INFECTION AND IMMUNITY, May 1995, p. 2079–2081 Vol. 63, No. 5
0019-9567/95/$04.00+0

Impact of Antigen-Presenting Cells on Cytokine Profiles of Human Th Clones Established after Stimulation with *Mycobacterium tuberculosis* Antigens

PETER CONRADT* AND STEFAN H. E. KAUFMANN

Department of Immunology, University of Ulm, D-89070 Ulm, Germany

Received 23 September 1994/Returned for modification 8 November 1994/Accepted 31 January 1995

Human T cells reactive with mycobacterial antigens are generally considered to correlate with a Th1 cytokine profile. Our data show that, in addition, Th0 and Th2 clones develop in bulk culture with appropriate antigen-presenting cells before cloning. $CD4^+$ blasts activated by mycobacterial antigens were cloned, and their mRNA patterns for the interleukins (IL) IL-2, IL-4, IL-5, IL-6, and IL-10 and gamma interferon were characterized by reverse-transcribed PCR. Nonadherent, nonrosetting, enriched peripheral blood mononuclear cells promoted development of Th0; after further depletion of monocytes and natural killer cells, Th2 clones were also found. Epstein-Barr virus-transformed B cells, with specificity for the stimulating antigen, increased the proportion of Th2 clones.

Although in initial reports, only Th0 clones had been identified in the human system, more recently clones with cytokine profiles resembling the murine Th1 and Th2 types were established from patients with various diseases (reviewed in reference 23). Thus, clones with specificity for mycobacterial antigens most often displayed a Th1 profile, whereas allergen-specific clones were Th2 (8). The mechanisms underlying the development of naive T cells into subsets with different cytokine profiles are still unclear. An example is the beneficial Th1 response in the tuberculoid form of leprosy and the detrimental role of Th2 cells in the lepromatous form (28). In vitro studies of the human system rely on the development of Th clones with distinct cytokine profiles from precursors responding to recall antigens like mycobacteria or allergens. Thus, preferential expansion of either the Th1 subset by interleukin-12 (IL-12) or alpha interferon (IFN-α) or alternatively of the Th2 subset by IL-4 has been described (16, 18, 19, 21). Experimental evidence for selective support of a single Th type by antigen-presenting cells (APC) has been obtained only in the murine system (13, 17).

Here, we analyzed the impact of different APC populations presenting mycobacterial antigen in short-term bulk culture on the cytokine profiles of cloned $CD4^+$ blasts. We compared the following three cell populations: (i) nonadherent nonrosetting peripheral blood mononuclear cells (PBMC), (ii) the same population after further depletion of monocytes and natural killer (NK) cells, and (iii) antigen-specific Epstein-Barr virus (EBV)-transformed autologous B cells (EBV-BCL). Since human clones secrete several cytokines, the method of reverse transcribed PCR (RT-PCR), which allows monitoring of a broad cytokine set, seemed to be appropriate. PBMC were prepared from buffy coats from healthy donors (German Red Cross Bloodbank Ulm, Germany) by flotation on Ficoll and an adherence step. The rosetting T-cell fraction was 90 to 95% $CD3^+$, and the nonadherent nonrosetting fraction was enriched for B cells ($CD20^+$) to about 50 to 60%, as determined by flow cytometry. Treatment of nonadherent nonrosetting PBMC with 5 mM L-leucine methyl ester for 45 min at room temperature (27) was followed by a separation over 43.5% Percoll. The high-density fraction was free of $CD14^+$ and $CD16^+$ cells (<1%), as assessed by flow cytometry. EBV-BCL were established from the nonadherent nonrosetting fraction by standard procedures; antigen-specific EBV-BCL were selected by testing culture supernatants in an anti-*Mycobacterium tuberculosis* H37Ra immunoglobulin enzyme-linked immunosorbent assay (ELISA) (data not shown). Antigen stimulation and expansion of the clones followed a published protocol (16). However, cloning was done at one cell per well with a FACSTAR Plus cell sorter (Becton Dickinson) and killed *M. tuberculosis* H37Ra (Difco, Stuttgart, Germany) was used as antigen. Actively growing $CD4^+$ blasts were cloned and were not observed in EBV-BCL cultures without antigen. Cloned T cells were characterized phenotypically as $CD4^+$ $CD3^+$ $CD8^-$ $TCR\gamma/\delta^-$. When antigen-specific proliferation of the clones to *M. tuberculosis* was assessed by [^{3}H]thymidine incorporation under autologous conditions, about 10% of the clones remained antigen specific with this protocol, as also shown by others (16). In order to exclude contribution of feeder cells to the cytokine profile, clones were stimulated with 2 ng of phorbol 12-myristate 13-acetate (PMA) per ml plus 1 μg of calcium ionophore A23187 per ml for 18 h at least 7 days after the last restimulation. The total RNA was isolated by the single-step method (5). RNA was reverse transcribed with a Strata script RT-PCR kit (Stratagene, Heidelberg, Germany). The cDNA was used in a PCR reaction with primer pairs for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and β-actin. Primers were from Stratagene or synthesized (10). PCR was performed for 35 cycles of denaturation (94°C, 1 min), annealing (60°C, 2 min), and extension (3 min, 72°C). RT-PCRs were performed on the clones after at least two independent stimulations and RNA preparations for each T-cell clone. The cytokine patterns were confirmed by PCR reactions from the same cDNA.

