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Plasma Cells and Nonplasma B Cells Express Differing IgE Repertoires in Allergic Sensitization

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The selection of allergen-specific B cells into the plasma cell (PC) pool is a critical step in the immune dysregulation that leads to the production of IgE in allergic diseases. We sought to characterize the murine IgE repertoire. In particular, we questioned whether the IgE repertoire of plasmablasts (PBs)/PCs differs from the IgE repertoire of non-PCs. Therefore, we sorted splenocytes from OVA-sensitized BALB/c mice into CD138^{pos} (PBs/PCs) and CD19^{pos}/CD138^{neg} (non-PCs) B cell fractions. Using reverse transcription PCR, we amplified, cloned, and sequenced IgE mRNA transcripts and analyzed the Ig H chain repertoire. As a reference, we characterized the IgM repertoire of the same animals. Compared to IgM, the IgE sequences contained a significantly higher level of somatic mutations and displayed an oligoclonal expansion with clonotype restriction. Interestingly, we found two phenotypically distinct IgE-producing B cell subpopulations that differed in their repertoire of H chain transcripts; IgE transcripts from PBs/PCs showed significantly more signs of Ag-driven selection than transcripts from non-PCs, including 1) a higher number of somatic mutations, 2) increased clustering of replacement mutations in the CDRs, and 3) biased third CDR of the heavy Ig chain composition. In conclusion, PBs/PCs and non-PCs from OVA-sensitized mice express distinct IgE repertoires, suggesting that 1) the repertoire of IgE-expressing PBs/PCs represents a highly biased selection from the global B cell repertoire and 2) Ag-driven affinity maturation is a major force that selects IgE-producing B cells into the CD138^{pos} PC pool. *The Journal of Immunology*, 2010, 184: 000–000.

In atopic diseases, part of the peripheral B cell compartment has undergone class switching to IgE (1). Bound to the surface of mast cells, IgE generates the interface between allergen and the immune system and hence represents the central effector molecule in allergic immune responses. The class switch to IgE is a tightly regulated process that requires a Th2-biased cytokine milieu and a concerted series of gene rearrangements and splicing events (2). Because plasma cells (PCs) are the source of IgE, recruitment and selection into the PC pool is a critical step in immune dysregulation that leads to the production of IgE. Whereas PCs as end-stage cells do not divide, plasmablasts (PBs)

in germinal centers still undergo cell divisions shortly before they become PCs (3, 4). PBs are CD138^{pos} cells that have begun to secrete Abs yet are still expressing characteristics of activated B cells that allow interaction with T cells. This ability is a prerequisite for clonal selection of those B cells that bear Ag receptors of the highest affinity, leading to affinity maturation during the secondary immune response (5). This Ag-driven selection leads to a targeted focusing of the immune response toward production of highly specific Abs.

The specificity of an Ig is defined by the classic Ag-binding site that is formed by the three hypervariable CDRs of the H and L Ig chains (6). Although the first (CDR-H1) and second CDR of the heavy Ig chain (CDR-H2) are encoded in the germline, the third CDR of the heavy Ig chain (CDR-H3) is created during the process of somatic recombination by the rearrangement of H chain variable (V_H), diversity (D), and joining (J_H) gene segments (7) and further diversified by addition of random N-nucleotides (8) and palindromic P-nucleotides (9), generating an almost unlimited variety of possible combinations. Due to its position at the center of the classic Ag-binding site, CDR-H3 often plays a determinative role in the recognition of the Ag (10).

Despite its pivotal role in defining the specificity of the Ab, little is known about the composition of the classic Ag-binding site of IgE. Using a gene targeted mouse strain with a limited CDR-H3 repertoire, we revealed that allergic sensitization and airway inflammation highly depend on the predominant BCR repertoire (11). Therefore, we now sought to systematically characterize the composition of the murine IgE repertoire. In particular, we questioned whether the IgE repertoire of murine CD138^{pos} B cells (PBs/PCs) differs from the IgE repertoire of CD19^{pos}/CD138^{neg} B cells (non-PCs). The current concept holds that non-PCs can be recruited into the PB/PC pool according to their ability to recognize Ag during a secondary challenge. Thus, the repertoire of Igs expressed by PBs/PCs might represent a biased selection from

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CDR-H1, first CDR of the heavy Ig chain; CDR-H2, second CDR of the heavy Ig chain; CDR-H3, third CDR of the heavy Ig chain; CDR_{rel}, average probability that a random mutation would allocate in the CDR; C_HE, outer IgE C region; ctrl, C_HE-positive control; D, diversity; FR, framework region; IgE_{non-PC}, IgE transcripts from CD138^{neg}/CD19^{pos} B cells; IgE_{PB/PC}, IgE transcripts from CD138^{pos} B cells; IgM_{Bcell}, IgM transcripts from B cells; J_H, joining; mIgE, membrane-bound IgE; M_v, total mutations in V region; neg, DNA-free negative control; PB, plasmablast; PC, plasma cell; R_{CDR}, replacement mutations in the CDR; R_f, sequence-inherent probability that a mutation in the CDR would be a replacement mutation; V_H, variable.

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those of non-PCs. Although this concept appears conclusive in IgM, IgG, and IgA (5, 12), it has never been confirmed for IgE, which differs from the other Ig isotypes in several aspects; serum IgE levels are usually below 0.1% of those of the other isotypes due to a significantly reduced number of IgE-producing PBs/PCs, a genetically programmed downregulation of IgE transcription, and a reduced half-life of serum IgE (13, 14). IgE can be produced as membrane-bound (mIgE) and secretory forms, which are attributed to memory cells and PCs, respectively (15). IgE memory cells appear to be extremely rare and could not yet be reliably visualized or quantified *in vivo* (16). However, recent data published by Achatz and coworkers (17, 18) suggest a role of mIgE-producing cells in the development and maintenance of allergies.

Our main hypothesis was that IgE-secreting PBs/PCs are selected during allergic sensitization due to their classic Ag-binding site, implying an active selection into the PB/PC pool. In the following, we present the hitherto most extensive collection of murine IgE transcripts, demonstrating for the first time that the IgE repertoire of murine splenic PBs/PCs differs from that of splenic non-PCs in having a privileged repertoire, enriched for long N1 regions and distal J_H genes. We found evidence of enhanced Ag-driven selection in PBs/PCs, indicating that IgE-producing splenic PBs/PCs are affinity-matured cells with numerous somatic mutations and signs of receptor revisions.

