

IL-4 AND IFN (α AND γ) EXERT OPPOSITE REGULATORY EFFECTS ON THE DEVELOPMENT OF CYTOLYTIC POTENTIAL BY Th1 OR Th2 HUMAN T CELL CLONES¹

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The cytolytic potential of a total number of 118 CD4⁺ human T cell clones specific for purified protein derivative (PPD) from *Mycobacterium tuberculosis*, tetanus toxoid, *Lolium perenne* group I allergen (Lol p I), *Poa pratensis* group IX allergen (Poa p IX), or *Toxocara canis* excretory/secretory antigen(s) (TES) was assessed by both a lectin (PHA)-dependent and a MHC-restricted lytic assay and compared with their profile of cytokine secretion. The majority of clones with Th1 or Th0 cytokine profile exhibited cytolytic activity in both assays, whereas Th2 clones usually did not. There was an association between the cytolytic potential of T cell clones and their ability to produce IFN- γ , even though IFN- γ produced by T cell clones was not responsible for their cytolytic activity. IL-4 added in bulk culture before cloning inhibited not only the differentiation of PPD-specific T cells into Th1-like cell lines and clones, but also the development of their cytolytic potential. The depressive effect of IL-4 on the development of PPD-specific T cell lines with both Th1 cytokine profile and cytolytic potential was dependent on early addition of IL-4 in bulk cultures. In contrast, the addition in bulk culture of IFN- γ enhanced both the cytolytic activity of PPD-specific T cell lines, as well as the proportion of PPD-specific T cell clones with cytolytic activity. The addition in bulk cultures before cloning of IFN- γ or IFN- α favored the development of TES-specific and Poa p IX-specific T cells into T cell clones showing a Th0 or even a Th1, rather than a Th2, cytokine profile. Accordingly, most of TES- and Poa p IX-specific T cell clones derived from cultures containing IFN- γ or IFN- α displayed strong cytolytic activity. These data indicate that the majority of human T cell clones that produce IFN- γ , but not IL-4 (Th1-like), as well as of T cell clones that produce IFN- γ in combination with IL-4 (Th0-like) are cytolytic. More importantly, they demonstrate that the addi-

tion of IFN (α and γ) or IL-4 in bulk cultures before cloning may influence not only the cytokine profile of human CD4⁺ T cell clones but also their cytolytic potential.

Activated murine CD4⁺ T cells comprise at least three functionally distinct subsets of cells: Th1 cells that secrete IL-2 and IFN- γ , but not IL-4 or IL-5, Th2 cells that produce IL-4 and IL-5, but not IL-2 or IFN- γ , and Th0 cells that can produce IL-2, IFN- γ , IL-4, and IL-5 simultaneously (1-3). Recently, human TCC³ with similar profiles of cytokine production have also been described (4). The majority of PHA-induced, alloreactive or tetanus toxoid-specific TCC display a Th0-like profile (5, 6). However, most TCC specific for allergens (7, 8) or helminth components (4, 9) exhibit a Th2-like profile, whereas TCC specific for mycobacterial Ag, *Borrelia burgdorferi*, nickel, and streptokinase usually exhibit a Th1-like profile (8-12). Interestingly, human Th1 and Th2 TCC were found to differ not only for their profile of cytokine production, but also for cytolytic potential and mode of B help for Ig synthesis (13, 14). The great majority of Th1, but only a minority of Th2, clones exhibited cytolytic activity against autologous Ag-presenting B cells (13).

There is general agreement that the different functional subsets of murine CD4⁺ T cells arise postthymically from a common pool of precursors, and as a consequence of activation by Ag (15-17). A number of possible factors that may influence CD4⁺ T cell differentiation in the murine system have been suggested (16). The clearest examples of such factors are IFN- γ and IL-4. IFN- γ promotes the differentiation of CD4⁺ T cells into Th1, both in vitro (18) and in the *Leishmania major* infection in vivo model (19-21). Evidence, again both in vitro (22-24) and in vivo (20), demonstrates that IL-4 has reciprocal activity, inducing differentiation into Th2 cells.

Recently, we have shown that the presence or the absence of exogenous IL-4 or IFN- γ in bulk cultures of PBMC before cloning may have strong regulatory effects on the in vitro development of human CD4⁺ T cells into Th1 or Th2 clones (25). First, IL-4 addition resulted in the development of PPD-specific TCC able to produce not only IFN- γ and IL-2 but also IL-4 and IL-5. Conversely, addition of IFN- γ and/or anti-IL-4 antibody markedly

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³ Abbreviations used in this paper: TCC, T cell clone; PPD, purified protein derivative; TT, tetanus toxoid; TES, *Toxocara canis* excretory/secretory Ag; TCL, T cell line; SN, supernatant; SR, spontaneous release; MR, maximum release.

inhibited the development of *Der p I*-specific CD4⁺ T cells into Th2-like clones (25). In our study, we asked whether addition in bulk cultures before cloning of IL-4, IFN- γ , or IFN- α influenced the cytolytic potential of TCC specific for different Ag. The results showed that IL-4 addition in bulk cultures can inhibit the development of PPD-specific CD4⁺ T cells with cytolytic activity, whereas IFN- γ or IFN- α favor the development of allergen- or helminth-specific CD4⁺ T cells into TCC possessing cytolytic potential.

