RESEARCH ARTICLE

Cytokine profiling of human peripheral blood CD4+ T lymphocytes reveals a new Th-subpopulation (Th6) characterized by IL-6

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Accepted for publication February 5, 2010

ABSTRACT. The number of functional subsets of CD4+ T lymphocytes distinguished by their cytokine production has been extended in the last decade. The *in vitro* generation of a T cell subset characterized by IL-6 production has resurrected the question of cytokine co-expression patterns in T cells. In order to delineate these cells as a specific functional subpopulation *in vivo*, we profiled the cytokine production pattern of human peripheral blood CD4+ T lymphocytes across established subsets. We provide evidence for a new T cell subset Th6, with an IL-6 signature. Freshly isolated PBMC were analyzed using intracellular cytokine detection (IDC). Cytokine co-expression patterns of up to three cytokines, as well as their correlation with selected transcription factors, were determined in CD4+ T lymphocytes. Co-expression of two of these signature cytokines used for the definition of functional subsets, *e.g.* IL-4, IFN-γ, IL-17 and IL-6 were observed, but nearly excluded the production of a third (or fourth) signature cytokine. In this respect, Th1 (key cytokine IFN-γ), Th2 (IL-4), Th6 (IL-6) and Th17 (IL-17) subsets can be defined, along with overlaps of any two of them. In contrast, TNF-α and IL-2 are not signature cytokines, but their absence or expression in single cells introduces further divisions across established subsets. Our study supports the concept of a further functional T cell Th6 subset, and contributes to the reference cytokine profiles of healthy individuals relevant to further studies in a variety of disease states.

Keywords: intracellular cytokine detection, flow cytometry, IL-2, IL-6, TNF-α, GATA-3

The understanding of the pathways involved in the immune response was considerably improved by the verification of postulated Th1 and Th2 subsets within CD4+ T lymphocytes by Mosmann and colleagues in 1986 [1]. Thus, Th1 cells are referred to as IFN-γ-producing cells, while the typical cytokine pattern of Th2 cells consists of the production of IL-4, IL-5 and IL-13. CD4+ T-cells showing a mixed cytokine profile are termed Th0 cells [2-4].

Nevertheless, this dichotomy of the T-helper (CD4+) cells left some questions unanswered: it has not been possible to explain all immuno-pathological conditions using the Th1/Th2 model, neither does a strictly polarized pattern reflect *in vivo* situations [4, 5]. Furthermore, several cytokines, *e.g.* IL-2 and TNF-α, originally assigned to Th1 or Th2, have never been confirmed as such unequivocally.

The spectrum of functional subpopulations has been expanded by the discovery of further subsets, *i.e.* regulatory T cells and (IL-17-producing) Th17 [6, 7]. Several transcription factors have been described as critical for the differentiation of these CD4+ subsets, *e.g.* T-bet (Th1), GATA-3 (Th2), RORγ(t) (Th17), FoxP3 (regulatory subsets), and they have been used to distinguish them [7]. A distinct population of IL-6-producing CD4+ T-cells has been described by our group in several studies [8-10]. This population of cells can be induced by calcitriol *in vitro* and does not show any typical Th1 or Th2 cytokine expression pattern. Originally, calcitriol has been described as inducing Th2 differentiation while inhibiting the Th1 pathway, and in the last few years, several studies have suggested the induction and increase in T cells with regulatory properties *in vitro* and *in vivo* [11-13].

doi: 10.1684/ecn.2010.0190

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In this study, we tried to provide evidence of an IL-6-producing CD4+ T cell subset in the peripheral blood of healthy humans, and focused on the distinction between Th6 and Th2 or regulatory T cell subsets, based upon their co-expression pattern of cytokines and transcription factors (GATA-3, FoxP3).
Taking advantage of multiparameter analysis, we were able to correlate the various cytokines and transcription factors at the single cell level.
Supported by the present experiments, we propose a novel Th subset of IL-6-producing cells, herein referred to as Th6.