Materials and Methods

Animals

BALB/c mice were obtained from Harlan Winkelmann (Borchen, Germany). All of the mice were housed in a specific pathogen-free environment and fed *ad libitum*. All of the animal care practices and experimental procedures were performed in accordance with the German animal protection law and were approved by the responsible governmental authority (Regierungspraesidium Giessen).

Protocol of allergic sensitization

Eight-week-old female mice were sensitized by i.p. administration of OVA (10 µg per injection) in PBS complexed with alum (1.5 mg per injection) at days 1, 14, and 21, according to a standardized protocol (19). On day 24, mice were sacrificed and spleens were removed.

Protocol of parasitic infection

Six-week-old wild-type mice were infected with L3 larvae of the nematode *Litomosoides sigmodontis* by bites of the tropical rat mite (*Ornithonyssus bacoti*). On day 60, mice were sacrificed and spleens were removed.

Flow cytometry and cell sorting

Single-cell suspensions were prepared by mincing the spleen in cold PBS. Cells were washed and resuspended in an appropriate volume of FACS buffer for counting and staining. Total splenocytes from individual mice were incubated with fluorescently labeled Abs (BD Biosciences, Heidel-

berg, Germany) according to the manufacturer's guidelines. Sorting was performed on a MoFlo instrument (DakoCytomation, Hamburg, Germany) in the FACS sorting core facility of the Philipps-University Marburg. Only mononuclear cells falling within the lymphocyte gate in the forward and side scatter were included. CD138^{pos} and CD138^{neg}/CD19^{pos} B cells were separately selected using the following mAbs: rat anti-mouse CD19 (PerCP-Cy5.5) and rat anti-mouse CD138 (PE) (BD Biosciences).

The non-PC fraction was further analyzed to ensure the presence of mIgE-expressing cells; before being stained for FACS analysis, single-cell suspensions from spleen were treated for 1 min with ice-cold acid buffer (0.01 M EDTA, 0.005 M KCl, 0.085 M NaCl, 0.05 M sodium acetate [pH 4]) to remove extrinsic IgE Abs noncovalently bound to B cells. The samples were then washed twice with FACS buffer before staining. The following reagents were used: rat anti-mouse IgE (R35-72) (FITC), rat anti-mouse CD19 (PerCP-Cy5.5), rat anti-mouse CD138 (PE) (BD Biosciences), and OVA (Cy5). In this experiment, cells were gated for lymphocytes in a forward and side scatter plot and for non-PCs (CD19^{pos} and CD138^{neg}).

Preparation of RNA and PCR

Cells were sorted directly into lysis buffer (2.5×10^4 cells for CD138^{pos}; 10^5 cells for CD138^{neg}/CD19^{pos} B cells). Total RNA was prepared using a RNA mini kit (Qiagen, Hilden, Germany) and eluted in 30 µl water. IgE transcripts were amplified by using seminested PCR. For reverse transcription, a multiplex primer specific for the IGHV1, IGHV2, IGHV3, IGHV5, and IGHV14 gene family was used as the forward primer [V_Hall (20)] together with an outer IgE C region (C_Hε)-1 reverse primer (Ce1-232r) (Table I). In a second PCR, aliquots of the first IgE PCR products were reamplified by using the V_Hall forward primer with an inner C_Hε1 reverse primer (Ce1-21r). IgM transcripts were amplified using the V_Hall forward primer together with an IgM C region 1 (C_Hμ1) reverse primer [Cμ2 (21)].

RT-PCR amplifications were carried out in a total volume of 25 µl containing 2 µl RNA eluate, 2.5 mM MgCl₂, 7.5 U recombinant RNase inhibitor, and 0.6 µM of each forward and reverse primer using a OneStep RT-PCR kit (Qiagen, Hilden, Germany). The following program was performed on a thermocycler (SensoQuest, Göttingen, Germany): 30 min at 50°C, 15 min at 94°C, followed by 40 cycles using a cycle profile of 30 s at 94°C, 30 s at 60°C (IgE) or 59°C (IgM), and 1 min at 72°C, followed by a final extension of 10 min at 72°C. The second PCR was carried out in a total volume of 25 µl containing 1.5 µl primary amplificate, 0.5 U Taq polymerase, 3.5 mM MgCl₂, and 0.3 µM of each forward and reverse primer. The following program was performed on a thermocycler: 2:30 min at 94°C, followed by 30 cycles using a cycle profile of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. As a control for RNA quality and for the presence of IgE transcripts, the IgE C region was amplified using C_Hε4 forward (Ce4-1219f) and C_Hε1 reverse (Ce1-200r) primers (Table I). PCR products were separated on a 1.5% agarose gel, and DNA of the expected m.w. (~400 bp) was extracted using a QIAquick gel extraction kit (Qiagen).

Cloning of PCR products

Ligation was performed according to the manufacturer's instructions (TOPO-TA cloning kit; Invitrogen, Karlsruhe, Germany). Successfully transformed cells were identified by blue/white color staining in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside. Plasmid DNA was purified from overnight cultures in lysogeny broth medium containing ampicillin (100 µg/ml), using QIAprep miniprep kit (Qiagen), and tested for the presence of an insert of the correct size by digestion with EcoR1.