Clones expressing various IL mRNA patterns were identified (Table 1). T-cell clones were negative for IL-6 mRNA. T-cell clones which were positive for mRNAs for the counteracting cytokines IL-4 and IFN-γ were defined as Th0. Clones which produced either mRNA for IL-4 but lacked those for IFN-γ or synthesized IFN-γ mRNA but lacked IL-4 mRNA were termed Th2 or Th1, respectively. Representative results

* Corresponding author. Mailing address: Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, D-89070 Ulm, Germany. Phone: 0731/502-3358 or -3361. Fax: 0731/502-3367. Electronic mail address: Stefan.Kaufmann@Medizin.Uni-Ulm.DE.

TABLE 1. Cytokine mRNA and Th phenotype of T-cell clones

Phenotype and cytokine combination[a]	Clone(s)		
	APC I[b]	APC II[c]	APC III[d]
Th1			
IFN-γ		6G3	
IL-2 + IFN-γ + IL-5		6D5	1D10
IL-2 + IFN-γ + IL-5 + IL-10	7F5		1G3
Th0			
IL-2 + IL-4 + IL-5 + IL-10 + IFN-γ	4F2, 4E4, 7B1[e], 7G1[e]	6F4, 6F9, 3C9	2F1, 2A1
IL-2 + IL-4 + IL-5 + IFN-γ	4H1, 4C2	6A1	1B9
IL-2 + IL-4 + IFN-γ	4F4		
Th2			
IL-5	7C6[e]		2A8
IL-2 + IL-4 + IL-5		3G5	1A3
IL-2 + IL-4 + IL-10			1D11
IL-2 + IL-4 + IL-5 + IL-10		3F3, 3C10	2G6
Naive			
IL-2		6A4	

[a] RT-PCR products from T-cell clones stimulated with PMA and an ionophore were evaluated on agarose gels.
[b] APC I, nonrosetting nonadherent PBMC.
[c] APC II, nonadherent nonrosetting PBMC depleted of monocytes and NK cells.
[d] APC III, autologous EBV-BCL.
[e] Clones from cultures supplemented with IL-4.

for Th0, Th1, and Th2 clones are shown in Fig. 1. The first group comprised two separate experiments (different donors) with nonadherent nonrosetting APC. Nine clones were investigated; six clones expressed IFN-γ mRNA. However, only one was found to be Th1 since five clones produced IL-4 mRNA and IFN-γ mRNA together and thus were Th0. Th1 clones did not develop under externally added IL-4 (50 U/ml) (Table 2).

In order to eliminate monocytes and NK cells as potential cytokine sources such as IL-12 or IFN-γ, the nonadherent nonrosetting fraction was depleted of cells with lytic potential with L-leucine methyl ester. From two separate experiments with these APC, 10 clones were characterized. The largest

