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Suppression of polyclonal and antigen-specific murine IgG$_1$ but not IgE responses by neutralizing interleukin-6 in vivo*

The crucial role of interleukin (IL)-4 in the induction of murine IgG$_1$ and IgE responses, which are coupled through the process of sequential isotype switching, has been well documented. Whereas IL-4 is obligatory for the induction of IgE responses, it enhances IgG$_1$ responses. In this study, using neutralizing antibodies, we provide evidence that, besides IL-4, also IL-6 is required for obtaining peak IgG$_1$ responses. The mRNA levels of these two cytokines are coordinately expressed in the spleen of mice immunized with trinitrophenol-keyhole limpet hemocyanin (TNP-KLH). No IL-6 requirement was observed for peak IgE responses. The IL-6 dependence of IgG$_1$ responses was found for both antigen-specific and polyclonal responses. Moreover, it was noted using TNP-KLH and goat anti-mouse (GAM) IgD as antigen that polyclonal IgG$_1$ responses are more dependent on IL-6 than antigen-specific responses. In vitro experiments revealed that exogenous IL-6 neither enhanced nor inhibited the IgG$_1$ and IgE production by naive B cells, suggesting that IL-6 did not interfere with the IL-4-induced isotype switch potential. Primary and memory IgG$_1$ responses were both similarly dependent on IL-6. These observations point to a role of IL-6 in the terminal differentation of B cells switched to IgG$_1$. Neutralization of IL-6 did not inhibit either antigen-specific or polyclonal IgE responses. Therefore, it was concluded that IL-6 is not involved in the terminal differentiation of B cells switched to IgE. These findings thus provide a distinct role for IL-6, besides IL-4, in regulating murine IgG$_1$ responses. The formation of IgE, however, is completely dependent on IL-4 alone.

1 Introduction

The crucial role of IL-4 in the regulation of IgE synthesis has been well established by studying mice that were made IL-4 deficient by gene targetting. No IgE synthesis was observed in these mice upon nematode infection [1]. Disrupting the IL-4 gene did not completely impair the IgG$_1$ production [1], indicating that IL-4 is not an absolute requirement for IgG$_1$. Functional studies have indicated IL-4 as a "switch-inducing factor" for both IgG$_1$ and IgE. It alters the chromatine structure of the Sγ_1 region [2] and induces accumulation of germ-line γ_1 and ϵ transcripts [3, 4], events that are associated with isotype class switching [5, 6].

In line with these results is the observation that IL-4 enhances the IgG$_1$ production, although less IL-4 is

required for peak IgG$_1$ responses than for peak IgE responses after LPS stimulation of B cells in vitro [7]. A linkage between IgG$_1$ and IgE responses has recently been demonstrated, in that γ_1-positive B cells can switch to ϵ-positive B cells with a $\gamma_1\epsilon$-double-positive B cell as an intermediate [8, 9]. Moreover, it has been suggested that these $\gamma_1\epsilon$-double-positive cells co-secrete IgG$_1$ and IgE [9].

The role of cytokines other than IL-4 in in vivo polyclonal and antigen-specific IgG$_1$ and IgE responses has not been well investigated. In the human system, IL-6 has been described as a cytokine that is involved in the IL-4-dependent IgE synthesis [10, 11]. Similar observations were made in mice, in that neutralization of IL-6 resulted in a inhibition of the number of IgE-secreting cells in the spleen [12]. Surprisingly, in that study an inhibition of the number of IgE-secreting cells was also observed when IL-6 was administered [12].

Because of these contradictory results we decided to study the role of both IL-4 and IL-6 in IgG$_1$ and IgE responses. In the study of Auci et al. [12] antigen had been given more than once. It is known that memory IgE responses differ from primary IgE responses with respect to their IL-4 requirement, in that memory IgE responses are partially IL-4 independent [13–15]. Therefore, we studied the effect of neutralizing IL-6 in vivo on memory IgG$_1$ and IgE responses as well as the effect of this treatment on polyclonal and antigen-specific primary IgG$_1$ and IgE responses. We also studied in in vitro cultures of splenic B cells whether IL-6 could enhance IgG$_1$ and/or IgE production.

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Collectively, our results show that IgG$_1$ and IgE responses not only differ in IL-4 dependence, but also deviate with respect to IL-6 in attaining peak levels. This finding thus provides a second regulatory mechanism of IgG$_1$ and IgE responses coupled through sequential isotype switching.

2 Materials and methods

2.1 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. The experiments were approved by the Animal Experiments Committee of the Erasmus University.

