

Impaired NK1.1⁺ T cells do not prevent the development of an IgE-dependent allergic phenotype

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Summary

Background The induction of TH₂ immune responses is critically dependent on initial IL-4. Although crucial, the source of this early IL-4 has not been identified. One candidate is a CD1 restricted NK1.1⁺ T cell subpopulation which is known to produce such early IL-4.

Objectives and methods The necessity of NK1.1⁺ T cells for the expression of an IgE-dependent phenotype was investigated in a NK1.1⁺ T cell deficient mouse model. The allergic phenotype was defined as immediate cutaneous hypersensitivity. It was induced by immunization of mice with ovalbumin. Mouse strains used were C57BL/6 mice and C57BL/6 mice homozygous for a targeted mutation of the β_2 microglobulin gene with consecutive loss of CD1 expression, which leads to a drastic reduction of NK1.1⁺ T cells. Manifestation of an allergic sensitization was assessed by intradermal allergen challenge after i.v. injection of Evans blue solution. The blue stained weal formations were quantified with the Bonitur method. In addition, the Th₂ response was confirmed by the measurement of cytokines and serum immunoglobulins. The capability to produce early IL-4 was tested through the assessment of IL-4 mRNA shortly after a single challenge.

Results Wild type and mutated mice did not differ in any of the immunological parameters measured.

Conclusion A single exposure to antigen with or without adjuvant induces early IL-4 production in C57BL/6 β_2 m^{-/-} mice. This early IL-4 is therefore independent of the presence of NK1.1⁺ T cells and functional MHC class I molecules and leads to IgE production and immediate cutaneous hypersensitivity.

Keywords: NK1.1⁺ T cells, β_2 -microglobulin, CD1, IL-4, TH₂, IgE, immediate cutaneous hypersensitivity, mouse system

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Introduction

Cytokines that are present in the early stage of antigenic stimulation can direct the differentiation of T cell precursors towards Th₁ or Th₂ effector cells resulting in two functionally different phenotypes. The cytokine IL-4 is critical for the development of Th₂ cells [1–3]; in mice this has most strikingly been demonstrated through gene disruption of either IL-4 [4] or STAT6, the signalling molecule activated

by IL-4 [5,6]. Such mice fail to generate Th₂ cells and the corresponding IgE response. The small NK1.1⁺ T cell subset has been recognized as a major source of early IL-4. Upon polyclonal stimulation of T cells with anti-CD3 antibodies *in vivo* they promptly produce large amounts of IL-4 [7]. Therefore this subpopulation has been hypothesized as promoting Th₂ responses.

In the mouse, NK1.1⁺ T cells are restricted by two non-classic MHC class Ib molecules, CD1.1 and CD1.2 [8,9]. The expression of these ligands is dependent on the expression of β_2 -microglobulin (β_2 m) [10]. Therefore mice with disruption of the β_2 m gene lack CD1 molecules and have drastically reduced NK1.1 T cell numbers. Such mice are a

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useful tool to test whether NK1.1 cells are necessary for the induction of TH₂ responses.

$\beta_2\text{m}^{-/-}$ mice on a C57BL/6 background, in contrast to the wild type mice, do not produce early IL-4 mRNA upon anti-CD3 treatment, or no IgE in response to anti-IgD antibodies, a polyclonal B cell stimulus [11]. This suggests that NK1.1 T cells provide the local cytokine milieu for initiating a TH₂ response.

To test this conclusion, two other routes for induction of TH₂ responses were investigated. First, $\beta_2\text{m}^{-/-}$ mice on either the BALB/c or the C57BL/6 background were infected with parasites. Remarkably, this did not affect the resistance to infection as compared with wild type mice [12,13]. Second, exposure to the protein antigens OVA or KLH also led to TH₂ responses with the production of similar levels of TH₂ cytokines and IgE in gene targeted and wild type mice [12,14].

As none of the phenotypes obtained was critically dependent on the presence of allergen-specific IgE we tested this dependency in immediate cutaneous hypersensitivity (ICHS). In this model allergen-specific IgE bound to mast cells is required for mast cell degranulation upon local allergen challenge, leading to plasma extravasation followed by intradermal weal formation. We demonstrate that the absence of NK1.1⁺ T cells in $\beta_2\text{m}^{-/-}$ mice does not inhibit induction and manifestation of this IgE dependent allergic response.