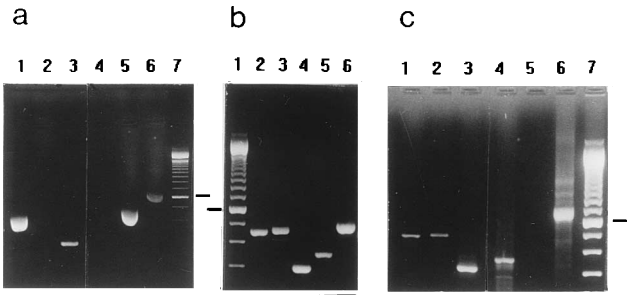

FIG. 1. Cytokine mRNA expression revealed by RT PCR after stimulation of Th1 clone 1D10 (a), Th0 clone 6F4 (b), and Th2 clone 3C10 (c) for 18 h with PMA and ionophore. Amplification was done with primers for IL-2 (expected size 458 bp) (a and c, lane 1; b, lane 2), IL-4 (456 bp) (a and c, lane 2; b, lane 3), IL-5 (291 bp) (a and c, lane 3; b, lane 4), IL-10 (351 bp) (a and c, lane 4; b, lane 5), IFN-γ (501 bp) (a and c, lane 5; b, lane 6), and β-actin (661 bp) (a and c, lane 6. The 100-bp ladder molecular weight marker is in lane 7 (a and c) or 1 (b). The bar denotes 600 bp. Analysis was done on a 2% agarose gel stained with ethidium bromide.

TABLE 2. Quantitative distribution of Th phenotypes[a]

Th phenotype	No. of clones[b]		
	APC I	APC II	APC III
Th1	1	2	2
Th0	5, 2[c]	4	3
Th2	1[c]	3	4
Naive		1	

[a] For further information, see Table 1.
[b] Total of 28 clones: APC I, 9; APC II, 10; APC III, 9.
[c] Clones from cultures supplemented with IL-4.

fraction (4 of 10) was Th0, while four clones lacked IFN-γ mRNA. Three clones were identified as Th2. IL-10 mRNA correlated with all Th types. One clone produced only IL-2 and was classified as naive (10) (Table 1). Therefore, further depletion of monocytes and NK cells from APC allowed the development of Th2 clones (Table 2).

The third set of experiments was set up to study the influence of B cells in the absence of other professional APC on T-cell development. For this purpose, autologous EBV-BCL were established. To take advantage of the potentiating effect of antigen-specific T-cell–B-cell interactions (14, 15), EBV-BCL secreting immunoglobulin against *M. tuberculosis* antigens were selected. Two separate experiments yielded nine $CD4^+$ clones. Seven clones expressed IL-4 mRNA; four of these clones had lost the ability to synthesize IFN-γ mRNA and hence were Th2, and three others were Th0. The remaining two clones expressed IFN-γ mRNA in combination with IL-2, IL-5, and/or IL-10 mRNA and thus were Th1 (Table 1). Therefore EBV-BCL promoted the highest number of Th2 clones (Table 2).

As yet, experiments showing influences of APC on cytokine patterns of clones in the human system have not been published to our knowledge. We chose the method of short-term antigen stimulation and subsequent cloning (7, 12, 16, 19, 20, 21, 24) followed by polyclonal expansion before analysis in order to avoid preselection of clones due to growth differences in long-term-antigen-stimulated lines. Once a Th1 or Th2 response has been established it appears to be stable; only IL-12 has been reported to induce qualitative changes in the cytokine profile of established clones (18, 30). However, such influences were excluded by omitting feeder cells during T-cell stimulation. Previous cloning experiments with mycobacterial antigens have indicated a preferential development of Th1 cells in the absence of exogenous cytokines (3, 12, 22), as assessed by cytokine determination with ELISA. By using the high sensitivity of the RT-PCR, we were more likely to detect a broader range of cytokines simultaneously for each clone. Indeed, most of our clones expressed various cytokine combinations. Consistent with published data (1–3, 7, 25, 29), we found that IL-2 and IL-10 mRNAs fail to correlate with a distinct Th profile. Using another sensitive detection technique, the ELISPOT assay, higher frequencies of IL-4-producing cells have been observed among PBMC from tuberculosis patients after purified protein derivative stimulation (11, 26). This method, however, fails to distinguish between Th0 and Th2 cells on the single-cell level. After stimulation in the presence of nonrosetting nonadherent PBMC, five of six clones expressed the Th0 profile and one expressed a Th1 profile. The additional elimination of cells with lytic potential, including NK cells, from the nonrosetting nonadherent fraction led to the development of Th0 as well as of Th2 clones.

