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ORIGINAL ARTICLE

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IL-4 down-regulates IL-2-, IL-3-, and GM-CSF-induced cytokine gene expression in peripheral blood monocytes*

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Summary IL-4, a product of the T-helper 0 (Th0) and 2 (Th2) subset, was originally described as a B-cell stimulatory factor and has subsequently been found to suppress IL-1α, IL-1β, IL-6, IL-8, and TNF-α gene expression in monocytes stimulated with LPS, and to upregulate IL-1 receptor antagonist (IL1-RA) gene expression. In this study we investigated the effect of IL-4 on the expression of cytokine genes in monocytes evoked by other T-helper cell cytokines: IL-2, IL-3, and GM-CSF. IL-4 down-regulated mRNA accumulation of the proinflammatory cytokines IL-1β, IL-8, and TNF-α in monocytes stimulated with IL-2, IL-3, and GM-CSF. IL-4 also suppressed the IL-2-induced IL-6 mRNA expression. Temporal analysis of the IL-4 down-regulatory effect on the IL-2-, IL-3-, or GM-CSF-induced proinflammatory cytokine gene expression in monocytes provided evidence that IL-4 acts predominantly on the post-transcriptional level. This was supported by the observation that the down-regulatory capacity of IL-4 appeared to be dependent on de novo protein synthesis. IL-4 did not exert significant influence on the induction of expression of IL-1-RA or various CSFs by IL-2, IL-3, and GM-CSF. We hypothesize that owing to its being a down-regulator of the expression of proinflammatory cytokines induced by IL-2, IL-3, and GM-CSF – products of activated Th0 or Th1 cells – together with its capacity as a B-cell stimulatory factor, IL-4 has an important role in directing the immune response.

Key words Cytokines · Monocytes · GM-CSF · IL-2 IL-3 · IL-4

Introduction

T lymphocytes and monocytes/macrophages are considered to be important sources of cytokines capable of regulating the immune and inflammatory response and hematopoiesis. Various subsets of T lymphocytes can be distinguished by their function and cytokine production profile [1]. Although initially characterized in mice, there is accumulating evidence for the existence of T-helper subsets with different cytokine profiles in human beings as well [2]. Specific to the T-helper 1 subset (Th1) is the production of IL-2 and γIFN upon activation, while the T-helper 2 (Th2) subset is characterized by the production of IL-4, IL-5, and IL-10. Both subsets have in common the production of IL-3 and GM-CSF. A third category of T-helper cells has been described (Th0) which combine the cytokine secretion profile of the Th1 and Th2 subset.

Monocytes have been shown to produce a variety of cytokines evoked by all kinds of stimuli including IL-2, IL-3, and GM-CSF. In the immune response T-helper cells and monocytes interact, resulting in the activation of T-helper cells. Lymphokines produced in this setting consequently influence cytokine production in monocytes. Previously, we evaluated the effect of IL-2, IL-3, GM-CSF, and γIFN on cytokine gene induction in monocytes [3]. IL-2 was found to induce the expression of the proinflammatory cytokine genes, including IL-1β, IL-6, IL-8, and TNF-α, and the IL-1 receptor antagonist (IL1-RA) gene. IL-2 appeared not to induce the gene expression of the colony-stimulating factors (CSF) G-CSF, GM-CSF, and M-CSF. In addition, IL-2 did not induce the expression of IL-10, a cytokine that has been shown to be produced in monocytes following stimulation by LPS. We showed that IL-3 and GM-CSF, simi-

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lar to IL-2, had the capacity to up-regulate the proinflammatory cytokine and IL1-RA genes, and also, in contrast to IL-2, appeared to induce M-CSF gene expression. IL-4, originally described as a B-cell stimulatory factor, was found to have a broad range of immunoregulatory and effector functions (reviewed in [4] and [5]). It was recently demonstrated that IL-4 may suppress IL-1α, IL-1β, IL-6, IL-8, and TNF-α gene expression in monocytes stimulated with LPS [6–8]. In contrast to the down-regulatory effect of IL-4 on proinflammatory cytokines, it was found to up-regulate the gene for IL1-RA both in vivo and in vitro [9–11]. Conflicting results have been reported regarding the ability of IL-4 to induce secretion of G-CSF and GM-CSF in human monocytes [12, 13].

