Natural Killer Cell Stimulatory Factor (Interleukin 12 [IL-12]) Induces T Helper Type 1 (Th1)-specific Immune Responses and Inhibits the Development of IL-4-producing Th Cells

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Summary

The effects exerted on the in vitro development of antigen-specific T cell lines and T cell clones by addition or neutralization of interleukin 12 (IL-12) in lymphocyte bulk culture were examined. T cell lines specific for *Dermatophagoides pteronyssinus* group I (Der p I) derived in the presence of IL-12 exhibited reduced ability to produce IL-4 and increased ability to produce interferon γ (IFN-γ), and developed into Der p I–specific CD4$^+$ T cell clones showing a T helper type 0 (Th0)- or Th1-, instead of Th2-, like cytokine profile. In contrast, purified protein derivative (PPD)-specific T cell lines derived in the presence of anti-IL-12 antibody exhibited an increased ability to produce IL-4 and developed into PPD-specific CD4$^+$ T cell clones showing a Th0-, instead of Th1-, like profile. The influence of IL-12 on the cytokine secretion profile of Der p I–specific T cell lines was not prevented by addition to lymphocyte bulk cultures of anti-IFN-γ antibody, but could be at least partially inhibited by the removal from bulk cultures of CD16$^+$ cells. Thus, IL-12 and CD16$^+$ cells appear to have inhibitory effects on the development of IL-4-producing cells and to play an inductive role in promoting Th1-like responses.

Differential cytokine production by CD4$^+$ Th cells during an immune response plays an important role in regulating the nature of the response. In the mouse, CD4$^+$ T cells that produce IL-2 and IFN-γ but little or no IL-4 and IL-5 (Th1 cells) induce the activation of macrophages to kill intracellular parasites, delayed-type hypersensitivity, and production of IgG2a, but not of IgG1 and IgE. In contrast, responses by CD4$^+$ T cells that predominantly produce IL-4 and IL-5 (Th2 cells) result in generation of IgG1- and IgE-secreting cells and eosinophilia (1). Human CD4$^+$ T cell clones specific for bacterial antigens (2–4) and allergen- or helminth-specific T cell clones (2, 3, 5, 6) have been found to exhibit Th1- or Th2-like cytokine production profiles, respectively, reminiscent of those described for mouse T cells. Both murine and human CD4$^+$ T cells with an intermediate cytokine profile (Th0) were also described (7–9). Th1 and Th2 cells may differentiate from a common pool of precursors (10) along differentiation pathways controlled, at least in part, by cytokines produced by lymphocytes or accessory cells. In the mouse, IFN-γ promotes differentiation of Th precursors into Th1 cells, both in vitro (11) and in the *Leishmania* model in vivo (12). IL-4 both in vitro (13) and in vivo (14) has opposite activity, inducing differentiation into Th2 cells. More recently, a reciprocal regulatory role of cytokines in the differentiation of human Th1 and Th2 cells was also demonstrated (15, 16). IL-4 added to bulk lymphocyte culture before cloning shifted the differentiation of purified protein derivative (PPD)-specific T cells from the Th1 to the Th0, or even to the Th2, phenotype (15). In contrast, either IFN-γ plus anti-IL-4 antibody or IFN-α induced allergen-specific T cells or T cells specific for helminth constituents to differentiate into Th0, or even Th1, instead of Th2 clones (15, 16).

Although IFN-γ is produced by both NK and T cells, resting NK cells are more efficient producers of IFN-γ than T cells in response to IL-2 and other stimuli, due to their rapid response and constitutive expression of the IL-2R chain (17, 18). A novel heterodimeric cytokine termed natural killer cell stimulatory factor (NKSF) or IL-12 (19, 20) was recently shown to be, together with IFN-γ and IL-2 (21), one of the small groups of cytokines able to rapidly enhance NK cell–mediated cytotoxicity. NKSF/IL-12, a product of phagocytic cells and B cells, is active on T and NK cells, affecting, in addition to cytotoxicity, cellular proliferation and produc-

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tion of lymphokine (19, 22–24). In particular, NKSF/IL-12 is a potent inducer of IFN-γ production, acting in this effect synergistically with IL-2 and several other inducing stimuli (19, 25).

