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Sequential class switching is required for the generation of high affinity IgE antibodies

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IgE antibodies with high affinity for their antigens can be stably cross-linked at low concentrations by trace amounts of antigen, whereas IgE antibodies with low affinity bind their antigens weakly. In this study, we find that there are two distinct pathways to generate high and low affinity IgE. High affinity IgE is generated through sequential class switching (μ→γ→ε) in which an intermediary IgG phase is necessary for the affinity maturation of the IgE response, where the IgE inherits somatic hypermutations and high affinity from the IgG1 phase. In contrast, low affinity IgE is generated through direct class switching (μ→ε) and is much less mutated. Mice deficient in IgG1 production cannot produce high affinity IgE, even after repeated immunizations. We demonstrate that a small amount of high affinity IgE can cause anaphylaxis and is pathogenic. Low affinity IgE competes with high affinity IgE for binding to Fcε receptors and prevents anaphylaxis and is thus beneficial.

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Abbreviations used: AID, activation-induced (cytidine) deaminase; CSR, class switch recombination; GC, germinal center; HA, hemagglutinin; HIES, hyper IgE recurrent infection syndrome; hMT, human metallothionein II_A; NP, Nitrophenyl; PCA, passive cutaneous anaphylaxis; qPCR, quantitative PCR; TBmc, TB monoclonal.

Antibodies belong to five classes (IgM, IgD, IgG, IgE, and IgA) depending on the constant regions of the heavy chain (C_H) they use. Class switch recombination (CSR) changes the C_H from Cμ to a downstream C_H (Cγs, Cε, or Cα) by DNA recombination and looping-out of the intervening sequences (Selsing, 2006). The irreversible DNA rearrangement in the C_H region occurs between two switch (S) regions, which lie upstream of each C_H region (except Cδ). CSR is initiated by activation-induced (cytidine) deaminase (AID), which is also essential for the introduction of somatic hypermutations in the variable regions of the BCR (Muramatsu et al., 2000).

IgE is the most stringently regulated secreted Ig, with the lowest serum concentration and the shortest half-life of about 2 d or less (Tada et al., 1975; Stone et al., 2010). In contrast, cell-bound IgE can remain for weeks on the surface of cells bearing Fcε receptors. Mast cells (as well as basophils) are prearmed with IgE through FcεRI molecules binding to the C region of IgE. Cross-linking of IgE molecules by multivalent antigen results in the activation of mast cells. Upon activation, mast cells degranulate, releasing histamine and other mediators that dilate blood vessels, initiate inflammation, and recruit other effector cells (Kinet, 1999; Kraft and Kinet, 2007; MacGlashan, 2008).

The affinity of IgE molecules for their antigens is crucial in determining the fate of IgE-decorated cells. It is possible that mast cells bearing IgE molecules with high affinity for the antigens could be degranulated in the presence of just minute amounts of antigens. In contrast, the high levels of antigen required to cross-link and degranulate mast cells covered by low affinity IgE molecules may not be physiologically achievable. Mast cells are important components of the homeostasis of tissues, and, in such role, they are beneficial. A series of papers have conclusively shown that IgE antibodies are capable of increasing survival of growth factor–deprived mast cells (Asai et al., 2001). There have been no studies comparing the behavior of mast cells covered by IgE antibodies that display low versus high affinity binding

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to a given antigen; instead, affinity correlations were performed by studying strong and weak cross-linking of FcεR in several alternative ways (Kalesnikoff et al., 2001; Gonzalez-Espinosa et al., 2003; Yamasaki et al., 2004; Kohno et al., 2005; Rivera et al., 2008). These studies showed that a high level of cross-linking of FcεRI receptors results in mast cell degranulation as well as cytokine/chemokine secretion, whereas a low level of cross-linking leads to cytokine and chemokine secretion but no degranulation. However, so far there is no conclusive evidence that the increasing affinity of a given antigen for IgE can turn the function of IgE from beneficial to pathogenic.

At the molecular level, there are two paths to produce IgE antibodies from IgM (Fig. 1 A). One is the direct switching pathway, in which recombination occurs between Sμ and Sε; the other is the sequential switching pathway, which is a two-step process observed in both mice and humans. In mice, Sμ first recombines with Sγ1, and the hybrid switch region undergoes a second recombination with Sε. At the end of sequential switching, Sγ1 remnants can be found in some of the rearranged Sμ-Sε junctions (Fig. 1 A). It has been reported that in mice up to 30% of the recombined regions contain Sγ1 remnants (Siebenkotten et al., 1992; Mandler et al., 1993). In human cells, the switch to ε in a lymphoid cell line was preceded by an Sμ-Sγ recombination (Mills et al., 1992). In B lymphocyte cultures from mice harboring a deletion in the IgM switch region, IgE^+ cells could be found at nearly normal levels (70%), and they were derived from CSR events involving Sγ1 (Zhang et al., 2010).

We previously reported that ε sterile transcripts and ε switched transcripts, which are generated before and after the switch to Cε, respectively, were abundantly found in $IgG1^+$ cells. Furthermore, purified $IgG1^+$ cells could switch to IgE in vitro and in vivo. In addition, affinity-enhancing somatic mutations found in the $IgG1^+$ cells were also found in IgE^+ B cells, although with some delay (Erazo et al., 2007). We proposed that sequential class switching through an IgG1 intermediate was a way to generate high affinity IgE antibodies. The IgE cells were absent from germinal centers (GCs), and we proposed that they inherited somatic mutations and affinity maturation from the preceding IgG1 phase.

Despite prior studies by multiple laboratories (Yoshida et al. [1990], Erazo et al. [2007], and references therein), it is unclear whether sequential switching to IgE through an IgG1 intermediate is an obligatory step to generate high affinity IgE antibodies. In this study, we demonstrate that, indeed, high affinity IgE antibodies in the mouse are generated through an IgG1 intermediate.

RESULTS

A simple assay to quantify sequential DNA switching in IgE^+ cells

Because our hypothesis is that high affinity IgE antibodies are generated through an IgG intermediate, we developed a PCR assay to quantify Sγ remnants in the Sμ-Sε junctions of IgE^+ cells. As schematized in Fig. 1 A, the finding of Sγ1 remnants is always indicative of sequential switching. In our assay, after we ran a PCR over the Sμ-Sε junctions, the products were subjected to quantitative PCR (qPCR) on the Sγ1 repeats.

We first validated the method using four IgE hybridomas and five subclones from one of them. PCR was performed using primers located at 5′ of Sμ and 3′ of Sε. The size of the PCR-amplified Sμ-Sε junctions was between 3 and 7 kb (Fig. 1 B). There was a dominant band for each hybridoma. The four hybridomas had different sized bands, and the five subclones had the same sized band as the original hybridoma (Fig. 1 B). 24 minipreps ran from cloned Sμ-Sε junctions

