Selection of a Human T Helper Type 1-like T Cell Subset by Mycobacteria

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Summary

Mycobacteria elicit a cellular immune response in their hosts. This response usually leads to protective immunity, but may sometimes be accompanied by immunopathology due to delayed type hypersensitivity (DTH). A striking example in man is tuberculoid leprosy, which is characterized by high cellular immunity to *Mycobacterium leprae* and immunopathology due to DTH. Skin lesions of patients suffering from this disease have the characteristics of DTH reactions in which macrophages and $CD4^+$ T lymphocytes predominate. In animal models, it has been shown that DTH responses are associated with the presence of a particular subset of $CD4^+$ T cells (T helper type 1 [Th1]) that secrete only certain cytokines, such as interleukin 2 (IL-2), interferon γ (IFN-γ), and lymphotoxin, but no IL-4 or IL-5. We studied the cytokine release of activated *M. leprae*–reactive $CD4^+$ T cell clones derived from tuberculoid leprosy patients. These T cell clones, which were reactive with mycobacterial heat shock proteins, exhibited a Th1-like cytokine secretion pattern with very high levels of IFN-γ. Half of these clones secreted low levels of IL-4 and IL-5, but the ratio of IFN-γ to IL-4 and IL-5 was much higher than that of T cell clones reactive with nonmycobacterial antigens. A Th1-like cytokine secretion pattern was also observed for T cell clones and polyclonal T cell lines from control individuals that recognized both heat shock and other mycobacterial antigens. The levels of IFN-γ secreted by these clones were, however, significantly less than those of patient-derived T cell clones. This Th1-like pattern was not found with T cell clones from the same patients and healthy individuals generated in the same manner, but reactive with nonmycobacterial antigens. Our data thus indicate that mycobacteria selectively induce human T cells with a Th1-like cytokine secretion profile.

Activated murine $CD4^+$ T cells comprise at least two functionally distinct subsets of cells (1, 2): Th1 cells that secrete IL-2 and IFN-γ upon activation but not IL-4 or IL-5, and Th2 cells that produce IL-4 and IL-5 but not IL-2 or IFN-γ. The differential cytokine secretion profile of these $CD4^+$ T cells correlates with different effector functions exerted by these cells: Th1 cells mediate delayed type hypersensitivity (DTH)[1] responses (3, 4), and Th2 cells provide superior help for antibody production by B cells (5, 6). There is some support for the notion that Th1 and Th2 cells are progeny of Th0 cells, which can produce IL-2, IFN-γ, IL-4, and IL-5 simultaneously (7–10).

Human T cell clones, so far, can not be classified into Th1 and Th2 cell types. Most T cell clones obtained from healthy subjects were able to produce IL-2, IFN-γ, IL-4, and IL-5 simultaneously after activation (11). This cytokine secretion profile was observed with randomly isolated T cell clones upon stimulation with mitogens, but was also found for alloreactive and tetanus toxoid–specific clones after stimulation with specific antigen. These findings suggest that most human T cell clones are comparable to murine Th0 cells. Recently, however, we observed that a series of T cell clones isolated from a patient suffering from severe combined immune deficiency (SCID) that was successfully transplanted with fetal tissue were unable to produce IL-4 upon activation, while normal levels of IL-2, IFN-γ, and IL-5 were detected in the supernatants of these clones (12). These findings indicated that in man also, $CD4^+$ T cell clones can emerge that display selective cytokine secretion profiles. Support for this notion was provided by results from Wierenga et al. (13), who described that $CD4^+$ T cell clones from atopic patients that were

[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; GM, granulocyte/macrophage; NIP, nitro-iodophenyl; PPD, purified protein derivative; RT, room temperature.

specific for a crude extract of house dust mite secreted IL-4 but undetectable levels of IFN-γ. These cells may be the human analogue of mouse Th2 cells. Thus far, however, human Th1 cells have not been observed.

It may be expected to find Th1 type cells or inflammatory T cells in those diseases that are characterized by T cell–mediated inflammations, the best candidates being infectious diseases, like leprosy and tuberculosis, that are caused by mycobacteria. These pathogens are known to elicit strong T cell–mediated immune responses sometimes accompanied by DTH reactions and granuloma formation. These features are typical for tuberculoid leprosy, a disease caused by *Mycobacterium leprae* (14). In the granulomatous skin lesions of patients suffering from tuberculoid leprosy, macrophages and $CD4^+$ T lymphocytes predominate. It is thought that these $CD4^+$ T cells and their secreted products are responsible for some of the features of the disease, like severe DTH responses. In the present study, we have investigated the cytokine secretion patterns of *M. leprae*–reactive T cell clones from tuberculoid leprosy patients and compared these patterns with those obtained from control individuals, also reactive with mycobacterial protein antigens. Both sets of mycobacteria-reactive T cell clones display a Th1-like pattern of cytokine release; after activation, many of these clones secreted (excessively) high levels of IFN-γ, while levels of IL-4 and IL-5 varied from normal to undetectable. This Th1-like pattern appeared to be specific for T cells reactive with mycobacterial antigens, because T cell clones from the same individuals reactive with non-mycobacterial antigens displayed a Th0-like profile with normal levels of IL-4 and IL-5.

