The E\(\mu\) Enhancer Region Influences H Chain Expression and B Cell Fate without Impacting IgV\(\text{H}\) Repertoire and Immune Response In Vivo

Marie Marquet, Armand Garot, Sébastien Bender, Claire Carrion, Pauline Rouaud, Sandrine Lecardeur, Yves Denizot, Michel Cogné and Eric Pinaud

*J Immunol* published online 25 June 2014
http://www.jimmunol.org/content/early/2014/06/25/jimmunol.1302868

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/06/25/jimmunol.1302868.DCSupplemental

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Eμ Enhancer Region Influences H Chain Expression and B Cell Fate without Impacting IgVH Repertoire and Immune Response In Vivo

Marie Marquet,* Armand Garot,*1 Sébastien Bender,*†,1 Claire Carrion,*
Pauline Rouaud,* Sandrine Lecardeur,* Yves Denizot,* Michel Cogné,*‡ and
Eric Pinaud*

The IgH intronic enhancer region Eμ is a combination of both a 220-bp core enhancer element and two 310–350-bp flanking scaffold/matrix attachment regions named MARSEμ. In the mouse, deletion of the core-enhancer Eμ element mainly affects VDJ recombination with minor effects on class switch recombination. We carried out endogenous deletion of the full-length Eμ region (core plus MARsEμ) in the mouse genome to study VH gene repertoire and IgH expression in developing B-lineage cells. Despite a severe defect in VDJ recombination with partial blockade at the pre–B cell stage, Eμ deletion (core or full length) did not affect VιH gene usage. Deletion of this regulatory region induced both a decrease of pre–B cell and newly formed B cell compartments and a strong orientation toward the marginal zone B cell subset. Because IgM H chain expression was decreased in Eμ-deficient pre–B cells, we propose that modification of B cell homeostasis in deficient animals was caused by “weak” pre–B cell and BCR expression. Besides imbalances in B cell compartments, Ag-specific Ab responses were not impaired in animals carrying the Eμ deletion. In addition to its role in VDJ recombination, our study points out that the full-length Eμ region does not influence VH segment usage but ensures efficient Igμ-chain expression required for strong signaling through pre–B cells and newly formed BCRs and thus participates in B cell inflow and fate. The Journal of Immunology, 2014, 193: 000–000.

Immunoglobulin H chain expression is critical for several major branch points of B lymphocyte development and is linked to a complex program of Ig gene activation. This B cell development program coordinates transcription and accessibility to major DNA remodeling events, including intragenic recombinations, within the IgH locus. At early stages of B cell development and independently of Ag stimulation, VDJ recombination assemblies segments encoding the IgH V region. Ag-dependent activation of mature B cells can lead to a second type of rearrangement called class switch recombination (CSR) that allows B cells to express IgH chains with different constant regions without altering the V region specificity.

*Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7276, Université d’Limoges, 87025 Limoges, France; †Centre Hospitalier Universitaire Dupuytren, Centre de Référence des Amyloses, 87042 Limoges, France; and ‡Institut Universitaire de France, 75005 Paris, France

Received for publication October 25, 2013. Accepted for publication June 2, 2014.

This work was supported by Association pour la Recherche sur le Cancer Grant and grants from the Conseil Regional du Limousin.

The online version of this article contains supplemental material.

Abbreviations used in this article: cEμ, core-enhancer element of the Eμ regulatory region; CSR, class switch recombination; ES cell, embryonic stem cell; FO, follicular; GC, germinal center; KO, knockout; MAR, matrix attachment region; MZ, marginal zone; neoR, neomycin-resistant; PNA, peanut agglutinin; SLC, surrogate L chain.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

Published June 25, 2014, doi:10.4049/jimmunol.1302868

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302868
of the intronic enhancer region on IgVH segment usage and consequences on B cell repertoire.

Most of the mouse models targeting the Eμ region supported the conclusion that its activity was restricted to the pre-B cell stage (10–13, 17). In contrast, a mouse model carrying a pre-rearranged VDJ segment knocked in the IgH locus led to the suggestion that Eμ (full-length region) was needed at the pre-B cell stage to maintain efficient IgH allelic exclusion (18). In mature B cells, Eμ deletion in the endogenous locus decreases but does not abolish CSR, while being totally dispensable for somatic hypermutation (13, 19, 20). In the present study we report that endogenous deletion of the Eμ region (full-length Eμ including flanking MARs or Eμ alone) in the mouse germline, although affecting as expected VDJ recombination in pro-B cells, had no associated effect on IgVH gene repertoire. Pre-B and immature-transitional B cells revealed an impact of this deletion on H chain expression, likely responsible for the strong reduction of newly formed B cell inflow and biased B cell fate. This last finding corroborated very recent data published by Peng and Eckhardt (21) underlining a role for the Eμ enhancer in selection at the pre-B to immature B cell transition. Despite imbalanced peripheral B cell subsets, Ig isotype–specific immune responses to both T-dependent and T-independent Ags were not impaired in animals carrying the full-length Eμ deletion.

Materials and Methods

Full-length Eμ and eμ KO mice

to generate the full-length Eμ KO model, a dedicated targeting vector was provided by Dr. F.W. Alt (12). The 5’ homology arm was a 1.7-kbp HindIII-XbaI genomic DNA fragment located upstream from the 5’ MARs; the 3’ homology arm was a 1-kbp XbaI-PvuII genomic DNA fragment located downstream from the 3’ MARs. For in vitro selection, a neomycin-resistant (neoR) cassette, flanked by loxP sites, was inserted between homology arms. neoR gene expression was driven by the Herpes simplex thymidine kinase promoter. Moreover, upstream from the 5’ homology arm, a thymidine kinase gene, driven by the phosphoglucomutase promoter, was included to permit negative selection against random integration. The linearized vector was used to transfect the mouse embryonic stem (ES) cell line E14 by electroporation. Transfected clones were selected in 100 μg/ml G418 and 100 μg/ml hygromycin B. One appropriately targeted clone showing homologous recombination was injected into C57BL/6 blastocysts to generate chimeric mice. Successful germline transmission was checked by Southern blot. Offspring positive for heterozygosity (EμneoR) were mated with cre-expressing mice to induce deletion of the neoR gene. Efficient cre-mediated deletion was tested by Southern blot and PCR. The heterogeneous EμneoR mice were bred to obtain homozygous EμΔ1α mice. Studies on homozygous (EμΔ1α mice) were performed on animals maintained in a mixed 129/C57BL/6 background; such mutants were compared with a wild-type (wt) strain of the same background (mixed 129/C57BL/6) carrying two IgH alleles.

Animal facilities and ethics

Experimental (wt and mutant) animals were housed at 21–23°C with a 12-h light/dark cycle. All experiments, approved as part of a protocol registered under no. CREAL 7-07-2012, were performed according to the guidelines of the Comité Régional d’Éthique de l’Expérimentation Animale du Limousin.

Southern blots

Genomic Southern blots were performed as follows: 20 μg genomic DNA was digested with SacI or BamHI and submitted to electrophoresis on a 0.7% agarose gel. DNA was transferred to nylon membranes (MP Biomedicals, Santa Ana, CA) by capillarity. Blots were hybridized with [32P]-labeled probes generated by random priming. 5’ (X, 0.8-kbp SacI-SphI fragment) and 3’ (Y, 0.8-kbp XbaI-BamHI fragment) probes are shown in Fig. 1. For IgH rearrangement status, B spleenocytes were purified by magnetic sorting with B220-coupled beads (Miltenyi Biotec) and 20 μg genomic DNA was digested by StuI and hybridized with a 0.468-kbp HindIII-Nael fragment containing the Jα segment and with a 0.8-kbp XbaI-BamHI fragment specific to the μ C region for normalization.