In this study, we analyzed whether IL-12 affects the in vitro differentiation of Th cell subsets. We show that *Dermatophagoides pteronyssinus* group I (Der p I)-specific T cell lines derived in the presence of IL-12 exhibited reduced ability to produce IL-4 and increased ability to produce IFN-γ, and developed into Der p I–specific T cell clones showing the Th0- or Th1-, instead of Th2-, like cytokine profile. In contrast, PPD-specific T cell lines derived in the presence of anti-IL-12 antibody exhibited an increased ability to produce IL-4 and developed into T cell clones showing Th0-, instead of Th1-, like phenotype. The influence of IL-12 on the cytokine production profile of Der p I–specific T cell lines was not apparently mediated by its ability to stimulate the production of IFN-γ, but could be at least partially inhibited by removal from bulk cultures of $CD16^+$ cells. Thus, IL-12 and NK cells seem to play an important role in determining the development of Th1-like responses.

Materials and Methods

Reagents. PPD was obtained from Istituto Sieroterapico e Vaccinogeno Sclavo (Siena, Italy). Der p I was obtained from Lofarma Allergeni (Milan, Italy). PHA was purchased from Gibco Laboratories (Grand Island, NY) and PMA was from Sigma Chemical Co. (St. Louis, MO). Anti-CD3 mAb was purchased from Ortho Pharmaceuticals (Raritan, NJ). Anti-CD4, anti-CD8, and anti-CD16 mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA). Human rIFN-γ and one of the three anti-IFN-γ mAbs (27.387.33) were purchased from Janssen (Beerse, Belgium). The other two mAbs were a generous gift of C. M. Liang (Biogen, Cambridge, MA). Human rIFN-α2b was purchased from Schering Co. (Kenilworth, NJ). Human rIL-2 was a generous gift of Eurocetus (Milan, Italy). Human rIL-12 was provided by Genetics Institute Inc. (Cambridge, MA) (22). Neutralizing anti-IL-12 mAb C8.6 was previously described (26).

Subjects. PBMC were obtained from healthy volunteers showing delayed-type hypersensitivity skin reaction to PPD, and from atopic house dust mite–sensitive subjects suffering from atopic dermatitis and/or extrinsic asthma and showing immediate-type skin reactivity to Der p I.

Generation of Antigen-specific T Cell Lines and T Cell Clones. PPD- and Der p I–specific T cell lines were generated according to a technique previously described (5, 15, 16). Briefly, 10^6 PBMC in 2 ml RPMI 1640 supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-ME, and 5% human serum (complete medium) were stimulated in 24-well flat-bottomed plates for 5 d with Der p I (10 μg/ml) in the absence or presence of IL-12 (100 U/ml) or with PPD (10 μg/ml) in the absence or presence of anti-IL-12 antibody (1 μg/ml), respectively. Human IL-2 (20 U/ml) was then added and cultures were continued for an additional 9 d. Viable T blasts were resuspended in complete medium and tested for their antigen specificity before cloning procedure. To assess the antigen specificity of T cell lines, 2×10^4 T blasts were seeded in microplates and cocultured for 48 h with irradiated (6,000 rad) autologous PBMC (10^5) in the presence of medium alone, Der p I (1 μg/ml), or PPD (1 μg/ml), respectively. After a 16-h pulse with 0.5 μCi [^{3}H]thymidine (Amersham International, Amersham, UK), cultures were harvested and radioactivity was measured by liquid scintillation. To generate T cell clones, T blasts obtained from antigen-specific T cell lines were seeded under limiting dilution conditions (0.3 cell/well) in 96 round-bottomed microwell plates containing 10^5 irradiated (6,000 rad) allogeneic PBMC cells (as feeder cells) and PHA (1% vol/vol) in a final volume of 0.2 ml complete medium supplemented with IL-2 (20 U/ml) and 10% FCS (Hyclone Labs., Logan, UT), as reported elsewhere (5, 15, 16). Growing microcultures were then supplemented, at weekly intervals, with IL-2 (20 U/ml) and 10^5 irradiated feeder cells. The phenotype of T blasts from T cell lines and T cell clones was examined by flow cytometry (16) and their antigen-specificity assessed by measuring [^{3}H]thymidine uptake after a 60-h stimulation with the appropriate antigen under MHC-restricted conditions (3, 5). When stimulation index (ratio between the mean cpm obtained in cultures stimulated with APC plus antigen and the mean cpm obtained in cultures with APC alone) was >10, responses were considered as positive. Clonal efficiency was evaluated according to Taswell (27).