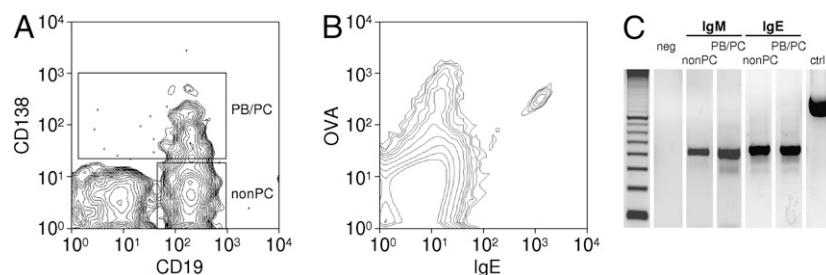


FIGURE 1. A, Representative FACS analysis for sorting of splenic B cell populations. CD138^{pos} cells (PBs/PCs) were sorted against CD138^{neg}/CD19^{pos} cells (non-PCs). Cells were gated for lymphocyte gate in a forward and side scatter plot. B, Representative FACS control demonstrating the presence of mIgE-expressing cells within the non-PC fraction after removal of extrinsic Abs noncovalently bound to B cells. Cells were gated for lymphocyte gate in a forward and side scatter plot and for non-PCs (CD138^{neg}/CD19^{pos}). C, Representative agarose gel showing the amplification of rearranged IgM and IgE H chain mRNA, yielding PCR products of $\sim 400 \pm 30$ bp. neg, DNA-free negative control; ctrl, C_Hε-positive control showing a 1043 bp PCR product.

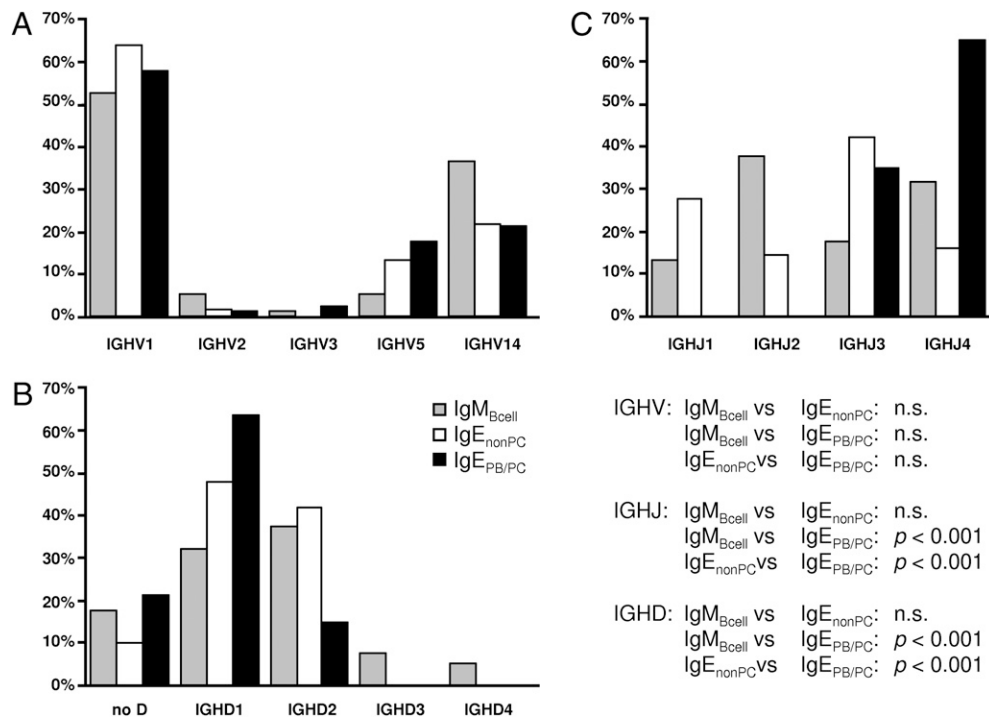


FIGURE 2. A–C, V_H gene (A), D gene (B), and J_H gene (C) family usage of IgM_{Bcell} (gray), IgE_{non-PC} (white), and IgE_{PB/PC} (black). The V_H gene segment usage did not differ significantly among the three populations, but usage of the IGHD1 gene family was significantly enhanced in IgE_{PB/PC} ($p < 0.001$, compared with IgE_{non-PC} and IgM_{Bcell}, respectively). A strong bias toward D distal J_H families was found in IgE_{PB/PC} ($p < 0.001$, compared with IgE_{non-PC} and IgM_{Bcell}, respectively). Only the two most distal J_H families IGHJ3 and IGHJ4 were used by IgE_{PB/PC}.

Gene segment identification and phylogenetic trees

Randomly selected cloned PCR products were commercially sequenced (GATC-Biotech, Konstanz, Germany) using an ABI 3730 XL capillary sequencer (Applied Biosystems, Darmstadt, Germany). Gene segments were assigned according to germline V_H, D, and J_H gene segments as listed in the ImMunoGeneTics database using the V-QUEST program (<http://imgt.cines.fr>) (22). Ig sequences were numbered according to the ImMunoGeneTics unique numbering (23). A minimum of six nonmutated nucleotides with at least two nonmutated nucleotides at each end was required to identify a D gene (24). The somatic mutation rate was calculated by dividing the sum of mutations in CDR-H1, framework region (FR)-H2, CDR-H2, and FR-H3 (compared with the germline V_H gene) by the total number of nucleotides. CDR-H3 was defined to include those residues located between the conserved cysteine (C104) of FR-H3 and the conserved tryptophan (W118) of FR-H4. To study the frequency of Ag-selected sequences, we used the algorithms of Lossos et al. (25) and Chang and Casali (26) to statistically analyze the distribution of replacement and silent mutations within V_H genes. Phylogenetic trees were compiled using Clustal X (www.clustal.org) (27) and TreeDyn (www.treedyn.org) (28).

Statistical analysis

Differences between populations were assessed by a two-tailed Student *t* test for normally distributed data or a Mann-Whitney *U* test for non-normally distributed data, respectively. A χ^2 test for homogeneity of variance was used for categorical data, and a two-tailed Fisher exact test was applied if one category was smaller than $n = 5$. Means are given with the SD. A $p < 0.05$ was accepted as significant. Statistical analyses were performed using Prism 5.0 (GraphPad, San Diego, CA).

Results

B cell sorting and RNA amplification

Splenic B cells from OVA-sensitized and *L. sigmodontis*-infected wild-type BALB/c mice that fell within the lymphocyte gate in the forward and side scatter were sorted on the basis of surface expression of CD138 (Syndecan-1) and CD19 into 1) PB/PC (CD138^{pos}) and 2) non-PC (CD138^{neg}/CD19^{pos}) B cell populations. Fig. 1A shows one representative of eight individual experiments.

