Prolonged *In Vivo* IL-4 Treatment Inhibits Antigen-Specific IgG_1 and IgE Formation

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IL-4 is obligatory for primary IgE responses, whereas primary IgG_1 and secondary IgE responses are partially IL-4 independent. To investigate the effect of IL-4 on the antigen-specific memory formation for these isotypes, BALB/c mice were treated after primary TNP-KLH immunization with recombinant IL-4 for a period of 4 months. This prolonged presence of a high IL-4 level resulted in increased serum levels of total IgG_1 and IgE, whereas total IgG_{2a} did not change. The expression of CD23, but not $I\text{-}A^d$, increased on the splenic B cells. IL-4 treatment did not affect the IL-4 production by Con A stimulated spleen cells, whereas it did decrease the IFN-γ production. In the same mice the TNP–specific IgG_1 and IgE serum levels, however, were decreased. Similar results were found when the antigen was continuously present during the IL-4 treatment. Furthermore, it was shown that IL-4 decreased the formation of IgG_1 and IgE memory cells. These results point to different effects of IL-4 in regulating antigen-specific and bystander responses.

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INTRODUCTION

IL-4 is a pleiotropic lymphokine, produced mainly by activated T cells, which has a number of activities on B cells [1]. For example IL-4 is known to enhance proliferation of B cells in response to soluble anti-Ig [2, 3]. Other effects of IL-4 are the increased expression of MHC class II [4–7] and CD23 ($Fc_\epsilon RII$) on resting B cells [8–10]. IL-4 also stimulates non-lymphoid haematopoietic cells, especially macrophages and mast cells [1, 11]. IL-4 is obligatory for IgE synthesis, whereas it has an enhancing effect on IgG_1 production. Functional studies indicate that IL-4 is a 'switch inducing' factor. It alters the chromatine structure of the $S\gamma_1$ region [12] and induces accumulation of germline γ_1 and ϵ transcripts [13–16]. Treatment of mice with neutralizing antibodies to IL-4 or IL-4 receptor completely inhibits the production of IgE, while the IgG_1 response to various antigens is only marginally affected [17–19]. Similarly IgE responses are undetectable in nematode infected mice which are made IL-4 deficient by gene targeting. IgG_1 can be detected in these mice, but the level is only one-sixth that of control mice [20].

Abbreviations: GAM, goat-anti-mouse; KLH, keyhole limpet hemocyanin; Ra, rat; RaAM, rat-anti-mouse; RARa, rabbit-anti-rat; RAM, rabbit-anti-mouse; SA-HRP, horse radish peroxidase-conjugated streptavidin; TD, thymus-dependent.

All the mentioned effects of IL-4 are inhibited by IFN-γ [1, 8, 10, 21]. On the other hand, IFN-γ is directly involved in stimulating IgG_{2a} synthesis, which can be inhibited by IL-4 [22–24]. Thus, the relative presence of functional IL-4 and IFN-γ determines whether and how much IgE can be produced after optimal stimulation. IgE memory responses are partially IL-4 dependent and thereby differ from primary responses that are completely dependent upon the presence of IL-4. It was hypothesized that this might be due to memory B cells that had already switched to IgE expression during the primary response [19].

The aim of this study was to investigate the effect of IL-4 on the antigen-specific memory formation for IgG_1 and IgE. To this end, BALB/c mice were treated continuously with recombinant IL-4 after primary TNP-KLH immunization for a period of 4 months. We used a method for cytokine administration that allowed persistent IL-4 levels for a prolonged period of time [25]. In this report we demonstrate that prolonged presence of a high IL-4 level increases the background level of IgG_1 and IgE, whereas IgG_{2a} is not influenced. On the contrary, IL-4 decreases the TNP-specific IgG_1 and IgE responses. Adoptive transfer experiments revealed also an inhibition of the formation of TNP-specific IgG_1 and IgE memory-B cells by IL-4. These results indicate that the level of functional IL-4 directs the balance between

the production of polyclonal and antigen-specific IgG_1 and IgE in TD immune responses.

MATERIALS AND METHODS

Mice. Female BALB/c mice were bred and maintained in the animal facilities of our own department. All mice were at an age of 12–16 weeks at the start of the experiments. Mice 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.

Immunization and anti-IL-4 treatment. KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 kDa of KLH (as determined spectrophotometrically) [26] by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA). Mice were injected with 0.2 ml containing 10 or 100 μg TNP-KLH adsorbed on 2 mg alum i.p.

IL-4 was neutralized by 10 mg rat MoAb specific for IL-4 (11B11) [27]. Rat MoAb specific for *E. coli* β-galactosidase (GL113) [25] was used as an IgG_1 isotype control.

IL-4 treatment. Mice were implanted i.p. with 2×10^6 CV-1/IL-4 cells encapsulated in alginate every 2 weeks as described earlier [25]. The monkey CV-1 cells were stably transfected with the murine IL-4 gene under control of the SV40 promotor. These cells were a gift of Dr N. Arai (DNAX Research Institute, Palo Alto, CA, USA). Briefly, cells were grown on Cytodex 3 beads (Pharmacia, Uppsala, Sweden). Fully covered beads were harvested after 2 days of culture and washed three times with sterile saline. The volume of the pellet was determined and 1 volume of saline followed by 2 volumes of a 1.2% sterile solution of cellprep alginate (FMC Bioproducts, Rockland, PA, USA) were combined in a syringe. This suspension was squirted through a 25-gauge needle into a fresh 80 mM $CaCl_2$ solution. The encapsulated cells were washed three times with saline and 2×10^6 encapsulated cells in 1 ml were injected i.p. with a 19-gauge needle. Empty beads encapsulated in alginate were used as control for the IL-4 treatment. No immunological effects were observed after injection of alginate encapsulated beads.