PCR genotyping of mutant animals

PCR experiments for CRE-mediated deletion were conducted with a specific forward (5’-TGGGGCCATTCCTTAGATTG-3’) and two reverse primers (5’-AATAGGCTTGCACATGACC-3’ and 5’-CGTAACCTCTCCTTCA-GACC-3’) located, respectively, in eμ and the neoR gene. DNA was denatured 5 min at 94°C and then submitted to 33 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Amplification products were analyzed on a 2% agarose gel. Expected sizes of amplified products were 556 and 249 bp, respectively, for wt and EμneoR alleles. PCR products for Eμ full-length deletion were conducted with a specific forward (5’-TGGGGCATTCTTCATAGTTG-3’) and two reverse primers (5’-AA-TAGGCTTGCACATGACC-3’ and 5’-AACATAAGCGGAGGCCGAG-3’) located, respectively, in the core Eμ and the μ exom. DNA was denatured 5 min at 94°C and then submitted to 33 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Amplification products were analyzed on a 2% agarose gel. Expected sizes of amplified products were 556 and 369 bp, respectively, for wt and EμΔ1α alleles.

Flow cytometry

Single-cell suspensions from fresh organs were washed with 2% FCS-PBS. Lymphoid cells from bone marrow, spleen, peritoneal cavity, and Peyer’s patches were labeled with various Abs. For bone marrow cells, Abs recognizing the following markers were used: B220-V450, IgM-FITC, CD117-PE, CD43-PE, and IgM-PE. When used for surface staining, the IgM mAb (clone DS-1, BD Biosciences, San Diego, CA) recognizes the “a” IgM allotype expressed at the membrane as a component of the BCR, but it does not recognize the Igμ chain as a component of the pre-BCR. For spleen cells, Abs were directed against B220-V450, CD21-PE, CD23-FITC, IgM-PE, IgM-FITC, IgM-PE, IgD-FITC, and CD3e-FITC. For the peritoneal cavity, Abs were directed against B220-V450, IgM-PE, and CD43-PE. For Peyer’s patches, Abs were directed against B220-V450, B220-allophycocyanin, IgA-FITC, IgM-PE, peanut agglutinin (PNA)–FITC, FAS-PE (SouthernBiotec [Birmingham, AL], ebioscience, Sigma-Aldrich [St. Louis, MO]), and BD Biosciences. Flow cytometry analysis was performed on a BD LSRFortessa cell analyzer (BD Biosciences).

For intracellular staining, bone marrow B-lineage cells were enriched by positive sorting with magnetic beads coupled to anti-B220 Ab (Miltenyi Biotec) for 15 min at 4°C after treatment with anti-CD16/CD32 (BD Biosciences). 2 × 10^6 cells were stained for surface IgM with FITC-labeled anti-IgM (clone DS-1, BD Biosciences) and PE-labeled anti-CD25 (clone PC61.5, eBioscience). After washing, cells were treated with a Cytofix/Cytoperm kit (BD Biosciences) and intracellular staining was performed with allophycocyanin-labeled anti-IgM H chain (clone II/41, ebioscience).

IgVH repertoire study

Three groups of five mice of each genotype (129/Sv wt and EμΔ1α) were used for this study. Bone marrow cells were enriched for Hardy’s fractions C′ and D (mostly large and small pre-B cell populations) expressing CD25 after magnetic sorting (Miltenyi Biotec). DNA was extracted and IgVH repertoire diversity was examined using the ImmunIg test (ImmunID Technologies, Grenoble, France) as described in Rouaud et al. (22). Multiplex PCR assays were performed with forward primers specific for all functional members of a given Vh family and reverse primers specific for a given Jh segment (http://www.immunid.com). This assay allows the simultaneous detection and resolution of 92 V-D-J rearrangements. For semiquantitative analysis, PCRAs were stopped at their exponential step.

Transcription studies

Bone marrow-enriched pre-B cell fraction (mostly large and small pre-B cells) was prepared with a CD25 isolation kit (Miltenyi Biotec) according to the manufacturer’s recommendations. Resting B cells from spleen of Eμ full-length or wt mice were purified using B220-coupled beads from Miltenyi Biotec according to the manufacturer’s recommendations.

Total RNA was extracted using TRI Reagent (Ambion). Reverse transcription was performed by using a high-capacity cDNA reverse transcription kit (Applied Biosystems) on 1 μg total RNA. Priming for reverse
transcription was done with random hexamers. Real-time PCR using TaqMan Universal or SYBR Green Master mix (Applied Biosystems) was performed on cDNA samples (25 ng per reaction). Data were analyzed by comparing threshold cycle (Ct) values according to the \( 2^{\Delta\Delta Ct} \) method.

The IgH primary transcripts were analyzed with IgH wt TaqMan probe located just downstream from the J4 segment (described in Ref. 23). Mature IgH transcripts were amplified with the following primers: \( \mu \) membrane forward (in \( \mu \) exon 4), 5′-TGGAACTCGGAGAGACCTA-3′; \( \mu \) membrane reverse (in \( \mu \) exon 7), 5′-ATCTTCATCCATTCACCTA-3′; relative mRNA levels were normalized to \( \beta 2 m \) transcripts with \( \beta 2 m \) TaqMan probe (Mm00432423_m1, Applied Biosystems). Mature Ig \( \kappa \) transcripts were amplified with the following primers: sense \( \kappa \) reverse \( 5^{\prime} \)-TGGCTGCTGCTGCTG-3′ and antisense \( \kappa \) reverse \( 5^{\prime} \)-TGGCTGCTGCTGCTG-3′; relative mRNA levels were normalized to \( G a p d h \) transcripts with \( G a p d h \) TaqMan probe (Mm99999915_g1, Applied Biosystems).

**Total serum Ig quantification by ELISA**

Sera from 8-week-old EμΔκ and wt mice were analyzed for the presence of different IgH classes and subclasses by ELISA. Assays were performed in polycarbonate 96-multiswell plates (MaxiSorp; Nunc, Roskilde, Denmark) coated overnight at 4°C (100 μl/well) with suitable capture Abs diluted in 0.05 M Na2CO3 buffer (2 μg/ml for IgM, IgG1, IgG2a, IgG2b, 3 μg/ml for IgG3, and 4 μg/ml for IgG1 and IgE). After three successive washes in 0.1% Tween 20 PBS (washing buffer), a blocking step was performed for 30 min at 37°C in 3% BSA/PBS. After three washes, 50 μl sera (first diluted to 1:50), supernatants, or isotypic standard IgGs was diluted into successive wells in 1% BSA/PBS buffer and incubated for 2 h at 37°C. The mouse standard panel included antisera specific for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA (SouthernBiotech) and for IgE (BD Biosciences). After three washing steps, 100 μl/well appropriate conjugated Abs was added and adsorbed during 1.5 h at 37°C. Alkaline phosphatase–conjugated goat antisera specific for mouse Ig classes (SouthernBiotech) were used in 0.1% Tween 20, PBS buffer, either at 1 μg/ml for IgM, IgG1, IgG2a, IgG2b, and IgG3 or at 2 μg/ml for IgE and IgA. After washing, phosphatase alkaline activity was assayed on 1 mg/ml AP substrate (Sigma-Aldrich) and blocked with the addition of 3 M NaOH. Absorbance was then measured at 405 nm with a Xenius spectrophotometer (SAFAS, Monaco). ELISA assay comparisons between mice were done using the Student \( t \) test.

**Specific Ig isotype response upon Ag stimulation**

Animal cohorts included EμΔκ and control wt mice. Eight-week-old mice (n = 5) were used for each experiment. Immunization experiments were performed three times with T-dependent Ag and twice with T-independent Ag with independent groups of mice.

To test T-dependent Ab responses, animals were immunized i.p. with 10 μg hen egg OVA per animal (Sigma-Aldrich). The first immunization was given in 30% CFA (Sigma-Aldrich); 14 d later, a second immunization was administered in 50% IFA (Sigma-Aldrich). Immunized mice were bled at various intervals during the immunization protocol, and sera were analyzed for the presence of OVA-specific IgM, IgG1, IgG2a, and IgG2b by ELISA.