On average, PBs/PCs and non-PCs represented 10.2 and 42.6% of the cells in the lymphocyte gate, respectively. Total RNA was extracted and amplified, using an IgE or IgM C region-specific primer in combination with a multiplex V_H primer (Table I). We cloned 279 productive V_HDJ_HC ϵ sequences (IgE transcripts), of which 153 were unique. Of these sequences, 80 were derived from sorted PBs/PCs (IgE_{PB/PC}, GenBank accession nos. FJ816282–FJ816361) and 73 from non-PCs (IgE_{non-PC}, GenBank accession nos. FJ816362–FJ816434). As a reference, 189 productive V_HDJ_HC μ sequences (IgM transcripts) of sorted PBs/PCs ($n = 58$) and non-PCs ($n = 56$) and unsorted splenocytes ($n = 75$) were cloned, of which 175 were unique (GenBank accession nos. FJ816435–FJ816609). IgM transcripts of PBs/PCs, non-PCs, and unsorted splenocytes were combined into one IgM group (IgM_{Bcell}) because no differences were observed among the three groups for all of the studied parameters. As a second reference, we obtained 34 IgE transcripts from sorted PBs/PCs and 35 IgE transcripts from sorted non-PCs of *L. sigmodontis*-infected mice (GenBank accession nos. GU906987–GU907055). Fig. 1B shows the control staining that was performed to ensure the presence of mIgE-positive cells within the non-PCs. A representative agarose gel, showing the amplification of IgM and IgE transcripts, is shown in Fig. 1C. In unsensitized mice, the IgE transcripts were below the detection limit of our RT-PCR protocol.

V_H, D, and J_H gene usage

V_H genes from the IGHV1, IGHV2, IGHV3, IGHV5, and IGHV14 family were amplified. Together, these families represent ~80% of all of the V_H genes (29). Usage of V_H gene families did not differ significantly among IgM_{Bcell}, IgE_{PB/PC}, and IgE_{non-PC} (Fig. 2A). In all three populations, the IGHV1 family was the most frequent (52.3% in IgM_{Bcell} to 63% in IgE_{non-PC}), followed by IGHV14 > IGHV5 > IGHV2 $\approx$ IGHV3. The number of individual V_H genes used was 42 in

Table I. Oligonucleotide primers

VH sense primer	VH α (20)	5'-AGG TSM ARC TGC AGS AGT CWG G-3'
CH antisense primer	Ce1-232r	5'-TTC GTT GAA TGA TGG AGG ATG TGT-3'
	Ce1-21r	5'-CCT TTA CAG GGC TTC AAG GGG TAG-3'
	C μ 2 (21)	5'-CAG GAG ACG AGG GGG AAG ACA TTT GG-3'
CH control primer	Ce4-1219f	5'-ACC AAG GCT CGT GGA TAT TGT TTT-3'
	Ce1-200r	5'-GCA AGT CAG CCA AGA ACT TCA CAT-3'

M = A or C; R = A or G; S = G or C; W = A or T.

IgM_{Bcell}, whereas 14 genes were used by both IgE_{PB/PC} and IgE_{non-PC}, respectively. Because the total number of functional V_H genes in the germline is ~200, the present sequence collection is not large enough for statistical analysis of individual V_H gene utilization. A D gene segment could be identified in 63 IgE_{PB/PC} (79%), 62 IgE_{non-PC} (90%), and 141 IgM_{Bcell} (82%). There was no significant difference in the D gene family usage between IgM_{Bcell} and IgE_{non-PC}. However, such differences were noted between IgE_{non-PC} and IgE_{PB/PC} ($p < 0.001$). Usage of IGHD1 family members was enhanced in IgE_{PB/PC} (63.8%) compared with those in IgE_{non-PC} (47.8%) and IgM_{Bcell} (32.2%) (Fig. 2B). All known 17 individual D genes were used by IgM_{Bcell}, whereas four and six genes were used by IgE_{PB/PC} and IgE_{non-PC}, respectively. Similar to D gene usage, J_H gene usage did not differ significantly between IgE_{non-PC} and IgM_{Bcell} but did differ between IgE_{non-PC} and IgE_{PB/PC}: 65.0% of IgE_{PB/PC} used the IGJ4

gene compared with 16.4% of IgE_{non-PC} and 31.4% of IgM_{Bcell} ($p < 0.001$) (Fig. 2C).

CDR-H3 length and composition

IgM_{Bcell} displayed a normally distributed and broad range of CDR-H3 length from 15–54 nts (Fig. 3A). In contrast, the CDR-H3 length of IgE_{non-PC} and IgE_{PB/PC} was limited to 27–48 nts (Fig. 3B, 3C). The average length of CDR-H3 was more than a codon shorter in IgM_{Bcell} (33.12 ± 8.31 bp) than that in IgE_{non-PC} (36.90 ± 7.24 bp, $p < 0.01$) and that in IgE_{PB/PC} (37.83 ± 6.29 bp, $p < 0.001$). To assess the relative contributions of the V_HDJ_H germline sequence, exonucleolytic nibbling, and N-nucleotide addition to the shifts in CDR-H3 length, we deconstructed the CDR-H3 sequence of those transcripts that contained identifiable D genes (Fig. 4). This analysis revealed that the differences in CDR-H3 length were primarily caused by an enhanced N-nucleotide addition between the V_H and D genes in IgE_{PB/PC} (7.73 bp) compared with those of IgE_{non-PC} (2.98 bp) and IgM_{Bcell} (3.40 bp) ($p < 0.001$). This increased N-nucleotide addition overcompensated for the reduced contribution of V_H germline sequence to the CDR-H3 in IgE_{PB/PC}, which was due to enhanced exonucleolytic nibbling. The contribution of the germline V_H sequence to CDR-H3 was significantly reduced in IgE_{PB/PC} (4.81 bp versus 6.15 bp in IgE_{non-PC} and 5.45 bp in IgM_{Bcell}, $p < 0.001$), whereas the contribution of the J_H gene to CDR-H3 was slightly smaller in IgE_{PB/PC} than in that IgE_{non-PC} (not significant). The CDR-H3 regions of sequences without an identifiable D_H gene were shorter in IgM transcripts ($n = 30$ sequences, 22.3 ± 4.9 bp) than those in IgE_{PB/PC} ($n = 17$, 29.1 ± 2.9) and in IgE_{non-PC} ($n = 7$, 30.0 ± 1.7 bp).