2.2 Immunization and infection

Mice were injected with 0.2 ml containing 10 μg TNP$_{25}$-KLH adsorbed on 2 mg alum i.p. [15] or 800 μg goat anti-mouse (GAM) IgD (Nordic Immunology,Tilburg,The Netherlands) i.v. or were infected with *Nippostrongylus brasiliensis* (Nb; a kind gift of Dr. R. L. Coffman, DNAX, Palo Alto, CA) by subcuteneously injecting 750 stage III Nb larvae, as indicated in Sect. 3.4

2.3 Isotype-specific ELISA

Total serum IgE and IgG$_1$ levels were measured by isotype-specific ELISA as described previously [15, 16]. Detection limits for the IgE and IgG$_1$ ELISA were 0.5 ng/ml and 0.2 ng/ml, respectively. TNP-specific IgG$_1$ and IgE were determined as previously described [15], with 0.2 ng/ml and 1 ng/ml as detection limit in the ELISA, respectively. Total serum IgG$_{2a}$ and IgM were determined essentially in the same way using either GAM IgG$_{2a}$ (Southern Biotechnology, Birmingham, AL) or GAM IgM (Southern Biotechnology) both at 1 μg/ml as coat and biotinylated GAM IgG$_{2a}$ or biotinylated GAM IgM both at 0.5 μg/ml as second step,with a detection limit of 0.3 ng/ml and 0.2 ng/ml, respectively.

2.4 Anti-cytokine treatment

Mice were treated *in vivo* by i.p. injection of purified neutralizing antibodies directed to IL-4 (11B11, rat IgG$_1$, 10 mg/mouse) [17], IL-5 (TRFK5, rat IgG$_1$ 2 mg/ml) [18] and IL-6 (20F3, rat IgG$_1$,2 mg/ml) [19]. Alternatively, mice were implanted with 2 × 10^6 alginate-encapsulated 11B11 and/or 20F3 hybridoma cells i.p. [20]. The hybridoma cells encapsulated in alginate were implanted in mice 3 days before immunization, the purified antibodies were given 2 h before immunization. The rat IgG$_1$ production by these 11B11 and/or 20F3 cells was determined in the serum using a rat IgG$_1$-specific ELISA as described previously [20]. Purified rat mAb specific for *E. coli* β-galactosidase (GL113) [21] or 2 × 10^6 alginate-encapsulated GL113 cells were used as an IgG$_1$ isotype control. The mAb were purified from culture supernatants by protein G-affinity chromatography [22]. The doses used have been widely

shown to be sufficient to neutralize the respective cytokine activities in a variety of systems. All the used hybridoma cells were assayed for their rat IL-6 production, and it was found that in all cases the average IL-6 production by 2 × 10^6 hybridoma cells/day was less than 8 U.This production was determined in a bioassay using B9 indicator cells as described [23], with a detection limit of 0.1 U/ml.

2.5 Semiquantitative reverse-transcription (RT)-PCR

RNA was isolated and reverse-transcribed after removing contaminating genomic DNA by DNase I, as previously described [20]. The cDNA was ^{2}log diluted in DEPC-treated H$_2$O.The separate dilutions were each subjected to PCR as described [24]. For amplification 35 cycles (1 min at 94 °C for denaturation, 2 min at 55 °C for primer annealing and 3 min at 72 °C for primer extension) were performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Primer sets for IL-4 resulting in a 180-bp product [24], IL-6 resulting in a 348-bp product [24], IFN-γ resulting in a 245-bp product [24] and hypoxanthine phosphatidyl ribosyltransferase (HPRT), a house keeping gene transcript resulting in a 176-bp product, [25] were used. Reverse-transcribed DNase I-treated CDC35 RNA and D1.1 RNA were used as positive control for Th2 and Th1 cytokines, respectively. Both were a kind gift of Dr. D. C. Parker and were maintained as previously described [26]. After amplification the reaction products were electrophorated in 2 % SeaKem LE agarose (FMC BioProducts, Rockland, ME). After staining with ethidium bromide the gels were photographed, and the photographs were subsequently scanned with a model 620 Video Densitometer (Bio-Rad Laboratories Inc., Hercules, CA).The reverse-transcribed mRNA amounts in the two cDNA preparations were compared in the linear part of the cDNA dilution curve, and were expressed as dilution of cDNA to obtain a similar optical density.

2.6 Adoptive transfer of spleen cells

Spleens cells (1 × 10^7) of mice, primed with TNP-KLH 3 months before, were adoptively transferred into naive irradiated (6 Gy) recipients as previously described [15]. All reconstituted mice were i.p. immunized with 10 μg TNP-KLH adsorbed on alum immediately after transfer.

2.7 T cell membrane stimulation

Spleen cells were cytotoxically depleted of T cells using anti-Thy-1.2 (clone F7D5; Serotec, Oxford, GB) and low-tox guinea pig complement (Cederlane, Hornby, Ontario, Canada) as described previously [16].The percentage of B220$^+$ B cells was routinely > 90 %, while the percentage of residual Thy-1$^+$ cells was < 2 %, as determined by flow cytometry.Viable T cell-depleted spleen cells at 2.5 × 10^5 cells/ml were cultured in flat-bottom microtiter plates together with 100 μg/ml T cell membrane fragments of activated H66 cells (Th1 clone), a kind gift of Dr. P. D. Hodgkin [27, 28] in 0.2 ml 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-mercaptoethanol, 35 ng/ml

purified IL-4, 1.75 ng/ml purified IL-5 and 5 μg/ml anti-IFN-γ (XMG 1.2) [29]. The cultures contained murine recombinant IL-6 (British Bio-technology Limited, Oxford, GB) 100 IU/ml as indicated in the results section. Cultures which did not contain membrane fragments of activated H66 cells served as controls. After 7 days of culture at 5 % CO_2 and 37 °C supernatants were harvested for ELISA.