Adoptive transfer of spleen cells. Spleens of contol treated and IL-4 treated mice were removed under aseptic conditions and single cell suspensions were prepared. Ten million cells were transferred via the tail vein into naive recipients. The recipients had been sub-lethally irradiated [6 Gy] with a Caesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) 1 day before cell transfer. All reconstituted mice were immunized with 10 μg TNP-KLH adsorbed on alum i.p. immediately after transfer.

Isotype-specific ELISA. Total serum IgE and IgG_1 levels were measured by isotype-specific ELISA as described previously [21]. Detection limits for the IgE and IgG_1 ELISA were 0.5 ng/ml and 0.2 ng/ml, respectively. TNP-KLH-specific IgG_1 was measured by direct ELISA. Plates were coated with TNP-KLH (3 μg/ml), blocked with 1% BSA and incubated with the appropriate dilutions of serum. Subsequent steps were biotin-conjugates GAM/IgG_1 (Southern Biotechnology, Birmingham, AL, USA), SA-HRP (Jackson Immunoresearch, West Grove, PA, USA) and the substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma, St. Louis, MO, USA). The detection limit of this ELISA was 0.2 ng/ml. TNP-specific IgE was quantified in a modified sandwich ELISA. Plates were coated with monoclonal RaAM/IgE (EM95, 2 μg/ml) [28] and incubated overnight at 4°C with diluted serum samples. TNP modified with alkaline phosphatase (kind gift of Dr A. J. M. van den Eertwegh, MBL-TNO, Rijswijk, The Netherlands) was used as second step. The ELISA was further developed by using Sigma 104 phosphatase substrate (Sigma). The detection limit of this ELISA was 1 ng/ml. Highly purified monoclonal anti-TNP antibodies of the appropriate isotypes were used for the standard curves in both antigen-specific assays.

In order to determine total serum IgG2a, plates were coated with GAM/IgG2a (Southern Biotechnology) blocked, washed and incubated with the appropriate dilutions of serum. The assay was further developed by applying biotinylated RaAM/IgG2a (Pharmingen, San Diego, CA, USA) as second step, SA-HRP and ABTS substrate. The detection limit was 0.3 ng/ml. Murine IgG_{2a} was used for the standard curves.

Flow cytometric analysis. Cells (2.5×10^5) were incubated on ice for 30 min with the appropriate MoAb, either as undiluted culture supernatant or carefully 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 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 unconjugated rat-anti-mouse MoAbs were used as undiluted culture supernatants: MT4 (CD4, clone H129.19, Ra IgG_{2a}), Lyt-2 (CD8, clone 53-6.7, Ra IgG_{2a}), B220 (CD45RA, clone RA3-6B2, Ra IgG_{2a}), Thy-1 (clone 59-AD2.2, Ra IgG_{2a}), CD3 (clone KT3, Ra IgG_{2a}), CD45 (clone 30-G12, Ra IgG_{2a}), F4/80 Ag (clone F4/80, Ra IgG_{2b}) and anti-granulocyte (clone RB6-8C5, Ra IgG_{2b}). All these MoAb's were kindly provided by Professor W. van Ewijk from our department. The following MoAbs were optimally titrated RaAM/I-A^d-FITC (Pharmingen) at 10 μg/ml, RaAM/CD23 (Pharmingen) at 10 μg/ml, GAM/Ig-PE (CLB, Amsterdam, The Netherlands, 1/50 diluted), and RARa/IgG, $F(ab')_2$-fragments-FITC conjugated (Cappel/Organon Technika, Oss, The Netherlands, 1/100 diluted).

Con A stimulation of splenocytes. Spleen cells (2×10^6/ml) were cultured in 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 in four replicate wells of a 24-well flat-bottom plate (1 ml/well) with 10 μg/ml Concanavalin A (Sigma). After 48 h culture supernatants were harvested and stored at −70°C before use.

Determination of cytokines. IL-4, IL-10 and IFN-γ were determined in ELISA as described previously [29–31]. IL-4 was also determined in a CT.4S bioassay (kind gift of Dr L. Nagelkerken, IVEG-TNO, Leiden) with a detection limit of 0.05 μg/ml [32]. The detection limits of the ELISAs were, 0.2 ng/ml, 3 U/ml and 0.2 ng/ml, respectively. IL-6 was determined in a bioassay using B9 indicator cells as described [33]. The detection limit of this bioassay was 0.1 U/ml.

RESULTS

Secondary IgE responses are partially IL-4 independent

Mice immunized with 10 μg TNP-KLH developed a primary total IgE response that reached a level of 3.7 μg/ml at day 14. Secondary immunization at 3 months after priming resulted in a total IgE response with a maximum level of 8.9 μg/ml at