Somatic mutation rate

In IgM_{Bcell}, a substantial amount of sequences contained no somatic mutations at all. In IgE transcripts by contrast, only a negligible part of the sequences were unmutated. The average mutational rate of IgE_{PB/PC} (51.5‰) was more than four times as high as that in IgM_{Bcell} (11.8‰, $p < 0.001$) (Fig. 5). Interestingly, the two IgE-producing B cell subsets also differed markedly, the IgE_{PB/PC} being significantly higher mutated than the IgE_{non-PC} (31.7‰, $p < 0.05$). To exclude a relevant biasing by Taq polymerase errors, we calculated the Taq error rate within the stretches

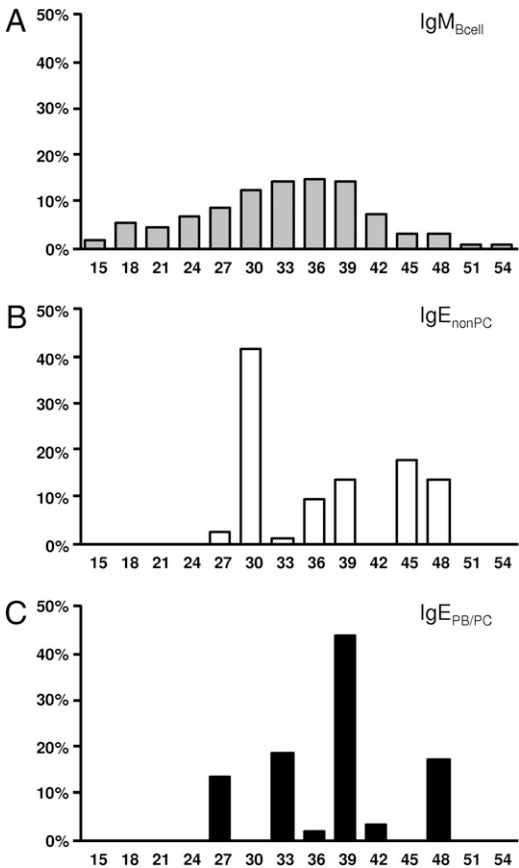


FIGURE 3. Distribution of CDR-H3 length in IgM_{Bcell} (gray), IgE_{non-PC} (white), and IgE_{PB/PC} (black). A, CDR-H3 length was normally distributed over a broad range from 15–54 nts in IgM_{Bcell}. B and C, In contrast, the CDR-H3 length of IgE_{non-PC} (B) and IgE_{PB/PC} (C) was limited to 27–48 nts. In IgM_{Bcell} (33.12 ± 8.31 bp), the average CDR-H3 length was more than a codon shorter than those in IgE_{non-PC} (36.90 ± 7.24 bp, $p < 0.01$) and IgE_{PB/PC} (37.83 ± 6.29 bp, $p < 0.001$).

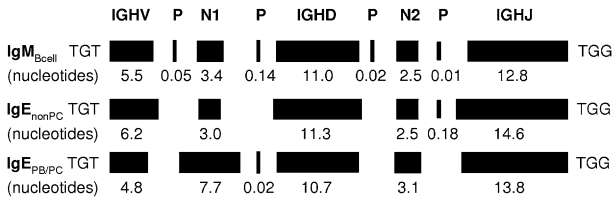
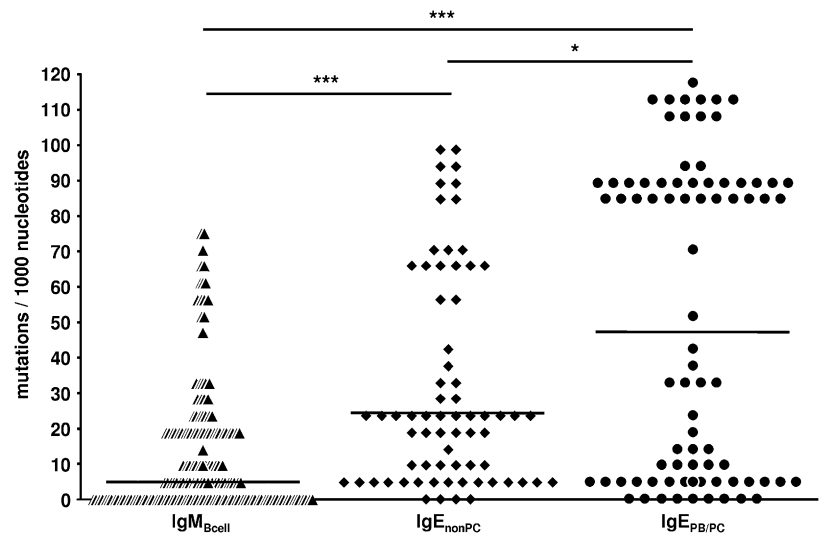


FIGURE 4. Relative CDR-H3 composition. Deconstruction of CDR-H3 sequences revealed that the differences in CDR-H3 length were primarily caused by an enhanced N-nucleotide addition between the V_H and D genes in IgE_{PB/PC} (7.73 bp) compared with those in IgE_{non-PC} (2.98 bp) and IgM_{Bcell} (3.40 bp) ($p < 0.001$). Only transcripts with identifiable D genes were included into the analysis.

FIGURE 5. Somatic mutation rate of IgM and IgE transcripts. In IgE_{PB/PC}, the average mutational rate (51.5‰) was more than four times as high as that in IgM_{Bcell} (11.8‰, $p < 0.001$). Interestingly, the two IgE-producing B cell subsets differed markedly, the IgE_{PB/PC} being significantly higher mutated than the IgE_{non-PC} (31.7‰, $p < 0.05$). Horizontal lines represent medians.



of the C region that were included in the amplicates. Taq error rates were 1.39/1000 nts in IgE_{non-PC}, 1.88/1000 nts in IgE_{PB/PC}, and 1.57/1000 nts in IgM_{Bcell} and therefore cannot explain the observed differences among the groups.