Materials and methods

Animals

Wild-type C57BL/6JOLA-Hsd were obtained from Harlan Winkelmann (Borchen, Germany). $\beta_2\text{m}^{-/-}$ mice homozygous for targeted disruption of the $\beta_2\text{m}$ gene and congenic on the C57BL/6J background were purchased from Taconic Farms (Germantown, NY, USA).

Induction of allergic sensitization

Mice were immunized by intraperitoneal injections of 10 μg

ovalbumin (OVA grade VI, Sigma Chemicals, Deisenhofen, Germany) with or without 150 μg aluminium hydroxide as adjuvant (Pierce, Rockford, IL, USA). In the case of immunization with adjuvant, mice were injected either once, twice on day 1 and 14, or three times on day 1, 14 and 21. In the absence of adjuvant, either three, four or five injections were given on day 1, 14, 21; 1, 14, 21, 28; 1, 14, 21, 28 and 35, respectively. Twelve days after the last injection skin tests were performed and blood and spleens collected post mortem.

Immediate cutaneous hypersensitivity

Mice were challenged intracutaneously with 75, 150, 300 and 600 ng of OVA. The mast cell degranulating compound 48/80 (Sigma Chemicals, Deisenhofen, Germany) was used as a positive control, PBS as a negative control. To visualize vascular leakage due to mast cell degranulation, 0.5% Evans blue solution was injected i.v. 15 min prior to allergen challenge. The Bonitur method (manuscript in preparation) is an estimation method to quantify complex traits. According to that method the size of skin weals is scored 0 as negative, and 3 as maximum. Addition of four scores for four OVA concentration reveals 0 as negative and 12 as maximum.

Quantification of total and OVA specific IgE

Sandwich ELISAs were performed with commercial antibodies (Binding Site, Heidelberg, Germany) according to manufacturer's instructions. For standardization a pool serum from OVA sensitized BALB/c mice was used with an assigned value of 2000 arbitrary laboratory units/mL (IU/mL).

Cytokine ELISA

Cytokine production was determined by culturing 10^7 splenic mononuclear cells in 1 mL RPMI plus FCS per well in 24-well plates at 37 °C and 5% CO₂. Cells were stimulated with either 2.5 μg ConA or 100 μg OVA. 20 h later supernatants were collected and IL-4, IL-5 and IFN γ

Table 1. Comparison of two different allergic sensitization protocols to achieve maximum immediate cutaneous hypersensitivity reactions. C57BL/6 wild type mice received 1, 2, or 3 injections of OVA plus adjuvant or 3, 4 or 5 injections of OVA without adjuvant. Twelve days after the last injection ICHS were performed

	OVA treatment i.p.	1 $\times$	2 $\times$	3 $\times$	4 $\times$	5 $\times$
ICHS score	With adjuvant	1 $\times$ 10 4 $\times$ 12	4 $\times$ 12	2 $\times$ 11 5 $\times$ 12	Not done	Not done
	Without adjuvant	Not done	Not done	5 $\times$ 0	5 $\times$ 12	5 $\times$ 12

were measured with commercial sandwich ELISAs (Pharmingen, San Diego, CA, USA) according to manufacturer's instructions.

