A Single D_H Gene Segment Is Sufficient for the Establishment of an Asthma Phenotype in a Murine Model of Allergic Airway Inflammation

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Key Words

Immunoglobulin E · Allergy · Allergic airway inflammation · Antigen-binding site · B cell response

Abstract

Background: We have previously shown that the allergic sensitization to ovalbumin does not represent a superantigen-like immune response. In gene-targeted mice (Δ D-iD) with a single modified *Diversity* gene segment (D_H) of the immunoglobulin heavy chain, enriched for charged amino acids, the asthma phenotype in a murine model was markedly alleviated compared to wild-type animals. **Objective:** We now sought to determine whether the confinement to a single D_H gene segment alone leads to a reduced allergic phenotype. **Methods:** We examined another gene-targeted mouse strain (Δ D-DFL) with a single D_H gene segment which encodes for neutral amino acids, thus reflecting the preferential repertoire in wild-type mice. Mice were sensitized intraperitoneally to ovalbumin. **Results:** Despite the constraint to a single D_H gene segment, Δ D-DFL mice mounted high total and allergen-specific IgG₁ and IgE serum levels after sensitization to ovalbumin. The affinity constants of aller-

gen-specific IgG₁ antibodies did not differ between Δ D-DFL and wild type. Following challenge with aerosolized allergen, a marked local T_H2 cytokine response and an eosinophilic airway inflammation developed. Quantitative histology revealed increased mucus production and intense goblet cell metaplasia which were identical to those in wild type. Moreover, Δ D-DFL mice developed an airway hyperreactivity to methacholine and to the specific allergen, which both did not differ from those in wild-type animals. **Conclusion:** A single D_H gene segment is sufficient for the establishment of the asthma phenotype in a murine model of allergic airway inflammation. Thus, the allergic phenotype depends on the amino acid composition and not on the diversity of the classical antigen-binding site. Copyright © 2011 S. Karger AG, Basel

Introduction

Bound to the surface of mast cells, immunoglobulin E (IgE) is the immunological interface between the allergen and the immune system [1]. Characterization of the molecular and structural factors that promote IgE responses

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to allergens is needed in order to gain a better understanding of the mechanisms that underlie the immune dysregulation of allergic diseases. This is of particular importance in the context of allergen-specific immunotherapy and of emerging anti-IgE therapies, which have 'redirected the spotlight' in allergy treatment to the B cell [2].

The antigen specificity of an antibody is typically determined by the classic antigen-binding site, which is created by the juxtaposition of three hypervariable complementarity determining regions (CDRs) from the heavy chain and three CDRs from the light chain [3]. The third CDR of the heavy chain (CDR-H3) has the greatest influence on the overall antibody diversity [4]. In contrast to the other five CDRs, which are largely or completely encoded by germline sequence, CDR-H3 includes random nontemplate (N) nucleotides [5] and palindromic (P) junction nucleotides [6] as well as portions of the *Variable* (V_H), *Diversity* (D_H) and *Joining* (J_H) gene segments [7]. Due to its position at the center of the classic antigen-binding site, CDR-H3 typically plays a defining role in the recognition of an antigen by IgM, IgG and IgA antibodies [4, 8].

Alternatively, superantigen-like reactions, which do not involve the classical antigen-binding site, can be seen in various immune responses. To test for this possibility in an IgE-dependent allergic reaction, we previously evaluated allergic sensitization in gene-targeted mice with a normal binding site for superantigens but a restricted repertoire of classical antigen-binding sites (ΔD -iD mice) [9]. In these mice with an altered CDR-H3 repertoire, the asthma phenotype was markedly alleviated compared to wild-type (WT) animals [10]. Following sensitization and aerosolic challenge with the hydrophobic allergen ovalbumin (OVA), ΔD -iD mice displayed significantly reduced allergen-specific IgE levels, eosinophilic airway inflammation and local T_H2 cytokine responses.

The *Diversity* locus of the heavy immunoglobulin chain (D_H) in ΔD -iD mice has undergone two major changes. First, the number of D_H gene segments was reduced from 13 to 1, thereby limiting combinatorial diversity. Second, the remaining D_H was altered to force use of positively charged amino acids in place of the normal preference for tyrosine and glycine [9]. Thus, although our findings demonstrated the critical importance of CDR-H3 diversity to the allergic response, these initial experiments could not differentiate between limitations in the number of available D_H gene segments or alteration of CDR-H3 amino acid patterns as the cause of the impairment in the allergic immune response.