3 Results

3.1 Cytokine mRNA expression in the spleen during a primary IgE response

In this study we were interested in the involvement of IL-4 and IL-6 in IgG_1 and IgE responses. Therefore, we investigated the cytokine mRNA expression in the spleen during a primary response against TNP-KLH, which predominantly induces IgG_1 and IgE responses in the given dose and the adjuvant employed. At days 0, 2, 4, 6, and 8 after immunization mRNA was isolated from the spleens and after DNase I treatment RT-PCR was performed. Maximum IL-4 and IL-6 mRNA expressions were seen at day 2 after immunization, whereas IFN-γ mRNA expression peaked at day 6 (Fig. 1). Moreover, it was found that the mRNA levels for IL-6 and IFN-γ were significant higher than those for IL-4. For IFN-γ mRNA a higher baseline level was observed when compared to the mRNA baseline levels for IL-4 and IL-6. The largest increase was seen for IL-4 mRNA expression (ninefold), whereas the mRNA levels for both IL-6 and IFN-γ increased twofold (Fig. 1). These results point to an important role of IL-4 and IL-6 in inducing IgG_1 and IgE responses upon TNP-KLH immunization.

3.2 Involvement of IL-4 and IL-6 in TNP-KLH-induced immune responses

Because of these results and former observations [10, 12] we decided to examine the involvement of both IL-4 and IL-6 in primary IgG_1 and IgE responses more closely. To this end, mice were treated with neutralizing antibodies directed to IL-4 and IL-6 prior to immunization with
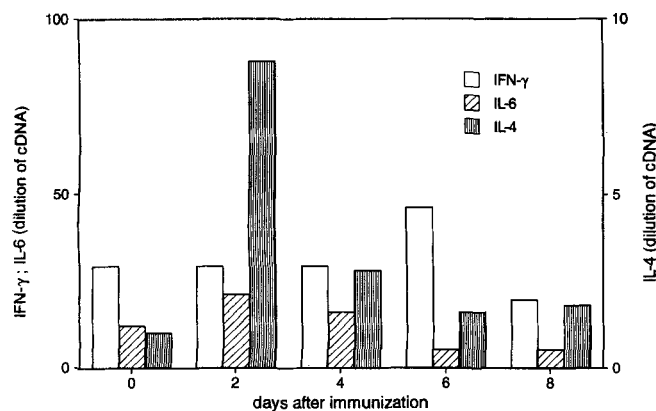

Figure 1. mRNA expression for IFN-γ, IL-6 and IL-4 in reverse-transcribed DNase I-treated total RNA preparations from spleens of mice at day 0, 2, 4, 6, and 8 after primary immunization with 10 μg TNP-KLH adsorbed on alum. Determined as described in Sect. 2.5.
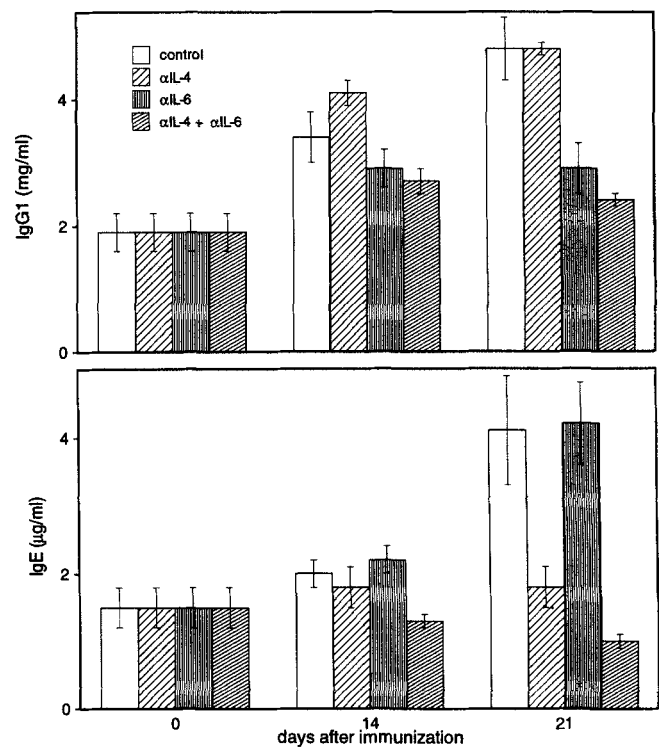

Figure 2. Effect of anti-(α)IL-4, anti-IL-6 or both on the primary total IgG_1 and IgE response. Mice were primed with 10 μg TNP-KLH adsorbed on alum i.p. 3 days after i.p. implantation of alginate-encapsulated anti-cytokine mAb producing hybridoma cells as indicated in Sect. 2.4. Serum levels are expressed as arithmetic mean ± SEM ($n = 5$).

