

Induction of IgE and allergic-type responses in fur mite-infested mice

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High serum IgE levels are characteristic of allergic diseases and immune responses to nematode parasites. A murine allergy model based on infestation with the fur mites *Myocoptes musculinus* and *Myobia musculi* was investigated. Analysis of mite infestation in various knockout mice revealed that IgE production in response to these ectoparasites was dependent on T cells, IL-4, and CD40L. Secretion of IL-4 by CD4⁺ T cells obtained from peripheral LN draining mite-infested skin sites was increased with progressing mite infestation and correlated with the serum IgE induction. A time course analysis of the mRNA expression of switched IgE, activation-induced cytidine deaminase (AID), and ϵ germ-line transcripts (ϵ GLT) suggested that switching to IgE in response to fur mites occurred initially in skin-draining LN. In addition, mite infestation induced mast cell degranulation in the skin as well as mast cell infiltration into skin-draining LN. Analysis of the immune response generated in mite-infested mice is a valuable model for the investigation of allergic disorders and provides information for better understanding of mechanisms involved in IgE induction and regulation in a physiological way of allergen-exposure resembling atopic sensitization in humans.

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Introduction

IgE is the main antibody isotype involved in atopic allergic disease and is characteristically elevated in response to infection with nematode parasites. The direct effects of IgE during an immediate-type hypersensitivity reaction are well characterized [1, 2]. However, many of the specific factors involved in the induction and regulation of IgE are yet to be determined and are of crucial importance for elucidating the etiology of allergic diseases.

Nematode infection models have been widely used to study IgE-related immune responses [3]. However, in contrast to allergic diseases, which are chronic, some frequently used parasites, such as *Nippostrongylus brasiliensis* in particular, induce Th2-type responses leading to protection. Other frequently employed methods to study allergic disorders have been based on immunization with purified protein antigens in adjuvants, which induce some of the symptoms of allergy or asthma [4]. While this can provide information on the effects of IgE after induction, it is important to investigate additional *in vivo* models, which could yield physiologically relevant information on the factors regulating the initiation of allergic responses.

Murine fur mite infestation was previously suggested to closely mimic the development of allergic type responses [5–7]. Most common mouse fur mite species are *Myocoptes musculinus* and *Myobia musculi* [8]. These mites are natural parasites of mice and live their entire life cycle on the same host [9]. Interestingly, despite the fact that these mites are classified as ectoparasites and do not penetrate the deeper layers of the skin, infested

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Abbreviations: AID: activation-induced cytidine deaminase ·

CSR: class-switch recombination · FRW: framework ·

GLT: germ-line transcript · MAUD: mite-associated ulcerative dermatitis · NC: Nishiki Cinnamon

mice develop greatly increased total serum IgE levels [6, 10]. Following prolonged periods of infestation, mice develop skin lesions known as mite-associated ulcerative dermatitis (MAUD), the severity of which is influenced by the genetic background [11, 12].

In the present study we characterized the initial requirements of IgE induction in mite-infested mice. We show that fur mite infestation represents a good animal model for investigation of allergic-type immune responses involving activation of Th2 cells and secretion of IL-4. T cell activation and B cell isotype switching to IgE appears to occur within skin-draining LN. Furthermore, mast cell degranulation was greatly increased in the skin, and mast cell infiltration in skin-draining LN was observed. These data suggest that mite antigens enter through the skin of infested animals, leading to induction of a Th2-type response and B cell switch to IgE in skin-draining LN. Murine mite infestation represents therefore an excellent physiological animal model in which to study induction of allergic responses.

Results

IgE induction by mite infestation

To investigate the antibody response induced by mite infestation, total serum IgE levels were analyzed in C57BL/6 and BALB/c mice infested with two common species of murine fur mites, *Myobia musculi* and *Myocoptes musculinus*. Up-regulation of total serum IgE was first observed 15 days after mite infestation (Fig. 1) followed by a gradual increase for about 10 weeks. Around 90 days after infestation IgE levels had increased significantly from 4 ± 1.8 ($n=15$) to 1908 ± 593 ng/mL ($n=11$; $p=0.0002$) in C57BL/6 mice and 59 ± 17 ($n=8$) to 9008 ± 7057 ng/mL ($n=7$; $p=0.0032$) in BALB/c mice. These high serum IgE levels remained stable up to 2 years after first contact with the ectoparasite (data not shown). Increased IgE levels were due to mite infestation, as naïve C57BL/6 mice housed in the same animal facility do not show increased IgE levels over the same amount of time [13]. In addition, total serum IgG₁ (Fig. 1) and IgG_{2a} (Fig. 1) levels were also determined. The kinetics for IgG₁ induction showed a similar course to that observed for IgE; however, the increase [from 548 ± 131 ($n=11$) to 2552 ± 515 µg/mL ($n=11$; $p=0.012$) in C57BL/6 and 435 ± 95 ($n=8$) to 9201 ± 2439 µg/mL ($n=7$; $p=0.002$) in BALB/c] was less prominent. As with IgE, serum IgG₁ levels did not increase in naïve C57BL/6 mice housed in the same facility over the same length of time (from 422 ± 182 to 419 ± 183 µg/mL ($n=3$; $p>0.5$)). Increases in IgE and IgG₁ likely reflect the induction of a Th2 response with secretion of IL-4.

Serum IgG_{2a} levels also increased over time: from 40 ± 9 ($n=11$) to 401 ± 75 µg/mL ($n=9$; $p=0.0001$) in C57BL/6 and 51 ± 11 ($n=8$) to 1646 ± 452 µg/mL ($n=7$; $p=0.0022$) in BALB/c. However, this increase was not mite induced as serum IgG_{2a} levels also increased the same magnitude in naïve C57BL/6 mice, from 47 ± 18 to 491 ± 445 µg/mL ($n=3$; $p=0.37$), over the same length of time. Thus, fur mite infestation parallels an allergic situation where high serum IgE levels are chronically induced in response to the constant presence of naturally occurring allergens.