Ag selection

We analyzed the distribution of replacement and silent mutations between FR and CDR to evaluate whether IgE sequences evolved under the influence of Ag selection. Using the method of Lossos et al. (25) we determined the replacement frequencies and the relative lengths of FR and CDR of each germline V_H gene. The average probability that a random mutation would allocate in CDR (CDR_{rel}) was calculated to be 0.24 ± 0.03 , and the sequence-inherent probability that a mutation in the CDR would be a replacement mutation (R_f) was estimated to be 0.78 ± 0.02 . Therefore, the chance for a random mutation to introduce a replacement mutation into the CDR was 0.18 ($q = CDR_{rel} \times R_f$). The binomial distribution method of Chang and Casali (26) was then used to calculate the 90 and 95% confidence limits for the ratio of replacement mutations in the CDR (R_{CDR}) to the number of total mutations in the V region (M_V). This confidence interval is shown as shaded area in Fig. 6. A data point falling outside these confidence limits represents a sequence that has a high proportion of replacement mutations in CDR. The probability that such a sequence has accumulated as many replacement mutations in the CDR by mere random mutation is <0.05 . Therefore, an allocation above the upper confidence limit was considered indicative of Ag selection. According to this definition, 99.4% of the IgM_{Bcell} showed no indications of Ag selection (Fig. 6A). More than half of the IgM transcripts had no replacement mutation in the CDR at all, the corresponding data points falling into the lower left corner. For IgE_{non-PC}, we found a similar distribution with only 1.4% of the sequences showing signs of Ag selection (Fig. 6B). Interestingly, the results for IgE_{PB/PC} differed strikingly, with 15% of the sequences falling outside the 95% confidence interval, IgE transcripts from PCs/PBs displayed a significant higher degree of Ag selection (Fig. 6C), the proportion of Ag-selected sequences being more than ten times as high in IgE-producing PBs/PCs as that in IgE-producing non-PCs.

Phylogenetic trees

To determine the clonal relationships of IgE_{PB/PC}, IgE_{non-PC}, and IgM_{Bcell} in terms of molecular evolution, we calculated genetic distance dendrograms for the nucleotide sequences (Fig. 7). Clones

were considered clonally related if they had an origin in the same V_H gene, a highly homologous CDR-H3, and identical length in CDR-H3. The 175 sequenced unique IgM_{Bcell} pertained to 165 different clonotypes (Fig. 7A). The IgE repertoire encompassed considerably fewer clonotypes but was composed of multiple related sequences. Among the 80 unique IgE_{PB/PC}, only 17 different clonotypes were identified (Fig. 7B), suggesting that many of the clones had an origin in the same precursor clone. Similarly, the 73 unique IgE_{non-PC} originated from only 22 different clonotypes (Fig. 7C). Interestingly, the clonotypic diversity of IgE transcripts from PBs/PCs and non-PCs of *L. sigmodontis*-infected mice was significantly higher than that from OVA-sensitized mice (Table II).

Discussion

IgE-producing PCs mediate long-term persistence of allergies by production of allergen-specific IgE (30). Therefore, a comprehension of the selective forces that shape the B cell repertoire during allergic sensitization is important to understand the dysregulation in allergic immune responses. In this work, we demonstrate evidence for two phenotypically distinct IgE-producing B cell subpopulations that differ in their repertoire of H chain transcripts. To the best of our knowledge, this is the first study to systematically characterize the repertoire of murine IgE transcripts.

In mice, Ab-secreting cells (PBs/PCs) express CD138 (Syndecan-1) (31). Therefore, we sorted splenic CD138^{pos} lymphocytes (PBs/PCs), which occur in sufficient numbers after allergic sensitization. We compared these PBs/PCs to sorted CD138^{neg}/CD19^{pos} lymphocytes (non-PCs), which represent a mixture of several B cell subsets, including marginal zone cells, follicular zone cells, and transitional B cells (32–34). However, by selectively amplifying IgE transcripts, we focused our study on class-switched B cells, which in other isotypes include memory cells. The presence of mIgE-expressing cells within the non-PC population was confirmed by surface staining for mIgE (Fig. 1B) and by PCR amplification of the membrane splice variant of the IgE C region (Supplemental Fig. 1). As a reference, IgM transcripts from the same mice were studied, representing the primary Ig repertoire that has undergone no or little Ag selection. This allowed us to characterize the selective pressure that acts during class switch and selection into the IgE-producing PB/PC and non-PC pools. We combined the IgM transcripts from PBs/PCs and non-PCs because they did not differ in any of the parameters studied.

Our study shows that the Ig repertoires of IgE- and IgM-producing B cells differ significantly; the utilization of germline segments is