Material and Methods

Antigens. *M. leprae* (CD104) was a kind gift of Dr. R.J.W. Rees (National Institute for Medical Research, London, UK). Hsp65 peptides amino acids 3–13, 418–427, and 412–425 were made by a solid phase peptide synthesis methodology and checked using analytical reverse phase HPLC and amino acid analysis (15). The peptides were kindly provided by Dr. D.C. Anderson (University of Washington, Seattle, WA). Purified protein derivative (PPD) was obtained from the Statens Seruminstitut (Copenhagen, Denmark), *M. tuberculosis* ultrasonicate preparation (H37RA) was kindly donated by Dr. P. Klatser from the Royal Tropical Institute (Amsterdam, The Netherlands), and tetanus toxoid was obtained from the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands).

T Cell Clones and Lines. T cell clones were prepared as described previously. Briefly, 10^6 PBL from tuberculoid leprosy patients (16), healthy individuals (17), and rheumatoid arthritis patients (18) were incubated with *M. leprae*, hsp65 peptide 3–13, tetanus toxoid, or PHA (Wellcome Diagnostics, Dartford, UK) for 5 d in IMDM (Gibco Laboratories, Paisley, Scotland) supplemented with 10% human serum (HS) or in Yssel's medium (19). The responding T cell blasts were expanded on IL-2 (kindly provided by Dr. R. Kastelein, DNAX, Palo Alto, CA). These activated T cells were then cloned by limiting dilution technique (0.3 cell/well) in 96-well flat-bottomed plates (Linbro; Flow Laboratories, McLean, VA) in the presence of a feeder cell mixture consisting of irradiated (30 Gy) allogeneic PBL (10^6/ml) and irradiated (50 Gy) EBV-transformed B cells (10^5/ml) and antigen. The clones were maintained in culture in the presence of IL-2 and were restimulated weekly with the feeder cell mixture containing also 0.1–1.0 μg/ml PHA (Wellcome Diagnostics). All T cell clones used in this study were $CD4^+$. The polyclonal T cell lines were generated by in vitro restimulation of PBMC with PPD or *M. leprae* for 5 d. Thereafter, the responding T cell blasts were expanded to large numbers by addition of exogenous rIL-2 (20 U/ml) to the cultures for another 7 d. The antigen specificity of the mycobacteria-reactive T cell clones and lines was tested using T cell immunoblot analysis (20, 21), as described in reference 18, and by Janson et al. (manuscript submitted for publication).

Stimulation for Lymphokine Secretion. The T cell clones and lines were stimulated for lymphokine secretion 8–12 d after the last stimulation with feeder cells. At that time point no feeder cells were present. In addition, T cells at this stage did not produce IL-2, IL-4, or IFN-γ spontaneously (11). The cells were washed twice with medium and placed in 1-ml volumes at a concentration of 10^6 cells/ml in Linbro 24-well tissue culture plates (Flow Laboratories). Polyclonal stimulation was performed using 20 μg/ml Con A (Sigma Chemical Co., St. Louis, MO) or with a 1:3,000 dilution of anti-CD3 mAb (ascites SPV-T3b) (11) plus 1 ng/ml PMA (Sigma Chemical Co.). The antigen-specific stimulation was performed using autologous or HLA-DR-matched APC ($3–5 \times 10^6$/ml) with an optimal dose of antigen. T cells and APC alone were used as controls. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 20–24 h. The supernatants were harvested and stored immediately at −20°C and thawed before testing.

Cytokine Detection Assays. IL-2 activity was assayed on the murine IL-2-dependent cell line CTLL-2, as described by Gillis et al. (22). The activity of the supernatants was compared with that of a standard IL-2 and calculated according to Gillis et al. (22). IL-4, IL-5, IFN-γ, and granulocyte/macrophage (GM)-CSF were quantified with immunoenzymatic assays (11, 12, 23) that were performed as follows. PVC plates (Nunc, Roskilde, Denmark) were coated with 10 μg/ml antibody (IL-4:rabbit IgG anti-IL-4 antiserum; IL-5:JES1-39D10.1; IFN-γ:3-6; GM-CSF:23B6.4) and incubated for 2 h at 37°C. The plates were washed in an automatic washer (Titertek, Flow Laboratories, Irvine, Scotland) with PBS supplemented with 0.1% BSA (Sigma Chemical Co.) and 0.5% Tween 20 (Sigma Chemical Co.). Standard recombinant lymphokines (IL-4, IL-5, IFN-γ, and GM-CSF, kindly provided by R. Kastelein [DNAX]) were diluted in medium, and the T cell supernatants were added to the plates and incubated at room temperature (RT) for 2 h. After three washes, a second antibody coupled to nitro-iodophenyl (NIP) was added (IL-4: 25D2.11-NIP; IL-5: JES1-5A10-NIP; IFN-γ: B27-NIP; GM-CSF: 21C11.3-NIP). The plates were incubated for 1 h at RT. After three washes, HR-peroxidase coupled to an anti-NIP antibody (J4) was added and the plates were incubated for another 1 h at RT. Enzymatic activity was determined with the substrate 2.2′ amino bis-3-ethyl-benzthiozolin sulfonic acid (ABTS) (Sigma Chemical Co.). The plates were read with a Vmax microplate reader (Molecular Devices, Redwood City, CA) coupled to a computer. Data were analyzed using specific software (Molecular Devices, Redwood City, CA).

