)J rearrangements (27, 28, 34). This cellular compartment is generally categorized as unswitched (IgM) memory B cells (30, 85) or, alternatively, “pre-diversified” progenitors of MZ B cells (28, 86). In HIGM1 patients, the mutated IgM+/IgD+CD27+ compartment develops relatively soon after birth (34), and perhaps even in the fetus (14), in the absence of detectable GC responses. Taken together, these observations are sufficiently surprising to imply a “second pathway” for VDJ SHM in the absence of cognate T-B cell interaction (27).

Despite reports of active SHM outside of GC (9–11, 14), examples of SHM and AID activity in non-GC B cells remain controversial, as genetically modified mice that coexpress fluorescent marker proteins and AID contain no marked immature B cells and few labeled cells not located in GC (87, 88). The absence of labeled developmentally immature B cells could reflect the low levels of AID message expressed by non-GC B cells (Figs. 4 and 5) (12, 87), the relative brevity of AID expression by im/T1 B cells, or both (11). Conversely, reports of AID expression and activity by developmentally immature B cells in humans and mice may reflect artifactual inclusion of Td or Ti GC B cells in isolated populations of immature and transitional B cells and/or atypical distributions of GC B cells in tissue.

In an attempt to understand the conditions that lead to SHM in the absence of GC, we previously demonstrated that both pro-/pre-B and im/T1 B cells of CD154−/− mice express elevated levels of AID and in im/T1 B cells at levels sufficient to support limited CSR (12). These observations raised the possibility that CD154−/− im/T1 B cells might undergo SHM as well. If so, CD154-deficient mice could offer insight into the origin(s) of the hyper-mutated IgM+/IgD+CD27+ B cells in HIGM1 patients and, more generally, SHM by non-GC B cells.

We found that in CD154−/− mice a potent Td immunogen does not elicit serum Ab (Fig. 1). In contrast to the robust Ab responses of normal congenic controls, CD154−/− mice did not mount specific IgM or IgG Ab responses after immunization with NP-CGG-alum. Whereas immunization did increase serum IgM in CD154−/− mice, these increases were nonspecific and likely represent a general immune activation driven by the inflammatory properties of alum (89). In addition to absent Ab responses, immunization of CD154−/− mice neither elicited GC nor increased the frequency of...
splenic B cells with the phenotype (B220<sup>hi</sup>GL-7<sup>hi</sup>) of GC B cells (Fig. 2). Thus, CD154 was required not only for the generation of GC architecture but also for the characteristic activation phenotype of GC B cells. Although we did not immunize with Ti Ag, CD154<sup>−/−</sup> mice respond to both type I and type II Ti Ag (18, 19), and it remained possible that any AID expression in these mice might be derived from cells recruited to Ti “GC” (16, 55, 90). This concern was mitigated by the absence of PP GC in CD154<sup>−/−</sup> mice, demonstrating that even prolonged exposure to the (T<sub>a</sub> and T<sub>i</sub> Ag of the gut flora is insufficient to generate GC or B cells with a GC phenotype in the absence of CD154 expression (Fig. 2). In this, the immunodeficiency of CD154<sup>−/−</sup> mice appears to be more severe than in some human HIGM1 patients (16). We conclude that any AID expression and activity in the B cell compartments of CD154<sup>−/−</sup> mice (Fig. 4) are highly unlikely to result from contamination by incidental T<sub>a</sub> or T<sub>i</sub> GC B cells.

Despite these differences in Ab production, immunization of BL/6 and CD154<sup>−/−</sup> mice with alum mobilizes BM CD93<sup>+</sup> im/T1 B cells to the spleen (64, 91) (Fig. 2). The mobilized CD93<sup>+</sup> cells localize to the border of the red pulp and splenic T cell zone in bridging channels (Fig. 3) (61), extra follicular sites where short-lived plasmacytes accumulate after immunization with T<sub>a</sub> Ag (62), and autoreactive B cells have been reported to undergo SHM (9). Similar mobilizations appear to occur in humans with systemic autoimmune disease, as these patients often exhibit inflammatory responses and the appearance of developmentally immature B cells in the periphery (92). The appearance of developing B cells in the periphery suggests a potential role for im/T1 B cells in autoreactive Ab production (93), and we speculate that autoreactive im/T1 B cells may persist or mature in autoimmune patients. Indeed, im/T1 B cells in autoimmune-prone mice are resistant to apoptosis after IgM cross-linking in vitro (94), whereas normal im/T1 B cells are exquisitely sensitive to BCR-induced apoptosis (95, 96).

