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Late Immature B Cells (IgM\textsuperscript{high}IgD\textsuperscript{neg}) Undergo a Light Chain Receptor Editing Response to Soluble Self-Antigen\textsuperscript{1}

Lina E. Tze, Keli L. Hippen, and Timothy W. Behrens\textsuperscript{2}

Receptor editing is an important mechanism to modify the Ag specificity of newly developing B cells that are reactive with self-Ags. Previous studies have suggested that late immature B cells, bearing high levels of IgM on their cell surface, are unable to initiate receptor editing and instead are deleted by apoptosis. Using the hen egg lysozyme transgenic system, we show that IgM\textsuperscript{high} late-immature B cells are fully capable of receptor editing to soluble self-Ag. This was demonstrated by the induction of new endogenous light chain locus rearrangements and by a single-cell flow cytometric assay using a recombination-activating gene 2-green fluorescence protein reporter transgene. These studies suggest that the developmental window available for immature B cells to edit their Ig receptors, at least in response to certain soluble Ags, extends through the IgM\textsuperscript{high} late immature B cell stage. The Journal of Immunology, 2003, 171: 678–682.

B cell development in the bone marrow (BM)\textsuperscript{3} is characterized initially by Ig rearrangements at H chain loci, followed by clonal expansion of cells with productively rearranged H chains, and then further rearrangements at L chain loci (1). Random Ig rearrangements provide a diversified Ab repertoire that is important for B cells to meet the challenges of adaptive immunity, but pose the problem of generating self-reactive B cells at a relatively high frequency. Newly generated self-reactive immature B cells are tolerated by several distinct mechanisms. Highly self-reactive cells recognizing membrane-bound self-Ags may undergo clonal deletion (2, 3), while self-reactive cells recognizing soluble self-Ags may enter a state of anergy, in which they respond poorly to subsequent B cell receptor (BCR) cross-linking (4). In addition, immature B cells with self-reactive receptors have the potential to initiate new rearrangements, particularly at L chain loci, in an attempt to modify the specificity of Ig in a process termed receptor editing (5, 6).

As many as 20% or more of all developing B cells undergo receptor editing in vivo (7); however, relatively little is known about the mechanisms that regulate this important pathway. Previous studies suggested strong developmental constraint on receptor editing. Using an in vitro model system employing anti-class I MHC 3–83 conventional transgenic (Tg) Ig B cells, Melamed et al. (8) provided evidence to suggest that immature B cells with low levels of IgM (IgM\textsuperscript{low}) were competent to edit in response to membrane-bound self-Ag, while IgM\textsuperscript{high} immature cells were incompetent, even when kept alive by virtue of a bcl-2 transgene.

We have recently used the hen egg lysozyme (HEL) Ig Tg system (4) to examine L chain receptor editing in response to soluble self-Ags (9). We tested whether the same developmental constraint reported for membrane-bound self-Ag also held for soluble Ag. Our results indicate that in the HEL Tg system, and in response to soluble Ag, IgM\textsuperscript{high} B cells are fully competent to undergo L chain editing in vitro.

Materials and Methods

Mice

MD4 HEL-Ig Tg mice (4) were originally provided by C. Goodnow (Australian National University, Canberra, Australia), while recombination-activating gene 2-green fluorescence protein (Rag2-GFP) reporter mice (10) were a gift from M. Nussenzweig (Rockefeller University, New York). The animals were intercrossed to generate Rag2-GFP/HEL-Ig double Tg mice. The mice were maintained in a specific pathogen-free facility at the University of Minnesota and were 4–8 wk of age at the time of the experiments.

IL-7 BM cultures

Single-cell suspensions of BM cells from HEL-Ig and HEL-Ig/Rag2-GFP mice were prepared as previously described (9). Cells were cultured at a concentration of 2 × 10\textsuperscript{5} cells/ml in complete medium consisting of 1/1 RPMI 1640:EHAA (Mediatech (Washington, DC) and Biofluids (Rockville, MD), respectively), 10% heat-inactivated FBS (Life Technologies, Gaithersburg, MD), 1-glutamine (BioWhittaker, Walkersville, MD), and penicillin and streptomycin (Mediatech) in the presence of 16 ng/ml recombinant murine IL-7 (R&D Systems, Minneapolis, MN) for 5 days (11–13). After sorting, cells were placed back into culture in complete medium on gamma-irradiated (2000 rad) S17 stroma (12, 14, 15). The cells were cultured in medium alone, HEL Ag (Sigma-Aldrich, St. Louis, MO), or HEL\textsuperscript{MUT}, a four-amino-acid site-directed mutant of HEL with a K\textsubscript{d} of ~10\textsuperscript{-7} M (~100-fold less affinity than wild-type HEL) for the HEL-Ig receptor (9, 16).