To distinguish between these possibilities, we now report an evaluation of allergic sensitization and airway hyperreactivity in mice limited to one single, normal D_H gene segment, DFL16.1 (ΔD -DFL mice). DFL16.1 is overutilized in the WT repertoire, contributing to approximately one fifth of rearrangements. Thus, ΔD -DFL mice essentially express a subset of the normal WT repertoire, with neutral amino acids accounting for about 75% of the total CDR-H3 region [11].

In the present paper we report that ΔD -DFL mice are able to mount a normal OVA-induced IgE response and to develop a T_H2 -mediated eosinophilic airway inflammation and an airway hyperreactivity that is similar to WT animals. Thus, an immunoglobulin locus with a single normal D_H gene segment is sufficient for the establishment of the asthma phenotype in a murine model of experimental allergic asthma, whereas a locus with a single altered D_H gene segment is not. These findings document a critical role for germline-encoded recognition of allergens in the allergic response.

Animals and Methods

Animals

We used a previously described mouse strain on a Balb/c background with a modified D_H locus [12]. Briefly, the D_H locus of the ΔD -DFL mouse strain has been changed by Cre-loxP gene targeting to limit the locus to a single D gene segment (DFL16.1). In both, WT and ΔD -DFL mice, the DFL16.1 segment is preferentially rearranged into reading frame 1, which encodes the neutral amino acids tyrosine, glycine, and serine [13–15]. As a result, the CDR-H3 regions of WT mice are enriched for tyrosine and glycine and other neutral amino acids, which in toto account for about 75% of the CDR-H3 loop [11]. ΔD -DFL mice were maintained in a homozygous breeding colony and were genotyped as previously described [12]. Balb/c WT animals were purchased from Harlan Winkelmann (Borchen, Germany). Animals were held specific pathogen free in single ventilated cages, fed an OVA-free diet and supplied with water ad libitum. The animal experiments were performed with the approval of the governmental authority (Regierungspräsidium Giessen).

Protocol of Allergic Sensitization

Four groups of mice were studied: (1) nonsensitized WT mice (WT PBS), (2) sensitized WT mice (WT OVA), (3) nonsensitized ΔD -DFL mice (ΔD -DFL PBS), and (4) sensitized ΔD -DFL mice (ΔD -DFL OVA). Mice were sensitized to OVA as previously described [16]. Ten micrograms of OVA grade VI (Sigma, Deisenhofen, Germany) was adsorbed to 1.5 mg $Al(OH)_3$ (Imject® Alum; Pierce, Rockford, Ill., USA) and administered intraperitoneally on days 1, 14 and 21. To induce an allergic airway inflammation, animals received three aerosol challenges with 1% (w/v) OVA grade V (Sigma), diluted in PBS, for 20 min on days 26, 27 and 28. Nonsensitized control mice received PBS alone intraperitoneally

on days 1, 14 and 21 and were challenged with aerosolic OVA on days 26, 27 and 28.

IgE-Polymerase Chain Reaction and Sequence Analysis

Total RNA was isolated from splenic tissue using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. IgE heavy chain transcripts were amplified and cloned as described previously [10]. Sequences were aligned with the ImMunoGeneTics (IMGT) VQUEST program (<http://imgt.cines.fr>) [17]. 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 [18].

Determination of Antibody Levels

Serum concentrations of total IgG₁ and total IgE on days 0 and 30 were determined by ELISA, as previously described [19]. Antibodies and standards were purchased from BD (Heidelberg, Germany). For OVA-sensitized mice, serum samples were diluted 1:100,000 (IgG₁) or 1:500 (IgE). For nonsensitized controls, the dilutions were 1:10 (IgG₁) or 1:2 (IgE). OVA-specific IgE antibody levels were determined as previously described [10]. As standard, we used an OVA-specific murine IgE (Serotec, Oxford, UK). Serum samples were applied in a dilution of 1:20 in washing buffer. 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).