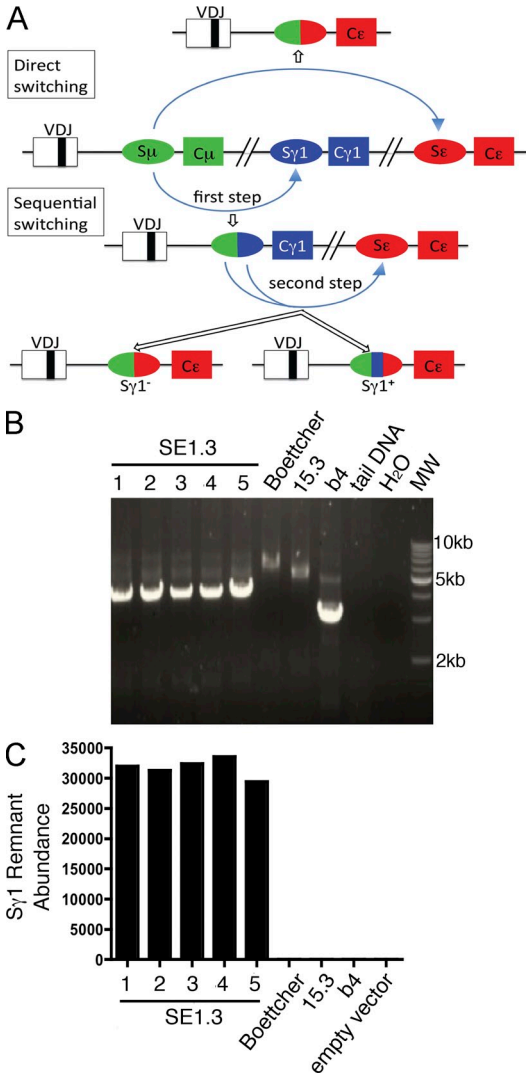

Figure 1. A simple PCR assay to detect sequential switching to IgE. (A) Schematic representation of direct and sequential switching together with the chromosomal products. Sγ1 remnants are always indicative of sequentially switched Sμ-Sε junctions. (B and C) Molecular characterization of the Sμ-Sε junction from IgE-producing hybridomas. (B) Sμ-Sε PCR on the DNA of hybridomas SE1.3, Boettcher, 15.3, and b4. (C) Sγ1 qPCR on miniprepped Sμ-Sε junctions. The abundance is calculated as the relative transcription normalized to neomycin resistance gene expression fixed at 10,000.

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of each hybridoma indicated that all inserts were of the same size, reassuring us that no major recombination events took place during the PCR and cloning steps.

The second PCR step was a quantitative real-time PCR on Sγ1 to target the characteristic 49-mer Sγ1 consensus sequences (Mowatt and Dunnick, 1986). Sγ1 contains a long (~11.5 kb) region characterized by >120 imperfectly repeated 49-mer sequences. Actual consensus Sγ1 sequence repeats perfectly only nine times, whereas the other repeats have small but complicated changes like overlaps and truncations. qPCR detected the Sγ1 remnant in one of the four hybridomas and all of its subclones. The other three hybridomas were negative (Fig. 1 C). Both positive and negative qPCR results were confirmed by DNA sequencing. Because it was apparent that the IgE-producing hybridomas only retained one Sμ-Sε allele, the Sγ1 qPCR yielded a quantitative signal of either >10,000 or 0, i.e., Sγ1 was either present or absent.

To evaluate the frequency of sequentially switched IgE cells within a population of cells during an immune response, we defined an Sγ1 ratio, which is the number of $S\gamma1^+$ junctions among the total number of Sμ-Sε junctions. Even though not all sequentially switched cells had Sγ1 remnants (Fig. 1 A), as a population, the frequency of $S\gamma1^+$ cells still provided a comparative measure of the extent of sequential switching. The Sγ1 ratio in hybridomas was either 0 or 1, and an in vivo IgE response will have a ratio anywhere between 0 and 1.

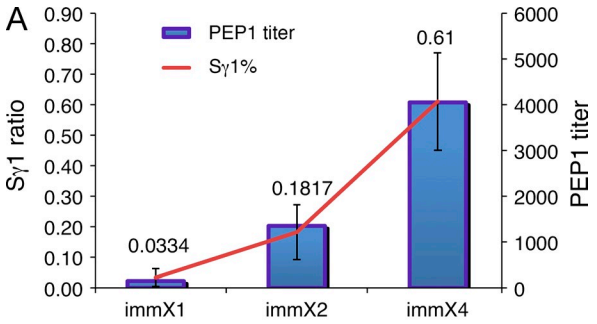
Increased sequential switching to IgE correlates with increased IgE affinity for antigen

For the present experiments on affinity maturation of the IgE response, we used two experimental systems. The TBmc system (Curotto de Lafaille et al., 2001) consists of heavy and light chain influenza hemagglutinin (HA)-specific Ig knockin mice crossed with OVA-specific TCR transgenic mice (D011.10) and RAG1 KO mice, all on a BALB/c genetic background. The HA-specific knockin Ig genes were derived from the high affinity BCR 17-9 hybridoma (Schulze-Gahmen et al., 1988), and as a consequence, the antibodies are intrinsically of high affinity for HA. Because of both the absence of natural regulatory T cells (T_{reg} cells) and the BALB/c background, these mice display a high HA-specific IgE response after one immunization with the cross-linked antigen OVA-HA. There is no increase in affinity-enhancing mutations of the anti-HA response after OVA-HA immunization (Erazo et al., 2007). To study affinity maturation, we used a single mutant HA peptide, PEP1, in which a phenylalanine residue replaced one of the tyrosine residues in HA. Binding of the endogenous anti-HA antibodies to PEP is 1,000 times weaker than to HA (Pinilla et al., 1993). In TB monoclonal (TBmc) mice, immunization with OVA-PEP1 allows the tracking of affinity maturation both by the appearance of PEP1-binding B cells (undetectable if mice are not immunized with PEP1) and PEP1 affinity–enhancing mutations in the Ig genes.

The second experimental system is a well-studied system of immunization of WT mice with the hapten Nitrophenyl (NP) conjugated with carrier proteins. The results using both experimental systems were in agreement, as shown in the following paragraphs.

Immunizing TBmc multiple times at 3-wk intervals with OVA-PEP1 enabled us to observe the gradual increase of IgE affinity to PEP1 (Fig. 2 A). After each immunization, we determined the serum titer against PEP1 and sorted $IgE^+IgG1^-Fas^{high}$ cells from the spleen (not depicted). As the number of immunizations of TBmc mice with OVA-PEP1 increased from one time to four times, the PEP1-specific IgE serum titer raised from 50 to 4,000 (Fig. 2 A). $IgE^+IgG1^-Fas^{low}$ cells were also sorted but did not contain amplifiable switch regions (not depicted); they were positive for FcεRI (not depicted), indicating that they were IgE-coated cells. As we reported previously, a mild acid treatment is sufficient to dislodge IgE molecules from CD23, the low affinity IgE receptor, but not from the high affinity FcεRI (Erazo et al., 2007).

DNA was extracted to perform Sμ-Sε PCR. We observed a smear of different sizes (not depicted). Interestingly, as the number of immunizations increased, we observed dominant Sμ-Sε bands, which was not seen in other experimental models such as the NP-immunized human metallothionein II_A (hMT) mice and the anti-IgD–treated WT mice (see below). We applied the aforementioned single colony qPCR assay to determine the Sγ1 ratio. As we increased the number of immunizations, the Sγ1 ratio went up from 0.03



B

Sγ1⁺ junctions / total Sμ-Sε junctions		
immX1	immX2	immX4
0/24	5/26	9/25
1/24	3/25	15/25
0/24	7/26	21/24

Figure 2. Sequential switching is correlated with increased IgE affinity for antigen. (A) TBmc mice were immunized with 100 μg OVA-PEP1 for one, two, and four times as indicated in the x axis. Serum was taken for ELISA to determine the IgE anti-PEP1 titer, and IgE^+ cells were sorted to prepare DNA, Sμ-Sε PCR, cloning, and Sγ1 qPCR assay. Blue bars indicate the anti-PEP1 IgE titer (right y axis), and the red line indicates the Sγ1 ratio of Sμ-Sε junctions (left y axis). Three mice from each group and at least 24 clones/mouse were randomly chosen for Sγ1 qPCR. SD is indicated. (B) Sγ1 ratio in A of each individual mouse by single colony qPCR.

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to 0.18 and then to 0.61 (Fig. 2, A and B), indicating that sequentially switched IgE was gradually enriched as the IgE affinity increased. To validate the sensitivity and specificity of Sγ1 qPCR, we randomly chose 40 Sμ-Sε junctions for sequencing and compared the sequencing data with qPCR results (Table S1). The Sγ1 qPCR assay was 100% sensitive and 100% specific. The minimal Sγ1 length that was detected was 108 bp. Many Sγ1 remnants had incomplete consensus sequences, indicating that the qPCR assay could deal with small Sγ1 fragments with imperfect 49-mer repeats.