In some experiments, T cell lines were derived from PBMC suspensions that had been depleted of $CD16^+$ cells by a double-step panning technique, as described by Zubler et al. (28).

Induction of Cytokine Production by Antigen-specific T Cell Lines and Clones. To induce the cytokine production, T blasts were resuspended at the concentration of 10^6/ml in complete medium and cultured for 24 h in the presence of PMA (10 ng/ml) plus anti-CD3 mAb (100 ng/ml). Cell-free culture supernatants were collected, filtered, and stored in aliquots at −70°C until used.

Quantitation of IFN-γ and IL-4. The quantitative determinations of IFN-γ and IL-4 were performed by a commercial RIA (Centocor Inc., Malvern, PA) and ELISA (Quantikine R & D Systems, Minneapolis, MN), respectively. Levels of cytokine 3 SD over those of control supernatants obtained by stimulation of irradiated feeder cells alone were regarded as positive.

Results

PBMC from 13 atopic donors were stimulated with Der p I in the absence or in the presence of IL-12. The resulting cell lines were assessed for antigen specificity, cell surface phenotype, and profile of cytokine production (Table 1). Both IL-12(−) and IL-12(+) Der p I–specific cell lines showed strong and comparable proliferative responses to Der p I under MHC-restricted conditions. However, despite the fact that total numbers of cells recovered were comparable, proportions of $CD8^+$ and $CD16^+$ cells were significantly higher in IL-12(+) than in IL-12(−) cell lines. The profile of cytokine production in response to stimulation with PMA plus anti-CD3 mAb was also different: Der p I–specific IL-12(+) cell lines produced significantly higher concentrations of IFN-γ and significantly lower concentrations of IL-4 than IL-12(−) cell lines (Table 1).

To test whether IL-12-induced changes in the cytokine profile of T cell lines really reflected a shift in the differentiation of Der p I–specific $CD4^+$ T cells toward the Th1-like profile, T blasts from the IL-12(−) and IL-12(+) T cell lines derived from one donor were cloned. 20 IL-12(−) and 22 IL-12(+) Der p I–specific $CD4^+$ T cell clones were obtained and assessed for their ability to produce IFN-γ and IL-4 in response to stimulation with PMA plus anti-CD3 mAb. As shown in Fig. 1 *A*, the proportion of IL-12(−) T cell clones

Table 1. *Antigen-induced Proliferative Response, Cell Surface Phenotype, and Cytokine Production by Der p I–specific T Cell Lines Obtained in the Absence or Presence of IL-12*

Cells cultured with:	Proliferative response*		Cell surface phenotype‡			Cytokine production§	
	APC	APC + Der p I	$CD4^+$	$CD8^+$	$CD16^+$	IL-4	IFN-γ
	cpm × 10^{-3}		*cells/ml × 10^{-6}*			*ng/ml*	*U/ml*
Der p I alone	2.1 ± 0.9	43.1 ± 12	1.7 ± 0.5	0.2 ± 0.1	0.2 ± 0.1	1.8 ± 0.3	56 ± 15
Der p I + IL-12	3.3 ± 1.6	37.9 ± 7.4	0.9 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	104 ± 21
p	NS	NS	<0.05	<0.05	<0.05	<0.001	<0.05

PBMC from 13 atopic donors were cultured with Der p I (10 μg/ml) alone or Der p I plus IL-12 (100 U/ml), and T cell lines were derived as described in Materials and Methods.

