Brief Definitive Report

Induction of Interleukin 4 (IL-4) Expression in T Helper (Th) Cells Is Not Dependent on IL-4 from Non-Th Cells

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Summary

Interleukin 4 (IL-4) is essential for the induction of immunoglobulin E (IgE) responses in mice. Recent in vitro studies have suggested that IL-4 derived from non T helper (Th) cells, in particular from mast cells and basophils, may be essential for triggering of IL-4 expression in Th cells and may directly contribute to IgE isotype switch induction. Here, we have generated mice carrying a functional IL-4 gene only in Th cells or non-Th cells, respectively, by reconstitution of IL-4–deficient mice (IL-4T mice) with $CD4^+$ or $CD4^-$ spleen cells from congenic wild-type animals. In mice in which only $CD4^+$ cells are able to express IL-4, antigen-specific IgE is produced in a T cell–dependent immune response. Thus, induction of IL-4 expression in Th cells can occur in the absence of IL-4 from non-Th cells, which suggests that at least some Th cells can express IL-4 in response to another signal which has yet to be identified. No IgE is detectable, however, in mice in which only $CD4^-$ cells can express IL-4, suggesting that Th cells are the primary, if not the only source of IL-4 for initial induction of IgE synthesis.

$CD4^+$ Th cells are generally considered the principle producers of IL-4 in an immune response (1). Recently, however, several groups have shown that other cell types, like mast cells, basophils, and $CD4^-$ T cells, can produce IL-4 as well (2–7). IL-4 itself has been shown to be a dominant factor for the induction of IL-4 expression in resting Th cells. In vitro, expression of IL-4 in activated Th cells can be induced by exogenous IL-4 (8, 9) and in vivo, development of IL-4–producing Th cells can be inhibited by anti-IL-4 antibody (10–12). For initial induction of IL-4 expression in Th cells, it has been speculated that IL-4 from non-Th cells, in particular from Fc receptor (FcR) triggered mast cells and basophils, may be required (3, 4, 6, 10, 13–15). Using mice in which only $CD4^+$ Th cells carry a functional IL-4 gene, we show here that expression of IL-4 can be induced in Th cells independent of IL-4 from non-Th cells. We reconstituted IL-4–deficient mice, which had been generated by targeted disruption of the IL-4 gene (IL-4T mice; 16) with highly purified $CD4^+$ spleen cells from congenic wild-type (wt) mice and $CD4^-$ spleen cells from IL-4T mice, i.e., with Th cells with and non-Th cells without a functional IL-4 gene. Expression of IL-4 was assayed by virtue of its unique capability to direct Ig class switching of B cells to IgE. IL-4T mice cannot produce IgE (16). In the reconstituted mice, with only $CD4^+$ cells capable of expressing IL-4, a specific IgE response was readily detectable upon immunization.

Materials and Methods

Mice. Homozygous C57BL/6 IL-4T mice were derived from the previously described (129/Ola × C57BL/6)F_2 mice homozygous for the IL-4T mutation (16) by six backcrosses to C57BL/6 wt mice and final interbreeding of the heterozygous offspring. Transmission of the IL-4T allele was followed through this breeding process by restriction analysis of tail DNA as shown before (16). Specific pathogen-free C57BL/6 wt mice were purchased from the Bomholtgard Breeding and Research Centre Ltd. (Ry, Denmark) and kept under sterile conditions. Both donor and recipient mice used in the cell transfer experiment were 13-wk-old.