More switch region mutations accumulate in junctions undergoing sequential switching than direct switching

It has been reported that somatic hypermutations in both VDJ regions and switch regions are driven by the same AID machinery (Petersen et al., 2001; Goodman et al., 2007; Maul and Gearhart, 2010). The mutation levels in switch regions can therefore be used to evaluate AID activity. To compare the level of AID activity during sequential and direct switching to IgE, we analyzed the mutations in Sμ-Sε junctions of PEP1-immunized TBmc mice. We found that >95% of the mutations in switch regions accumulated in a 1,000-bp region flanking the actual junction (500 bp each side). There were significantly more mutations in $S\gamma1^+$ than in $S\gamma1^-$ Sμ-Sε junctions (5.7 vs. 12.4; P = 0.02; Fig. 3, A and B). 67% of the $S\gamma1^+$ clones (sequentially switched) had >10 mutations, whereas only 19% of the $S\gamma1^-$ clones had >10 mutations. Conversely, two thirds of $S\gamma1^-$ junctions had zero to four mutations, whereas only 17% of the $S\gamma1^+$ junctions had such low mutation levels (Fig. 3 B). Over 70% of the mutations had the AID target motif WGCW (Goodman et al., 2007; Han et al., 2011). The difference in the number of mutations between $S\gamma1^-$ and $S\gamma1^+$ junctions increased as the number of immunizations augmented (5.7 in the Sμ-Sε direct switching junctions vs. 9.8 in the Sμ-Sγ1 sequential switching junctions, after four immunizations; P = 0.04; Fig. 3 C). Furthermore, the distribution of the number of mutations in $S\gamma1^-$ junctions was discontinuous, with the appearance of a bimodal distribution (Fig. 3 B, left). It is possible that the highly mutated $S\gamma1^-$ sequences represent sequential switching events in which the Sγ1 remnants were absent (Fig. 1 A). However, these mutations were particularly enriched in intra-switch recombination events, as four out of six $S\gamma1^-$ sequences with >20 point mutations had intra-switch recombination in Sμ; therefore, we cannot be sure that the highly mutated $S\gamma1^-$ sequences represent sequential switching events.

The lower mutation frequency in Sε compared with Sμ could be explained, at least partially, by the lower number and density of AID target motifs in the Sε than in the Sμ region; the preferred AID motif WGCW is present 415 times in Sμ (density of 134.7/kb), 120 times in Sε (density of 41.9/kb), and 401 times in Sγ1 (density of 34.9/kb). Collectively, these results suggest that sequential switching correlates with a higher AID activity or two separate AID events from the two-step recombination process.

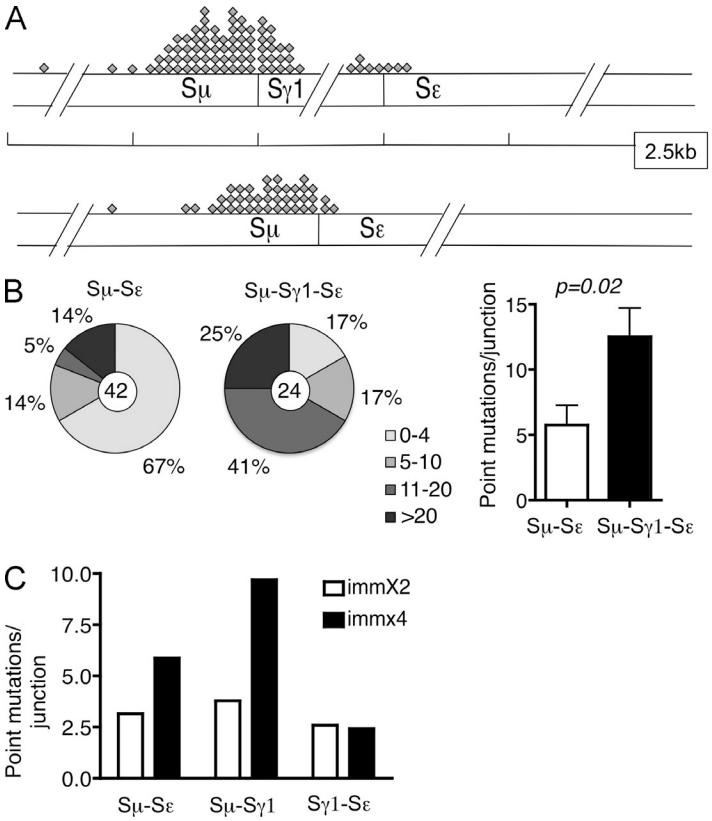

Figure 3. Sequentially switched Sμ-Sε junctions harbor a large number of mutations. (A) TBmc mice were immunized two or four times with OVA-PEP1. The DNA was extracted, and the Sμ-Sε junctions were amplified. 33 clones from each group were sequenced. Diamonds show the approximate position of the mutations at the junction. (B) $S\gamma1^-$ and $S\gamma1^+$ junctions from mice immunized four times were sequenced and analyzed using pies (left) to show the distribution of point mutation frequency and columns (right) to show the overall mutation frequency. Error bars indicate SEM. (C) Mean number of point mutations at the junctions after two and four immunizations. $S\gamma1^+$ junctions were split into two junctions, Sμ-Sγ1 and Sγ1-Sε, and counted separately.

IgG1-deficient hMT mice produce normal levels of IgE but have impaired affinity maturation of the IgE response

If, as the correlations show, high affinity IgE antibodies are generated through sequential switching through an IgG1 intermediate, then IgG1 deficiency should have an impact on the affinity maturation of the IgE response. hMT mice have a replacement at the Iγ1 region (5′ of Sγ1) by an hMT promoter, which doesn't respond to a variety of inductive stimuli or get involved in any recombination process. As a result, these hMT-deficient mice cannot generate sterile γ1 transcripts and therefore lack $IgG1^+$ cells and IgG1 antibodies (Lorenz et al., 1995). It has been shown that these mice can still have

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normal levels of total IgE (Jung et al., 1994), indicating that IgE does not necessarily derive from sequential switching. However, we propose that the IgE affinity maturation in hMT mice is impaired.

We immunized hMT and their WT littermates with $NP_{19}KLH$ four times at 3-wk intervals and collected serum to assess the NP-specific antibody responses. Plates were coated with NP_4BSA and $NP_{23}BSA$. NP_{23} is a highly conjugated molecule that binds both low and high affinity antibodies, whereas NP_4 has a low degree of cross-linking and binds only high affinity antibodies. A higher NP_4/NP_{23} ratio indicates a higher affinity to NP (Herzenberg et al., 1980; Allen et al., 1988; Smith et al., 1994; Chen et al., 2000; Shimizu et al., 2003; O'Connor et al., 2006). IgE antibodies from hMT mice had a much lower NP_4/NP_{23} ratio than IgE antibodies from WT mice (Fig. 4 A, left). Absolute absorbance of NP_{23}-bound IgE was similar between hMT and WT mice, but the binding of NP_4 was much weaker in hMT IgE than in WT IgE (Fig. 4 A, right). As expected, total IgE levels were similar in hMT and WT (Fig. 4 B), indicating that the low NP_4/NP_{23} ratio in hMT mice was not caused by the deficiency of IgE production but rather by the affinity maturation of the IgE response. We applied the same ELISA technique to study the IgG2a and IgG1 responses in the same mice. IgG2a constitutes a good control because the Sγ2a region is, like Sε, located downstream Sγ1, and it is at 5′ next to Sε. Thus, IgG2a is a good isotype to identify whether any nonspecific effect on IgE could be taking place. hMT mice did not have any IgG1, whereas IgG1 of high affinity was readily detected in WT mice (Fig. 4 C, left). The affinity maturation of the IgG2a response was the same in hMT and in WT mice (Fig. 4 C, right), indicating that IgG2a affinity maturation occurs independent of γ1 sequential switching.