MATERIALS AND METHODS

Reagents. PPD and TT were obtained from Istituto Sieroterapico e Vaccinogeno Sclavo (Siena, Italy). Purified *Lolium perenne* group I (*Lol p I*) was kindly provided by Dr. A. Ansari (Johns Hopkins University, Baltimore, MD). Recombinant *Poa pratensis* group IX (*Poa p IX*) was kindly provided by Dr. S. Mohapatra (University of Manitoba, Winnipeg, Canada). TES was provided by Dr. P. Falagiani (Lofarma Allergeni, Milan, Italy). PHA was purchased from GIBCO Laboratories (Grand Island, NY). OKT3 (anti-CD3), OKT4 (anti-CD4), and OKT8 (anti-CD8) mAb were purchased from Ortho Pharmaceutical Co. (Raritan, NJ). Leu 11 (anti-CD16) was purchased from Becton Dickinson (Mountain View, CA). Human IFN- γ was purchased from Janssen (Beerse, Belgium). One of the three anti-IFN- γ mAb (1598-00) (1000 neutralizing units/ μ g) was purchased from Genzyme Corporation (Cambridge, MA). The other two mAb were a generous gift of Dr. C. M. Liang (Biogen, Cambridge, MA). Human rIFN- α 2b was purchased from Schering Co. (Kenilworth, NJ). Human rIL-2 was generous gift of Eurocetus (Milano, Italy). Human rIL-4 and the polyclonal anti-IL-4 antibody were a generous gift of Dr. J. Banchereau (Dardilly, France). Human rIL-5 was purchased from Amgen Biologicals (Thousand Oaks, CA). TNF- α , TNF- β , anti-TNF- α , anti-TNF- β , and anti-IL-2 antibodies were purchased from Genzyme Co.

Subjects. PBMC were obtained from healthy volunteers showing delayed type hypersensitivity skin reaction to PPD, lymphoproliferative response in vitro to TES, or who had been vaccinated with TT, as well as from atopic grass-sensitive subjects suffering from atopic dermatitis and extrinsic asthma and showing immediate type skin reactivity to *Lol p I* and/or *Poa p IX*.

Generation of Ag-specific TCL and TCC. PPD-, TT-, *Lol p I*-, *Poa p IX*-, and TES-specific TCL were generated according to a technique previously described (6, 9, 25). Briefly, 10⁶ PBMC in 2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2 $\times$ 10⁻⁵ M 2-MER, and 5% human serum (complete medium) were stimulated with Ag (2 μ g/ml) in 24-well flat-bottomed plates for 5 days in the absence or in the presence of IFN- γ (500 U/ml), IL-4 (200 U/ml), polyclonal anti-IL-4 antibody (10 μ g/ml), or a mixture of three anti-IFN- γ mAb (2500 neutralizing units/ml final concentration). Human IL-2 was then added and cultures continued for additional 9 days. Viable T blasts were resuspended in complete medium and tested for their Ag specificity before cloning procedure. To assess the Ag specificity of TCL, 4 $\times$ 10⁴ T blasts were seeded in microplates and cocultured for 48 h with irradiated (6000 rad) autologous PBMC (8 $\times$ 10⁴) in the presence of medium alone, PPD, TT, TES, *Lol p I*, or *Poa p IX* (1 μ g/ml), respectively. After a 16-h pulse with 0.5 μ Ci [³H]TdR, cultures were harvested and radioactivity measured by liquid scintillation. T blasts from TCL were analyzed for CD4, CD8, and CD16 expression by flow cytometry. In some experiments, TCL were depleted of CD8⁺ and CD16⁺ cells using the panning technique described by Zubler et al. (26). To generate TCC, T blasts obtained from Ag-specific TCL were seeded under limiting dilution conditions (0.3 cell/well) in round-bottomed microwell plates containing 10⁵ irradiated (6000 rad) allogeneic PBMC (as feeder cells) and PHA (1% v/v) in a final vol of 0.2 ml complete medium supplemented with IL-2 (20 U/ml) and 10% FCS (HyClone Laboratories Inc., Logan, UT), as reported elsewhere (6, 9, 25). Growing microcultures were then supplemented, at weekly intervals, with IL-2 (20 U/ml) and 10⁵ irradiated feeder cells. The phenotype of T blasts of TCC was examined by flow cytometry and their Ag-specificity assessed by measuring [³H]TdR uptake after 60 h stimulation with the appropriate Ag under MHC-restricted conditions, as reported elsewhere (8, 9). When stimulation index (ratio between the mean cpm obtained in cultures stimulated with APC plus Ag 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). Ag-specific TCC were maintained in culture and expanded for 3 to 4 wk before being evaluated for cytokine secretion profile and cytolytic activity. Last supply with IL-2 and feeder cells was performed 1 wk before the assays.

Induction of cytokine production by Ag-specific TCC. To induce

the cytokine production by stimulation with the appropriate Ag, T blasts from each TCC were washed three times with FCS-supplemented medium, counted, and 10⁶ cells cocultured in 1 ml complete medium with 5 $\times$ 10⁵ irradiated autologous non-T cells (8, 9) in the presence of the appropriate Ag (2 μ g/ml). After 48 h, culture SN were collected, filtered, and stored in aliquots at -70°C until used.

Quantitation of IL-2, IFN- γ , IL-4, and IL-5 in TCC SN. To evaluate the ability of TCC to produce IL-2, 0.2 ml SN was added at different concentrations (1/2 to 1/16) to 4 $\times$ 10³ indicator cells (CTLL-2 murine cell line), as previously described (9). A semiquantitative estimate of IL-2 produced by each TCC was obtained by a standard curve of human rIL-2. 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. For the measurement of IL-5, the murine LyH7.B13 cell line was used as a source of indicator cells (kind gift of Dr. R. Palacios, Basel, Switzerland) (28). A semiquantitative estimate of IL-5 produced was obtained by a standard curve of human rIL-5 (9). Values of the cytokine content 3 SD over those of control SN obtained by stimulation of irradiated feeder cells alone, were regarded as positive.