In this study we investigated the effect of IL-4, a product of Th0 and Th2 cells, on the expression of cytokine genes in monocytes evoked by Th1 or Th0 cytokines IL-2, IL-3, and GM-CSF.

Materials and methods

Purification and culture of monocytes

Normal peripheral blood monocytes were isolated from pooled buffy coats from healthy blood donors. Mononuclear cells were first isolated by standard Ficoll-Isopaque separation, washed in PBS, and fractionated by centrifugal counterflow elutriation on a Heraeus Sepatech elutriator (Heraeus Separationstechnic, Ostenrode, Germany) as described by Plas et al. [14]. The different cell fractions were analyzed on a Sysmex microcellcounter F-800 (Toa Medical Electronics Co. Ltd., Kobe, Japan). Fractions containing fewer than 15% lymphocytes were further analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer after labeling with the anti-CD14 mAb Leu M3 (Becton Dickinson). Fractions containing more than 85% monocytes were used for experiments. These cells were cultured at a concentration of $2–2.5 \times 10^6$ cells/ml in hepes-buffered RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 m*M*/l L-glutamine. To prevent activation of monocytes by possible traces of endotoxin (LPS) in the culture medium, 5 μg/ml polymixin-B was added to all culture and washing media in all experiments. Special tissue culture flasks (no. 690190 Greiner BV, Alphen a. d. Rijn, The Netherlands) that prevented adherence of the cells were used. In all experiments medium controls were performed simultaneously with drug treatment on the same preparation of monocytes.

Reagents

Recombinant IL-2 was obtained from the Cetus Corporation (Amsterdam, The Netherlands). Recombinant IL-3 and GM-CSF were kind gifts from Sandoz Pharma BV (Uden, The Netherlands). Human recombinant IL-4 was a kind gift from the Schering Plough Corp. (Bloomfield, NJ, USA). In some experiments, to block intracellular protein synthesis, cells were treated with cycloheximide (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 5 μg/ml.

RNA extraction and Northern blotting

Total cellular RNA was isolated by the guanidium-thiocyanate-phenol-chloroform extraction procedure with minor modifications as described previously [15]. Equal amounts of RNA (25 μg unless otherwise indicated) were fractionated in 1% agarose gels containing 2.2 *M* formaldehyde. RNA was transferred to nylon membranes and fixed by short-wave ultraviolet radiation. Prehybridization, hybridization, and autoradiography were performed using standard techniques. The probes were radiolabeled with [α-^{32}P] dCTP using a random primer DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). For β-actin, IL-1β, IL-6, IL-8, TNF-α, IL1-RA, G-CSF, and GM-CSF hybridization, PCR-generated cDNA probes were used [16]. An M-CSF probe containing a 1642-kb insert was kindly provided by Dr. P. Ralph (Cetus Corp., Emeryville, CA, USA). As a control for loading equivalent amounts of RNA per lane, expression of β-actin gene was measured. mRNA expression was quantitated by scanning using an Ultroscan XL densitometer (Pharmacia LKB, Uppsala, Sweden). Values for cytokine mRNA expression obtained by densitometric scanning were corrected for differences in β-actin mRNA expression.

Results

IL-4 down-regulates IL-2-, IL-3-, and GM-CSF-induced cytokine gene expression

Co-stimulation of monocytes with IL-2 (3000 IU/ml) and IL-4 (10 ng/ml) resulted in significantly decreased mRNA accumulation of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α in comparison to stimulation with IL-2 alone (Fig. 1). As measured by densitometric scanning, the down-regulatory effect of IL-4 as a percentage of IL-2-induced proinflammatory cytokine