RT-PCR analysis of IL-4 mRNA

Spleens from C57BL/6 and C57BL/6 $\beta_2\text{m}^{-/-}$ mice were

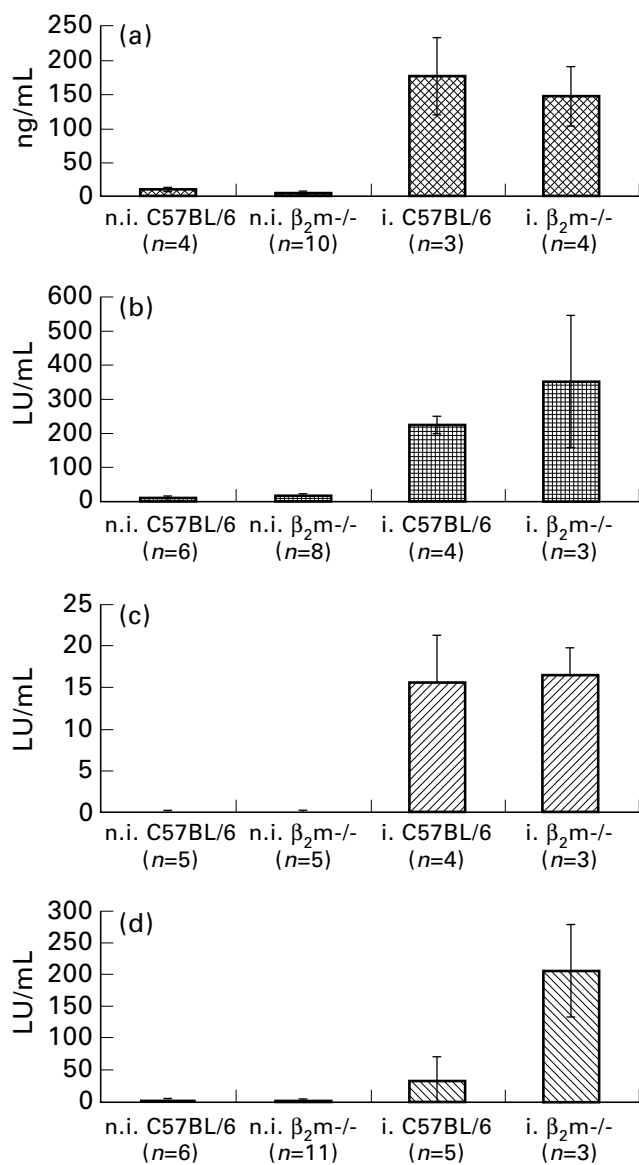


Fig. 1. Serum immunoglobulin concentrations after a single immunization of C57BL/6 and C57BL/6 $\beta_2\text{m}^{-/-}$ mice. Mice were immunized with 10 μg OVA and aluminium hydroxide. 12 days later blood was collected and serum concentrations of total IgE and OVA specific IgE, IgG1, IgG2 were measured by ELISA. (a) total IgE; (b) OVA-specific IgE; (c) OVA-specific IgG1; (d) OVA-specific IgG2a.

collected 1, 8 and 16 h after a single antigen exposure. PCR amplification was performed as described previously [15]. Briefly, total RNA was purified using RNeasy (AGS, Heidelberg, Germany), following the manufacturer's instructions. A 2.5 μg aliquot of total cellular RNA was reverse transcribed using random hexanucleotide as primer and a SUPERScript II amplification kit (Gibco BRL, Gaithersburg, Germany). A cDNA equivalent of 0.5 ng RNA was amplified in a 20 μL reaction containing 250 mM of each dNTPs, 200 nM primers, 2 μL 10-fold PCR buffer and 1 U Taq DNA polymerase ('Amplitaq', Perkin-Elmer, Weiterstadt, Germany) during 40 cycles (1 min at 94°C, 2 min at 60°C, 2 min at 72°C).