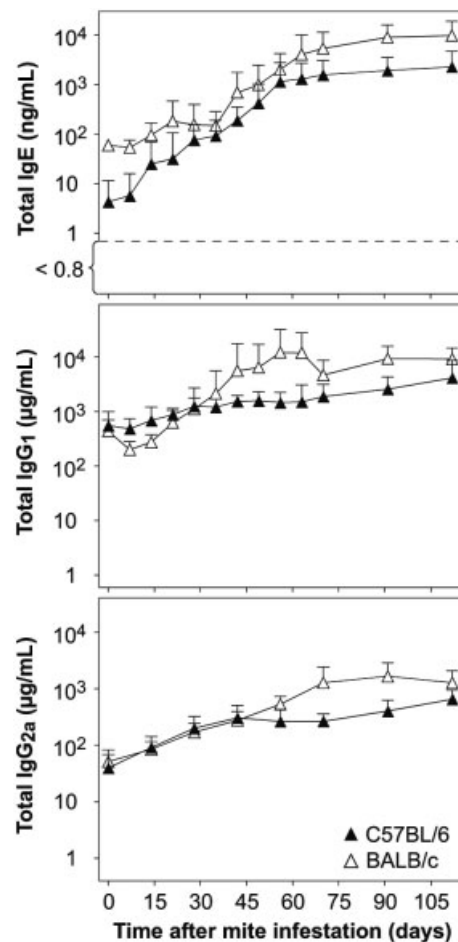


Fig. 1. Mite infestation induces IgE and IgG₁. C57BL/6 mice (closed symbols) or BALB/c mice (open symbols) were infested with fur mites at day 0. Blood was collected at weekly intervals and total serum IgE (upper panel), IgG₁ (middle panel) and IgG_{2a} (lower panel) were determined by ELISA. The data points represent the mean $\pm$ SD of 11 C57BL/6 and 8 BALB/c mice. Results are representative of five separate experiments. The dotted line in the upper graph indicates the lower limit sensitivity (0.8 ng/mL) of the IgE ELISA.

Requirement for T cells, CD40L, and IL-4 in the induction of IgE in response to mite infestation

To investigate which factors are required for the immune response induced against mites, several different knock-out mice were infested. At various time points thereafter, blood was collected and total serum IgE, IgG₁ and IgG_{2a} concentrations were analyzed. A requirement for T cells for mite-induced IgE was confirmed by investigating T cell-deficient TCR β ^{-/-} mice, as IgE levels remained below the detection limit throughout the time of mite infestation (Fig. 2, upper panel). To investigate whether T cell help was required to provide cytokines or for direct cognate interaction with B cells, mice deficient in CD40L, IL-4 or IL-5 were infested with mites and serum IgE levels monitored (Fig. 2, upper panel). As expected, no increase in serum IgE levels was observed in either IL-4^{-/-} or CD40L-deficient mice. However, the absence of IL-5 did not alter IgE production in response to mites. IgG₁ measurements led to comparable observations (Fig. 2, middle panel). In contrast, while baseline levels of IgG_{2a} were lower in TCR β ^{-/-} and

CD40L^{-/-} mice, following mite infestation they increased with the same kinetics as observed in WT mice (Fig. 2, lower panel).

These data suggest that mite infestation induces B cells to switch to IgE or IgG₁ under the influence of signals through CD40 and the Th2-type cytokine IL-4 as observed for IgE induction during allergic reactions. Therefore, mite infestation may serve as a suitable model for investigation of allergy.

Mite infestation induces IL-4 secretion in skin-draining LN

To characterize the immune response mounted against fur mites, we investigated the site of T cell activation, which could provide insight into the site of antigen entry. For this purpose, we determined the cytokine profile of T cells in spleen, lung, and LN draining the skin (brachial and cervical LN), the gut (mesenteric LN), and the lung (mediastinal LN). Cells were isolated at several time points after mite infestation and restimulated *in vitro* with PMA and ionomycin. Percentages of IL-4⁺,

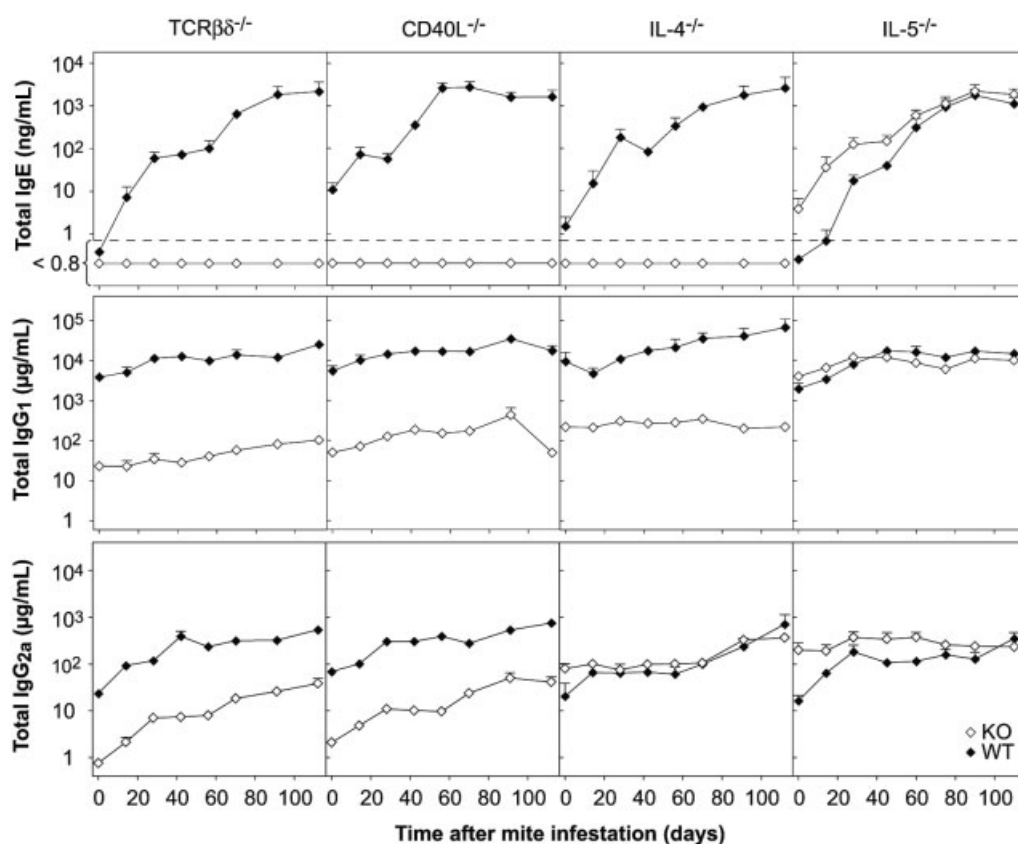


Fig. 2. Absence of IgE or IgG₁ induction in response to mite infestation in mice deficient in T cells, CD40L or IL-4. Knockout (open symbols) and WT control mice (closed symbols) were infested with mites and housed together in the same cage. Total serum IgE (upper panel), IgG₁ (middle panel) and IgG_{2a} (lower panel) of TCR β ^{-/-}, CD40L^{-/-}, IL-4^{-/-}, and IL-5^{-/-} mice were determined by ELISA at different time points after infestation. The data points represent the mean $\pm$ SD of four to five mice per group. Results are representative of two separate experiments. The dotted line in the upper panel indicates the lower limit sensitivity (0.8 ng/mL) of the IgE ELISA.