DONORS AND METHODS

Donors and sample preparation

Venous peripheral whole blood was collected from healthy donors. In total, four male and four female subjects (age 25-48 years) were enrolled after informed consent. The present study was approved by the ethics committee of the Medical University of Vienna.
Peripheral blood mononuclear cells (PBMC) were isolated by means of density centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). After isolation, PBMC were seeded at a density of 2 x 10^6 cells/mL of Ultra Culture Medium (Bio Whittaker, Cambrex Cooperation, East Rutherford, NJ, USA) supplemented with 2 mM L-glutamine, 10^5 U/L penicillin G sodium, 10^5 µg/L streptomycin sulphate, 50 mg/L gentamicin and 3.5 µl/L mercaptoethanol (all purchased from Invitrogen Gibco, Carlsbad, CA, USA).

Stimulation

PBMC (six donors) were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA), 1.25 µM ionomycin and 10 µg/mL Brefeldin A (all purchased from Sigma-Aldrich, Saint Louis, MO, USA), for four hours, at 37°C, in a saturated atmosphere containing 5% CO_2.
For selected experiments, PBMC (three donors) were stimulated overnight with CD2/CD3/CD28 beads from a T Cell Activation/Expansion Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. Brefeldin A was added during the last four hours.
Throughout this study, data refer to PMA/ionomycin stimulation, unless otherwise stated.

Long-term culture (selected experiments)

For selected experiments, PBMC (six donors) were cultured for 21 days as described previously [10]. Nine mL of peripheral blood were collected. PBMC were isolated as described above, seeded at a density of 1 x 10^6 /mL of Ultra Culture Medium, and initially stimulated for three days with 1% phytohemagglutinin (Invitrogen Gibco). From day three to day 21, PBMC were cultured in the presence of IL-2 (Roche, Indianapolis, IN, USA), at a concentration of 20 U/mL, to achieve cell proliferation and maintain viability. Cells were re-stimulated and harvested on day 21, as described above.

Intracellular detection of cytokines (IDC) and transcription factors (TF)

Expression of surface markers, intracellular cytokines and transcription factors were assessed using a multi-color flow cytometry technique. All monoclonal antibodies (mAb) used were diluted as recommended by the manufacturers (Miltenyi Biotec; BD, San Jose, CA, USA; BD Pharmingen, San Diego, CA, USA; eBioscience, San Diego, CA, USA), and listed in *table 1B*. Anti-CD3, anti-CD-4 and anti-CD8 antibodies were used for surface staining in order to identify T cell subsets. To identify any possible relationship between Th6 and regulatory T cells, an anti-CD25 mAb, as well as an anti-IL-10 mAb, were applied in selected experiments. In the protocol used for the assessment of transcription factors, the surface VioBlue dyes proved to be more stable and were therefore applied preferentially.
PBMC were harvested and processed on ice until fixation. For IDC, cells were fixed with formaldehyde at a final concentration of 2% and permeabilized with 0.1% saponin (Sigma-Aldrich). Cytokine staining was performed according to the panel shown in *table 1A*.
For TF, the Foxp3 Staining Buffer Set (Miltenyi Biotec) was used for fixation and permeabilization. PBMC were processed according to the protocol provided by the manufacturer, and stained for intracellular cytokines (IL-4, IL-6 or IFN-γ) in combination with specific T-cell-subsets TF (T-bet, GATA-3 and FoxP3).

Flow cytometric analysis

Cells were analyzed using multi-color flow cytometry (FACSAria; FACSCanto II; BD). Lymphocytes were gated according to their specific scatter properties. Acquisition was stopped when at least 2 x 10^5 events had been counted within the lymphocyte gate. Lymphocytes defined in the light scatter plot were further gated as CD4+CD8- and verified as T cells by CD3 expression. Further analysis of the cytokine production pattern was based on these CD4+ T lymphocytes.
Data analysis was performed using FACSDiva software.

Statistics

Data are presented as mean values and standard deviations from independent measurements (the number of experiments is indicated in the figure legends).
Statistically significant differences mentioned in the text ($p < 0.05$) were assessed by Student's t test (paired, two-tailed).