10 μg/ml TNP-KLH, by implanting i.p. alginate-encapsulated 11B11 cells, which produce anti-IL-4 antibodies, and 20F3 cells, which produce anti-IL-6 antibodies. Both 11B11 and 20F3 are of rat origin, and produce antibodies of the IgG_1 isotype. Alginate-encapsulated GL113 cells producing rat IgG_1 antibodies directed to *E. coli* β-galactosidase served as isotype control. At day 21 maximum total IgG_1 and IgE responses were observed in the serum of immunized mice treated with control antibody, 4.8 mg/ml and 4.1 μg/ml, respectively (Fig. 2). Treatment of mice with anti-IL-4 completely abolished the increase in serum total IgE, whereas this treatment did not affect the increase in serum total IgG_1. Treatment with anti-IL-6 had an opposite effect on these two isotypes. It did not disturb the IgE response, whereas it inhibited the increase in serum total IgG_1 by 66 % (Fig. 2). For total serum IgE it was observed that the combination of encapsulated anti-IL-4-producing 11B11 and anti-IL-6-producing 20F3 cells decreased the serum level below the day 0 level, indicating an effect on the background production (Fig. 2).

With respect to the TNP-specific IgG_1 serum levels, other patterns were observed. These responses peaked at day 14. It was observed that neutralization of IL-6 alone did not inhibit the increase of TNP-specific serum IgG_1 (144 μg/ml at day 0 to 455 μg/ml on day 14). The increase in TNP-specific IgG_1 was inhibited by 80 % by treating mice with a combination of encapsulated 11B11 and 20F3 cells. At day 21 no significant inhibition was observed in this situation (data not shown). The production of anti-cytokine mAb by the encapsulated cells was monitored by measuring

rat IgG₁ in the serum of the mice in which they were implanted i.p. At day 7, 87 μg/ml, 108 μg/ml 319 μg/ml and 675 μg/ml rat IgG₁ was observed in animals treated with GL113, 11B11, 20F3 or 11B11 together with 20F3 cells encapsulated in alginate, respectively (data not shown). After day 14 a decline in these rat IgG₁ serum levels was observed. This decline could explain the difference between the TNP-specific IgG₁ serum levels found on day 14 and 21 in mice that were implanted i.p. with both encapsulated 11B11 and 20F3 cells.

3.3 Involvement of IL-4 and IL-6 in GAM IgD-induced immune responses

The results found with respect to the TNP-KLH immunization suggested that IL-6 had a more important role in polyclonal IgG₁ responses than in antigen-specific primary IgG₁ responses. To study this role more precisely we immunized mice with 800 μg GAM IgD i.v. which is known to elicit strong polyclonal IgG₁ and IgE responses [30]. Immunization of mice resulted in an increase in the serum of 24 mg/ml for total IgG₁ and 4 μg/ml for total IgE at day 7 (Fig. 3). As in the TNP-KLH response, treatment of mice with alginate-encapsulated anti-IL-4-producing 11B11 cells completely abolished the IgE response in serum, but in this experiment inhibition (42 %) of the IgG₁ response was also observed (Fig. 3). Neutralization of IL-6 by treating mice with 20F3 cells encapsulated in alginate inhibited the increase in serum total IgG₁ by 80 %, resulting in a serum level of total IgG₁ of 6 mg/ml (Fig. 3). The increase in serum total IgE was not influenced by this treatment. Moreover, no synergistic effect was seen for anti-IL-4 mAb

Table 1. Effect of anti-IL-6 treatment on IgE levels during Nb infection[a]

Antibody injected	Dose	IgE μg/ml	
		Day 0	Day 11
None		1.5 ± 9.4	41 ± 9.8
Anti-IL-4	1 mg	1.5 ± 0.4	16.5 ± 1.8
Anti-IL-6	1 mg	1.5 ± 0.4	25.9 ± 3.9
Anti-Il-4 + anti-IL-6	1 mg + 1 mg	1.5 ± 0.4	17.9 ± 2.1
Anti-IL-4	10 mg	1.5 ± 0.4	2.9 ± 0.7
Control	1 mg	1.5 ± 0.4	40.5 ± 5.4

a) Mice were infected with 750 stage III larvae s.c. at day 0. Antibodies were given i.p. in doses of 1 or 10 mg/mouse as indicated. Results are presented as arithmetic mean ± SD (*n* = 5).

and anti-IL-6 mAb in the GAM IgD-induced IgG₁ response (Fig. 3). GAM IgD injection neither induced an IgM nor an IgG₂ₐ response, showing the isotype specificity of the induced response. These responses were not changed by the various anti-cytokine treatments (Fig. 3).