Various cytokines are produced by adherent cells in re-

sponse to whole mycobacteria or their cell wall constituents (2, 6, 25). Monocyte-derived cytokines like IL-12 and IFN-α, when added in the same stimulation protocol shifted the phenotype towards the Th1 type (16, 19, 21). A similar influence has been proposed for IL-12-induced IFN-γ production by NK cells (19). Unfractionated PBMC were used in most other studies. Depletion of adherent cells and cells with lytic potential in two of our experimental settings could have favored Th0 and Th2 clones.

Almost half of the clones which developed in response to mycobacteria presented by antigen-specific syngeneic EBV-BCL expressed a characteristic Th2 cytokine profile. The remaining clones segregated into Th0 and Th1 cells. Cytokine regulation in antigen-specific T-cell–B-cell interactions is largely unknown. IL-10 is produced by EBV-BCL (4) but has been shown to equally influence development of both Th1 and Th2 cells (7, 9). In conclusion, our data reveal that the clonal expression of the human helper profile is markedly influenced in vitro by the type of APC presenting *M. tuberculosis*. IFN-γ-producing T cells are considered essential for protective immunity against tuberculosis. The high frequency of Th1 cells in vitro has been taken as evidence that they preponderate in *M. tuberculosis* infection in vivo. Our data show that both Th1 and Th2 cells can be activated by *M. tuberculosis* antigens.

This work received financial support from the Bundesministerium für Forschung und Technologie.

We thank Jürgen Hess for helpful discussions and critically reading the manuscript.