RESULTS

Cytokine profiles of previously described, functional CD4+ subpopulations

PBMC were treated according to the PMA/ionomycin stimulation protocol described in Methods and materials. Previously described functional subpopulations of CD4+ gated T lymphocytes are presented in *figure 1*. CD4+ T lymphocytes producing IFN-γ and not IL-4

Table 1
Antibody panels used for the characterization of cytokine (co-)expression patterns (**A**) and antibodies and labels used in the study (**B**)

A) Cytokine staining panel				
	FITC	**PE**	**APC**	**Alexa Fluor® 647**
1	IFN-γ	IL-6		IL-17
2	IFN-γ	IL-17	IL-4	
3	IFN-γ	TNF-α		IL-17
4	IFN-γ	IL-4	TNF-α	
5	IFN-γ	IL-6	IL-4	
6	IFN-γ	IL-6	TNF-α	
7	IFN-γ	IL-2		IL-17
8	IFN-γ	IL-2	TNF-α	
9	IFN-γ	IL-2	IL-4	
10	IFN-γ	IL-13	IL-4	
11	IFN-γ	IL-13	IL-2	
12	IFN-γ	IL-6	IL-2	
13	IFN-γ	IL-13	TNF-α	
14	IFN-γ	IL-13		IL-17

B) Antibodies		
mAb	**Fluorochrome**	**Company**
CD3	VioBlue	Miltenyi Biotec
CD3	APC Cy7	Pharmingen
CD4	PE Cy7	Pharmingen
CD4	VioBlue	Miltenyi
CD8	PerCP Cy5.5	BD
CD 25	PE Cy7	Pharmingen
IFN-γ	FITC	Pharmingen
IFN-γ	APC	Miltenyi
TNF-α	PE	Pharmingen
TNF-α	APC	BD
IL-2	PE	Pharmingen
IL-2	APC	Pharmingen
IL-4	PE	Pharmingen
IL-4	APC	Pharmingen
IL-6	PE	Pharmingen
IL-10	PE	Pharmingen
IL-10	APC	Pharmingen
IL-13	PE	Pharmingen
IL-17	PE	eBioscience
IL-17	Alexa Fluor®647	eBioscience
T-bet	Alexa Fluor®647	eBioscience
GATA-3	Alexa Fluor®488	Pharmingen
FoxP3	APC	Miltenyi Biotec

Surface staining was performed as described in "Donors and methods". Antibodies were diluted according to manufacturers' protocols.

have been established as Th1 cells; those producing IL-4 and not IFN-γ as Th2 cells, and T lymphocytes co-expressing both cytokines have been classified as Th0 cells *(figure1A)*.
CD4+ T lymphocytes producing IL-17 and not IFN-γ are designated Th17, and those co-expressing IL-17 and IFN-γ as Th17/Th1 *(figure 1B)*.
CD4+ T lymphocytes producing IL-6, but not IFN-γ, are referred to as Th6 throughout this study *(figure 1C)*.
To obtain an extended cytokine profile, cells were gated according to the dot plot quadrants, and the percentages of cells co-expressing further cytokines were evaluated. This revealed minimal co-expression with IL-17 or IL-6 for Th1, Th2 or Th0.

The Th2-like cytokine IL-13 is highly associated with IL-4 production in Th2 (IL-4+IFN-γ-), but only weakly with the Th0 (IL-4+IFN-γ+) subset *(figure 1A)*.
Th17 cells do not co-express IL-4 or IL-13 to any great extent. However, around 10% of Th17 cells also produce IL-6 *(figure 1B)*. Accordingly, a significant proportion of Th6 cells co-express IL-17 *(figure 1C)*. In contrast, minimal co-expression of IL-4 can be detected for Th6.

Co-expression patterns of the Th2, Th6 and Th17 cytokines IL-4, IL-6 and IL-17

PBMC were treated according to the PMA/ionomycin stimulation protocol described in Methods and materials. A very strict separation can be observed between CD4+ T lymphocytes producing IL-4 and those expressing either IL-6 *(figure 2A)* or IL-17 *(figure 2C)*. However, a small but significant subpopulation co-expresses IL-6 and IL-17 *(figure 2B)*. This co-expression pattern is even more evident after culturing cells for three weeks as described in Methods and materials *(figure 3)*.