As expected (12), pro-/pre-B and im/T1 B cells from adult CD154<sup>−/−</sup> and BL/6 mice expressed low but significant levels of AID, whereas message was undetectable in the MF B cell compartment of both control and knockout animals (Fig. 4). Quantitative RT-PCR results indicate that AID expression by im/T1 B cells ranged from 2 to 4% of that observed in primary, splenic GC B cells and was greater than or equal to a collection of AMuLV-transformed B cell lines (Fig. 4).

Significant AID expression by pro-/pre-B and im/T1 B cells from murine fetal liver was also observed (Fig. 5). Pro-/pre-B cells from E16 fetal liver expressed AID message at levels equivalent to pro-/pre-B cells from adult BM, while AID expression in E19 fetal liver pro-/pre-B cells was 7-fold higher and in E19 im/T1 B cells was 2-fold higher than in pro-/pre-B and im/T1 cells from adult BM (Figs. 4 and 5).

AID expression by human im/T1, T2, and MF B cells from umbilical cord blood mirrored the patterns observed in mice with significant AID expression present in im/T1 cells but not the T2 and MF compartments (Fig. 6). Human im/T1 B cells from cord blood express quantities of AID (3–6% of tonsil tissue; Fig. 6) that are similar to those of murine im/T1 B cells (2–4% of GC; Fig. 4). In mice, this level of AID is sufficient to support both CSR (11, 12) and SHM (Table I) (10, 11). AID message was also abundant in two human fetal liver RNA libraries (Fig. 6). Indeed, AID expression (relative to Igβ) in 20-wk human fetal liver was equivalent to that of the Ramos human GC B cell line and about half that of human tonsil, a tissue rich in GC B cells (83, 84) (Fig. 6).

In contrast to our findings (Figs. 4–6) (12) and those of Imanishi-Kari and colleagues (10, 11), Papavasiliou and colleagues (97, 98) reported negligible levels of AID expression in developing B cells but significantly increased AID expression in developing B lymphocytes after viral transduction/infecction. We also observed significant levels of AID message in cloned AMuLV-transformed B cell lines (Fig. 4), but the levels of AID message in AMuLV transformants were similar to im/T1 B cells freshly recovered from BM (Fig. 4). Interestingly, mutation frequencies for the J<sub>H</sub>4 and A<sub>S</sub> genes (6.8 and 11.9 × 10<sup>−4</sup>bp, respectively) (97) in AMuLV-transformed pro-B cells said to express high levels of AID are below those of VH mutations in CD154<sup>−/−</sup> im/T1 B cells (16.3 × 10<sup>−4</sup>; Table I) and comparable to those observed in BL/6 im/T1 B cells (5.1 × 10<sup>−4</sup>; Table I). Thus, by either the measure of quantitative AID expression (Fig. 4) or Ig mutation frequencies (Table I), we were unable to confirm virally induced elevations of AID expression and SHM in AMuLV-transformed B cells (97).

Although AID expression was equivalent in BL/6 and CD154<sup>−/−</sup> mice, significantly elevated VDJ mutation frequencies were observed only in CD154-deficient mice (Table I). Whereas this restriction of VH mutation may mirror the relationship of HIGM1 patients and normal individuals, the mechanism(s) responsible for this bias is unclear. In im/T1 B cells, the measured VH mutation frequency (16.3 × 10<sup>−4</sup>) was 10% of GC B cells (161 × 10<sup>−4</sup>; Table I) and, unlike GC B cells, mutations were present in only about a third (27%) of CD154<sup>−/−</sup> im/T1 B cells and showed little or no evidence for Ag-driven selection (Table I). VH mutations in CD154<sup>−/−</sup> im/T1 B cells were characterized by low ratios of replacement-to-silent mutations (Table I), broad dispersal within the VH gene segments, and appearance in diverse VH<sub>4</sub> gene segments (supplemental Fig. S3). Taken together, these characteristics led us to conclude that Ag does not expand specific (auto-reactive?) clones of im/T1 B cells, although Ig signaling may promote SHM (11).