To test for T-independent Ab responses, mice were immunized i.p. with 50 μg animal 2,4-DNP–aminooethyl carbamoyl–Ficoll (Biosearch Technologies, Novato, CA). The first and second 2,4-DNP–aminooethyl carbamoyl–Ficoll immunizations (14 d later) were given in 50% IFA (Sigma-Aldrich). Animal sera were analyzed at various intervals during the immunization protocol for the presence of DNP-specific IgM and IgG3 by ELISA.

ELISA assays were performed in polycarbonate 96-multiswell plates (MaxiSorp; Nunc) coated overnight at 4°C with T-dependent or T-independent Ag. Control sera included sera from 8-week-old wt mice (50 μl/well egg OVA [Sigma-Aldrich] in 0.05 M Na2CO3 and 100 μl 10 μg/ml albumin-DNP [Sigma-Aldrich]) in 0.05 M Na2CO3, respectively). After three successive washing steps in 0.1% Tween 20 PBS (washing buffer), a blocking step was performed for 30 min at 37°C with 150 μl 100 μg/ml gelatin (Sigma-Aldrich) in PBS. After three washes, 50 μl assay sera or control serum (diluted in the appropriate range) was diluted into successive wells in 3 mg/ml gelatin in PBS and incubated 2 h at 37°C. The control serum consisted of a pool of sera from OVA– or DNP–Ficoll–immunized wt mice; the same control sera were used in all ELISA assays. After three washing steps, 100 μl/well appropriate conjugated Abs was added and adsorbed during 1.5 h at 37°C. Alkaline phosphatase–conjugated goat antisera specific for mouse Ig isotypes IgM, IgG1, IgG2a, and IgG3 (SouthernBiotech) were used at 1 μg/ml diluted in 0.1% Tween 20 PBS. After washing, alkaline phosphatase activity was assayed on 1 mg/ml AP substrate (Sigma-Aldrich) during 15 min at room temperature. Enzymatic reactions were stopped with 30 μl/well 3 M NaOH and absorbance was measured at 405 nm in a Xenius spectrophotometer (SAFAS).

For each serum, a ratio to the control sera (assayed on the same 96-multiswell plate) was calculated and gave the OVA-specific Ig titration. Each ELISA titration was done twice. ELISA assay comparisons between mice were done using the Student \( t \) test.

**Results**

**Replacement and deletion of the full-length Eμ region in the mouse germline**

The vector used for ES cell targeting (described in Ref. 12) replaces the 995-bp Xbal genomic fragment (encompassing both the core Eμ element and its flanking matrix attachment regions) with a pgk-neo gene flanked by loxp recombination target sequences (Fig. 1A, 1B). After transfection and selection of the E14 ES cell line, three independent clones were identified by Southern blot as correctly targeted by homologous recombination. Hybridization with 5′ and 3′ probes located outside the targeting construct revealed fragments of the size expected for the Eμneo allele, that is, replacement of the full-length Eμ region by the neoR cassette (Fig. 1B). Two of these clones were injected into C57BL/6 blastocytes and implanted into foster mothers to derive mouse somatic chimeras. Animals allowing germline transmission of the Eμneo mutant allele were bred with a transgenic mouse strain CMV-cre, 129/Sv background (24) to delete the pgk-neoR via loxp/cre recombination in the germline. Efficient and complete deletion of the pgk-neoR cassette in the offspring was also verified by Southern blot with the same probes (Fig. 1C). The allele carrying the deletion of the whole Eμ region was named EμΔκ. Heterozygous EμΔκ animals were bred to obtain the homozygous mouse line EμΔκ/Δκ. For the needs of this study, additional breeding to wt C57BL/6 was done to generate F1 heterozygous animals EμΔκ/Δκ.

**Deletion of the full-length Eμ region impairs but does not prohibit B cell development**

It was questionable whether endogenous deletion of the full-length Eμ region, larger than the previously described cEμ deletion (13), could support, even partially, complete B lineage development. In our study we compared B-lineage cell subsets in 2-mo-old 129/Sv wt and homozygous EμΔκ mice. Early B cell development was severely impaired but not abolished in EμΔκ animals. B-lineage cell numbers in the bone marrow were decreased ~2-fold (9 × 10⁶ compared with almost 21 × 10⁶ in wt; Tables I, II). EμΔκ mice harbored a partial blockade at pre–B to pre–B cell transition with an increased proportion and number of pre-pro–B precursors (220+/CD117+) and pre–B cells (IgM+ / B220+/CD43high) (Fig. 2A, Table I).

To evaluate impairment of VDJ recombination linked to deletion of the whole Eμ region, we analyzed IgM allotype expression by flow cytometry in bone marrow B-lineage cells and splenic B cells of F1 heterozygous EμΔκ/Δκ and EμΔκ/Δκ control mice. The total lack of IgM+ expressing cells observed in EμΔκ/Δκ animals (Fig. 2B, Table II) implied that the allele carrying Eμ deletion was always nonfunctional, likely due to an impaired VDJ recombination process.

Southern blot analysis performed on purified splenic B cell DNA confirmed a VDJ recombination defect in EμΔκ/Δκ animals; indeed, all DNA samples from mutant B cells retained a germline IgH restriction fragment (3.9 kb) whereas all wt samples did not (Fig. 2C). This simple assay revealed that an important proportion of IgH genes in homozygous mutant animals B cells was unarranged, in contrast to normal peripheral B cells that have almost all undergone D(μ) to J(μ) recombination on both alleles (25). This finding that full-length Eμ deletion impaired VDJ recombination was not surprising but entirely consistent with previous models of endogenous deletions that encompass at least the cEμ region.
However, the fact that the entire B cell development could be completed in the absence of the full-length Eμ region was not reported in the context of the endogenous locus devoid of any prerearranged VDJ exon. Animals devoid of full-length or core Eμ region harbor a diversified preimmune IgH repertoire

Because deletion of the full-length Eμ region in the mouse germline did not fully block B cell development and allowed significant levels of VDJ recombination, the preimmune IgVH repertoire could be examined in bone marrow precursor B cells to avoid a bias generated by Ag selection. Enrichment in pre–B cells was a mandatory step for this experiment because this population is drastically reduced in Eμ-deficient mice. For this purpose, isolation of bone marrow Hardy’s fraction C9 and D (large and small pre–B cells) was performed by magnetic sorting based on surface expression of CD25 as previously described (22), resulting in 70–90% enrichment of the population of interest. A 92-primer combination semiquantitative PCR assay, performed on DNA prepared from enriched pre–B cell samples, amplified VDJ rearrangements occurring between a broad variety of IgVH segments (including distal, middle, and proximal segments; see Fig. 3A, 3C) to the four JH segments. The ratio of observed amplifications to theoretical amplifications estimates the diversity of VDJ rearrangements. Even if not fully quantitative, this PCR rearrangement assay referenced as Immun’Ig test previously provided a clear estimation of both VDJ recombination efficiency and VH gene usage based on ImMunoGeneTics classification (http://www.imgt.org) in KO mice models (22). The study was first performed by comparing the repertoires of wt 129/Sv and EμΔ/Δ mice (Fig. 3) and second on wt 129/Sv and cEμΔ/Δ mice as an additional comparison (Supplemental Fig. 1). Consistent with the VDJ recombination defect observed in both EμΔ/Δ (13) and Eμλ/λ (this study) B-lineage cells, the Immun’Ig test revealed lower rearrangement efficiency in pre–B cells devoid of the Eμ region when compared with wt pre–B cells (Fig. 3B, Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, upper panel). In EμΔ/Δ all distal (VH1 family, formerly called VHJ558) and middle (VH3, VH4, and VH7–16 families) VH segments were represented in proportions similar to wt (Fig. 3D, lower panel). When performed on cEμ mice, the repertoire assay also revealed normal usage of most IgVH families, with all being represented in proportions similar to wt (Supplemental Fig. 1B). Nevertheless, when IgVH family frequencies were compared with their wt counterparts, EμΔ/Δ pre–B cells harbored a diversified IgVH preimmune repertoire; most distal, middle, and proximal VH segments were significantly rearranged (Fig. 3B, 3D, lower panel).
gene targeting) known to display a 120-kbp deletion within the proximal V_{H} region (28). Indeed, usage of proximal V_{H} segments was normal in E_{M}-deficient mice (Supplemental Fig. 1D), probably because the cE_{M} deletion was performed in TC1 ES cells that derive from the 129/Sv strain (13). Overall, repertoire studies did not reveal any obvious differences in IgV_{H} segment usage in the absence of the E_{M} region.

