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Secondary IgE Responses *In Vivo* are Predominantly Generated Via $\gamma_1\epsilon$-Double Positive B Cells

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We have recently developed a model in which mice were treated with IL-4 after primary immunization, resulting in elevated total serum IgG_1 and IgE levels, but decreased antigen-specific levels and memory formation for these isotypes. In this report, we describe that these effects of IL-4 are mediated at the B cell and not the T-cell level. Major changes occurred in the $\gamma_1\epsilon$-double positive B-cell population which is increased as a result of IL-4 treatment. Moreover, it is shown that $\gamma_1\epsilon$-double positive B cells can develop *in vitro* out of γ_1-positive primed B cells and that these double positive cells can differentiate into IgG_1- and IgE-secreting cells. The existence of $\gamma_1\epsilon$-double positive memory B cells can explain the differences in cytokine dependence of TNP-specific memory IgG_1 and IgE responses found after adoptively transferring primed spleen cells into irradiated naive recipients. Whereas the IL-4 independent TNP-specific memory IgG_1 responses could be blocked efficiently by neutralizing IL-5 and IL-6, TNP-specific memory IgE responses were virtually not susceptible to such treatment. These IgE responses were also not susceptible to IFN-γ, used in doses that could inhibit the primary IgE response. Inhibition of the TNP-specific memory IgG_1 response by neutralizing IL-5 and IL-6 is accompanied by a 10-fold increase of the IL-4 independent TNP-specific IgE memory response. These data indicate that secondary IgE responses primarily result from B cells that are either switched to IgG_1, or are double positive for IgG_1 and IgE, thereby suggesting a minor role for ϵ-single positive B cells in secondary IgE responses.

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INTRODUCTION

Interleukin-4 (IL-4) has been found to be necessary for the induction of IgE synthesis on account of its ability to induce the expression of ϵ-germline transcripts [1, 2]; the inhibition of parasite-induced IgE responses by neutralizing antibodies against IL-4 [3]; the absence of IgE responses in nematode-infected mice that were made IL-4-deficient by gene targeting [4], and the IgE hyperproduction in IL-4 transgenic mice [5–7]. Although IL-4 induces accumulation of germline transcripts for γ_1 [8, 9], which correlates with an increase in IgG_1-secreting cells [10], it is not required for IgG_1 synthesis as was shown in IL-4 deficient mice [4].

Functional studies indicate that IL-4 is a 'switch inducing' factor. It alters the chromatin structure of the $S\gamma_1$ region [9] and induces accumulation of germline γ_1 and ϵ transcripts [1, 8, 11, 12]. The induction of γ_1 germline transcripts is inhibited by interferon-γ (IFN-γ) [8], which also inhibits the *in vivo* production of IgG_1 and IgE [13]. IL-4 and IFN-γ act reciprocally in the determination of IgG_1 and IgE responses [14], whereas IL-5 seems to act synergistically with IL-4 in that it specifically enhances the accumulation of productive γ_1 and ϵ transcripts [15]. IL-5 and IL-6 are both cytokines involved in the maturation of B cells to become Ig-secreting plasma cells [16, 17]. However, we have recently shown that primary and secondary antigen-specific and total IgG_1, but not IgE responses are dependent on IL-6 to obtain peak levels [18].

Like IgG_1 responses, secondary IgE responses are partially IL-4-independent [18–21], although IL-4 seems to be still important in sustaining *in vivo* IgE responses [22]. The IL-4-independent component of a secondary IgE response developed only when IL-4 was present during the primary response [3, 19], indicating that such response was based on B cells that have already switched to IgE. Such cells were, although in low frequency, found after immunization and can produce IgE upon subsequent culturing [23].

Recently, evidence for sequential isotype switching from μ

to ϵ via γ_1 has been reported [2, 24]. This isotype switching mechanism could explain why, during the process of switching to IgE, many cells express both γ_1 and ϵ on their membranes [25]. It was further suggested that individual $\gamma_1\epsilon$-double positive B cells can co-secrete these two isotypes [24].

Most of the studies on the involvement of IL-4 in IgE responses used MoAb to neutralize IL-4. However, recently IgE regulation in IL-4 transgenic mice has been described [5–7]. These mice are exposed to high levels of IL-4 during development which can lead to aberrant situations. Therefore, we used an alginate encapsulation method in which an IL-4 producing cell line is encapsulated and subsequently implanted in the peritoneal cavity of normal developed mice, to modulate *in vivo* IL-4 levels [19]. The aim of this study was to investigate the effect of thus modulated IL-4 levels on the memory formation for IgG_1 and IgE responses *in vivo* with respect to B and T cells. It was found that the previously observed IL-4-induced decreased antigen-specific memory formation for IgG_1 and IgE [19] was mediated at the B-cell and not the T-cell level. Flow cytometry revealed that IL-4 exerted its effect mainly on the pool of $\gamma_1\epsilon$-double positive B cells. It was found *in vitro* that these $\gamma_1\epsilon$-double positive B cells developed out of γ_1-positive B cells. Moreover, data are presented that suggest that $\gamma_1\epsilon$-double positive B cells can either develop into IgG_1- or IgE-secreting cells, leaving open the question of whether both isotypes are produced at the same time by one B cell. *In vivo* the existence of those cells 3 months after priming is indirectly shown by differences in cytokine requirement of memory B cells. This cytokine dependence is not changed by IL-4 treatment during the memory formation, indicating that IL-4 mediates its effects on pools of B cells, not inducing intrinsic changes in B cells leading to different cytokine requirements.

MATERIALS AND METHODS

Mice. Female BALB/c mice were bred and maintained at the Department of Immunology of the Erasmus University. All mice were at an age of 12–16 weeks at the start of the experiments. They were held in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*. The microbiological status of the mice fulfilled the standard of 'specific pathogen free V' according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University.

Immunization. KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 Da of KLH (as determined spectrophotometrically) [26] by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA). Mice were injected with 0.2 ml containing either 10 or 100 μg TNP-KLH adsorbed on 2 mg alum i.p., as indicated in the Results section.

Cytokine treatment. Mice immunized with 100 μg TNP-KLH adsorbed on alum were implanted i.p. with 2×10^6 CV-1/IL-4 cells encapsulated in alginate every 2 weeks for a period of 4 months [19, 27, 28]. The CV-1/IL-4 cells were a kind gift of Dr N. Arai (DNAX Research institute, Palo Alto, CA, USA]. Empty beads encapsulated in alginate were used as control. No immunological effects were induced by this treatment in all experiments. This control is sufficient, since it has been shown that the IL-5-producing cell line CV-1/IL-5 encapsulated in alginate could not restore the impaired IgE synthesis in SJA/9 mice (data not shown). Encapsulated CV-1/IL-4 cells *in vivo* produce IL-4 for a period of at least 2 weeks [28]. The IFN-γ treatment consisted of two injections of 25 μg of purified IFN-γ dialysed and diluted in PBS +10 mM cysteine per day on days 1–5 of the primary response. During the secondary response two injections of 40 μg per day were given i.p. on days 1–3.