Inhibition ELISA

Antibody affinity constants were determined by ELISA, as previously described [20]. Briefly, pooled serum was diluted by 0.1% BSA solution in PBS to give a final concentration of 2 ng/ml. Diluted serum samples were incubated with different amounts of OVA (0, 0.025, 0.05 and 0.1 μM). Mixtures were diluted 2, 4, 8, 16 and 32 times, and resulting samples were incubated for 18–20 h at room temperature. Free antibodies were determined by ELISA in OVA-coated wells using biotin-conjugated anti-IgG₁ (BD) and streptavidin peroxidase (Calbiochem, Bad Soden, Germany). POD-BM blue (Roche) was used as chromogenic substrate. Absorption was measured at 450 nm against 690 nm with a photometric ELISA reader (Asys).

Passive Cutaneous Anaphylaxis

Mice were passively sensitized by intravenous injection with 30 μl pooled serum samples of OVA-sensitized WT mice or ΔD-DLF mice. After 24 h, they were injected again with 30 μl pooled serum. After an additional 3 h, mice were challenged by topical application of 20 μl PBS alone to the right ear and 20 μl OVA (100 μg/ml) in PBS to the left ear. Ear-swelling responses were assessed by measurement of ear thickness with a digital gauge (B110T; Kroplin, Schlüchtern, Germany).

Lung Function Analysis

To assess the lung function, we used head-out body plethysmography, as previously described [21]. This system allows for the simultaneous evaluation of the breathing pattern of nonanesthetized, 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), that is, the expiratory airflow (in milliliters per second) at 50% tidal volume. To assess airway responsiveness, we determined the dose-dependent response to inhaled methacholine (Sigma). As vehicle control, the diluent PBS was nebulized. For the statistical comparison of experimental groups, the provocation concentration 50 (PC₅₀) was calculated. This is the methacholine dose (in milligrams per milliliter) needed to cause a 50% decline in MEF₅₀. 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

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 Bronchoalveolar Lavage Fluids

The levels of interleukin-4 (IL-4), IL-5, IL-13, IL-6, and IL-10 in bronchoalveolar (BAL) fluids were measured by bead-based FlowCytomix assay (Bender MedSystems, Vienna, Austria), according to the manufacturer's protocol using a FACScalibur® flow cytometer (BD). 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, 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 as described previously [22]. Systematic uniform random samples of lung tissue were taken according to standard methods. Airway epithelial mucus was identified, using periodic acid Schiff (PAS) staining. The surface area of mucus-containing goblet cells (S_{gc}) per total surface area of airway epithelial basal membrane (S_{ep}) and the volume of PAS-stained epithelial mucus (V_{mucus}) per S_{ep} were determined using a computer-assisted stereology tool box (CAST-Grid 2.0®; Olympus, Albertslund, Denmark) according to the following formulas:

$$\frac{S_{gc}}{S_{ep}} = \frac{\sum I_{gc}}{\sum I_{ep}} \\ \frac{V_{mucus}}{S_{ep}} = LP \times \frac{\sum P_{mucus}}{2} \times \frac{\sum I_{ep}}{\sum I_{ep}}$$

where $\sum I_{gc}$ is the sum of intersections of test lines with goblet cells, $\sum I_{ep}$ is the sum of all intersections of test lines with epithelial basal membrane, $\sum P_{mucus}$ is the sum of all points hitting mucus and LP is the test line length at final magnification.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 (La Jolla, Calif., USA) and SPSS 17.0 (SPSS Inc., Chicago, Ill., USA). For the assessment of normality distribution, we used a Kolmogorov-Smirnov test. Differences between populations were assessed by a two-tailed Student t test for normally distributed data or a Mann-Whitney U test for nonnormally distributed data. A p value < 0.05 was accepted as significant. For de-

nomination in figures, the following symbols were used: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are presented as box plots with traditional Tukey whiskers, showing 1.5 times the inter-quartile distance. The data in figure 5 are presented as means $\pm$ SEM.

Results

Limited CDR-H3 Diversity Is Preserved in IgE Transcripts from Δ D-DFL Mice

The restricted primary immunoglobulin repertoire (IgM) of Δ D-DFL mice was previously described by Schelonka et al. [12]. To determine whether the limited diversity of the immunoglobulin repertoire is preserved in IgE, we analyzed IgE transcripts from Δ D-DFL mice. Using a semi-nested PCR, we gained 13 unique sequences of ϵ -heavy chain variable region transcripts from OVA-sensitized Δ D-DFL mice (GenBank accession No. GQ457520 through GQ457532).

