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Natural IgE Production in the Absence of MHC Class II Cognate Help

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Summary

IgE induction by parasites and allergens is antigen driven and cognate T cell help dependent. We demonstrate that spontaneously produced IgE in T cell-deficient and germ-free wild-type (wt) mice is composed of natural specificities and induced by a mechanism independent of MHC class II (MHC II) cognate help. This does not require secondary lymphoid structures or germinal center formation, although some bystander T cell-derived IL-4 is necessary. The pathway of spontaneous IgE production is not inhibited by regulatory T cells and increases with age to constitute significant serum concentrations, even in naive animals.

Introduction

IgE is present at low concentrations in the serum of mice or man living in hygienic conditions (Sutton and Gould, 1993). The specificity and function of this IgE is unclear, although it appears to be functionally important. Hapten-irrelevant IgE or preimmune spontaneous IgE normally present in mice bound to FcεRI on mast cells is required to prolong mast cell survival (Asai et al., 2001; Kalesnikoff et al., 2001) and to sensitize mice to contact sensitivity (Bryce et al., 2004) through mast cell modulation of dendritic cell migration or function. Although mast cells certainly function as effector cells during allergic responses, they also represent an important component of innate immunity at skin and mucosal surfaces (Marshall and Jawdat, 2004; Wedemeyer et al., 2000). In this paper, we dissect the mechanisms of induction of spontaneously produced natural IgE.

IgE has usually been studied during responses to parasitic infection or in individuals suffering from atopic allergic diseases, including asthma, eczema, and hayfever. IgE binds to the high-affinity IgE receptor FcεRI on mast cells and basophils and, upon antigen-specific crosslinking, induces mast cell and basophil degranulation (Kinet, 1999). Class switch recombination (CSR) of B cells and generation of parasite or allergen-specific IgE has been shown to require cognate interactions between B cell MHC II molecules and the T cell receptor (TCR)-CD3 complex on helper $CD4^+$ T cells (Vercelli et al., 1989a, 1989b). Activated T cells upregulate CD40L and secrete interleukin-4 (IL-4) and IL-13, promoting germ-line transcription of the ε heavy chain to initiate B cell class switching (Coffman and Carty, 1986; Coffman et al., 1986; Finkelman et al., 1986, 1988; Jabara et al., 1990; Punnonen et al., 1993; Shapira et al., 1992; Zhang et al., 1991). This model of cognate T cell help for induction of specific IgE has been substantiated by observations made in vivo (Kawabe et al., 1994; Kopf et al., 1993; Kuhn et al., 1991).

In contrast to mechanisms of specific IgE induction, the mechanism for production of base-line IgE in the sera of naive mice kept in pathogen-free conditions is not understood. The requirement for T cell help may differ as high levels of IgE have been observed in unmanipulated athymic mice (Ito et al., 1979; Savelkoul et al., 1989) and strains deficient for $\alpha\beta^+$ T cells (Wen et al., 1994) or the linker for activation of T cells (LAT) adaptor protein (Aguado et al., 2002). In man, T cell immunodeficiency can also be associated with high serum IgE, for example in Hyper-IgE syndrome (Buckley, 2001), Wiskott-Aldrich syndrome (WAS) (Ochs, 2001; Ramesh et al., 1999), DiGeorge anomaly (Buckley, 2002), and Ommen's syndrome (Villa et al., 1998), usually without a clear infective cause.

We show here that natural IgE is spontaneously produced in the serum of wt germ-free animals and becomes elevated in T cell-deficient mouse strains. The mechanism of natural IgE production is MHC II-mediated T cell cognate independent, although bystander T cells and IL-4 production are still required. This relatively primitive pathway of IgE production is not inhibited by regulatory T cells and increases with age to constitute significant serum concentrations, even in naive animals, and may function as a component of mast cell mediated innate responses.

Results and Discussion

Elevated Natural IgE in Mouse Strains with Impaired T Cell Function

Although class switch recombination to IgE is conventionally viewed as highly dependent on T cell help, we found that baseline serum IgE concentrations were spontaneously and dramatically increased in strains with reduced T cell numbers or absence of cognate T

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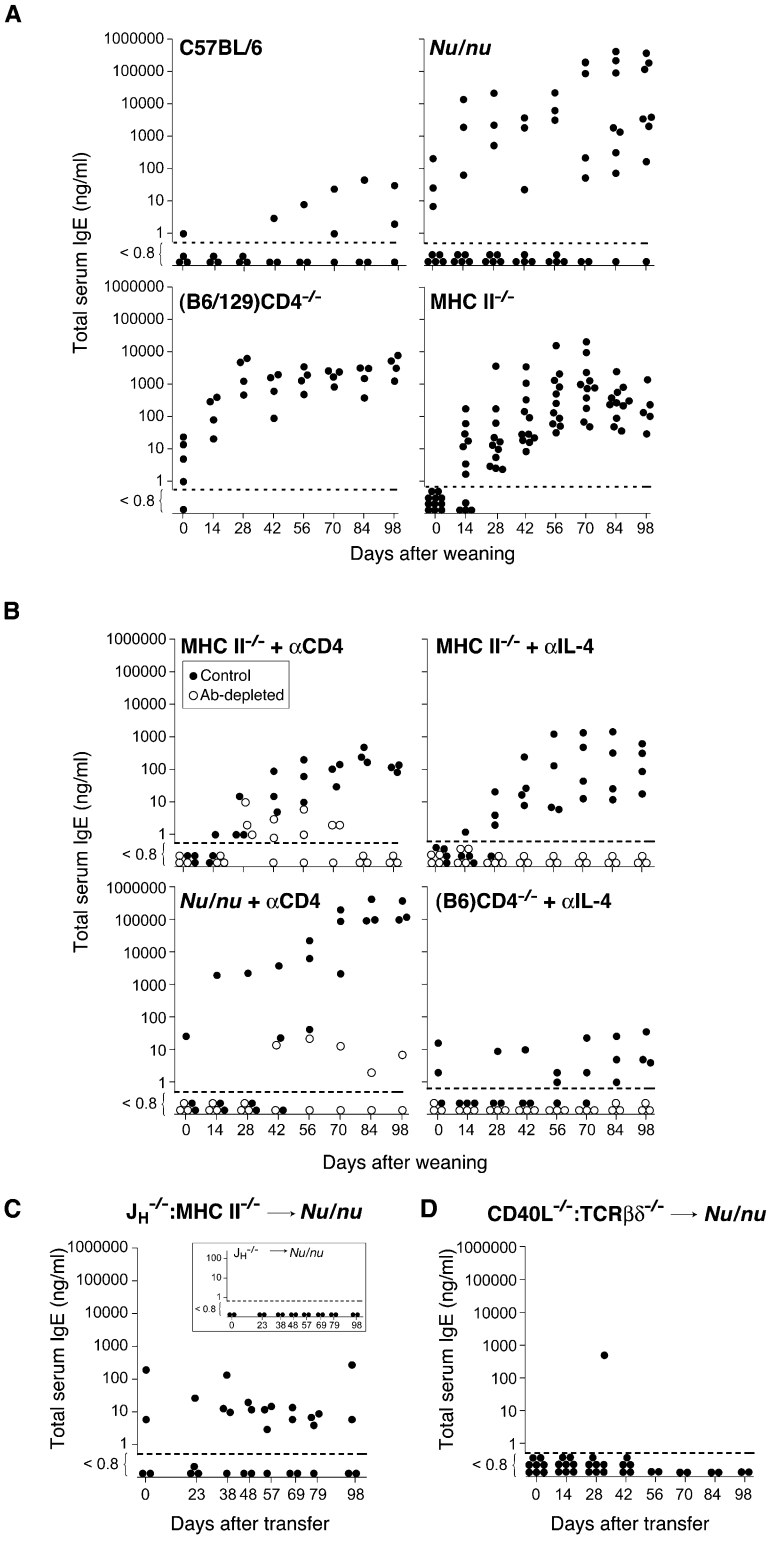

Figure 1. Total Serum IgE Levels in T Cell-Immunodeficient Mice Increase Spontaneously after Weaning

Mice were weaned on day 18 after birth, indicated as day 0.