Full-length E_{M} deletion impairs IgH expression in pre-B and newly formed B cells

At the cellular level, the developmental defect observed in E_{M}^{ΔΔ} mutant mice induced both a strong blockade at the pre-B cell stage (IgM+ /B220+/CD43high) and a dramatic reduction (∼5-fold) of the absolute number of pre-B cell precursors (IgM+/ /B220+/CD43low, Table I). If the pro-B cell accumulation could easily be linked to the VDJ recombination defect, the decrease in the pre-B cell compartment was surprising and unsolved. A reasonable explanation for such reduced pre-B cell numbers was a lack of pre-B cell compartment expansion due to impaired pre-BCR signaling (for review, see Refs. 29–31; also, see Discussion). Other studies have reported similar drastic pre-B cell depletion in mice devoid of SLC components that express directly an IgM BCR complex at the pre-B cell stage (32–34), but also in a mouse model carrying a “hypomorphic IgH-chain” expressing low levels of the Ig_{M}-chain at the pre-B cell stage (35). We then sought to verify whether deletion of the E_{M} full-length region could lead to similar effects: premature expression of IgM BCR or decreased expression of pre-BCR?

Because E_{M} full-length deletion affected VDJ recombination efficiency, it was possible that delayed H chain rearrangements in E_{M}^{ΔΔ} precursors led to premature IgL chain rearrangements. In the first case, a functional IgL chain could compete with SLCs and could eventually lead to premature expression of a complete IgM-BCR, similar to what was observed in SLC-deficient mice (32–34). In this case, mimicking SLC-deficient models, “IgM-expressing pre-B-like cells” could be pushed toward immature B cell stages. To test this hypothesis we first analyzed, in sorted wt and E_{M}^{ΔΔ} pre-B cells, the proportion of cells expressing intracellular L chains by flow cytometry. Although reduced in numbers, E_{M}^{ΔΔ} pre-B cells did not display increased proportions of Igk-expressing cells (Fig. 4A), suggesting that IgL chain rearrangements were not occurring prematurely in the absence of the full-length E_{M} region. To confirm this, we looked for the presence of N nucleotide insertions in rearranged L chain junctions, a hallmark of TdT enzyme activity expressed mostly at the pre-B cell stage (36, 37). When compared to wt controls, Vk-Jk junctions cloned out of E_{M}^{ΔΔ} bone marrow CD25+ precursors did not show increased proportions of N nucleotide insertions, suggesting that L chain rearrangements did not occur prematurely

| Table I. Absolute numbers of B-lineage cell subsets in bone marrow, spleen, peritoneal cavity, and Peyer’s patches of 8-wk-old wt and E_{M}^{ΔΔ} mice |

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>wt Mice (×10⁶)</th>
<th>E_{M}^{ΔΔ} Mice (×10⁶)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>50.78 ± 2.79 (6)</td>
<td>37.18 ± 3.25 (6)</td>
<td>0.015</td>
</tr>
<tr>
<td>B220⁺/IgM⁺</td>
<td>20.87 ± 3.69 (6)</td>
<td>9.00 ± 1.60 (6)</td>
<td>0.015</td>
</tr>
<tr>
<td>IgM⁺/B220⁺/CD43low pre-B cells (×10⁶)</td>
<td>9.24 ± 2.12 (4)</td>
<td>1.84 ± 0.45 (4)</td>
<td>0.014</td>
</tr>
<tr>
<td>B220⁺/CD117⁺ cells (×10⁶)</td>
<td>3.61 ± 0.68 (4)</td>
<td>7.46 ± 1.05 (4)</td>
<td>0.022</td>
</tr>
<tr>
<td>B220⁺/CD117⁻ cells (×10⁶)</td>
<td>1.65 ± 0.23 (6)</td>
<td>2.63 ± 0.33 (6)</td>
<td>0.035</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>134.30 ± 9.80 (11)</td>
<td>68.85 ± 6.60 (11)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B220⁺/IgM⁺</td>
<td>56.39 ± 5.71 (11)</td>
<td>13.21 ± 1.90 (11)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgD⁺/B220⁺/IgM⁺ cells (×10⁶)</td>
<td>35.64 ± 3.99 (6)</td>
<td>10.50 ± 3.23 (6)</td>
<td>0.001</td>
</tr>
<tr>
<td>IgD⁺/B220⁺/IgM⁺ cells (×10⁶)</td>
<td>35.84 ± 3.57 (4)</td>
<td>10.37 ± 3.87 (4)</td>
<td>0.009</td>
</tr>
<tr>
<td>B220⁺/CD21high/CD23⁺ MZ B cells (×10⁶)</td>
<td>7.24 ± 1.05 (9)</td>
<td>4.65 ± 0.94 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>B220⁺/CD21high/CD23high FO B cells (×10⁶)</td>
<td>42.01 ± 4.29 (9)</td>
<td>5.32 ± 0.86 (9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Peritoneal cavity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>2.90 ± 0.38 (6)</td>
<td>3.36 ± 0.47 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>B220⁺/CD117⁺ cells (×10⁶)</td>
<td>1.40 ± 0.30 (6)</td>
<td>1.24 ± 0.18 (7)</td>
<td>NS</td>
</tr>
<tr>
<td>CD5⁺/IgM⁺ cells (×10⁶)</td>
<td>0.34 ± 0.07 (6)</td>
<td>0.53 ± 0.08 (7)</td>
<td>NS</td>
</tr>
<tr>
<td>Peyer’s patches</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>11.36 ± 1.82 (6)</td>
<td>6.39 ± 0.77 (6)</td>
<td>0.0306</td>
</tr>
<tr>
<td>B220⁺/CD117⁺ cells (×10⁶)</td>
<td>7.11 ± 0.97 (6)</td>
<td>1.76 ± 0.29 (6)</td>
<td>0.0004</td>
</tr>
<tr>
<td>B220⁺/IgM⁺ cells (×10⁶)</td>
<td>0.72 ± 0.14 (5)</td>
<td>0.58 ± 0.06 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>B220⁺/IgM⁺ cells (×10⁶)</td>
<td>5.56 ± 1.02 (5)</td>
<td>0.75 ± 0.16 (6)</td>
<td>0.0006</td>
</tr>
<tr>
<td>B220⁺/IgM⁺/Igα⁺/Igβ⁺ naive B cells (×10⁶)</td>
<td>5.01 ± 0.78 (6)</td>
<td>0.75 ± 0.13 (6)</td>
<td>0.0003</td>
</tr>
<tr>
<td>B220⁺/PNAb/A/PNS⁺ naive B cells (×10⁶)</td>
<td>1.27 ± 0.19 (6)</td>
<td>0.83 ± 0.15 (6)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Means ± SEM are reported; significance was assessed by a Student t test.

| Table II. Absolute numbers of B-lineage cell subsets expressing IgM⁺ or IgM⁻ allotypes in bone marrow and spleen of heterozygous 8-wk-old E_{M}^{ΔΔ}/wt and wt/wt mice |

