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Composition of the immunoglobulin classic antigen-binding site regulates allergic airway inflammation in a murine model of experimental asthma

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Abstract

Background—When bound to mast cell FcεRI, IgE serves as antigen receptor for allergic reactions, permitting specific identification of the allergen. Although the core of the classic antigen-binding site is heavy chain *complementarity determining region 3* (CDR-H3), recent studies suggest that allergens might also bind IgE in a superantigen-like fashion outside the classic antigen-binding site.

Objective—We sought to evaluate the contribution of the classic CDR-H3-centric antigen-binding site to the development of an allergic phenotype.

Methods—Using a murine model of experimental asthma, we characterized a gene targeted mouse strain expressing an altered range of CDR-H3s (ΔD-iD mice) in response to the hydrophobic allergen ovalbumin. Mutant and wildtype (*wt*) mice were sensitized intraperitoneally with ovalbumin; non-sensitized mice served as controls.

Results—We found the composition of the classic CDR-H3-centric antigen-binding site to be critical for the development of characteristic aspects of allergic asthma: (i) Compared to *wt* animals, ΔD-iD mice showed a significantly less pronounced ovalbumin-induced rise in allergen-specific IgE levels and hence in total serum IgE levels. (ii) In addition, ΔD-iD mice demonstrated a significant reduction in eosinophilic airway inflammation, as well as in IL-4, IL-5, and IL-13 levels in BAL fluids.

Conclusion—Allergic sensitization and airway inflammation depend on the composition of the predominant CDR-H3 repertoire, suggesting that the classic CDR-H3-centric antigen-binding site plays a crucial role in creating the immunological interface between allergen and IgE. Our results further emphasize a central role of IgE, not only in mediating but also in regulating the allergic immune response.

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Disclosures

The authors declare that they have no financial or commercial conflict of interest.

Keywords

IgE; allergy; asthma; allergic airway inflammation; classic antigen binding-site; CDR-H3; B cell response

Introduction

Immunoglobulin E (IgE) is a central effector molecule for allergic immune responses. When bound to mast cell FcεRI, IgE functions as the immunological interface between the allergen and the immune system [1]. Cross-linkage of two adjacent FcεRI-bound IgE molecules triggers mast cell degranulation, leading to the release of the proinflammatory mediators that orchestrate allergic inflammation (reviewed in [2]). The studying of this interface between the allergen and adaptive immune recognition is promising to improve our understanding of the mechanisms that underlie clinical allergic responses and might open the way to new therapeutic options, such as anti-idiotypic antibodies against the allergen-binding site of IgE.

From the traditional point of view, the specificity of an immunoglobulin is defined by the classic antigen-binding site at the tip of the variable domain. This site is created by the juxtaposition of three hypervariable *complementarity determining regions* (CDR) from the heavy chain and three CDRs from the light chain [3]. Of the six CDRs, the third CDR of the heavy chain (CDR-H3) has the greatest influence on the overall antibody diversity [4]. In contrast to CDR-H1 or CDR-H2, which are contained entirely within the V_H gene segment, CDR-H3 is created by the rearrangement of V_H-, D_H-, and J_H- segments and by addition of random N nucleotides during the process of somatic recombination [5], generating an almost unlimited variety of possible combinations. Coupled with its position at the center of the classic antigen-binding site, CDR-H3 often plays a determinative role in the recognition and binding of the antigen to immunoglobulin [4, 6].