Immortalization of B cells with EBV. PBMC, obtained from the same donors of TCC, were depleted of T lymphocytes by a double step of rosetting with SRBC followed by C'-dependent cytotoxicity with anti-CD3 mAb plus fresh rabbit serum. T cell-depleted suspensions were then incubated for 48 h with SN of the EBV-producing marmoset cell line B95.8 and subsequently expanded in complete medium supplemented with 15% FCS, as described (13).

Assays for cytolytic activity. Cytolytic activity of lines or clones was assessed as reported elsewhere (13). Briefly, T blasts were washed three times, resuspended, counted, and divided into two aliquots. One aliquot was tested for cytolytic activity against murine ⁵¹Cr-labeled P815 mastocytoma cells in the presence of PHA (1% v/v) (lectin-dependent cytolytic assay) with E:T ratio ranging from 20:1 to 5:1. After 4 h at 37°C, 0.1 ml SN was removed for measurement of ⁵¹Cr release. MR was obtained by treating target cells with 0.1 ml 1 M HCl. SR was determined in control microcultures without effector cells. Specific lysis was calculated according to the formula: percent specific lysis = 100 $\times$ (experimental release - SR) / (MR - SR). Cultures in which ⁵¹Cr release exceeded the mean SR by >5 SD were considered positive. To assess the Ag-induced MHC-restricted cytolytic activity of TCL or TCC against autologous APC, EBV-B cells were incubated overnight with the specific Ag, pelleted, resuspended, and labeled with ⁵¹Cr (1 h at 37°C). After three washes, 10⁴ target cells in 0.1 ml were distributed in triplicate wells of 96-well V-bottomed plates. The second aliquot of washed T blasts from each TCL or TCC was then added in 0.1 ml at E:T ratios ranging from 20:1 to 5:1. After centrifugation, microplates were incubated for 4 h at 37°C, and 0.1 ml SN was removed for measurement of ⁵¹Cr release. The percent specific lysis was calculated using the formula reported above.

RESULTS

Cytolytic potential of CD4⁺ human TCC is related to their ability to produce IFN- γ . The cytolytic activity of a total number of 118 CD4⁺ human T cell clones specific for five different Ag and showing different profiles of cytokine secretion was examined. Inasmuch as IL-2 was not detectable in culture supernatants of most clones in response to Ag stimulation, probably due to the consumption of this cytokine by the growing cells, the definition of the cytokine profile of clones under study was based on the secretion of IFN- γ , IL-4, and IL-5. On the basis of this criterion, 25 clones producing IFN- γ , but not IL-4 and IL-5, were classified as Th1, 41 producing IFN- γ , IL-4, and/or IL-5 as Th0, and 52 producing IL-4 and/or IL-5, but not IFN- γ as Th2. Twelve of 25 Th1 clones were specific for PPD and 13 for TT. Three of 41 Th0 clones were specific for PPD, 14 for TT, 9 for *Lol p I*, 8 for *Poa p IX*, and 7 for TES. Five of 52 Th2 clones were specific for TT, 9 for *Lol p I*, 23 for *Poa p IX*, and 15 for TES (Table I). The cytolytic potential of all clones was tested by both a lectin (PHA)-dependent and a MHC-restricted lytic assay using the P815 murine mastocytoma cell line and EBV-transformed autologous B cells in the presence of the specific Ag, respectively. The results

TABLE I

Summary of Ag specificity, cytokine profile, and cytolytic activity of 118 CD4⁺ human T cell clones^a

Ag Specificity	Cytokine ^b Profile	No. of Clones	No. of Cytolytic ^c Clones
PPD	Th1	12	9 (75%)
	Th0	3	2 (67%)
TT	Th1	13	9 (69%)
	Th0	14	10 (71%)
	Th2	5	1 (20%)
Lol p I	Th0	9	6 (67%)
	Th2	9	0
Poa p IX	Th0	8	5 (63%)
	Th2	23	2 (9%)
TES	Th0	7	5 (71%)
	Th2	15	2 (13%)

^a PPD- and TT-specific TCC were derived from PBL of a TT-vaccinated healthy donor showing delayed type hypersensitivity reaction to PPD. Lol p I- and Poa p IX-specific TCC were derived from PBL of two atopic patients with grass pollen-induced asthma. TES-specific TCC were derived from a healthy donor showing lymphoproliferative response in vitro to TES Ag.

^b Cytokine profile was assessed by measuring IL-4, IL-5, and IFN- γ production by TCC stimulated for 48 h with the specific Ag under MHC-restricted conditions. TCC able to produce IFN- γ , but not IL-4/IL-5, were defined as Th1; TCC able to produce IL-4/IL-5, but not IFN- γ , were defined as Th2; TCC able to produce at the same time IL-4/IL-5 and IFN- γ were defined as Th0.

^c Cytolytic activity of each TCC was tested against ⁵¹Cr-labeled P815 target cells at an E:T ratio of 5:1 in the presence of PHA. Specific lysis was calculated as described in *Materials and Methods*.