Adoptive transfer of spleen cells. Spleens of control-treated and IL-4 treated mice were removed under aseptic conditions and single cell suspensions were prepared. Then 1×10^7 spleen cells were transferred via the tail vein into naive recipients. The recipients had been sublethally irradiated (6 Gy) with a caesium-137 source (Gamma-cell 40, Atomic Energy of Canada, Ottawa, Canada) 1 day before cell transfer. All reconstituted mice were i.p. immunized with 10 μg TNP-KLH adsorbed on alum immediately after transfer.

Anti-cytokine treatment. Mice were treated *in vivo* by i.p. injection of neutralizing antibodies directed to IL-4 (11B11, rat IgG_1, 10 mg/mouse) [29], IL-5 (TRFK-5, rat IgG_1, 2 mg/mouse) [30], IL-6 (20F3, rat IgG_1, 2 mg/mouse) [31], IFN-γ (XMG1.2, rat IgG_1, 10 mg/mouse) [32], alone or in combination. Rat MoAb specific for *E. coli* β-galactosidase (GL113) [27] was used as an IgG_1 isotype control. The MoAb were purified from culture supernatants by protein G affinity chromatography [33]. These doses of antibody have been widely shown to be sufficient to neutralize the respective cytokine activities in a variety of systems.

Isotype-specific ELISA. Total serum IgE, IgG_1 and IgG_{2a}, levels were measured by isotype-specific ELISA as described previously [19, 34]. Detection limits for the IgE, IgG_1 and IgG_{2a} ELISA were 0.5 ng/ml, 0.2 ng/ml and 0.3 mg/ml, respectively. TNP-specific IgG_1 and IgE serum levels were determined as previously described [19], with 0.2 ng/ml and 1 ng/ml as respective detection limits in the ELISA.

ELISA-spot assay. Nitrocellulose bottomed 96-well Multiscreen HA plates (Millipore, Bedford, MA, USA) were coated with either EM95 [35], 2 μg/ml, or goat anti-mouse IgG_1, 1 μg/ml (Southern Biotechnology), and blocked with PBS containing 1% BSA. The plates were then incubated with spleen cell samples for 4 h in a humidified and vibration free 5% CO_2 incubator. Plates were washed once with 0.05% Tween 20 in distilled water, to remove the cells and twice with PBS containing 0.1% BSA and 0.05% Tween 20. Subsequently, the plates were treated as in a normal ELISA. Development was done by using AEC substrate which was prepared by dissolving 25 mg of 3-amino-9-ethyl carbazole (AEC) (Sigma Chemical, St Louis, MO, USA) in 2 ml dimethylformamide, followed by addition of 95 ml of 0.05 M acetate buffer, pH 5.0 and 40 μl of 30% H_2O_2. The substrate solution was filtered (0.2 μm) to remove particulate matter. Developed plates were dried and the red spots were enumerated under low magnification (10×), using a dissecting microscope (Stemi SV 6, Zeiss, Oberkochen, Germany) equipped with a coaxial reflected light source.

Preparation of B cells and T cells. Splenic B cells were prepared from control and IL-4 treated mice. T cells were cytotoxically eliminated by treating spleen cells with anti-Thy-1.2 (clone F7D5; Serotec, Oxford, UK) and low-tox guinea pig complement (Cederlane, Hornby, Ontario, Canada) in a two-step procedure at 0°C and 37°C, respectively [36]. For the preparation of T cells, B cells and granulocytes were eliminated by a similar procedure using anti-B220 (clone RA3-6B2) and anti-granulocyte (clone RB6-8C5). Both

MoAb were kindly provided by Professor Dr W. van Ewijk from our department. Viable cells were isolated by flotation on Histopaque 1119 (Sigma). The percentage of residual Thy-1$^+$-cells was < 2% and B220$^+$-cells < 5% as determined by flow cytometry.

T-B cell culture. CDC35, an I-A^d restricted rabbit Ig-specific Th2 clone [37], a kind gift of Dr D. C. Parker, was maintained by stimulation, every 2 weeks, with irradiated BALB/c spleen cells (30 Gy) and 50 μg/ml rabbit IgG (RIgG) (Sigma) in complete RPMI-1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercapto-ethanol and 20 IU/ml IL-2. Prior to culture with B cells the CDC35 cells were washed and viability was assessed by trypan blue exclusion. Routinely, viability was > 98%.

T cell-depleted spleen cells, at 2.5×10^5 cells/ml were cultured in eight replicate wells of flat-bottomed microtitre plates, together with 5×10^4 cells/ml of irradiated CDC35 cells (30 Gy) in 0.2 ml of complete RPMI-1640 medium at 5% CO_2 and 37°C. The following antigens were used for stimulation: 30 ng/ml TNP-RIgG, 10 ng/ml TNP-KLH + 10 ng/ml RIgG, 10 ng/ml RIgG, 10 ng/ml rabbit anti-mouse IgE (RAM/IgE) [38], or 10 ng/ml rabbit anti-mouse IgG_1 (RAM/IgG_1) [39], 10 ng/ml rabbit anti-mouse $IgG_{2a/2b}$ ($RAM/IgG_{2a/2b}$) (Nordic Immunology, Tilburg, The Netherlands). These rabbit anti-mouse isotype-specific antibodies were tested extensively for isotype specificity. After 5 days of culture, cells were harvested for ELISA-spot assay and supernatants for ELISA. For large-scale experiments, essentially similar cultures were performed at 2 ml in 24-well flat-bottomed plates. On day 4 of the culture, cells were harvested and stained for FACScan analysis.