REFERENCES

1. **Barnes, P. F., J. S. Abrams, S. Lu, P. A. Sieling, T. H. Rea, and R. L. Modlin.** 1993. Pattern of cytokine production by Mycobacterium-reactive human T-cell clones. Infect. Immun. **61:**197–203.
2. **Barnes, P. F., D. Chatterje, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin.** 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. J. Immunol. **149:**541–547.
3. **Boom, W. H., R. S. Wallis, and K. A. Chervenak.** 1991. Human *Mycobacterium tuberculosis*-reactive $CD4^+$ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. Infect. Immun. **59:**2737–2743.
4. **Burdin, B., C. Peronne, J. Banchereau, and F. Rousset.** 1993. Epstein-Barr virus transformation induces B lymphocytes to produce interleukin 10. J. Exp. Med. **177:**295–304.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guadinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **D'Andrea, A., M. Rengaraju, N. M. Valiente, Y. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, E. Nickbarg, R. Chizzonite, S. F. Wolf, and G. Trinchieri.** 1992. Production of natural killer cell stimulatory factor (interleukin-12) by peripheral blood mononuclear cells. J. Exp. Med. **176:** 1387–1398.
7. **Del Prete, G. F., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti, and S. Romagnani.** 1993. Human IL-10 is produced by both type 1 helper (TH1) and type 2 helper (TH2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J. Immunol. **150:**353–360.
8. **Del Prete, G. F., M. De Carli, M. Mastromauro, R. Biagiotti, D. Maccia, P. Falagiani, M. Ricci, and S. Romagnani.** 1991. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. J. Clin. Invest. **88:**346–350.
9. **De Waal Malefyt, R., H. Yssel, and J. E. De Vries.** 1993. Direct effects of IL-10 on subsets of human $CD4^+$ T cell clones and resting T cells. J. Immunol. **150:**4754–4765.
10. **Ehlers, S., and K. S. Smith.** 1991. Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. J. Exp. Med. **173:**25–36.
11. **ElGhazali, G. E. B., S. Pauli, G. Andersson, I. Hansson, G. Holmquist, J.-B. Sun, T. Olson, H. P. Ekre, and M. Troye-Blomberg.** 1993. Number of interleukin-4-and interferon-γ-secreting human T cells reactive with tetanus toxoid and the mycobacterial antigen PPD or phytohemagglutinin: distinct response profiles depending on the type of antigen used for activation. Eur. J. Immunol. **23:**2740–2745.
12. **Haanen, J. B. A. G., R. De Waal Malefijt, P. C. M. Res, E. M. Kraakman, T. H. M. Ottenhoff, R. R. P. De Vries, and H. Spits.** 1991. Selection of a human T helper type 1-like T cell subset by mycobacteria. J. Exp. Med. **174:**583–592.
13. **Hsieh, C.-S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. M. Murphy.** 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an αβ T-cell-receptor transgenic system. Proc. Natl. Acad. Sci. USA **89:**6065–6069.
14. **Lanzavecchia, A.** 1985. Antigen-specific interaction between T and B cells. Nature (London) **314:**537–539.
15. **Lanzavecchia, A., B. Parodi, and F. Celada.** 1983. Activation of human B lymphocytes: frequency of antigen-specific B cells triggered by alloreactive or by antigen-specific T cell clones. Eur. J. Immunol. **13:**733–738.
16. **Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M.-P. Piccini, F. S. Rugiu, M. De Carli, M. Ricci, and S. Romagnani.** 1992. Reciprocal regulatory effects of gamma interferon (IFN-γ) and interleukin-4 (IL-4) on the in vitro development of human T helper 1 (TH1) and TH2 clones. J. Immunol. **148:**2142–2147.
17. **Magylavy, D. B., F. W. Fitch, and T. F. Gajewski.** 1989. Murine hepatic accessory cells support the proliferation of TH1 but not TH2 helper T lymphocyte clones. J. Exp. Med. **170:**985–990.
18. **Manetti, R., F. Gerosa, G. Giudizi, R. Biagotti, P. Parronchi, M. Piccini, S. Sampognaro, E. Maggi, and G. Trinchieri.** 1994. Interleukin 12 induces stable priming for interferon γ production during differentiation of human T helper cells and transient IFN γ production in established Th2 cell clones. J. Exp. Med. **179:**1273–1283.
19. **Manetti, R., P. Parronchi, M. G. Giudizi, M.-P. Piccini, E. Maggi, G. Trinchieri, and S. Romagniani.** 1993. 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. J. Exp. Med. **177:** 1199–1204.
20. **Paliard, X., R. De Waal Malefijt, H. Yssel, D. Blanchard, I. Chretien, J. S. Abrams, J. De Vries, and H. Spits.** 1988. Simultaneous production of IL-2, IL-4, and IFN-γ by activated human $CD4^+$ and $CD8^+$ T cell clones. J. Immunol. **141:**849–855.
21. **Parronchi, P., M. De Carli, R. Manetti, C. Simonelli, S. Sampognaro, M.-P. Piccini, M. Maccia, E. Maggi, G. F. Del Prete, and Sergio Romagnani.** 1992. IL 4 and IFN (α and γ) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. J. Immunol. **149:**2977–2983.
22. **Parronchi, P., M. Maccia, M.-P. Piccini, P. Biswas, C. Simonelli, E. Maggi, M. Ricci, A. A. Ansari, and S. Romagniani.** 1991. Allergen and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. Proc. Natl. Acad. Sci. USA **88:**4538–4542.
23. **Romagniani, S.** 1994. Lymphokine production by human T cells in disease states. Annu. Rev. Immunol. **12:**227–257.
24. **Rotteveel, F. T. M., I. Kokkelink, R. A. W. Van Lier, B. Kuenen, A. Meager, F. Miedema, and C. J. Lucas.** 1988. Clonal analysis of functionally distinct human $CD4^+$ T cell subsets. J. Exp. Med. **168:**1659–1673.
25. **Sieling, P. A., J. S. Abrams, M. Yamamura, P. Salgame, B. Bloom, T. H. Rea, and R. L. Modlin.** 1993. Immunosuppressive roles for IL-10 and IL-4 in human infection. J. Immunol. **150:**5501–5510.
26. **Surcel, H. M., M. Troye-Blomberg, S. Paulie, G. Andersson, C. Moreno, and G. Pasvol.** 1994. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology **81:**171–176.
27. **Thiele, D. L., and P. E. Lipsky.** 1985. Modulation of human natural killer cell function by L leucine methyl ester: monocyte dependent depletion from human peripheral blood mononuclear cells. J. Immunol. **134:**786.
28. **Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B. R. Bloom, and R. L. Modlin.** 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science **254:**277–279.
29. **Yssel, H., R. De Waal Malefijt, M.-G. Roncarolo, J. S. Abrams, R. Lahesmaa, H. Spits, and R. De Vries.** 1994. IL-10 is produced by subsets of human $CD4^+$ T cell clones and peripheral blood T cells. J. Immunol. **149:**2378–2384.
30. **Yssel, H., F. Fasler, J. E. de Vries, and R. de Waal Malefyt.** 1994. IL-12 transiently induces IFN-γ transcription and protein synthesis in human $CD4^+$ allergen-specific Th2 T cell clones. Int. Immunol. **6:**1091–1096.