IL-5-, and IFN- γ -producing cells were determined by intracellular staining. Representative dot plots of IL-4⁺CD4⁺ T cells are shown in Fig. 3A. A clear increase in IL-4 secretion in response to mite infestation was observed in CD4⁺ T cells from skin-draining LN, mediastinal LN, and lung, while the spleen and mesenteric LN showed a weaker response (Fig. 3B and data not shown). Importantly, whereas significant increase in IL-4 production in mediastinal LN and lung occurred only late after mite infestation (>150 days), the increases in IL-4 levels in skin-draining LN was observed already 30 days after infestation, corresponding to the time of IgE up-regulation. These data suggest that the IL-4 responsible for B cell switch to IgE in mite-infested mice was produced in skin-draining LN. In contrast, CD4⁺ T cell secretion of IL-5 was slightly increased above background levels only at very late time points (>150 days, data not shown). This correlated well with the levels of peripheral blood eosinophilia observed in infested mice. In our animal facility the percentage of eosinophils in peripheral blood of naïve C57BL/6 mice averages $0.6 \pm 0.8\%$ ($n=38$) and was increased to $2.5 \pm 1.3\%$ ($n=38$) on day 60 after

infestation. Although such levels of eosinophilia are low compared to that observed after helminth parasite infection [14], the observed increase was significant ($p<0.0001$). After this time eosinophil levels remained stable or even declined. T cell secretion of IFN- γ remained stable at background levels at least until 60 days post infestation and then became elevated at late time points (Fig. 3B, lower panel).

To ensure that the increase in IL-4 and IL-5 was due specifically to mite infestation and did not merely reflect an age-related increase, the numbers of IL-4- and IL-5-secreting cells in skin draining LN and spleen of day 400 mite-infested and age-matched naïve C57BL/6 mice (housed in the same animal facility) were determined by ELISPOT. While mite-infested mice had high levels of both IL-4- (LN, 176 ± 26.7 ; spleen, 93.3 ± 20.7) and IL-5-secreting cells (LN, 149 ± 25.6 ; spleen, 89 ± 24.7), age-matched naïve mice had very low levels of both IL-4- (LN, 2.0 ± 2.2 ; spleen, 18.5 ± 5.9) and IL-5-secreting (LN, 3.7 ± 2.9 ; spleen, 6.3 ± 3.4) cells.

Taken together, these results suggest that mite antigens enter initially *via* the skin and not through other possible routes such as the lung or the gut.

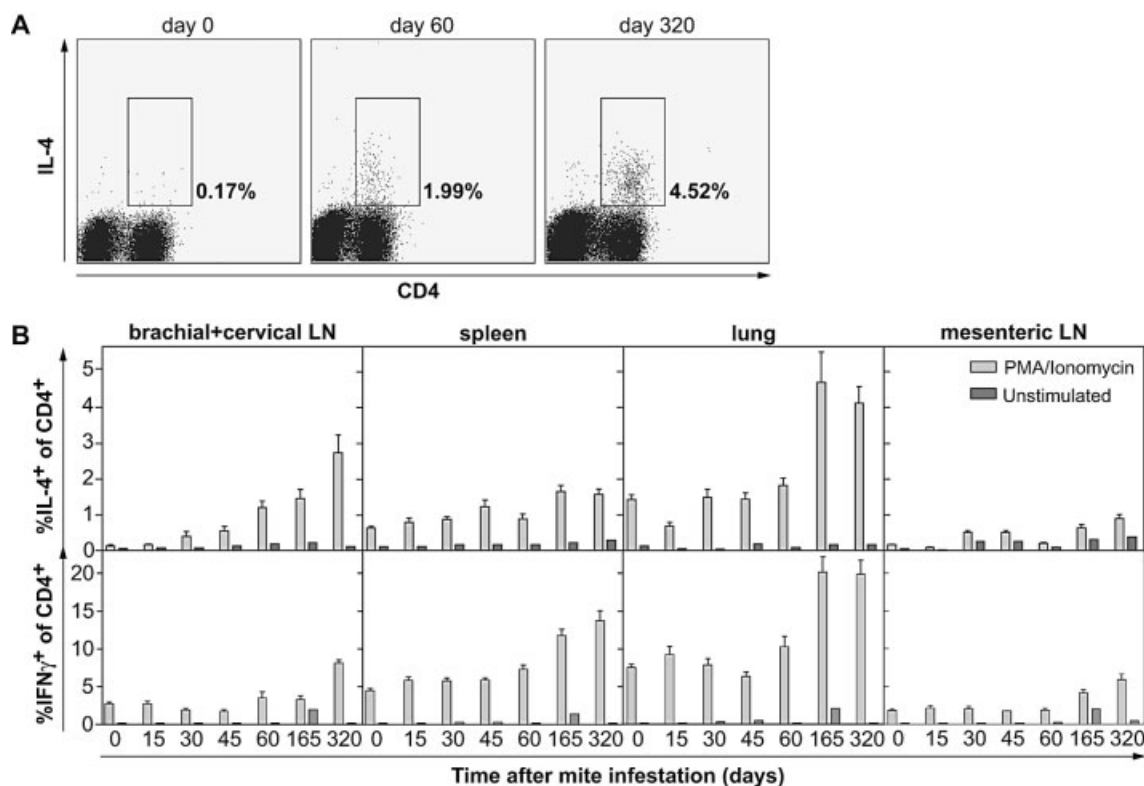


Fig. 3. CD4⁺ T cells in skin-draining LN produce IL-4 in response to mite infestation. (A) Representative dot plots of brachial and cervical LN isolated on days 0, 60, and 320 after mite infestation. The percentage of PMA/ionomycin-restimulated IL-4⁺CD4⁺ cells is indicated. (B) Cells isolated from LN, spleen, and lung on indicated time points after mite infestation and stimulated with PMA/ionomycin (light bars) or with medium alone as controls (dark bars). Gates were set on live CD4⁺ cells and IL-4 (upper panel) and IFN- γ (lower panel) expression was analyzed. Results represent the mean $\pm$ SD of five mice per group and are representative of two separate experiments.

Moreover, skin-draining LN were increased in size following mite infestation, while the size of other LN remained stable (data not shown).

Switching to IgE preferentially induced in skin-draining LN

To determine the sites at which B cell switching to IgE was occurring, B cells were purified from the spleen and the LN draining the skin, lung, and gut of naïve and mite-infested mice. RT-PCR was then performed to detect ϵ germ-line transcripts (GLT), μ GLT, mature switched IgE, and activation-induced cytidine deaminase (AID). The presence of sterile ϵ GLT, which lack the V(D)J region and result from the activation of the I ϵ promoter, provides evidence for the initial steps leading to IgE class-switch recombination (CSR). AID has been shown to play a crucial role in CSR [15], and its expression indicates initiation of CSR. Mature IgE transcripts, resulting from DNA excision and ligation of the V(D)J sequences to the constant ϵ locus, indicate the presence of IgE-switched B cells. μ GLT expression, which characterizes functional B cells, was used as an internal control. Our results show an up-regulation of ϵ GLT and mature IgE transcripts induced in B cells isolated from skin-draining and mesenteric LN, while in B cells from spleen the expression of all transcripts remained comparable over the time of infestation (Fig. 4). However, background levels of ϵ GLT were slightly increased in mesenteric LN. AID expression was also up-regulated in skin-draining and mesenteric LN but to a lesser extent. Importantly, the levels of μ GLT remained

constant, indicating that B cell isolation and cDNA synthesis were comparable in all samples (Fig. 4).