Flow cytometry and cell sorting

BM cells grown in IL-7 cultures were stained in FACS buffer (1× PBS and 10% FBS) with FITC-, PE-, or CyChrome-conjugated mAbs to IgM\textsuperscript{a} and IgD\textsuperscript{a} (BD PharMingen, San Diego, CA) and IgD\textsuperscript{b} (BD PharMingen, San Diego, CA) and were sorted by FACS Vantage (BD Biosciences, Mountain View, CA). Sorted B cells from secondary cultures were harvested and stained with FITC-, PE-, or CyChrome-conjugated mAbs to IgM\textsuperscript{a} and IgD\textsuperscript{a} and B220 (BD PharMingen) and assayed by FACSCalibur (BD Biosciences). In some staining conditions, annexin V-PE and/or 7-AAD (BD PharMingen and Calbiochem (San Diego, CA)) were used to exclude dead cells. For the quantitation of cell numbers, a known number of PKH26 reference microbeads (Sigma-Aldrich) was added to the cell suspensions just before FACS analysis. Flow cytometric

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\textsuperscript{3}Abbreviations used in this paper: BM, bone marrow; 7AAD, 7-amino-actinomycin D; BCR, B cell receptor; Tg, transgenic; HEL, hen egg lysozyme; Rag2-GFP, recombination-activating gene 2-green fluorescence protein reporter transgene. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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analyses were performed using CellQuest (BD Biosciences) and Flowjo (Treestar, San Carlos, CA) software.

**PCR analyses**

Genomic DNA was extracted from sorted BM cells before and after 2-day secondary cultures in either the presence or the absence of soluble HEL or HEL_MUT. Genomic DNA from the equivalent of 4000 cells was then subjected to semiquantitative PCR amplifications using primers specific for HEL-Ig and V-Jc1 as previously described (9, 17) (http://fcsnr.anu.edu/group_pages/mge/PCR.html). The HEL-Ig PCR was slightly modified and performed using primers IgHFl (5'-GGGACTCCATCTACCCAGGAT-3') and IgHR1 (5'-ACCACAGACCACAGGACAAG-3') and using the following cycling conditions: 1 min at 94°C, 1 min at 59°C, and 1.5 min at 72°C for 25 cycles. The V-Jc1 PCR was designed to detect the rearrangements of various Vκ gene segments to Jκ1 by using degenerate Vκ primers and a primer downstream of the Jκ1 gene segment. Recombining sequence (RS) recombinations were detected with the following primers: VDEG1, 5'-GGGACTCCATCTACCCAGGAT-3'; VDEG2, 5'-GGGACTCCATCTACCCAGGAT-3'; VDEG3, 5'-GGGACTCCATCTACCCAGGAT-3'; and reverse primer (RS3'), 5'-CTCAAACTAGGCTCAACTG-3', using the following cycling conditions: 45 s at 94°C, 1 min at 64°C, and 1 min at 72°C for 27 or 30 cycles (18). This RS PCR was designed to detect rearrangements occurring from Vκ regions to a conserved heptamer-nonamer recombination signal sequence found downstream of Cκ and 3' enhancer, resulting in the deletion of all Jκ and Cκ regions (19). PCR products were resolved on 1% agarose gels and transferred onto nitrocellulose blots. Specific bands were detected by Southern hybridization of nitrocellulose blots with radiolabeled internal oligonucleotides and autoradiography.

**Results**

Fresh bone marrow from HEL-Ig mice was grown in IL-7 for 5 days, resulting in cell populations enriched for immature HEL-Ig Tg B cells (IgM+ IgDneg-low; Fig. 1A). These cells were then sorted by flow cytometry into highly purified IgMlow (IgMlow/IgDneg) and IgMhigh (IgMhigh/IgDneg) cell subpopulations (IgMhigh purities averaging 94%). Surface staining showed that the purified IgMhigh cells expressed slightly higher levels of the maturation markers B220, CD21, and CD23 and slightly lower levels of CD43 (Fig. 1B). Previous experiments showed that IgMhigh cells are the precursors of IgMhigh cells (8), and we confirmed this relationship in the current system (data not shown and see Fig. 2B).