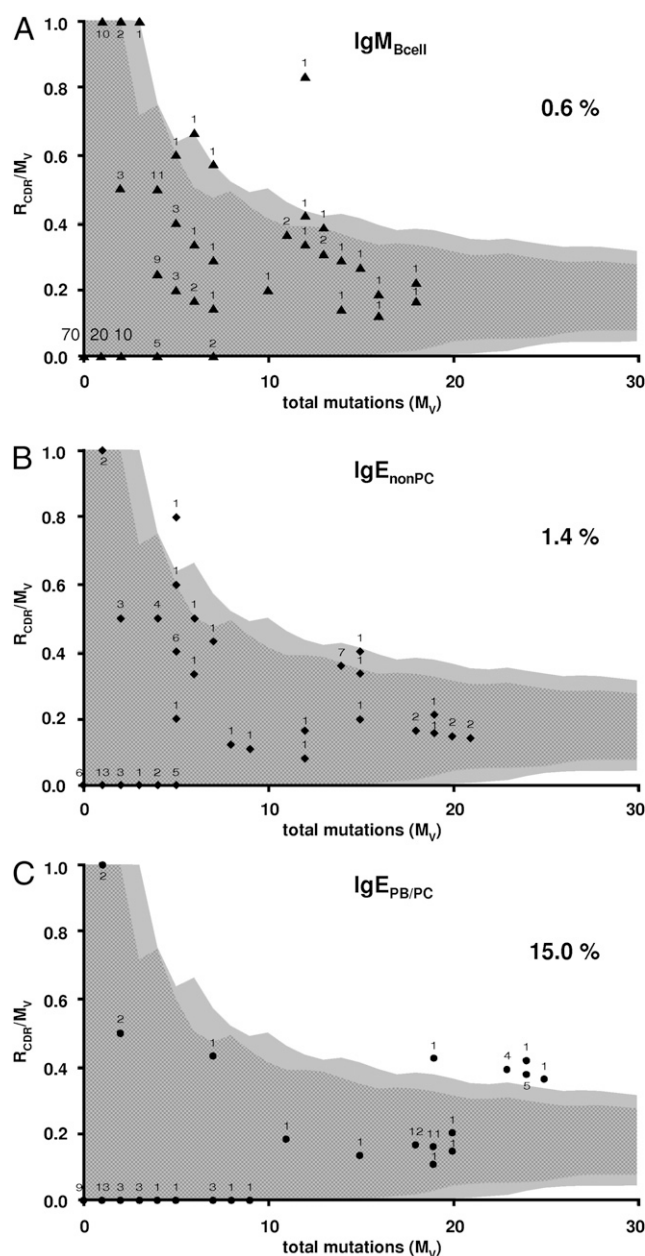


FIGURE 6. Inference of Ag selection. A–C, The ratio of replacement mutations in CDR-H1 and CDR-H2 (R_{CDR}) to total number of mutations (M_V) is plotted against M_V in IgM_{Bcell} (A), IgE_{non-PC} (B), and IgE_{PB/PC} (C). The light shaded areas represent the 95% confidence limits, and the dark shaded areas represent the 90% confidence limits for the probability of random mutations. A data point falling outside these confidence limits represents a sequence that has a high proportion of replacement mutations in CDR and is considered indicative of Ag selection. Data points are accompanied by their observed frequency. Whereas 99.4% of IgM_{Bcell} and 98.6% of IgE_{non-PC} were not Ag-selected, 15% of IgE_{PB/PC} displayed signs of a significant Ag selection. Hence, the proportion of Ag-selected sequences was more than ten times as high in IgE-producing PBs/PCs as that in IgE-producing non-PCs.

restricted in IgE-producing cells (IgE_{PB/PC} and IgE_{non-PC}), whereas the somatic mutation rate and the CDR-H3 length are significantly elevated compared with IgM_{Bcell}. The most interesting finding of our study is that the two IgE-producing subsets also differ significantly in pivotal aspects; IgE_{PB/PC} exhibit more signs of Ag-driven selection, including 1) increased number of somatic mutations, 2) augmented clustering of replacement mutations in the CDR, 3) focused CDR-H3 composition, and 4) signs of enhanced receptor revision.

Surprisingly, only the two D distal J_H gene segments (IGHJ3 and IGHJ4) were used in IgE_{PB/PC}, suggesting secondary D–J_H rearrangements during allergic sensitization (35, 36). Receptor revision represents a possible attempt of the immune system for secondary tolerance induction in B cells of unwanted specificity (e.g., in autoimmunity) (reviewed in Refs. 37, 38). The healthy immune system subjects autoreactive B cells to anergy or clonal deletion (39). A biologically more economical way to escape this fate is secondary modification of the Ag receptor by receptor revision. By altering the Ag receptor specificity, the formerly autoreactive B cell might be rendered nonautoreactive. As in autoimmunity, a major hallmark in the pathogenesis of allergies is the loss of peripheral B cell tolerance (39). Thus, our results raise the question whether enhanced secondary D–J_H rearrangements in IgE_{PB/PC} are the result of futile attempts to induce tolerance in allergen-specific cells. An alternative explanation for the preferred usage of IGHJ3 and IGHJ4 in IgE_{PB/PC} could be a selection for particular structural properties of the CDR-H3 loop. Interestingly, we found in our study that in mice usage of IGHJ3 is a prerequisite for the formation of an extralinked base structure according to Shirai's "H3 rules" (40). An extralinked base structure retracts the tip of the CDR-H3 loop into the Ag binding groove and can therefore influence Ag binding properties of the Ab (6, 41, 42). The predictable percentage of extralinked base structures in IgE_{PB/PC} was 7-fold higher than that in IgE_{non-PC} or IgM_{Bcell} (data not shown). Therefore, the preferred usage of certain IGHJ genes in IgE_{PB/PC} might reflect the selection for structural stability in the CDR-H3 base.

Beyond the characterization of the genetically determined composition of the IgE repertoire, a major aim of our study was to evaluate the putative selective pressure that coined the expressed repertoire. It is subject to ongoing discussion whether allergic sensitization represents a misled but classical Ag-driven and oligoclonal B cell response (43, 44). However, recent observations of a heavily biased use of specific V_H gene families in IgE transcripts from allergic patients (45–49) have led to the hypothesis that some allergens might also act in a superantigen-like fashion (superallergen hypothesis, reviewed in Refs. 50, 51). If OVA should act as a superallergen, then we would have expected a highly biased distribution of V_H gene usage. On the contrary, we found no differences in V_H gene utilization among IgE_{PB/PC}, IgE_{non-PC}, and IgM_{Bcell} and hence no indication of a superantigen-like action of OVA.

However, we found evidence for classical Ag-driven selection during the OVA-induced IgE response; compared with IgM sequences from the same animals, IgE sequences showed a high level of somatic hypermutation, clustering in the CDRs. In our study, only 0.6% of the IgM_{Bcell} but 15% of the IgE_{PB/PC} displayed signs of Ag selection. Moreover, although almost all unique IgM_{Bcell} belonged to clonally unrelated and hence different clones, we found an oligoclonal expansion with a distinct clonotype restriction in IgE. Therefore, we conclude that the murine IgE sequences in our study are consistent with an Ag-driven B cell response of the adaptive B-2 B cell repertoire. This is in harmony with a murine study from Luger et al. (52) and with several studies in allergic patients, suggesting that allergic sensitization represents an Ag-driven, affinity-matured, and oligoclonal B cell response (reviewed in Refs. 1, 53). Erazo et al. (16) postulate a "programmatic link" between the high-affinity IgE response and PC differentiation. Interestingly, in our present study IgE_{non-PC} displayed significantly fewer signs of somatic hypermutation and Ag selection than IgE_{PB/PC}. Although twice as high as in IgM_{Bcell}, the somatic mutation rate in IgE_{non-PC} was only 50% of that in IgE_{PB/PC}. Moreover, IgE_{PB/PC} showed much more evidence for significant Ag selection than IgE_{non-PC}.