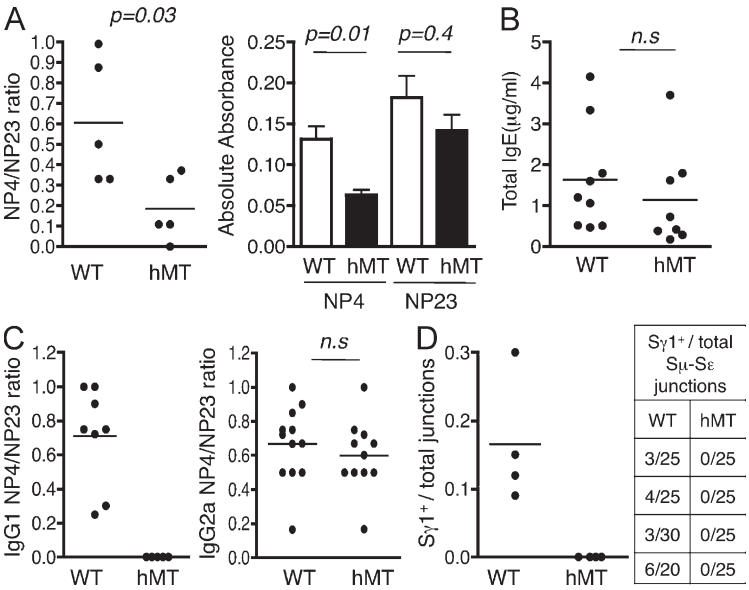
We sought to demonstrate that the high affinity NP-specific IgE response was accompanied by molecular remnants of sequential switching through IgG1. We sorted IgE^+ cells and ran the Sμ-Sε PCR in the same way we did on TBmc mice. In contrast to the TBmc situation, dominant Sμ-Sε bands were not observed after four immunizations in hMT mice or the WT littermates. We then calculated the Sγ1 ratio based on 100 cloned Sμ-Sε junctions derived from four WT mice and a similar number of hMT mice. The Sγ1 qPCR assay showed that, depending on the mice, 10–30% of WT Sμ-Sε junctions had Sγ1 remnants (Fig. 4 D). Interestingly, Sμ-Sε junctions from hMT mice not only lacked Sγ1 remnants, which was expected, but contained no other intervening S region (not depicted). Thus, in hMT mice, IgE antibodies are exclusively generated through direct switching.

Somatic mutations that enhance affinity to NP are not present in IgE genes from IgG1-deficient mice

In the C57BL/6 (B6) mouse strain, the repertoire of B cells responding to NP has been extensively studied (Cumano and Rajewsky, 1986; Furukawa et al., 1999; Weiser et al., 2011). It was determined that $V_H186.2$ was preferentially used in the anti-NP response, paired with IgLλ1.

The maturation of the NP-specific response includes two different mutation paths: in one of them, the first junctional residue in the CDR3 is Y99. In those B cells, affinity to NP is greatly increased by a W33L mutation. Additional mutations do not further enhance the affinity for NP in the Y99-type cells. The second path involves heavy chains with a junctional CDR3 residue G99, encoded by N nucleotides. In this case, high affinity is achieved through the accumulation of several mutations, mainly K59R, G57D, S66N, and other contributors, and allows for much higher affinities (Furukawa et al., 1999; Weiser et al., 2011). However, there is a timing trade-off: it has been determined that the Y99/W33L type of cells appears relatively early during the anti-NP response, whereas the G99/K59R type of cells appears late, but the latter type takes over upon secondary immunization as it accumulates additional affinity-enhancing mutations (Jacob et al., 1993; Takahashi et al., 2001; Murakami et al., 2010; Nishimura et al., 2011).

In contrast to the B6 strain, knowledge on the molecular basis of affinity maturation of the NP response in BALB/c mice is scanty. Because of the fact that B6 mice make poor IgE responses, our work was performed in hMT mice (and littermates) backcrossed



Sγ1+ / total Sμ-Sε junctions	
WT	hMT
3/25	0/25
4/25	0/25
3/30	0/25
6/20	0/25

Figure 4. IgE affinity is impaired in IgG1-deficient mice. (A) WT and hMT mice were immunized with $NP_{19}KLH$ four times. Serum was analyzed by ELISA with NP_4- and NP_{23}-coated plates. (left) NP_4/NP_{23} ratio. (right) Absolute absorbance value. Error bars indicate SEM. Each dot represents one mouse. (B) Same sera used in A were subjected to total IgE ELISA. (C) IgG1 and IgG2a ELISA with NP_4/NP_{23} plates. (D) Sγ1 ratio in WT and hMT mice using the Sγ1 qPCR assay. Each dot represents one animal (four animals per group). The number of $S\gamma1^+$ junctions in each mouse is shown in the right panel, divided by the total number of Sμ-Sε sequences. (A–D) Horizontal bars indicate the mean.

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onto the BALB/c background. Our characterization of the NP-specific response in mice of BALB/c background indicated that the preferred V_H gene was homologous to V_H186.2 and that the λ IgL chain also dominated the NP response in BALB/c mice (not depicted). We immunized BALB/c background mice with NP_{19}KLH, sorted $IgG1^+$ cells that did or did not bind NP-PE (not depicted), and sequenced the VDJ segments. Interestingly, the Y99/W33L path was not found on >300 DNA sequences from NP^+ B cells, at any time point ranging from day 7 to day 80 after immunization (Fig. 5 A). Instead, acquisition of NP binding was accompanied by an increase in the frequency of the K59R mutation, which in approximately half of the cases had G57D and/or S66T/N mutations. G99 was found only in some of these antibodies, but all the junctions contained N nucleotides (Table S2). Thus, the pathway that confers the highest NP binding affinity in B6 mice is the one that operates in BALB/c mice.

With this knowledge in hand, we sought to analyze the affinity-enhancing mutations in IgE, IgG2a, and IgG1 in WT and hMT mice immunized with NP_{19}KLH. We amplified V_H1.1 sequences (homologous to the B6 V_H186.2) in IgE^+, $IgG2a^+$, and $IgG1^+$ cells. We then studied the mutations in 96 DNA sequences for IgE, 82 sequences for IgG2a, and 62 sequences for IgG1 (the latter only from WT mice). The IgE VDJ repertoire in hMT mice had significantly fewer mutations than WT mice (Fig. 5, B and C). Only 8% of the IgE cDNAs from hMT mice had 20–24 nucleotide mutations, and no cDNA had 25–29 mutations. In contrast, in WT mice, 13% and 17% of the IgE VDJ regions contained 20–24 and 25–29 mutations, respectively. The percentage of sequences with high frequency of mutations in IgE genes from WT mice was slightly lower than that of IgG1 genes from the same mice (Fig. 5 B), consistent with the observation in TBmc anti-PEP1 response (Erazo et al., 2007). In contrast with IgE, the frequency of mutations in IgG2a VDJ genes was similar between hMT and WT mice (Fig. 5 B). Note that the reduction in the frequency of mutations is less pronounced than the reduction in affinity, indicating that the selection of high affinity mutants is more affected than the overall level of mutations.

Next we analyzed the typical mutations that confer higher binding affinity to NP in mice of BALB/c background. In IgE genes, the K59R mutation was found only in WT IgE but not hMT IgE (Fig. 5 A). In contrast, the K59R mutation had a similar frequency in IgG2a from both WT and hMT mice. About half of the K59R IgE clones in WT mice had the additional G57D and/or S66T/N mutations (Fig. 5 A).