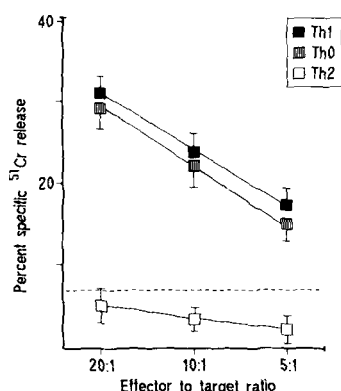


Figure 1. Ag-induced cytolytic activity against ⁵¹Cr-labeled EBV-transformed autologous B cells by Th1, Th0, and Th2 CD4⁺ human TCC. Twenty-five Th1 TCC specific for PPD or TT, 41 Th0 TCC specific for PPD, TT, Lol p I, Poa p IX or TES, and 52 Th2 TCC specific for TT, Lol p I, Poa p IX, or TES were assayed at different E:T ratios for cytolytic activity against ⁵¹Cr-labeled EBV-transformed autologous B cells in the presence of the specific Ag. Specific lysis was calculated as described in *Materials and Methods*. Cytokine profile was assessed by measuring IL-4, IL-5, and IFN- γ production by TCC stimulated for 48 h with the specific Ag under MHC-restricted conditions, as indicated in the legend to Table I. The dotted line represents 5 SD above the mean spontaneous release of target cells. Results represent mean values $\pm$ SE.

of these experiments are summarized in Table I and Figure 1. The majority of both Th1 and Th0 clones showed cytolytic activity in both assays, whereas Th2 clones did not. As shown in Table I, there was a strict association between the cytolytic potential of TCC and their ability to produce IFN- γ . Moreover, a correlation ($R = 0.62$, $p < 0.001$) was found between the degree of lytic activity and the amount of IFN- γ produced by 49 randomly selected T cell clones (Fig. 2). In contrast, in a group of 8 Th1 and 17 Th0 clones producing detectable amounts of IL-2, no correlation between the degree of cytolytic activity and the amount of IL-2 produced was found. IFN- γ produced by TCC, however, was probably

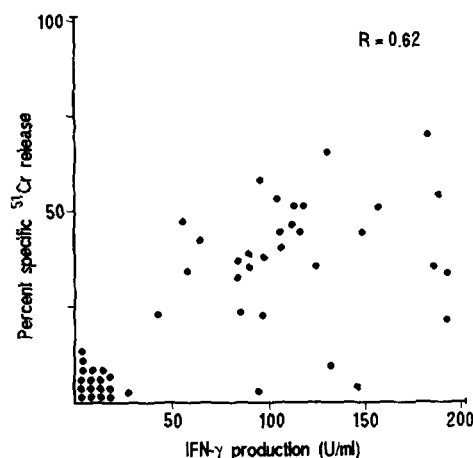


Figure 2. Relationship between cytolytic activity of TCC against ⁵¹Cr-labeled EBV-transformed autologous B cells in the presence of the specific Ag and their ability to produce IFN- γ upon Ag stimulation under MHC-restricted conditions. Seventeen Th1, 15 Th0, and 17 Th2 TCC were assayed for cytolytic activity against ⁵¹Cr-labeled EBV-transformed autologous B cells in the presence of the specific Ag at an E:T ratio of 10:1. Specific lysis was calculated as described in *Materials and Methods*. IFN- γ production by TCC stimulated for 48 h with the specific Ag under MHC-restricted conditions was assessed as detailed in *Materials and Methods*.

not responsible for their cytolytic activity. In fact, concentrations as high as 50 μ g/ml (50,000 neutralizing units/ml) of an anti-IFN- γ mAb displayed no inhibitory effect on the lytic activity of TCC in both assays. More importantly, the addition of IFN- γ (500 U/ml) and/or IL-2 (25 U/ml) in the Ag-dependent cytolytic assay did not enable noncytolytic Th2 clones to express lytic activity (data not shown).

IL-4 has inhibitory effect on development of PPD-specific T cell lines and T cell clones displaying cytolytic activity. We have recently shown that IFN- γ and IL-4 added in bulk cultures of PBMC may influence the cytokine secretion profile of human PPD- or Der p I-specific CD4⁺ T cells (25). We asked, therefore, whether the addition of IFN- γ or IL-4 before cloning had a similar influence on the development of their lytic potential. To do this, PBMC from 12 normal individuals were stimulated with PPD in the absence or in the presence of IL-4 (200 U/ml) or IFN- γ (500 U/ml). Five days later, cultures were supplemented with identical concentrations of IL-2 and expanded for 9 additional days. On day 14, the T cell lines so obtained were assessed for their phenotype, as well as for their ability to lyse autologous EBV-transformed B cells in the presence of PPD. The results of cytolytic assays are summarized in Figure 3. PPD-specific T cell lines derived from PBMC stimulated with PPD in the absence of both IL-4 and IFN- γ were cytolytic and those derived in the presence of IFN- γ exhibited an even higher cytolytic activity irrespective of the E:T cell ratios used ($p < 0.05$). In contrast, the cytolytic potential of PPD-specific T cell lines derived from PBMC stimulated with PPD in the presence of IL-4 was significantly lower ($p < 0.05$). When PBMC from the same donors were stimulated with PPD in the presence of other T cell-derived cytokines, such as TNF- α , TNF- β , or IL-5, no inhibition or enhancement of the cytolytic potential of PPD-specific T cell lines was observed (data not shown). The depressive effect of IL-4 on the development of PPD-specific T cell lines with cytolytic potential was dependent on early addition of IL-4 in bulk cultures. As shown

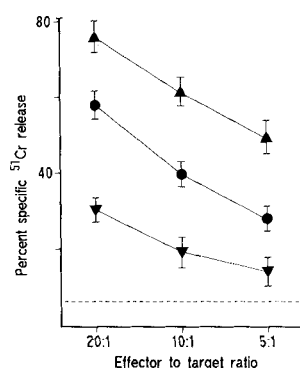


Figure 3. Effect of the addition in bulk culture of IL-4 or IFN- γ on the development of PPD-specific T cell lines with cytolytic activity against ^{51}Cr -labeled EBV-transformed autologous B cells in the presence of the specific Ag. PBMC from 12 normal individuals were stimulated with PPD in the absence (●) or in the presence of IL-4 (200 U/ml) (▲) or IFN- γ (500 U/ml) (■). Five days later, bulk cultures were supplemented with IL-2 and expanded for 9 additional days. On day 14, T blasts from each PPD-specific TCL were assessed for their ability to lyse autologous EBV-transformed B cells in the presence of PPD (10 $\mu\text{g}/\text{ml}$), as detailed in *Materials and Methods*. The dotted line represents 5 SD above the mean spontaneous release of target cells. Results represent mean values $\pm$ SE.