FACScan. The cells (2.5×10^5) were incubated on ice for 30 min with the appropriate MoAb, either as undiluted culture supernatant or optimally titrated purified MoAb, followed by a triple wash with PBS containing 1% BSA and 0.1% azide. After using unconjugated MoAb, another 30 min incubation was performed with a conjugated isotype-specific second-step MoAb on ice. After a triple wash the cells were taken up in isotonic fluid and analysed on a FACScan analyser (Becton-Dickinson, Mountain View, CA, USA). A life gate was used to gate out rare dead cells and erythrocytes. The following MoAb were used for staining: biotin conjugated goat anti-mouse IgG_1 (Southern Biotechnology) at 12.5 μg/ml; rat anti-mouse IgE (Pharmingen, San Diego, CA, USA), at 10 μg/ml; rat anti-mouse B220 (clone RA3-6B2) and rat anti-mouse Thy-1 (clone 59-AD2.2), both as undiluted culture supernatant. Rabbit anti-rat IgG, F(ab')2-fragments FITC-conjugated (Cappel/Organon Technika, Oss, The Netherlands, 1/100 diluted), and R-phycoerythrin conjugated streptavidin (Caltag, San Francisco, CA, USA, 1/20 diluted) were used as second step reagents. To prevent cytophilic binding of the immunoreagents to FcγR, staining was performed after mild acid treatment in the presence of 2% normal goat serum (Dako A/S, Glostrup, Denmark). Stainings with rabbit anti-rat IgG, F(ab')2-fragments-FITC conjugated (Cappel/Organon), and R-phycoerythrin conjugated streptavidin (Caltag) alone were used as control.

Acid treatment. To remove cytophilic bound immunoglobulin molecules, spleen cells were treated with 0.05 M acetate buffer (pH 4.0) containing 0.085 M NaCl, 0.005 M KCl, and 1% FCS for 1 min on ice as described by Kumagai *et al.* [40]. To neutralize the acid to the range of pH 7.2, PBS, supplemented with 0.1 M hepes (Gibco Life Technologies, Paisley, UK), was added to the suspension. Next the cell suspension was underlain with FCS. The cells were pelleted and washed two times with HBSS. No decrease in viability was observed as determined by trypan blue exclusion.

Statistical analysis. Differences between groups were analysed using the Student's *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

IL-4 inhibits memory formation of TNP-KLH-specific B cells but not T cells

In a previous study we have shown that prolonged IL-4 treatment induced decreased TNP-specific IgG_1 and IgE responses after primary immunization, accompanied by a decreased memory formation for these two isotypes [19]. In order to determine whether IL-4 exerted this inhibitory effect on the TNP-KLH-specific B cells, T cells or both, we reconstituted irradiated mice with TNP-KLH-primed B cells from control-treated mice and TNP-KLH-primed T cells from IL-4 treated mice and vice versa. The B cells (> 98% B220$^+$ cells) and T cells (> 95% Thy-1$^+$ cells) were isolated 3 months after the last of 10 IL-4 or control administrations, to exclude direct effects of exogenous IL-4, for it is to be expected that no exogenous IL-4 is produced by this time. Furthermore, at this time no differences were found in the percentages and numbers of T and B cells between control and IL-4 treated mice (data not shown).

Irradiated control mice reconstituted with primed B cells from IL-4 treated mice displayed, after immunization with TNP-KLH, significantly lower TNP-specific IgG_1 and IgE levels when compared to mice reconstituted with primed B cells from control mice. These levels were not influenced by using T cells from either control or IL-4 treated mice (Fig. 1). Apparently, T cells from IL-4 treated mice were not able to inhibit or amplify memory B cells from control treated mice to give rise to TNP-specific IgG_1 or IgE production. Moreover, T cells from control mice failed to amplify the induction of TNP-specific B cells from IL-4-treated mice to produce TNP-specific IgG_1 or IgE levels comparable to those of control treated mice.

These results suggest that IL-4 exerted its inhibitory effect on the TNP-specific memory formation for IgG_1 and IgE at the B cell level, not influencing the memory T cell development.

$\gamma_1\epsilon$-Double positive B cells are increased in IL-4 treated mice after 10 IL-4 administrations

To study the effect at the B cell level more precisely, we examined the number of switched B cells in the spleens of control- and IL-4-treated mice by FACScan analysis. At days 2 and 3 after the last of 10 administrations the numbers and percentages of γ_1-single, ϵ-single and $\gamma_1\epsilon$-double positive B cells were determined (Table 1). On these two days only the number of $\gamma_1\epsilon$-double positive B cells were significantly increased (two-fold) in the IL-4-treated mice when compared with control mice. No major differences were seen when comparing the number of γ_1-single or ϵ-single positive B

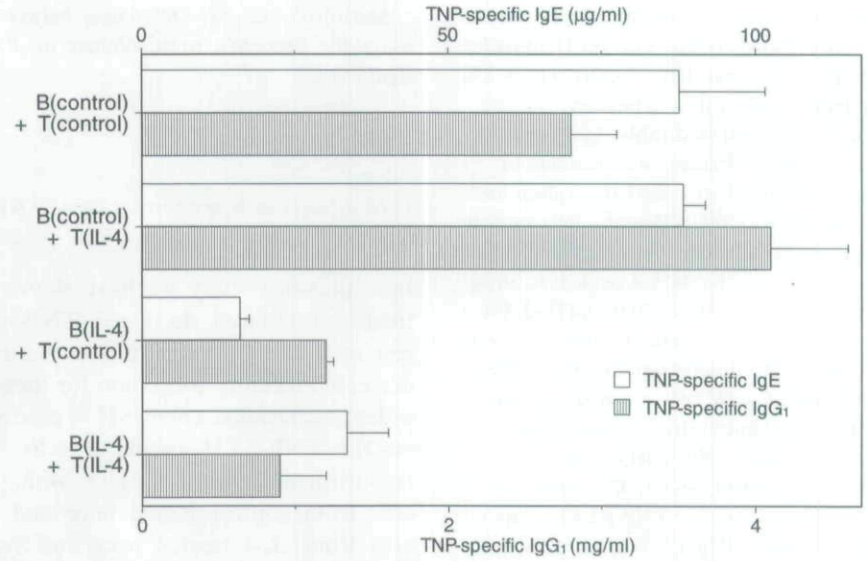

Fig. 1. Irradiated mice (6 Gy) were reconstituted with combinations of 4×10^6 purified B cells and 4×10^6 purified T cells from control and IL-4 treated mice as indicated. All reconstituted mice were boosted with 10 μg TNP-KLH at day 0. TNP-specific IgG_1 and IgE serum levels were determined at day 9. Results are represented as arithmetic mean ± SEM ($n = 4$–6). TNP-specific IgG_1 is expressed in mg/ml and TNP-specific IgE is expressed in μg/ml.

cells in the spleens of IL-4 treated mice and control mice at days 2 and 3 after the last of 10 administrations. Both in control and IL-4 treated mice, 40% of the γ_1-positive B cells in the spleen were also μ-positive (data not shown).

These results show that IL-4 treatment caused its effect mainly by increasing the pool of $\gamma_1\epsilon$-double positive B cells.