Results

Lymphokine Secretion Profile of T Cell Clones from a Tuberculoid Leprosy Patient. *M. leprae*–reactive $CD4^+$ T cell clones were obtained from tuberculoid (TT) leprosy patient R. The antigen specificity and the HLA-DR restriction has been determined and described previously (24, 25). The T cell clones recognized either the mycobacterial 65-kD heat shock pro-

tein (hsp65) (26), or another protein (M_r = 67–80 kD hsp of *M. leprae*), as determined by immunoblot technique (Janson et al., manuscript submitted for publication), and were restricted by HLA-DR2 or DR3. The T cell epitopes within hsp65 were mapped recently (26). The HLA-DR2-restricted clones recognize an *M. leprae*–specific epitope (amino acids 418–427, LQAAPALDKL). The majority of the HLA-DR3-restricted T cell clones recognizes an epitope on the NH_2 terminus of hsp65 (amino acids 3–13, KTIAYDEEARR) that is immunodominant for HLA-DR3-restricted hsp65-reactive T cells (27); two other clones were reactive with another hsp (M_r = 67–80 kD). Clones that recognize the same epitope restricted by the same HLA-DR molecule can use different TCRs as determined by Southern blot analysis and Vβ gene segment sequencing (van Schooten et al., manuscript submitted for publication).

To analyze the cytokine secretion profile (IFN-γ, IL-2, IL-4, and IL-5) of these T cell clones, the cells were stimulated with APC and the appropriate antigen. As a source of antigen, we used synthetic peptides corresponding to the T cell epitopes of hsp65 in case of hsp-65-specific clones, or *M. leprae* for clones R1E4 and R1F3. Upon stimulation with antigen, most *M. leprae*–reactive clones from this TT leprosy patient secreted high levels of IFN-γ, and some clones showed extremely high IFN-γ secretion (>1,000 ng/ml). In contrast, the levels of IL-4 and IL-5 produced by these clones were either undetectable, low, or in the normal range (Table 1). In those supernatants where normal levels of IL-4 and IL-5 were detected, the levels of IFN-γ were very high, resulting in IFN-γ to IL-4 ratios (96–28,000) that were disproportionate compared to such ratios detected in the supernatants of most normal T cell clones. These normal values for secretion of IFN-γ, IL-4, and IL-5 were obtained by screening a large panel (n = 38) of $CD4^+$ T cell clones isolated from four healthy individuals. We measured the ratio of IFN-γ to IL-4 secretion as a marker for Th cytokine secretion profiles. The antigen specificity of the T cell clones varied, including tetanus toxoid and HLA class II alloantigens, but no mycobacterial antigens were recognized. Mean values for IFN-γ secreted upon stimulation with Con A or specific antigen ranged from 3 to 54 ng/ml, for IL-4 from 200 to 3,200 pg/ml, and the IFN-γ to IL-4 ratios varied from 1 to 62. These ratios are included in Fig. 1, where we compare ratios of IFN-γ to IL-4 secretion between all T cells reactive with nonmycobacterial antigens used in this study. When measured, the level of IL-2 and GM-CSF secretion by these clones was comparable to those observed with tetanus toxoid and alloreactive clones from healthy individuals (data not shown). Importantly, the same Th1-like cytokine secretion pattern with excessive high levels of IFN-γ and undetectable to normal levels of IL-4 and IL-5 was observed for five DR1-restricted, hsp65-specific T cell clones from two other tuberculoid leprosy patients (data included in Fig. 1).

To investigate whether the Th1-like cytokine secretion pat-

Table 1. *Cytokine Secretion Profiles T Cell Clones reactive with Mycobacterial hsp and Nonmycobacterial Antigens from Tuberculoid Leprosy Patient R*

Donor	Clone	Restricted by:	Specificity	IFN-γ	IL-4	IL-5	Ratio of IFN-γ/IL-4
				ng/ml	*pg/ml*	*pg/ml*	
R	2F10	HLA-DR2	[418-427]	1,300.0 ± 78	1,080 ± 38	965 ± 46	1,203
	2B61	HLA-DR2	[418-427]	99.2 ± 22	370 ± 100	<50	268
	1F9	HLA-DR3	[3-13]	1,400.0 ± 42	<50	<50	>28,000
	3E10	HLA-DR3	[3-13]	77.5 ± 2	<50	<50	>1,550
	5E10	HLA-DR3	[3-13]	48.9 ± 6	<50	<50	>978
	515	HLA-DR3	[3-13]	192.4 ± 24	1,995 ± 415	<50	96
	518	HLA-DR3	[3-13]	1,100.0 ± 54	2,110 ± 130	>5,000	521
	1E4	HLA-DR3	hsp 67-80 kD	5.9 ± 2	<50	<50	>118
	1F3	HLA-DR3	hsp 67-80 kD	44.3 ± 1	<50	<50	>886
R	TT003	HLA-DR2,3	Tet. tox.	1.4 ± 1	3,600 ± 310	1,175 ± 240	0.4
	TT004	HLA-DR2,3	Tet. tox.	10.2 ± 1	1,100 ± 220	2,375 ± 210	9.3
	TT005	HLA-DR2,3	Tet. tox	6.4 ± 1	2,000 ± 100	ND	3.2
	TT006	HLA-DR2,3	Tet. tox	3.6 ± 1	4,300 ± 100	ND	0.8
	TT008	HLA-DR2,3	Tet. tox	3.4 ± 1	1,650 ± 100	ND	2.1
	TT011	HLA-DR2,3	Tet. tox	37.0 ± 1	2,400 ± 100	ND	15.4
	TT014	HLA-DR2,3	Tet. tox	20.0 ± 1	5,000 ± 200	ND	4
	A101	ND	JY	6.3 ± 1	270 ± 40	6,230 ± 310	23
	A109	ND	JY	28.2 ± 1	165 ± 40	835 ± 175	34