Cell Sorting. $CD4^+$ and $CD4^-$ murine spleen cells were separated by high gradient magnetic cell sorting using the MiniMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The MACS technology has been described in detail (17). Briefly, cells are labeled with superparamagnetic microparticles conjugated to mAbs, and labeled cells are separated from unlabeled cells on a MiniMACS column inserted into the MiniMACS magnet. The magnetic cells are retained in the column while the nonmagnetic cells pass through. Labeled cells can be eluted when the column is removed from the magnet. For magnetic labeling of Th cells, spleen cells were incubated with anti CD4 microbeads (Miltenyi Biotec GmbH). To evaluate the efficiency of the cell separation, aliquots of unseparated cells and from the magnetic and nonmagnetic cell fractions were stained with the fluoresceinated anti-CD4 mAb GK-1.5 (18) and analyzed by flow cytometry, using a FACScan® (Becton Dickinson & Co., Mountain View, CA). Propidium iodide (0.4 μg/ml) was used for exclusion of dead cells.

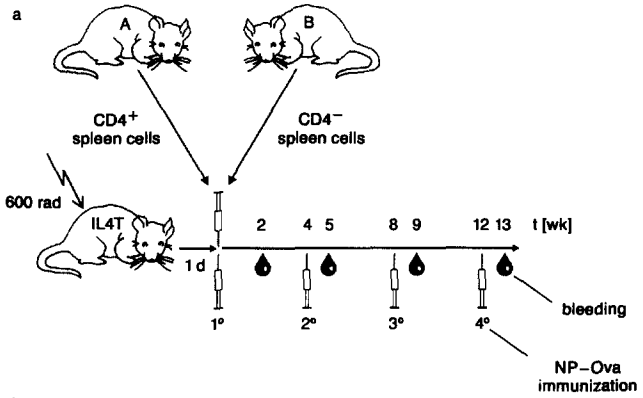
Cell Transfer. IL-4T mice were X-irradiated with 600 rad 1 d before transfer. Irradiated IL-4T mice were reconstituted by injection of 2 × 10^6 $CD4^+$ spleen cells and 8 × 10^6 $CD4^-$ spleen cells in 200 μl of PBS into the tail vein.

Immunization. Reconstituted IL-4T mice were immunized by intraperitoneal injection of alum-precipitated (4-hydroxy-3-nitrophenyl)acetyl $(NP)_6$-OVA (10 μg in 100 μl PBS per mouse) immediately, 4, 8, and 12 wk after cell transfer. Blood was collected from tail or retro-orbital veins 2 wk after primary (1°) immunization and 1 wk after each boost.

ELISA. Serum concentrations of total and NP-specific Ig of various isotypes, were determined by ELISA, as described previously (16). For quantification of NP-specific γ1-, γ2a-, κ-, and λ1-bearing antibodies, 96-well plastic plates were coated with (4-hydroxy-5-iodo-3-nitrophenyl)acetyl $(NIP)_{15}$-BSA, blocked, and then incubated with serial dilutions of serum. Bound Ig was detected with biotinylated monoclonal rat anti-mouse IgG1 (Miltenyi Biotec GmbH), anti-mouse $IgG2a^b$ (mAb G12-47/30; 19), anti-mouse κ (mAb R33-18-10-1; 20), and anti-mouse λ1 (mAb LS136; 21), respectively. The plates were developed with streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) and substrate (4-nitrophenylphosphate; Merck, Darmstadt, Germany). Serum concentrations were calculated using NP-specific mAb of the various isotypes as standards. To quantify NP-specific IgE, plates were coated with anti-mouse IgE (mAb 95.3; 22), then serially diluted serum was added and NP-binding IgE was detected with NIP_{15}-BSA, followed by biotinylated anti-NP antibody (mAb N1G9; 23), streptavidin-alkaline phosphatase, and substrate. Concentrations of NP-binding IgE were calculated relative to a serum from a C57BL/6 mouse that had been immunized four times at 4-wk intervals with NP-OVA. Total IgG2a and IgE in the sera was measured using anti-mouse $IgG2a^b$ (mAb G12-47/30) and anti-mouse IgE (mAb 95.3) as coat, respectively, and biotinylated anti-mouse $IgG2a^b$ (mAb G12-47/30) or anti-mouse IgE (mAb R35-92; Pharmingen, San Diego, CA) for detection. Total IgG1 and total λ1-bearing antibodies were quantified by an ELISA inhibition assay. Plates were coated with a γ1- or λ1-bearing mAb and a 1:1 mixture of serially diluted serum and biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) or anti-mouse λ1 (mAb LS136) was added. Finally, the plates were incubated with streptavidin-alkaline phosphatase and substrate. Serum concentrations of total IgG2a, IgE, IgG1, and λ1-bearing Ig were calculated using mAb of the various isotypes as standards.