$\gamma_1\epsilon$-Double positive B cells can develop in either IgG_1- or IgE-secreting cells

To investigate whether the increase in $\gamma_1\epsilon$-double positive B cells would result in increased numbers of IgG_1- and IgE-secreting cells after isotype-specific stimulation, we used a cognate T–B cell culture system [37]. In this system T cell-depleted splenocytes from control and IL-4-treated mice were stimulated with antigen and CDC35 cells, a Th2 cell line specific for rabbit IgG (RIgG). In this culture system rabbit anti-mouse IgE (RAM-IgE), and rabbit anti-mouse IgG_1 ($RAM\text{-}IgG_1$) were used to selectively stimulate ϵ- and γ_1-positive B cells through their membrane-bound isotypes. TNP coupled to RIgG (RIgG-TNP) was used to stimulate TNP-specific B cells, whereas TNP-KLH and RIgG served as controls for the *in vitro* stimulation. Both TNP-KLH and RIgG were used as a control because stimulation by these two antigens gave a measurement of non-cognate activation of B cells in this system.

In all experiments this non-cognate activation leading to IgG_1- and IgE-producing cells at day 5 of the culture gave rise

Table 1. Number of switched B cells in spleens of control and IL-4 treated mice

	Control-treated		IL-4-treated	
Surface Ig expression	Day 2	Day 3	Day 2	Day 3
γ_1-single$^+$	58740 (53)	69300 (63)	59500 (54)	89600 (60)
ϵ-single$^+$	178 (0.2)	59 (0.1)	85 (0.1)	51 (0.1)
$\gamma_1\epsilon$-double$^+$	4450 (4.0)	5742 (5.2)	8500 (7.7)	8192 (5.5)

Surface marker expression evaluated by FACScan analysis of total spleen cells from control and IL-4-treated mice at days 2 and 3 after the last of 10 alginate administrations. Cell suspensions were pooled from two mice. Rare dead cells and erythrocytes were gated out. Results are the absolute number of positive cells (thousands). Number of lymphocytes per spleen based on the forward scatter/ side scatter plot were 1.1×10^8 in control mice both on days 2 and 3. In spleens of IL-4 treated mice the number of lymphocytes were 1.1×10^8 and 1.5×10^8 on days 2 and 3, respectively. Numbers in parentheses represent percentage of lymphocytes.

to less than 3% of the number of IgG_1- or IgE-producing cells that developed after specific stimulation. Addition of IL-4 (100 IU/ml) to the cultures resulted in a two-fold increase of this non-cognate activation (data not shown). Moreover, addition of IL-4 failed to increase the number of IgG_1- and IgE-secreting cells after 5 days of culture, when compared with the situation in which no IL-4 was added (data not shown). This indicated that the CDC35 cells themselves produced enough IL-4 after cognate stimulation to give rise to IgG_1- and IgE-secreting cells.

Kinetic studies had shown that a culture period of 5 days was optimal for the development of IgG_1- and IgE-secreting cells, when B cells were used from primed mice (data not shown). After 5 days of culture the numbers of IgG_1- and IgE-secreting cells, were determined by ELISA-spot assay. Results representative for two experiments showed that upon stimulation of T cell-depleted spleen cells, from IL-4 treated mice with the Th2 cell line in the presence of RAM-IgG_1, more than twice as many IgG_1-secreting cells were formed, as compared to the situation in which splenic B cells from control treated mice were used (Fig. 2C). A similar increase was seen when RAM-IgE was used as antigen in these cultures (Fig. 2C). That these differences were not caused by an increased proliferation of T cell depleted spleen cells from IL-4 treated mice is shown in Fig. 2A. The number of IgE-secreting cells was dramatically increased when spleen cells were used from mice treated with IL-4 in combination with RAM-IgE and, to a lesser extent with RAM-IgG_1, as antigen (Fig. 2B). These data suggested an increase in B cells already switched to IgG_1 (more than two-fold) and IgE (seven-fold) as a result of prolonged IL-4 treatment.

When rabbit anti-mouse $IgG_{2a/2b}$ (RAM-$IgG_{2a/2b}$) was used as stimulating antigen, 5585 IgG_1- and 288 IgE-secreting cells per culture of T-cell depleted spleen cells of control treated mice on day 5 were found. Upon culturing T cell-depleted spleen cells of IL-4-treated mice with RAM-$IgG_{2a/2b}$, 4296 IgG_1- and 169 IgE-secreting cells per culture were found on day 5. This result makes clear that the observed differences in IgG_1- and IgE-secreting cells were not caused by anti-Ig reactivity or FcR-mediated effects of RAM-IgG_1 and RAM-IgE. In that case, similar increases would have been found upon culturing with RAM-$IgG_{2a/2b}$.

The increase in IgG_1- and IgE-secreting cells is supported by the cumulative immunoglobulin levels produced during 5 days of culture. When stimulating ϵ-positive B cells in the pool of T cell-depleted spleen cells from control mice, using RAM-IgE, 56 ng/ml of IgE was produced. During the same period cells from IL-4 treated mice produced 217 ng/ml IgE (Table 2). As a result of the IL-4 treatment, an increase in the IgG_1 production was also seen when the γ_1-positive B cells of the T cell-depleted spleen cells were stimulated with RAM-IgG_1 (19 μg/ml when cells were used from control mice and 29 μg/ml when cells were used from IL-4 treated mice) (Table 2). Only marginal increases of IgG_{2a} production were found when T cell-depleted spleen cells from IL-4-treated mice instead of from control treated mice were stimulated with RAM-IgG_1 or RAM-IgE (Table 2). Addition of IL-4 to the cultures did not markedly increase the production of the measured isotypes (Table 2).

Collectively, these results point to a $\gamma_1\epsilon$-double positive B-cell population that can produce both IgG_1 and IgE upon stimulation via their isotype, and that is increased as result of IL-4 treatment.