3.4 Involvement of IL-6 in an Nb-induced IgE response

We next used a Nb infection model to study the effects of neutralizing IL-4 and/or IL-6 on the induction of a different, but also strong, polyclonal IgE response. Nb infection induced an increase in the total serum IgE level from 1.5 μg/ml on day 0 to 41.9 μg/ml on day 11. Neutralizing IL-4 with 10 mg purified 11B11 inhibited the IgE response by 97 %, whereas neutralizing IL-6 resulted in a 40 %
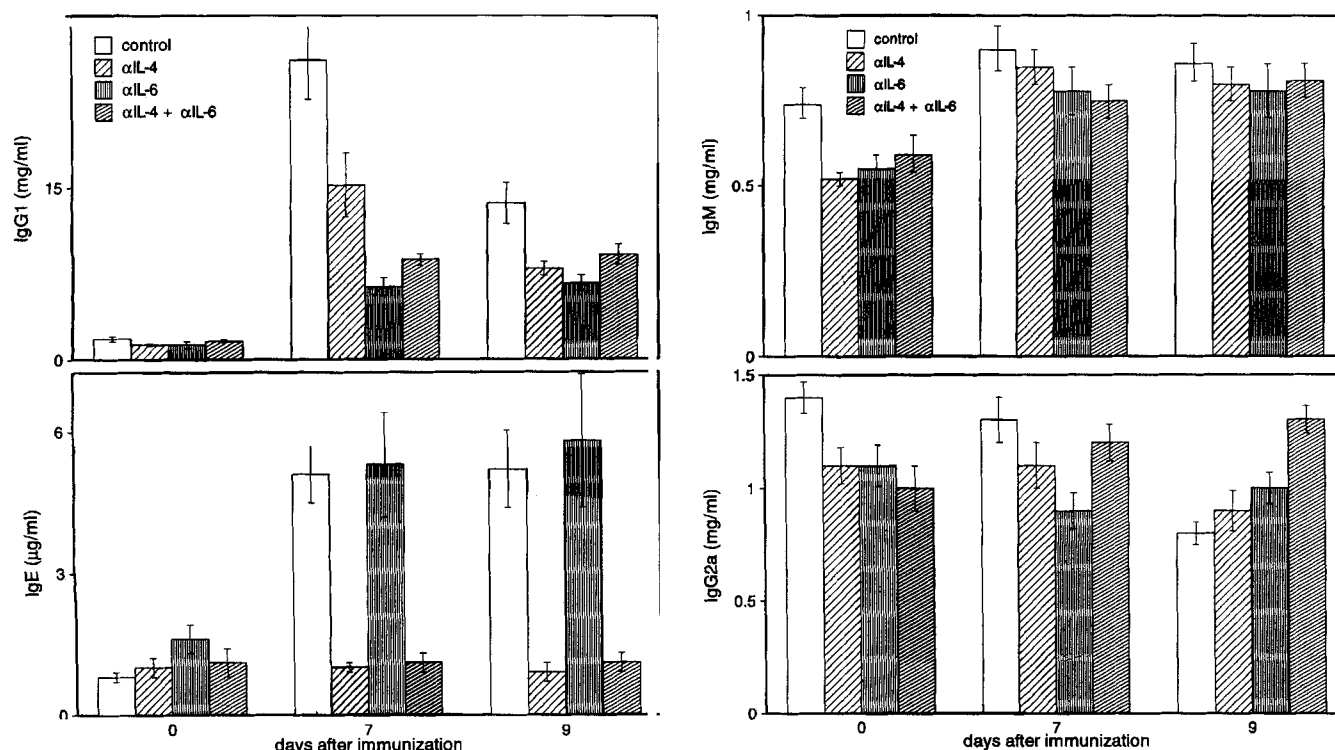

Figure 3. Effect of anti-(α)IL-4, anti-IL-6 or both on GAM IgD induced polyclonal IgG₁, IgE, IgM, and IgG₂ₐ responses. Mice were immunized with 800 μg GAM IgD i.v. At day − 3 alginate-encapsulated anti-cytokine mAb producing hybridoma cells were implanted i.p. as indicated in the Sect. 2.4. Serum levels are expressed as arithmetic mean ± SEM (*n* = 5).

inhibition of the IgE increase (Table 1). These results indicate an involvement of IL-6 in obtaining a strong polyclonal increase in IgE. To study whether IL-4 and IL-6 worked synergystically in this response, an experiment was performed in which IL-4 was suboptimally neutralized (1 mg 11B11 i.p.), resulting in a 63 % inhibition. Neutralization of IL-6 at the same time did not increase the percentage of inhibition (Table 1), indicating that IL-4 and IL-6 do not synergize in this polyclonal IgE response.

3.5 IgG$_1$ production by naive B cells stimulated with T cell membrane fragments is not potentiated by IL-6

To study whether IL-6 can potentiate IgG$_1$ production by naive B cells we performed in vitro experiments in which naive splenic B cells were polyclonally stimulated with membrane fragments of activated Th1 cells in the presence or absence of exogenous IL-6. IL-4, IL-5 and anti-IFN-γ were present in all cultures to facilitate optimal responses [27, 28]. Addition of IL-6 neither enhanced nor inhibited the production of IgG$_1$ and IgE during the 7 days of culture (Fig. 4). In both stimulation conditions similar amounts of IgG$_1$ and IgE were produced; 20 μg/ml and 2.8 μg/ml, respectively. This result indicated that enough IL-6 was present in the culture system to facilitate peak IgG$_1$ and IgE production by naive B cells upon polyclonal stimulation.
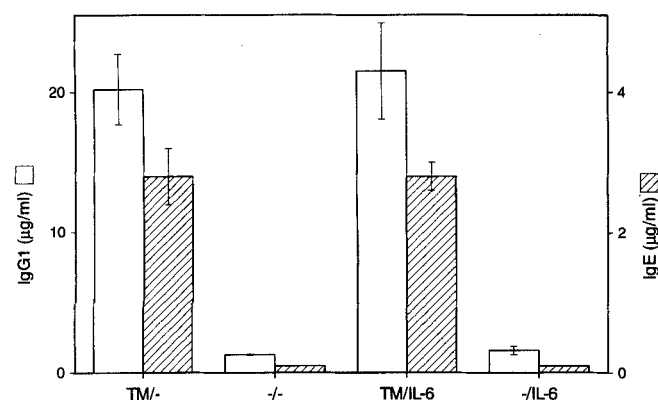