Results and Discussion

Cell Type–specific Expression of IL-4 In Vivo. IL-4T mice were irradiated and reconstituted with 2 × 10^6 $CD4^+$ and 8 × 10^6 $CD4^-$ spleen cells either from IL-4T mice or from congenic wt mice (Fig. 1). The $CD4^+$ and $CD4^-$ spleen cells had been isolated by high gradient magnetic cell sorting (17). The purity of $CD4^+$wt cells in the mice that received $CD4^+$wt and $CD4^-$IL-4T cells ($CD4^+$wt/$CD4^-$IL-4T mice), as determined by flow cytometry (Fig. 2), was about 99% (Table 1). Thus, the 2 × 10^6 $CD4^+$wt Th cells transferred into each mouse contained about 2 × 10^4 (1%) $CD4^-$wt cells. According to light scatter, about 80% of the contaminating $CD4^-$wt cells were lymphocytes (data not shown). Since the frequency of B and T cells responding to a particular antigen is on the order of $1/10^5$ (24, 25), it is extremely unlikely that contaminating wt B and $CD4^-$ T cells could be activated, much less produce IL-4, in a specific immune response. In view of the fact that production of IL-4 by B cells has been demonstrated only for murine B cell lym-



b

designation of mouse group	donor of transferred cells	
	$CD4^+$ (A)	$CD4^-$ (B)
$CD4^+$ IL4T / $CD4^-$ IL4T	IL4T	IL4T
$CD4^+$ IL4T / $CD4^-$ wt	IL4T	wt
$CD4^+$ wt / $CD4^-$ IL4T	wt	IL4T
$CD4^+$ wt / $CD4^-$ wt	wt	wt

Figure 1. Experimental outline. (*a*) 13-wk-old IL-4T mice were X-irradiated (600 rad) and reconstituted one day later by intravenous injection of 2 × 10^6 $CD4^+$ spleen cells (donor *A*) and 8 × 10^6 $CD4^-$ spleen cells (donor *B*), either from 13-wk-old IL-4T mice or from congenic wt mice. Immediately after transfer (*1°*) and every 4 wk thereafter (*2°*, *3°*, *4°*), the mice were immunized with alum-precipitated NP-OVA (10 μg i.p. per mouse). Concentrations of total and NP-specific Ig of various isotypes in the sera were determined 2 wk after primary immunization and 1 wk after each boost. (*b*) Donor of transferred $CD4^+$ (*A*) and $CD4^-$ (*B*) spleen cells of the various experimental groups. (*IL4T*) C57BL/6 mice homozygous for a mutation that inactivates the IL-4 gene; (*wt*) C57BL/6 mice.