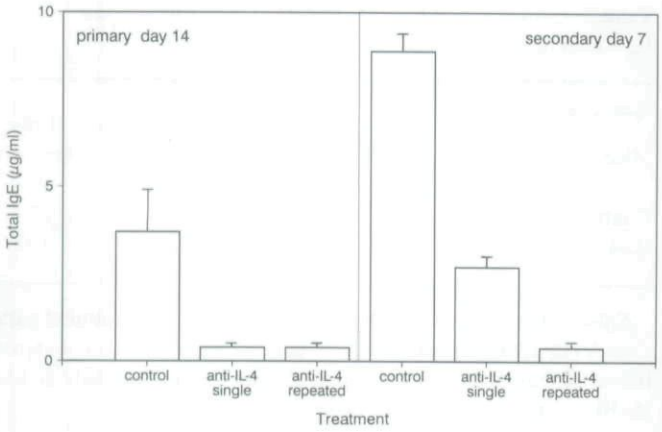

Fig. 1. Primary and secondary serum peak IgE responses in presence and absence of IL-4. Mice were primed and boosted with 10 μg TNP-KLH adsorbed on alum and were injected with either 10 mg GL113 (rat IgG_1 isotype control) or 10 mg 11B11 (anti-IL-4) once, on the day of immunization, or every 2 weeks starting at primary immunization till the end of the secondary response. Results are expressed as arithmetic mean ± SEM ($n = 5$).

day 7. The primary IgE response could be completely blocked by anti-IL-4 treatment indicating the absolute requirement of IL-4 in the induction of a primary IgE response (Fig. 1). Anti-IL-4 treatment at the start of a secondary immune response did not completely inhibit the secondary IgE response. At day 7 a maximum IgE level of 2.7 μg/ml, 30% of the control situation, was observed. On the other hand, mice that were continuously treated with anti-IL-4 did not show primary or secondary serum IgE responses (Fig. 1).

Phenotypic analysis of IL-4 treated mice

In order to investigate the effects of IL-4 on secondary TNP-specific IgG_1 and IgE responses mice were treated continuously with IL-4 for 4 months after primary immunization with 100 μg TNP-KLH. Treatment did not result in any symptoms of morbidity in all experiments. After this period the cellular composition of the spleen of control and IL-4 treated mice was analysed by flow cytometry. The results (Table 1) show that prolonged IL-4 treatment consistently reduced the absolute numbers of splenic $CD3^+$ (by 20%) and Thy-1 (by 15%) T cells as compared to control treated mice. Staining for CD4 and CD8 showed that this decrease was primarily due to a decrease in the number of $CD4^+$ cells. IL-4 did not influence the absolute number of $B220^+$ and slg^+ cells. On the other hand, the absolute numbers of macrophages ($F4/80^+$) and granulocytes increased by 300% and 75%, respectively. Prolonged IL-4 treatment apparently led to disturbances in cell numbers of the main cell populations in the spleen. The alterations in cell populations and their localization was confirmed by immunohistological stainings of spleen sections of IL-4 and control treated mice (data not shown).

In IL-4 treated mice, no IL-4 could be detected in the serum as determined by both IL-4 specific ELISA and bioassay (data not shown). In order to measure IL-4 driven phenomena other than IgE induction, the expression of CD23 and $I\text{-}A^d$ on lymphocytes was studied by flow cytometric analysis. To this end spleen cells were stained with anti-$I\text{-}A^d$ and anti-CD23 antibodies at day 1, 2 and 3 after last CV-1/IL-4 administration (Table 2). FACSCAN analysis showed that in the IL-4 treated group the number of CD23 middle and high expressing lymphocytes gated on the basis of forward scatter/side scatter plot was increased at the expense of CD23 low expressing lymphocytes. In the spleens of IL-4 treated mice, the $I\text{-}A^d$ expression was increased particularly in the intermediate expressing lymphocytes at the expense of the low expressing population. No change in $I\text{-}A^d$ expression was observed in the high expressing lymphocyte subpopulation (Table 2).

To analyse the effects of IL-4 on the $I\text{-}A^d$ and CD23 expression of B cells double-stainings with GAM/Ig and

Table 1. Phenotype of splenocytes after prolonged IL-4 treatment

	Positive cells		Number of cells ($\times 10^7$)	
Antigen	Control	IL-4	Control	IL-4
B220	30.4	26.1	3.8	3.5
slg	25.8	27.9	3.2	3.8
Thy-1	52.7	41.3	6.6	5.6
CD3	52.7	39.3	6.6	5.3
CD4	33.8	25.6	4.2	3.5
CD8	14.5	11.5	1.8	1.6
CD45	85.1	74.6	10.6	10.1
F4/80	0.9	3.1	0.1	0.4
Granulocyte	3.4	5.4	0.4	0.7

Surface marker expression evaluated by FACSCAN analysis of total spleen cells from control or IL-4 treated mice. Cell suspensions were pooled from two mice. Rare dead cells and erythrocytes were gated out. The results are representative for three independent experiments.

Table 2. Effect of prolonged IL-4 treatment on CD23 and $I\text{-}A^d$ expression of lymphocytes

		Expression					
		Low		Middle		High	
Treatment	Ag	Per cent	MCF	Per cent	MCF	Per cent	MCF
Control	CD23	61	146	13	282	26	493
IL-4	CD23	42	161	21	281	36	546
Control	$I\text{-}A^d$	51	163	18	313	31	549
IL-4	$I\text{-}A^d$	35	178	32	309	33	551

Percentage of splenic lymphocytes gated on the forward scatter/side scatter plot. MCF is mean channel fluorescence. Cell suspensions were pooled from two mice. The results are representative for five independent experiments.