Distribution of IL-2 production among functional CD4+ subpopulations

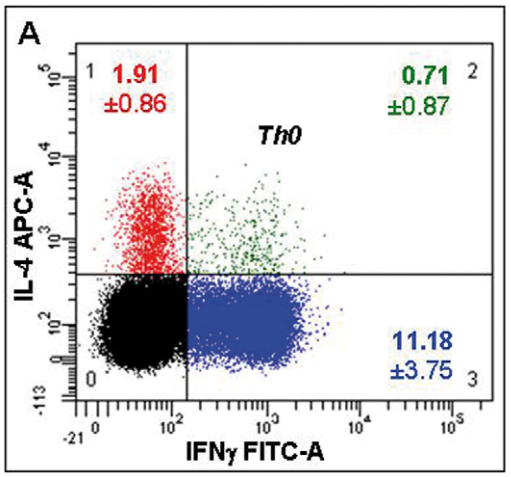
PBMC were treated according to the PMA/ionomycin stimulation protocol described in Methods and materials. Approximately two thirds of all CD4+ T lymphocytes produce IL-2. Within their functional subpopulations, a substantial majority also expresses IL-2 *(figure 1)*. The least co-expression is seen in the Th6 subset *(figure 1C)*. Evaluation of the co-expression pattern of IL-2 against the various other cytokines reveals that the majority of cells positive for a certain cytokine correlate with a high IL-2 expression signal *(figure 4)*. The cell population exerting intermediate signals for IL-2 also contains a cell fraction positive for IL-4, IL-6, IL-13, IL-17, IFN-γ or TNF-α, respectively. Remarkably, a distinct population of cells positive for any of these cytokines always appears to be IL-2-negative *(figure 4)*.

Distribution of TNF-α production among functional CD4+ subpopulations

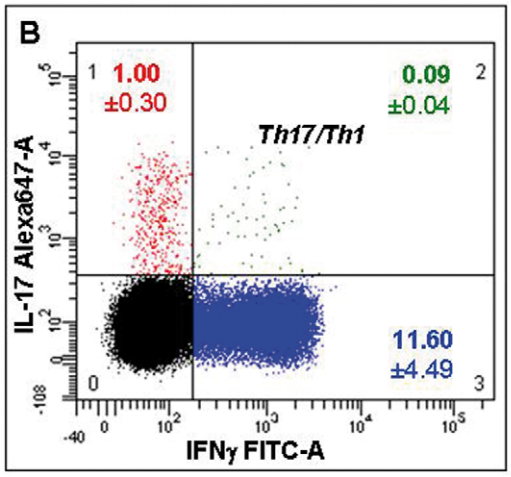
PBMC were treated according to the PMA/ionomycin stimulation protocol described in Methods and materials. Staining for TNF-α shows a continuum from negative to highly positive. Approximately 50% of all CD4+ T lymphocytes stain positive for APC-labeled TNF-α, whereas PE-labeled antibodies exert higher sensitivity, leading to approximately 70% positive cells *(figure 5)*. Expression of IL-4, IL-17 and IFN-γ correlates with high TNF-α production. This is also true, but less obvious for IL-13. In contrast, a substantial proportion of IL-6-producing cells appear negative for TNF-α *(table 2)*. A significantly lower co-expression of TNF-α is also evident in the Th6 (IL-6+IFN-γ-) subpopulation *(figure 1C)*. The mean co-expression for the other subsets is around or above 90% *(figure 1)*.

Co-expression of IL-2 and TNF-α with IFN-γ

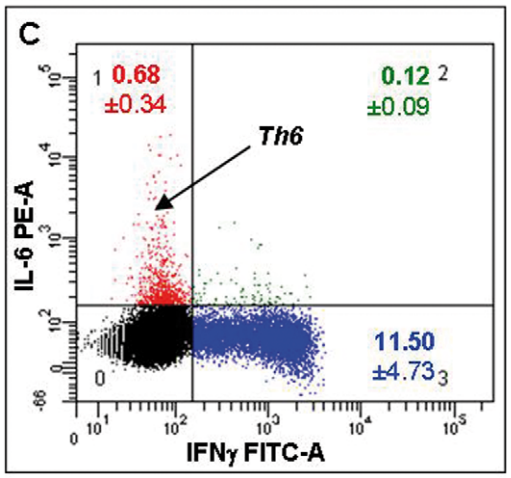
PBMC were treated according to the PMA/ionomycin stimulation protocol described in Methods and materials. The co-expression patterns of IL-2 or TNF-α with other



	IL4+IFNγ-	IL4+IFNγ+	IL4-IFNγ+
IL2	88.55	79.47	84.87
±	5.46	13.88	3.83
IL6	0.56	0.00	0.91
±	1.36	0.00	0.86
IL13	51.91	9.17	0.60
±	8.26	10.74	0.67
IL17	0.00	0.00	0.97
±	0.00	0.00	0.61
TNFα	82.42	91.25	91.80
±	11.00	9.97	4.65