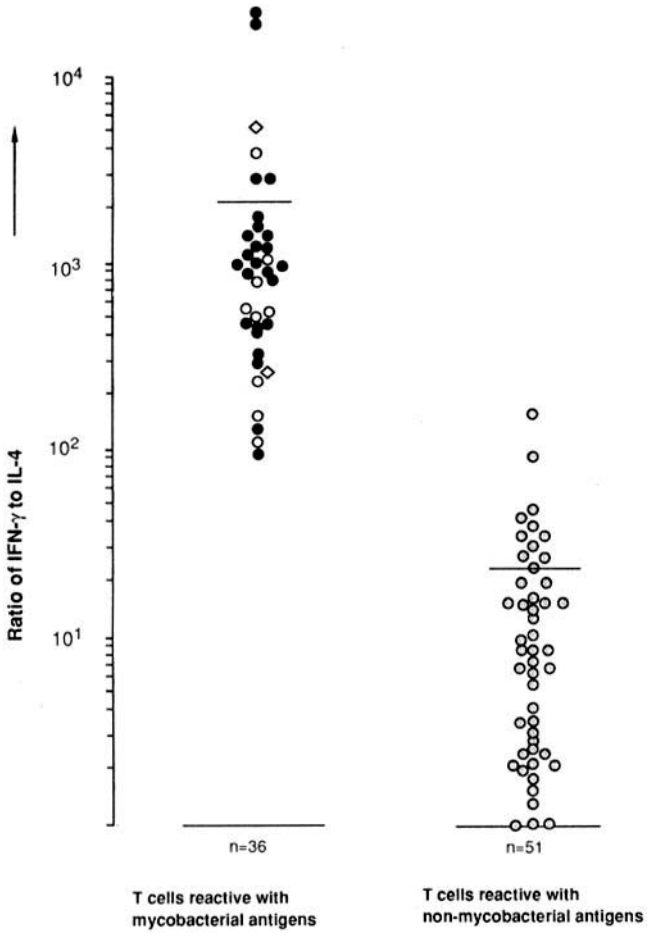

Figure 1. (●) T cell clones reactive with mycobacterial hsp antigens; (○) T cell clones reactive with mycobacterial non-hsp antigens; (◇) Polyclonal T cell lines reactive with mycobacterial non-hsp antigens; (◎) T cell clones reactive with nonmycobacterial antigens, including tetanus toxoid and allo-antigens. The IFN-γ to IL-4 ratios of the majority of the T cells reactive with mycobacterial antigens are higher than the depicted values (see Tables 1, 2, and 3).

terns we observed were related to recognition of mycobacterial antigens, we also analyzed the cytokine secretion profiles of a number of alloreactive and tetanus toxoid–specific T cell clones from tuberculoid leprosy patient R. In the supernatants of eight tetanus toxoid–specific and two alloreactive clones from this patient, we detected moderate levels of IFN-γ, IL-4, and IL-5, all within the normal range. Also, the IFN-γ to IL-4 ratios, varying from 0.4 to 34, are clearly different from those of *M. leprae*–reactive T cells. Thus, in this tuberculoid leprosy patient, the cytokine secretion profile of $CD4^+$ T cell clones reactive with nonmycobacterial antigens is not affected by the disease, but is comparable with the cytokine secretion patterns of tetanus toxoid and alloreactive T cell clones from healthy individuals.

The Cytokine Secretion Profile of T Cell Clones from a Healthy Individual. Our findings suggest that *M. leprae*–reactive T cell clones in these tuberculoid leprosy patients underwent a selection process resulting in a Th1-like cytokine secretion profile, characterized by high ratios of IFN-γ to IL-4 secretion. To investigate whether this selection process is associated with inflammatory diseases like tuberculoid leprosy, we tested four hsp65 peptide 3–13-specific T cell clones from healthy individual HY. In none of the supernatants of the hsp65 peptide 3–13-specific T cell clones could IL-4 be detected after activation (Table 2). In supernatants of two clones, very low levels of IL-5 were measured. In one T cell culture, >100 ng/ml IFN-γ was detected, while the other T cell clones secreted IFN-γ in normal or slightly higher levels than normal. Importantly, IFN-γ to IL-4 ratios were in the same range as those of *M. leprae*–reactive T cell clones from tuberculoid leprosy patients. IL-2 and GM-CSF levels were in the normal range (data not shown). In addition to hsp65 peptide 3–13-specific T cell clones from this healthy individual, HY, six other hsp65 peptide 3–13-specific T cell clones from two different healthy individuals were tested. The cytokine secretion profile of these hsp65 peptide 3–13-specific clones were identical to those obtained from clones of individual HY (data