γ_1-Positive secondary B cells can undergo sequential isotype switching to $\gamma_1\epsilon$-positive B cells

We next studied whether $\gamma_1\epsilon$-double positive B cells, also developed during the *in vitro* T–B cell cultures, as well as *in vivo*. Therefore, we examined, by flow cytometry, the

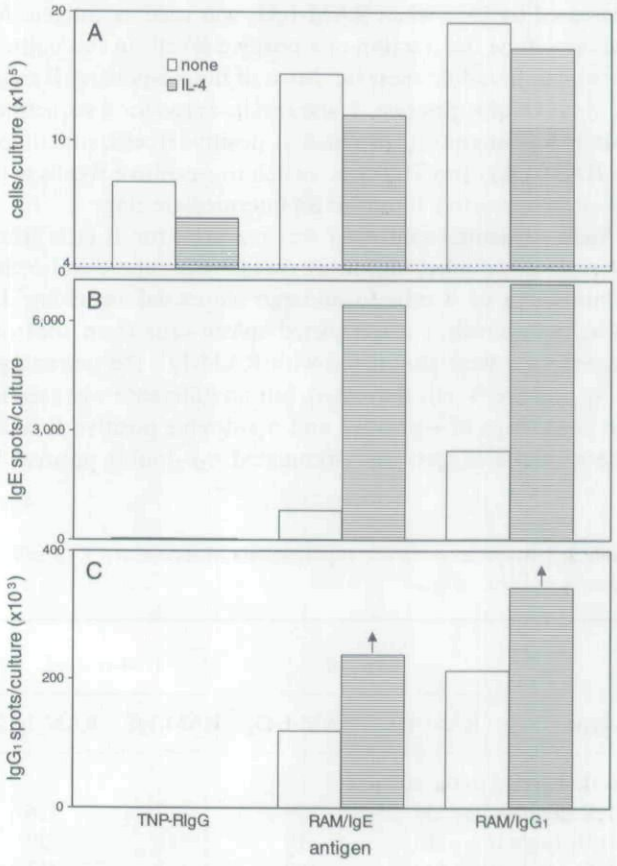

Fig. 2. IL-4 treatment increases the number of B cells switched to IgE and IgG_1 in the spleen, as compared to control mice. T cell-depleted spleen cells were cultured with irradiated (30 Gy) CDC35 cells in the presence of either 30 ng/ml TNP-RIgG, 10 ng/ml RAM-IgE or 10 ng/ml RAM-IgG_1. The total number of cells per culture (average number per eight pooled wells of 200 μl) at day 5 (A). The dashed line indicates the maximum number of cells in cultures stimulated with TNP-KLH and RIgG (A). The numbers of (B) IgE-producing cells and (C) IgG_1-producing cells, per culture (average number per eight pooled wells of 200 μl) were determined at day 5.

percentage of switched B cells in the pool of $B220^+$ cells at days 0 and 4 upon culturing in the presence of antigen and CDC35 cells (Table 3). To rule out a significant contribution of cytophyllic IgG_1 or IgE to the estimation of the percentage of γ_1-positive and ϵ-positive cells, cells were treated with acid before staining. This procedure has previously shown to remove all cytophilically FcR bound isotypes [40]. Moreover, all stainings were carried out in the presence of 2% normal goat serum, to prevent aspecific cytophillic binding of the immunoreagents to FcγR. At day 0 the percentages of ϵ-positive, γ_1-positive and $\gamma_1\epsilon$-double positive B cells were determined, and these cells were stimulated with RAM-IgG_1 or RAM-IgE. At day 0, no differences were seen in the percentages of ϵ-positive and $\gamma_1\epsilon$-double positive B cells when comparing control mice and IL-4 treated mice. IL-4 treatment had increased the percentage of γ_1-positive B cells by 16%. By day 4 the percentage of γ_1-positive B cells decreased by 13% when RAM-IgG_1 was used as antigen. At the same time the fraction of ϵ-positive B cells in this culture showed a three-fold increase. Most of these ϵ-positive B cells, were $\gamma_1\epsilon$-double positive. These results argue for a sequential switch in these cultures in which γ_1-positive B cells, stimulated by RAM-IgG_1 and Th2 cells, switch to ϵ-positive B cells with $\gamma_1\epsilon$-double positive B cells as an intermediate stage.

Such sequential switching was not seen for B cells from control treated mice, indicating that IL-4 is important for the commitment of B cells to undergo sequential switching. In cultures in which T cell-depleted spleen cells from control-treated mice were stimulated with RAM-IgE the percentage of γ_1-positive B cells decreased, but no difference was seen in the percentage of ϵ-positive and $\gamma_1\epsilon$-double positive B cells. These results suggest that stimulated $\gamma_1\epsilon$-double positive B cells differentiate to IgG_1- and/or IgE-secreting cells that do not express sIg, and are therefore no longer detectable by flow cytometry.

Table 2. Isotype levels in the supernatants of secondary T–B cell cultures *in vitro*

	Control		IL-4-treated	
Isotype	RAM/IgE	RAM/IgG_1	RAM/IgE	RAM/IgG_1
No IL-4 added to the cultures				
IgE (ng/ml)	56	279	217	426
IgG_1 (μg/ml)	10	19	11	29
IgG_{2a} (ng/ml)	130	160	183	313
IL-4 (100 IU/ml) added to the cultures				
IgE (ng/ml)	147	286	322	406
IgG_1 (μg/ml)	13	18	15	25
IgG_{2a} (ng/ml)	96	86	255	175

2.5×10^5/ml T cell-depleted spleen cells from control and IL-4-treated mice and 5×10^4/ml irradiated (30 Gy) CDC35 cells were cultured for 5 days in 8 wells of 200 μl in the presence of 10 ng/ml RAM-IgE or RAM-IgG_1. Results represent supernatant levels as determined by ELISA.

$\gamma_1\epsilon$-double positive B cells can explain the different cytokine requirements of memory IgG_1 and IgE responses

We have shown in adoptive transfer experiments that TNP-specific IgG_1 and IgE memory responses were not differentially influenced by T cells from control and IL-4 treated mice (Fig. 1). Moreover, it was shown that $\gamma_1\epsilon$-double positive B cells can develop both in IgG_1- and IgE-secreting cells *in vitro* (Fig. 2). Therefore, we next studied the cytokine dependence of memory B cells to become IgG_1- or IgE-secreting cells *in vivo*.

To this end, irradiated mice were reconstituted with TNP-primed spleen cells from control treated mice (Table 4). Neutralizing the presence of IL-4 by anti-IL-4 MoAb in these mice did not reduce the production of TNP-specific IgG_1, whereas it did partially reduce the production of TNP-specific IgE (Table 4). This production of TNP-specific IgE was 1820-fold higher than the IgE levels found in immunized non-reconstituted irradiated mice, and is 15% of the TNP-specific IgE level in reconstituted mice that received control MoAb. This indicated that the TNP-specific IgE response after immunization, of irradiated mice reconstituted with spleen cells from control-treated mice, was at least in part (15%) IL-4 independent. Using neutralizing MoAb against IL-5 and IL-6, TNP-specific IgG_1 and IgE memory responses were analysed in reconstituted recipient

Table 3. Surface Ig phenotype of B cells in secondary T–B cell cultures *in vitro*

	Surface Ig (sIg)				
	ϵ Total	ϵ^*	$\gamma_1\epsilon$	γ_1 Total	γ_1^*
Day 0					
Control	3	0	4	84	80
IL-4-treated	3	2	1	100	99
Day 4					
RAM/IgG_1 stimulation					
Control	2	1	1	82	81
IL-4-treated	9	2	7	87	80
RAM/IgE stimulation					
Control	0	0	1	87	86
IL-4-treated	4	2	2	78	76

ϵ^* = total $sIgE^+ - \gamma_1\epsilon$; γ_1^* = total $sIgG_1^+ - \gamma_1\epsilon$.