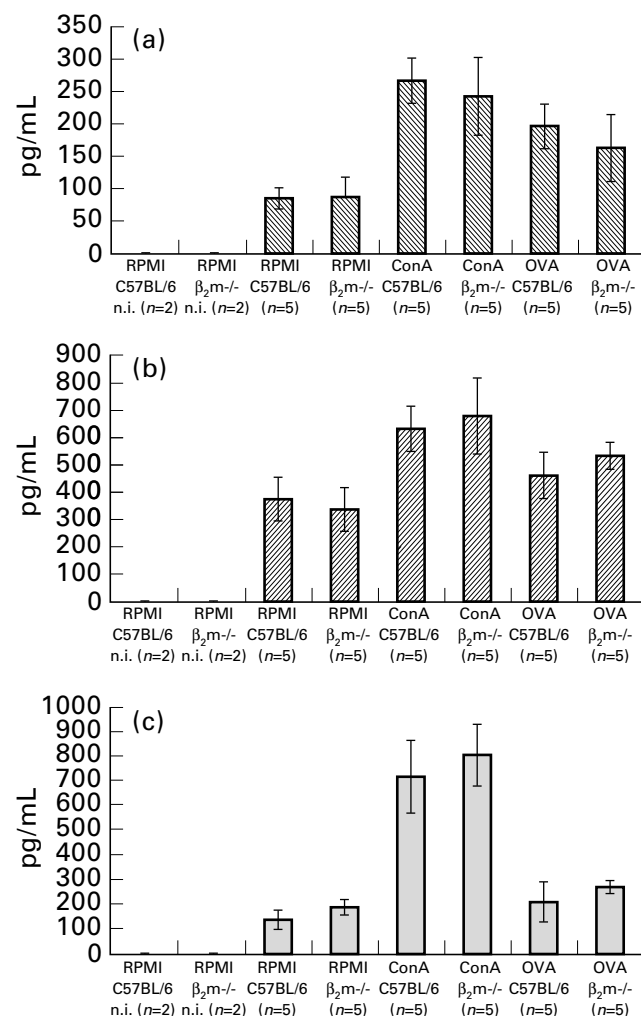


Fig. 2. Cytokine concentrations after a single immunization of C57BL/6 and C57BL/6 $\beta_2\text{m}^{-/-}$ mice. Splenic mononuclear cells were prepared from identical mice as in Fig. 1. 1×10^7 cells/mL medium were stimulated with either 2.5 $\mu\text{g}/\text{mL}$ ConA or 100 $\mu\text{g}/\text{mL}$ OVA and supernatants were collected 20 h later. IL-4, IL-5 and IFN γ concentrations in the supernatants were analysed with ELISA. (a) IL-4; (b) IL-5; (c) IFN γ .

Primer sequences were as follows: IL-4 sense: 5'-CGA AGA ACA CCA CAG AGA GTG AGC T3'; IL-4 antisense: 5'-GAC TCA TTC ATG GTG CAG CTT ATC G3'; -actin sense: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C3'; β -actin antisense: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G3'. In all experiments a mock PCR (without DNA) was included to exclude contamination.

Results

Allergic sensitization strategies

It is possible that induction of TH₂ responses is dependent on NK1.1⁺ T cells in the case of a single challenge and that this requirement is abrogated with secondary and additional challenges. To test this, C57BL/6 wild type mice were immunized i.p. with ovalbumin in the presence or absence of aluminium hydroxide as adjuvant one to three times. In the presence of adjuvant a single dose of antigen was sufficient to induce total IgE and OVA specific IgE, IgG1 and IgG2a and maximum immediate cutaneous hypersensitivity reaction. In the absence of adjuvant four injections were required to obtain similar positive skin reactions (Table 1).

To use minimum immunization strategy all further experiments were performed after applying a single dose of OVA plus adjuvant.

Evaluation of TH₂ responses

Twelve days after a single exposure to OVA immunoglobulin levels were measured in the serum of C57BL/6 β_2 m^{-/-} and wild type mice (Fig. 1). Both strains had similar increases of total IgE and OVA specific IgE and IgG1 as compared with untreated animals. In addition to the TH₂ dependent immunoglobulin isotypes IgE and IgG1 the TH₁ dependent immunoglobulin IgG2a was produced. Therefore, the immune response for both strains has to be regarded as a mixed Th₀ response. Whether the higher levels of IgG2a in β_2 m^{-/-} mice were due to the lack of MHC class I molecules as described elsewhere [16] was not further investigated.

Furthermore, a characteristic Th₀ cytokine distribution was obtained in supernatants from splenic mononuclear cells (SMC) of immunized mice of both strains. SMC were cultured in RPMI and stimulated *in vitro* with antigen or mitogen. As controls, SMC from non-immunized mice were cultured under the same conditions. There were