Finally, we also studied the J and D segment usage in these clones. In C57BL/6 mice, the early NP-specific B cells (Y99 type) have highly enhanced usage of the Dfl16.1-J_H2 segments, and even the late-arising NP-specific B cells (G99 type) predominantly use the Dfl16.1 segment (Furukawa et al., 1999). In the BALB/c background mice, there was no strict preference for the usage of certain J_H (not depicted) or D_H (Table S2) segments; however, there was a preference of J_H3 in NP^+IgG1^+ cells, whereas NP^-IgG1^+ cells used more the J_H4 segment (P = 0.0001; not depicted). Thus, J_H3 usage correlates with higher affinity for NP in the BALB/c background. In accordance with this, the J_H usage in total IgE^+ cells from hMT mice showed preference for J_H4 over J_H3, whereas the higher affinity IgE^+ cells from WT mice showed preference for J_H3 over J_H4 (P = 0.0001; not depicted). In summary, these data strongly support the model in which high affinity IgE is generated through sequential switching involving Sγ1.

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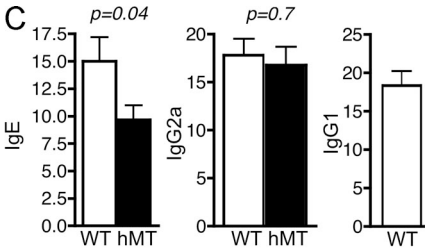
A

	WT IgE	hMT IgE	WT IgG2a	hMT IgG2a	NP high IgG1	NP low IgG1
K59R	7/82	0/80	5/60	6/69	9/50	1/50
K59R+G57D/ S66N	3/7	-	2/5	3/6	4/9	0/1



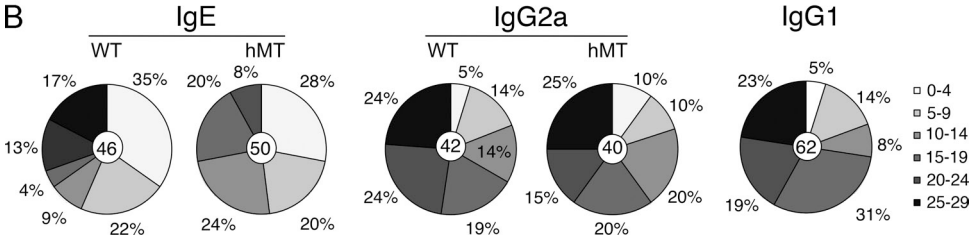


Figure 5. Mutations in the VDJ region of IgG1-deficient mice and their WT littermates. (A) Total splenocytes from the same hMT and WT littermate mice used in Fig. 4 A were prepared for cDNA. PCR was performed using a common V_H1.1 forward primer and Cε and Cγ2a reverse primers. NP^{high} and NP^{low} $IgG1^+$ cells were sorted by staining with NP-PE. cDNA was prepared, and V_H1.1- Cγ1 PCR was performed. After sequencing, the NP affinity–enhancing mutations were tabulated. (B and C) Total nucleotide mutation analysis of the same sequences described in A (B) and the analysis of total mutation frequency (C). Error bars indicate SEM.

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Direct switching to IgE upon antigen-independent B cell stimulation

After confirming the importance of sequential class switching to IgE for the affinity maturation of two different antigen-driven T cell–dependent responses, we investigated the role of sequential switching to a known polyclonal stimulator of IgE and IgG1 production, anti-IgD serum (Finkelman et al., 1989). We injected the WT BALB/c with goat anti-IgD antibodies and, as a control, with one $NP_{19}KLH$ immunization. Anti-IgD–treated mice produced a substantial IgE response (Fig. 6 A, left). However, the $IgE^{+}Fas^{high}$ fraction contained <10 oligoclonal Sμ-Sε bands (Fig. 6 B). This oligoclonality was not the consequence of antigen-driven selection (Yoshimoto et al., 1995; Morris et al., 1998) but probably the result of the proliferation of a limited number of B cells. The Sγ1 qPCR assay showed that 79 out of 80 clones from four anti-IgD–treated mice lacked Sγ1 remnants. Thus, only about 1% of the IgE was sequentially switched, compared with 14% in mice immunized only once with $NP_{19}KLH$ (Fig. 6 C). Despite the lack of sequential switching to IgE, IgG1 levels were high in anti-IgD–treated mice (Fig. 6 A, right), indicating that the absence of sequential switching was not caused by impaired Sμ-Sγ1 switching. We believe that the sequential switching pathway, which is associated with affinity maturation, was not activated by the anti-IgD treatment. Consistent with this, we observed a much lower number of mutations in Sμ-Sε junctions from anti-IgD–treated mice than in junctions obtained from mice immunized with antigens (Fig. 6 D).

Antigen-driven high affinity but not low affinity IgE causes anaphylaxis

The differential pathological function of IgE antibodies with high versus low affinity for their antigens has been proposed but not fully explored. Anaphylaxis is a life-threatening reaction caused by antigen-induced cross-linking of IgE molecules bound to mast cells. In this study, we used a local passive cutaneous anaphylaxis (PCA) assay in the ears to quantify the effect of high and low affinity IgE antibodies in vivo. The amount of IgE, from IgG- and IgA-depleted serum, was normalized in all cases. First, mice were injected in the ears with anti-HA IgE, followed by cross-linked HA (OVA-HA) together with Evans blue by systemic route. This combination induced strong extravasation of Evans blue into the ears (Fig. 7 A, left). However, there was virtually no Evans blue leakage when injection of the same anti-HA IgE antibodies was followed by low affinity antigen OVA-PEP1 (Fig. 7 A, middle left). As indicated above (Erazo et al., 2007), HA-specific antibodies are ~1,000-fold less reactive to OVA-PEP1 than to OVA-HA. When IgE affinity to PEP1 increased through affinity maturation caused by repeated immunizations, these IgE antibodies displayed the capability to cause Evans blue extravasation upon OVA-PEP1 challenge (Fig. 7 A, middle right). As expected, unconjugated monovalent soluble PEP1 could not cross-link anti-PEP1 IgE and did not cause the PCA (Fig. 7 A, right). The results were confirmed by spectrophotometric determination of the leaked dye upon cutting and dissolving the affected and control ears (Fig. 7 B).

Next, we titrated the amount of injected IgE in the ears. In serial dilution experiments, very diluted anti-HA IgE (1:100 dilution) caused a strong PCA reaction by OVA-HA, and the same happened in the case of anti-PEP1 IgE with OVA-PEP1. However, in the presence of OVA-PEP1, anti-HA IgE did not cause anaphylaxis, even when injected undiluted (Fig. 7 C). To illustrate the importance of affinity maturation of IgE in anaphylaxis, we studied IgE obtained after