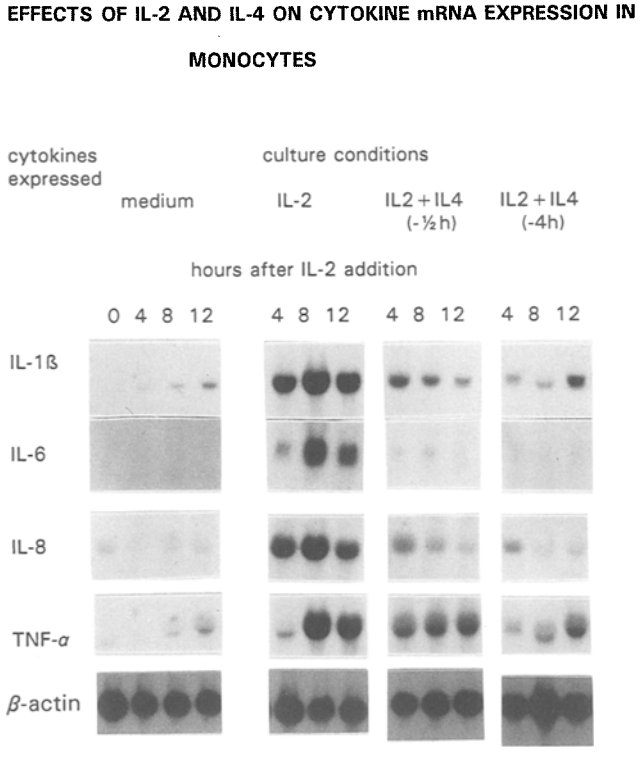

Fig. 1 Cytokine gene mRNA accumulation measured by Northern blot analysis. Monocytes were cultured for 4, 8, or 12 h in medium alone, with IL-2 (3000 IU/ml), or with IL-2 (3000 IU/ml) and IL-4 (10 ng/ml). IL-4 was added to the monocyte cultures ½ h (*−½ h*) or 4 h (*−4 h*) before the addition of IL-2

Table 1 Down-regulation by IL-4 of IL-2-induced proinflammatory cytokine mRNA expression in monocytes. Mean ± standard deviation of three experiments

Incubation time (hours)[a]	Down-regulation of IL-2-induced mRNA expression by IL-4 (%)			
	IL-1β	IL-6	IL-8	TNF-α
4	90± 7	85±10	88± 6	54±21
8	83±14	86±11	80±15	87±11
12	74±18	94± 1	88± 6	71±13

[a] Cells were cultured for 4, 8, or 12 h with IL-2 (3000 IU/ml) and IL-4 (10 ng/ml)

Table 2 Down-regulation by IL-4 of IL-3- or GM-CSF-induced proinflammatory cytokine mRNA expression in monocytes. Mean ± standard deviation of three experiments

Incubation time (hours)[a]	Inducing cytokine	Down-regulation of mRNA expression by IL-4 (%)		
		IL-1β	IL-8	TNF-α
4	IL-3	77±24	78±25	67±11
	GM-CSF	91± 2	70± 7	52± 4
8	IL-3	84± 4	65±20	68±19
	GM-CSF	84± 9	82± 4	56±13
24	IL-3	78±21	81± 3	78±11
	GM-CSF	87±17	79±16	85± 6

[a] Cells were cultured for 4, 8, or 24 h with IL-3 (250 ng/ml) or GM-CSF (50 ng/ml) and IL-4 (10 ng/ml)

expression at 4, 8, and 12 h incubation was 90, 83, and 74 for IL-1β, 85, 86, and 94 for IL-6, 88, 80, and 88 for IL-8, and 54, 87, and 71 for TNF-α, respectively (Table 1). As was found for IL-2 and IL-4 separately, co-stimulation of the cells with IL-2 and IL-4 did not result in the expression of G-CSF, GM-CSF, and M-CSF genes (data not shown). IL-4 had only a weak effect on the expression of the IL1-RA gene and was not found to up- or down-regulate the IL-2-induced IL1-RA gene expression in monocytes (data not shown).

IL-4 also showed a profound down-regulatory effect on the IL-3- or GM-CSF-induced expression of the IL-1β, IL-8, and TNF-α genes (Table 2). The IL-3- and GM-CSF-induced expression of IL-6 and M-CSF was too weak to allow a reliable quantitative assessment of the down-regulatory effect of IL-4 on these genes. Stimulation of monocytes with IL-3, GM-CSF, or IL-4 separately or in combinations did not result in the expression of G-CSF and GM-CSF genes. No significant additive effect was found of IL-4 on the IL-3- or GM-CSF-induced IL1-RA expression (data not shown).