Results are expressed as percentages of $B220^+$ B cells in cultures with RAM-IgE or RAM-IgG_1 as stimulating antigen; 2.5×10^5/ml T cell-depleted spleen cells and 5×10^4/ml irradiated (30 Gy) CDC35 cells were cultured in 10 wells of 2 ml.

Table 4. Influence of cytokines on TNP-specific secondary IgG_1 and IgE responses after adoptive transfer

Treatment	TNP-specific IgG_1		TNP-specific IgE	
	Day 9	Day 12	Day 9	Day 12
Control	2847 ± 333	6720 ± 697	87.6 ± 14	61.9 ± 67
+ αIL-4	1376 ± 293	5366 ± 584	4.5 ± 1.0	9.1 ± 1.7
+ αIL-4 + αIL-5	2183 ± 554	4127 ± 1070	8.5 ± 0.4	12.4 ± 1.5
+ αIL-4 + αIL-6	1934 ± 513	3999 ± 463	6.6 ± 1.0	5.8 ± 0.7
+ αIL-5 + αIL-6	1483 ± 150	2553 ± 362	92.8 ± 8.9	65.0 ± 7.7
+ αIL-4 + αIL-5 + αIL-6	1593 ± 286	2332 ± 361	110.4 ± 12.4	57.1 ± 7.2
Irradiation control	66 ± 12	35 ± 0	0.015 ± 0.011	0.005 ± 0.001

Irradiated mice (6 Gy) were reconstituted with 1×10^7 TNP-KLH primed spleen cells from control-treated mice. Antibody treatment: control (GL113, 4 mg/mouse i.p.); αIL-4 (11B11, 10 mg/mouse i.p.); αIL-5 (TRFK-5, 2 mg/mouse i.p.), and αIL-6 (20F3, 2 mg/mouse i.p.). All reconstituted mice were boosted with 10 μg TNP-KLH adsorbed on alum i.p. Results are represented in μg/ml as arithmetic mean ± SEM ($n = 5$).

mice. The TNP-specific IgE memory responses turned out to be IL-5- and IL-6-independent, whereas the TNP-specific IgG_1 production was markedly decreased after these treatments.

Moreover, these results also suggest an additive IL-5 and IL-6 dependence of the TNP-specific IgG_1 production during memory responses. In the presence of these neutralizing antibodies the complete TNP-specific IgE response was IL-4-independent. This may indicate that γ_1-positive and/or $\gamma_1\epsilon$-double positive TNP-specific memory B cells, that are blocked to differentiate to IgG_1-secreting cells in the absence of IL-5 and IL-6 [18], can develop in IgE-secreting cells independently from IL-4.

As it was shown that IL-4 can commit B cells to undergo sequential isotype switching, by which $\gamma_1\epsilon$-double positive cells are formed, we also examined the cytokine requirement of TNP-specific memory B cells from IL-4-treated mice for production IgG_1 and IgE. As stated before, no differences were seen in the percentages of splenic T and B cells between control- and IL-4-treated mice at this point. It was found that even prolonged IL-4 treatment for 4 months after primary immunization did not induce differences in the cytokine dependence of the TNP-specific memory B cells generated (Table 5). IL-4 treatment reduced the generation of TNP-specific IgG_1 and IgE memory cells, but no differences were seen in the cytokine requirement for secondary TNP-specific IgG_1 and IgE responses in irradiated recipient mice reconstituted with either spleen cells from control or IL-4-treated mice. These results indicate that IL-4 treatment did not induce intrinsic changes in the TNP-specific memory B cells with respect to their cytokine dependence.

Memory B cells are less dependent on IL-4 and resistant to IFN-γ when becoming IgE-secreting cells in vivo

To more fully determine the role of cytokines in a non-adoptive transfer model, we immunized naive mice and mice primed 3 months previously with TNP-KLH during which either IL-4 was neutralized, or IFN-γ administered. The effect of neutralizing IL-4 on TNP-specific memory IgE responses has already been described [22], although in that study mice were boosted already 3 or 6 weeks after priming. However, for IFN-γ comparable studies were not described.

Immunization of mice with 10 μg TNP-KLH resulted in a primary TNP-specific IgE response starting at a level $\ll 0.03$ μg/ml at day 0 and reaching a level of 0.48 μg/ml

Table 5. Effect of prolonged IL-4 treatment on the cytokine dependence of TNP-specific memory B cells

Treatment	Days after transfer		
	7	9	12
TNP-specific IgG_1			
Control	130 ± 22	925 ± 348	1612 ± 394
+ αIL-4	77 ± 20	515 ± 101	1949 ± 379
+ αIL-5 + αIL-6	79 ± 12	517 ± 74	808 ± 123
+ αIL-4 + αIL-5 + αIL-6	72 ± 10	493 ± 71	609 ± 87
TNP-specific IgE			
Control	7.0 ± 1.7	33.4 ± 6.8	14.6 ± 1.6
+ αIL-4	0.2 ± 0.04	2.7 ± 0.6	4.0 ± 1.2
+ αIL-5 + αIL-6	2.9 ± 0.7	33.8 ± 7.7	20.1 ± 2.5
+ αIL-4 + αIL-5 + αIL-6	3.6 ± 1.5	29.0 ± 7.8	17.2 ± 4.5

Irradiated mice (6 Gy) were reconstituted with 1×10^7 TNP-KLH primed spleen cells from IL-4-treated mice. Antibody treatment: control (GL113, 4 mg/mouse i.p.); αIL-4 (11B11, 10 mg/mouse i.p.); αIL-5 (TRFK-5, 2 mg/mouse i.p.), and αIL-6 (20F3, 2 mg/mouse i.p.). All reconstituted mice were boosted with 10 μg TNP-KLH adsorbed on alum i.p. Results are represented in μg/ml as arithmetic mean ± SEM ($n = 5$).