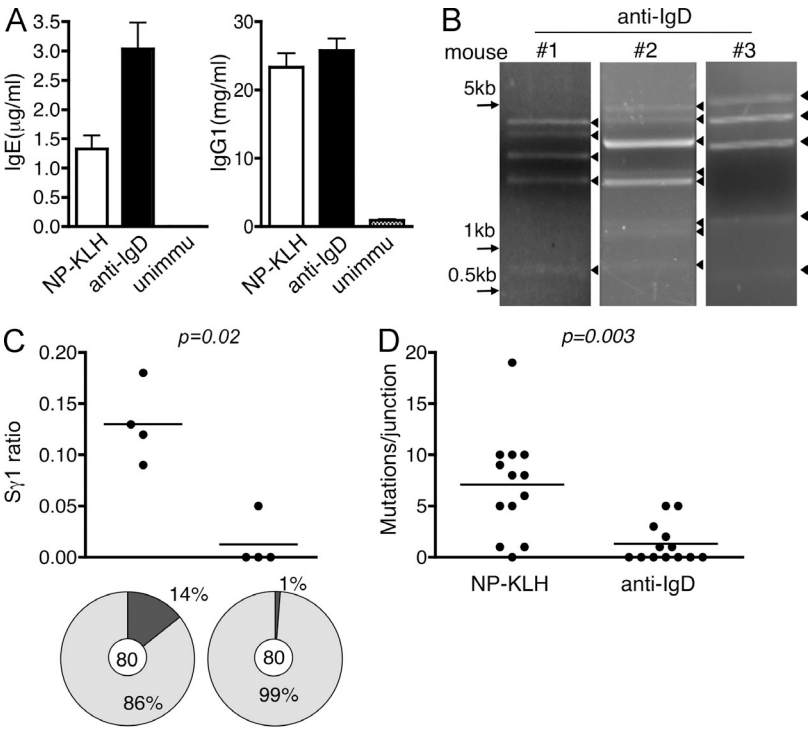

Figure 6. Antigen-independent IgE responses lack Sγ1 remnants. (A) BALB/c mice were injected intravenously with 100 μg of goat anti-IgD serum or $NP_{19}KLH$. Total IgE and IgG1 levels were determined by ELISA. Error bars indicate SEM. (B) Approximately 10^4 purified IgE^{+} cells from anti-IgD–treated mice were used for Sμ-Sε PCR. Amplified Sμ-Sε junctions from three different mice are shown. Arrowheads indicate the dominant Sμ-Sε bands. (C) Sγ1 ratio measured by the Sγ1 qPCR assay. Each dot represents one mouse (four animals per group; 20 clones per mouse). The overall Sγ1 ratio among the 80 junctions is shown in the pie chart (bottom). (D) Sμ-Sε junctions from anti-IgD– or $NP_{19}KLH$-treated mice were sequenced, and the number of mutations of each junction was plotted. Each dot represents a junction (13 junctions from three mice per group). (C and D) Horizontal bars indicate the mean.

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zero, one, two, and four immunizations with OVA-PEP1, using normalized amount of total IgE. Only IgE from mice immunized four times was capable of causing the full anaphylactic reaction (Fig. 7 D), which was confirmed by spectrophotometric measurement (Fig. 7 E).

We next performed PCA competition experiments between high and low affinity IgE and also IgM and IgG1 antibodies. Our experiments showed that when injected with high affinity anti-PEP1 IgE, only anti-HA IgE could eliminate the OVA-PEP1–induced anaphylaxis reaction, in a dose-dependent manner. Neither IgM nor IgG1 could reduce the OVA-PEP1–mediated anaphylactic reaction (Fig. 7, F and G). It is thus clear that the potential for causing pathology of low affinity and high affinity IgE antibodies is very different. In summary, the affinity maturation of the IgE response requires an IgG intermediate, and the consequence of this process is the generation of antibodies capable of eliciting anaphylaxis.

DISCUSSION

Sequential switching $\mu \rightarrow \gamma \rightarrow \varepsilon$ was discovered >20 yr ago (Yoshida et al., 1990). However, previous studies did not investigate whether Sγ remnants represented transient markers of an instant sequential switching event or there was a cellular IgG1 phase. Our data showed that affinity-enhancing mutations in VDJ segments from IgE cDNAs but not from the IgG2a cDNA, whose C region lies next to Cε, were greatly reduced in IgG1-deficient mice. Thus, affinity maturation of IgE can occur through a GC IgG1 phase. Whether these $IgG1^+$ cells that switch to IgE^+ cells are immediate post-GC cells, memory cells, or cells committed to become plasmablasts remains the subject of future studies. We previously showed that GC IgG1 cells can express sterile as well as switched ε transcripts, but double expressing $IgG1^+/IgE^+$ cells produced in homozygous knockin TBmc mice do not have a GC phenotype (Erazo et al., 2007).

It has been known for a long time that the dose and frequency of immunizations have an impact on the amount and ratio of IgE and IgG1 antibodies (Vaz et al., 1971). It is likely that at higher doses, T_{reg} cells can be recruited to the response and suppress the IgE response (Curotto de Lafaille et al., 2001).

We previously reported that IgE cells were absent from GCs during *Nippostrongylus brasiliensis* infection as well as during immunization of TBmc with cross-linked OVA-HA (Erazo et al., 2007). In fact, the evidence that GCs are not favorable structures for IgE^+ cells is mounting. Bcl6-deficient

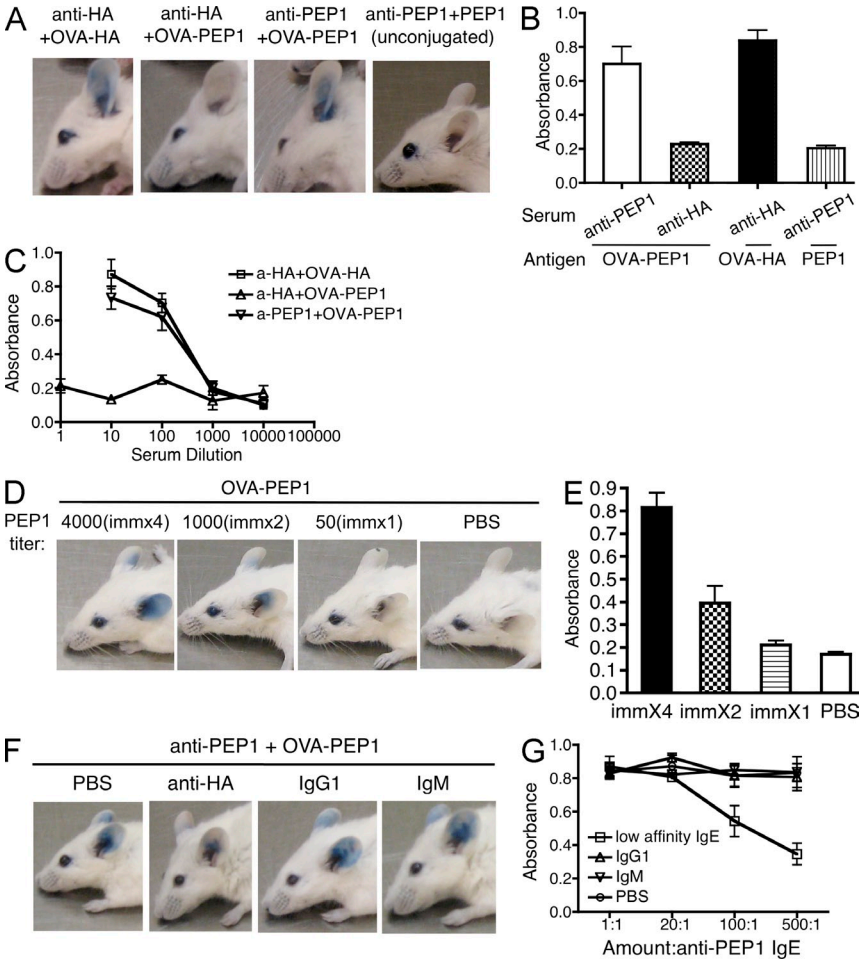

Figure 7. Anaphylaxis caused by IgE of high affinity to its antigen can be competed by IgE of low affinity for the same antigen. TBmc mice were immunized with OVA-HA or OVA-PEP1 two times, and sera were collected and depleted of IgG and IgA. IgE concentration was measured by ELISA. 25 ng of total IgE was injected intradermally into the ear of recipient BALB/c mice. 24 h later, 50 µg OVA-HA, OVA-PEP1, or unconjugated PEP1 was injected intravenously together with 1% Evans blue. (A and B) 30 min later, mice were sacrificed, and the extent of Evans blue leakage was determined either visually (A) or spectrophotometrically (B; three mice per group). (C) Before injecting into the ears, sera were diluted as indicated. (D and E) Similar PCA assay was performed using sera from TBmc mice immunized with OVA-PEP1 one, two, or four times. The PEP1-specific IgE titer is shown above the photos. A total of 25 ng IgE was injected per ear. 24 h later, recipient BALB/c mice were injected intravenously with OVA-PEP1 and Evans blue. The ears are shown in D, and Evans blue was measured spectrophotometrically in E. (F) 6 ng of high affinity anti-PEP1 IgE was mixed with 100 times more PBS, anti-HA IgE, IgG1, or IgM to perform the PCA assay mediated by OVA-PEP1. The extent of Evans blue leakage was determined visually. (G) Quantification of Evans blue leakage by spectrophotometry in the ears injected with 1, 20, 100, and 500 times more IgM, IgG, or low affinity IgE (three mice each group). (B, C, E, and G) Error bars indicate SEM.