Unlike the CDRs, the *framework regions* of the antibody molecule are usually not involved in antigen binding. Instead they form a supportive scaffold for the classic antigen-binding site [3]. There is a minority of antigens that are capable of interacting with the immunoglobulin molecule via these framework regions. Antigens that are recognized through this archaic “innate-like” mechanism are referred to as *B cell superantigens*. Due to their ability to bind V gene segments directly, superantigens are often very potent inducers of immune activation and have been suggested to play a role in autoimmune diseases, immunodeficiencies, and allergies [7, 8]. Many findings support the view that allergic sensitization represents a misled classical affinity-driven immune response towards the production of allergen-specific IgE under the influence of a T_H2-biased cytokine milieu [9-11]. However, there are recent observations that have challenged this view. First, evidence of a biased use of specific V_H families in IgE transcripts has been reported in patients with allergic rhinitis [12, 13], allergic asthma [14, 15], and atopic dermatitis [16, 17]. Second, unlike classic IgG responses, IgE heavy chain sequences from allergic individuals have in some cases shown no evidence of enrichment for replacement mutations in the CDRs, arguing against a classical affinity maturation (reviewed in [18]). Therefore, it was hypothesized that some allergens might also act in a superantigen-like fashion (reviewed in [8]). This thought led to the *superallergen hypothesis* [19]. However, allergens are not the only candidates for a superantigen-like interaction with IgE. Recent studies suggest a role for bacterial and viral superantigens in the activation and perpetuation of allergic inflammation (reviewed in [8]). Namely superantigens produced by *Staphylococcus aureus* have been implicated in the pathogenesis of allergic inflammation by providing unspecific stimulation to polyclonal naïve B and T cells, leading to a proliferation, driving

somatic recombination, and facilitating the production of allergen-specific IgE by activated B cells [20].

It remains unclear to what extent allergens are recognized as classical antigens or as superantigens or as both. We sought to determine the role of the classic CDR-H3-centric antigen-binding site in a murine model of allergic sensitization and allergic asthma. The main hypothesis in our study was, that gene targeted mice with preferentially charged amino acids within their CDR-H3 regions (Δ D-iD) are impaired in their ability to develop an allergic immune response to the allergen ovalbumin (OVA), which has allergenic epitopes of high hydrophobicity [21, 22]. This would indicate that the CDR-H3 acts as a major site of allergen/immunoglobulin interaction and would argue against a solely superallergen-like action of OVA. Otherwise, should the allergic immune response to OVA be independent from a modified hydrophobicity of the classic antigen binding-site, this would argue for a potential superantigen-like action of this allergen.

Material and Methods

Animals

We used a previously described gene targeted mouse strain with a modified immunoglobulin heavy chain *Diversity* (D_H) gene segment locus [23]. In Δ D-iD mice, the D_H locus has been replaced by a single, modified D_H containing inverted DSP2.2 gene segment sequence. In *reading frame 1*, this D_H encodes arginine, histidine, and asparagine. Thus, Δ D-iD mice preferentially generate B cell receptors enriched for positively charged amino acids in CDR-H3. Prior to experimentation, all mice were genotyped as previously described [23]. BALB/c *wt* controls were purchased from Harlan Winkelmann (Borchen, Germany). Animals were held under specific pathogen-free conditions in single ventilated cages, fed an ovalbumin-free diet, and supplied with water *ad libitum*. The animal experiments were performed with the approval of the local governmental authority (Regierungspraesidium Giessen, Germany).

IgE-PCR and sequence analysis

Total RNA was isolated from splenic tissue using the RNeasy minikit (Qiagen; Hilden, Germany) according to manufacturer's instruction. IgE specific heavy chain transcripts were amplified by a semi-nested reverse transcriptase PCR, using the following primers:

The following program was performed on a thermocycler (Sensoquest; Gottingen, Germany): 30 min at 50°C, 15 min at 94°C, followed by 40 cycles consisting of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C, followed by a final extension of 10 min at 72°C. The second (semi-nested) PCR comprised another 30 cycles with the same conditions. The PCR products were gel purified, extracted, cloned into *E. coli* using the TOPO-TA cloning kit (Invitrogen; Karlsruhe, Germany), and sequenced commercially by GATC-Biotech (Konstanz, Germany). Sequences were aligned with the ImMunoGeneTics (IMGT) VQUEST program (<http://imgt.cines.fr>) [24]. The CDR-H3 was defined to include those residues located between the conserved cysteine (C104) of FR3 and the conserved tryptophan (W118) of FR4. The average hydrophobicity of CDR-H3 was calculated using the normalized Kyte-Doolittle index [25].