	IL17+IFNγ-	IL17+IFNγ+	IL17-IFNγ+
IL2	72.44	100.00	83.82
±	6.99	0.00	4.34
IL4	0.00	0.00	1.01
±	0.00	0.00	0.64
IL6	11.01	0.00	0.99
±	8.29	0.00	0.59
IL13	1.85	0.00	1.93
±	4.54	0.00	0.37
TNFα	91.60	100.00	97.71
±	6.72	0.00	2.01



	IL6+IFNγ-	IL6+IFNγ+	IL6-IFNγ+
IL2	62.58	90.00	85.92
±	8.74	22.36	3.65
IL4	1.39	0.00	4.09
±	3.40	0.00	4.23
IL13	nd	nd	nd
±			
IL17	7.66	0.00	0.49
±	4.04	0.00	0.59
TNFα	70.91	100.00	89.57
±	9.15	0.00	7.14

Figure 1

PBMC were treated according to the PMA/ionomycin stimulation protocol decribed in Methods and materials. Previously described functional subpopulations of CD4+ gated T lymphocytes are illustrated as representative dot plots according to their cytokine profile: Th1, Th2 and Th0 (**A**), Th17 and Th17/Th1 (**B**) and Th6 (**C**). Values within quadrants are means of percentages within CD4+ T cells ± standard deviations of six experiments. Extended cytokine profiles for each quadrant (1-3) are illustrated in table form as mean values of percentages within a given (quadrant-) subset (= 100%) ± standard deviations of six experiments. Surface markers and cytokines are labeled as shown in *table 1*. nd: not determined.

cytokines are usually not applied for the characterization of established functional T lymphocyte subpopulations. Subsets of CD4+ T lymphocytes arising from the co-expression of IL-2 or TNF-α with IFN-γ were analyzed further for expression of the other cytokines studied.

The subset producing IFN-γ, but not IL-2 (IL-2-IFN-γ+) is virtually negative for IL-6, IL13, and IL-17, however, a substantial proportion (with individual variation) may co-express IL-4 (6.46 ± 4.79%). A completely different distribution is detectable for TNF-α: about 80-90% of