Kinetics of the down-regulatory effect of IL-4 on cytokine gene expression

We investigated whether the addition of IL-4 to monocyte cultures before or after the addition of IL-2, IL-3, or GM-CSF influenced the down-regulatory effect of IL-4. When IL-4 was added 4 h prior to, simultaneously with, or 1–4 h after stimulation with IL-2, similar down-regulation of the proinflammatory cytokine gene expression was observed 8 h after IL-2 stimulation (Table 3). Addition of IL-4 at various intervals during monocyte stimulation with IL-2 for 4 or 12 h resulted in down-regulatory effects on proinflammatory cytokine mRNA expression similar to those observed for 8 h stimulation with IL-2 (data not shown). Because the down-regulatory effect of IL-4 added 2 h after the addition of IL-2 was still profound when measured at 4 h after the addition of IL-2, it can be concluded that less than 2 h were necessary for IL-4 to exhibit its inhibitory effect.

When IL-4 was added prior to, simultaneously with, or up to 2 h after the addition of IL-3 or GM-CSF to the monocyte culture, the same degree of suppression of IL-1β, IL-8, and TNF-α mRNA accumulation was measured at 4, 8, and 24 h (the data for 8 h stimulation with IL-3 or GM-CSF are shown in Table 4). The addition of IL-4 4 h after the addition of GM-CSF did not

Table 3 Temporal analysis[a] of the IL-4 down-regulatory effect on IL-2-induced cytokine mRNA accumulation[b]

Time of addition of IL-4	Down-regulation of IL-2-induced mRNA expression by IL-4 (%)			
	IL-1β	IL-6	IL-8	TNF-α
−4	75	83	76	84
−0.5	77	79	71	80
0	83 (14)[c]	86 (16)	80 (15)	87 (11)
+1	85 (18)	88 (11)	81 (19)	87 (8)
+2	97	95	93	95
+4	89 (13)	93	81 (22)	94

[a] IL-4 (10 ng/ml) was added to the monocyte cultures from 4 h before (−4) to 4 h (+4) after the addition of IL-2
[b] Cells were cultured for 8 h in IL-2 (3000 IU/ml)
[c] Mean of two experiments (standard deviation in parentheses)

Table 4 Temporal analysis[a] of the IL-4 down-regulatory effect on IL-3- or GM-CSF-induced cytokine mRNA accumulation[b]

Time of addition of IL-4	Down-regulation of mRNA expression by IL-4 (%)					
	IL-1β		IL-8		TNF-α	
	IL-3	GM-CSF	IL-3	GM-CSF	IL-3	GM-CSF
−4	85	n.d.	92	n.d.	47	n.d.
−0.5	75	76	81	65	39	48
0	83	81	86	85	62	61
+1	92	n.d.	92	n.d.	77	n.d.
+2	82	85	81	57	60	43
+4	n.d.	70	n.d.	61	n.d.	41

[a] IL-4 (10 ng/ml) was added to the monocyte cultures from 4 h before (−4) to 4 h (+4) after the addition of IL-3 or GM-CSF
[b] Cells were cultured for 8 h in IL-3 (250 ng/ml) or GM-CSF (50 ng/ml)

Table 5 Effects of the protein synthesis inhibitor cycloheximide (*CX*) on the down-regulation by IL-4 of IL-2 induced proinflammatory cytokine gene expression in monocytes[a]

Culture condition	Cytokine gene expression as % of control (IL-2)			
	IL-1β	IL-6	IL-8	TNF-α
Medium	4± 1	7± 3	13± 2	10± 3
IL-2	100	100	100	100
IL-2+IL-4	3± 1	5± 2	4± 2	12± 5
IL-2+IL-4+CX	114±12	169± 63	107±61	27±10
CX	16±12	14± 11	22±20	9± 2
IL-2+CX	92±18	197±112	104±47	41± 4
IL-4+CX	12±11	15± 12	5± 3	6± 2
IL-4	3± 1	4± 3	4± 2	18±12

[a] Monocytes were cultured for 8 h in IL-2 (3000 IU/ml) and/or IL-4 (10 ng/ml) and/or CX (5 μg/ml)

change the down-regulation of IL-1β, IL-8, and TNF-α gene expression in comparison to earlier addition of IL-4. These data also show that the time necessary for IL-4 to exert its down-regulatory effect was less than 2 h and that the down-regulatory capacity of IL-4 was preserved even when IL-4 was added to the monocyte cultures up to 4 h after the exposure to GM-CSF.