Table 2. *Cytokine Secretion Profile T Cell Clones Reactive with Mycobacterial hsp and Nonmycobacterial Antigens from Healthy Individual HY*

Donor	Clone	Restricted by:	Specificity	IFN-γ	IL-4	IL-5	Ratio of IFN-γ/IL-4
				ng/ml	*pg/ml*	*pg/ml*	
HY	1	HLA-DR3	[3-13]	26.4 ± 0.5	<50	<50	>528
	3	HLA-DR3	[3-13]	150.0 ± 12	<50	130 ± 40	>3,000
	6	HLA-DR3	[3-13]	87.0 ± 1	<50	<50	>1,740
	7	HLA-DR3	[3-13]	44.6 ± 1	<50	205 ± 35	>892
	827	HLA-DR3	Tet. tox.	43.5 ± 1	230 ± 50	530 ± 50	189
HY	15	ND	Autoantigen	0.8 ± 0.5	1,435 ± 120	6,325 ± 325	0.6
	606	ND	Alloantigen	1.6 ± 1	720 ± 45	ND	2.2
	837	ND	Alloantigen	28.1 ± 1	3,320 ± 320	5,320 ± 50	8.4

included in Fig. 1). As a control for cloning technique and antigen-related cytokine secretion patterns, we also included four nonmycobacteria-reactive T cell clones from healthy individual HY, established in the same manner. These T cell clones with allo or undefined specificity were capable of secreting IFN-γ, IL-4, and IL-5 simultaneously and thus clearly differed from the mycobacteria-reactive Th1-like cells.

This Th1-like pattern of cytokine secretion found in T cells from healthy individuals is similar to that of the T cell clones from tuberculoid leprosy patients, although our data suggest that the patient-derived T cell clones are capable of producing more IFN-γ than the clones and lines from healthy individuals. Statistical analysis (Mann-Whitney U-Test) comparing the IFN-γ secretion of hsp65 peptide 3–13-specific clones from patients with that of healthy controls (χ^2 = 9.01, and p = 0.002) indicated that at least for reactivity to this peptide the difference in IFN-γ secretion between patients and controls is significant. In conclusion, the secretion of high levels of IFN-γ and low to negligible levels of IL-4 and IL-5 (Th1-like pattern) seems to be related to the antigen specificity of T cells per se, irrespective of the health or disease status of the donor.

Cytokine Secretion Profiles of T Cell Clones and Lines Reactive with Mycobacterial Proteins Different from Heat Shock Proteins. So far, all heat shock protein-specific T cell clones obtained from both tuberculoid leprosy patients and healthy individuals exhibited a Th1-like cytokine secretion profile as determined by the high IFN-γ to IL-4 and IL-5 ratios. Although heat shock proteins are major mycobacterial antigens and strong immunogens, it needed to be emphasized whether the Th1-like cytokine profiles were confined to T cells reactive with heat shock proteins. Therefore, we tested a panel of T cell clones and polyclonal T cell lines that were generated against either *M. tuberculosis*, PPD, or *M. leprae*. The specific antigen definition was determined by T cell immunoblot analysis and revealed that protein antigens other than hsp were recognized (Table 3). The *M. tuberculosis*–reactive T cell clones (n = 6) were obtained from the synovial fluids of two rheumatoid arthritis patients, whereas *M. leprae*–reactive T cell clones (n = 3) and the PPD-reactive polyclonal T cell lines (n = 2) were derived from three healthy donors. The T cell clones employed the TCR-α/β for recognition of antigen and were positive for the CD4 surface molecule. After stimulation with antigen, in all T cell cultures IFN-γ could be detected. The levels varied from normal (5 ng/ml) to very high (825 ng/ml). In only one of the supernatants, however, low level of IL-4 (200 pg/ml) could be detected. IL-5 levels, when tested, did not exceed 350 pg/ml. In those supernatants where we tested for IL-2 and/or GM-CSF, normal values were obtained.

Our results show that mycobacteria-reactive T cells, independent of their antigen specificity or the health or disease status of the donor, display a Th1-like cytokine secretion profile. Interestingly, even in short-term bulk cultures generated by in vitro restimulation of PBMC with mycobacteria, this Th1-like response can easily be detected. We defined this Th1-like cytokine secretion profile by calculating the ratios of IFN-γ to IL-4 levels secreted by the T cells.