Protocol of Allergic Sensitization

We studied four groups of mice: (1) non-sensitized *wt* mice (*wt* PBS), (2) sensitized *wt* mice (*wt* OVA), (3) non-sensitized Δ D-iD mice (Δ D-iD PBS), and (4) sensitized Δ D-iD mice (Δ D-iD OVA). Mice were sensitized to OVA as previously described [26]. Ten micrograms of OVA grade VI (Sigma, Deisenhofen, Germany) were adsorbed to 1.5 mg Al(OH)₃ (Imject® Alum; Pierce, Rockford (IL), USA) and administered intraperitoneally (i. p.) on

days 1, 14, and 21. To induce allergic airway inflammation, the animals received three aerosol challenges with 1% (wt/vol) OVA grade V (Sigma, Deisenhofen, Germany) diluted in PBS for 20 min on days 26, 27, and 28. Non-sensitized control mice received PBS alone i. p. and were challenged with aerosolic OVA on days 26, 27, and 28. Therefore, all data presented in the results section are from mice which have been challenged with aerosolic OVA on days 26, 27, and 28.

Determination of Antibody Levels

Serum concentrations of total IgE, IgM, and IgG₁ were measured by ELISA as previously described [27]. Antibodies and standards were purchased from BD (Heidelberg, Germany). Serum samples were diluted 1:500 (IgE), 1:10,000 (IgM), and 1:100,000 (IgG₁) respectively for OVA-sensitized mice. For non-sensitized controls, the dilutions were 1:2 (IgE), 1:10 (IgM), and 1:10 (IgG₁) respectively.

For determination of OVA-specific IgE antibody levels, we established a novel ELISA assay. ELISA plates (Nunc, Roskilde, Denmark) were coated with rat anti-murine IgE (BD, Heidelberg, Germany) in a dilution of 1:500 overnight. After blocking with 3% bovine serum albumin in PBS, serum samples were applied in a dilution of 1:20 in washing buffer. As standard, we used an OVA-specific murine IgE (Serotec, Oxford, UK). Samples and standard were incubated overnight. The plates were then incubated with OVA °VI (20 µg/ml) for 2 hours at room temperature. To detect the bound allergen, we applied a murine anti-OVA specific antibody of the isotype IgG₁ (Sigma, Deisenhofen, Germany) and used biotin-conjugated anti-IgG₁ (BD, Heidelberg, Germany) as the secondary antibody. After incubation with streptavidin peroxidase (Calbiochem, Bad Soden, Germany), POD-BM blue (Roche, Mannheim, Germany) was used as chromogenic substrate. Absorption was measured at 450 nm against 690 nm with a photometric ELISA reader (Asys, Eugendorf, Austria).

Assessment of Lung Function

To assess the lung function, we used head-out body plethysmography as previously described [28]. This system allows for the simultaneous evaluation of the breathing pattern of non-anesthetized, spontaneously breathing mice in response to different stimuli. The lung function analysis was performed on day 29.

For the determination of bronchoconstriction, we measured the midexpiratory airflow (MEF₅₀), i. e. the expiratory airflow (in ml/s) at 50% tidal volume. To assess airway responsiveness, we determined the dose-dependent response to inhaled methacholine (Sigma, Deisenhofen, Germany). As vehicle control, the diluent PBS was nebulized. We calculated the methacholine dose (in mg/ml) needed to cause a 50% decline in MEF₅₀, the provocation concentration 50 (PC₅₀). For the evaluation of allergen-induced bronchoconstriction, mice were challenged intranasally with OVA (1% w/v in PBS). Airflow obstruction was assessed by measurement of MEF₅₀.

Bronchoalveolar Lavage (BAL)

On day 30, trachea was cannulated and airways were lavaged with 1.6 ml ice-cold PBS, containing proteinase inhibitor (Complete®; Boehringer, Mannheim, Germany). Total cell numbers were determined by a pulse area analysis cell counter (Casy®; Schärfe, Reutlingen, Germany). Cells were cytocentrifuged onto slides, differentially stained with DiffQuik® (Behring, Marburg, Germany), and classified by light microscopy.

Determination of cytokine levels in BAL fluids

The levels of interleukin-4 (IL-4), IL-5, IL-13, IL-6, IL-10, and interferon- γ (IFN- γ) were measured by bead-based FlowCytomix assay (Bender MedSystems; Vienna, Austria), according to the manufacturer's protocol using a FACScalibur[®] flow cytometer (BD; Heidelberg, Germany). The detection limits were 0.7 pg/ml for IL-4, 4.0 pg/ml for IL-5, 9.3 pg/ml for IL-13, 6.5 pg/ml for IFN- γ , 5.4 pg/ml for IL-10, and 2.2 pg/ml for IL-6.