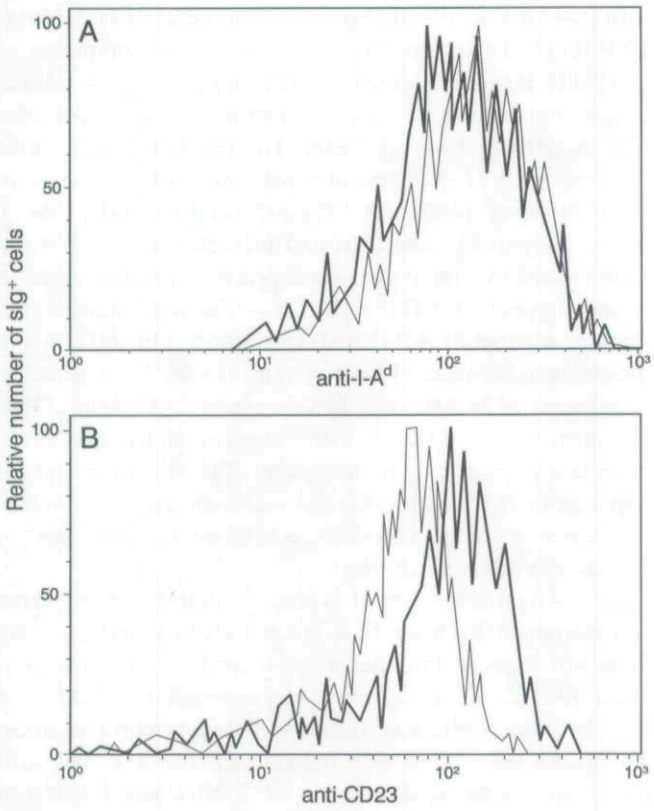

Fig. 2. Expression of CD23 and I-A^d on B cells on day 1 after the last of 10 IL-4 treatments. (A) Histogram representing staining of slg^+ spleen cells with anti-I-A^d antibody. (B) Histogram representing staining of slg^+ spleen cells with anti-CD23 antibody. The thick line represents the staining of cells from IL-4 treated mice and the thin line that of control mice. Cell suspensions from two mice were pooled. The results are representative for three individual experiments.

respectively anti-I-A^d and anti-CD23 were done. This study revealed that B cells from control treated mice were already positive for I-A^d. Apparently IL-4 treatment did not result in a further increase of this expression, whereas expression of CD23 on these B cells showed a significant increase (Fig. 2). The number of intermediate I-A^d positive B cells increased as a result of prolonged IL-4 treatment, as was also seen after staining of lymphocytes with anti-I-A^d.

IL-4 treatment reduces the basal IFN-γ production in the spleen

The influence of prolonged treatment on the cytokine production profile was determined by measuring the production of IL-4, IL-6, IL-10, and IFN-γ in cultures of Con A stimulated spleen cells at day 1 after the last injection of encapsulated CV-1/IL-4 cells. At that time, IL-4 treated mice displayed elevated total IgE and total IgG_1 serum levels. In IL-4 treated mice the concentration of IFN-γ was reduced 4.5-fold from 1.71 ng/ml in the sup of control mice to 0.38 ng/ml the sup of IL-4 treated mice. No such reduction was observed for the cytokines IL-4, IL-6, and IL-10 as shown in Table 3. Similar results were obtained at days 2 and 3 after the last of 10 CV-1/IL-4 administrations (data not shown).

Table 3. Cytokine profile of splenocytes 1 day after the last of 10 IL-4 administrations

Mice	IL-4 (ng/ml)	IL-6 (U/ml)	IL-10 (U/ml)	IFN-γ (ng/ml)
Control	0.24	701	19.2	1.71
IL-4	0.22	632	21.2	0.38

Spleen cells (2×10^6 ml) pooled from two mice were cultured with Con A (10 μg/ml) for 48 h in four replicate wells. The supernatants harvested from these wells were pooled prior to cytokine analysis. 1 U IL-10 is approximately 1 pg.

Primary TNP-specific IgG_1 and IgE responses are inhibited by IL-4 treatment

Previous studies have demonstrated the involvement of IL-4 in IgE and IgG_1 responses *in vivo* and *in vitro*. In order to study the effect of continuous IL-4 treatment during primary immunization on the antigen-specific and total serum IgG_1 and IgE responses, BALB/c mice were immunized with either 100 μg TNP-RIgG or 100 μg TNP-KLH. Mice were treated with IL-4 by injecting alginate encapsulated CV-1/IL-4 cells every 2 weeks. This treatment was carried out for 4 months. During this period the serum levels for IgG_1 and IgE were monitored. Immunization with 100 μg TNP-KLH led to a response of approximately 2 mg total IgG_1, both in control and IL-4 treated mice at day 14. The total IgE response at this time point in control and IL-4 treated mice was 0.5 and 1.5 μg/ml, respectively. As a result of the IL-4 treatment an increase in total serum IgG_1 was observed during the 4 months of treatment: control mice exhibited an IgG_1 plateau level of 3.2 mg/ml whereas IL-4 treated mice displayed an IgG_1 level of 13.7 mg/ml (Fig. 3A). The total IgE levels in the serum of IL-4 treated mice immunized with 100 μg TNP-KLH displayed a more profound increase (from 1.0 to 12.3 μg IgE/ml during the first month), which reached a plateau of 153.6 μg IgE/ml after 4 months of IL-4 treatment (Fig. 3B). In the same period, the total serum IgE levels in control mice increased to 3.8 μg IgE/ml (Fig. 3B).

In order to determine whether the increase in serum IgG_1 was antigen-specific, a TNP-specific IgG1 ELISA was performed. IL-4 treatment resulted in a seven-fold decrease of TNP-specific IgG_1 at day 86 of treatment (Fig. 3C). A similar decrease was seen for TNP-specific IgE (Fig. 3D). No increase in total serum IgG_{2a} was observed after immunization with TNP-KLH and prolonged IL-4 treatment (data not shown).