The analysis of these sequences showed: (1) the DFL16.1 segment was preserved in all sequences (online suppl. table 1, www.karger.com/doi/10.1159/000323527) with reading frame 1 used by all but one sequence; (2) the most abundant amino acids in the CDR-H3 of IgE transcripts were tyrosine (23%), serine (16%) and glycine (13%), leading to an average hydrophobicity of -0.14 according to the normalized Kyte-Doolittle hydrophobicity scale [18].

Thus, the characteristics of the CDR-H3 repertoire in Δ D-DFL mice, as described by Schelonka et al. [12] for IgM [that is, most abundant amino acids in the CDR-H3: tyrosine (25%), serine (14%) and glycine (16%)], were preserved in IgE transcripts, thereby reflecting the preferential repertoire in WT mice.

Mice with a Single D_H Gene Segment Mount Normal IgG₁ and IgE Levels

To assess the allergic sensitization, serum levels of IgG₁ and IgE were determined. While nonsensitized controls showed total IgG₁ and IgE levels near the detection limits (fig. 1a, b), we found a considerable induction of both isotypes by OVA sensitization, which did not differ significantly between WT and Δ D-DFL mice ($p < 0.001$ compared to PBS controls for IgG₁ and IgE in both genotypes). This was reflected in OVA-specific IgG₁ and IgE levels (fig. 1c, d), which did not differ significantly between WT and Δ D-DFL mice. The sensitization-induced rise in OVA-specific IgG₁ and IgE levels was significant in both genotypes ($p < 0.01$ and $p < 0.001$, compared to PBS controls).

Table 1. Affinity constants of OVA-specific IgG₁ antibodies

	K_a , M ⁻¹	SEM, M ⁻¹	p
WT	2.99×10^7	0.32×10^7	0.724
DFL	3.57×10^7	0.61×10^7	
mAb [20]	1.10×10^8	0.13×10^7	

Affinity of Induced Antibodies Does Not Differ between the Two Genotypes

To rule out the possibility that, despite the statistically not differing serum immunoglobulin levels, the allergen-induced antibodies in Δ D-DFL mice might be of lower affinity than their WT counterparts, we determined the affinity constants (K_a) of OVA-specific IgG₁ antibodies by means of inhibition ELISA. In OVA-sensitized and OVA-challenged animals, K_a values did not differ significantly between the two genotypes ($p = 0.72$) (table 1). The K_a of allergen-specific immunoglobulins in our study lay in the range of one third of the K_a of previously published monoclonal OVA-specific IgG₁ antibodies [20].

Airway Inflammation Is Unaltered in Δ D-DFL Mice

To assess the local inflammatory response in the airways, we determined the cellular influx into BAL fluids. In nonsensitized but OVA-challenged mice, no eosinophils were detectable (fig. 2). OVA-sensitized and OVA-challenged animals of both genotypes developed a strong allergic airway inflammation, characterized by the marked immigration of eosinophils ($p < 0.001$). Of note, the magnitude of the eosinophilic influx in Δ D-DFL mice was identical to that in WT mice.

Local T_H2 Cytokine Response Is Maintained in Δ D-DFL Mice

In order to reveal whether Δ D-DFL mice develop a local T_H2 cytokine response, we determined the levels of cytokines in BAL fluids by cytometric bead assay (fig. 3). In nonsensitized (but OVA-challenged) controls of both genotypes, IL-4, IL-5 and IL-13 levels were below or near detection limits. Following OVA sensitization and OVA challenge, BAL fluid levels of IL-4, IL-5 and IL-13 rose significantly in both WT and Δ D-DFL mice ($p < 0.01$). The increase in IL-4 and IL-13 did not differ between Δ D-DFL and WT mice. For IL-5, however, the rise was slightly weaker in Δ D-DFL than in WT mice ($p < 0.05$). The level of IL-6, on the other hand, was increased in OVA-sensitized mice with a single D_H gene segment ($p <$

Fig. 1. Serum immunoglobulin levels. Sensitization to OVA induced a significant rise in IgG₁ (a) levels ($p < 0.001$) and IgE (b) levels ($p < 0.001$) in WT mice and in mice with a single D_H gene segment (Δ D-DFL). This was reflected in allergen-specific IgG₁

(c) and IgE (d) levels which did not differ significantly between WT and Δ D-DFL. Box plots with traditional Tukey whiskers, showing 1.5 times the interquartile distance. A nonparametric U test was used for determining p values.