(A) Blood was collected from naive C57BL/6, *nu/nu*, $CD4^{-/-}$, and MHC $II^{-/-}$ mice and analyzed for total serum IgE.

(B) MHC $II^{-/-}$ and *nu/nu* mice were depleted of $CD4^+$ T cells by weekly i.p. treatment with an anti-CD4 mAb. For neutralization of IL-4, MHC $II^{-/-}$ and $CD4^{-/-}$ mice were treated i.p. weekly with an anti-IL-4 mAb.

(C) Recipient *nu/nu* mice were lethally irradiated (950 rad) and reconstituted with equal numbers of bone marrow cells from $J_H^{-/-}$ and MHC $II^{-/-}$ mice (1:1 ratio of donor cells). The inset shows IgE levels in irradiated *nu/nu* mice reconstituted with bone marrow from $J_H^{-/-}$ mice.

(D) Recipient *nu/nu* mice were lethally irradiated (950 rad) and reconstituted with equal numbers of bone marrow cells from $CD40L^{-/-}$ and $TCR\beta\delta^{-/-}$ mice (1:1 ratio of donor cells).

In (A)–(C), all animals were housed under identical conditions. Blood was collected at weekly intervals and total serum IgE levels determined by ELISA. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each data point indicates a single animal, and results are representative of at least two separate experiments. We have also reconstituted MHC $II^{-/-}$ mice with B6, and these mice also get IgE, as would be expected as the resulting chimeric mice are also lymphopenic and lack MHC II-selected $CD4^+$ T cells.

cell help. Total serum IgE levels became elevated starting from the day of weaning in B6 *nu/nu* (nude), MHC $II^{-/-}$, and $CD4^{-/-}$ mice compared with C57BL/6 wt controls (Figure 1A). IgE in the serum and switched IgE transcripts in splenic B cells of these strains were confirmed by Western blot and RT-PCR, respectively (Figure S1 available in the Supplemental Data with this article online). Athymic hairless *nu/nu* mice (Pantelouris, 1968) carry a single base pair deletion in exon 3 of the winged helix nude gene *whn* (Nehls et al., 1994) and lack peripheral $CD4^+$ and $CD8^+$ T cells of thymic origin. MHC II-deficient mice have severely decreased numbers of peripheral $CD4^+$ T cells due to the absence of MHC II-mediated positive selection in the thymus (Cosgrove

et al., 1991; Grusby et al., 1991). Similarly, CD4 deficiency limits MHC II-restricted T cell selection (Rahemtulla et al., 1991), although some MHC II-restricted $CD8^+$ T cells may be present.

Spontaneous elevation of IgE was not determined by genetic background, as BALB/c and ICR *nu/nu* mice also displayed high serum IgE levels (2593 ± 1248 ng/ml, n = 21 and 16,472 ± 4695 ng/ml, n = 11, respectively) equivalent to C57BL/6 *nu/nu* mice (6488 ± 4546 ng/ml, n = 32). We also found increased serum IgE levels (4 log) in another T cell immunocompromised mouse strain, which expresses a transgenic TCR α chain (Vα2/JαTA31) exhibiting specificity for the H-$2D^b$-restricted LCMV gp33 epitope (TCRα Tgn) (Brandle et al., 1991; Pircher et al., 1987) and which therefore has a very limited $CD4^+$ T cell repertoire (Figure S2). Taken together, these observations suggested that the pathway of spontaneous class switch recombination to IgE giving baseline levels may be distinct, in particular less dependent on conventional T cell help, compared with the parasite or allergen induction pathways that are normally studied: this is further addressed experimentally in the next section.

There was no evidence that the elevated IgE in these strains was due to subclinical infection, and the effect was reproduced within various animal facilities. Moreover, the spontaneous production of IgE was heterogenous, and mice with no detectable IgE were housed in the same cage as littermates that developed high levels of IgE, supporting the interpretation that IgE production was not linked to hygiene status. Even germ-free mice, which are also relatively lymphopenic with poor lymphoid organization (Crabbe et al., 1970), had elevated serum IgE with levels ~2.5 log greater than those detected in specific pathogen-free C57BL/6 mice derived from the same germ-free stock after colonization with a simple Schaedler bacterial flora (267 ± 54 ng/ml, n = 16 and 1.0 ± 0.4 ng/ml, n = 26, respectively). This spontaneous IgE detected under germ-free conditions was, by definition, likely to be composed of natural specificities, raising the possibility that IgE in the T cell-compromised mice could also be natural antibody.

We first confirmed that spontaneous IgE had the undiversified sequence characteristics of natural specificities by analysis of over 100 IgE V_H-D-J_H rearrangements in B cells isolated from germ-free C57BL/6, pathogen-free C57BL/6, *nu/nu*, MHC $II^{-/-}$, $CD4^{-/-}$, and TCRα transgenic mice. The IgE repertoire was polyclonal, as >90% of IgE clones isolated were unique and there was no bias in the usage of V_H, D_H, or J_H families (Tables 1 and 2 and Tables S1–S5). There was also no sequence evidence for somatic hypermutation, because accumulation of mutations within the CDRs was low and equivalent to the frame-work regions, and in cases of the same VDJ recombination event, we could see that mutations had not been acquired sequentially as one would expect for a T-dependent germinal center response selecting clones with the highest affinities (Table S6). The natural IgE repertoire appears to be diverse, although we cannot exclude some oligoclonality from the sample of sequences we have determined. Importantly, the diverse repertoire and lack of somatic hypermutation were similar to characteristics of the spontaneous natural IgE found in germ-free C57BL/6 mice (as well as the base-line IgE found in naive C57BL/6 mice). These observations support spontaneous IgE in T cell immunocompromised mice being a class switched natural antibody.

Table 1. In-Frame V(D)J Cε Rearrangements in C57BL/6 and T Cell Immunocompromised Mice: V_H Family Usage And Overall Mutation Rates

		Number of Mutations		R/S	
Clone	V_H Family[a]	FR	CDR	FR	CDR
GF B6-1[b]	V_H1	25	9	1.8	1.3
GF B6-2	V_H5	10	8	0.7	3.0
GF B6-3	V_H1	11	6	2.7	5.0
GF B6-4	V_H1	18	6	2.0	1.0
GF B6-5	V_H1	17	5	0.7	1.5
B6-1[c]	V_H10	10	0	2.3	NA
B6-2	V_H8	81	24	1.5	0.8
B6-3	V_H1	11	5	1.2	4.0
B6-4	V_H14	19	11	1.7	1.2
B6-5	V_H1	18	6	2.0	NA
nu/nu-1[d]	V_H1	10	0	2.3	NA
nu/nu-2	V_H1	1	1	NA	NA
nu/nu-3	V_H1	10	0	4.0	NA
nu/nu-4	V_H1	12	0	1.4	NA
nu/nu-5	V_H1	6	3	2.0	NA
MHC $II^{-/-}$-1[e]	V_H5	14	4	3.7	NA
MHC $II^{-/-}$-2	V_H1	25	3	2.6	NA
MHC $II^{-/-}$-3	V_H1	10	4	4.0	NA
MHC $II^{-/-}$-4	V_H7	6	1	2.0	NA
MHC $II^{-/-}$-5	V_H5	15	3	0.9	2.0
$CD4^{-/-}$-1[f]	V_H1	19	4	5.3	NA
$CD4^{-/-}$-2	V_H5	6	4	2.0	3.0
$CD4^{-/-}$-3	V_H3	24	6	1.2	2.0
$CD4^{-/-}$-4	V_H1	15	4	1.1	3.0
$CD4^{-/-}$-5	V_H7	15	2	2.8	NA
TCRα Tgn-1[g]	V_H1	9	7	2.0	2.5
TCRα Tgn-2	V_H2	13	5	0.9	NA
TCRα Tgn-3	V_H1	21	3	1.8	2.0
TCRα Tgn-4	V_H1	20	4	2.3	1.0
TCRα Tgn-5	V_H5	1	3	NA	2.0