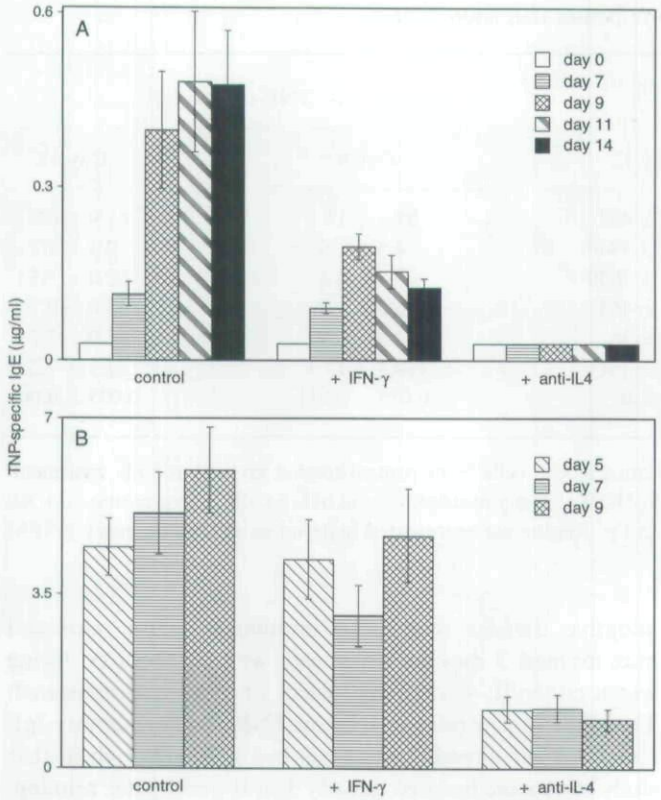

Fig. 3. Effect of anti-IL-4 and IFN-γ on (A) the primary and (B) the secondary TNP-specific IgE response. Mice were primed and boosted with 10 μg TNP-KLH adsorbed on alum, followed by either a control, anti-IL-4 or IFN-γ treatment as described in the Materials and Methods section. Serum levels of TNP-specific IgE are expressed as arithmetic mean ± SEM ($n = 5$).

at day 14. This increase could be blocked completely by neutralizing IL-4, indicating the IL-4 requirement of the primary TNP-specific IgE response. This anti-IL-4 treatment was more efficient in blocking the TNP-specific IgE response than was IFN-γ which inhibited the response up to 27% (Fig. 3A). On the other hand, the secondary IgE response could only be inhibited up to 24% at day 7 by treatment with anti-IL-4, suggesting a partial IL-4 independence of this response (Fig. 3B). TNP-KLH immunization in combination with a control treatment gave rise to 5.1 μg/ml TNP-specific IgE at day 7 as compared to 1.2 μg/ml that occurred in the presence of anti-IL-4. Moreover, this secondary TNP-specific IgE response could not be inhibited to any significant extent by IFN-γ treatment (Fig. 3B), pointing to the minor role that IFN-γ plays during the secondary IgE response.

DISCUSSION

In this paper we show that the effects of prolonged IL-4 treatment during memory formation after priming with TNP-KLH, that we have previously described [19], are primarily mediated at the B cell level, leaving the T cells unchanged. Major changes occur in the $\gamma_1\epsilon$-double positive B cell population which is increased significantly as a result of IL-4 treatment. Moreover, *in vitro* T–B cell cultures in which primed B cells were stimulated in an isotype-specific manner by rabbit anti-mouse isotype antibodies and a rabbit Ig-specific Th2 cell clone suggested the existence of a $\gamma_1\epsilon$-double positive B cell population that can develop into both IgG_1- and IgE-secreting cells.

The number of B cells switched to IgG_1 and IgE increased *in vivo* as a result of prolonged IL-4 treatment. This was found both at the membrane level and by stimulating B cells *in vitro*, independently of their antigen specificity, but isotype-specifically with rabbit anti-mouse isotype-specific antibodies, that were tested extensively for isotype specificity, and a rabbit Ig-specific Th2 cell clone. Moreover, it was found that upon stimulating γ_1-positive cells, IgE-secreting cells could be detected on day 5 of culture. These IgE-secreting cells can either originate from γ_1-positive cells that have undergone sequential isotype switching [2, 24], or can be the result of pre-existing $\gamma_1\epsilon$-double positive B cells [25] that differentiate to IgE-secreting cells after stimulation. When ϵ-positive B cells are isotype-specifically stimulated, both IgG_1- and IgE-secreting cells are found. The latter are most probably the result of $\gamma_1\epsilon$-double positive cells that have the ability to differentiate either into IgG_1- or IgE-secreting cells. These results imply that B cells exist that express IgE on their membrane, but have not yet undergone sequential isotype-switching. Moreover, they show that these B cells can be stimulated via this membrane IgE to develop in IgG_1-secreting cells. Another possibility could be that these $\gamma_1\epsilon$-double positive B cells, upon stimulation, can develop in cells that cosecrete these isotypes, for which evidence has been presented by Mandler *et al.* [24]. In all cases more IgG_1- and IgE-secreting cells per culture were found after culturing T cell depleted spleen cells of IL-4 treated mice than upon culturing T cell depleted spleen cells of control treated mice. These results are not caused by anti-Ig activity of the rabbit anti-mouse isotype specific antibodies used, because opposite effects were found upon culturing with RAM-$IgG_{2a/2b}$ as antigen. It has been described by Snapper *et al.* that Thy-1 positive B cells are highly enriched in IgE-secreting cells [41]. In this study we stimulated Thy-1-depleted spleen cells and determined the number of B cells that became IgG_1- and IgE-secreting cells. Therefore, Thy-1 depletion will not influence the numbers of IgE-secreting cells that were found as result of stimulation. In fact, Thy-1 depletion could have had a positive effect in lowering the background levels of cells that already secrete IgE independent of specific stimulation.