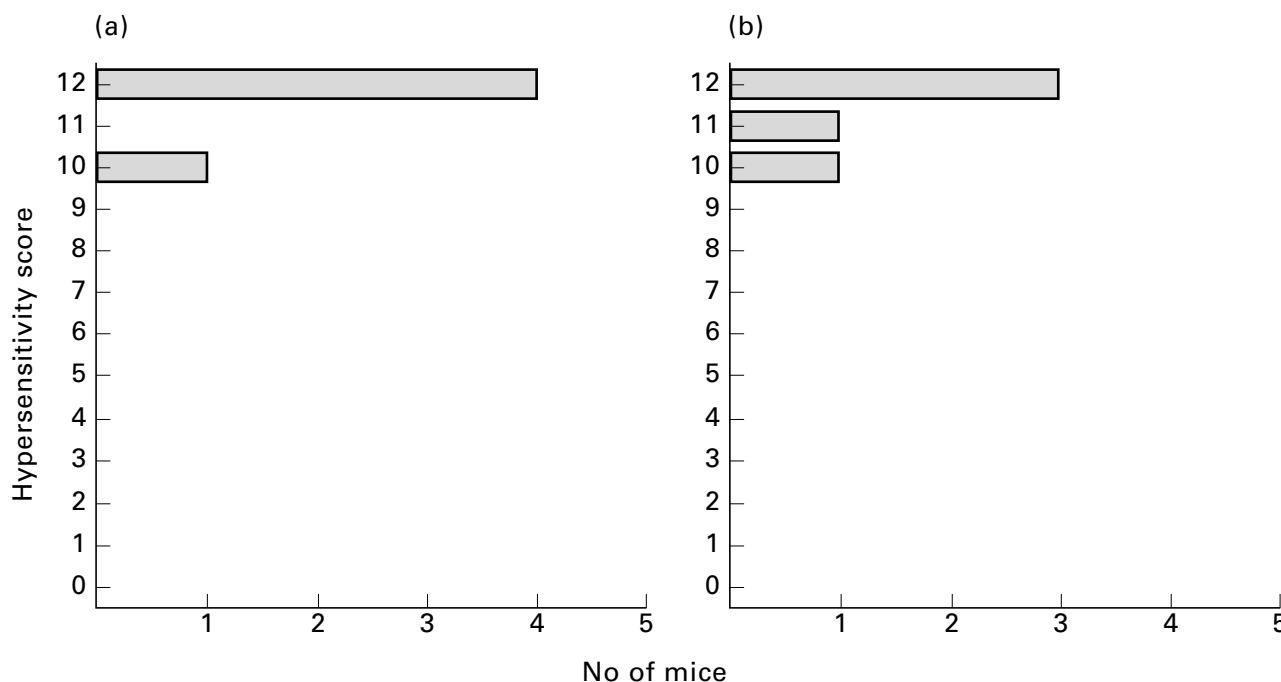


Fig. 3. Immediate cutaneous hypersensitivity reactions after a single immunization of C57BL/6 and C57BL/6 β_2 m^{-/-} mice. Mice were immunized with 10 μ g OVA and aluminium hydroxide. Twelve days later mice were challenged intracutaneously with 75, 150, 300 and 600 ng of OVA. The mast cell degranulating compound 48/80 was used as a positive control, PBS as a negative control. To visualize vascular leakage due to mast cell degranulation, 0.5% Evans blue solution was injected i.v. 15 min prior to allergen challenge. According to the Bonitur method the size of skin weals was scored 0 as negative, and 3 as maximum. Addition of scores for each OVA concentration revealed 0 as negative and 12 as maximum. (a) C57BL/6; (b) β_2 m^{-/-}.

significant increases of IL-4, IL-5 and IFN γ in supernatants of cells derived from immunized C57BL/6 and $\beta_2\text{m}^{-/-}$ mice as compared with untreated animals (Fig. 2). Addition of OVA to the culture system did not increase the amount of secreted cytokines over spontaneous secretion; none of these TH₀ cytokines was detectable in supernatants of non-immunized mice (data not shown). No differences in cytokine levels were apparent in C57BL/6 and C57BL/6 $\beta_2\text{m}^{-/-}$ mice.

Assessment of immediate cutaneous hypersensitivity reactions

One consequence of an allergic sensitization is the development of positive skin reactions upon local allergen challenge. ICHS were induced by injection of four different concentrations of OVA. Negative responses were scored 0

and maximum responses were scored 12 thus allowing a differentiation into low, intermediate and high responses (Fig. 3). None of the hypersensitivity scores in $\beta_2\text{m}^{-/-}$ mice was of intermediate or low response and therefore did not demonstrate a significantly weaker manifestation of allergic sensitization.

NK1.1⁺ T cells are not required for early IL-4 production upon allergic sensitization

A main property of NK1.1⁺ T cells is the rapid burst of IL-4. The read-outs described above reflect the immune response 12 days after allergen challenge. At this time point a strain difference could be compensated by other IL-4 secreting cell populations. Therefore, we measured the production of early IL-4 mRNA. C57BL/6 and C57BL/6 $\beta_2\text{m}^{-/-}$ mice received a single dose of OVA with or without adjuvant. Spleens were taken 1, 8 and 16 h afterwards and IL-4 mRNA was investigated (Fig. 4). C57BL/6 and C57BL/6 $\beta_2\text{m}^{-/-}$ mice expressed comparable amounts of IL-4 mRNA after 8 and 16 h. In addition, IL-4 mRNA expression was detectable as early as after 1 or 4 h in wildtype mice (Fig. 4a, lanes 1 and 5). However, this difference did not influence any of the immune response parameters tested. Our results thus suggest the presence of additional cell populations which can provide early IL-4.