Down-regulation of IL-2-induced cytokine gene expression by IL-4 is dependent on de novo protein synthesis

To analyze whether IL-4 exerts down-regulation of cytokine genes by the intermediate synthesis of a regulatory protein, monocytes were stimulated with IL-2 and IL-4 in the presence or absence of a protein synthesis inhibitor, cycloheximide. The addition of cycloheximide alone to monocyte cultures resulted in induction of the proinflammatory cytokine genes detectable after 4 h of incubation, but not thereafter. When analyzed 8 h after stimulation of the monocytes in the presence of cycloheximide, IL-2-induced expression of IL-1β, IL-6, and IL-8 appeared not to be dependent on protein synthesis, in contrast to TNF-α expression, which was found to be partially inhibited by cycloheximide. IL-4 did not shown down-regulation of IL-2-induced proinflammatory cytokine gene expression in the presence of cycloheximide, indicating that protein synthesis is essential for this IL-4 activity (Table 5).

Discussion

Previously we showed that IL-2, IL-3, and GM-CSF were capable of inducing the expression of the proinflammatory cytokine genes and the IL1-RA gene in monocytes. We showed that IL-3 and GM-CSF, but not IL-2, induced M-CSF gene expression and that IL-3, GM-CSF, and IL-2 had no up-regulatory effect on the G-CSF and GM-CSF genes in monocytes cultured under serum-free, LPS-free, and nonadherent conditions. Here, we investigated the effect of IL-4 on the gene expression of the proinflammatory cytokines induced by IL-2, IL-3, and GM-CSF. In addition, we studied the effect of IL-4 on the expression of the colony-stimulating factor (CSF) genes and on the IL1-RA gene in monocytes.

Previously, it was shown that IL-4 suppressed LPS-induced proinflammatory cytokine gene expression and cytokine secretion by monocytes [6–8]. In this study we demonstrated that IL-4 also strongly down-regulated the level of proinflammatory cytokine mRNA in monocytes stimulated by IL-2, IL-3, or GM-CSF. The IL-2-, IL-3-, or GM-CSF-induced IL1-RA expression appeared not to be influenced by IL-4, in contrast to the recent observation that IL-4 up-regulates the LPS-induced IL1-RA gene expression [9, 10]. The observation that in the presence of the protein synthesis inhibitor cycloheximide the down-regulatory effect of IL-4 on the IL-2-induced proinflammatory cytokine gene expression was largely abrogated may indicate interference of IL-4 at the post-transcriptional level. Such a molecular mechanism for the IL-4 effect on proinflammatory cytokine genes has recently been shown for LPS-induced IL-1β and IL-6 expression, as it was demonstrated that IL-4 reduced the IL-1β mRNA half-life from 2–3 to 0.5–1 h and the IL-6 mRNA half-life from 4 h to less than 1 h [9, 10]. Based on the observation that IL-1β, IL-6, IL-8, and TNF-α mRNA transcripts are rich in adenine and uridine nucleotide sequences (AU sequences) at their 3′ untranslated region, which renders them more susceptible to degradation, it has been speculated that IL-4 mediates its effect by the induction of a ribonuclease that specifically degrades cytokine mRNA [8–10, 17–19]. In support of such a hypothesis is the finding that IL1-RA mRNA, which does not contain AU-rich sequences, was not down-regulated by IL-4. Although it was shown by Donnelly et al. that IL-4 can reduce the transcriptional rate of the IL-1β gene in LPS-stimulated monocytes, these authors also suggested that the predominant IL-4-induced effect occurs at the post-transcriptional level [17].

Kinetic analysis showed that IL-4 exhibited its suppressive effect irrespective of whether IL-4 was added to the monocyte cultures before, at the same time, or up to 4 h after the addition of IL-2, IL-3, or GM-CSF and that less than 2 h were necessary for IL-4 to attain maximal down-regulation. These findings are in line with the previously observed reduction of IL-1β and IL-6 mRNA half-lives to less than 1 h and strongly support a predominant post-transcriptionally regulated inhibitory effect of IL-4 on the expression of proinflammatory cytokines.