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>wt⁻/wt⁺ Mice (n)</th>
<th>E_{M}^{ΔΔ}/wt⁻ Mice (n)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220⁺/IgM⁺ cells (×10⁶)</td>
<td>2.90 ± 0.62 (3)</td>
<td>0.06 ± 0.01* (3)</td>
<td>0.0104</td>
</tr>
<tr>
<td>B220⁺/IgM⁻ cells (×10⁶)</td>
<td>2.91 ± 0.66 (3)</td>
<td>5.25 ± 0.62 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220⁺/IgM⁺ cells (×10⁶)</td>
<td>11.70 ± 1.58 (3)</td>
<td>0.03 ± 0.01* (3)</td>
<td>0.0018</td>
</tr>
<tr>
<td>B220⁺/IgM⁻ cells (×10⁶)</td>
<td>9.31 ± 0.71 (3)</td>
<td>22.22 ± 1.81 (3)</td>
<td>0.0027</td>
</tr>
</tbody>
</table>

Means ± SEM are reported; asterisks indicate values below background; significance was assessed by a Student t test.
and B220+CD43low (pre-B) cell populations are shown after staining with anti-IgMa Abs and VDJ recombination. (IgMa-negative population to exclude BCR-expressing cells. B220+IgMlow/+ (immature), B220+/highIgMhigh (transitional), and B220high (pro- and pre-B), and structure. Third row Bone marrow B-lineage cells defined as B220+IgM high/low (pre-B and transitional I B cells from the spleen (CD19+/CD23-/CD21+) showed a clear reduction of IgM expression level in the absence of the Eμ region (Fig. 4B, top right panel). Our data clearly indicated that deletion of the whole Eμ region led to decreased expression of the newly generated IgM H chain in pre–B and newly formed B cells. We also evaluated IgH expression in mature naive B cells subsets from the spleen by surface staining and FACS analysis. In contrast to pre–B and transitional I B cells, splenic marginal zone (MZ) and follicular (FO) B cells subsets from wt and EμΔ/Δ animals expressed similar levels of surface IgM (Fig. 4B, bottom panels), confirming that the full-length Eμ region no longer modulated IgH expression in mature B cells.

To determine whether transcription variations accounted for the observed decrease in IgM protein expression in pre–B cells, IgH primary transcripts were monitored by quantitative RT-PCR on RNA prepared from either a bone marrow–enriched pre–B cell fraction or resting splenic B cells. Once normalized to the Cd79a gene encoding the Igα molecule, both the primary IgH transcript initiated at the promoter of the rearranged VDJ segment (monitored with a probe located in the intron downstream from the JH4 segment) and the mature transcript encoding the membrane form of the IgM–chain were decreased in pre–B cells sorted from EμΔ/Δ animals (Fig. 4C). The same quantitative RT-PCR assays performed on RNA from splenic resting mature B cells showed that both primary and mature IgM H chain transcripts were found in similar proportions (Fig. 4C). This confirmed that the full-length Eμ region modulates H chain transcription and expression specifically at the pre–B and transitional cell stages of B cell development.

FIGURE 2. Full-length Eμ deletion leads to impaired B cell development and VDJ recombination. (A) Bone marrow cells of wt and EμΔ/Δ mice were stained with fluorescent Abs. Top row, B220+CD43high (pro-B) and B220+CD43low (pre-B) cell populations are shown after staining with V450–anti-B220, FITC–anti-IgMa, and PE–anti-CD43 Abs, gated on the IgM-negative population to exclude BCR-expressing cells. Second row, Bone marrow B-lineage cells defined as B220+IgM+ (pro- and pre-B), B220+IgMhigh (immature), B220+IgMlow (transitional), and B220+IgM high IgM+ (mature recirculating) were stained with V450–anti-B220, FITC–anti-IgMa, and PE–anti-IgMb Abs and gated on developing lymphocytes based on their size and structure. Third row, B220+/CD117+ (pro-pre– and pro–B cells) and B220+/CD117− (pre–B, immature, transitional, and recirculating B cells) were stained with V450–anti-B220 and PE–anti-CD117 Abs and gated on developing lymphocytes based on their size and structure. Fourth row, IgM allotype expression in bone marrow cells from heterozygous mutant EμΔ/Δwta/wtb (129/C57BL/6 F1) animals. Cells were labeled with allophtoocyain–anti-B220, FITC–anti-IgM+, and PE–anti-IgMb Abs and gated on developing lymphocytes based on their size and structure. (B) IgM allotype expression in splenic B cells from heterozygous mutant EμΔ/Δwta/wtb and control wta/wtb (129/C57BL/6 F1) animals. Cells were labeled with allophtoocyain–anti-B220, FITC–anti-IgM+, and PE–anti-IgMb Abs and monitored on developing lymphocytes based on their size and structure. (C) Estimation of IgH allele rearrangement status in peripheral B cells. DNA prepared from positively sorted splenic B cells was digested with Stul. Fragments corresponding to the germline configuration (4.9 and 3.9 kbp, respectively, for wt and EμΔ alleles) were detected by Southern blotting using a 0.47-kbp HindIII-NaeI fragment containing the JH4 segment as a probe. On the same blot, DNA normalization was performed by hybridization of the 5.3-kbp unrearranged μ C region using a 0.8 kbp XhoI-BamHI fragment as a probe.
Deletion of the full-length Eμ region modifies peripheral B cell populations

Eight-week-old EμΔ/Δ mice displayed a drastic reduction in the number of peripheral B cells in the spleen and Peyer’s patches (~25% of the absolute number when compared with wt, Table I). This B cell lymphopenia is consistent with the drastic reduction of the B/T cell ratio observed by flow cytometry (Fig. 5A). Such an overall defect in peripheral mature B cells has also been observed in animals carrying germline deletion of the cEμ enhancer region (13).

In the peritoneal cavity, despite a decreased proportion of CD5+ B cells (Fig. 5B), absolute numbers of the different B cell subsets were not significantly affected in EμΔ/Δ mice (Table I).

In Peyer’s patches of EμΔ/Δ mice, when the proportion of IgA-expressing B cells was increased (35% in mutants compared with 15% in wt, Fig. 5C), the absolute number of such switched cells remained comparable to wt counts (Table I). In contrast, both IgM+ cell proportions and counts were drastically decreased (Fig. 5C, Table I). Collectively, these data suggested a strong reduction in the inflow of mature B cells in Peyer’s patches. Independently, IgA-switched cells did accumulate normally in this organ, suggesting that class switching to IgA was not affected. Examination of germinal center (GC) B cells and their naive counterparts in Peyer’s patches corroborated this hypothesis: mutant animals displayed reduced proportions and numbers of PNA+i/FAS−low naïve B cells.
(mostly) in favor of cells undergoing GC reaction PNA\textsuperscript{high}/FAS\textsuperscript{high} that displayed increased proportions but remained unchanged in numbers (Fig. 5C, Table I).

Interestingly, both proportion and absolute numbers of FO B cells (B220\textsuperscript{+}/CD21\textsuperscript{+}/CD23\textsuperscript{high}) were strikingly reduced when the Em\textsuperscript{Δ} allele supported B cell development (Fig. 5A, bottom panel, Table I). In contrast to the FO subset diminishment, the number of splenic MZ B cells (B220\textsuperscript{+}/CD21\textsuperscript{high}/CD23\textsuperscript{+}) was normal in Em\textsuperscript{Δ}/Δ mice (Table I) such that their proportion was increased relative to FO cells (Fig. 5A, bottom panel).
The lack of the full-length Eμ region led to a noticeable decrease in peripheral functional B cells, especially FO B cells, with most resting EμΔμ peripheral B cells displaying an MZ phenotype. The decreased Igμ H chain expression found in EμΔμ bone marrow precursor B cells could explain such consequences in peripheral organs (see Discussion).