Similar results were obtained when mice were immunized with 100 μg TNP-RIgG. Total serum IgE of control mice stayed at a level of 1.8 μg/ml whereas in IL-4 treated mice it

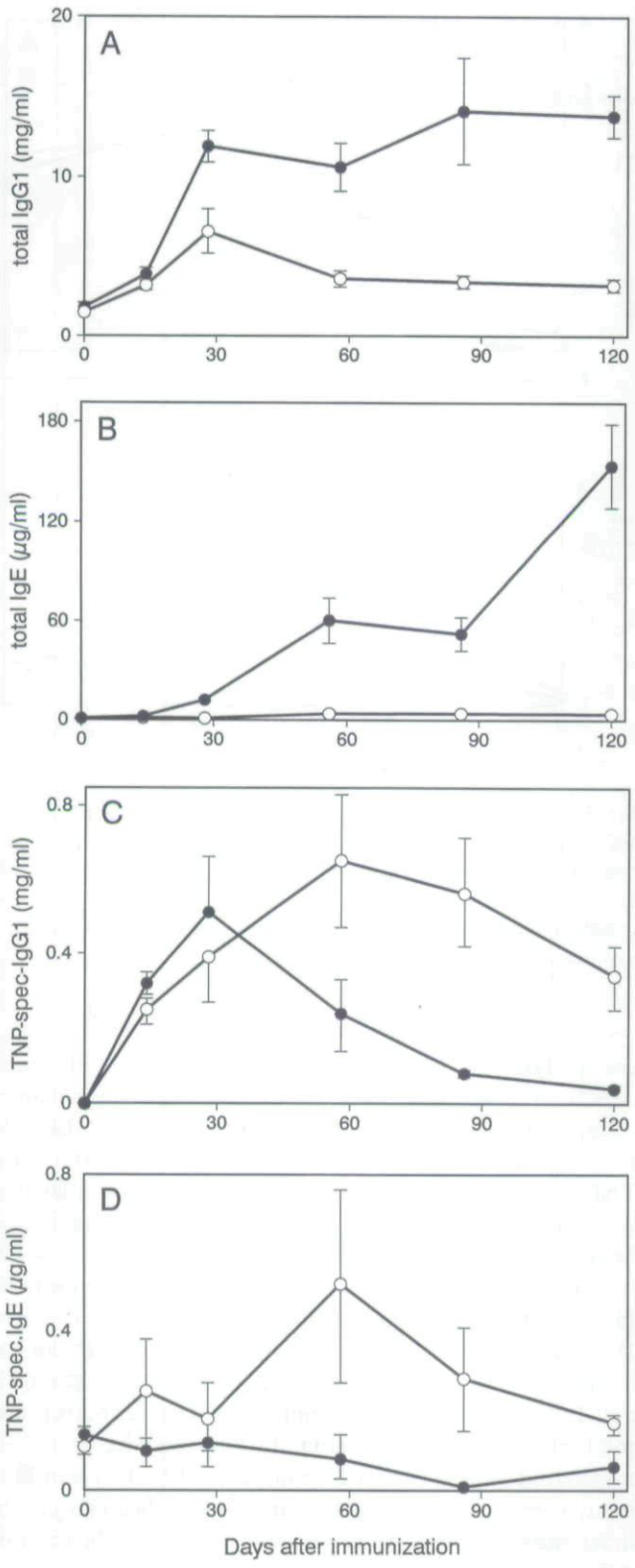

Fig. 3. Effect of IL-4 on total and TNP-specific IgE and IgG_1 levels. Mice were immunized with 100 μg TNP-KLH adsorbed on alum followed by a control treatment (○) or a prolonged IL-4 treatment (●) every 2 weeks. Serum levels of total IgG_1 (A), and IgE (B), and TNP-specific IgG_1 (C) and IgE (D) serum levels were determined by ELISA, and are expressed as arithmetic mean±SEM ($n = 5$).

reached a level of 29.0 μg/ml after 4 months treatment (data not shown). The IgG_1 serum levels were significantly elevated by IL-4 treatment (after control treatment 2.1 mg/ml and IL-4 treatment 14.5 mg/ml) (data not shown).

Treatment of mice with encapsulated CV-1/IL-4 cells every 2 weeks, without immunization with TNP-KLH, resulted also in high levels of total serum IgG_1 and IgE, respectively 17.9 mg/ml and 57.4 μg/ml at day 70 (data not shown). These levels are comparable with the levels of total serum IgG_1 and IgE that occur after treatment with CV-1/IL-4 cells in combination with immunization with TNP-KLH. IL-4 treatment alone increased the serum levels for TNP-specific IgG_1 from 22 μg/ml (day 0) to 93 μg/ml (day 70) (data not shown). These results indicate that prolonged IL-4 treatment leads to a strong polyclonal activation of B cells, part of which are specific for TNP.

IL-4 inhibits the hyperexpression of TNP-specific IgG_1 and IgE induced by repeated antigen exposure

In order to investigate whether the inhibition of the TNP-specific IgG_1 and IgE responses by IL-4 was the result of a lack of antigen, the IL-4 treatment was repeated under conditions of repeated antigen exposure. To this end mice received a primary immunization with 100 μg TNP-KLH on alum on day 0 and starting at day 14 100 μg TNP-KLH in saline every 2 weeks in conjunction with a control- or IL-4 treatment. Repeated antigen exposure itself resulted in high levels of total serum IgG_1 (20.0 mg/ml). Moreover, because of this repeated antigenic exposure practically 100% of this serum IgG_1 was antigen-specific at day 56. IL-4 treatment of mice subjected to continuous antigen exposure resulted in 15.5 mg/ml total serum IgG_1, with only 56% being TNP-specific (Fig. 4, upper part). IL-4 treatment resulted in a higher serum level of total IgE (65.9 μg/ml) when antigen was continuously present (after control treatment: 8.5 μg/ml). The TNP-specific fraction, however, decreased to 0.4% after IL-4 treatment (after control treatment 21%; Fig. 4, lower part).