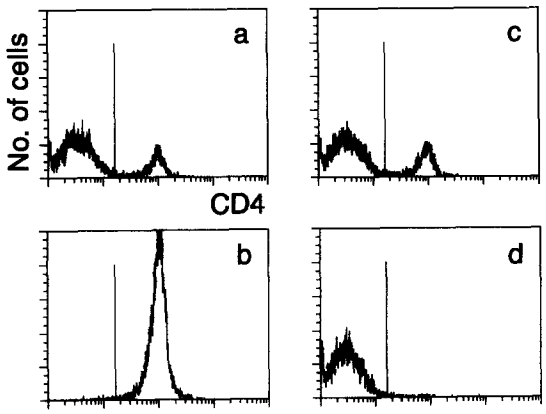


Figure 2. Purification of $CD4^+$ and $CD4^-$ murine spleen cells. Histograms of CD4 staining of cell fractions before (*a* and *c*) and after (*b* and *d*) magnetic cell separation. Live cells were gated according to light-scatter signals and propidium iodide fluorescence. (*b*) Purified $CD4^+$wt spleen cells transferred into $CD4^+$wt/$CD4^-$IL-4T mice. (*d*) $CD4^-$wt spleen cells transferred into $CD4^+$IL-4T/$CD4^-$wt mice. The threshold for determination of frequencies of positive and negative cells is indicated. The frequencies are given in Table 1.

Table 1. *Efficiency of Magnetic Cell Separation*

Group	Purity of transferred cells	
	CD4+	CD4-
	%	
CD4+IL-4T/CD4-IL-4T	98.2*	99.6
CD4+IL-4T/CD4-wt	97.3	99.3
CD4+wt/CD4-IL-4T	99.0	99.5
CD4+wt/CD4-wt	98.7	97.1

* The purity of transferred CD4+ and CD4- spleen cells was determined by flow cytometry as shown in Fig. 2.

phomas, but not for normal activated murine B cells (26), it is doubtful that B cells can produce IL-4. Of non-B, non-T cells from spleen, cells capable of producing IL-4 have been found exclusively among cells that express the high affinity FcεR (27). Such cells are mainly basophils and mast cells and comprise about 0.5% of non-B, non-T cells in the spleen (2, 27, 28). About 2.5% of the FcεR+ spleen cells, i.e., 0.0125% of the splenic non-B, non-T cells, are capable of producing IL-4 (27). Accordingly, 2×10^6 CD4+ cells of 99% purity and 0.2% contaminating nonlymphocytes contain at most 0.5 non-B, non-T cells which could produce IL-4. Thus, it is highly unlikely that the 2×10^6 CD4+ cells transferred into IL-4T mice contained any CD4- cells that could express IL-4 in a specific immune response.

The purity of the 8×10^6 CD4-wt cells in the mice that received CD4+IL-4T and CD4-wt cells (CD4+IL-4T/CD4-wt mice), was >99% (Table 1). Thus, $<8 \times 10^4$ CD4+wt cells were cotransferred. On the other hand, these 8×10^6 CD4- cells comprise about 1/5 of the CD4- cells of a spleen, including all CD4- cells from spleen reported to be capable of expressing IL-4, such as mast cells and basophils.

Reconstituted mice were immunized four times at 4-wk intervals with alum-precipitated NP_6-OVA, first directly after cell transfer. Mice were bled 2 wk after 1° immunization and 1 wk after each boost (Fig. 1). The overall antibody response to NP-OVA, as determined by the concentration of NP-specific κ- and λ-bearing antibodies in the sera, and production of NP-binding IgG2a, were similar in all four groups of mice, except for the CD4+IL-4T/CD4-IL-4T mice in which the titers of NP-specific λ-antibodies were somewhat lower (Fig. 3). Given the complexity of the experimental