TABLE II

Effect of early or delayed addition of IL-4 on development of cytolytic activity and IFN- γ production by PPD-specific T cell lines^a

Cytokine Added	Time of Addition	Mean ($\pm$ SD) percent ^b Specific ^{51}Cr Release	Mean ($\pm$ SD) IFN- γ ^c Production (U/ml)
None	Day 0	51 $\pm$ 12	312 $\pm$ 42
IL-4	Day 0	27 $\pm$ 9	78 $\pm$ 19
IL-4	Day 1	23 $\pm$ 5	162 $\pm$ 28
IL-4	Day 3	36 $\pm$ 7	248 $\pm$ 39
IL-4	Day 5	49 $\pm$ 12	304 $\pm$ 27

^a PPD-specific T cell lines were derived from PBL of four healthy donors showing delayed type hypersensitivity reaction to PPD. PBMC were stimulated for 5 days with PPD (2 $\mu\text{g}/\text{ml}$) in the presence of medium or IL-4 (200 U/ml) added at different time intervals. Growing T blasts were cultured for additional 9 days in the presence of IL-2 (20 U/ml) and then assayed for both cytolytic activity against autologous EBV-transformed B cells in the presence of PPD and PPD-induced IFN- γ production under MHC-restricted conditions.

^b Cytolytic activity of each T cell line was tested against ^{51}Cr -labeled EBV-transformed autologous B cells at an E:T ratio of 10:1 in the presence of PPD (10 $\mu\text{g}/\text{ml}$). Specific lysis was calculated as described in *Materials and Methods*.

^c T blasts ($10^6/\text{ml}$) from each T cell line were cultured in 1 ml complete medium with 5×10^5 irradiated autologous non-T cells in the presence of PPD (2 $\mu\text{g}/\text{ml}$). After 48 h, culture supernatants were collected and assayed for IFN- γ production, as detailed in *Materials and Methods*.

in Table II, the depressive effect was still observed when IL-4 was added in bulk cultures 24 h after the addition of PPD, but it could no longer be observed when IL-4 was added later than day 2. Interestingly, the IL-4-induced change on the IFN- γ secretion by PPD-specific T cell lines showed a similar behavior (Table II).

To evaluate whether the cytolytic activity of PPD-specific TCL was influenced by the presence of different numbers of CD8⁺ and/or CD16⁺ cells, three PPD-specific TCL were assessed for their ability to lyse EBV-transformed autologous B cells in the presence of PPD before and after depletion of CD8⁺ and CD16⁺ cells. Despite the fact that after panning with anti-CD8 and anti-CD16 antibodies the proportions of CD8⁺ and CD16⁺ cells were reduced to less than 1 and 5%, respectively, and the proportions of CD4⁺ T cells ranged between 91 and 98%, the Ag-induced cytolytic activity of unfractionated and CD4⁺-enriched TCL was comparable (Table III).

We then asked whether the different degree of cytolytic potential of PPD-specific T cell lines derived under different bulk culture conditions actually reflected the pres-

TABLE III

Ag-dependent cytolytic activity of unfractionated or CD4-enriched T blasts of PPD-specific TCL derived from PBMC stimulated with PPD in absence or in presence of IFN- γ or IL-4

Bulk Culture ^a Conditions	Percent of Positive Cells ^b			Percent of Specific ^{51}Cr Release ^c	
	CD4 ⁺	CD8 ⁺	CD16 ⁺	Unfractionated	CD8 ⁺ CD16 ⁺
PPD + medium	44 $\pm$ 7	33 $\pm$ 6	33 $\pm$ 7	34 $\pm$ 6	29 $\pm$ 7
PPD + IFN- γ	38 $\pm$ 8	38 $\pm$ 6	41 $\pm$ 9	48 $\pm$ 9	51 $\pm$ 11
PPD + IL-4	56 $\pm$ 6	29 $\pm$ 4	12 $\pm$ 3	15 $\pm$ 3	12 $\pm$ 3

^a PPD-specific TCL were derived from PBMC of three healthy donors showing delayed type hypersensitivity reaction to PPD. PBMC ($10^6/\text{ml}$) were stimulated with PPD (2 $\mu\text{g}/\text{ml}$) for 5 days in the absence or in the presence of IFN- γ (500 U/ml) or IL-4 (200 U/ml). Human IL-2 (20 U/ml) was then added and cultures continued for additional 9 days before being assessed for their phenotype and Ag-dependent cytolytic activity.

^b T blasts of PPD-specific TCL derived from bulk cultures performed under different conditions were examined for CD4, CD8, and CD16 expression by flow cytometry. Results represent the mean percent values ($\pm$ SD) of positive T blasts from nine different TCL derived from three donors.

^c CD4⁺ T blasts of PPD-specific TCL derived from three donors under different bulk culture conditions were enriched by depletion of CD8⁺ and CD16⁺ cells by panning technique (26). Unfractionated and CD4⁺CD8⁺CD16⁺ T blasts of PPD-specific TCL were assayed for Ag-dependent cytolytic activity against ^{51}Cr -labeled EBV-transformed autologous B cells at an E:T ratio of 10:1 in the absence or in the presence of PPD (10 $\mu\text{g}/\text{ml}$). In the absence of PPD or in the presence of an unrelated Ag (TT, 10 $\mu\text{g}/\text{ml}$), the percent ^{51}Cr release ranged between 1.3 and 3.4. When ^{51}Cr -labeled EBV-transformed allogeneic MHC-incompatible B cells were used, either in the absence or in the presence of PPD, the percent ^{51}Cr release ranged between 1.9 and 4.3. Results represent the mean ($\pm$ SE) percent values of specific lysis calculated as described in *Materials and Methods*.