EμΔμ animals exhibit normal Ab production and immune responses in vivo

Serum Ab quantification in 8-wk-old EμΔμ animals revealed a modest but significant effect on the major circulating isotypes IgM and IgG1. When compared with wt mice, homozygous mutant mice showed a 2-fold increase in serum IgM (1534 ± 306 μg/mL for mutants, 646 ± 97 μg/mL for wt) and a 3-fold decrease in serum IgG1 (174 ± 29 µg/mL for mutant, 576 ± 103 µg/mL for wt) (Fig. 6A). For all other isotypes, no significant differences were observed using the Student t test.

Two independent cohorts of homozygous mutant animals and age-matched wt controls were immunized with T-independent (DNP-Ficoll) and T-dependent (OVA) Ags to evaluate Ag-specific immune responses. After DNP-Ficoll immunization, IgM and IgG3 Ab responses against DNP were measured by ELISA in the serum of challenged mice. In this case, EμΔμ mutant mice responded as well as did their wt counterparts (Fig. 6B). Similarly, upon OVA challenge, IgM, IgG1, and IgG2a responses were quantified by ELISA in mouse sera. For these T-dependent Ags, no significant differences were observed between wt and EμΔμ mice (Fig. 6C). Immunization with both Ags revealed that EμΔμ mutant mice exhibited normal Ag response in vivo.

Discussion

Numerous mutant models have been created to study the role of the Eμ intronic enhancer region, each one providing a part of the puzzle. Because our model of full-length Eμ deletion is the first, to our knowledge, carried out in the germline, it specifies some new insights into multiple functions of the intronic enhancer at early stages of B cell development. Our study confirmed that the full-length Eμ region is required to initiate efficient DΗ to JΗ rearrangements: first, in a heterozygous context (EμΔμhΔμ animals), B cell development was supported by the wt IgH locus only; second, homozygous mutant animals exhibited a severe (but incomplete) blockade at the pre-B to pre-B cell transition and their peripheral B cells retained unrearranged IgH alleles. Indeed, these consequences of initial steps of VDJ recombination were expected and reminiscent of models devoid of either cEμ alone or both the 5′ MAR plus cEμ (13, 14). Despite this blockade, full-length Eμ deletion did not abrogate VDJ recombination. Some B cells arose in the peripheral organs but in significantly decreased numbers, arguing for a strong reduction in the dynamic inflow of B cells able to give birth to peripheral B cells.

One notable finding of our study is that the decreased efficiency of VDJ recombination does not result in any compromised Ig VH region usage. This specific point is somehow unexpected because Eμ has long been suggested to participate in the long-distance control of accessibility along the V gene cluster, a prerequisite for the V to DJH recombination step (10–12). More recently, KO models in the mouse germline refined the activity of the core Eμ enhancer region as a positive regulatory element for D to J

The Journal of Immunology 9

Discussion

Numerous mutant models have been created to study the role of the Eμ intronic enhancer region, each one providing a part of the puzzle. Because our model of full-length Eμ deletion is the first, to our knowledge, carried out in the germline, it specifies some new insights into multiple functions of the intronic enhancer at early stages of B cell development. Our study confirmed that the full-length Eμ region is required to initiate efficient DJH to JH rearrangements: first, in a heterozygous context (EμΔμhΔμ animals), B cell development was supported by the wt IgH locus only; second, homozygous mutant animals exhibited a severe (but incomplete) blockade at the pre-B to pre-B cell transition and their peripheral B cells retained unrearranged IgH alleles. Indeed, these consequences of initial steps of VDJ recombination were expected and reminiscent of models devoid of either cEμ alone or both the 5′ MAR plus cEμ (13, 14). Despite this blockade, full-length Eμ deletion did not abrogate VDJ recombination. Some B cells arose in the peripheral organs but in significantly decreased numbers, arguing for a strong reduction in the dynamic inflow of B cells able to give birth to peripheral B cells.

One notable finding of our study is that the decreased efficiency of VDJ recombination does not result in any compromised Ig VH region usage. This specific point is somehow unexpected because Eμ has long been suggested to participate in the long-distance control of accessibility along the V gene cluster, a prerequisite for the V to DJH recombination step (10–12). More recently, KO models in the mouse germline refined the activity of the core Eμ enhancer region as a positive regulatory element for D to J

The lack of the full-length Eμ region led to a noticeable decrease in peripheral functional B cells, especially FO B cells, with most resting EμΔμ peripheral B cells displaying an MZ phenotype. The decreased Igμ H chain expression found in EμΔμ bone marrow precursor B cells could explain such consequences in peripheral organs (see Discussion).

EμΔμ animals exhibit normal Ab production and immune responses in vivo

Serum Ab quantification in 8-wk-old EμΔμ animals revealed a modest but significant effect on the major circulating isotypes IgM and IgG1. When compared with wt mice, homozygous mutant mice showed a 2-fold increase in serum IgM (1534 ± 306 μg/mL for mutants, 646 ± 97 μg/mL for wt) and a 3-fold decrease in serum IgG1 (174 ± 29 µg/mL for mutant, 576 ± 103 µg/mL for wt) (Fig. 6A). For all other isotypes, no significant differences were observed using the Student t test.

Two independent cohorts of homozygous mutant animals and age-matched wt controls were immunized with T-independent (DNP-Ficoll) and T-dependent (OVA) Ags to evaluate Ag-specific immune responses. After DNP-Ficoll immunization, IgM and IgG3 Ab responses against DNP were measured by ELISA in the serum of challenged mice. In this case, EμΔμ mutant mice responded as well as did their wt counterparts (Fig. 6B). Similarly, upon OVA challenge, IgM, IgG1, and IgG2a responses were quantified by ELISA in mouse sera. For these T-dependent Ags, no significant differences were observed between wt and EμΔμ mice (Fig. 6C). Immunization with both Ags revealed that EμΔμ mutant mice exhibited normal Ag response in vivo.

Discussion

Numerous mutant models have been created to study the role of the Eμ intronic enhancer region, each one providing a part of the puzzle. Because our model of full-length Eμ deletion is the first, to our knowledge, carried out in the germline, it specifies some new insights into multiple functions of the intronic enhancer at early stages of B cell development. Our study confirmed that the full-length Eμ region is required to initiate efficient DJH to JH rearrangements: first, in a heterozygous context (EμΔμhΔμ animals), B cell development was supported by the wt IgH locus only; second, homozygous mutant animals exhibited a severe (but incomplete) blockade at the pre-B to pre-B cell transition and their peripheral B cells retained unrearranged IgH alleles. Indeed, these consequences of initial steps of VDJ recombination were expected and reminiscent of models devoid of either cEμ alone or both the 5′ MAR plus cEμ (13, 14). Despite this blockade, full-length Eμ deletion did not abrogate VDJ recombination. Some B cells arose in the peripheral organs but in significantly decreased numbers, arguing for a strong reduction in the dynamic inflow of B cells able to give birth to peripheral B cells.

One notable finding of our study is that the decreased efficiency of VDJ recombination does not result in any compromised Ig VH region usage. This specific point is somehow unexpected because Eμ has long been suggested to participate in the long-distance control of accessibility along the V gene cluster, a prerequisite for the V to DJH recombination step (10–12). More recently, KO models in the mouse germline refined the activity of the core Eμ enhancer region as a positive regulatory element for D to J

The Journal of Immunology 9

Discussion

Numerous mutant models have been created to study the role of the Eμ intronic enhancer region, each one providing a part of the puzzle. Because our model of full-length Eμ deletion is the first, to our knowledge, carried out in the germline, it specifies some new insights into multiple functions of the intronic enhancer at early stages of B cell development. Our study confirmed that the full-length Eμ region is required to initiate efficient DJH to JH rearrangements: first, in a heterozygous context (EμΔμhΔμ animals), B cell development was supported by the wt IgH locus only; second, homozygous mutant animals exhibited a severe (but incomplete) blockade at the pre-B to pre-B cell transition and their peripheral B cells retained unrearranged IgH alleles. Indeed, these consequences of initial steps of VDJ recombination were expected and reminiscent of models devoid of either cEμ alone or both the 5′ MAR plus cEμ (13, 14). Despite this blockade, full-length Eμ deletion did not abrogate VDJ recombination. Some B cells arose in the peripheral organs but in significantly decreased numbers, arguing for a strong reduction in the dynamic inflow of B cells able to give birth to peripheral B cells.