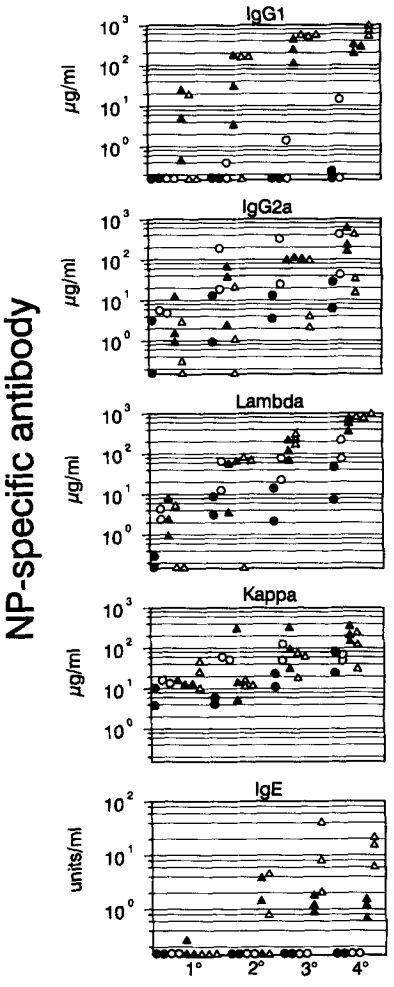


Figure 3. NP-specific antibody response. Serum concentrations of NP-binding antibodies of the indicated isotypes as determined by ELISA. Each symbol represents one animal at one time point. (●) CD4+IL-4T/CD4-IL-4T mice; (○) CD4+IL-4T/CD4-wt mice; (▲) CD4+wt/CD4-IL-4T mice; and (△) CD4+wt/CD4-wt mice.

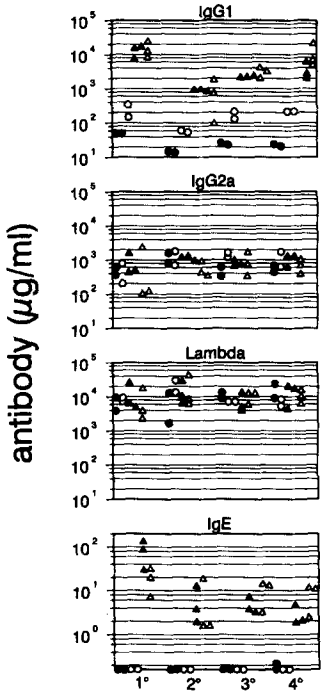


Figure 4. Serum concentrations of Ig isotypes. Serum titers of the indicated isotypes as determined by ELISA. Each symbol represents one animal. (●) CD4+IL-4T/CD4-IL-4T mice; (○) CD4+IL-4T/CD4-wt mice; (▲) CD4+wt/CD4-IL-4T mice; and (△) CD4+wt/CD4-wt mice.

procedure and the low number of CD4+IL-4T/CD4−IL-4T mice analyzed, we cannot be sure that this difference is significant. Production of total λ-bearing antibodies and of IgG2a was very similar in all animals (Fig. 4).

IL-4–producing Th Cells Are Generated Independent of IL-4 from non-Th Cells. Production of IL-4 in the reconstituted mice was assayed functionally by its exclusive ability to induce class switching to IgE. It had been shown before, that IL-4T mice produce no IgE and only little IgG1 in a T cell–dependent immune response (16). Also here, upon immunization with NP-OVA, NP-specific IgE (<0.15 U/ml) and IgG1 (<0.16 U/ml) were not detectable in the sera of IL-4T mice reconstituted with CD4+ and CD4− spleen cells from IL-4T mice (CD4+IL-4T/CD4−IL-4T mice), except for one animal in which NP-binding IgG1 was just above the limit of detection (0.24 μg/ml) after the fourth (4°) immunization (Fig. 3). In contrast, both NP-specific IgE and IgG1 were readily detectable in CD4+wt/CD4−IL-4T mice. From the tertiary (3°) response on, the sera contained about 1.5 U/ml NP-binding IgE and about 260 μg/ml NP-specific IgG1. Thus, with respect to the limit of detection, titers of NP-specific IgE were at least 10 times and those of IgG1 about 1,600 times higher than those of CD4+IL-4T/CD4−IL-4T mice.