No differences in the amount of KLH-specific IgE were found both in control and IL-4 treated mice, when using KLH modified with horseradish peroxidase instead of TNP modified with alkaline phosphatase in ELISA (data not shown).

Mice with elevated serum IgG_1 and IgE levels still respond to a booster immunization

To determine whether IL-4 treated TNP-KLH immunized BALB/c mice with elevated serum IgE and IgG_1 levels could still respond to a secondary immunization, such mice were boosted with 10 μg TNP-KLH. This dose of TNP-KLH is known to give an optimum IgE response. On day 7, an increase in TNP-specific serum IgG_1 was observed which reached a plateau of approximately 2.6 mg/ml at day 21. This plateau did not differ significantly for the IL-4 treated and control groups (Fig. 5A). The TNP-specific IgE levels also did not differ between the control and IL-4 treated group

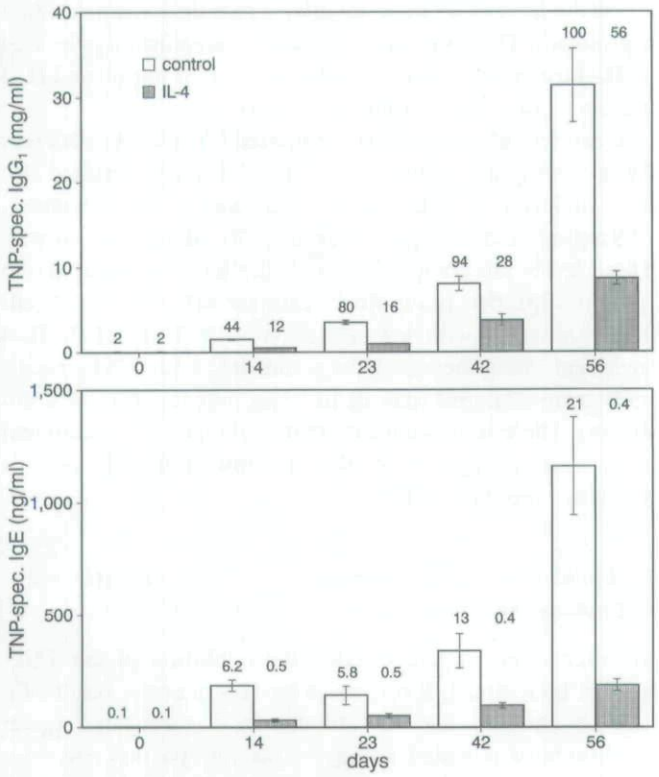

Fig. 4. Mice were i.p. immunized with 100 μg TNP-KLH adsorbed on alum on day 0 followed by injection of 100 μg TNP-KLH in saline every 2 weeks. The control group received a control treatment every 2 weeks, whereas the IL-4 group received CV-1/IL-4 cells encapsulated in alginate every 2 weeks. Results are expressed as arithmetic mean ± SEM ($n = 5$). The numbers in the Fig. represent the percentage TNP-specific serum Ig versus total serum Ig.

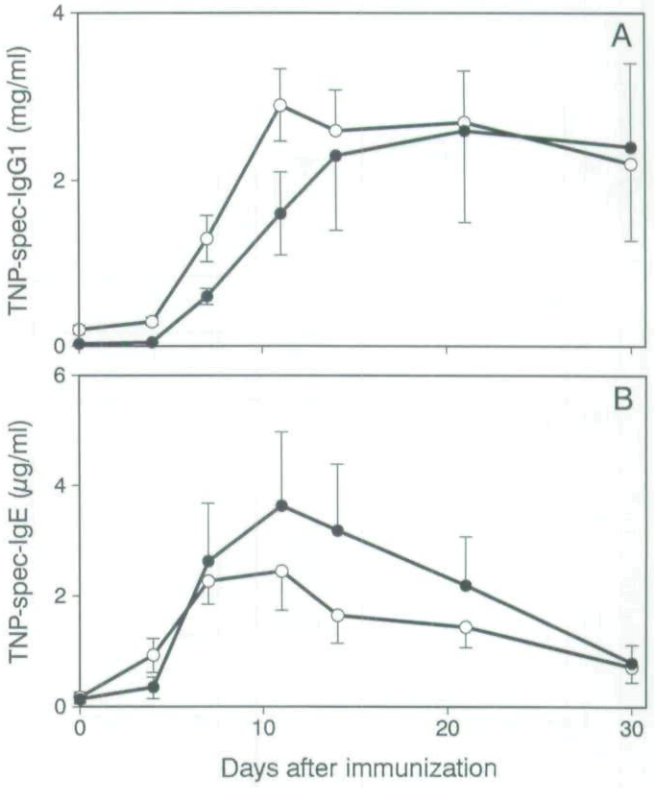

Fig. 5. Effect of IL-4 treatment on the TNP-specific secondary IgG_1 and IgE response. Primed IL-4 treated (●) and control treated mice (○) were boosted with 10 μg TNP-KLH adsorbed on alum on days 0, 3 months after priming. Serum levels of TNP-specific IgG_1 (A) and IgE (B) were determined by ELISA and are expressed as arithmetic mean ± SEM ($n = 5$).