[a] The closest germline V_H family of isolated clones was assigned by using the DNAPLOT program (http://www.genetik.uni-koeln.de/dnaplot).
[b] 5/7 representative sequences from naive germ-free C57BL/6 mice. 1/7 sequences was isolated in two clones after PCR reaction.
[c] 5/6 representative sequences from naive C57BL/6 mice. 1/6 sequences was isolated in two clones after PCR reaction.
[d] 5/38 representative sequences from naive C57BL/6 *nu/nu* mice, 1/38 sequences was isolated in two clones, and 1/38 was isolated in five clones after PCR reaction.
[e] 5/8 representative sequences from naive MHC $II^{-/-}$ mice, 2/8 sequences were isolated in two clones after PCR reaction.
[f] 5/31 representative sequences from naive $CD4^{-/-}$ mice, 2/31 sequences were isolated in two clones after PCR reaction.
[g] 5/33 representative sequences from naive TCRα TCR transgenic mice. Abbreviations: FR, frame-work; CDR, complementarity-determining region; NA, not applicable; and R/S, ratio of replacement to silent mutations.

We obtained two additional lines of evidence to confirm the spontaneous production of natural IgE in immunodeficient mice. First, we found that spontaneous IgE from *nu/nu* mice also contained the characteristic natural specificity antiphosphorylcholine (anti-PC) (Kearney, 2005) by specific ELISA (Figure 2). In contrast, IgE from parasite-infected wt mice, which displayed equally high total IgE levels, did not contain anti-PC specificity (Figure 2). Second, we tested whether targeted VDJ insertion (knockin) mice of defined specificity (and precisely known heavy chain sequence) would produce

Table 2. In-Frame V(D)J Cε Rearrangements in C57BL/6 and T Cell Immunocompromised Mice: D_H and J_H Usage

Clone	V_H[a]	(P)N	D_H	(P)N	J_H	D_H	J_H
GF B6-1	TGTGCA	C	CTATGAT		TTTGCTTAC	DSP2.2	J_H3
GF B6-2	TGTGCAAG	GG	ATGATGGTTACTAC	G	ACTACTTTG	DSP2.9	J_H2
GF B6-3	TGTGCAAGA	AGGG	GATGGTTTCTTC	C	GTTTGCTTA	DSP2.9	J_H3
GF B6-4	TGTGCGCGA	TCGG	CGGCT	GGGGC	CTTTGACTA	DFL16.2	J_H2
GF B6-5	TGCGCAA	CCC	ATGGT		TTTCCTTAC	DSP2.x	J_H3
B6-1	TGTGCAAGA	GGG	AACT	GGGC	CTTTGA	DSP2.7	J_H2
B6-2	TGTGCTCGA	ATGGGGG	GGATTACGTC	GAA	ACTACT	DSP2.2	J_H2
B6-3	TGTGCAAGA		TTTACTGCGGTAGTAGGTAC	GG	TTGACT	DFL16.1	J_H2
B6-4	TGTGCTAGA	GAGCTGA	TTTACTACGGTTAT	AA	CTACAG	DSP2.4	J_H1
B6-5	TGTGCAAGA		GGG		GCTTAC	?	J_H3
nu/nu-1	TGTGCAAGA	GAGGGTG	ACTACGGTAGTAGC	CCT	TACTGG	DFL16.1	J_H1
nu/nu-2	TGTGCAAGA	TC	TTATTACTACGGTAGTAG	AAGG	TACTTT	DFL16.1	J_H2
nu/nu-3	TGTGCAAGA	AGGT	TCTACTATGGTTACGAC	GTCCCC	TTTGCT	DSP2.4	J_H3
nu/nu-4	TGTGCAAG	C	TTACGAC	TGAGG	TACTGG	DSP2.4	J_H2
nu/nu-5	TGTGCAAGA	GGGGGCCCTAGAGGTAA	CTACTGGGGC	T	CCTGGT	DQ52	J_H3
MHC $II^{-/-}$-1	TGTGCACG	CCACCGCCT	TCTAT	C	CTGGTA	DSP2.9	J_H1
MHC $II^{-/-}$-2	TGTGCAAGA	GGGGGAAAA	TATTACTACGGT	CTT	TGGTTC	DFL16.1	J_H1
MHC $II^{-/-}$-3	TGTGCAAGA		TATTGGGGTTACTAC	GCCCC	TGACCA	DSP2.9	J_H2
MHC $II^{-/-}$-4	TGTGCAAGA	GCTG	ACTGGGAC	G	TCTACT	DQ52	J_H2
MHC $II^{-/-}$-5	TGTGCAAGACG	GG	ACTATGATTACGAC	GAC	GACTAC	DSP2.2	J_H4
$CD4^{-/-}$-1	TGTGCAAGA	TCGA	ACTACGGTAGTAGCTTC		TTTGAC	DFL16.1	J_H2
$CD4^{-/-}$-2	TGTGCAAGA		CATCCTATGATTA	GGA	ACTATG	DSP2.6	J_H4
$CD4^{-/-}$-3	TGTGCAAGA	TTAGGATC	TTACTACGGC	C	TTGACT	DFL16.2	J_H2
$CD4^{-/-}$-4	TGTGCAAG	G	TCATTACTACGGCTAC	G	CTGGTA	DFL16.2	J_H1
$CD4^{-/-}$-5	TGTGCAAGA	GA	CTATGATGGTTACTAC	CCCG	CCTGGT	DSP2.9	J_H3
TCRα-1	TGTGCAAGA	CACGAAGGA	TATGGTTACGAC	C	CCTGGT	DSP2.6	J_H3
TCRα-2	TGTGCCAGA	AAGG	ACGATAGTGGCTTC	GTAGGGGT	TTACTA	DFL16.1	J_H4
TCRα-3	TGTGCAAGA	AGA	ACTGGGAC	TGG	CTTTGA	DQ52	J_H2
TCRα-4	TGTGCAAGA	GGGGGAA	TCTACTATGGTAA		CTACAG	DSP2.5	J_H1

The closest germline V_H family of isolated clones was assigned as described in Table 1.
[a] Differences with the closest germline sequence are underlined.

undiversified IgE of that specificity on the MHC $II^{-/-}$ background. This was tested in two different MHC II-deficient immunoglobulin knockin mice: TgH(KL25) expressing the V_H-D-J_H chains of KL25 neutralizing monoclonal antibody against lymphocytic choriomeningitis virus and TgH(VI10)xYEN expressing V_H-D-J_H heavy chains of VI10, which neutralizes vesicular stomatitis virus (Hangartner et al., 2003). IgE was detected in 11/13 naive TgH(KL25)xMHCII$^{-/-}$ mice with total serum IgE levels of 123 ± 93 ng/ml (n = 11), whereas 8/8 naive (TgH[VI10]xYEN)xMHCII$^{-/-}$ mice had detectable IgE with total serum levels of 221 ± 67 ng/ml (n=8). This spontaneously induced IgE is definitely of natural knockin specificity because in both mouse strains the IgE was found to be of transgene origin by anti-idiotypic ELISA (titers of TgH[KL25]xMHCII$^{-/-}$: 1/23 ± 1/30, n = 11; (TgH[VI10]xYEN)xMHCII$^{-/-}$: 1/395 ± 1/242, n = 7; and C57BL/6 <1/1, n = 5), and sequence analysis of ε heavy chains in TgH(KL25)xMHCII$^{-/-}$ revealed no evidence for hypermutation as the sequences analyzed corresponded exactly to the knocked-in sequence (Table S7; 12 sequences analyzed).