The numbers of VH<sub>4</sub> mutations in mutated im/T1 sequences (averages, 1.6; range, 1–4) were about one-third those observed in GC B cells (average, 4.8; range, 1–12), but they were easily distinguished from matched AID<sup>−/−</sup> controls, pro-/pre-B cells, and MF B cells (Table I). The absence of a CD27-like marker in mice precludes a directed search for any descendants of mutated im/T1 B cells, but it is clear that the great majority of IgM<sup>+</sup>IgD<sup>−</sup> MF B cells, even in CD154<sup>−/−</sup> mice, do not carry mutated Ig genes (Table I). It is possible that mutated im/T1 B cells enter a compartment that we did not directly sample (e.g., the peritoneum or the splenic MZ). If BCR signaling promotes SHM in im/T1 B cells (11), physiologic selection for im/T1 B cells with low affinity for self-Ags (99) could bias im/T1 B cell differentiation to the MZ (96) or peritoneal B cell compartments (100).

It is possible that AID<sup>+</sup> im/T1 B cells have few or no descendants. Activated im/T1 B cells are highly sensitive to apoptotic signals, and it is plausible that some CD154-dependent process normally eliminates AID<sup>+</sup> im/T1, perhaps as a consequence of genotoxic stress (101). Of note, the survival and/or proliferation of im/T1 B cells is augmented by BAFF (12), a cytokine that is often elevated in autoimmune patients (102). The concatenation of BCR signaling and BAFF-enhanced survival of autoreactive im/T1 B cells may represent a prepathologic condition that promotes autoimmunity in peripheral tissues.

It remains unclear when and where human IgM<sup>+</sup>IgD<sup>−</sup>CD27<sup>−</sup> B cells acquire Ig mutations. The mutation frequency of IgM<sup>+</sup>IgD<sup>−</sup>CD27<sup>−</sup> B cells increases during first year of life (28), indicating that these B cells undergo SHM during that period. Recently, Scheeren et al. have provided evidence that human IgM<sup>+</sup>IgD<sup>−</sup>CD27<sup>−</sup> B cells become mutated in utero when they recovered mutated IgM<sup>+</sup>IgD<sup>−</sup>CD27<sup>−</sup> B cells from fetal spleen (14). Among fetal tissues, AID message is detected in mesenteric lymph node and liver (Fig. 6) (14, 84) but not in spleen or BM (14); therefore,
it is likely that B cells undergo SHM in fetal liver or fetal mesenteric lymph node during ontogeny.

AID expression and SHM in mouse CD154−/− im/T1 B cells (Fig. 4 and Table I) suggest a potential relationship between murine im/T1 B cells and the mutated IgM/IgD CD27+ B cells in HIGM1 patients (27, 28). These B cell subsets share characteristically diverse Ig repertoires and, to lesser and greater degrees, Ig SHM that is independent of Tα and Tβ immune responses (10, 34) (Figs. 1 and 2 and Table I). AID message is present in mouse im/T1 B cells from fetal liver, BM, and spleen (Figs. 4 and 5) (10–12) and in human im/T1 B cells from cord blood (Fig. 6) and in fetal hematopoietic tissue (Fig. 6) (14). We conclude that it is entirely plausible that human im/T1 B cells, or even earlier B cell types in fetal tissues, could undergo SHM to produce the mutated IgM/IgD CD27+ B cells present in HIGM1 patients.