One notable finding of our study is that the decreased efficiency of VDJ recombination does not result in any compromised Ig VH region usage. This specific point is somehow unexpected because Eμ has long been suggested to participate in the long-distance control of accessibility along the V gene cluster, a prerequisite for the V to DJH recombination step (10–12). More recently, KO models in the mouse germline refined the activity of the core Eμ enhancer region as a positive regulatory element for D to J...
recombination (13, 14), replacing the observed V to DJ recombination defect as an outcome of the previous defect (14). Stepwise IgH accessibility to VDJ recombination has also been proven to be dependent on the core Eμ enhancer element (16), although the RAG-deficient backgrounds used for such studies could only report on chromatin status in unrearranged precursor B cells. Current models propose that modifications of three-dimensional IgH structures in precursor B cells (loops and locus contraction) orchestrate VDJ recombination (38, 39). Sense and antisense germline transcription within IgVH regions has been observed prior VH to DJH rearrangement and is proposed to regulate accessibility of such regions (40, 41). Beyond germline transcription accessibility, recombination of distal VH segments is modulated by several chromatin-modifying enzymes such as EZH2 (42, 43) or transcription factors including PAX5, IKAROS, and YY1 (44–46). YY1 was shown to bind Eμ (47) and was proposed to mediate VH region contraction through this interaction (45); beyond its effect within the IgH locus, YY1 has also recently been shown to interact with EZH2 and modulate the Igκ L chain repertoire in developing B cells (48). Supporting this hypothesis, a recent study reported YY1-dependent long-range interactions in RAG-deficient pro-B cells between Eμ and some PAIR elements located in distal VH regions (49). It was then tempting to speculate that Eμ could regulate usage of distal VH segments through long-distance interactions mediated by YY1. Surprisingly, our study clearly demonstrated that this is not the case because both full-length Eμ and cEμ-deficient pre-B cells display similar proportions of rearranged distal VH1 family segments (also known as VHJ558) to wt pre-B cells. According to our results, it is likely that VH segments (including distal families) come into close proximity to the DJH rearranged segment independently of the full-length Eμ region (with both MARs and the cEμ enhancer being dispensable). Although we cannot assert that physical interactions do not exist between Eμ and accessible VH segments, our findings can be interpreted, in a provocative hypothesis, that Eμ-dependent loops are irrelevant for V to DJ recombination. This hypothesis is sustained by studies carried out in bone marrow RAG-deficient precursors devoid of either cEμ alone or cEμ plus its 5’ MAR, showing that the Eμ region does not influence germline transcription of distal VH segments (15, 16, 49). As an alternative looping process, V to JH linking (in RAG-deficient precursors) could be the result of CTCF/cohesin-mediated loops (28, 39, 50) (because this complex does not bind to the Eμ region). Another hypothesis could be that YY1 does enhance physical interactions between V and DJ segments but that anchoring of the rearranged DJH segment does not take place within the Eμ region (39, 49).

Regarding B-lineage cell homeostasis, deletion of the full-length Eμ region in the mouse germline specifies a critical role for Eμ in rearranged H chain transcription (and expression) in B-lineage precursors. Our study extends the window of activity for the

### FIGURE 6.

Serum Ig and Ag-specific responses in wt and EμΔΔ animals. (A) Analysis of serum Ig isotype by ELISA for Ig secretion in 8-wk-old mice sera. Wt littermates are shown on the left part of graphs, followed by homozygous EμΔΔ. Means and SEM are indicated for each case. Significant differences are indicated with p values, according to the Student t test. (B) Specific-Ab response in serum of wt and EμΔΔ mice immunized with DNP-Ficoll. Ab levels, detected by ELISA, are expressed in arbitrary units by comparison with a control serum. Time after immunization is indicated in days. Arrows correspond to Ag injection at days 1 and 14. Each point is the mean (±SEM) of serum determinations from five mutant and five wt mice. One representative experiment out of two is shown. (C) Specific-Ab response in the serum of wt and EμΔΔ mice immunized with OVA Ab levels, detected by ELISA, are expressed in arbitrary units by comparison with a control serum. Time after immunization is indicated in days. Arrows correspond to Ag injection at days 1 and 14. Each point is the mean (±SEM) of serum determinations from five mutant and five wt mice. One representative experiment out of three is shown.
Eμ region beyond the pre-pro-B and pro-B cell stages where VDJ rearrangements take place, with substantial activity at the pre-B, immature B, and transitional B cell stages. This emphasizes an important role for this regulatory element on pre-BCR and newly formed BCR expression, with consequences on pre-B cell compartment expansion.

As a reasonable hypothesis, the reduced μ-chain expression at the pre-B cell stage (in newly formed large pre-B cells corresponding to Hardy’s C’ fraction) observed in our model would lead to decreased surface pre-BCR complexes with, as a consequence, impaired pre-B cell clonal proliferation and, hence, reduced numbers of precursors able to rearrange L chains. However, our model points out that L chain rearrangements (at least κ-chains) do not occur prematurely in the absence of the Eμ region. This confirms that κ loci rearrangements occur in an autonomous manner and are driven by their own regulatory elements. At the pro-B cell stage, lack of the Eμ region led to a modest but significant decrease in μ-chain transcription (2- and 3-fold decrease for primary and mature IgH transcripts, respectively), similar to what was observed in mouse models carrying pre-rearranged B1-8 VH genes published by Eckhardt and colleagues (18, 21). Using these dedicated models, Li and Eckhardt (18) proposed that the Eμ region is necessary to maintain allelic exclusion, providing a sufficient level of expression of the newly assembled IgH genes. In a very recent study using the same knock-in mouse models, Peng and Eckhardt (21) also demonstrated that the Eμ region influenced H chain expression in pre-B cells and pointed out a role for this region in pre-B to immature B cell transition. Interestingly, an Eμ-deficient B1-8 allele did not display any H chain expression defect at immature B cell stages (21). In contrast, our present study points out a clear decrease in H chain expression in transitional B cells upon full-length Eμ deletion (Fig. 4B). Indeed, although we could not evaluate immature bone marrow B cells, which were almost absent in Eμ-deficient animals, we clearly showed decreased surface IgM expression in Eμ-deficient transitional (T1) spleen B cells. It can thus reasonably be postulated that a similar decrease affects the immediately upstream immature bone marrow B cells. One possible explanation for such a striking difference could be that the Eμ-deficient B1-8 allele remains unrearranged and therefore might retain some regulatory elements or chromatin marks that could influence H chain expression at immature stages. Additionally, normal H expression in Eμ-deficient B1-8 immature B cells might be linked to the particular B1-8 transgene-associated promoter whose expression might be differently influenced by Eμ at this stage of B cell development. Although low pre-BCR expression (similar to our model) led to decreased signaling, Eμ-deficient B1-8 mice displayed normal pre-B cell counts (18, 21). This last finding was quite unexpected because efficient pre-BCR expression and signaling are required for the burst of pre-B cell proliferation (29, 51). This point is difficult to interpret in a model where B cell development is driven by a single type of H chain. Unlike Eμ mutations in the B1-8 model, our full-length Eμ KO animals obviously displayed a drastic decrease in pre-B cell numbers. The origin of this defect remains difficult to identify because of the scarcity of such cells. Nonetheless, several hypotheses can be considered. First, this decrease could mainly originate from the severe but incomplete blockade at the pre-pro-B cell transition as a consequence of VDJ recombination. In this case, only very few pro-B cells undergoing IgH functional VDJ rearrangements could enter the pre-B compartment. Despite pre-B cell clonal expansion, this would keep pre-B cell numbers quite low and this shortage would last until immature stages. As a second hypothesis, VDJ recombination blockade might not be as drastic, but the limited numbers of pre-B cells arising would fail to proliferate owing to low levels of pre-BCR expression. In this case, the reduced inflow of newly formed B cells would be consecutive to a lack of pre-B cell expansion. Even if difficult to confirm, this second hypothesis is supported by several data. First, a significant defect in pre-B cell expansion was observed in mice carrying the hypomorphic IgH allele that lowered H chain expression at this stage (35), although VDJ recombination was not affected in this model. Second, we could speculate that if a proliferative burst occurred on the few pre-B cells arising in our Eμ-deficient mice, the IgH preimmune repertoire would be limited, at a given time, to only a few VH segments. Our data clearly demonstrate that this is not the case because VH54 segments were broadly used in our Eμ KO mice (Fig. 3). Third, partial blockade at the pro-B cell stage might yield significant numbers of pre-B cells because it is admitted that Eμ mostly affects D to Jμ and not V to DJμ recombination (13, 14). Our findings that VH gene usage was not affected by Eμ deletion supports this statement. All this strongly suggests that pre-BCR expression and consequently pre-B cell expansion is controlled by the Eμ regulatory region.