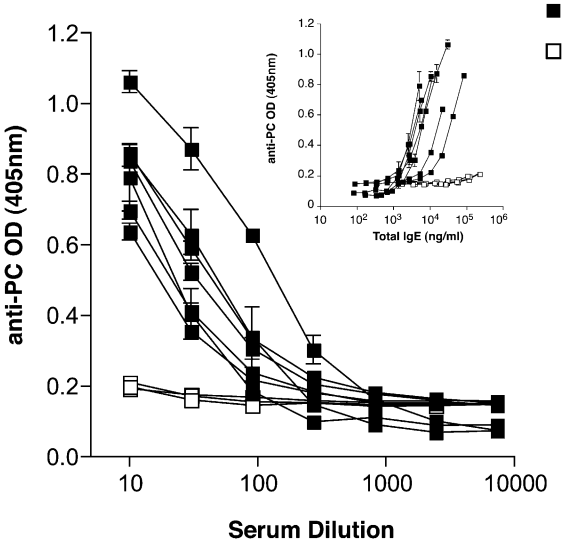

Figure 2. Anti-PC IgE Is Present in *nu/nu* Mice

Anti-PC-specific IgE was analyzed from blood collected from BALB/c *nu/nu* mice with high total IgE (23,665 ± 10,410 ng/ml, n = 7) (black squares) and Hp-infected BALB/c wild-type (wt) mice with high total IgE (151,363 ± 38,700 ng/ml, n = 3) (open squares). Values represent the mean ± SD of duplicate measurements and each line represents one mouse. Inset, total IgE levels are plotted against anti-PC titers.

IgE Production in Immunocompromised Mice Requires T Cells and IL-4

We next addressed the requirement for T cells in the pathway for spontaneous IgE production. Although all the immunocompromised mice displaying high IgE levels had reduced numbers of $CD4^+$ T cells, they were not entirely T cell deficient. Despite the absence of a thymus, low percentages of both single-positive CD4 ($0.36 \pm 0.09\%$, n = 10) and CD8 ($3.4 \pm 0.61\%$, n = 4) T cells could be detected in peripheral blood of *nu/nu* mice. Analysis of MHC $II^{-/-}$ mice also revealed the presence of small numbers of peripheral blood $CD4^+$ T cells ($0.8 \pm 0.07\%$, n = 17) and a correspondingly increased percentage of $CD8^+$ T cells ($31.1 \pm 1.6\%$, n = 17). Although $CD4^{-/-}$ mice lack $CD4^+$ T cells, it has been reported that the CD8 population in these mice contains MHC II-restricted T cells (Tyznik et al., 2004). Thus, in all of these mouse strains, it was possible that the low numbers of $CD4^+$ T cells, or class II-restricted $CD8^+$ T cells (Tyznik et al., 2004), were responsible for driving B cell switching to IgE.

To assess whether the spontaneous IgE production pathway required the presence of CD4 cells in these mouse strains with reduced T cell repertoires, we carried out CD4 depletion experiments. These showed that the elevated IgE in *nu/nu* and MHC $II^{-/-}$ mice was dependent on $CD4^+$ T cells (Figure 1B), as the IgE response was abrogated by CD4 depletion. Similarly, IL-4 neutralization experiments also showed that elevated IgE was dependent on IL-4 (Figure 1B). Although the fact that the response is abrogated by either CD4 or IL-4 depletion does not prove that the IL-4 is derived from CD4 cells in itself, we showed by intracellular cytokine flow cytometry that mice with high spontaneous IgE had residual bystander activated $CD4^+$ T cells with increased levels of IL-4 production and increased mRNA production of IL-4 or IL-13 (Figures S3A–S3D). IL-4 production is not confined to CD4 T cells in this setting: those *nu/nu* and MHC $II^{-/-}$ mice with high serum IgE levels also had increased basophil numbers compared to mice with low serum IgE or naive C57BL/6 mice and between 70% and 100% of all basophils analyzed produced IL-4 after stimulation with PMA and ionomycin (Figure S3). Nevertheless abrogation of the response by CD4 depletion alone suggests that the IL-4 contributed by basophils probably reflects a positive feedback mechanism that drives IgE production even higher, with the activated IL-4-producing $CD4^+$ T cells responsible for initiating the spontaneous IgE production.

NK cells did not produce IL-4 in any of the mouse strains tested (data not shown). MHC $II^{-/-}$ mice did exhibit increased NKT cell IL-4 production, but this was not found in *nu/nu* mice (MHC $II^{-/-}$ $7.1 \pm 2.4\%$, *nu/nu* $0.3 \pm 0.16\%$ IL-4^+NKT cells). Many of the eosinophils, identified as $CCR3^+Gr\text{-}1^+$, died in response to PMA/ionomycin stimulation; however, those remaining were not found to produce IL-4 (data not shown).

Natural Spontaneous IgE Production Is Independent of Cognate T Cell Help

Demonstration that spontaneous natural IgE production was dependent on CD4 T cells in mice with limited T cell repertoires raised the issue of whether class switch recombination to natural IgE occurs in vivo with bystander rather than cognate CD4 help. Indeed, the presence of elevated IgE in MHC $II^{-/-}$ mice suggested that cognate T cell help is not required for induction of this IgE. To verify this, we generated mixed bone marrow chimeras in which only B cells lacked MHC II expression. *Nu/nu* mice were lethally irradiated and reconstituted with equal numbers of bone marrow cells from $J_H^{-/-}$ and MHC $II^{-/-}$ mice ($J_H^{-/-}$:MHC $II^{-/-}$ → *nu/nu*). Despite the inability of B cells to present MHC II-restricted peptides, $J_H^{-/-}$:MHC $II^{-/-}$ → *nu/nu* chimeric mice nonetheless exhibited spontaneous production of serum IgE (Figure 1C). *nu/nu* mice that only received bone marrow cells from $J_H^{-/-}$ mice (and therefore lacked B cells) ($J_H^{-/-}$ → *nu/nu*) did not have measurable IgE in their serum (Figure 1C, inset), confirming that radio-resistant IgE-producing cells did not confound these results. Interestingly, $CD4^+$ T cell-dependent but cognate-independent induction of IgA (Sangster et al., 2003) and anti-PC IgG (Khan et al., 2004; Wu et al., 2002) have previously been reported, and through the TNF family members BAFF and APRIL, dendritic cells can deliver CSR signals independently of CD40-CD40L interactions (Castigli et al., 2005; Litinskiy et al., 2002). In addition, noncognate $CD4^+$ T cell help has been implicated in the IgG anti-PC response to *S. pneumoniae*. To investigate whether CD40-CD40L interactions were required for induction of natural IgE, we generated mixed bone marrow chimeras in which all T cells lacked CD40L expression. *nu/nu* mice were lethally irradiated and reconstituted with bone marrow cells from $CD40L^{-/-}$ and $TCR\beta\delta^{-/-}$ mice ($CD40L^{-/-}$:$TCR\beta\delta^{-/-}$ → *nu/nu*). Absence of CD40L on T cells completely abolished the spontaneous production of IgE in *nu/nu* mice (Figure 1D), indicating that induction of natural IgE was dependent on CD40-CD40L interactions. Using flow cytometry, we confirmed that all T cells in this chimera were CD40L deficient, whereas basophils retained expression of CD40L (data not shown). This observation further suggests that basophils were unable to initiate the spontaneous induction of IgE.