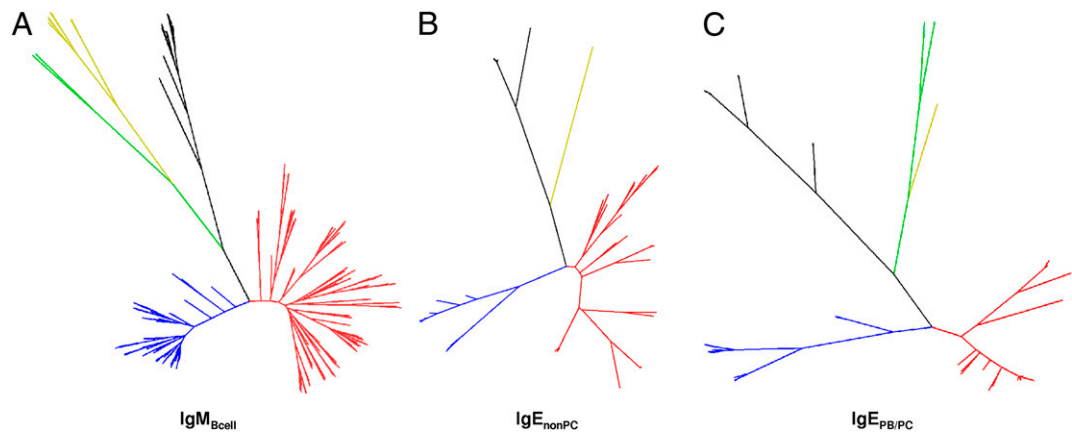


FIGURE 7. A–C, Phylogenetic trees showing the evolutionary relationships among transcripts from (A) IgM_{Bcell}, (B) IgE_{non-PC}, and (C) IgE_{PB/PC} (red, IGHV1; yellow, IGHV2; green, IGHV3; black, IGHV5; blue, IGHV14). Almost every unique IgM-sequence pertained to a different clonotype, whereas the IgE repertoire encompassed markedly fewer clonotypes and was clonally restricted.

Thus, IgE-producing PBs/PCs from OVA-sensitized mice appear to be a highly specialized and thoroughly selected cell population, suggesting that 1) Ag-driven affinity maturation is a major force that selects IgE-producing B cells into the CD138^{pos} PC pool and that the Ig repertoire of PCs represents a biased selection from the global B cell repertoire. Perhaps our observation that a group of highly mutated sequences and a group of low mutated sequences are present within IgE_{PB/PC} and within IgE_{non-PC} indicates that each of these IgE-producing cell populations could be divided into further subsets, which remain to be characterized in future studies. To address this point, we are currently studying the IgE response in mice that were infected with the parasitic nematode *L. sigmodontis*. Initial results indicate that the IgE response during this parasitic infection is much more diverse than during allergic sensitization with OVA (Table II). Thus, the allergic sensitization to OVA apparently represents a more focused immune response than the parasitic infection with *L. sigmodontis*. We conclude that our study in OVA-sensitized mice must be interpreted carefully, because the IgE response might vary between differing Ags, mouse strains, or species.

Taken together, we found evidence for at least two distinct IgE-expressing B cell subsets, namely, a CD138^{pos} (PBs/PCs) and a CD138^{neg}/CD19^{pos} (non-PCs). The IgE transcripts of PBs/PCs are significantly higher mutated with a pattern of Ag-driven selection, indicating classical affinity maturation. The CDR-H3 composition, which typically influences the Ag recognition properties of an Ab, differs between IgE transcripts from PBs/PCs and non-PCs. We conclude that during allergic sensitization, IgE-secreting PCs are recruited due to their classic Ag-binding site, implying an Ag-driven selection into the PC pool.

Table II. Clonotypic diversity of the IgE-producing PBs/PCs and non-PCs in *L. sigmodontis*-infected versus OVA-sensitized mice

		Infected	Sensitized	<i>p</i> Value, Two-Tail χ^2 Test	
PBs/PCs	Functional sequences	34	80	0.0002	
	Clonotypes	20	17		
	Clonotypic diversity	0.59	0.21		
Non-PCs	Functional sequences	35	73	0.011	
	Clonotypes	20	22		
	Clonotypic diversity	0.57	0.30		

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Disclosures

The authors have no financial conflicts of interest.

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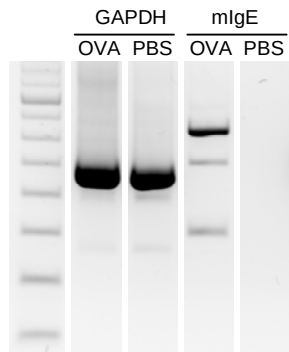
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SUPPLEMENTAL DATA:

To confirm the presence of mIgE-bearing cells within the nonPC fraction, an additional control experiment was performed:



Supplemental Figure 1: Cells in the nonPC fraction of OVA-sensitized mice express transcripts for the membrane splicing variant of IgE (mIgE).

NonPC cells (CD138^{neg}/CD19^{pos} lymphocytes) were sorted according to the method given in the manuscript (Figure 1A), and RNA was extracted from the sorted nonPC cells. mIgE transcripts were amplified by reverse transcriptase-PCR using an IgE constant region 3 (CH ϵ 3) forward primer (C ϵ 3-820f, 5' GCCAAGGACTGGATTGAAGG 3') together with an IgE membran region 2 reverse primer (M ϵ 2-1432r, 5' CCCTGCTCTGGAGGATGTTG 3').

RT-PCR amplifications were carried out in a total volume of 25 μ l containing 2 μ l of RNA, 2.5 mM MgCl₂, 6 U recombinant RNase inhibitor, and 0.6 μ M of each forward and reverse primer using a OneStep RT-PCR kit (Qiagen; Hilden, Germany). The following program was performed on a thermocycler (Sensoquest; Göttingen, Germany): 30 min at 50°C, 15 min at 94°C followed by 40 cycles using a cycle profile of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. GAPDH gene products were amplified using mGAPDH-1 (5' GGGGTGAGGCCGGTGCTGAGTAT 3') and mGAPDH-2 (5' CATTGGGGGTAGGAACACGGAAGG 3') primers.