* Der p I specificity of T cell lines was assessed by culturing the cells (2 × 10^4) for 3 d with irradiated autologous PBMC (APC) alone (10^5) or in the presence of Der p I (10 μg/ml). 16 h before harvesting, triplicate cultures were pulsed with [^{3}H]thymidine. Results expressed as mean values (± SE) of cpm obtained with the 13 T cell lines.

‡ Cell surface phenotype was evaluated by flow cytometer analysis. Mean values (± SE) of absolute numbers of different subsets are reported.

§ T blasts (10^6/ml) were stimulated with PMA (10 ng/ml) plus anti-CD3 antibody (100 ng/ml) and the 24-h culture supernatants assessed for cytokine content as described in Materials and Methods.

able to produce IL-4 (18/20) was significantly higher than that (7/22) of IL-12(+) clones (p <0.005). Likewise, the mean IL-4 concentrations produced by IL-12(−) clones were significantly higher than that of IL-12(+) clones (2.1 ± 0.4 vs. 0.8 ± 0.4 ng/μl; p <0.05). In contrast, no significant differences between IL-12(−) and IL-12(+) clones in either the proportion of clones able to produce IFN-γ (17/20 vs. 17/22) or the mean concentration of IFN-γ produced (84 ± 10 vs. 91 ± 17 U/μl) were observed.

To analyze whether endogenously produced IL-12 can influence the cytokine profile of antigen-specific T cells, PPD-specific T cell lines were derived from PBMC of four normal donors in the presence or absence of neutralizing anti-IL-12 antibody (Table 2). The ability of PPD-specific T cell lines to produce IFN-γ was not influenced by culturing the cells in presence of anti-IL-12 mAb, but their ability to produce IL-4 was significantly enhanced. 8 and 11 PPD-specific T cell clones were obtained from the cell lines originated from one normal donor in the absence or presence of anti-IL-12 mAb, respectively. As shown in Fig. 1 *B*, all PPD-specific T cell clones produced high concentrations of IFN-γ in response to stimulation with PMA plus anti-CD3 mAb, but only two of those derived in the absence of anti-IL-12 mAb produced IL-4. In contrast, all PPD-specific T cell clones derived in presence of anti-IL-12 antibody produced high IL-4 concentrations.

The possibility that the inhibitory effect of IL-12 on the development of IL-4-producing $CD4^+$ T cells was mediated

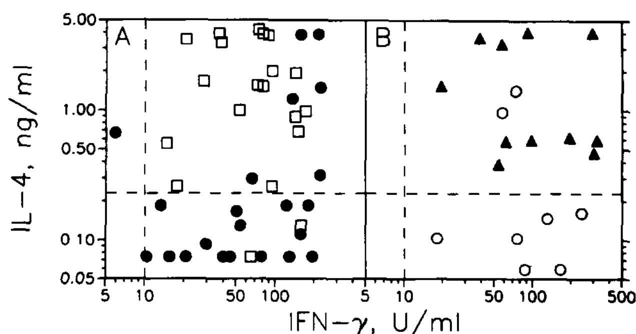

Figure 1. Production of IL-4 and IFN-γ by (*A*) Der p I-specific $CD4^+$ T cell clones derived from PBMC cultured with Der p I in the absence (□) or presence of IL-12 (●) and by (*B*) PPD-specific T cell clones derived from PBMC cultured with PPD in the absence (○) or presence of anti-IL-12 antibody (▲). Clonal T blasts (10^6/ml) were stimulated for 24 h with PMA (10 ng/ml) plus anti-CD3 antibody (100 ng/ml) and supernatants were assessed for IL-4 and IFN-γ content, as described in Materials and Methods. Dotted lines represent the mean values plus 3 SD of cytokine concentrations produced by irradiated feeder cells alone in response to PMA plus anti-CD3 antibody.