Quantitative Histology

On day 30, lungs were fixed by instillation with 6% phosphate-buffered paraformaldehyde. Systematic uniform random samples of lung tissue were taken according to standard methods [29]. Airway epithelial mucin was identified, using periodic acid Schiff (PAS) staining. The surface area of mucin-containing goblet cells (S_{gc}) per total surface area of airway epithelial basal membrane (S_{ep}) and the volume of PAS-stained epithelial mucin (V_{mucin}) per S_{ep} were determined using a computer-assisted stereology tool box (CAST-Grid 2.0[®]; Albertslund, Olympus, Denmark) [30, 31] according to the formulas:

$$S_{gc}/S_{ep} = \sum I_{gc} / \sum I_{ep}$$

$$V_{mucin}/S_{ep} = LP \times \sum P_{mucin} / 2 \times \sum I_{ep}$$

$\sum I_{gc}$ = sum of intersections of test-lines with goblet cells; $\sum I_{ep}$ = sum of all intersections of test-lines with epithelial basal membrane; $\sum P_{mucin}$ = sum of all points hitting mucin; LP = test-line length at final magnification.

Statistical Analysis

Results are presented as mean $\pm$ SEM in histograms. To determine the level of significance, a two-tailed Student's *t* test was used. Values of $p < 0.05$ were considered significant. For denomination in figures, the following symbols are used: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistical analyses were performed using SPSS[®] v.12.0 (Chicago (IL), USA) and GraphPad Prism4.0[®] (El Camino Real, San Diego (CA), USA).

Results

IgE repertoire of ΔD -iD mice preserves limited diversity

Ippolito et al. described the limited diversity of the CDR-H3 repertoire in ΔD -iD mice for the primary immunoglobulin repertoire (IgM) [23]. To assess whether this limitation is preserved in the IgE repertoire, we analyzed sequences of IgE transcripts from ΔD -iD mice. Using a novel semi-nested PCR techniques which allows for the high sensitive amplification of murine IgE, we gained 84 productive sequences of IgE heavy chain variable region transcripts from OVA-sensitized ΔD -iD mice. Of these 84 IgE transcripts, 34 were unique (GenBank accession numbers FJ150835 to FJ150868).

The analysis of the CDR-H3 repertoire of these sequences revealed: (i) The ΔD -iD segment (inverted DSP2.2) [23] was preserved in all IgE transcripts. (ii) In all productive sequences, the charged first reading frame (RF 1) was utilized (supplemental figure 1). (iii) The most abundant amino acids used in CDR-H3 of IgE were asparagine (41%), arginine (15%), and aspartic acid (15%) (supplemental figure 2), leading to an average hydrophobicity of -0.71 according to the normalized Kyte-Doolittle hydrophobicity scale [25]. In summary, the

hydrophobic properties of the CDR-H3 repertoire in Δ D-iD mice, as described by Ippolito et al. for IgM [23], were found to be preserved in IgE transcripts.

Allergic sensitization is impaired in Δ D-iD mice

For the assessment of the primary immune response, we determined total IgM levels and found no significant differences between the experimental groups (data not shown). To assess the allergic sensitization, serum levels of total IgG₁ and total IgE were determined. For IgG₁, we found a significant increase after sensitization to OVA ($p < 0.001$). The magnitude of this increment did not differ between the genotypes (Figure 1A). However, we found considerable differences between the genotypes in the extent of IgE production. Before sensitization, all animals showed non-detectable or very low levels of total IgE (Figure 1B). These levels rose markedly during allergic sensitization in both genotypes ($p < 0.001$). In *wt* animals, the total IgE levels rose above 10,000 ng/ml after sensitization. However, this rise was significantly alleviated in Δ D-iD mice ($51\% \pm 9\%$ compared to *wt*; $p < 0.05$). The attenuation of total IgE production was reflected in OVA-specific IgE levels (Figure 1C), which were significantly reduced in Δ D-iD mice ($36\% \pm 18\%$ compared to *wt*; $p < 0.05$). Figure 1D illustrates the composition of total IgE: whereas the levels of non OVA-specific IgE (dark proportion in Figure 1D) did not differ markedly between the genotypes, the differences in total IgE were almost exclusively due to the significant reduction of OVA-specific IgE (light proportion in Figure 1D) in Δ D-iD mice. This reduction lead to a shift of ratios between the two fractions, resulting in a percentage of OVA-specific IgE of 60% in the *wt* mice, whereas in Δ D-iD mice only 41% of the total IgE were allergen-specific.