Thus, these data suggest that B cell switching to IgE in response to mite infestation occurs in skin-draining LN, corresponding to the site of initial IL-4 production, although switching likely also occurs at more distal sites.

Mite-induced IgE production is partially antigen driven

Parasite infections are known to induce an increase in total IgE, although the levels of parasite-specific IgE are usually low [16–18]. This implies that parasites may induce a polyclonal nonspecific response. To investigate the diversity of the IgE repertoire induced by mite infestation, we determined the usage of the V, D, and J segments of IgE heavy chains present in B cells isolated from bone marrow, spleen, and skin-draining LN of mite-infested mice. Forty-four IgE sequences containing an open reading frame and originating from 29 distinct clones (from 17 different mice) were aligned to the closest germ-line sequence using the DNAPlot database. The V_H, D_H, and J_H gene usage as well as the number of mutations within the framework (FRW) and the CDR of these sequences are shown in Table 1.

The V_H gene usage in the analyzed sequences revealed a possible overrepresentation of V_H5 and, to a lesser extent, the V_H1 gene family members. The genome of C57BL/6 mice is known to encode 92 functional V_H gene segments belonging to 15 families [19]. As the V_H5 family has 8 members, if there was no specific selection, one would expect 9% of the 29 obtained sequences to belong to the V_H5 family. In contrast, 21% of the analyzed sequences were found to be of the V_H5 family. The V_H1 family also appeared to be overrepresented with 59% of the analyzed sequences belonging to V_H1 compared to an expected rate of 45%. While we cannot rule out a potential primer binding bias for certain V_H family members, previous sequence analysis of IgE found in naïve C57BL/6 mice did not reveal a bias towards the V_H5 family, although a similar overrepresentation of V_H1 was observed [13]. Interestingly, V_H5 predominance has been reported in patients with atopic dermatitis [20], asthma [21], and most recently in allergic rhinitis [22], while V_H1 overabundance has been observed in subjects with peanut allergy [23]. Biased usage of a V_H family has been proposed to derive from B cell superantigens binding to FRW regions [21, 22].

All analyzed nucleotide sequences carried somatic mutations with comparable distributions within both the FRW and CDR in the range of about 5% (Table 1). Seven sequences (25%) displayed a ratio of replacement to silent mutations in the CDR substantially higher than would be expected by chance, which might indicate

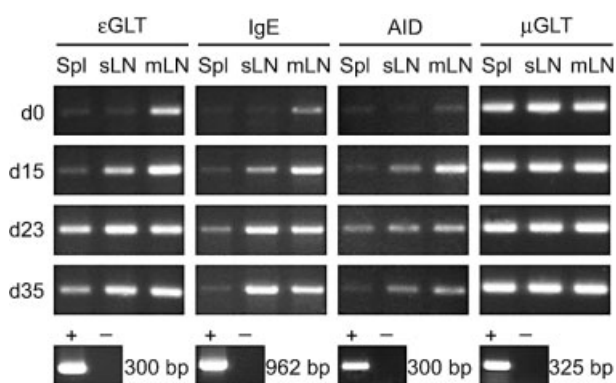


Fig. 4. Messenger RNA expression of ϵ GLT and mature switched IgE transcripts is up-regulated in skin-draining LN upon mite infestation. B cells were isolated from spleen (Spl), brachial and cervical LN (sLN), and mesenteric LN (mLN) of naïve or fur mite-infested C57BL/6 mice at 15, 23, and 35 days after infestation. Positive controls (+) derive from RNA isolated from B cells stimulated *in vitro* with IL-4 and anti-CD40 for 40 h. Negative controls (–) are H₂O. Results are representative of two independent experiments.

Table 1. V(D)J C ϵ rearrangements in mite-infested C57BL/6 mice

No.	V _H family ^{a)}	D _H family ^{a)}	J _H family ^{a)}	% Mutations ^{b)}		R/S ^{c)}	
				FRW	CDR	FRW	CDR
1 ^{d)}	V _H 1	DQ52	J _H 2	3.6	2.5	1.7	na
2 ^{d)}	V _H 1	DQ52	J _H 3	5.5	7.5	1.4	3.0
3 ^{d)}	V _H 1	DFL16.1	J _H 1	0.9	0.8	1.0	na
4 ^{d)}	V _H 5	DFL16.1	J _H 4	2.3	1.7	4.0	1.0
5 ^{e)}	V _H 5	DFL16.1	J _H 4	2.7	7.5	1.5	2.0
6 ^{e)}	V _H 5	DSP2.9	J _H 1	3.2	2.5	2.5	0.5
7 ^{f)}	V _H 1	DFL16.1	J _H 4	5.5	5.8	1.4	2.5
8 ^{g)}	V _H 1	DFL16.1	J _H 2	5.9	5.8	3.3	1.3
9	V _H 3	DFL16.1	J _H 3	5.9	5.8	1.2	6.0
10	V _H 1	DFL16.1	J _H 2	6.8	7.5	1.5	2.0
11	V _H 14	DQ52	J _H 2	11.4	7.5	1.5	2.0
12	V _H 1	DSP2.X	J _H 4	6.4	3.3	1.8	3.0
13	V _H 3	DFL16.1	J _H 1	7.7	6.7	1.1	7.0
14	V _H 10	DFL16.1	J _H 1	1.8	0.0	3.0	na
15	V _H 1	DFL16.1	J _H 2	5.5	1.7	1.4	1.0
16	V _H 1	DFL16.2	J _H 3	8.2	2.5	0.8	2.0
17	V _H 1	DSP2.X	J _H 4	5.9	11.7	2.2	3.7
18	V _H 1	DQ52	J _H 3	5.9	1.0	5.0	1.5
19	V _H 1	DFL16.1	J _H 4	0.9	0.8	na	na
20	V _H 1	DFL16.1	J _H 2	5.9	5.8	3.3	6.0
21	V _H 1	DFL16.X	J _H 3	4.5	3.3	1.5	3.0
22	V _H 5	DSP2.9	J _H 2	0.0	1.7	na	na
23	V _H 3	DSP2.X	J _H 3	6.8	11.7	1.1	2.5
24	V _H 1	DFL16.1	J _H 4	4.1	3.3	0.8	1.0
25	V _H 1	DFL16.1	J _H 2	2.7	5.0	5.0	5.0
26	V _H 1	DQ52-C	J _H 2	3.2	5.8	0.8	6.0
27	V _H 5	DSP2.9	J _H 4	4.5	7.5	9.0	10.0
28	V _H 5	DFL16.1	J _H 4	16.4	14.2	0.7	3.0
29	V _H 7	DFL16.1	J _H 2	2.3	0.0	1.5	na

a) Closest germ-line V_H, D_H, and J_H families of clones were assigned using the DNAPlot database.