The sorted cell subpopulations were further cultured in the absence or the presence of soluble HEL (sHEL) self-Ag or HEL_MUT, a four-amino acid mutant HEL protein with ~100-fold reduced affinity for the HEL-Ig BCR (16), for 2 days on S17 BM stromal cells. The sorted cells were sensitive to cell death, as determined by forward light scatter and the incorporation of 7-amino-actinomycin D (7AAD) at the end of a 48-h culture (Fig. 2A) as well as by viable cell counts (see below). As previously noted in the 3–83 Tg B cell model system (8), the IgMhigh immature B cell population was more susceptible to cell death than the IgMlow immature B cells even in the absence of Ag. Fig. 2B shows that both IgMlow and IgMhigh immature B cells down-regulated surface IgM levels following incubation with Ag to a similar degree, with more extensive surface IgM down-regulation observed in the presence of sHEL than sHEL_MUT. In the absence of Ag, IgMlow cells became IgMhigh during the 2-day culture period.

Cells were harvested at the end of the secondary Ag culture and assayed for new endogenous V-Jc1 and RS rearrangements, markers for receptor editing in this system. As shown in Fig. 3A, both the IgMlow and IgMhigh immature B cell subpopulations were equally capable of receptor editing in response to sHEL, as determined by the detection of new endogenous V-Jc1 and RS rearrangements. The receptor editing responses of both subpopulations were more vigorous in the presence of HEL than in the presence of HEL_MUT, consistent with previous observations in unfractionated cells (9) (Fig. 3B). As noted previously (9), we did not detect surface λ light chain-expressing cells in these cultures as a marker for editing, possibly due to the inability of λ-chains to pair well with the HEL-Ig H chain or to an inability to displace the endogenous HEL L chain (data not shown). It was also technically difficult to determine whether there was a loss of HEL reactivity in this system as a consequence of editing due to strong down-regulation of overall Ig receptor levels upon Ag encounter and the fact that the anti-HEL L chain is not replaced during editing because it is integrated into chromosomal DNA outside the normal κ locus.

We next bred mice carrying a Rag2-2-GFP reporter transgene (10) with HEL-Ig mice to generate double-Tg mice. BM from these animals was cultured in IL-7 and sorted into IgMlow and IgMhigh immature B cell populations, and cells were then incubated with Ag. This single-cell analysis showed that a significant proportion of both IgMlow and IgMhigh immature B cells up-regulate Rag2 in response to sHEL (Fig. 4A). These data suggest that a large percentage of the viable IgMhigh cell population was undergoing the editing response, rather than vigorous editing in a small subpopulation.

It was important to rule out the possibility that the few contaminating IgMlow B cells (averaging 6.0% in five experiments) in the sorted IgMhigh populations could account for the receptor editing response observed in the sorted IgMhigh cell populations. Viable cells in the cultures were quantified using trypan blue exclusion and a flow cytometric bead-based method (see Materials and