FACScan analysis revealed that, upon IL-4 treatment, the pool of $\gamma_1\epsilon$-double positive cells is profoundly increased *in vivo* at days 2 and 3 after IL-4 administration. On the other hand, no major changes occurred in the pool of ϵ-single positive B cells after IL-4 treatment, suggesting that the effect previously found, that IL-4 increased the total IgE serum levels [19], was mediated by increasing the pool of $\gamma_1\epsilon$-double

positive B cells. The results of both the *in vitro* T–B cell culture system and the *in vivo* adoptive transfer experiments provide evidence for such double positive B cells from which IgE-secreting cells originate, through the sequential isotype switching which has been reported by Yoshida *et al.* [2]. These authors provided molecular evidence for a sequential class switching from μ to ϵ via γ_1 [2]. This could explain why, during the process of switching to IgE in the LPS system, 75% of the ϵ-positive B cells co-express γ_1, representing 9% of the total B cell population [25]. In our study further evidence is provided for such a sequential class switching, detectable at the membrane level. When selectively stimulated, isotype-specifically via γ_1, with a rabbit Ig-specific Th2 clone and RAM-IgG_1 antibody as antigen, the number of γ_1-positive splenic B cells decreased, while there was an increase in the percentage of $\gamma_1\epsilon$-double positive B cells from 1 to 7% of all $B220^+$ B cells. This was only seen when B cells were obtained from mice that were repeatedly treated with IL-4. We therefore conclude that IL-4 commits γ_1-positive B cells to undergo a sequential switch to ϵ-positive B cells. However, it is also possible that IL-4 increases B cell populations, resulting in the possibility of detecting sequential isotype switching at the membrane level. A successive DNA deletion could explain the simultaneous expression of $sIgG_1$ and sIgE, if the γ_1 mRNA expression is stable and functional even after the switch to ϵ. Other mechanisms which allow the existence of $\gamma_1\epsilon$-double positive cells are the alternative splicing of long nuclear RNA and trans-splicing [42]. In both mechanisms, switching occurs without DNA recombination.

When studying lipopolysaccharide-activated murine B cells, IL-4-induced IgE class switching occurred predominantly through sequential isotype switching [24]. In this system it was also shown that LPS stimulation in the presence of IL-4 resulted in $\gamma_1\epsilon$-double positive cells that could produce IgG_1 as well as IgE. Recently, evidence for a successive class switching was reported in the human system by comparing $S\mu$–$S\epsilon$ junctions in IL-4 treated human B lymphoblastoid cells [43]. These composite switch regions contained $S\gamma$ sequences, indicating that sequential switching had occurred. The data presented in our study show that sequential isotype switching is also likely to occur during secondary responses. It is shown that γ_1-positive primed B cells upon stimulation develop in $\gamma_1\epsilon$-double positive B cells that can differentiate in both IgG_1- and IgE-secreting cells.

The fact that memory B cells can undergo sequential isotype switching makes it possible that they are not tightly controlled by T cells. This could be the reason that the TNP-specific IgG_1 and IgE responses in irradiated control mice are not influenced by using T cells from control or IL-4 treated mice. Using an adoptive transfer system, we studied the cytokine dependence of TNP-specific secondary IgG_1 and IgE responses. Such a system was chosen, because it is known that secondary IgE responses are preferentially enhanced after transferring spleen cells in irradiated control mice [19, 44]. It was found that IL-5 and IL-6 were necessary for maximum *in vivo* TNP-specific IgG_1 responses. IL-4 treatment during the primary response did not change this cytokine dependence. These results, with respect to IgG_1, are in line with the observation that IL-4 can induce membrane bound IgG_1 on B cells, whereas IL-2 and IL-5 are necessary for subsequent IgG_1 secretion [45]. By using the adoptive transfer system it was clearly shown that the TNP-specific secondary IgE response is partially IL-4-independent in that it could not be completely inhibited by neutralizing antibodies against IL-4. This IL-4-independent IgE level is even higher than the IL-4 independent IgE response in boosted mice (Fig. 3B). Surprisingly, in the absence of IL-5 and IL-6 no inhibition of the secondary TNP-specific IgE response by anti-IL-4 MoAb was detectable. A possible explanation for these results is that TNP-specific IgG_1 memory B cells do not mature to IgG_1-secreting plasma cells in the absence of IL-5 and IL-6, since these two cytokines are likely to be involved in this process [16–18]. The γ_1-positive, or $\gamma_1\epsilon$-double positive B cells that are arrested could subsequently switch to become ϵ-positive B cells for which they need less IL-4, or eventually can switch independently of the presence of IL-4. These B cells do not appear to be dependent on IL-5 and IL-6 to become IgE-secreting cells [18]. Other factors that induce or potentiate secretion of IgE, like IL-13 in the human system [46], and that are not analysed in our study could be involved in the development of IgE-secreting cells. Although it has not yet been ascertained that IL-13 induces IgE secretion by mouse B cells, this might be possible under certain circumstances.

Recently, it has been described that neutralizing anti-IL-4 antibodies, when complexed with IL-4, can serve as reservoirs for long-term (at least 9 days) delivery of cytokines *in vivo* [47]. It is possible that, in the absence of IL-5 and IL-6, a persistent low dose of IL-4, as a result of IL-4–anti-IL-4 complexes, is sufficient to allow differentiation of γ_1-positive and/or $\gamma_1\epsilon$-double positive B cells in IgE-secreting cells. The presence of additional IL-4 in combination with neutralization of IFN-γ did not increase the TNP-specific secondary IgG_1 and IgE response (data not shown). This indicates that the endogenous production of IL-4 was already sufficient to result in a maximum response in irradiated mice reconstituted with spleen cells from control treated mice.

We next studied the dependence on IL-4 and the susceptibility to IFN-γ of primary and secondary TNP-specific IgE responses. With respect to IFN-γ, no studies have been described in which its role in memory IgE responses is established. We found that the primary TNP-specific IgE response is completely IL-4-dependent and can be inhibited extensively by IFN-γ, whereas the secondary TNP-specific IgE response is partially IL-4-independent, and cannot be inhibited by IFN-γ in a concentration that does inhibit the primary TNP-specific IgE response. The IL-4-independent part of the IgE response is probably the result of memory B cells already switched to IgE, whereas the IL-4-dependent component can result from B cells switched to IgG_1, which

need IL-4 to switch further to IgE. Finkelman *et al.* described that memory IgE responses for TNP-KLH are, like primary responses, dependent on IL-4 [22]. This discrepancy with our results is most probably the result of the different resting periods between priming and boosting used in these studies. Using sheep red blood cells as thymus dependent antigen it was found that the maximum memory formation for IgG_1 was achieved only 12 weeks after priming [48]. It is quite possible that during the 3 weeks after priming, memory B cells switched to IgE are not formed to an extent that allowed detection of IgE in the absence of IL-4.

Overall, these results make clear that sequential isotype switching not only plays an important role in the generation of primary, but also in secondary IgG_1 and IgE responses. Such sequential isotype switching, most probably, causes the differences in cytokine requirements that are observed when examining the capacity of unprimed naive B cells versus memory B cells to become IgG_1- or IgE-secreting cells. Furthermore, it is shown that *in vivo* also the IgE-inducing capacity of IL-4 is mediated through the generation of $\gamma_1\epsilon$-double positive cells, that can be stimulated to become both IgG_1- and IgE-secreting cells.

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