b) Percentages of mutations within the FRW and the CDR were determined.

c) Ratio of replacement to silent mutations (R/S).

d) Sequence was isolated in two clones after PCR.

e) Sequence was isolated in three clones after PCR.

f) Sequence was isolated in four clones after PCR.

g) Sequence was isolated in five clones after PCR.

na = not applicable.

positive selection by antigen [24]. Remarkably most of the sequences with a high ratio of replacement/silent mutations in the CDR did not belong to the V_H5 family. This suggested that some B cell clones might have been selected due to an antigen binding in the FRW leading to the preferential usage of the V_H5 family, while other

clones might have undergone somatic hypermutation due to antigenic pressure resulting in increased levels of replacement mutations within the CDR.

Several clonally related sequences (Table 1, nos. 1–8) containing the same V_H, D_H, and J_H gene elements and identical V/D and D/J junctions were identified. Thus,

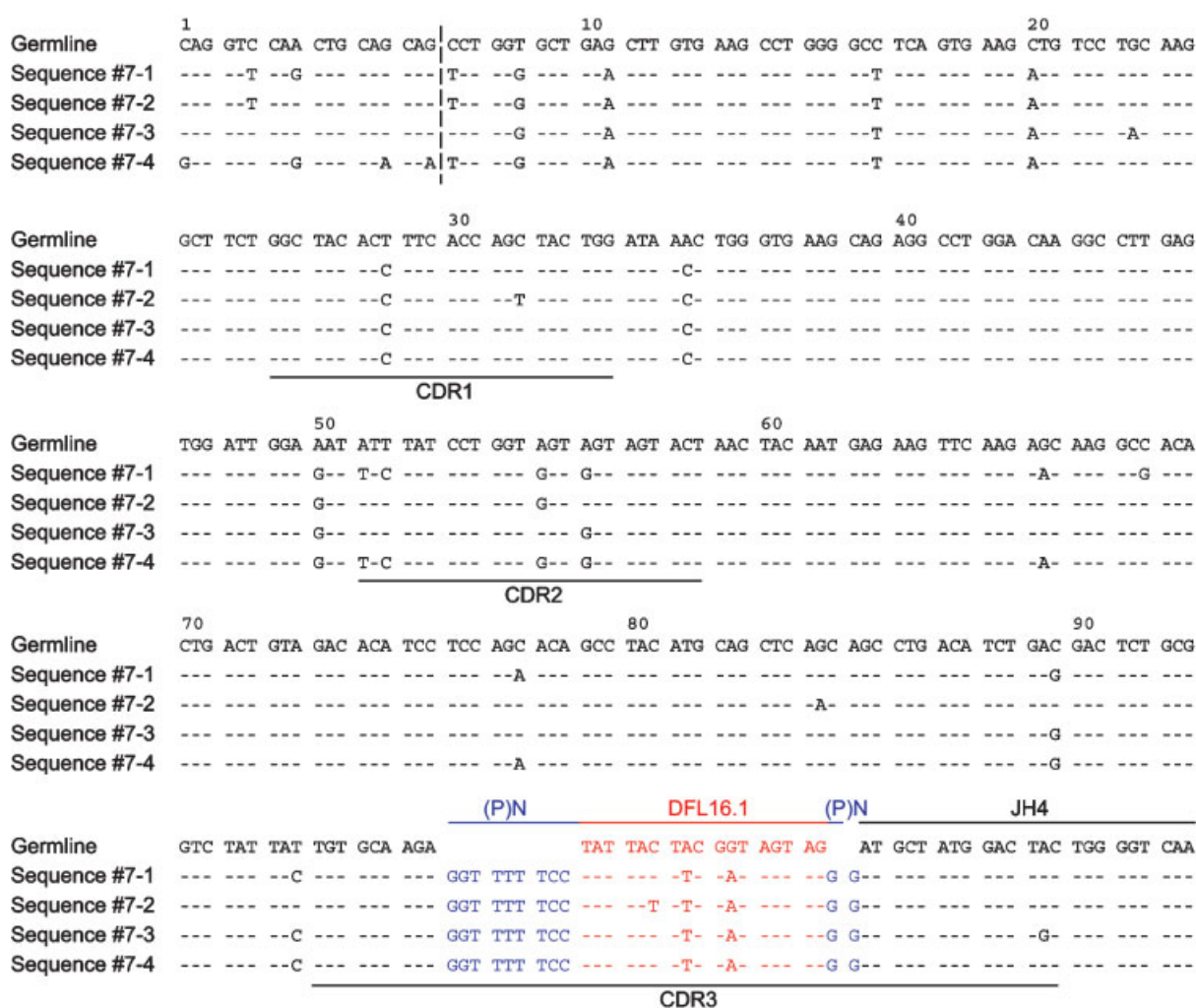


Fig. 5. Clonally related IgE-producing B cells were identified in mite-infested mice. Nucleotide sequences of V, D, and J segments of IgE heavy chain cDNA including putative nucleotide insertions (P)N (blue) of representative clonally related sequences were aligned to the corresponding germ-line sequence using the DNAPLOT database (www.dnaplot.de). Codons 1–98 represent the V_H5 germ-line sequence M37621, the D_H segments were aligned to the germ-line DFL16.1 (red) and the J_H segments to the germ-line J_H4 gene. The first six codons, separated by a dashed vertical line, belong to the binding site of the degenerative primer mix [38] and mutations in this region were not considered in the analysis. Horizontal dashes: nucleotide identity.

the corresponding B cells derived from a common precursor, which had undergone clonal expansion. The nucleotide alignment of a representative clone (Table 1, no. 7) to the germ-line sequence is shown in Fig. 5. Identical nucleotide insertions indicate the common origin while differences in the mutations provide evidence for transcripts derived from diverged IgE-bearing B cells.

Together these data indicate that the antibody response to mite infestation resulted in production of both specific IgE induced by antigen-driven selection, as indicated by clonally related B cells and increased replacement mutations in the CDR, and antigen-nonspecific IgE derived from polyclonal B cell class switching, as indicated by several unrelated sequences with low mutational frequencies.