Discussion

Previous findings suggested that NK1.1⁺ T cells might provide the source of initial IL-4 for driving naive CD4⁺ T cells into the TH₂ phenotype. Mice deficient in this T cell subpopulation, either through disruption of $\beta_2\text{m}$ with consecutive deficiency of CD1 surface expression, or genetically as in SJL mice, do not respond to anti-CD3 or anti-IgD antibodies with elevated IL-4 and IgE levels *in vivo* [11,17]. To determine the importance of NK1.1⁺ T cells for development of TH₂ responses to stimuli other than polyclonal B and T cell activators, we and others [12–14] have treated $\beta_2\text{m}^{-/-}$ mice on a C57BL/6 or BALB/c background with either parasites or protein antigens. In all cases the immune response did not differ from that in wild type mice, suggesting that $\beta_2\text{m}^{-/-}$ dependent T cells are not required to polarise the immune response towards the TH₂ type.

To test whether there are even quantitative differences in allergic sensitization, we investigated the immediate cutaneous hypersensitivity as an IgE-dependent phenomenon. We could not detect any substantial difference between wild type and $\beta_2\text{m}^{-/-}$ mice, suggesting that this, like most other TH₂ dependent immune responses, is $\beta_2\text{m}^{-/-}$ independent and thus distinct from the responses induced by anti-CD3 and anti-IgD antibody treatments.

One possibility is that a residual population of NK1.1⁺ T

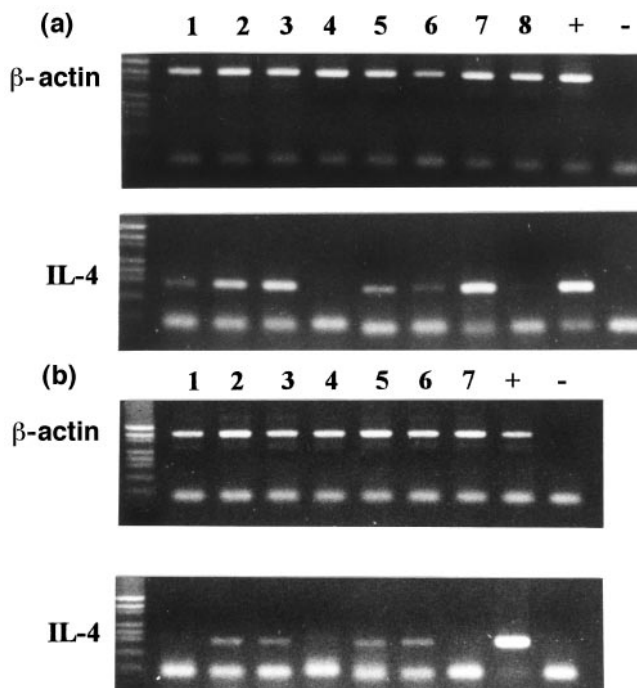


Fig. 4. RT-PCR analysis. (a) Expression of IL-4 in C57BL/6 mice. Analyses were performed 1, 8 and 16 h after a single dose of antigen in the presence (lanes 1–3) or 1, 4, 8, 16 h (lanes 4–7) in the absence of adjuvant. Lane 8 shows IL-4 analysis in spleen cells of an untreated animal, followed by the positive and negative control. (b) Expression of IL-4 in C57BL/6 $\beta_2\text{m}^{-/-}$ 1, 8 and 16 h after a single antigen exposure in the presence (lanes 1–3) or absence (lanes 4–6) of adjuvant. Lane 7 shows IL-4 analysis in spleen cells of an untreated animal, followed by the positive and negative control. The amount of cDNA analysed was similar in different samples as shown by PCR amplification of β -actin cDNA. Analyses were performed with three mice in each group.

cells can produce enough IL-4 under most conditions to induce TH₂ responses, but that activation through anti-CD3 and anti-IgD antibodies has exceptionally high requirements for IL-4. Recent work with $\beta_2\text{m}^{+/+}$, CD1^{-/-} mice has shown that CD1 restricted NK1.1⁺ T cells are not required for IgE production in the anti-IgD induction model [18–20]. Therefore it is possible that another $\beta_2\text{m}$ -dependent subpopulation is able to compensate for the loss of CD1 restricted NK1.1⁺ T cells, and that the requirement of such a compensating $\beta_2\text{m}$ -restricted population can be overcome by stronger TH₂ inducing stimuli than anti-IgD.

In summary, these results suggest that TH₂ responses and IgE dependent allergic phenotypes are IL-4 dependent, but independent of $\beta_2\text{m}$ and CD1 expression. This also implies that MHC class I restricted CD8⁺ T cells are dispensable in the TH₂ response to most biological stimuli.

Other cell types, long considered a possible source of initial IL-4, such as mast cells, basophils and CD4⁺ T cells remain as possible mediators of ICHS.

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