Concentrations of total IgE in CD4+IL-4T/CD4−IL-4T mice were also below the limit of detection, with one exception of doubtful significance, and total IgG1 titers were low (<50 μg/ml; Fig. 4). In CD4+wt/CD4−IL-4T mice, however, concentrations of IgE were at least 20 times and of IgG1 about 100 times higher. Compared with IL-4T mice that received CD4+ and CD4− cells both from wt animals, titers of NP-specific IgG1 and titers of total IgE and IgG1 were very similar in CD4+wt/CD4−IL-4T mice. Concentrations of NP-specific IgE were also similar in CD4+wt/CD4−IL-4T and CD4+wt/CD4− wt mice after 1° and 2° immunization, but after 3° and 4° immunization, the titers did not increase in CD4+wt/CD4−IL-4T mice, whereas they increased about 10-fold in CD4+wt/CD4−IL-4T mice. Thus, our results show clearly that IL-4 from CD4− cells is not required for induction of IL-4 expression in Th cells and does not significantly contribute to induction of IgE class switching. In memory responses, however, IL-4 from CD4− cells may amplify IL-4 expression in Th cells and directly contribute to IgE isotype switch induction. Once antigen-specific IgE or IgG has been produced, subsequent antigenic challenge could trigger expression of IL-4 in FcεR+ mast cells or basophils by FcεR or FcγR cross-linking (27, 29). It remains unclear, how expression of IL-4 is induced in CD4+ Th cells. Our results imply that at least some CD4+ cells are capable of producing IL-4–independent of IL-4 itself. Memory Th cells are a candidate for such cells. Since the CD4+wt Th cells used for reconstitution were derived from specific pathogen-free mice that had not been exposed to NP-OVA, only memory Th cells specific for other, possibly cross-reactive, antigens, could have produced IL-4 in the CD4+wt/CD4−IL-4T mice. However, although it cannot be excluded at the present time, it appears to be highly unlikely that IL-4 from memory Th cells could be essential for triggering of IL-4 expression in naive Th cells, inasmuch as expression of IgE and largely also of IgG1 would be entirely a function of the history of an immune system, and would not depend on the nature of the antigen, as has been demonstrated (30). Alternatively, some, if not all naive CD4+ Th cells could produce IL-4 in response to a signal other than IL-4. The putative signal remains to be identified. It could be derived from APCs, as we and others have shown for the signals for induction of IFN-γ expression in CD4+ Th cells, which are derived primarily from macrophages (31–34).

IgE Synthesis Is Not Induced if IL-4 Can Be Expressed Only by Transferred Non-Th Cells. In IL-4T mice that were reconstituted with CD4+IL-4T and CD4−wt spleen cells (CD4+IL-4T/CD4−wt mice), titers of total (<0.16 mg/ml) and NP-specific IgE (<0.15 U/ml) were always below the limit of detection (Fig. 3). Thus, the 8×10^6 CD4− cells transferred into each mouse did not produce sufficient IL-4 to induce measurable amounts of IgE.

In one of two CD4+IL-4T/CD4−wt mice, serum concentrations of NP-binding IgG1 were above the limit of detection from the 2° response on, but even after 4° immunization, the titer was more than 20-fold lower as in CD4+wt/CD4−IL-4T mice (Fig. 3). Also, the titers of total IgG1 were about 20-fold lower in both CD4+IL-4T/CD4−wt mice than in CD4+wt/CD4−IL-4T mice (Fig. 4). It is known that some IgG1 can be induced independent of IL-4 (16). However, the serum concentrations of total IgG1 in CD4+IL-4T/CD4−wt mice were consistently about six times higher than in CD4+IL-4T/CD4−IL-4T mice, indicating that the CD4− cells in the CD4+IL-4T/CD4−wt mice produced some IL-4 which contributed to IgG1 synthesis but was not sufficient to induce production of detectable amounts of IgE. These findings are compatible with the notion that Th cells are the major, if not the only source of IL-4 for initial induction of IgE isotype switching in B cells.

We are grateful to Shinsuke Taki for helpful discussions and to Gertrud von Hesberg for expert technical help.

This work was supported by the Bundesministerium für Forschung und Technologie (Genzentrum Köln, group 2.07).

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Received for publication 29 November 1993 and in revised form 12 January 1994.

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