![FIGURE 1.](http://www.jimmunol.org/) Isolation and cell surface phenotype of IgMlow and IgMhigh immature B cell populations. A, IgMlow/IgDneg and IgMhigh/IgDneg-low immature B cells were sorted from 3-day IL-7 cultures of bone marrow from HEL-Ig Tg mice (sort purities averaging 98% for IgMlow and 94% for IgMhigh cell populations). B, Flow cytometric analysis of B cell surface markers on IgMlow and IgMhigh immature B cell populations. Plots show cells gated on the basis of forward light scatter and B220 expression. The dark line represents IgMhigh cells, and the light line represents IgMlow cells. Data are representative of six independent experiments.
Methods). As noted above, there was a higher rate of cell death in cultured IgM<sub>high</sub> B cells compared with the IgM<sub>low</sub> cells in the presence of sHEL (Fig. 4B). On the average, however, 18% of the starting IgM<sub>high</sub> B cells were alive at the end of the culture, and ~40% of these were Rag2-GFP positive (Fig. 4A). After initial cell sorting, there was an average of 6.0% contaminating IgM<sub>low</sub> cells in the IgM<sub>high</sub> cell populations. Since the average viability of IgM<sub>low</sub> cells in the presence of sHEL was 80%, they would constitute 26.7% of the viable cell population in the sHEL treated IgM<sub>high</sub> cultures. Because only 30% of viable IgM<sub>low</sub> cells up-regulate Rag2-GFP in the presence of sHEL, only 8% (30% × 26.7%) of the 40%, for a total of 20% of the Rag2-GFP<sup>+</sup> cells found in the IgM<sub>high</sub> cultures after incubation with Ag = 26.7%). Because only 30% of viable IgM<sub>low</sub> cells up-regulate Rag2-GFP in the presence of sHEL, only 8% (30% × 26.7%) of the 40%, for a total of 20% of the Rag2-GFP<sup>+</sup> cells found in the IgM<sub>high</sub> cultures, could be derived from contaminating IgM<sub>low</sub> cells (Fig. 4A). Even in a worst case scenario, where all contaminating IgM<sub>low</sub> cells were viable, and 50% were Rag2-GFP<sup>+</sup>, they could only account for less than half of the Rag2-GFP<sup>+</sup> cells in the IgM<sub>high</sub> cultures. An expansion of contaminating IgM<sub>low</sub> cells is also very unlikely,
since there was no appreciable proliferation of cells in these Ag-treated cultures, as determined by \([\text{\textsuperscript{3}H}}\]thyidine incorporation or CFSE labeling (9) (data not shown). We conclude that IgM\textsuperscript{high} cells are capable of undergoing receptor editing in vitro in response to soluble self-Ag.

**Discussion**

The experiments described here clearly demonstrate the ability of IgM\textsuperscript{high} late immature B cells generated from HEL-Ig Tg bone marrow to undergo a receptor editing response following incubation with soluble Ag. These results contrast with the previously reported in vitro experiments in the 3-83 Tg system (8), which suggested significant developmental constraint on the editing process, by which IgM\textsuperscript{high} immature B cells were shown to be incapable of editing. There are a number of experimental factors that may account for the disparate results obtained in the two systems. First, these are very distinct Tg systems. The HEL-Ig BCR has a much higher affinity for HEL Ag (\(K_d = 10^{-7} \text{M}\)) than the 3-83 receptor has for class I MHC (\(K_d = 10^{-6} \text{M}\)), and it is possible that higher affinity interactions are required to potently initiate the signaling required to induce editing in IgM\textsuperscript{high} immature B cells. Arguing against this possibility is our finding that lower affinity Ag (HEL\textsuperscript{MUT}, \(K_d = 10^{-7} \text{M}\)) for the HEL-Ig receptor was also capable of inducing editing in IgM\textsuperscript{high} cells. Other factors relating to the Tg receptors, such as differences in BCR density and differing abilities to down-regulate BCR levels in response to Ag encounter, may also play a role.

A clear difference between the systems is the nature of the Ag. For the reported 3-83 experiments, the class I MHC Ag was recognized as an abundant, high density, membrane-bound Ag on Op42 stromal cells (8), while the experiments described here involve soluble self-Ag on S17 stromal cells. The avidity of the 3-83 receptor/Ag interaction together with unknown interactions between surface proteins on the Op42 stromal cell line and the IgM\textsuperscript{high} B cells may have combined to block the ability of these B cells to initiate an editing response. On the other hand, the S17 cell line in some fashion might remain permissive for receptor editing in IgM\textsuperscript{high} late immature B cells, particularly in response to the lower levels of cross-linking observed in the HEL system in response to soluble Ag. In experiments not shown we attempted to examine the editing of IgM\textsuperscript{high} immature B cells in response to membrane-bound HEL, by incubating sorted cells with BM stroma derived from membrane-bound HEL mice (3). However, these experiments were inconclusive due to the very small number of viable cells remaining at the end of a 2-day culture.

In summary, the current study suggests that the window of time available for immature B cells to revise their receptors following an encounter with self-Ag may extend to the late IgM\textsuperscript{high} stage, at least for certain Ags. Given the increased sensitivity of IgM\textsuperscript{high} cells to cell death, it is unlikely that these cells would have a lengthy period of time available for revising receptors after contact with self-Ag. However, this mechanism may allow for “11th hour” salvage of self-reactive receptors before B cell clonal deletion.

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**References**