The picture emerging from a comparison of the IFN-γ to IL-4 ratios between all mycobacteria-reactive T cells and

Table 3. *Cytokine Secretion Profile of T Cell Clones and Lines Reactive with Mycobacterial Protein Antigens Different from hsp*

Donor	Clone	Restricted by:	Specificity (M_r)	IFN-γ	IL-4	IL-5	Ratio of IFN-γ/IL-4
			kD	*ng/ml*	*pg/ml*	*pg/ml*	
W	5	DR1	14-15	12.5 ± 1.0	<50	340 ± 65	>250
	9	DR4(Dw14)	16-23	190.0 ± 9	<50	<50	>3,800
	13	DR4(Dw14)	30/31	26.0 ± 1	<50	300 ± 85	>520
	15	DR4(Dw14)	30/31	45.0 ± 14	<50	<50	>900
K	IIB8	DR4(Dw4)	27-29	5.1 ± 1	<50	350 ± 80	>102
	IVB6	DR4(Dw4)	ND	40.0 ± 11	<50	170 ± 50	>880
CAA	p1517	DR3	41-45	19.8 ± 3	200 ± 45	175 ± 50	>110
	p1518	DR3	57-67	26.8 ± 2	<50	<50	>530
	p1510	DR3	<15	25.7 ± 2	<50	<50	>510
vEs	TCL_{ppd}	HLA-DR3,5	30/31	26.0 ± 1	<100	ND	>260
Hass	TCL_{ppd}	HLA-DR3,5	30/31	825 ± 12	<100	ND	>5,000

Donors W and K are rheumatoid arthritis patients. The six *M. tuberculosis*–reactive T cell clones are derived from the synovial fluid of these patients. None of the *M. tuberculosis* protein fractions recognized by these clones comprise hsp. Donors CAA, vEs, and Hass are healthy PPD responders. p1517, p1518, and p1510 are HLA-DR3-restricted T cell clones, and TCL_{ppd} are short-term bulk cultures generated against *M. tuberculosis* (PPD), respectively. TCL_{ppd} from these individuals respond predominantly to a secreted non-hsp antigen with M_r 30/31 kD.

nonmycobacteria-reactive T cells used in this study (Fig. 1), illustrates the clearcut difference in cytokine secretion profiles within these groups.

Cytokine Secretion Profile of M. leprae–reactive T Cell Clones after Different Modes of Activation. The effect of mitogenic stimulation on the cytokine secretion of these *M. leprae*–reactive T cell clones was compared with antigen-specific stimulation. Stimulation for 22 h with either the lectin Con A or a combination of an anti-CD3 mAb plus phorbol ester PMA has been shown to be a powerful cytokine secretion inducer in T cells (11). The influence of mitogenic stimuli on the cytokines secretion was studied especially in those cases where IL-4 and IL-5 release after peptide stimulation was undetectable. As shown in Table 4, treatment of T cells with polyclonal stimuli did not induce the ability to secrete detectable levels of IL-4 or IL-5. The levels of IFN-γ released by the T cell clones varied with different modes of activation, but the highest values were obtained using antigen-specific stimulation. Thus, the Th1-like cytokine secretion pattern seems to be an intrinsic property of activated *M. leprae*–reactive T cell clones.

The Effect of Different APC on the Cytokine Secretion of M. leprae–reactive T Cell Clone R1F9. Clone R1F9 was studied to examine the role of different sources of APC on the cytokine secretion pattern. In the mouse, it was shown that the different $CD4^+$ helper T cell subsets may require different accessory signals (28–30). We compared the capacity of PBMC (with monocytes as the major source of APC) and EBV-transformed B cells to induce cytokine secretion in this representative T cell clone. As shown in Table 5, both types of APC were able to induce secretion of IL-2, IFN-γ, and GM-CSF, although differences in the levels of IL-2 and GM-CSF induced by PBMC or EBV-B cells were found. Neither PBMC nor EBV-B cells were able to induce secretion IL-4 and IL-5, again indicating that the cytokine secretion pattern of an activated T cell clone is an intrinsic property of the clone. Thus, at

Table 4. *Cytokine Secretion Profile Is an Intrinsic Property of Activation T Cell Clones*

Clone	RD	Stimulation	IFN-γ	IL-4	IL-5
			ng/ml	*pg/ml*	*pg/ml*
R1F9	DR3	Con A	17.4 ± 2	<50	<50
R1F9	DR3	Anti-CD3/PMA	7.0 ± 1	<50	<50
R1F9	DR3	APC + [3-13]	1,400.0 ± 42	<50	<50
R1F9	DR3	APC	<1.0	<50	<50
R3E10	DR3	Con A	18.6 ± 3	<50	<50
R3E10	DR3	Anti-CD3/PMA	22.5 ± 5	<50	<50
R3E10	DR3	APC + [3-13]	77.5 ± 2	<50	<50
R3E10	DR3	APC	<1.0	<50	<50
R5E10	DR3	Con A	12.4 ± 2	<50	<50
R5E10	DR3	Anti-CD3/PMA	5.3 ± 1	<50	<50
R5E10	DR3	APC + [3-13]	49.9 ± 6	<50	<50
R5E10	DR3	APC	<1.0	<50	<50
R1F3	DR3	Con A	27.6 ± 3	<50	<50
R1F3	DR3	Anti-CD3/PMA	56.1 ± 3	<50	<50
R1F3	DR3	APC + *M. eprae*	44.2 ± 3	<50	<50
R1F3	DR3	APC	<1.0	<50	<50
N4H1	DR1	Con A	7.6 ± 1	<50	<50
N4H1	DR1	Anti-CD3/PMA	4.3 ± 1	<50	<50
N4H1	DR1	APC + [412-425]	78.0 ± 2	<50	<50
N4H1	DR1	APC	<1.0	<50	<50
W2G2	DR1	Con A	42.1 ± 4	<50	<50
W2G2	DR1	Anti-CD3/PMA	68.6 ± 3	<50	<50
W2G2	DR1	APC + [412-425]	1,250.0 ± 56	<50	<50
W2G2	DR1	APC	<1.0	<50	<50

Six *M. leprae*–reactive T cell clones were stimulated in same experiment with either Con A, anti-CD3 mAb + PMA, or with specific antigen in the presence of HLA-DR matched APC for 20–24 h, after which the supernatants were tested for cytokine release by the T cells.