Figure 4. Thy-1-depleted spleen cells of naive mice were stimulated with T cell membrane fragments of activated Th1 cells (TM) in the presence or absence of IL-6. At day 7 the supernatants were harvested and the total IgG$_1$ and IgE levels determined. Results are expressed as arithmetic mean ± SD (n = 5).

anti-cytokine mAb-producing hybridoma cells. At day 5 the rat IgG$_1$ serum levels of mice implanted with encapsulated GL113, 11B11, 20F3 or 11B11 combined with 20F3 cells were determined and found to be 185 μg/ml, 149 μg/ml, 320 μg/ml and 397 μg/ml, respectively. On day 7 the 108 μg/ml rat IgG$_1$ measured in serum of mice implanted with in alginate-encapsulated anti-IL-4-producing 11B11 cells completely abolished the primary IgE response. Anti-IL-4 and anti-IL-6 treatment inhibited the TNP-specific IgG$_1$ response on day 7 by 34 % and 23 %, respectively. When these two treatments were combined, the TNP-specific IgG$_1$ response was inhibited by 57 %, indicating that both IL-4 and IL-6 are required for an optimal memory TNP-specific IgG$_1$ response (Fig. 5).
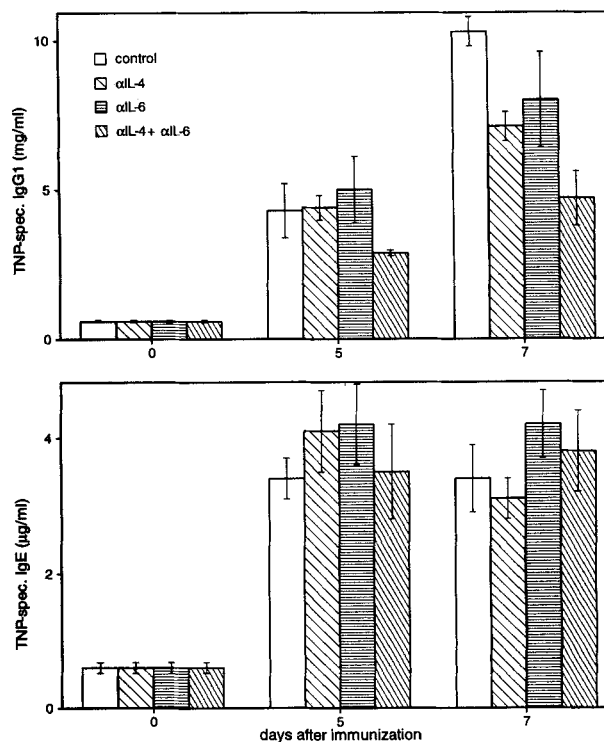

Figure 5. Involvement of IL-4 and IL-6 in memory IgG$_1$ and IgE responses. Mice that had been primed with 10 μg TNP-KLH were boosted 3 months later with 10 μg TNP-KLH. At day − 3 alginate-encapsulated anti-cytokine mAb producing hybridoma cells were implanted i.p. as indicated in Sect. 2.4. Serum levels are expressed as arithmetic mean ± SEM (n = 5).

3.6 Involvement of IL-4 and IL-6 in memory IgG$_1$ and IgE responses

We next studied the involvement of IL-4 and IL-6 in the memory TNP-specific IgG$_1$ and IgE responses. For this, BALB/c mice that had been primed with 10 μg TNP-KLH adsorbed on alum 3 months before were boosted with 10 μg TNP-KLH adsorbed on alum. Maximum TNP-specific IgG$_1$ responses were seen on day 7, whereas a maximum TNP-specific IgE response was already observed on day 5 (Fig. 5). This TNP-specific IgE response was neither inhibited by either anti-IL-4 or anti-Il-6 alone, nor by the combination of these two anti-cytokine mAb. Treatment was performed by implantation of alginate-encapsulated