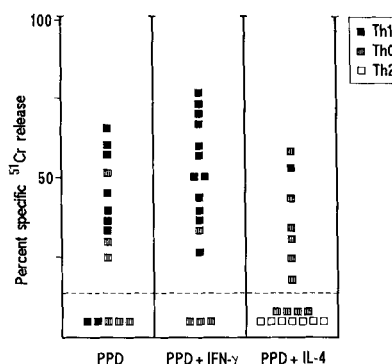


Figure 4. Cytokine profile and cytolytic potential of CD4⁺ PPD-specific TCC derived from PBMC stimulated with Ag in the absence or in the presence of IFN- γ or IL-4. PPD-specific TCC were derived from PBL of a healthy donor showing delayed type hypersensitivity reaction to PPD. PBMC were stimulated for 5 days with PPD (2 $\mu\text{g}/\text{ml}$) in the absence or in the presence of IFN- γ (500 U/ml) or IL-4 (200 U/ml). Growing T blasts were cultured for additional 9 days in the presence of IL-2 (20 U/ml). PPD-specific TCL were then cloned under limiting dilution conditions in the presence of irradiated feeder cells, PHA, and IL-2, as detailed in *Materials and Methods*. The cloning efficiency ranged between 26 and 31%. CD4⁺ PPD-specific TCC were assessed for both cytokine secretion profile (as detailed in *Materials and Methods*) and cytolytic activity against ^{51}Cr -labeled EBV-transformed autologous B cells in the presence of medium alone, PPD (10 $\mu\text{g}/\text{ml}$), or TT (10 $\mu\text{g}/\text{ml}$) as unrelated Ag, as detailed in *Materials and Methods*. The dotted line represents 5 SD above the mean spontaneous release of target cells. The mean ($\pm$ SD) percent release of ^{51}Cr in the presence of medium alone was 3.1 ± 1.9 and that induced by an unrelated Ag (TT) was 3.7 ± 1.3 .

ence of different numbers of PPD-specific cytolytic CD4⁺ T cells. Therefore, T blasts from three PPD-specific T cell lines derived from the same donor under different bulk culture conditions (with PPD alone, with PPD plus IL-4, or with PPD plus IFN- γ) were cloned with PHA, in the presence of IL-2 and irradiated feeder cells. A total number of 49 PPD-specific T cell clones were obtained and assessed for their phenotype and ability to lyse EBV-transformed autologous B cells in the presence of PPD. As shown in Figure 4, the great majority of Th1 and Th0 PPD-specific CD4⁺ TCC derived in the absence of IL-4 or

IFN- γ (67%) or in the presence of IFN- γ (81%) displayed cytolytic activity. In contrast, only 39% of PPD-specific CD4⁺ TCC derived in the presence of IL-4, all showing Th0 phenotype, exhibited cytolytic activity.

IFN (α and γ) favor development of allergen- and helminth-specific T cells into cytolytic T cell clones. Unlike PPD-specific TCC that exhibit a Th1-like profile of cytokine production, TCC specific for allergens or helminth components usually have a Th2-like profile and do not display cytolytic potential (7, 8, 13, 25). However, the development of allergen (*Der p 1*)-specific Th2-like TCC can be significantly inhibited by the addition in bulk culture of IFN- γ and/or anti-IL-4 antibody (25). Therefore, we asked whether addition in bulk culture of IFN- α , IFN- γ , IL-4, or anti-IL-4 antibody could result in the development of allergen- or helminth-specific TCC exhibiting different cytolytic potential. To this end, PBMC from a normal donor showing in vitro lymphoproliferative response to TES were stimulated with TES in the absence or in the presence of IFN- γ or IL-4 and both the cytokine secretion profile and the cytolytic activity of the corresponding TES-specific TCC was assessed. The results of these experiments are summarized in Figure 4. A total number of 38 TES-specific CD4⁺ TCC were obtained from PBMC cultures stimulated with TES in the absence of exogenous IFN- γ or IL-4. Twenty-five were Th2, 13 Th0, but none of them had a Th1 cytokine profile. Only 11 TCC (8 Th0 and 3 Th2) were cytolytic against autologous EBV-transformed B cells in the presence of TES Ag. Twenty-six TES-specific clones were obtained when PBMC were stimulated with TES in the presence of IFN- γ . However, only five were Th2, whereas 17 showed a Th0 and 4 a Th1 cytokine profile. Accordingly, 19 TES-specific TCC obtained under these experimental conditions (1 of the 4 Th2, 14 of the 17 Th0, and all 4 Th1 TCC) exhibited cytolytic activity. In contrast, none of the 26 Th2, but all the 6 Th0, TES-specific clones derived from PBMC stimulated with TES in the presence of IL-4 showed Ag-dependent lytic activity against autologous EBV-B cells (Fig. 5).

In a subsequent series of experiments, PBMC from a grass-sensitive atopic donor were stimulated with *Poa p IX* in the absence or in the presence of IFN- α or a rabbit

polyclonal anti-IL-4 antibody. *Poa p IX*-specific clones were then derived and assayed for both their cytokine secretion phenotype and cytolytic capacity under the experimental conditions reported above. As shown in Figure 6, a total number of 31 *Poa p IX*-specific CD4⁺ TCC were obtained from bulk cultures stimulated in the absence of IFN- α and 22 from bulk cultures stimulated in the presence of IFN- α . A total of 23 of 31 clones derived in the absence of IFN- α were Th2, 8 were Th0, and only 7 of them (2 Th2 and 5 Th0) were cytolytic. In contrast, only 2 of 22 *Poa p IX*-specific clones derived in the presence of IFN- α were Th2, 13 being Th0 and 7 Th1. More importantly, as many as 17 of the 22 clones (10 of 13 Th0 and all 7 Th1) displayed cytolytic activity. Only five *Poa p IX*-specific CD4⁺ TCC were obtained from bulk cultures stimulated with *Poa p IX* plus anti-IL-4 antibody. They all showed a Th0 cytokine profile and three were cytolytic (data not shown).