Airway inflammation is markedly alleviated in mice with modified CDR-H3

To assess the local inflammatory response in the airways, we determined the cellular influx into BAL fluids. In non-sensitized mice but OVA-challenged, no eosinophils were detectable (Figure 2). OVA-sensitized and -challenged animals of both genotypes developed a strong allergic airway inflammation, characterized by the immigration of eosinophils ($p < 0.001$). However, in sensitized Δ D-iD mice, the eosinophilic influx was half that observed in *wt* controls ($48\% \pm 15\%$, compared to *wt*, $p < 0.01$).

Local cytokine response is reduced in Δ D-iD mice

In order to reveal whether the reduced eosinophilic influx in Δ D-iD mice might reflect a decrease in local cytokine activity, we determined the levels of cytokines in BAL fluids (Figure 3). Following OVA-sensitization and -challenge, BAL fluid levels of IL-4, IL-5, and IL-13 rose significantly in both *wt* and Δ D-iD mice compared to PBS-treated (but OVA-challenged) controls. However, in OVA-sensitized Δ D-iD mice, this rise was significantly reduced for IL-4 ($60\% \pm 6\%$, compared to *wt*, $p < 0.05$), for IL-5 ($42\% \pm 4\%$, compared to *wt*, $p < 0.01$), and for IL-13 ($9\% \pm 2\%$, compared to *wt*, $p < 0.05$). In contrast to levels of T_H2 cytokines, IFN- γ levels in BAL fluids from OVA-sensitized Δ D-iD mice were significantly elevated ($155\% \pm 5\%$, compared to *wt*, $p < 0.01$). The IL-6 response did not differ significantly between the two genotypes. The observed difference between PBS-treated *wt* and Δ D-iD mice was statistically not significant ($p = 0.37$). For IL-10, we found elevated levels in BAL fluids from OVA-sensitized Δ D-iD mice. However, this difference did not reach statistical significance ($258\% \pm 81\%$, compared to OVA-sensitized *wt*, $p = 0.15$). The difference between PBS-treated *wt* and Δ D-iD mice also lacked statistical significance ($p = 0.24$).

Goblet cell hyperplasia is unaffected

To evaluate inflammation induced structural changes in the airways, we performed quantitative histology. Compared to PBS control animals, OVA-sensitized and challenged mice developed an intense goblet cell hyperplasia (Figure 4A). The quantification revealed a significant increase in the percentage of goblet cells (Figure 4B). In addition, a strong rise in mucin volume was observed (Figure 4C). These structural changes were highly significant compared to non-sensitized control animals ($p < 0.001$), but did not differ between the two genotypes.

Airway hyperreactivity is uncoupled from inflammation

Methacholine-response—For the determination of airway reactivity, we analysed the response to inhaled methacholine. Non-sensitized mice showed a dose-dependent decline in MEF₅₀ in response to inhaled methacholine (Figure 5A). This response was enhanced in OVA-sensitized animals (Figure 5B), indicating a state of airway hyperreactivity. To compare the groups statistically, we calculated the PC₅₀, i. e. the methacholine concentration (in mg/ml) needed to cause a 50% decrease in MEF₅₀ (Figure 5C). Compared to non-sensitized controls, the PC₅₀ were significantly reduced in OVA-sensitized mice ($p < 0.05$ for *wt* and $p < 0.01$ for ΔD -iD mice respectively). However, the extent of airway hyperreactivity did not differ between the two genotypes.