Table 5. *Effect of Different APC on the Cytokine Secretion Profile*

Clone	APC	[3-13]	IL-2	IFN-γ	IL-4	IL-5	GM-CSF
		μg/ml	*U/ml*	ng/ml	*pg/ml*	*pg/ml*	*ng/ml*
R1F9	MNC	0.001	0.0	15.8	<50	<50	15.9
	MNC	0.01	0.0	31.2	<50	<50	18.8
	MNC	0.1	0.0	51.7	<50	<50	2.1
	MNC	0.3	0.0	96.3	<50	<50	1.7
	MNC	1.0	0.2	211.0	<50	<50	6.6
	MNC	10.0	0.5	750.0	<50	<50	8.3
	MNC	30.0	1.0	1,300.0	<50	<50	13.2
R1F9	EBV-BCL	0.001	0.0	<1.0	<50	<50	1.9
	EBV-BCL	0.01	0.0	3.9	<50	<50	1.6
	EBV-BCL	0.1	0.2	5.5	<50	<50	2.3
	EBV-BCL	0.3	0.3	40.0	<50	<50	3.4
	EBV-BCL	1.0	3.7	140.0	<50	<50	13.9
	EBV-BCL	3.0	9.3	610.0	<50	<50	28.6
	EBV-BCL	10.0	17.4	1,100.0	<50	<50	58.2
	EBV-BCL	30.0	33.6	1,600.0	<50	<50	98.1

Clone R1F9 was stimulated with its specific antigen *M. leprae* hsp65 peptide 3-13 in increasing doses using either irradiated PBMC or EBV-BCL as APC. The number of T cells (10^6) and APC (3×10^6) was kept constant. T cells were stimulated in such a manner for 22 h. Supernatants were tested the presence of IL-2, IFN-γ, IL-4, IL-5, and GM-CSF.

least for this established human T cell clone, accessory signals necessary for secretion of IL-2, IFN-γ, and GM-CSF can be provided by different types of APC.

Discussion

Several groups have reported that the majority of human T cell clones established in vitro are able to produce IL-2, IL-4, IL-5, IFN-γ, and other cytokines after stimulation (11, 31). Those T cell clones were established using different methodologies and had various antigen specificities or no clearly defined specificities when generated with mitogens like PHA. No evidence was found in these earlier studies that human T cell clones can be classified into Th1 and Th2, as reported for mouse helper T cells. In fact, most human T cell clones analyzed thus far, including many alloreactive, tetanus toxoid, and Herpes Simplex Virus (HSV)-specific clones, seem to resemble murine Th0 cells. Recently, human T cell clones have been described that displayed cytokine secretion profiles distinct from the Th0 pattern, indicating that also in man functionally distinct $CD4^+$ T cell subsets may exist (12, 13). In this report, we describe a group of human T cell clones with common characteristics that bear close resemblance to murine Th1 cells. The common characteristic of these T cell cones is that they recognize mycobacterial antigens. These mycobacterium-reactive T cell clones show very high IFN-γ to IL-4 ratios as compared to T cell clones specific for alloantigen or for tetanus toxoid. It should, however, be noted that in contrast to what has been observed with murine Th1 clones, a number of mycobacteria-specific clones do produce IL-4 and IL-5, and many of those clones that do not secrete detectable amounts of IL-4 and IL-5 proteins do express IL-4 and IL-5 transcripts, as determined with the highly sensitive PCR method (data not shown). Nevertheless, the ratios of the secretion of IFN-γ to IL-4 of these IL-4- and IL-5-producing T cell clones that are reactive with mycobacterial antigens clearly sets them apart from the Th0-like tetanus toxoid and alloantigen-specific clones.

The mycobacteria-reactive clones recognized different antigens. T cell clones isolated from three different tuberculoid leprosy patients recognized hsp65 and hsp70. The hsp-65-specific clones recognized different T cell epitopes in the context of HLA-DR1, DR2, or DR3. The Th1-like pattern of cytokine secretion was, however, not restricted to T cell clones reactive with mycobacterial hsp. Several T cell clones, isolated from the synovial fluid of patients suffering from rheumatoid arthritis that were reactive with different mycobacterial proteins not belonging to the hsp family, also showed a Th1-like cytokine secretion profile. Furthermore, this Th1-like pattern was not a consequence of a particular disease of the donors from whom these T cell clones were isolated. First, T cell clones from a tuberculoid leprosy patient that were specific for nonmycobacterial antigens like tetanus toxoid and alloantigens, secreted levels of IFN-γ, IL-4, and IL-5 comparable to those secreted by clones described so far (11, 13, 31), with similar antigen specificities, including tetanus toxoid, alloantigens, *Candida albicans*, or HSV isolated from healthy individuals. Second and more importantly, T cell clones reactive