Table 2. *IL-4 and IFN-γ Production in Response to Stimulation with PMA plus Anti-CD3 Antibody by PPD-specific T Cell Lines Derived in Absence or in Presence of Anti-IL-12 Antibody*

Cells cultured with:	IL-4	IFN-γ
	ng/ml	*U/ml*
PPD	0.04 ± 0.01	148 ± 19
PPD + anti-IL-12 antibody	0.6 ± 0.1	155 ± 10
p	<0.05	N.S.

PBMC from four normal donors were cultured in presence of PPD (10 μg/ml) alone or PPD plus anti-IL-12 antibody (1 μg/ml) and PPD-specific T cell lines then derived as described in Materials and Methods. T blasts (10^6/ml) from PPD-specific T cell lines were stimulated with PMA plus anti-CD3 antibody and the 24-h culture supernatants were assessed for IL-4 and IFN-γ content by ELISA and RIA, respectively, as described in Materials and Methods. Mean values ± SE (n = 4) are reported.

at least in part by its activity on NK cells was investigated. To this end, IL-12(−) and IL-12(+) Der p I–specific T cell lines were derived from two atopic donors by using unfractionated PBMC or PBMC that had been depleted of $CD16^+$ cells (Table 3). In both donors, the IL-12-induced inhibitory effect on the development of IL-4-producing cells was lower when Der p I–specific T cell lines were derived from $CD16^-$ PBMC than when derived from total PBMC.

To test whether the inhibitory effect of IL-12 on the development of Der p I–specific T cells into IL-4-producing T cell lines and clones was mediated by the ability of IL-12 to stimulate IFN-γ production by either NK or T cells, Der p I–specific T cell lines were derived by adding IL-12 in the absence or presence of anti-IFN-γ antibody. In five experiments, anti-IFN-γ antibodies did not prevent the inhibitory activity of IL-12 on the development of Der p I–specific T cells into IL-4-producing T cell lines (data not shown).

Discussion

The results of the present study indicate that IL-12 promotes the in vitro differentiation of Th lymphocytes into cells showing a Th1-like profile of cytokine production. Exogenous rIL-12, added to bulk lymphocyte cultures from atopic donors during in vitro stimulation with Der p I, favored the differentiation of antigen-specific T cell lines and clones producing high concentrations of IFN-γ and no or low concentrations of IL-4, unlike the T cell lines and clones generated in the absence of IL-12, which usually produce high amounts of IL-4 and no or low concentrations of IFN-γ. On the other hand, the addition in bulk culture of anti-IL-12 Ab to neutralize endogenously produced IL-12 favored the differentiation of PPD-specific T cells from normal donors (which usually develop into T cell lines and clones producing high concentrations of IFN-γ but no IL-4) into T cells producing noticeable amounts of IL-4, as well. The results clearly indicate that IL-12 in vitro and, possibly, in vivo is a physiological inducer of the Th1 type of immune responses, with a negative effect on the development of IL-4-producing Th cells.

Table 3. *Removal from Bulk Cultures of $CD16^+$ Cells Partially Prevents the Ability of IL-12 to Inhibit the Development of Der p I–specific T Cells into IL-4-producing T Cell Lines*

Bulk culture conditions	IL-4 produced by Der p I–specific T cell lines	
	Exp. 1	Exp. 2
	ng/ml	
Total PBMC + Der p I	4.0	0.5
Total PBMC + Der p I + IL-12	0.1	0.02
$CD16^-$-PBMC + Der p I	4.0	0.5
$CD16^-$-PBMNC + Der p I + IL-12	1.5	0.3

Unfractionated PBMC or PBMC depleted of $CD16^+$ cells were cultured with Der p I (10 μg/ml) alone or Der p I plus IL-12 (100 U/ml) and T cell lines were derived as described in Materials and Methods. T blasts (10^6/ml) from Der p I–specific T cell lines were stimulated with PMA (10 ng/ml) plus anti-CD3 antibody (100 ng/ml) and the 24-h culture supernatants were assessed for IL-4 content by ELISA, as described in Materials and Methods.