Ovalbumin-response—To further assess airway reactivity, we determined the lung function in response to the specific allergen. After local administration of OVA, non-sensitized mice displayed a short dip in MEF₅₀ (Figure 5D), but recovered within a few minutes and reached baseline values again. On the other hand, OVA-sensitized mice showed a marked and persistent decline of MEF₅₀ in response to the OVA (figure 5E). This allergen-induced bronchoconstriction did not differ between the two genotypes.

Discussion

The purpose of our study was to evaluate the contribution of the classic CDR-H3-centric antigen-binding site to the development of an allergic phenotype. Using a murine model of experimental asthma, we characterized the response to the hydrophobic allergen OVA in a gene targeted mouse strain expressing a polyclonal globally altered CDR-H3-repertoire (ΔD -iD mice). ΔD -iD mice preferentially generate B cell receptors enriched for positively charged amino acids in CDR-H3. The amino acid composition of this mouse strain represents a distinct subset that normally comprises $< 5\%$ of the *wt* mature B cell repertoire [32]. Notably, except for the reduction of germline D_H genes from 13 to one, all mechanisms of antibody diversification, class switch recombination, and B-cell selection are intact in ΔD -iD mice [23]. We characterized three fundamental aspects of the phenotype in allergic asthma: (i) allergic sensitization, (ii) allergic airway inflammation, and (iii) airway hyperreactivity. In summary, we found the global hydrophobicity of the classic CDR-H3-centric antigen-binding site to be critical for the development of two of these characteristic features. First, compared to *wt* animals, ΔD -iD mice had a significantly less pronounced OVA-induced rise in allergen-specific IgE levels and hence in total serum IgE levels. Second, ΔD -iD mice showed a significant reduction in eosinophilic airway inflammation and in IL-4, IL-5, and IL-13 levels in BAL fluids compared to *wt*.

The observed relationship between IgE and eosinophils is concordant with the current concept that bronchial eosinophilia is a consequence of the inflammatory cascade that follows crosslinking of IgE and mast cell degranulation [2]. However, the close correlation of IgE levels with T_H2 cytokine levels and eosinophilia in BAL fluids we observed is surprising and suggests a dose-dependent regulation of bronchial inflammation.

Nevertheless, these results are in line with recent data from clinical trials that have tested the anti-inflammatory effects of the monoclonal anti-IgE antibody omalizumab [33, 34]. Such studies have shown that treatment with omalizumab can reduce bronchial eosinophilia, airway inflammation, and the number of B and T cells in the bronchial submucosa. These and our findings indicate that the suppression of IgE is followed by a downregulation of multiple inflammatory effector cells. These results further emphasize the central role of IgE, not only in mediating but also in regulating the allergic immune response.

Much to our surprise, the altered cytokine response was not reflected in mucus production and goblet cell hyperplasia. Despite markedly reduced IL-13 levels in ΔD -iD mice, the mucus production was unaffected. This result is insofar surprising as IL-13 is considered one of the major regulator for mucus production in allergic airway inflammation [35]. However, our results are in line with some other recent studies, which also found that mucus production can be induced independently of IL-13 ([36, 37]). Nath and colleagues for example found the goblet cell hyperplasia only partly inhibited in IL-13 $-/-$ mice. They concluded that there are various redundancies and complementarities of T_H2 cytokines in the induction of airway remodeling. Although we found a significant reduction in T_H2 cytokine levels in OVA-sensitized ΔD -iD mice compared to wt mice, these levels were not completely nullified. In our experimental setting, the observed cytokine levels seem to suffice for the induction of goblet cell metaplasia and mucus production. However further studies are required to clarify the complex contribution of various cytokines to goblet cell metaplasia.

Like mucus production, the airway reactivity to methacholine and to inhaled OVA remained unaffected in ΔD -iD mice despite the reduced inflammatory response. This finding again is concordant with the anti-IgE studies in asthmatic patients. Unlike airway inflammation, the bronchial hyperresponsiveness to methacholine was not affected by a 16 week treatment with omalizumab [33]. These findings offer further support for the concept that airway inflammation and airway hyperreactivity can be dissociated in allergic immune responses [38, 39].