with mycobacterial antigens, isolated from healthy subjects, secreted IFN-γ but almost no IL-4 and IL-5. It seems, therefore, that mycobacteria select for $CD4^+$ T cells that produce IFN-γ, but no or disproportionally low levels of IL-4 and IL-5. This cytokine secretion profile was observed with T cell clones reactive with mycobacteria regardless of the antigen recognized, the HLA-DR restriction element, and the TCR Vβ gene segment usage, or whether or not the donor suffered from a mycobacterium-inflicted disease. In addition, this Th1-like pattern of cytokine secretion was an intrinsic property of the mycobacterium-reactive clones, since this pattern was not affected by the way the clones were stimulated in vitro.

It is likely but not sure that the Th1-like T cell clones are representative for the T cells that are involved in the cellular immune response against mycobacteria. It needs to be demonstrated that, for example, T cells present in lesions inflicted by *M. leprae* produce IFN-γ but not IL-4. Th1 cells have been shown to protect mice against lethal infections with the intracellular parasite *Leishmania donovani* (32). The mechanism of protection is not entirely clear but involves IFN-γ. Injection of anti-IFN-γ antibodies abrogates resistance of C3H mice to infection with *L. major* (33). On the other hand, IL-4 has an adverse effect on protection against Leishmania infection, since anti-IL-4 mAbs can cure susceptible BALB/c mice infected with *L. major* (34). IFN-γ induces killing of the obligatory intracellular amastigote form of the parasite (35–37). IL-4 seems to inhibit this IFN-γ-induced killing activity of macrophages (38). It is likely, therefore, that IFN-γ-induced macrophage killing activity is involved in the protection against infections with Leishmania, although there is evidence for other T cell–dependent mechanisms that play a role in this protection (33). Mycobacteria are obligatory intracellular organisms, and similar mechanisms may be implicated in containment of mycobacterial and Leishmania infections. In that case, Th1-like cells may pose a distinct advantage above Th0- or Th2-like cells in protection against mycobacterial infections. However, in tuberculoid leprosy, there is evidence for the occurrence of pathological immune responses against *M. leprae*. This disease is characterized by the occurrence of granulomatous skin lesions with heavy infiltration of $CD4^+$ T lymphocytes around activated macrophages, usually accompanied by nerve destruction. In these lesions, few if any bacilli can be found, indicating a successful eradication of the mycobacteria. There is, however, evidence that the prominent nerve damage is the result of this cellular immune response against *M. leprae* (14). Thus, the Th1-like cells in tuberculoid leprosy patients may also be involved in some of the immunopathological manifestations. In this respect, it is perhaps relevant that T cell clones specific for the hsp65 peptide 3-13 isolated from a tuberculoid leprosy patient secreted much more IFN-γ than T cell clones with exactly the same antigen specificity (hsp65 peptide 3-13) isolated from healthy individuals. This suggests that a feedback mechanism is operating in tuberculoid leprosy patients that results in an enhanced capacity to produce IFN-γ. It is presently unknown how the balance between a normal protective immune response and a immunopathological immune response is disturbed in tuberculoid leprosy.

Thus far, it is not known how T cells with different cytokine secretion patterns are selected. Studies that address the question how naive $CD4^+$ T cells mature to memory cells may help to unravel the mechanism underlying the selective induction of distinct helper T cell subsets. It has been reported that murine $CD4^+$ T cells that were considered functionally immature are able to produce IL-2 but almost no IFN-γ or IL-4 (39, 40). Likewise, in man, $CD45RA^+$ T cells, considered to be naive, and cord blood T cells, which should be immunologically immature, are able to produce IL-2 and perhaps low levels of IFN-γ but no IL-4 (41–45). On the other hand, $CD45R0^+$ T cells, thought to include memory T cells, do produce IL-2, IFN-γ, and IL-4 after activation in vitro (41, 46). These findings suggest that the ability to produce IFN-γ, IL-4, and several other cytokines is acquired upon maturation of naive to memory T cells (45). Mosmann and Coffman (47) have proposed two mechanisms for maturation of TH1 and Th2 cells. One postulates that Th1 and TH2 cells develop from a common precursor Th0 cell that is able to produce all cytokines simultaneously. Based on the findings with naive and memory T cells discussed above, this precursor Th0 cell should be a memory cell that has already been primed with antigen. In that case, it should be assumed that tetanus toxoid–specific clones are arrested in a Th0 phase, while T cells reactive with mycobacterial antigens mature further to Th1-like cells. Another perhaps more plausible hypothesis is that Th1, Th2, and TH0 cells mature from naive T cells independently. Insight in the mechanism of priming of naive T cells with mycobacteria may provide an answer to the question why T cells reactive with mycobacteria develop into Th1-like cells.

The data presented here indicate that mycobacteria select for a certain immune response. It would be interesting to know whether other pathogenic bacteria can also induce maturation of distinct subsets of $CD4^+$ T cells.

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