DISCUSSION

Human T lymphocytes have been subdivided into two main subpopulations on the basis of expression of CD4 or CD8 Ag. A relationship exists between these T cell subsets and the MHC gene products recognized by their TCR. CD4⁺ T lymphocytes interact with target cells expressing MHC class II gene products, whereas CD8⁺ T cells interact with target cells expressing class I MHC molecules (29). Generally, CD4⁺ cells are regarded as helper cells and CD8⁺ as cytotoxic cells. A functional distinction between CD4⁺ and CD8⁺ T cells, however, is not absolute, because cloned CD4⁺ T lymphocytes that have a cytotoxic capacity have been described (30, 31).

Our recent work and from other laboratories has shown that human CD4⁺ T lymphocytes, in analogy to the murine system (3), can be divided into at least three distinct subsets based on their lymphokine secretion profile (4, 5, 9, 12). One type of CD4⁺ clones (Th1) produces IL-2 and IFN- γ , but not IL-4 and IL-5, a second type (Th2) produces IL-4 and IL-5 but not IL-2 and IFN- γ , and a third type (Th0) produces IL-2, IFN- γ , IL-4, and IL-5 (4, 9, 12). Interestingly, human Th1 and Th2 clones were found to differ not only for their profile of cytokine production, but also for cytolytic potential (12, 13, 32). The

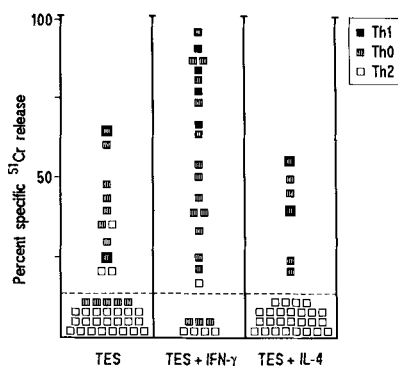


Figure 5. Effect of the addition in bulk culture of IFN- γ or IL-4 on the development of cytokine secretion phenotype and cytolytic activity of TES-specific CD4⁺ T cell clones. PBMC from a normal donor showing in vitro lymphoproliferative response to TES were stimulated with TES in the absence or in the presence of IFN- γ (500 U/ml) or IL-4 (200 U/ml) and the corresponding CD4⁺ TES-specific TCC were assessed for both cytokine secretion profile and cytolytic activity against ⁵¹Cr-labeled EBV-transformed autologous B cells in the presence of TES Ag, as detailed in *Materials and Methods*. The dotted line represents 5 SD above the mean spontaneous release of target cells.

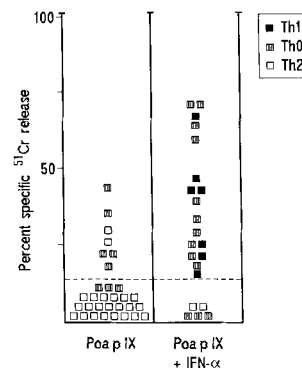


Figure 6. Effect of the addition in bulk culture of IFN- α on the development of cytokine secretion phenotype and cytolytic activity of *Poa p IX*-specific CD4⁺ T cell clones. PBMC from a grass-sensitive atopic subject were stimulated with *Poa p IX* in the absence or in the presence of IFN- α (1000 U/ml) and the corresponding CD4⁺ *Poa p IX*-specific TCC were assessed for both cytokine secretion profile and cytolytic activity against ⁵¹Cr-labeled EBV-transformed autologous B cells in the presence of *Poa p IX*, as detailed in *Materials and Methods*. The dotted line represents 5 SD above the mean spontaneous release of target cells.

great majority of Th1, but only a minority of Th2, clones exhibited anti-CD3-mediated (32), lectin-dependent (3, 12), and MHC-restricted cytotoxic activity (13). We demonstrate that the majority of Th0 clones also exhibit both lectin-dependent and MHC-restricted cell cytotoxicity. A correlation between cytotoxic activity and lymphokine release by T cells has been suggested (32, 33), whereas others did not find such a correlation (34, 35). We have demonstrated a correlation not only between cytotoxic activity and production of IFN- γ , but also between the degree of cytotoxic activity and the amount of IFN- γ produced by CD4⁺ T cell clones. However, IFN- γ itself, as well as other Th1-restricted or-related cytokines such as IL-2, TNF- β , and TNF- α , were apparently not responsible for the cytotoxic activity of CD4⁺ clones because high concentrations of anti-IFN- γ antibodies or mixtures of antibodies specific for IL-2, IFN- γ , TNF- β , and TNF- α did not affect the Ag-induced cytotoxicity of Th1 or Th0 clones against autologous EBV-B cells (data not shown) (13). More importantly, neither addition of IFN- γ nor addition of both IFN- γ and IL-2 enabled noncytolytic Th2 clones to express cytolytic activity. Thus, it is reasonable to suggest that the association between IFN- γ production and cytotoxic activity by CD4⁺ human clones does not reflect a cause-effect relationship.