3.7 Effect of neutralization of IL-4 and IL-6 on memory B cells to become IgG$_1$ or IgE-secreting plasma cells

In other studies we have shown that adoptive transfer of primed spleen cells to irradiated control mice leads to enhanced memory IgG$_1$ and IgE responses after boosting the reconstituted mice [15]. Therefore, we reconstituted 6 Gy-irradiated mice with 1 × 10^7 TNP-KLH primed spleen cells and studied the effect of neutralizing IL-4 and IL-6 by injecting mice i.p. with 10 mg anti-IL-4, 10 mg anti-IL-4 + 2 mg anti-IL-6. Previously, IL-5 was reported to have an important role in inducing IgG$_1$-positive B cells to secrete IgG$_1$ [31]. For this reason we also studied the involvement of both IL-5 and IL-6 in memory IgG$_1$ and IgE responses in
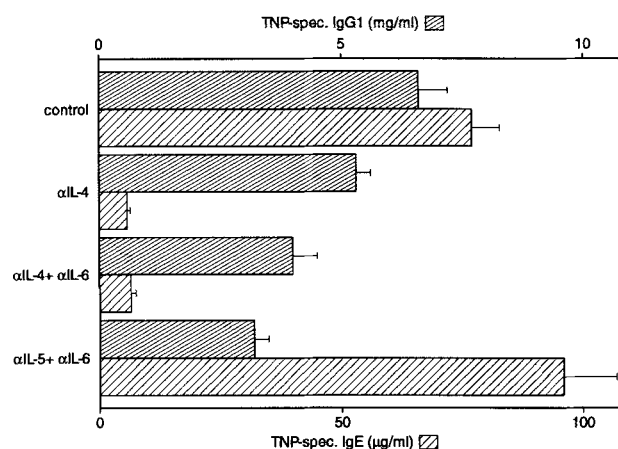
Figure 6. Effect of neutralization of IL-6 and IL-5 on peak levels of memory IgG$_1$ and IgE responses after adoptive transfer. Irradiated mice (6 Gy) were reconstituted with TNP-KLH primed spleen cells. Mice were injected i.p. with the following antibodies: control (GL113, 4 mg/mouse); anti-(α)IL-4 (11B11, 10 mg/mouse); anti-αIL-5 (TRFK-5, 2 mg/mouse); and anti-αIL-6 (20F3, 2 mg/mouse). All reconstituted mice were boosted with 10 µg TNP-KLH adsorbed on alum i.p. Results represent arithmetic mean ± SEM (n = 5–15).

reconstituted mice. To this end mice were injected i.p. with 2 mg anti-IL-5 + 2 mg anti-IL-6. Control mice received 4 mg rat IgG$_1$. Subsequently all mice were boosted with 10 µg TNP-KLH i.p., resulting in a peak response for IgE on day 9 and for IgG$_1$ on day 12. Neutralization of IL-4 inhibited the IgE memory response by 90 %, whereas it inhibited the IgG$_1$ response by 20 % (Fig. 6). Neutralization of both IL-5 and IL-6 significantly inhibited the IgG$_1$ memory response by 52 %. This treatment did not influence the memory IgE response (Fig. 6). No synergistic effect was observed for IL-4 and IL-6 with respect to the memory IgE response, suggesting that also in the memory response no IL-6 is required for IgE production, even not in the absence of IL-4.

4 Discussion

This study shows that murine IgG$_1$ and IgE responses differ in their requirement for IL-6 in reaching peak levels. Neutralization of IL-6 resulted in inhibition of polyclonal IgG$_1$ responses. Primary and memory antigen-specific IgG$_1$ responses were slightly inhibited by neutralizing IL-6, but neutralization of both IL-4 and IL-6 resulted in a marked decrease. These results show that these two cytokines act synergystically during a primary and memory antigen-specific IgG$_1$ responses. However, IL-6 neutralization did not inhibit antigen-specific and polyclonal IgE responses both after primary and secondary immunization. For human B cells a synergistic effect of IL-6 in combination with IL-4 in generating IgE has been described, by showing that anti-IL-6 antibodies strongly inhibited the IL-4-dependent IgE production *in vitro* [10, 11]. In mice we did not observe such synergistic effect. Therefore, we conclude that murine B cells and human B cells differ with respect to the need for IL-6 to give rise to peak levels of IgE.

Neutralization of IL-4 after primary immunization with TNP-KLH resulted in an abrogation of the increase in total

serum IgE, whereas it did not result in an inhibition of the increase in total serum IgG$_1$, an effect that has been well documented by us and other investigators [15, 30, 32]. However, we found that IgG$_1$ and IgE responses also differ in their need for IL-6 to obtain peak levels after primary immunization with TNP-KLH. Neutralization of IL-6 resulted in an inhibition of total serum IgG$_1$, whereas it did not have any effect on the induced total serum IgE response. No inhibition of the increase in antigen-specific IgG$_1$ was observed as result of IL-6 neutralization, but neutralization of both IL-4 and IL-6 resulted in 80 % inhibition of the antigen-specific IgG$_1$ response. This suggests that polyclonal IgG$_1$ responses are more dependent on IL-6 than antigen-specific IgG$_1$ responses, most likely as the result of preactivated B cells that only need IL-6 to become IgG$_1$-secreting plasma cells. Moreover, with respect to IL-4 the opposite can be concluded in that antigen-specific IgG$_1$ responses are more dependent on IL-4 than polyclonal IgG$_1$ responses, suggesting that the polyclonal response originates from B cells already switched to IgG$_1$. The induction of an IgE response, both antigen specific and polyclonal, could be inhibited by neutralizing IL-4, whereas neutralization of IL-6 did not have such effect. Furthermore, no synergistic effects were observed with respect to inhibition of polyclonal and antigen-specific IgE responses, when both IL-4 and IL-6 were neutralized.