B2 Cells Contribute to the Production of Natural IgE

IgM natural antibodies are thought to be produced mainly by B1 cells (Baumgarth et al., 2005). We therefore investigated whether the spontaneously produced IgE in T cell-deficient mice was also derived from B1 cells. Total B1 cell numbers, identified as $CD5^+B220^{int}IgM^{int}CD3^-$, within the peritoneal cavity and spleen of MHC $II^{-/-}$ and *nu/nu* mice were comparable to that found in C57BL/6 mice (Figure 3A). We therefore isolated $CD19^+$ B cells from the spleen and peritoneal cavity, sorted the cells into peritoneal $CD5^+B220^{int}IgM^{int}$ (B1 cells) and splenic $CD5^-B220^{high}IgM^{high}$ (B2 cells) populations by flow cytometry, and analyzed for the presence of εGLT. εGLTs were detected in the $CD5^-B220^{high}$ IgM^{high} (B2 cell) population purified from the spleen of both MHC $II^{-/-}$ and *nu/nu* mice (Figure 3B). No εGLT was detected within the $CD5^+B220^{int}IgM^{int}$ population (B1 cells) (Figure 3B). To further investigate the B cell origin of the natural IgE, mixed bone marrow chimeric mice were generated where B1- or B2-derived IgE could be distinguished. Lethally irradiated *nu/nu* mice were reconstituted with bone marrow from B6.Cg.Igh^a mice and purified peritoneal B1 cells from C57BL/6 mice such that

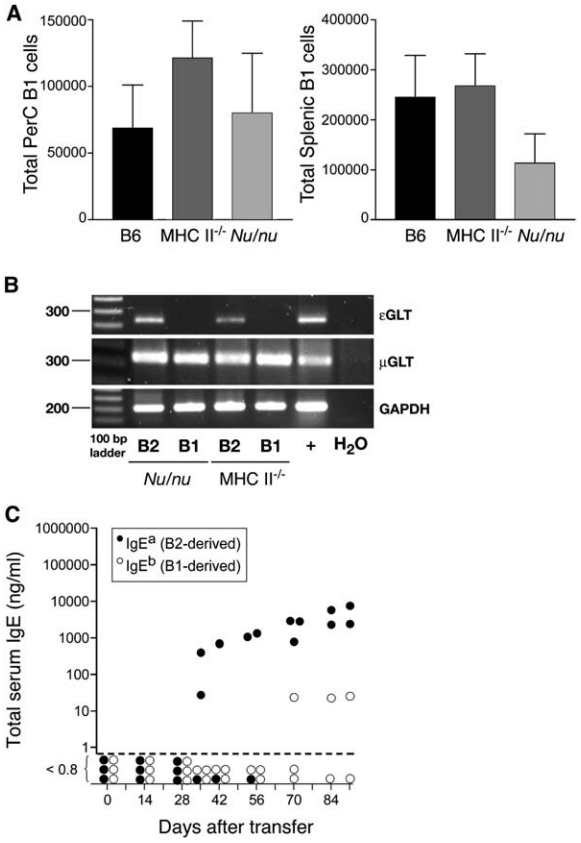

Figure 3. B2 B Cells Undergo CSR to IgE in MHC $II^{-/-}$ and *nu/nu* Mice

(A) Total B1 ($CD5^+B220^{int}IgM^{int}CD3^-$) cell numbers within the peritoneal cavity (PerC) and spleen are shown for C57BL/6 wt (B6), MHC $II^{-/-}$, and *nu/nu* mice, as indicated. Bars represent the mean ± SEM of two to four mice per group.

(B) Total RNA was isolated from purified peritoneal B1 ($CD5^+B220^{int}IgM^{int}$) and splenic B2 ($B220^{high}IgM^{high}CD5^-$) B cells, reverse transcribed into cDNA, and germ line transcripts specific for IgE (εGLT) and IgM (μGLT) were amplified by PCR. The expected sizes of the εGLT and μGLT PCR products are 219 bp and 325 bp, respectively. GAPDH transcripts are shown in the bottom panel.

(C) Lethally irradiated *nu/nu* mice were reconstituted with equal numbers of bone marrow cells from C57BL/6J-$Igh^aThy1^aGpi1^a$ mice and purified $CD5^+B220^{int}IgM^{int}$ peritoneal B1 cells from C57BL/6 mice. Blood was collected at weekly intervals and total serum IgE levels determined by ELISA. Each data point indicates a single animal, and results are representative of at least two separate experiments.

all B2 cells will produce IgE^a and all B1 cells will produce IgE^b. The IgE produced in these mice was found to be largely IgE^a, although a small amount of IgE^b appeared at later time points (Figure 3C). Thus, IgE production in MHC $II^{-/-}$ and *nu/nu* mice appears to be largely derived from B2 cells, although B1 cells may provide some contribution at later time points.

Absence of Helminth-Specific IgE in Mouse Strains with Impaired T Cell Function

To verify that the natural IgE pathway is functionally distinct from the classical cognate T-dependent IgE, we also investigated whether the various strains of T cell immunocompromised mice could mount an antigen-specific IgE response after infection with the helminth parasite *Heligmosomoides polygyrus*. Control C57BL/6 and BALB/c mice both responded to parasite infection with a characteristic 3–4 log increase in total serum IgE, with specific IgE antibodies against *H. polygyrus* excretory-secretory proteins (HES) (248 ± 23 and 2289 ± 2351 U/ml, respectively). In contrast, MHC II-deficient, CD4-deficient, and *nu/nu* mice all failed to generate detectable parasite-specific IgE antibodies (<1 U/ml) nor did they exhibit significant increases in total serum IgE titers over the 30 day period of infection. This confirms that the natural IgE pathway is distinct from the normal cognate T-dependent induction and, unlike the parasite-specific pathway, is not driven by exogenous antigen.

Organized Secondary Lymphoid Tissues Are Not Required for Natural IgE Production

The existence of a T cognate-independent IgE pathway suggested that classical organized secondary lymphoid structure with germinal centers might not be required. To test this, we used alymphoblastic (aly/aly) mice, which lack lymph nodes and Peyer's patches, exhibit disorganized splenic and thymic structures, and have defective B cell responses and germinal center formation (Miyawaki et al., 1994; Nanno et al., 1994; Yin et al., 2001). Interestingly, similar to *nu/nu* and MHC $II^{-/-}$, aly/aly mice also exhibit defective T cell responses to immunogens and pathogens (Karrer et al., 1997; Miyawaki et al., 1994), and those T cells present display an activated phenotype with IL-4 production (Figure S4). We observed that aly/aly mice, which cannot make IgE, exhibited numerous features of allergic disease, including elevated blood and skin eosinophilia, and airway hyperresponsiveness (Figure S4). To overcome the intrinsic B cell defects in aly/aly mice, we reconstituted lethally irradiated mice with wt bone marrow, in these circumstances lymphoid structures are not regenerated nor do they recover germinal center formation or normal immune responses to viral pathogens (Karrer et al., 2000). Nevertheless, B6 → aly/aly bone marrow chimeras showed elevated IgE as compared with control B6 → B6 chimeras (Figure 4A). Histological analysis of lung and liver in B6 → aly/aly chimeric mice revealed large numbers of IgE-producing plasma cells residing within mononuclear cell aggregates; however, these aggregates did not contain germinal centers or follicular dendritic cell networks (Figure 4B). IgE production in B6 → aly/aly chimeric mice was dependent on $CD4^+$ T cells but did also not require MHC II expression on B cells (data not shown). These data indicate that elevated production of cognate-independent IgE can occur in the virtual absence of conventional secondary lymphoid structures and did not require germinal center formation. IgE production in the absence of germinal center formation is also consistent with our demonstration of the absence of somatic hypermutation in natural IgE.