Several mechanisms might be responsible for the alleviated allergic sensitization in terms of reduced IgE levels in ΔD -iD mice. One could hypothesize that the epitope/paratope interaction is impaired in ΔD -iD mice. Binding of an antibody to its antigen is a reversible noncovalent interaction, involving a variety of forces. Hydrophilic/hydrophobic interactions between epitope and paratope are a major contributor to these forces [40]. In our murine model of allergic airway inflammation, OVA is used as the allergen. OVA has five immunodominant epitopes which have been shown to be critical for IgE binding *in vitro* [21, 22]. These five epitopes contain a large fraction of highly hydrophobic amino acids (25/46 or 54% of residues were classified as hydrophobic), and most of the critical residues belong to the hydrophobic group. Mine *et al.* found that substitution of a single amino acid could lead to the loss of IgE-binding to OVA. However, these experiments were not designed to study the physiological consequences of reduced OVA binding by IgE. In our study, we took the complementary approach in a model that allows for the correlation with the pathophysiological phenotype. As ΔD -iD mice preferentially generate B cell receptors enriched for positively charged amino acids in CDR-H3 [23], they might be handicapped in their interaction with the hydrophobic antigen OVA. Ippolito *et al.* showed that the enrichment for charged amino acids in ΔD -iD mice results in a distinct limitation of the diversity of the IgM repertoire [23]. To rule out the possibility that naïve B cells that produce highly charged CDR-H3 regions would be excluded from the class switch to IgE, we studied splenic IgE-transcripts in OVA-sensitized ΔD -iD mice. In summary, we found no evidence of selection against sequences that contain the charged iD gene segment. The hydrophobic properties of the CDR-H3 repertoire in ΔD iD mice are preserved in IgE

transcripts and the preferred use of the charged reading frame (RF-1) is maintained. Therefore, the diversity of the CDR-H3 repertoire in Δ D-iD mice is indeed also limited in IgE transcripts.

This limited repertoire of antigen binding sites in Δ D-iD mice might have severe impact on the clonal selection during the process of allergic sensitization, in which positive selection results in the proliferation of B cells expressing an antigen receptor with high affinity to the given antigen [41]. The reduction in circulating IgE could thus reflect reduced numbers of plasma cells due to a diminished number of mature B cells able to recognize OVA, or reduced numbers due to an altered pattern of positive selection and proliferation that might reflect difficulty in generating optimal CDR-H3-centric antigen-binding sites of the proper affinity. As due to the short half-life of IgE, serum levels roughly reflect the number of plasma cells committed to IgE synthesis [18], this would lead to reduced levels of circulating IgE. Arguing against a reduction in the number of precursor B cells are the observations that the level of IgG₁ and IgM were almost equal in Δ D-iD and *wt* animals. Moreover, we found a normal induction of IgG₁ in response to systemic immunization with OVA. These results would argue that the diminished IgE response reflects the effect of the altered CDR-H3-centric antigen-binding site repertoire on positive selection for IgE bearing cells, rather than a restriction in starting B cell numbers.

In support of this latter hypothesis, we found that the percentage of OVA-specific IgE in total IgE was also markedly reduced in Δ D-iD mice. As this shifted ratio between OVA-specific and non-OVA-specific IgE will be reflected by the IgE molecules bound to the surface of mast cells [18], this would lower the probability of crosslinking two adjacent specific IgE molecules. Therefore, the central effector function of the allergic early phase response, mast cell degranulation, would be predicted to be reduced in the Δ D-iD mice, leading to a reduction in the release of proinflammatory mediators. This hypothesis is supported by our observation of reduced cytokine levels and diminished eosinophilic influx into the airways in Δ D-iD mice. Moreover, as mediators derived from IgE-activated mast cells can direct IgE synthesis in a positive feedback loop [1], reduced mast cell activity might also contribute to the reduction of IgE levels. This would suggest an impaired allergen-mediated “pump priming” [1] of the allergic response in Δ D-iD mice.

To the best of our knowledge, this is the first study to show that the level of IgE production can be regulated by the composition of the antigen-binding sites of the antibody repertoire. We hypothesize that the lower IgE levels in Δ D-iD mice result from decreased affinity for OVA among IgE bearing cells that leads to a decrease in the number of IgE plasma cells, as well as from an impaired positive feedback loop after mast cell degranulation.