Mite-induced mast cell degranulation in skin and mast cell recruitment to skin-draining LN

To investigate the effects of the high IgE concentrations induced upon mite infestation, we performed histochemical analysis of mast cells by staining sections of several organs with toluidine blue. While total mast cell numbers in skin sections did not change throughout the time of mite infestation (Fig. 6A), the numbers of degranulated mast cells increased greatly upon exposure to mites (Fig. 6B). The dramatic increase in degranulation is clearly visible by diffusion of granules in the skin of wild-type mice, while no such increase was observed in mice deficient in T cells or CD40L, even late after mite infestation (Fig. 6C). Induction of mast cell degranulation suggested the presence of mite-specific

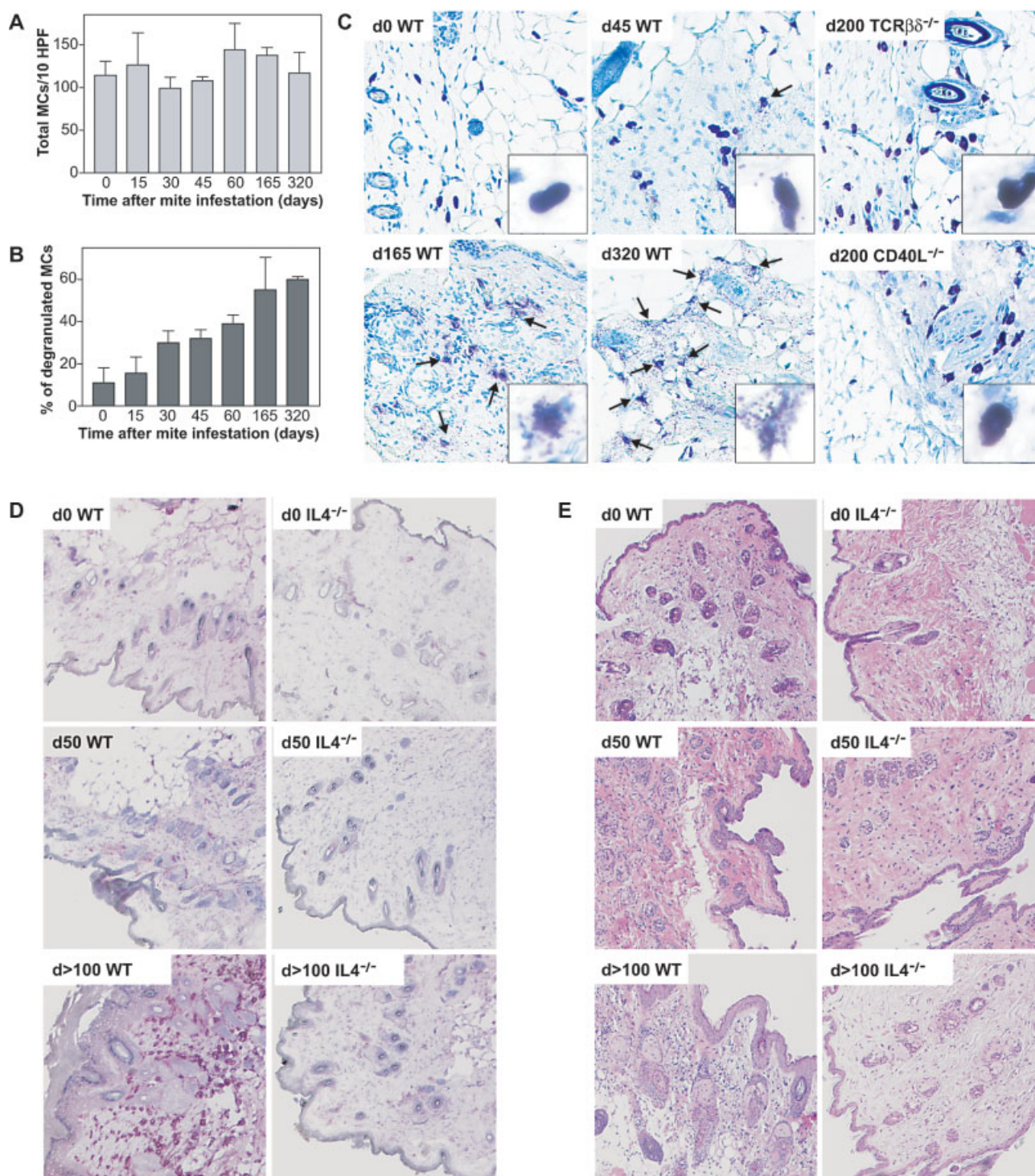


Fig. 6. Mast cell degranulation and inflammation occurs in skin of mite-infested mice. Total numbers $\pm$ SD of mast cells per ten high-power fields (HPF) of a 20 $\times$ magnification (A) and the percentage $\pm$ SD of degranulated mast cells (B) were determined in skin sections of WT C57BL/6 mice at different time points after mite infestation. (C) Toluidine blue-stained skin sections (20 $\times$ magnification) from naïve, mite-infested WT C57BL/6 or mite-infested TCR $\beta\delta$ -deficient and CD40L-deficient mice at various time points post infestation are shown. Arrows indicate examples of degranulated mast cells, while insets show 50 $\times$ magnification of a representative mast cell from each section. (D, E) Skin sections from naïve or mite-infested WT C57BL/6 and IL-4-deficient mice (as indicated) were analyzed for (D) IgE or (E) stained with hematoxylin and eosin. Photographs show sections at 6.25 $\times$ magnification and are representative of at least two mice from two separate experiments.

IgE, which would be cross-linked by mite antigens. It also demonstrated that scratching alone resulting from mite presence on the fur cannot be responsible for mast cell degranulation as scratching was also observed in $\text{TCR}\beta^{-/-}$ and $\text{CD40L}^{-/-}$ mice. Thus, our results strongly suggest that the immune response resulting from mite infestation was directed against specific antigens. Together with mast cell degranulation there was a dramatic increase in the number of IgE^{+} cells in the skin of wild-type B6 mice following mite infestation (Fig. 6D; left panel), which was not observed in $\text{IL-4}^{-/-}$ mice (Fig. 6D; right panel). The presence of IgE^{+} cells and mast cell degranulation correlated with development of a local inflammatory response, evidenced by leukocyte infiltration into the dermis and epidermal hyperplasia (Fig. 6E; left panel). In contrast, a local inflammatory response was not observed in $\text{IL-4}^{-/-}$ mice, which lack IgE (Fig. 6E; right panel). Moreover, we observed an accumulation of mast cells in skin-draining LN (Fig. 7A–D) but not in gut-draining LN (Fig. 7E, F)