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mice have severely impaired GC formation and no affinity maturation, but they harbor an increased number of IgE^+ cells (Ye et al., 1997). Similarly, deficiency of Dock8 (dedicator of cytokinesis 8) caused unstable GCs in mice (Randall et al., 2009) and hyper IgE syndrome in humans (Engelhardt et al., 2009; Zhang et al., 2009). IL-21 is a critical cytokine produced by T_{FH} cells; similarly to Bcl6 and Dock8, IL21R-deficient mice have higher IgE and lower IgG1 level (Ozaki et al., 2002). Thus, the incompatibility between IgE and GCs can be explained by our sequential switching model, in which $IgG1^+$ intermediate cells undergo the GC phase.

As the number of immunizations increased, the frequency of Sμ-Sε junctions that contain Sγ1 remnants increased, in parallel with an increase in IgE affinity. It is remarkable that during the course of immune responses we did not see S remnants other than Sγ1 in Sμ-Sε junctions. The reason for the strong Sγ1 dominance is that sterile transcripts from Iγ1 and Iε promoters share some of the triggers, such as IL-4/STAT6, which could turn on Sγ1 and Sε in the same cell, whereas other sterile transcripts such as Iγ2a or Iγ2b would remain silent. If this is correct, in other IgE responses in which IgG1 is not activated, we may possibly find remnants other than Sγ1 in Sμ-Sε junctions.

We did not find evidence of the contribution of the memory IgM^+ B cells to the high affinity IgE response (Dogan et al., 2009; Tomayko et al., 2010; Pape et al., 2011), as we found almost no affinity maturation in the absence of IgG1. However, it is possible that memory IgM^+ B cells are prone to undergo switching to IgE via IgG1 rather than through the direct switching route.

In B6 mice, the high affinity to NP first comes from a combination of V_H186.2 and IgL λ1. Of the two paths that further increase V_H186.2 affinity for NP, the first path (Y99/W33L) does not have N nucleotides in the VD junctions, whereas the second path (G99/K59R) uses N nucleotides. In BALB/c mice, we found NP-binding cells were also highly enriched in IgLλ, and the K59R with other affinity-enhancing mutations were found. Interestingly, there were many N nucleotides in these junctions, even though they did not necessarily encode G99. These observations show that the mechanism used by BALB/c mice to produce high affinity anti-NP IgE is very similar to the second path in B6 mice, which can be time consuming, but end up with a higher affinity than the first path.

IgE antibodies are spontaneously increased in mice with T cell immunodeficiencies such as nu/nu, $CD4^{-/-}$, MHC class $II^{-/-}$; these IgEs have been referred to as natural IgE antibodies (McCoy et al., 2006). Like the conventional IgE antibodies, natural IgE antibodies are dependent on T cells, CD40–CD40L interactions, and IL-4; however, organized lymphoid structures such as GC are not required, and natural IgE antibodies do not accumulate affinity-enhancing somatic hypermutations (McCoy et al., 2006). Importantly, lymphopenia-induced proliferation in T cell–deficient mice induces T cells that develop predominantly along the Th2 pathway (Milner et al., 2007). The high IgE found in Lat_{Y136F} knockin mice could have a similar genesis (Aguado et al., 2002). It thus appears that during some T cell lymphopenic disorders, IgE switching resembles much of what we showed here for directly μ→ε switched antibodies. A nonexclusive alternative is that natural IgE antibodies may be the result of switching to IgE at the immature B cell developmental stage. It has recently been shown that immature B cells in bone marrow and spleen switch to IgE in a direct μ→ε CSR, whereas the mature B cells have a propensity to switch via an IgG1 intermediate (Wesemann et al., 2011). In contrast to natural IgE generation, conventional IgE responses, which involve affinity maturation, are generated through a different mechanism, involving a key IgG phase.

Natural IgE antibodies may be involved in conditions such as hyper IgE recurrent infection syndrome (HIES), Wiskott–Aldrich syndrome, Omenn syndrome, and DiGeorge syndrome. The combination of immunodeficiency and Th2 bias sometimes results in the production of extremely high levels of IgE (50,000 U/ml) without, however, allergic symptoms such as rhinitis, asthma, urticaria, and anaphylaxis (Grimbacher et al., 2005; Ozcan et al., 2008; Minegishi, 2009; Freeman and Holland, 2010). If all these IgEs had undergone affinity maturation to bind environmental antigens with high affinity, these patients would have severe allergic reactions upon minimal exposure to antigen. Thus, it is likely that hyper IgE responses in HIES patients are of low affinity to environmental antigens and are directly switched μ→ε, like natural IgE responses. Compared with HIES patients, children with allergic asthma have lower levels of IgE; however, these IgEs showed hypermutations and affinity maturation (Kerzel et al., 2010).

IgE responses have traditionally been considered important in the clearance of some helminthes, which induce a massive IgE production caused by very efficient Th2 differentiation induced by parasite components. Interestingly, although some of the IgE antibodies are specific for the helminthes (Jarrett and Haig, 1976; Pochanke et al., 2007), the vast majority of the IgE antibodies do not react to the parasites. Evolutionarily, for the parasite, it could result in a delayed rejection, as low affinity IgE competes with parasite-specific IgE for FcεRI receptors and cross-linking of host effector cells would be perturbed (Pritchard, 1993). For the host, helminth nonspecific IgE binding to mast cells would reduce immunopathology and prevent serious systemic allergic reactions.

It has been long known that recombinant Fc fragments and synthetic peptides from Cε can inhibit antigen-specific IgE reactions when present at 200- or 1,000-fold excess, respectively (Geha et al., 1985; Burt and Stanworth, 1987; Nio et al., 1992). We observed inhibition of high affinity IgE-mediated anaphylaxis by the addition of intact IgE of low affinity for the antigen, although complete inhibition of anaphylaxis required large molar excess. It clearly demonstrates the beneficial and pathogenic functions of low and high affinity IgE, respectively.

Besides the effect on basophil and mast cell degranulation, IgE antibodies have also been shown to facilitate antigen

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presentation, an effect which is antigen-specific. Thus, high affinity recognition of antigens by IgE could also increase T cell proliferation, as was described both in mice (Oshiba et al., 1997) and humans (Turcanu et al., 2010).

There are large health implications of the affinity maturation of IgE antibodies. By describing a distinct mechanism for the generation of the high affinity IgE response, our study makes possible the detection of high affinity responses even when triggering antigens are unknown, using Sγ remnants in switch regions as indicators of affinity maturation. The existence of a distinct mechanism also allows the design of therapeutic interventions specifically targeted to affect sequential generation of IgE^+ cells, an intervention which would preserve the positive effects of low affinity IgE antibodies on tissue homeostasis.

MATERIALS AND METHODS

Mice, immunization, and injection. TBmc mice were previously described (Curotto de Lafaille et al., 2001). Heterozygous heavy and light chain knockin mice were used. Mice were immunized by intraperitoneal injection of 100 μg OVA-HA (YPYDVPDYASLRS) or with 100 μg OVA-PEP1 (YPYDVPDFASLRS) in alum. hMT mice (Lorenz et al., 1995) were provided by C. Jacob (University of Southern California, Los Angeles, CA). Mice were backcrossed to BALB/c genetic background and immunized by intraperitoneal injection of 50 μg $NP_{19}KLH$ (purchased from Biosearch) in alum. BALB/c mice were purchased from the Jackson Laboratory. Mice were injected intraperitoneally with 200 μl of goat anti–mouse IgD serum (eBioscience). All procedures involving mice were approved by New York University's Institutional and Animal Care Use Committee.