Looking at peripheral B cell compartments in our full-length Eμ deletion model, one obvious observation was the strong orientation of B cell development toward MZ B cell subsets in the spleen at the expense of the FO B cell subset. We assumed that this subset preference could also be the consequence of variations in IgH expression at early (immature/transitional) stages of B cell development. Many reports supported the hypothesis that peripheral B cell fate depends on pre-BCR and BCR signal strength at early stages (52). It was proposed that weak BCR signaling, above and beyond the Ag-independent tonic signal, prompted developing B cells to assume MZ phenotypes. This was observed in mouse models leading to a decrease in pre-BCR or BCR signaling (53, 54). In our full-length Eμ precursor cells, the newly formed bone marrow B cells expressing low levels of Igμ H chains would provide weak BCR signals (below the tonic BCR threshold), supporting mostly differentiation into MZ B cell subsets. Such preference for the MZ compartment was also reported in a mouse model carrying a GFP reporter gene knocked-in downstream from Eμ (35). In this model called the “hypomorphic IgH-chain”, the authors described reduced Igμ H chain expression in pre-B and immature B cells that logically led to MZ B cell fate. Interestingly, the weakly expressed B1-8 VH hypomorphic allele (devoid of MARs and cEμ regions) also oriented newly formed B cells toward the MZ compartment (19). Surprisingly, even in the absence of SLCs, B lineage cells still progressed through the pre-BCR checkpoint and developed (in reduced numbers) into mature cells that also exhibited a preference for MZ fate (55).

In contrast to our full-length Eμ deletion, the hypomorphic IgH chain mutation confers a defect in Ab response (35), and this effect could logically be linked to decreased IgH expression at mature B cell stages observed in this model. In our full-length Eμ deletion model, decreased IgH expression was no longer observed in resting B cells from the spleen, confirming that full-length Eμ region activity was mostly restricted to immature stages. Indeed, at mature B cell stages, it is admitted that the 3’ regulatory region takes the relay for optimal H chain expression, CSR, and somatic hypermutation events (56, 57).

Although some noticeable effects of Eμ deletion on CSR efficiency have been previously reported, it is admitted that Eμ is dispensable for such a process in vivo (13, 19, 20, 58). Because our full-length Eμ KO model modified peripheral B cell fate, we expected Ab responses to be affected. Surprisingly, this was not the case, demonstrating that peripheral B cells devoid of both MARs and cEμ enhancer are fully competent to enable efficient
T-depleted and -independent Ab responses. This suggests, once more, that regulatory elements involved in Ab responses are within the 3′ regulatory region (59, 60).

In conclusion, our mouse model clarifies some important and under-rated functions regarding the Eμ regulatory region, beyond its direct role in promoting D to J recombination. Our study specifies that VH segments (including distal families) come into close proximity to the DJH rearranged segment independently of the core or the full-length Eμ region, but once VDJ recombination has occurred, the rearranged VDJ segment comes under the control of Eμ. At this stage, Eμ acts as a critical regulatory element that enhances μ H chain expression in pre-B and newly formed bone marrow B cells, supporting pre-B cell expansion and promoting FO B cell fate.

Acknowledgments
We are very grateful to Dr. Frederick W. Alt (Howard Hughes Medical Institute, Children’s Hospital, and Harvard Medical School, Boston, MA) for providing the full-length Eμ targeting construct and the core Eμ-deficient mouse line to proceed to preimmune IgVH repertoire analysis. We are very grateful to all members of the Service Commun d’Animalerie and Plateforme de Transgenose of Limoges University for mouse work. We are very grateful to Catherine Ouk-Martin and the FACS sorting facility of Limoges University. We are also very grateful to Dr. Jeanne Cook-Moreau for critical reading of this manuscript. We thank Drs. Laurent Delphy, Christophe Sirac, and Sandrine Le Noir for discussions and helpful comments.

Disclosures
The authors have no financial interests of conflict.

References


Supplementary Figure 1: IgH pre-immune V<sub>H</sub> repertoire in wt and core-Eµ-deficient mice.

A. VH segments clusters

B. Immun’Ig® test panel

C. Correspondence between VH family segments detected by the Immun’Ig® test, IMGT® classification and historical VH nomenclature.

D. 129wt

Legend to supplementary Figure 1:(A) IgH locus, the V<sub>H</sub> region is divided into three major clusters including distal, middle and proximal V<sub>H</sub> segments. (B) Semi-quantitative V(D)J recombination assay performed on pre-B cell DNA from core-Eµ<sup>ΔΔ</sup> (Perlot, T., F. W. Alt, C. H. Bassing, H. Suh, and E. Pinaud. 2005. Elucidation of IgH intronic enhancer functions via germ-line deletion. Proc. Natl. Acad. Sci. U.S.A 102: 14362–14367.) and 129 wt mice. For each genotype, experiments were performed on 2 independent DNA samples, each one prepared from a pool of CD25+ sorted bone marrow cells from 2 to 3 age-matched mice (enriched with C’ and D Hardy’s fractions that correspond to large and small pre-B populations). (C) Correspondence between VH family segments detected by the Immun’Ig® test, IMGT® classification and historical VH nomenclature. (D) VH family segment usage in pre-B cells from core-Eµ<sup>ΔΔ</sup> and 129/SV wt mice. Data from section B are represented as pie chart diagrams, showing proportions (in percentage) of each VH family segment (according to the IMGT® nomenclature).
**Supplementary Table 1**: Igκ L chain junctional diversity in wt and full length Eμ KO mice.

<table>
<thead>
<tr>
<th>DNA source</th>
<th>n</th>
<th>% out of frame junction</th>
<th>%N⁺</th>
<th>%Pᵥ⁺</th>
<th>%Pᵢ⁺</th>
<th>CDR3 length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt CD25⁺ bone marrow</td>
<td>55</td>
<td>62</td>
<td>11</td>
<td>0</td>
<td>3.6</td>
<td>26.4</td>
</tr>
<tr>
<td>Eμ⁺ CD25⁺ bone marrow</td>
<td>44</td>
<td>54</td>
<td>14</td>
<td>3</td>
<td>4.5</td>
<td>26.9</td>
</tr>
</tbody>
</table>

Legend to supplementary table 1: κ junctional sequences were cloned out of bone marrow pre-B cell DNA samples from 3 animals of each genotype. Junction analysis was performed using the IMGT V-QUEST software (http://www.imgt.org/IMGT_vquest/share/textes/). n indicates the number of analyzed independent sequences, percentage of out of frame sequences was determined, N and P nucleotide insertions and CDR3 length were reported.