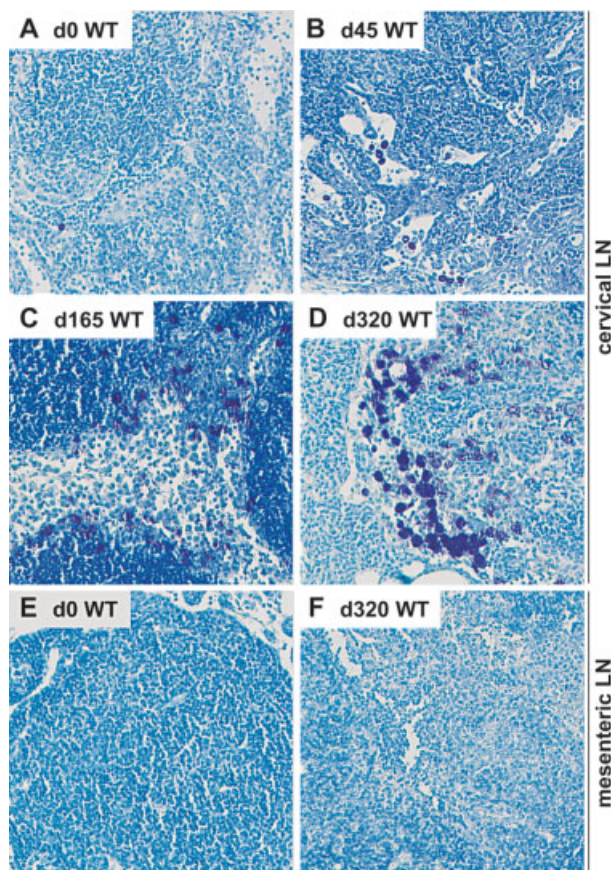


Fig. 7. Mast cell infiltration occurs in skin-draining lymph nodes after mite infestation. Cervical LN of naïve (A) or mite-infested WT C57BL/6 mice on day 45 (B), 165 (C), and 320 (D) after mite infestation and mesenteric LN of naïve (E) or mite-infested WT C57BL/6 mice on day 320 (F) after mite infestation. Results are representative of three mice per group and two independent experiments.

nor in skin-draining LN of $\text{TCR}\beta^{-/-}$ or $\text{CD40L}^{-/-}$ mice (data not shown). No mast cell infiltration was found in any other organs such as spleen, lung, jejunum, ileum or colon (data not shown). Mast cells infiltrating skin-draining LN upon mite infestation may interact with lymphocytes and influence mite-induced immune responses. However, the relevance of such interactions remains to be determined.

Lack of mucus accumulation in the lungs of mice at various time points after mite infestation (data not shown) indicated absence of lung inflammation in this model.

Induction of mast cell degranulation in the skin as well as infiltration of mast cells into skin-draining LN, in agreement with the observations of IL-4 production and B cell switch to IgE, provided strong evidence that after mite infestation antigen uptake took place in the skin and an immune response was induced in skin-draining LN.

Discussion

This study showed that murine ectoparasite infestation serves as an excellent model to investigate allergic disorders. The results demonstrated that mite infestation induced a strong Th2-type response, resulting in persistent high serum IgE levels. The fact that mites caused a chronic IgE-associated response was of particular interest because of its resemblance to chronically induced atopic reactions in humans that cannot be easily reproduced in mice by common allergy models based on peptide immunizations.

Induction of the immune response against fur mites, involving IL-4 secretion by CD4^{+} T cells and B cell switching to IgE, occurred in LN draining mite-infested skin indicating mite antigen being taken up *via* the skin. Although other antigen entry sites, such as ingestion through the gut or inhalation through the lung, may also be possible, the absence of marked signs of inflammation in these organs indicated that priming of the allergic responses occurred in other tissues. Up-regulation of the expression of ϵGLT and switched IgE transcripts during the course of mite infestation was explicitly shown in B cells isolated from skin-draining LN. Some increase of ϵGLT and switched IgE transcription was also observed in the mesenteric LN; however, this increase was only detected on day 15 after mite infestation, while in skin-draining LN the up-regulation of ϵGLT and IgE expression was maintained until at least day 35. The induction of high serum IgE levels observed between 15 and 60 days after mite infestation correlates therefore with the CSR to IgE in skin-draining LN. This provides additional evidence for antigen entry occurring *via* the skin.

So far, no mite antigen has been identified and, therefore, we could not directly prove antigen specificity of the IgE produced in response to mite infestation. Nevertheless, the highly increased mast cell degranulation in skin of infested mice strongly suggested that at least part of the total IgE induced was mite antigen specific. This view is supported by a previous report in which passive and active cutaneous anaphylaxis as well as mast cell degranulation in the skin have been demonstrated in response to mite extract [6]. Moreover, as discussed in the results, the analysis of V(D)J C ϵ nucleotide sequences revealed the occurrence of clonally related B cells and the presence of increased replacement mutations in the CDR in some clones, providing additional evidence for an antigen-driven IgE response to mites. Despite this evidence, we cannot rule out the possibility that in our mite-infested mice mast cell degranulation might have occurred through alternative non-IgE-mediated mechanisms, such as in response to complement product activation or TLR ligation [25].

Histochemical analysis of organ sections isolated from mite-infested mice revealed not only mite-dependent mast cell degranulation in the skin but also accumulation of large numbers of mast cells in skin-draining LN. The role of mast cell accumulation in skin-draining LN remains controversial. Mite-induced mast cell infiltration has been previously reported by Jungmann *et al.* [10], who speculated that mast cells might re-circulate into the lymphatics and contribute to antigen transport and presentation in draining LN. Several studies indicated that mast cells can enhance the development of T cell-associated responses. For example, mast cells have been shown to be able to present antigen *in vitro* through MHC class I- and class II-dependent pathways [26, 27]. Moreover, mast cells have been demonstrated to express a number of costimulatory and adhesion molecules that may allow them to contribute to B cell switching to IgE by inducing IL-4- and CD40L-mediated pathways [28]. Interestingly, mast cell migration to LN has also been demonstrated during dinitrofluorobenzene-induced contact hypersensitivity in mice [29]. Wang *et al.* [29] suggested that mast cells may contribute to the induction of primary immune responses by migration from the site of antigen encounter to draining LN, where they may promote T cell recruitment by secretion of the chemokine macrophage inflammatory protein-1 β . In fur mite-infested mice, mast cell infiltration was observed exclusively in skin-draining LN. The absence of mast cell accumulation in other organs suggested the involvement of mite antigens taken up through the skin, causing activation of immune responses in the skin-draining LN. Further investigation is required to evaluate the significance of mast cell accumulation in

the skin-draining LN and its possible involvement in mite-induced responses.

In recent years, fur mites have been investigated in the context of atopic dermatitis in Nishiki Cinnamon (NC) mice. NC mice were initially believed to spontaneously develop dermatitis-like syndromes accompanied by high serum IgE levels and were therefore suggested to be a good model to study human atopic dermatitis [30]. Later, however, the fur mites *Myocoptes musculinus* and *Myobia musculi* were reported to be the cause of skin lesions and increased IgE production in NC mice [31, 32]. The NC strain appears to be particularly susceptible to MAUD showing rapid development of severe skin lesions (at ~12 weeks of age) [30]. In the present study, we investigated mite infestation mainly on C57BL/6 mice. MAUD symptoms were observed in about 5% of these mice when mite-infestation was maintained for at least one year, which is comparable to previously published data [12].