0.01, compared to OVA-sensitized WT). IL-10 levels in BAL fluids from OVA-sensitized mice were elevated compared to nonsensitized controls ($p = 0.11$ for WT and $p < 0.05$ for Δ D-DFL). We found no difference between the two genotypes.

Quantitative Histology

To correlate the inflammatory response in BAL fluids with inflammation-induced structural changes in the airways, we performed quantitative histology. In contrast to nonsensitized control animals, OVA-sensitized and

OVA-challenged mice developed a strong goblet cell metaplasia (fig. 4a). Stereologic quantification revealed a significant increase in the percentage of airway epithelial basal surface covered by goblet cells ($p < 0.001$, compared to nonsensitized controls; fig. 4b). In addition, an intense rise in mucus volume per unit epithelial basal surface was observed ($p < 0.001$, compared to nonsensitized controls) (fig. 4c). In OVA-sensitized Δ D-DFL mice, these structural changes were highly significant compared to nonsensitized controls ($p < 0.001$) and were identical to OVA-sensitized WT animals.

Fig. 2. Content of eosinophils in BAL fluids. In nonsensitized but OVA-challenged animals, no eosinophils were detectable in BAL fluids. In OVA-sensitized and OVA-challenged WT animals, a strong recruitment of eosinophils into the airways was found ($p < 0.001$). This eosinophilic influx in response to OVA challenge was identically inducible in Δ D-DFL mice ($p < 0.001$). Box plots with traditional Tukey whiskers, showing 1.5 times the interquartile distance. A nonparametric U test was used for determining p values.

Lung Function Analysis

Airway Hyperreactivity to Methacholine Is Inducible in Δ D-DFL Mice

For the determination of airway reactivity, we analyzed the response to inhaled methacholine. Nonsensitized mice showed a dose-dependent decline in MEF_{50} in response to inhaled methacholine (fig. 5a). This response was enhanced in OVA-sensitized animals (fig. 5b), indicating a state of airway hyperreactivity. To compare the groups statistically, we calculated the PC_{50} , that is, the methacholine concentration (in milligrams per milliliter) needed to cause a 50% decrease in MEF_{50} (fig. 5c). Compared to nonsensitized controls, the PC_{50} was significantly reduced in OVA-sensitized mice ($p < 0.05$). In Δ D-DFL mice, similar airway hyperreactivity as in WT mice could be induced.

Δ D-DFL Mice Display Allergen-Induced Bronchoconstriction

To further assess airway reactivity, we determined the lung function in response to the specific allergen. After local administration of OVA, sensitized mice showed a marked and persistent decline in MEF_{50} , which was significantly enhanced compared to nonsensitized controls ($p < 0.05$), indicating airway hyperreactivity (fig. 6). The allergen-induced bronchoconstriction in gene-targeted

mice with a single D_H gene segment (Δ D-DFL) was equivalent to that in WT animals.

Passive Cutaneous Anaphylaxis

We passively sensitized WT mice by intravenous injection of serum of OVA-sensitized WT or Δ D-DFL mice and then challenged them by topical application of PBS alone to the right ear and OVA in PBS to the left ear. Ear thickness increased from 0.110 ± 0.002 to 0.145 ± 0.004 mm ($p < 0.001$) in WT serum-treated mice, and from 0.105 ± 0.003 to 0.150 ± 0.005 mm ($p < 0.001$) in Δ D-DFL serum-treated mice (fig. 7). The ear swelling did not differ significantly between mice that were injected with serum of OVA-sensitized WT or Δ D-DFL mice.