$CD4^+CD25^+$ T Cells Cannot Inhibit IgE Production in T Cell Immunocompromised Mice

Because deficient T regulatory (Treg) cell function has been reported to lead to hyper-IgE in IPEX syndrome patients (Wildin et al., 2002), we investigated the possibility that a lack of regulation of $CD4^+$ T cell activity by Treg

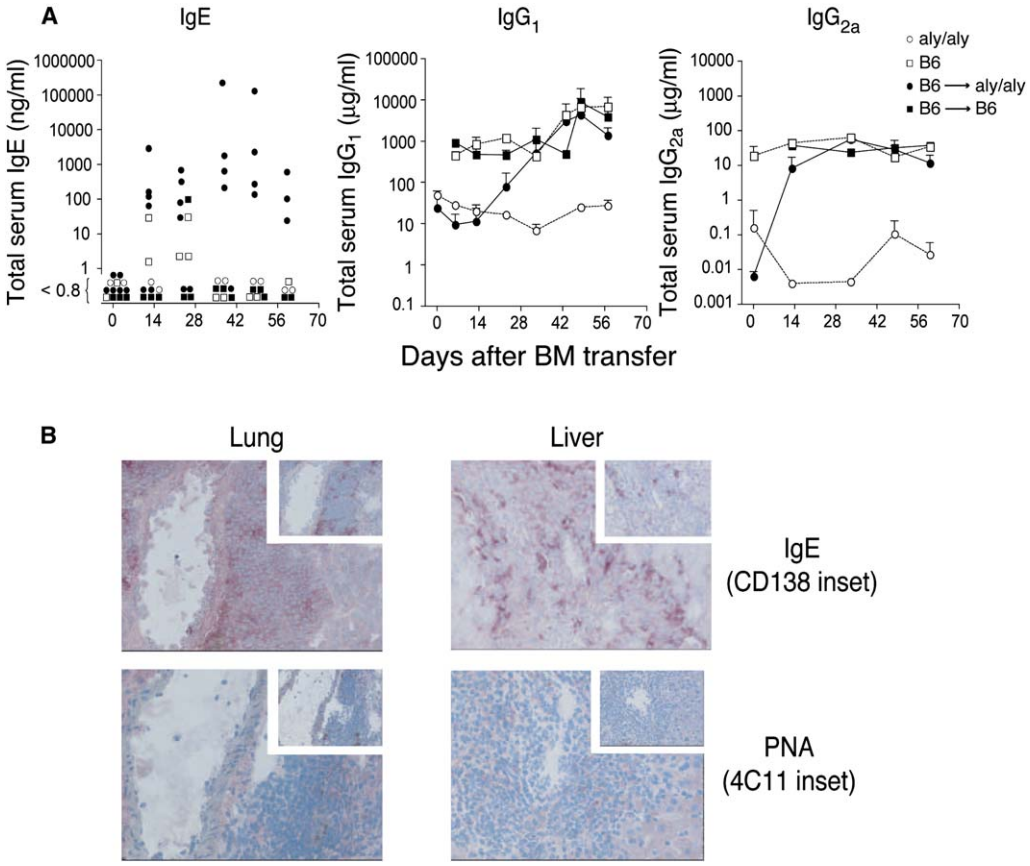

Figure 4. IgE Production in the Absence of Conventional Secondary Lymphoid Tissues

(A) Individual titers for total serum IgE, or average serum titers (mean ± SEM of two to six mice) for total IgG_1 and IgG_{2a}, are shown for B6 (open squares) and aly/aly mice (open circles), or for B6 → B6 (closed squares) and B6 → aly/aly (closed circles) bone marrow chimeras at various times after bone marrow transfer. All mice were splenectomized at least 2 weeks prior to irradiation. To control for possible GVHD, lethally irradiated and splenectomized aly/aly mice were also reconstituted with bone marrow from heterozygous aly/+ mice. These mice also exhibit increased IgE levels, therefore showing that the response is not due to GVHD.

(B) Lung and liver were taken from B6 → aly/aly bone marrow chimeric mice exhibiting high serum IgE at day 50–60 after bone marrow transfer and analyzed for IgE, CD138 (marker for plasma cells), PNA (marker for germinal center formation), or 4C11 (marker for follicular dendritic cells). Photographs show sections at 20× magnification and are representative of at least five mice from three separate experiments.

cells in T cell immunocompromised mice could explain the observed elevations in IgE. Flow cytometric analysis showed that the ratio of $CD25^+$ to $CD25^-$ cells was either similar (*nu/nu*) or increased (MHC $II^{-/-}$ and TCRα transgenic) compared with naive B6 wt mice (Table S8). We next checked for Treg function in these mice by purifying $CD4^+CD25^+CD45RB^{low}$ Treg cells from MHC $II^{-/-}$ and *nu/nu* mice and analyzed the ability of these cells to inhibit anti-CD3-induced proliferation of $CD4^+CD25^-CD45RB^{hi}$ naive T cells from wt mice in vitro. Treg cells from MHC $II^{-/-}$ mice were able to inhibit T cell proliferation as well as wt Treg cells (Figure 5A). In contrast, Treg cells from *nu/nu* mice were not able to inhibit T cell proliferation as well as wt Treg cells (Figure 5A). There was no consistent change in potential T regulatory cell function as determined by the levels of Foxp3 mRNA transcripts in purified $CD4^+CD25^+CD45RB^{low}$ (Treg cells) or $CD4^+CD25^-CD45RB^{hi}$ (naive T cells) populations from C57BL/6, MHC $II^{-/-}$, and *nu/nu* mice (data not shown). To determine directly whether Treg cells could attenuate elevated IgE production by MHC II-deficient mice, we injected mice with purified $CD4^+CD25^+CD45RB^{low}$ T cells (that are protective against colitis [Mottet et al., 2003] and Figure 5, inset), or $CD4^+CD25^-CD45RB^{hi}$ (naive) cells and monitored serum IgE levels on a weekly basis. Neither $CD4^+CD25^+CD45RB^{low}$ (Treg) nor control $CD4^+CD25^-CD45RB^{hi}$ (naive) cells were able to suppress the spontaneous induction of IgE in MHC $II^{-/-}$ mice (Figure 5).

In summary, we have identified a pathway for the induction of natural IgE. This IgE differs from antigen-driven IgE in that it occurs independently of T cell cognate help, does not require the presence of organized lymphoid structures, and does not exhibit evidence of somatic hypermutation. It also includes specificities directed against self-antigens, so this is probably a primitive means of IgE production driven by activated bystander CD4 T cells.

Although natural IgE is considerably increased in mice with limited T cell repertoires, the explanation for the increase in proportions of activated IL-4-producing CD4 cells responsible for triggering the effect remains unclear. However, in humans, similar effects are seen. The paradoxical observations of high serum IgE titers

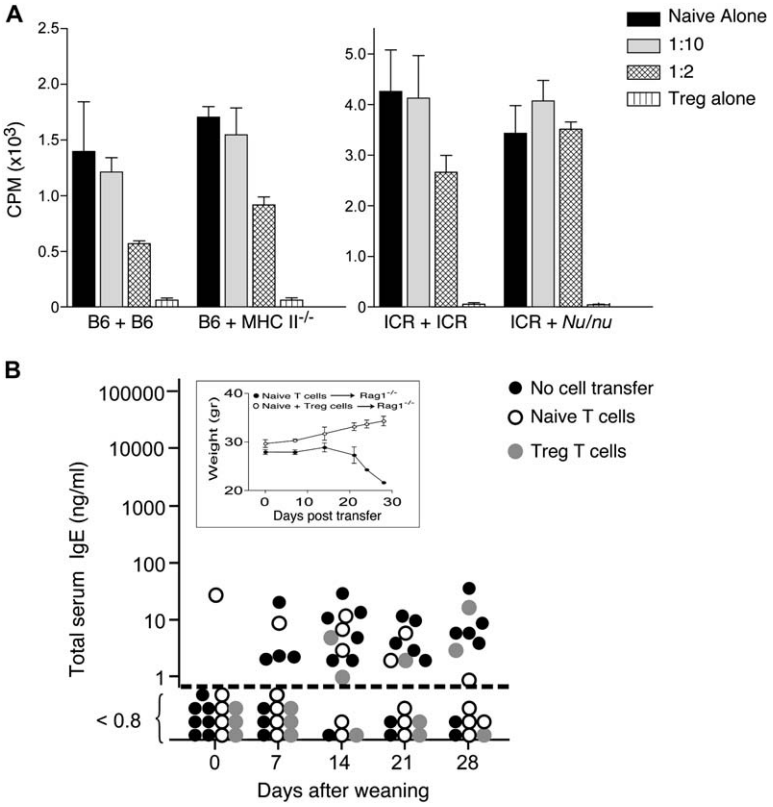

Figure 5. $CD4^+CD25^+CD45RB^{low}$ Treg Cells Do Not Inhibit the Spontaneous Induction of Natural IgE