(Fig. 5B). Comparable results were found after TNP-RIgG booster immunization (data not shown). Moreover, IL-4 treatment during priming did not result in an increase or decrease of total and TNP-specific serum IgG_{2a} after booster immunization (data not shown). Thus IL-4 treatment did not disturb the antigen-specific isotype regulation during a secondary immune response. These results also indicate that TNP-specific IgE can be detected in a large pool of total serum IgE using our TNP-specific IgE ELISA.

Continuous IL-4 treatment inhibits the TNP-specific IgG_1 and IgE memory formation

In order to investigate the influence of continuous IL-4 on the TNP-specific IgG_1 and IgE memory formation an adoptive transfer system was used. Irradiated recipient mice were reconstituted with TNP-KLH primed spleen cells from control and IL-4 treated mice isolated on day 0 and 3 months after the last of nine injections of encapsulated CV-1/IL-4 cells. All recipient mice were immunized with TNP-KLH immediately after reconstitution. On days 7, 9 and 12 the TNP-specific IgG_1 and IgE serum levels were determined. In two independent experiments, the TNP-specific IgG_1 and IgE levels in mice reconstituted with spleen cells from IL-4 treated mice were significantly lower than in mice reconstituted with spleen cells from control mice (Table 4). Prolonged IL-4 treatment of the donors apparently reduced the TNP-specific IgG_1 and IgE memory formation. No differences were seen in the percentages of $B220^+$ and Thy-1^+ spleen cells between IL-4 treated- and control mice 3 months after the last injection of encapsulated CV-1/IL-4 cells (data not shown). So the difference in the percentage of T cells in the spleens from IL-4 treated mice at day 0 and three months after the last administration of CV-1/IL-4 cells did not influence the IgG_1 and IgE production after adoptive transfer (Table 4). The total IgE levels at day 9 did not differ between mice adoptively transferred with spleen cells from control treated and IL-4 treated mice in both experiments. This indicates that the differences in TNP-specific IgE levels are not influenced by differences in total IgE levels.

DISCUSSION

The main finding from this study is that prolonged IL-4 treatment after primary immunization with a TD antigen led to a strong increase in polyclonal but not in antigen-specific IgG_1 and IgE formation. This was based on the

Table 4. TNP-specific serum IgG_1 and IgE responses after adoptive transfer of spleen cells from control and IL-4 treated mice

	TNP-spec. IgG_1 (mg/ml)			TNP-spec. IgE (μg/ml)		
	7	9	12	7	9	12
A						
Control	1.2 ± 0.1	4.4 ± 1.0	8.0 ± 1.7	52.9 ± 8.3	80.1 ± 12.7	50.5 ± 10.5
IL-4	0.4 ± 0.1	2.0 ± 0.4	3.9 ± 0.8	20.6 ± 4.8	55.1 ± 16.0	38.9 ± 9.6
B						
Control	0.5 ± 0.05	2.8 ± 0.3	6.7 ± 0.7	21.6 ± 0.4	87.6 ± 14.0	61.9 ± 6.7
IL-4	0.1 ± 0.02	0.9 ± 0.5	1.6 ± 0.4	7.0 ± 1.7	33.4 ± 6.8	14.6 ± 1.6

Irradiated mice (6 Gy) were reconstituted with 1×10^7 TNP-KLH primed spleen cells from control and IL-4 treated mice at the end of the treatment (A) and 3 months after treatment (B). All reconstituted mice were boosted with 10 μg TNP-KLH on day 0. Serum levels of TNP-specific IgG_1 and IgE were determined on days 7, 9 and 12. Results are represented as arithmetic mean $\pm$ SEM ($n = 5$).

finding that TNP-specific IgG_1 and IgE responses were consistently reduced upon treatment with IL-4. The IgG_{2a} response was not influenced, indicating that the observed effects of IL-4 were specific for the IgG_1 and IgE isotypes. The decrease in the TNP-specific IgG_1 and IgE responses by IL-4 is accompanied by a decrease in the TNP-specific memory formation for these isotypes as is seen in the adoptive transfer experiments. The question remained whether IL-4 exerted its effect directly on the B cells or indirectly via other cell types, such as T cells.

It has been suggested that IL-4, when persistently present, can inhibit the IL-4 receptor expression on T cells [34]. Since IL-4 can act as a proliferation factor of activated T cells [35] it is tempting to speculate that IL-4 receptor down-regulation can lead to decreased T cell proliferation, resulting in a reduced availability of T cells. Indeed, we did find a decrease in the number of T cells in the spleens of IL-4 treated mice (Table 1), and we cannot exclude that the observed decrease in $CD4^+$ T cells of IL-4 treated mice is to some extent due to a (selective) inhibition of antigen-specific T_h2 cells. However, no difference in the IL-4 production by spleen cells after Con A stimulation was observed as a result of the IL-4 treatment.

On the other hand, the possibility of an indirect effect of IL-4 on the B cells seems unlikely, because the TNP-specific IgG_1 and IgE responses in the adoptive transfer experiments were not influenced by differences in the percentage of T cells in the spleen cell samples of IL-4 treated mice used to reconstitute the irradiated mice (Table 4). These results indicate that a more likely explanation for the observed phenomena is a direct negative effect of IL-4 on the antigen-specific B cell consistent with the findings of Asano *et al.* [36]. Furthermore, the adoptive transfer results show that the observed inhibition of the TNP-specific memory formation for IgG_1 and IgE by IL-4 is a long term effect as the same degree of inhibition is found 3 months after the last administration of IL-4 (Table 4).