Discussion

The primary question of this study was whether a single normal D_H gene segment is sufficient for the establishment of an asthma phenotype in a murine model of experimental asthma. We characterized three fundamental aspects of the phenotype in allergic asthma: (1) allergic sensitization, (2) allergic airway inflammation and (3) airway hyperreactivity. In summary, we found that despite the absence of 12 of the 13 D_H gene segments, each of which normally creates its own constituent repertoire, Δ D-DFL mice are able to mount an IgE response of normal intensity, to develop T_H2 cytokine-mediated eosinophilic airway inflammation and to display airway hyperreactivity, which in quality and magnitude are similar to those in WT Balb/c mice. Despite minor differences in the cytokine expression between sensitized WT and Δ D-DFL mice, these results indicate that the entire antibody variety is not necessary for the induction of an allergic phenotype, but that 20% of the global antibody repertoire by contribution [13] or 8% by genotypic complement suffice.

Fig. 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 nonsensitized controls ($p < 0.01$ and $p < 0.001$, respectively). Although the IL-5 levels were slightly reduced in Δ D-DFL mice, the induction of a local T_H2 cytokine response was maintained in transgenic with a single D_H gene segment. **d** IL-10 levels were elevated in OVA-sensitized animals ($p = 0.11$ in WT and $p < 0.01$ in Δ D-DFL). The two genotypes did not differ. **e** For IL-6, we found an elevation in Δ D-DFL ($p < 0.01$, compared to WT). Box plots with traditional Tukey whiskers, showing 1.5 times the interquartile distance. Dotted lines indicate the respective detection limit. A nonparametric U test was used for determining p values.

Fig. 4. Quantitative histology of airway inflammation. **a** OVA-sensitized and OVA-challenged mice developed an intense goblet cell metaplasia which was absent in PBS control animals. Violet color indicates PAS-positive staining. $\times 1,700$. Quantification revealed a significant increase in the percentage of epithelial base-membrane surface covered by goblet cells (**b**) and a strong

rise in mucus volume per surface unit (**c**). These changes were highly significant compared to nonsensitized and OVA-challenged control animals ($p < 0.001$), and were also present in Δ D-DFL mice. Box plots with traditional Tukey whiskers, showing 1.5 times the interquartile distance. A nonparametric U test was used for determining p values.

Fig. 5. Lung function analysis – methacholine response. **a** Nonsensitized (but OVA-challenged) mice showed a dose-dependent decline in MEF₅₀ in response to inhaled methacholine. **b** In OVA-sensitized and OVA-challenged animals, this response was markedly enhanced, indicating a state of airway hyperreactivity. **c** Compared to nonsensitized controls, PC₅₀ was significantly reduced in OVA-sensitized WT mice ($p < 0.05$). This airway hyperreactivity was equally inducible in mice with a single D_H gene segment ($p < 0.05$). Mean $\pm$ SEM. A parametric t test was used for determining p values.

Our findings are in line with Schelonka et al. [12], who showed that the D-limited DFL16.1 repertoire is sufficiently plastic to allow normal numbers of mature B cells and to achieve normal levels of serum IgM, IgG and IgA in untreated mice. However, the authors described some differences in the mounting of humoral immune responses. While the IgG response to the T-dependent antigen (NP19-CGG) [23] in ΔD-DFL was identical to the response in WT littermates [11], the primary immune response (IgM) to the T-independent bacterial polysaccharide antigen $\alpha(1 \rightarrow 3)$ -dextran [24, 25] was significantly reduced [12]. The authors hypothesized that this impaired response might be due to a depletion of short CDR-H3 sequences, which in WT mice are preferentially derived from 1 or more of the 12 deleted D_H gene segments.

To the best of our knowledge the present work with ΔD-DFL mice and our previous work with ΔD-iD mice represent the first studies testing the role of the composition of the antibody repertoire on the allergic immune response. In previous work, we could demonstrate that allergic sensitization to OVA does not represent a superantigen-like immune response and that the classic antigen-binding site plays a crucial role in creating the immunological interface between allergen and IgE [10]. Using a murine model of experimental asthma, we found that allergic sensitization and airway inflammation depend on the composition of the predominant CDR-H3 repertoire. In gene-targeted mice with a restricted and predominantly charged CDR-H3 repertoire (ΔD-iD mice) [9], the asthma phenotype was markedly alleviated compared to WT mice [10]. Following sensitization to the relatively hydrophobic allergen OVA, ΔD-iD mice displayed a significantly reduced induction of allergen-specific IgE levels. Moreover, after aerosolic challenge with OVA, eosinophilic airway inflammation and local T_H2 cytokine response were reduced in ΔD-iD mice. The airway hyperresponsiveness, however, was unaffected in ΔD-iD, supporting the concept that airway inflammation and airway hyperreactivity can be dissociated in allergic immune responses.