The ability of IL-12 to promote the in vitro development of Th1-like cells is at least in part due to its stimulatory effect on NK cells. This conclusion is suggested by the fact that: (*a*) both relative and absolute proportions of $CD16^+$ were increased in T cell lines derived in the presence of IL-12; and (*b*) removal of $CD16^+$ cells from PBMC bulk cultures partially prevented the inhibitory effect of IL-12 on the development of IL-4-producing Der p I–specific T cells. Because IL-12, particularly in association with IL-2, stimulates IFN-γ production by both T cells and NK cells (19, 25) and IFN-γ inhibits both the development of T cells into IL-4-producing cells (15, 16) and the proliferation of Th2 clones (11), the IL-12-induced T cell differentiation into Th1-like cells could be indirect and mediated by induced IFN-γ production. However, neutralizing antibodies to IFN-γ failed to overcome the IL-12-mediated suppression of IL-4-producing cell development. Although these negative results could be explained by the difficulty to block completely the biological activity of endogenously produced IFN-γ, the possibility that IL-12 acts via an IFN-γ-independent mechanism should be considered.

IL-12 is produced predominantly by macrophages and other MHC class II–positive cells, cell types that have an important role in antigen presentation to Th cells (26). Class II–positive PBMC also produce IFN-α (29), which has similar effects on the Th differentiation (16). Thus, it is reasonable to suggest that, given the capacity of viruses and bacteria to stimulate PBMC production of IFN-α and IL-12 (26, 29), Th cells may be simultaneously presented with processed antigen plus cytokines that induce them to differentiate towards the Th1 phenotype. Indeed, when influenza virus, poly-I-C, or Bacillus Calmette-Guerin, agents known to stimulate endogenous IFN-α production and possibly IL-12 secretion, were added together with allergen, T cell differentiation was again pushed from the Th2 to Th0, or even to Th1, profile (30, and Manetti, R., P. Parronchi, R. Biagiotti, M.-P. Piccinni, E. Maggi, and S. Romagnani, manuscript in preparation). Interestingly, the poly-I-C-induced Th0 or Th1 differentiation of allergen-specific T cells could be driven to the Th2 profile only by the simultaneous addition of IL-4, plus antibodies reactive with IFN-γ, IFN-α, and IL-12 (30, and Manetti et al., manuscript in preparation). These data strongly suggest that viruses and intracellular bacteria induce Th1-like responses because the profile of the natural immune response they evoke provides optimum conditions (high concentrations of IFN-γ and absence of IL-4) for the development of Th1 cells (30). We have recently obtained evidence indicating that IL-12 production by accessory cells is a requirement for optimal production of IFN-γ by lymphocytes and that the Th2 products IL-4 and IL-10 are powerful inhibitors of IL-12 production (26, and D'Andrea, A., M. Aste, M. Kubin, and

G. Trinchieri, manuscript in preparation). In particular, we have shown that IL-12 and TNF produced by accessory cells are the cytokines responsible for IFN-γ production by NK cells and T cells in response to bacterial stimulation, and that the inhibitory effect of IL-10 on IFN-γ production is mediated by its ability to suppress production of these two cytokines (D'Andrea et al., manuscript in preparation). It is therefore likely that IL-12 represents a key component in the network of cytokines, with complex positive and negative feedback regulatory mechanisms that are able to determine the type of Th immune response at the very early stages of antigenic challenge.

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