CSF genes in monocytes appeared not to be affected by IL-4 as a single stimulus or by co-stimulation with IL-2, IL-3, or GM-CSF. The observation by others that IL-4 can also suppress LPS-induced G-CSF and GM-CSF production in monocytes in vitro suggests that IL-

4 may play a role as a negative regulator of inducible hematopoiesis [13].

The finding that IL-4 can antagonize the proinflammatory effects of IL-2, IL-3, and GM-CSF, products of activated Th0 or Th1 cells, together with its capacity as a B-cell stimulatory factor, suggests a specific role for IL-4 in determining the mode of the immune response. However, the pathophysiologic relevance of the observed antiinflammatory capacity of IL-4 as an inhibitor of gene expression and production of the proinflammatory cytokines in response to LPS is not clear. Several lines of evidence indicate that the sequelae of endotoxemia are probably mediated by proinflammatory cytokines [20–26]. As yet there are no in vivo studies demonstrating a role for IL-4 as a protective factor in the setting of endotoxemia. In view of the fact that IL-4 is produced mainly by activated T cells, it even may be doubtful whether IL-4 takes part in the maintenance of homeostasis when endotoxemia occurs, because antigen-specific T-cell activation is probably a process too slow to counteract the immediate effects of endotoxin [23, 27]. Other microbial products, in particular, the so-called superantigens, can cause severe clinical conditions, like the toxic shock syndrome (TSS), where IL-1β and TNF-α are thought to play an important pathophysiologic role. Superantigens are among the most powerful T-cell mitogens known that can induce the release of large amounts of cytokines, including IL-2, γIFN, and TNF-α (reviewed in [28]). No data are reported on the induction of IL-4 production or gene expression in T cells by superantigens. The recent observation that the induction of significant amounts of IL-1β and TNF-α by the *Staphylococcus* TSS toxin 1 occurred in the presence of both monocytes and T cells suggests that also in this setting the anti-inflammatory effect of IL-4 might be of less influence [29]. Furthermore, it has been observed, in a model to investigate the dynamics of inflammation in vivo using skin lesions induced by suction, that the levels of IL-1β, IL-6, IL-8, and TNF-α were markedly elevated after 24 h, while there was little accumulation of IL-4 [30]. Although it remains to be determined whether IL-4 has an important physiologic anti-inflammatory function, the recent observation that monocytes from cancer patients treated with IL-4 expressed increased IL1-RA mRNA suggests that IL-4 may have clinical significance as an anti-inflammatory agent [11]. It can be speculated that the observation that IL-4 can suppress the expression of proinflammatory cytokine genes induced by various stimuli in vitro is merely the reflection of the post-transcriptional mechanism by which IL-4 probably exerts its effect.

In conclusion, it was shown that IL-4 can down-regulate in monocytes IL-2-, IL-3-, and GM-CSF-induced expression of the proinflammatory cytokines IL-1β, IL-8, and TNF-α, and that IL-4 also can suppress the IL-2-induced IL-6 mRNA expression. The down-regulatory capacity of IL-4 was found to be preserved even when IL-4 was added to the monocyte cultures 4 h after the exposure to IL-2, IL-3, of GM-CSF. This finding, together with the observation that the down-regulatory effect of IL-4 was dependent on de novo protein synthesis and that the time necessary for IL-4 to exert its down-regulatory effect appeared to be less than 2 h, while proinflammatory cytokine mRNA half-lives have been reported to be 2–4 h, suggests that this IL-4 effect probably is exerted predominantly at the post-transcrptional level. IL-4 appeared not to have a significant influence on the induction of IL1-RA or CSF gene expression by IL-2, IL-3, and GM-CSF. We hypothesize that the down-regulatory effect of IL-4 on the expression of proinflammatory cytokines induced by IL-2, IL-3, or GM-CSF, products of activated Th0 or Th1 cells, together with its capacity as a B-cell stimulatory factor, indicates a specific role for IL-4 in directing the immune response.

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