This finding is widely in accordance with the observations of Hamelmann et al. [26, 27], who found that IgE plays an important role in the development of airway inflammation under conditions in which limited IL-5 is induced, but showed that IgE is not required for the development of airway hyperresponsiveness in mice systemically sensitized and challenged via the airways. The aspect of allergic sensitization in terms of allergen-specific antibody responses has understandably not been addressed by the studies with B cell-deficient mice.

Fig. 6. Lung function analysis – OVA response. **a** After local allergen challenge, OVA-sensitized WT animals displayed a marked and persistent decline in MEF_{50} , which was significantly enhanced compared to nonsensitized controls ($p < 0.05$). **b** An equivalent allergen-specific airway hyperreactivity was inducible in ΔD -DFL mice, which also showed a significantly enhanced reactivity in OVA-sensitized animals compared to nonsensitized controls ($p < 0.05$). A nonparametric U test was used for determining p values.

Fig. 7. Passive cutaneous anaphylaxis. Ear edema in response to cutaneous OVA challenge emerged in naive WT mice passively sensitized with serum of OVA-sensitized WT mice. This increase in ear thickness was identical in naive WT mice passively sensitized with serum of OVA-sensitized ΔD -DFL mice. Box plots with traditional Tukey whiskers, showing 1.5 times the interquartile distance. A non-parametric U test was used for determining p values.

However, our previous and the present study add novel insights into this not unimportant aspect of the allergic phenotype. In our previous study we were unable to determine whether the reduced induction of allergen-specific IgE reflects (1) the inclusion of charged amino acids in the CDR-H3 or (2) the loss of CDR-H3 diversity, represented by the deletion of 12 of the 13 normal D_H gene segments. To rule out the possibility that the confinement to a single D_H gene segment alone led to the observed differences, we have now analyzed the allergic phenotype in mice that are also restricted to a single D_H gene, but one enriched for the neutral amino acids that commonly comprise the majority of the CDR-H3 repertoire [12] instead of the positive charged amino acids that predominate the CDR-H3 repertoire in Δ D-iD mice [9].

In contrast to Δ D-iD, the Δ D-DFL mice did not differ from WT animals in their ability to perform an allergic immune response. Neither serum immunoglobulin levels nor allergic airway inflammation, passive cutaneous anaphylaxis or airway reactivity in the lung function differed between Δ D-DFL and WT Balb/c mice. These findings are further sustained by our observation that the affinity constants (K_a) of the allergen-induced IgG₁ antibodies did not differ between Δ D-DFL and WT mice. Interestingly, the K_a of allergen-specific immunoglobulins in our study lay in the same order of magnitude as the K_a of previously published monoclonal OVA-specific IgG₁ antibodies [20].

We thus conclude that the changes in allergic immune response in Δ D-iD mice are not due to the limitation imposed by a single D_H gene locus but to the change in CDR-H3 content. These findings provide support for our previous hypothesis that the epitope/paratope interaction between the allergen and the B cell receptor, which are primarily conveyed by noncovalent hy-

drophobic-hydrophilic interactions [28], are impaired in Δ D-iD mice [10]. In our murine model of allergic airway inflammation, OVA is used as the allergen. OVA has 5 immunodominant epitopes, which have been shown to be critical for IgE binding in vitro [29, 30]. These 5 epitopes contain a large fraction of neutral or even highly hydrophobic amino acids (36/46 or 78%). Unlike in Δ D-DFL mice and WT Balb/c animals, the B cell receptors in Δ D-iD mice are hence a priori handicapped in the hydrophobic-hydrophilic interaction with OVA, which consecutively leads to the reduced allergic immune response with all the subsequent effects as outlined elsewhere [10].

In consideration of our previous findings that the phenotype in a model of experimental asthma was markedly alleviated in gene-targeted mice with a restricted and predominantly charged CDR-H3 repertoire [10], we conclude that classical antigen-antibody interactions are crucial for the development of the allergic immune response in this model of experimental asthma. To the best of our knowledge, this is the first study to positively prove that a small subset of the naturally occurring antibody repertoire is sufficient for the induction of allergic sensitization and for the establishment of asthma.

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