(A) Purified $CD4^+CD25^-CD45RB^{hi}$ naive (5×10^4) and $CD4^+CD25^+CD45RB^{low}$ Treg (2.5×10^4) T cells from C57BL/6, MHC $II^{-/-}$, ICR wt, and ICR *nu/nu* mice were cultured alone, or the two populations were mixed together at the indicated ratios (1:10 or 1:2 Treg to naive T cells). All mixtures were assayed for proliferation in the presence of irradiated and T cell-depleted C57BL/6 or ICR wt splenocytes and anti-CD3 (1 μg/ml). Bars represent the mean ± SD of triplicate cultures.

(B) $CD4^+CD25^+CD45RB^{low}$ Treg (gray symbols) and $CD4^+CD25^-CD45RB^{high}$ naive T cells (open symbols) were purified from C57BL/6 naive mice and adoptively transferred by i.v. injection into MHC $II^{-/-}$ mice on the day of weaning. Each animal received either 2×10^6 Treg or 3×10^6 naive T cells. Control mice (black symbols) did not receive any cells. Blood was collected at weekly intervals, starting day 0 prior to cell transfer, and total serum IgE levels determined by ELISA. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each data point indicates a single animal, and results are representative of two separate experiments. Inset, purified Treg cells were able to protect $Rag1^{-/-}$ mice from colitis induced by transfer of naive T cells. $Rag1^{-/-}$ mice were injected i.v. with 2×10^5 $CD4^+CD25^-CD45RB^{high}$ naive T cells alone (black symbols) or together with 2×10^5 $CD4^+CD25^+CD45RB^{low}$ Treg cells (open symbols) on day 0 and weight loss monitored. Symbols represent the mean ± SEM of two to three mice per group.

in DiGeorge (Buckley, 2002) or Omenn's syndrome (Buckley, 2002) patients suffering from congenital T cell deficiencies are resolved by our data.

IgE has a short half-life in the serum (Tada et al., 1975), but it exhibits an extremely high binding affinity for the FcεRI (Kulczycki and Metzger, 1974), which is expressed on the surface of mast cells and basophils, and this interaction prolongs the half-life of IgE to 1–2 weeks (Wan et al., 2002). Monomeric IgE has been demonstrated to act in an antigen-independent manner to promote mast cell survival (Asai et al., 2001; Kalesnikoff et al., 2001) and cytokine production (Kalesnikoff et al., 2001) in vitro. In vivo, IgE-deficient mice have been used to show that hapten-irrelevant IgE or preimmune spontaneous IgE normally present in mice is required, bound to FcεRI on mast cells, to sensitize mice to contact sensitivity (Bryce et al., 2004) through mast cell modulation of dendritic cell migration or function. Our sequence data show that unmanipulated C57BL/6 wt mice have undiversified Igε heavy chain repertoires similar to the natural Igε heavy chain sequences of natural antibodies of mice (Tables 1 and 2), and it is likely that the noncognate induction mechanisms are also similar. Thus, the probable function for natural IgE, present at low levels even in naive mice, germ-free mice, or nonallergic individuals, is to regulate mast cell and basophil homeostasis. Mast cells not only function as effector cells during allergic responses but also represent an important component of innate immunity at skin and mucosal surfaces. Bacterial, viral, parasitic, and fungal pathogens have all been reported to result in the immediate activation of mast cells with the resulting release of cytokines, chemokines, leukotreines, and histamine that modulate leukocyte migration and vascular permeability (reviewed in Marshall and Jawdat [2004] and Wedemeyer et al. [2000]). IgE has previously been considered a highly adaptive immunoglobulin. Natural IgE, with specificities determined by evolution may in fact represent an essential component in mast cell mediated innate responses.

Experimental Procedures

Mice and Antibodies

C57BL/6, C57BL/6-Sv129 $CD4^{-/-}$ (Rahemtulla et al., 1991), BALB/c *nu/nu*, ICR *nu/nu*, $J_H^{-/-}$ (Chen et al., 1993), aly/aly, $TCR\beta\delta^{-/-}$, $CD40L^{-/-}$, C57BL/6J-$Igh^aThy1^aGpi1^a$ (originally obtained from The Jackson Laboratory), and TCRα TCR transgenic mice were bred at the Labortierkunde of the University of Zürich. C57BL/6 $CD4^{-/-}$, C57BL/6 *nu/nu*, MHC $II^{-/-}$ ($I\text{-}A^{b-/-}$) (Kontgen et al., 1993), IL-$4^{-/-}$ (Kopf et al., 1993), TgH(KL25)xMHC $II^{-/-}$, and (TgH[VI10]xYEN)x MHC $II^{-/-}$ mice were bred and maintained at the Biologisches Zentrallabor of the University of Zurich. BALB/c mice were obtained from Harlan, Netherlands. Germ-free C57BL/6 mice were maintained under gnotobiotic conditions at the Labortierkunde of the University of Zürich. aly/aly mice were purchased from CLEA, Japan on a C57BL/6xAEJ (*H-2*^{b}) background and maintained by breeding heterozygous females to homozygous males. Animal experiments were performed according to institutional guidelines and to Swiss federal and cantonal laws on animal protection. 11B11 is a monoclonal

antibody (Rat IgG_1) directed against mouse IL-4 (Finkelman et al., 1986), 100 μg was injected intraperitoneally (i.p.) at weekly intervals for neutralization of IL-4. YTS 191.1.2 is a monoclonal antibody (Rat IgG) directed against mouse CD4 (Galfre et al., 1979), 100 μg was injected i.p. at weekly intervals for depletion of $CD4^+$ cells.

ELISA Assays

Antibody serum titers were determined by ELISA, as previously described (Macpherson et al., 2001). Total serum IgE or helminth-specific IgE were measured in a similar manner after coating with 5 μg/ml rat anti-mouse IgE (clone 6HD5), or 5 μg/ml HES (excretory/secretary products collected from adult L5 *H. polygyrus* cultured for a period of 2 days in RPMI plus antibiotics and 1% glucose, followed by concentration to ≥0.5 mg/ml by centrifugation through a 10,000 MWCO cellulose membrane, Centriprep, Millipore, MA). To calculate IgE concentrations, a mouse myeloma IgE (clone TIB-141) or an internal standard consisting of serum from mice infected two times with *H. polygyrus* were used. For detection of anti-PC IgE, total IgE was captured with anti-IgE (clone 6HD5) and anti-PC-specific IgE was detected by addition of PC-BSA (50 μg/ml) followed by an HRP-labeled anti-PC Ab. For detection of allotype-specific IgE, total IgE was captured with anti-IgE^b (clone JKS-6) or anti-IgE^a (clone UH297) and detected with an HRP-labeled anti-IgE (clone HMK-12) (Miyajima et al., 1996). For detection of anti-idiotypic IgE, total IgE was captured with anti-IgE (clone 6HD5) and idiotypic IgE detected with biotin-conjugated anti-KL25 (clone IIIC4) or anti-VI10 (clone 35.61) idiotypic antibodies and HRP-conjugated streptavidin (Hangartner et al., 2003). Titers were calculated as the dilution that gave an Abs (405 nm) equal to 2× the Abs (405 nm) of the background. Results given in text are mean ± SD.