The similar patterns of AID expression during B cell development in mice and humans (10–12, 14, 84) suggest that developmentally regulated AID expression may represent a developmental program shared by most, and perhaps all, vertebrate species (103). It is interesting to consider the possibility that the Ag-driven GC response evolved from this more primitive developmental program. If so, understanding the physiologic onset, site, triggers, and significance for developmentally regulated AID expression constitutes significant and novel goals in understanding B cell biology.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

4. Burleigh, J. H., J. H. Akira, K. Calame, B. Beutler, E. Selsing, and C. R. Maliszewski. 1994. AID deficiency in mice and humans (10–12, 14, 84) suggest that developmentally regulated AID expression may represent a developmental program shared by most, and perhaps all, vertebrate species (103). It is interesting to consider the possibility that the Ag-driven GC response evolved from this more primitive developmental program. If so, understanding the physiologic onset, site, triggers, and significance for developmentally regulated AID expression constitutes significant and novel goals in understanding B cell biology.

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References


46. Lentz, V. M., and T. Manser. 2001. Cutting edge: germinal centers can be

47. Toellner, K. M., W. E. Jenkinson, D. R. Taylor, M. Khan, D. M. Sze,


49. /H9260

50. 36: 807–816.

51. 63. Garcia De Vinuesa, C., A. Gulbranson-Judge, M. Khan, P. O’Leary,


53. 30: 807–816.

54. 42. Alt, F., N. Rosenberg, S. Lewis, E. Thomas, and D. Baltimore. 1981. Organi-


56. 73. Rogozin, I. B., and N. A. Kolchanov. 1992. Somatic hypermutagenesis in im-


58. 64. Ueda, Y., K. Yang, S. J. Foster, M. Kondo, and G. Kelsoe. 2004. Inflammation
to (4-hydroxy-3- nitrophenyl)acetyl, II: A common clonal origin for periarte-

59. 77. Dorshkind, K., and E. Montecino-Rodriguez. 2007. Fetal B-cell lymphopoiesis

60. 78. Gathings, W. E., A. R. Lawton, and M. D. Cooper. 1977. Immunofluorescent

61. 48: 379–388.


63. 418 – 423.

64. 41: 253–261.

65. 59. Yuan, X., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most pe-

66. 365:

67. 90. de Vinuesa, C. G., M. C. Cook, J. Ball, M. Drew, Y. Sunners, M. Cascalho,

68. 20: 329 –339.

69. 61. Gu, H., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most pe-

70. 76. Allen, D., A. Cumanio, R. Dildrop, C. Kocks, K. Rajewsky, N. Rajewsky,

71. 36: 1665–1673.

72. 52. Alt, F., N. Rosenberg, S. Lewis, E. Thomas, and D. Baltimore. 1981. Organi-

73. 75. Martin, A., P. D. Bardwell, C. J. Woo, M. Fan, M. J. Shulman, and M. M. Sze,

74. 71. Gu, H., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most pe-


76. 327: 301–305.

77. 40: 301–305.

78. 195: 593–616.

79. 204:

80. 30: 807–816.

81. 32: 3384–3396.

82. 29: 1370–1371.


84. 1171: 11–18.


86. 60. 66. Gauchat, J. F., S. Henchou, G. Mazzei, J. P. Aubry, T. Brunner, H. Blassey,

87. 51. 36: 1665–1673.

88. 201: 4390–4398.


90. 35: 593–616.

91. 32: 3384–3396.

92. 78. 4390–4398.


96. 249: 361–363.

97. 28: 3384–3396.

98. 199: 1115–1135.


100. 249: 361–363.

101. 33: 1370–1381.

102. 1145–1156.

103. 239: 124–125.

104. 199: 1115–1135.

105. 4390–4398.

106. 1107–1108.

107. 5: 804–810.

108. 35: 593–616.

109. 1145–1156.

110. 1107–1108.

111. 5: 804–810.

112. 35: 593–616.

113. 1107–1108.

114. 5: 804–810.

115. 35: 593–616.

116. 35: 593–616.

117. 35: 593–616.

118. 35: 593–616.

119. 35: 593–616.
mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity* 11: 201–212.