It was noted, by studying the mRNA expression for IL-4, IL-6 and IFN-γ in the spleen after TNP-KLH immunization, that IL-4 and IL-6 are expressed simultaneously. After peaking at day 2 the mRNA levels for IL-4 and IL-6 returned to baseline level by day 6. At this time point a peak level for the IFN-γ mRNA expression was found. These results suggest that IL-4 and IL-6 act in concert during a TNP-KLH-induced IgG$_1$ and IgE response. The up-regulation of IFN-γ mRNA and, at same time, down-regulation of the mRNA levels of IL-4 and IL-6 suggest an active role for IFN-γ in this process. This observation could very well provide the basis for the well-documented inhibition of IL-4-induced IgG$_1$ and IgE synthesis mediated by IFN-γ [32]. Svetic et al. [33] also found after immunization with GAM IgD a coordinate expression of IL-4 and IL-6 mRNA.

To study the involvement of IL-4 and IL-6 in a strong polyclonal response we did experiments in which we used GAM IgD to evoke large polyclonal IgG$_1$ and IgE responses *in vivo* [30]. The GAM IgD-induced serum peak IgG$_1$ levels could be inhibited for 80 % by neutralizing IL-6, indicating that IL-6 is important for the induction. As expected, the induced increase in total serum IgE could be completely inhibited by neutralizing IL-4. No inhibition of the IgE response was found upon neutralizing IL-6. Also in the GAM IgD-induced response no synergistic effect was found when both IL-4 and IL-6 were neutralized. Two possibilities can account for this new phenomenon. It has been described that IL-6 is required for terminal differentiation of B cells to Ig-secreting plasma cells [34]. Here, we show that this observation holds for IgG$_1$- but not IgE-secreting cells. This suggests that B cells switched to IgE are already in a further differentiation stage than B cells switched to IgG$_1$. This might be a direct consequence of IL-4 which is an absolute requirement for the formation of B cells switched to IgE, but not for the induction of B cells

switched to IgG$_1$ [1]. More speculative is the possibility that B cells switched to IgE do not express the receptor for IL-6. This could explain the observation that such B cells produce less antibody than B cells switched to IgG$_1$, as it has been described that IL-6 enhances the murine antibody response [35]. However, the *in vitro* experiments, revealed that both the IgG$_1$ and IgE production were not influenced by exogenous IL-6. It is possible that no enhancement by IL-6 is observed because enough endogenous IL-6 is produced in the *in vitro* cultures to obtain peak production. Nevertheless, this study makes clear that neither the IgG$_1$ nor the IgE production is inhibited by exogenous IL-6.

For *Mesocestoides corti*-infected mice it was described that neutralization of IL-6 resulted in a marked inhibition of the IgG$_1$ serum levels, which are known to increase as result of infection [36]. This is in line with our results. However, *M. corti* infection does not lead to an increase in serum IgE. Therefore, it could not be determined whether IL-6 was necessary for peak IgE responses upon parasite infection. In this study we show that IL-6 in part is necessary for the large increase in polyclonal IgE responses after infection with Nb. However, no synergistic effect of neutralizing IL-4 and IL-6 with respect to the inhibition of the IgE response was seen when IL-4 was suboptimally neutralized.

We also examined the IL-6 dependence of antigen-specific IgG$_1$ memory responses, because it was shown by Hilbert et al. [37] that primary influenza virus-specific antibody responses were IL-6 dependent, whereas secondary antigen-specific antibody responses were IL-6 independent. In that study, however, no distinction has been made between the individual isotype responses that account for the antigen-specific antibody responses after priming and boosting with the antigen. In our study no difference was seen with respect to IL-6 dependence between the antigen-specific primary and memory IgG$_1$ response. For the antigen-specific memory IgG$_1$ response no synergistic effect was observed when both IL-4 and IL-6 were neutralized. As found in former studies, the antigen-specific memory IgE response was not completely inhibited by neutralizing IL-4. However, the amount of anti-IL-4 antibody present in the serum of treated mice was sufficient to completely inhibit the primary IgE response. Hence, it is possible that the amount of anti-IL-4 antibodies present in the serum is not enough to completely neutralize IL-4 after secondary immunization. This in combination with a decreased IL-4 dependence of memory B cells to secrete IgE could account for a normal antigen-specific IgE response after secondary immunization.

The adoptive transfer experiments were performed because it is known that in this sort of experiments upon boosting the secondary IgE responses are preferentially enhanced [15, 38]. In these experiments, it was found that only neutralizing IL-4 inhibited the memory IgE response, leaving 10% of the response intact. This part of the response is also independent of IL-6, and is most likely the result of B cells switched to IgE. In addition, neutralization of both IL-5 and IL-6 did not influence the IgE memory response, whereas it did inhibit the IgG$_1$ memory response, showing that both IL-5 and IL-6 are involved in the IgG$_1$, but not the IgE memory response.

Collectively, these results show that IgG$_1$ and IgE responses, both primary and secondary, differ in IL-6 dependence.

Whereas IgG$_1$ responses are inhibited *in vivo* by neutralizing IL-6, IgE responses are not influenced. The difference in IL-6 necessity to obtain peak IgG$_1$ and IgE responses provides a new regulatory mechanism, besides IL-4, for the murine IgG$_1$ and IgE responses coupled to each other by sequential isotype switching.

5 References

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