No differences were observed between the TNP-specific secondary IgG_1 and IgE responses in IL-4 and control treated mice, indicating that the isotype-specific regulation in IL-4 treated mice was obviously not disturbed. This despite the fact that these IL-4 treated mice had total IgG_1 and IgE levels that were increased five-fold and 12-fold, respectively over control mice. In the adoptive transfer experiments a decrease in the TNP-specific memory formation resulting from the IL-4 treatment was observed. These adoptive transfer experiments were carried out to study the TNP-specific memory responses in a more isolated way, without the possible interference of residual systemic effects induced in the treated donor mice. Furthermore, the resulting TNP-specific IgE responses were much larger in this system and therefore potential differences between IL-4 and control treatment became visible.

The markedly elevated bystander responses, that is responses to other antigens than TNP-KLH, in IL-4 treated mice could be the result of enhanced differentiation or prolonged survival of pre-activated B cells [37, 38]. As it is shown in Figs 1 and 4, immunization with TNP-KLH adsorbed on alum induces enough endogenous IL-4 to mount an IgE and IgG_1 response. The effect of CV-1-secreted IL-4 may be enhancement of responses to 'e.g., environmental antigens' that do normally not induce levels of IL-4 high enough to result in IgE or IgG_1 responses, possibly by reversing the Fc receptor-mediated inhibition of B-cell activation [39]. This could lead to activation of B cells which can subsequently switch to IgG_1 and IgE, causing high levels of these isotypes.

The high polyclonal IgG_1 and IgE levels themselves could have a negative effect on TNP-specific IgG_1 and IgE formation. The high level of polyclonal IgG_1 alone could possibly also inhibit the TNP-specific IgE response, by inhibiting the TNP-specific IgG_1 levels, as it is described that the IgG_1 response is coupled to the IgE response [40].

The lack of sufficient antigen and/or antigen-specific helper T cells for TNP-specific B cells when IL-4 is excessively present could also be an explanation for the observed inhibition of TNP-specific IgG_1 and IgE. Both would lead to a limited cognate T–B cell interaction that is obligatory for antigen-specific antibody responses against TD antigens [41].

However, our results indicate that a lack of antigen is not the explanation for decreased antigen-specific antibody responses since IL-4 also inhibits TNP-specific IgG_1 and IgE responses when antigen is repeatedly injected.

Our results indicate that antigen and IL-4 should be in balance for maximum antigen-specific IgG_1 and IgE responses. Disturbance of this balance by administration of IL-4 leads to an inhibition of the antigen-specific responses together with an elevation of the bystander responses. On the other hand, antigen-specific responses could need locally present antigen in conjunction with the crucial amount of IL-4. So in general, antigen-specific and bystander responses are differentially regulated. These results extend the *in vitro* findings of Asano *et al.* [36] who reported an inhibitory effect of high doses of antigen on the antigen-specific IgG_1 response. This inhibition was found to be mediated by IL-4, which exerted its effect only on the cognate pathway.

Besides the effect on immunoglobulin secretion mediated by prolonged IL-4 treatment we observed that the number of splenic T cells slightly decreased, largely due to a decrease in $CD4^+$ cells as was also seen in some IL-4 transgenic mice [42]. The IgE and IgG_1 hyperproduction that we observed in our IL-4 treated mice was also seen in some IL-4 transgenics [42, 43]. In all described IL-4 transgenic mice the MHC class II expression on B cells increased [43, 44]. We could not detect an increase in $I\text{-}A^d$ expression on the splenic B cells of IL-4 treated mice. However, B cells of IL-4 treated mice showed an increased CD23 expression. This might be due to the increased IL-4 level, but could also be the result of the elevated serum IgE levels [8–10]. All of the observed phenomena could be explained by different levels of expression of IL-4 in transgenic and IL-4 treated mice.

The observed effects on total serum IgG_1 and IgE levels, and CD23 expression on the splenic B cells in mice that were repeatedly treated with IL-4 provide evidence for the presence of increased levels of functional IL-4 *in vivo*. This occurred despite the fact that no IL-4 could be detected in the circulation of such IL-4 treated mice. Previous studies have shown that injection of 2×10^6 alginate encapsulated CV-1/IL-4 cells resulted in a consistent IL-4 production for periods up to 2 weeks. This treatment proved to be sufficient to transform IgE non-responder SJA/9 mice into IgE high-responder mice [25]. Treatment of mice with the IL-5 producing CV-1/IL-5 cell line did not result in any of the effects described in this article, although eosinophilia occurred (data not shown).

As a result of prolonged IL-4 treatment the IFN-γ production by Con A stimulated spleen cells decreased, while the production of the T_h2 cytokines (IL-4, IL-6 and IL-10) was not affected (Table 3). IL-4 treatment apparently did not dysregulate the endogenous production of T_h2 cytokines. A possible explanation for the decreased IFN-γ production by the IL-4 treatment could be a decrease in the development of T_h1 cells from T_h0 cells [45–49]. Another possibility is a direct effect of the applied IL-4 on the IFN-γ production by $CD4^+$ and/or $CD8^+$ T cells. For the human system this has been described by Vercelli *et al.* [50] who reported a decreased IFN-γ production and mRNA expression in mixed lymphocyte cultures and Con A stimulated peripheral blood mononuclear cells as a result of exposure to IL-4. It might be that IL-4 decreases the transcription and/or stability of IFN-γ mRNA.

Our results underline the peculiar effects of IL-4 on the proliferative and isotype switching capacity of B cells. Moreover, they point to differences in the effect of IL-4 on antigen-specific and bystander responses. This indicates that caution has to be taken when IL-4 administration is considered to increase the induction of IgG_1 or IgE specific for a particular antigen.

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