Hybridomas. IgE-producing hybridomas (Böttcher et al., 1980; Rudolph et al., 1981) were grown in RPMI medium supplemented with FBS, Hepes, L-glutamine, β-mercaptoethanol, penicillin, and streptomycin.

ELISA. Total and antigen-specific IgE and IgG1 antibodies were quantified by ELISA as described previously (Curotto de Lafaille et al., 2001). To measure antigen-specific IgE in serum, IgG and IgA were depleted by incubating with protein G–Sepharose (GE Healthcare) overnight at 4°C. The plate was coated with 2 μg/ml PEP1 for anti-PEP1 ELISA or with 10 μg/ml NP_4BSA and 10 μg/ml $NP_{23}BSA$ separately for anti-NP ELISA. Absorbance was detected by spectrometer at 450 nm. Serum titer was defined as the lowest dilution that rendered an optical density value (OD450) >0.1 after background subtraction.

Flow cytometry. Before staining for FACS analysis, single-cell suspensions from spleen were treated for 1 min with ice-cold acid buffer (0.085 M NaCl, 0.005 M KCl, 0.01 M EDTA, and 0.05 M NaAcetate, pH 4) to remove extrinsic IgE antibodies noncovalently bound to CD23. The samples were then neutralized with a large volume of cell culture medium and washed twice before staining. The following reagents were used: FITC–anti-IgG1 (A85-1), biotin–anti-IgE (R35-72), PerCP–anti-B220 (RA3-6B2), PE–anti-Fas (jo2), PE-Cy7–anti-Fas (jo2), NP-PE (Biosearch), and APC-streptavidin (BD).

Sγ1 qPCR. Cells were sorted by a MoFlo cytometer. DNA was extracted from the cells with lysis buffer where proteinase K at 50 μg/ml was freshly added. The following primers were used to amplify Sμ-Sε junctions: SμF, 5′-TTGGGGAAGGGAAAATAAA-3′; and SεR, 5′-TAGGGCTGTTGGTCATAGAT-3′. Amplified junctions were cloned into TA vector (Invitrogen) and miniprepped. qPCR was performed using the following Sγ1 primers: Sγ1F, 5′-AGCCAGGACAGGTGGAAGT-3′; and Sγ1R, 5′-CTGCTCTGCCTGGGTCA-3′.

DNA sequence analysis. For VDJ sequencing, total RNA extraction and cDNA synthesis were performed by standard procedures. PCR reactions were performed with high fidelity ExTaq Polymerase (Takara Bio Inc.) and the common forward primer VH1.1 in combination with primers specific for IgE, IgG1, or IgG2a constant regions (Erazo et al., 2007). For switch region sequencing, PCR-amplified junctions were cloned into TA vector and miniprepped. All the sequences were performed by Macrogen Corp. Point mutations in Sγ1 regions with length of <1,000 bp were counted in the first half and second half of Sγ1 separately and were included in the overall mutation frequencies of the Sμ-Sγ1 junction and the Sγ1-Sε junction, respectively.

PCA assay. 10–20 μl IgE-containing serum was injected intradermally into the ears of BALB/c mice. 24 h later, mice were injected intraperitoneally with antigen and 1% Evans blue. 30 min later, mice were sacrificed. The ears were cut and digested in proteinase K–containing lysis buffer. Supernatant was subjected to spectrometry at 650 nm to quantify Evans blue.

Statistical analysis. The two-tailed χ^2 test was performed to J_H preference.

Online supplemental material. Table S1 shows that the sequencing results validate the sensitivity and specificity of the Sγ1 qPCR assay, which is related to Fig. 3. Table S2 shows the usage of D segments and sequences of K59R-contaning sequences, which is related to Fig. 5. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20111941/DC1.

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SUPPLEMENTAL MATERIAL

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Table S1. Sequencing results validate the sensitivity and specificity of the Sγ1 qPCR assay

Clone no.	Sμ	Sγ1	Sε	Signal from Sγ1 qPCR assay
	bp	*bp*	*bp*	
#1	1,483	348	1,241	>10,000
#2	1,782	0	528	0
#3	1,743	0	544	0
#4	2,054	0	2,385	0
#5	2,100	675	863	>10,000
#6	1,117	127	1,281	>10,000
#7	1,288	764	548	>10,000
#8	1,322	0	255	0
#9	2,049	0	1,024	0
#10	2,020	160	931	>10,000
#11	1,279	0	830	0
#12	1,995	233	2,215	>10,000
#13	2,241	0	96	0
#14	1,926	260	1,522	>10,000
#15	1,147	0	1,093	0
#16	1,657	0	1,247	0
#17	1,470	170	591	>10,000
#18	2,021	0	585	0
#19	3,423	0	2,571	0
#20	1,596	246	218	>10,000
#21	782	0	1,147	0
#22	639	0	1,011	0
#23	1,506	0	1,216	0
#24	932	0	588	0
#25	1,687	0	1,042	0
#26	933	0	589	0
#27	2,046	0	1,032	0
#28	1,957	321	116	>10,000
#29	2,200	570	350	>10,000
#30	517	109	823	>10,000
#31	625	0	1,254	0
#32	1,733	0	417	0
#33	1,483	0	1,496	0
#34	779	0	1,145	0
#35	2,010	0	997	0
#36	1,490	0	2,382	0
#37	1,775	0	1,313	0
#38	537	109	837	>10,000
#39	1,766	108	1,332	>10,000
#40	1,596	246	218	>10,000

40 clones from 12 TBmc mice were randomly picked out for sequencing and aligning with germline DNA. The length of the Sμ, Sγ1, and Sε segments is shown. The signal from the Sγ1 qPCR assay is listed. The indicated signal represents the absolute abundance of Sγ1 DNA divided by the absolute abundance of plasmid DNA input (neo-resistance gene), multiplied by 10,000.

Table S2. Usage of D segments and sequences of K59R-contaning sequences

Amino acid sequence	Nucleotide sequence	D segment
IgG1		
RLLSN	5′-CGACTACTATCCAAT-3′	Dsp2.1/2/3/4/5/6/7
YRYYYGTS	5′-TACCGTTATTACTACGGTACTAGT-3′	Dfl16.1
YTYGRTST	5′-TATACCTACGGTCGTACCTCGACT-3′	Dfl16.1
SYYDYDVP	5′-AGCTACTATGATTACGACGTCCCC-3′	Dsp2.2
YRLARY	5′-TATAGACTTGCGAGGTAT-3′	unclear
YRLARY	5′-TATAGACTTGCGAGGTAT-3′	unclear
KVITTR	5′-AAGGTGATTACGACCAGG-3′	Dsp2.2
DYYDSSYV	5′-GATTACTACGATAGTAGCTACGTG-3′	Dfl16.1
KDFGTNF	5′-AAGGACTTCGGTACTAACTTT-3′	unclear
YGNYQRGNYFT	5′-TATGGTAACTACCAACGGGGAAATTACTTTACT-3′	Dsp2.1/5/7/8
IgE		
VNYYGSS	5′-GTGAATTACTACGGTAGTAGCTACATT-3′	Dfl16.1
EGDWLLP	5′-GAGGGAGACTGGTTACTCCCT-3′	Dsp2.3/4
PYRYEG	5′-CCCTATAGGTACGAAGGAGAC-3′	Dsp2.1/5
GYYGY	5′-GGATACTACGGCTAC-3′	Dfl16.2
GFYAYDS	5′-GGGTTCTTGCTTACGACAGTGGG-3′	Dsp2.3/4/6
YLLSH	5′-TACCTTCTTTCGCAC-3′	Dfl16.2
GSQYPYG	5′-GGTTCCCAATATGGTTACCCC-3′	Dsp2.3/4/6