In conclusion, we were able to demonstrate that in our murine model of experimental asthma the levels of allergen-specific IgE depend on the predominant CDR-H3 repertoire, suggesting that the classic CDR-H3-centric antigen-binding site plays a crucial role in creating the immunological interface between the allergen and IgE. Moreover, the close correlation between specific IgE and airway inflammation further emphasizes a central role of IgE, not only in mediating but also in regulating the allergic immune response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Serum immunoglobulin levels

(A) Sensitization to OVA induced a significant rise in IgG₁ levels ($p < 0.001$), which did not differ between the genotypes. (B) *Wt* mice showed a strong induction of total IgE after sensitization, which was significantly attenuated in $\Delta D-iD$ mice ($p < 0.05$). (C) Allergen-specific IgE levels were significantly reduced in $\Delta D-iD$ mice ($p < 0.05$). (D) Composition of total IgE: (i) non OVA-specific (dark proportion), (ii) OVA-specific (light proportion). Mean + SEM. All mice were sensitized to and challenged with OVA.

Figure 2. Content of eosinophils in BAL fluids

In non-sensitized but OVA-challenged animals no eosinophils were detectable in bronchoalveolar lavage fluids. In OVA-sensitized and OVA-challenged *wt* animals, a strong recruitment of eosinophils into the airways was found. In contrast, in Δ D-iD mice, the number of eosinophils recruited into the airways was significantly reduced ($p < 0.01$). Mean + SEM.

Figure 3. Cytokine levels in BAL fluids

(A – C) In OVA-sensitized mice, IL-4, IL-5, and IL-13 levels were significantly elevated compared to non-sensitized controls. However, this rise was significantly attenuated in sensitized $\Delta D-iD$ mice compared to *wt* ($p < 0.05$ (IL-4/IL-13); $p < 0.01$ (IL-5)). (D) IFN- γ levels were elevated in $\Delta D-iD$ mice ($p < 0.01$ compared to *wt*). (E) Levels of IL-10 were also elevated in $\Delta D-iD$ mice. However, this difference did not reach statistical significance ($p = 0.015$). (F) IL-6 levels did not differ between the genotypes. All mice were challenged with aerosolic OVA. Mean + SEM. The dotted line indicates the detection limit. U/DL: under detection limit.

Figure 4. Quantitative histology of airway inflammation

(A) OVA-sensitized and challenged mice developed an intense goblet cell hyperplasia which was absent in PBS control animals. *Violet color* indicates PAS positive staining.

Magnification: $\times 1,700$. (B) Quantification revealed a significant increase in the percentage of goblet cells and (C) a strong rise in mucin volume. These changes were highly significant compared to non-sensitized and OVA-challenged control animals ($p < 0.001$), but did not differ between the two genotypes. Mean + SEM.

Figure 5. Lung function analysis – methacholine and OVA response

(A) Non-sensitized (and OVA-challenged) mice showed a dose-dependent decline in MEF₅₀ in response to inhaled methacholine. (B) In OVA-sensitized and -challenged animals, this response was markedly enhanced, indicating a state of airway hyperreactivity. (C) Compared to non-sensitized controls, PC₅₀ was significantly reduced in OVA-sensitized *wt* mice ($p < 0.05$) and $\Delta D-iD$ mice ($p < 0.01$). The extent of airway hyperreactivity to methacholine did not differ between the genotypes. (D) After local administration of OVA, non-sensitized (and OVA-challenged) mice displayed a short dip in MEF₅₀, but recovered within a few minutes and reached baseline values again. (E) OVA-sensitized and -challenged mice showed a marked and persistent decline of MEF₅₀ in response to the OVA. This allergen-induced bronchoconstriction did not differ between the two genotypes. Mean + SEM.

Table 1

Oligonucleotide primers for IgE PCR

V _H forward	5'-AGG TSM ARC TGC AGS AGT CWG G-3'
Ce1 reverse	5'-TTC GTT GAA TGA TGG AGG ATG TGT-3'
Ce2 reverse	5'-CCT TTA CAG GGC TTC AAG GGG TAG-3'

(M = A or C; R = A or G; S = G or C; W = A or T)