The fur mite-infestation model presented in this report could serve to evaluate the relevance of infectious burden in childhood for protection against atopic reactions, as suggested by epidemiological data, which showed that the increase in the prevalence of allergy correlates with westernized lifestyle [33]. Mite infestation could be investigated following exposure to viral or bacterial pathogens to study their effect in naturally occurring IgE-mediated immune responses. We expect the mite allergy model to provide new insights into factors regulating the development of atopic disease.

Materials and methods

Mice and mite infestation

C57BL/6, CD40L^{-/-} [34], IL4^{-/-} [35], IL5^{-/-} [36], and TCR β ^{-/-} [37] mice were bred and maintained at the Institute of Laboratory Animal Science of the University of Zurich. BALB/c mice were purchased from Harlan (Netherlands). All animals were used in accordance with the guidelines of the Kanton Zurich and the Swiss Veterinary Office Animal Protection Laws.

For mite infestation, female mice at 6–8 weeks of age were put into a cage with a previously infested mouse carrying a mixed population of *Myocoptes musculinus* and *Myobia musculi*. Hereafter infestation occurred by direct contact. Transgenic mice were housed in the same cage with respective control mice in order to avoid cage-cage variation. None of the mite-infested mice analyzed in this study had skin lesions.

Total serum IgE, IgG₁ and IgG_{2a} measurement

Blood was collected weekly and total serum IgE, IgG₁ and IgG_{2a} concentrations were determined by ELISA. ELISA plates were coated with 5 μ g/mL rat anti-mouse IgE (clone 6HD5) or with 1 μ g/mL goat anti-mouse IgG₁ or IgG_{2a} (Southern Biotechnol-

ogies) in 0.1 M NaHCO₃ (pH 9.6) and blocked with 5% BSA. Serially diluted serum or standards, IgE (clone TIB-141), IgG₁ (Zymed) or IgG_{2a} (Zymed), were added and incubated for 2 h. Bound IgE was detected with biotinylated anti-mouse IgE (clone RIE-4) at 2.5 µg/mL followed by HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories) at 1 µg/mL. Bound IgG₁ or IgG_{2a} were detected by HRP-conjugated anti-mouse IgG (Sigma). ABTS peroxidase solution (2,2'-azino-bis-[3-ethylbenzthiazidine-6-sulfonic acid] 0.1 mg/mL, 0.05% H₂O₂, and 0.1 M NaH₂PO₄ pH 4.0) was used for the peroxidase-catalyzed color reaction. Color reaction was read at 405 nm in a Bio-Rad microplate reader and standard curves and antibody concentrations analyzed with Microplate Manager III software (Bio-Rad).

Flow cytometry

To obtain single-cell suspensions, LN and spleens were smashed through nylon or metal grids, whereas lungs were digested for 1 h at 37°C in DNase I (50 µg/mL, Fluka Chemie) and collagenase (500 µg/mL, Invitrogen). Cells were restimulated for 6 h with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of Brefeldin A (5 µg/mL). Collected cells were surface-stained with anti-CD4-FITC (5 µg/mL, BD Biosciences), fixed with 2% paraformaldehyde and permeabilized in 0.1% saponin. Intracellular staining was performed with allophycocyanin-conjugated (-APC) anti-IL-4 (2 µg/mL, BD Biosciences), anti-IL-5-PE (2 µg/mL, BD Biosciences), or anti-IFN-γ-APC (1 µg/mL, BD Biosciences).

IL-4 and IL-5 ELISPOT

Single-cell suspensions of skin-draining LN and spleen were added to ELISPOT plates coated with anti-CD3 (clone 2C11; 10 µg/mL) and anti-IL-4 (clone 11B11; 20 µg/mL) or anti-IL-5 (PharMingen; 20 µg/mL). Cells were added at an initial concentration of 1×10^6 /mL, then serially diluted twofold over eight steps and incubated for 48 h (37°C, 5% CO₂). Cells were washed off and plates incubated successively with biotin-conjugated anti-IL-4 (PharMingen; 1:1000) or anti-IL-5 (clone TRFK-4; 5 µg/mL) and streptavidin-alkaline phosphatase (Promega; 1:1000) before development of alkaline phosphatase color reactions using the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Values given represent the mean $\pm$ SD of the number of spot-forming cells/ 1×10^6 total cells from triplicate replicates of three mice per group.

Histochemistry

For detection of mast cells, freshly removed organs were immersed in Carnoy's fixative (60% ethanol, 20% chloroform, 20% acetic acid). Sections of 5-µm thickness were cut in paraffin, and stained with toluidine blue using standard procedures. For hematoxylin and eosin staining or detection of IgE, tissues were immersed in 4% formalin or snap frozen in Hanks' balanced salt solution using liquid nitrogen, respectively, and immunohistochemistry was performed as previously described [13].

RT-PCR and sequencing

B cells were purified to > 95% from spleen and LN by MACS (Miltenyi Biotec) using anti-B220 microbeads. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. First-strand cDNA synthesis of 1 µg total RNA was performed with random hexamers and SuperScript II reverse transcriptase (Invitrogen Life Technologies). Of the resulting cDNA, 1 µL was amplified by PCR using Taq polymerase. Primer sequences and PCR conditions were as follows: mature IgE switched transcripts (962 bp): 5'-CCTGCCCTCGGTTCTGA-3' and 5'-CTAGGCGAC-TGAAGATGAAG-3' (94°C for 5 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; followed by 72°C for 10 min), εGLT (300 bp): 5'-GCAGAAGATGGCTTCGAATAAG-AACAGT-3' and 5'-TCGTTGAATGATGGAGGATGTGTACAGT-3' (94°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; followed by 72°C for 10 min), µGLT (325 bp): 5'-CTCTGGCCCTGCTTATTGTTG-3' and 5'-ATGGTGCTGGG-CAGGAAGTC-3' (94°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; followed by 72°C for 10 min), AID (300 bp): 5'-GGCTGAGGTTAGGGTTCATCTCAG-3' and 5'-GAGGGAGTCAAGAAAGTCACGCTAGA-3' (94°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; followed by 72°C for 10 min).

For sequence analysis, cDNA was synthesized from B cells purified from bone marrow, spleens, and skin-draining LN of mite-infested mice about 10 months after initial contact with mites. V(D)J_H-C_ε transcripts were amplified by PCR using a degenerative primer mix [38] specific for the V_H region and a reverse primer that anneals within exon 1 of the C_ε gene (IgEc, 5'-TCTGAATACCAGGTCACAGTC-3'). PCR products were purified using the agarose gel extraction kit (Qiagen) and subcloned directly into the pGEM-T vector (Promega) for automated sequencing. DNA was isolated from individual clones and sequences were then aligned to the closest germline sequence using the DNAPLOT database (www.dnaplot.de) to determine the V_H, D_H, and J_H gene usage and the number of mutations within the FRW and the CDR.

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