Our results also demonstrate that the development of CD4⁺ clones with a given profile of cytokine production and the expression of cytolytic activity are similarly regulated. In the murine system, a number of possible factors that may influence CD4⁺ T cell differentiation into one or another cytokine subset have been suggested (16, 36, 37). So far, however, the most clear-cut requirements for generation of murine Th1 or Th2 cells include IFN- γ and IL-4. IFN- γ promotes the differentiation of CD4⁺ T cells into Th1, both in vitro (18) and in vivo (19–21). In vitro (22–24) and in vivo (20) evidence demonstrates that IL-4 has reciprocal activity, inducing differentiation into Th2 cells. Recently, we have shown that the presence or the lack of IL-4 or IFN- γ in bulk cultures of PBMC before cloning may have strong regulatory effects on the in vitro development of human CD4⁺ T cells into Th1 or Th2 clones (25). First, IL-4 addition resulted in the development of PPD-specific TCC able to produce not only IFN- γ and IL-2, but also IL-4 and IL-5. Conversely, addition of IFN- γ and/or anti-IL-4 antibody markedly inhibited the development of *Der p* 1-specific CD4⁺ T cells into Th2-like clones (25). In our study, we report that IL-4 addition in bulk cultures before cloning also inhibits the differentiation of PPD-specific T cells into T cell clones with cytolytic potential. However, IFN- γ addition in bulk culture before cloning resulted in the development of CD4⁺ clones specific for helminth components that differed from those derived without addition of this cytokine not only for their ability to produce IFN- γ in combination with IL-4 and IL-5, but also for the expression of cytotoxic capacity. Similarly, addition in bulk culture of IFN- α enabled allergen-specific T cells to develop into CD4⁺ clones producing IFN- γ and exhibiting strong cytolytic potential.

Why and how IL-4 promotes the in vitro development of PPD-specific CD4⁺ T cells into IL-4-producing cells lacking cytolytic activity is unknown. In the murine system, IL-4 appears to exert dramatic effects on the phenotypes of effectors that develop in vitro (22–24). Added

at the beginning of cultures, IL-4 promotes development of effectors that can be restimulated to secrete IL-4/IL-5 and strongly suppresses development of IL-2-secreting effectors. At a dose two times the dose required for optimum development of IL-4/IL-5-secreting effectors, IL-4 dramatically suppresses the development of IFN- γ -producing cells (37). In the human system, rIL-4 was found to suppress the production of IFN- γ by PBMC (38). However, the question of whether IL-4 acted directly on T cells or its activity was mediated by other cell types was not tackled. More recently, we have found that human IL-4 selectively promotes the proliferative response and enhances IL-4 and IL-5 production by Th2 clones, without apparently affecting both proliferation and cytokine production by Th1 clones⁴.

The mechanisms by which IFN- γ and IFN- α favor the development of allergen- or helminth-specific T cells into T cell clones that produce IFN- γ in addition to IL-4/IL-5 and exhibit cytolytic potential are also unclear. In the murine system, IFN- γ inhibits the proliferation of Th2, but not Th1, clones (39) and favors the development of CD4⁺ T cells into Th1 clones (16, 18). Recently, we have found that human IFN- γ inhibits both Ag-induced proliferation and Ag-induced cytokine production by Th2, but not Th1, clones (see footnote 4). IFN- α was even more potent than IFN- γ in inhibiting both Ag-induced proliferation and cytokine production by Th2 clones, whereas it enhanced IFN- γ production by Th1 clones (data not shown). Similar activities by IFN- α have recently been observed in the murine system in vivo (40). Thus, IFN- α may favor the differentiation of T cells into the Th1 direction, as well as into cells that exhibit cytolytic potential, by both inducing IFN- γ production and inhibiting IL-4 production.

It is not easy to settle the physiologic relevance of these in vitro pathways. However, because IFN- α is predominantly produced by macrophages that play an important role in the presentation of Ag to T cells, one might speculate that the capacity of a given Ag to stimulate IFN- α production by macrophages is crucial in determining the subset of T cells that is activated. IFN- α production occurs indeed soon after macrophage and Ag first come into contact, allowing virgin T cells to simultaneously be presented with processed Ag, that leads to their activation, plus a cytokine that induces them to differentiate in a Th1 direction. This might explain why responses against viruses or intracellular bacteria, that induce large production of IFN- α , usually involve Th1 cells. However, both Th1 lymphokine-mediated effector activity and Th1-mediated cytotoxicity probably represent highly effective mechanisms in the protection against these infectious agents. Furthermore, the activity of Th1 clones in mediating killing of APC might down-regulate specific B cell responses, accounting for short-lived antibody production frequently observed in successful inflammatory reactions associated with vigorous delayed-type hypersensitivity.

By contrast, repeated or chronic stimulation by inhaled allergens, that do not result in strong macrophage activation and therefore do not evoke high IFN- α production,

⁴Del Prete, G. F., M. De Carli, F. Almerigogna, and S. Romagnani. 1992. Interleukin-10 (IL-10) and viral IL-10 inhibit antigen-specific proliferation and cytokine production by both type 1 helper (Th1) and type 2 helper (Th2) human T cell clones. *J. Immunol.* submitted for publication.

can allow activation of T cells that produce IL-4 but little, if any, IFN- γ . It cannot be excluded that additional IL-4 produced by cells belonging to the mast cell or basophil lineage upon cross-linkage by immune complexes of their FcR (41, 42) may further contribute to the maintenance and amplification of Th2-restricted responses against allergens. Finally, the inability of allergen-specific Th2 cells to kill their APC might explain, at least in part, the chronicity of IgE antibody production occurring in atopic patients. Our finding that IFN- α is not only able to influence the cytokine production profile of allergen-specific T cells, but also enables them to kill the APC might be of great interest in view of new approaches to the therapy of IgE-mediated allergic disorders.

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