Generation of Bone Marrow Chimeras

Recipient C57BL/6, *nu/nu*, or aly/aly mice were lethally irradiated (9.5 Gy) by using a ^{60}Cobalt source and were injected i.v. with 5–10 × 10^6 bone marrow (BM) cells that were collected from tibias and femurs of donor mice. For generation of mixed BM chimeras in which all B cells were deficient in MHC II, equal numbers of MHC $II^{-/-}$ and $J_H^{-/-}$ BM cells were injected. Control chimeras were generated by reconstituting *nu/nu* mice with $J_H^{-/-}$ BM alone. For generation of mixed BM chimeras in which all T cells were deficient in CD40L, equal numbers of $CD40L^{-/-}$ and $TCR\beta\delta^{-/-}$ BM cells were injected. Both aly/aly and control C57BL/6 mice were reconstituted with C57BL/6 BM. Both aly/aly and C57BL/6 control recipient mice were splenectomized at least 2 weeks prior to irradiation. Blood was collected every 7–14 days for determination of total IgE.

RT-PCR and Sequencing

B cells were purified to >95% from the spleen by MACS (Miltenyi Biotech) using anti-B220 microbeads. RNA was then isolated from splenic B cells or total bone marrow cells by using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA was produced by using random hexamers and Superscript II RT (Invitrogen Life Technologies) according to the manufacturer's instructions. Detection of mature IgE-switched transcripts was performed by PCR using primers (0.2 μM) annealing within exon 1 (5′-CCTGCCCTCGGTTCTGA-3′) and exon 4 (5′-CTAGGCGACTGA AGATGAAG-3′) of the Cε gene and the following cycling conditions: 94°5′[94°1′, 58.1°1′, 72°1′] × 35, 72°7′. V_H-Cε transcripts were amplified by PCR using a degenerative primer mix (Krebber et al., 1997) specific for the V_H region and a reverse primer (0.2 μM) that anneals within exon 1 of the Cε gene (5′-TCTGAATACCAGGTCACAGTC-3′) and the following cycling conditions: 94°5′[94°1′, 55.5°1′, 72°1′] × 35, 72°7′. PCR products were purified by agarose gel electrophoresis and the agarose gel extraction kit (Qiagen) and subcloned directly into the pGEM-T vector (Promega) for automated sequencing. For IgE and IgM germ line and GAPDH transcripts, cDNA was produced from FACS-purified B1 or B2 cells by using specific primers (εGLT, 5′-TCGTTGAATGATGGAGGATGTGTCACGT-3′; μGLT, 5′-AT GGTGCTGGGCAGGAAGTC-3′; and GAPDH, 5′-CATCAAGAAGGT GGTGAAGC-3′) and Superscript II RT (Invitrogen Life Technologies) according to the manufacturer's instructions. For the detection of εGLT, a nested PCR approach was applied in order to increase sensitivity and confirm negative results. μGLT and GAPDH were detected with a standard PCR. Primer sequences and conditions for each PCR were as follows: $\varepsilon GLT\ 1^{ST}$ PCR, upper 5′-GCGGCCCCTA GGTACTACCA-3′ and lower 5′-TCGTTGAATGATGGAGGATGTGT CACGT-3′, 94°5′[94°1′, 60.0°1′, 72°1′] × 35, 72°7′; εGLT 2nd PCR, upper 5′-GCAGAAGATGGCTTCGAATAAGAACAGT-3′ and lower 5′-CC GAGGGCAGGGAAGTTCACA-3′, 94°5′[94°1′, 55.0°1′, 72°1′] × 35, 72°7′; μGLT PCR, upper 5′-CTCTGGCCCTGCTTATTGTTG-3′ and lower 5′-ATGGTGCTGGGCAGGAAGTC-3′, 94°5′[94°1′, 60.0°1′, 72°1′] × 35, 72°7′; and GAPDH PCR, upper 5′-CCTGTTGCTGTAGCCGT ATT -3′ and lower 5′-CATCAAGAAGGTGGTGAAGC -3′, 94°5′ [94°30″, 55.0°45″, 72°30″] × 35, 72°7′.

Purification of B and T Cells and Transfer of Treg

To identify B1 and B2 cells, peritoneal and spleen mononuclear cells were isolated by MACS using anti-CD19 microbeads. Cells were further stained with monoclonal antibodies directed against B220, CD5, CD3, and IgM and subpopulations isolated by FACS sorting using a FACSAria (Becton Dickinson). Splenic $CD4^+$ cells were purified to >90% from C57BL/6 mice by MACS (Miltenyi Biotech) using anti-CD4 microbeads. $CD4^+CD25^+CD45RB^{low}$ (Treg) and $CD4^+CD25^-CD45RB^{high}$ (naive T cells) T cells were then purified to >95% by FACS sorting using a FACSAria (Becton Dickinson). All antibodies used for flow cytometry were obtained from PharMingen (Becton Dickinson Biosciences). Purified cells were then injected i.v. (2 × 10^6 for Treg cells and 3 × 10^6 for naive T cells) into recipient MHC $II^{-/-}$ mice on the day of weaning. Blood was collected from recipient mice every 7 days for determination of total IgE levels. To test whether the purified Treg cells were functional, $CD4^+CD25^-CD45RB^{high}$ naive T cells (2 × 10^5) were injected (i.v.) alone or together with $CD4^+CD25^+CD45RB^{low}$ Treg cells (2 × 10^5) into naive $RAG1^{-/-}$ recipient mice. Mice were then analyzed for the development of colitis by weighing every 4–7 days.

Proliferation Assay

For in vitro proliferation assays, purified $CD4^+CD25^-CD45RB^{high}$ naive T cells (5 × 10^4) from C57BL/6 or ICR wt mice were cultured in 96-well plates with 5 × 10^4 T cell-depleted irradiated splenocytes (3000 R) from C57BL/6 or ICR wt mice in the presence of 1 μg/ml of anti-CD3 mAb (2C11). Purified $CD4^+CD25^+CD45RB^{low}$ Treg cells from C57BL/6, MHC $II^{-/-}$, ICR wt, or ICR *nu/nu* mice were titrated in at 5 × 10^3 or 2.5 × 10^4 to give a 1:10 or 1:2 ratio of Treg:naive cells. Each $CD4^+$ population was also cultured alone with splenocytes and anti-CD3. All cultures were performed in triplicate, incubated for 72 hr, and pulsed with ^{3}H-thymidine for the last 6 hr before harvesting.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Macpherson et al., 2001). Briefly, tissues were immersed in 4% formalin or snap frozen in Hanks Balanced Salt Solution by using liquid nitrogen. For formalin-fixed samples, tissues were embedded in paraffin, sectioned, and stained with hemalum and eosin. For frozen samples, tissue sections of 5 μm thickness were fixed with acetone for 10 min and stored at −70°C. Rehydrated sections were then incubated with rat monoclonal antibodies against IgE (clone 3-11; C. Heusser, Novartis Pharma; dilution 1:800), CD138 (clone 281-2, BD Biosciences, San Diego, CA; dilution 1:500), or 4C11 (clone FDC-M1, BD Biosciences; dilution 1:50). Antibody binding was revealed by sequential incubation with goat antibodies to rat immunoglobulins (Caltag Labs, San Francisco, CA; dilution 1:150) and alkaline phosphatase-labeled donkey antibodies to goat immunoglobulins (Jackson ImmunoResearch Labs, West Grove, PA; dilution 1:80). For the staining of germinal center cells, biotinylated PNA (Vector Labs, Burlingame, CA; dilution 1:50) was applied and revealed by the ABC/alkaline phosphatase method (DakoCytomation, Glostrup, Denmark). Alkaline phosphatase was visualized by using naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate and sections counterstained with hemalum.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and eight tables and can be found with this article online at http://www.immunity.com/cgi/content/full/24/3/329/DC1/.

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