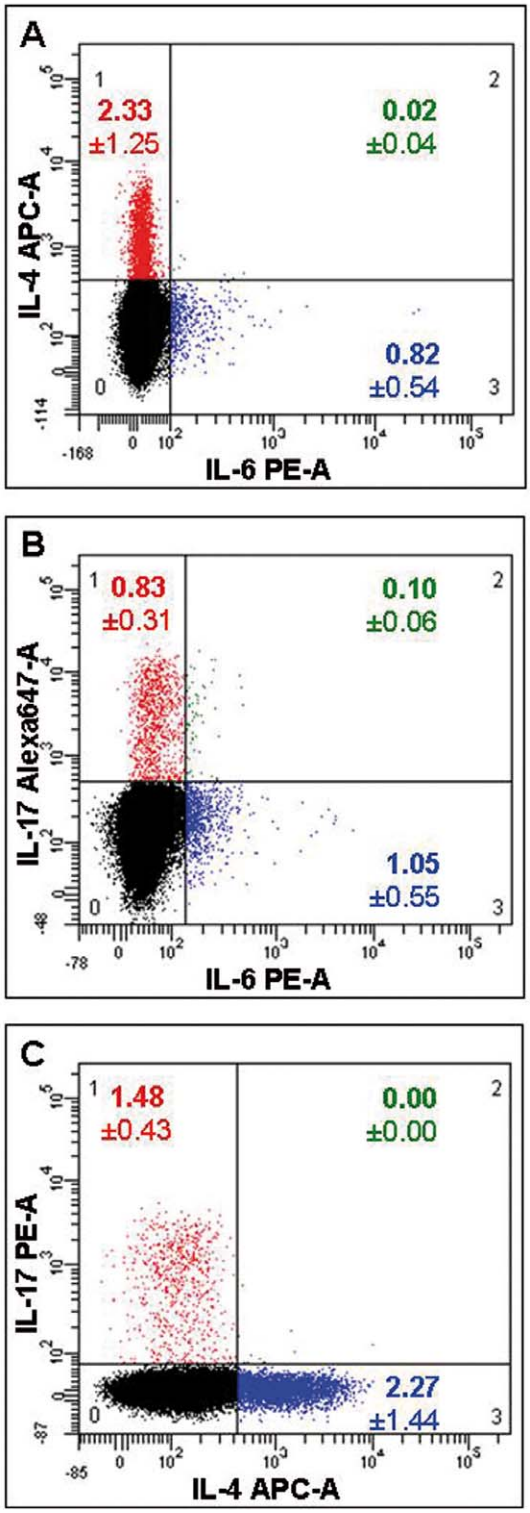


Figure 2
Co-expressions of signature cytokines on CD4+ gated T lymphocytes are illustrated as representative dot plots: IL-4/IL-6 (**A**), IL-17/IL-6 (**B**), and IL-17/IL-4 (**C**). Values within quadrants are means of percentages within CD4+ T cells ± standard deviations of six experiments. Surface markers and cytokines are labeled as shown in *table 1*.

IL-2-IFN-γ+ and IL-2+IFN-γ+ also produce TNF-α, whereas less than 50% of IL-2+IFN-γ- co-express TNF-α (data not shown).

Within the IFN-γ-producing CD4+ T lymphocytes, only a negligible proportion is negative for TNF-α *(figure 5)*. In this TNF-α-IFN-γ+ subset, virtually no IL-6, IL-13 or

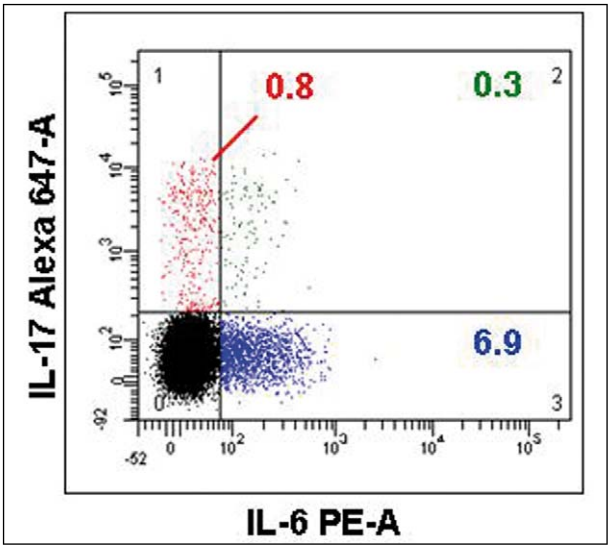


Figure 3
Co-expression of IL-17/IL-6 on CD4+ gated T lymphocytes after three weeks of culture from a representative experiment is illustrated as dot plot. Culture and staining were performed as described in Methods and materials.

IL-17 can be detected. Expression of IL-2 or IL-4 is also significantly lower than in TNF-α+IFN-γ+ or TNF-α+ IFN-γ- cells.

Likelihood of co-expression of cytokines

An overview of cytokine co-expressions is presented in *table 2*. CD4+ T lymphocytes were subgated according to their expression of a specific cytokine (= 100%) as indicated in the headings. The proportions of these cells simultaneously producing a specific, second cytokine are presented in columns. For example, an IL-13+ CD4+ T lymphocyte has a > 50% chance of additionally producing IL-4, whereas the likelihood of an IL-17+ lymphocyte doing so is virtually zero.

Comparison of PMA/ionomycin versus *CD2/CD3/CD28 stimulation protocol*

Data from experiments with the two different stimulation protocols described in Methods and materials are presented in *figure 8*. Incubation with CD2/CD3/CD28 clearly resulted in lower frequencies of CD4 T lymphocytes producing IL-4, IL-17 or IFN-γ, and this effect was obvious in all subsets defined by the various co-expression patterns in a similar way. Also, frequencies for IL-2, TNF-α appeared lower with this protocol (data not shown). Interestingly, as the only exception from this trend, the proportion of CD4 T lymphocytes positive for IL-6 happened to be and remained, at least, stable.

Lack of GATA3 or FoxP3 expression in Th6 cells

PBMC were stimulated with PMA/ionomycin and processed as described in Methods and materials. Expression of cytokines and transcription factors is shown in *figure 6*. Whereas T cells showing a typical Th2 cytokine production pattern (IL-4+/IFN-γ-) were positive for GATA-3, those considered as Th0 (IL-4+/IFN-γ+) did not express

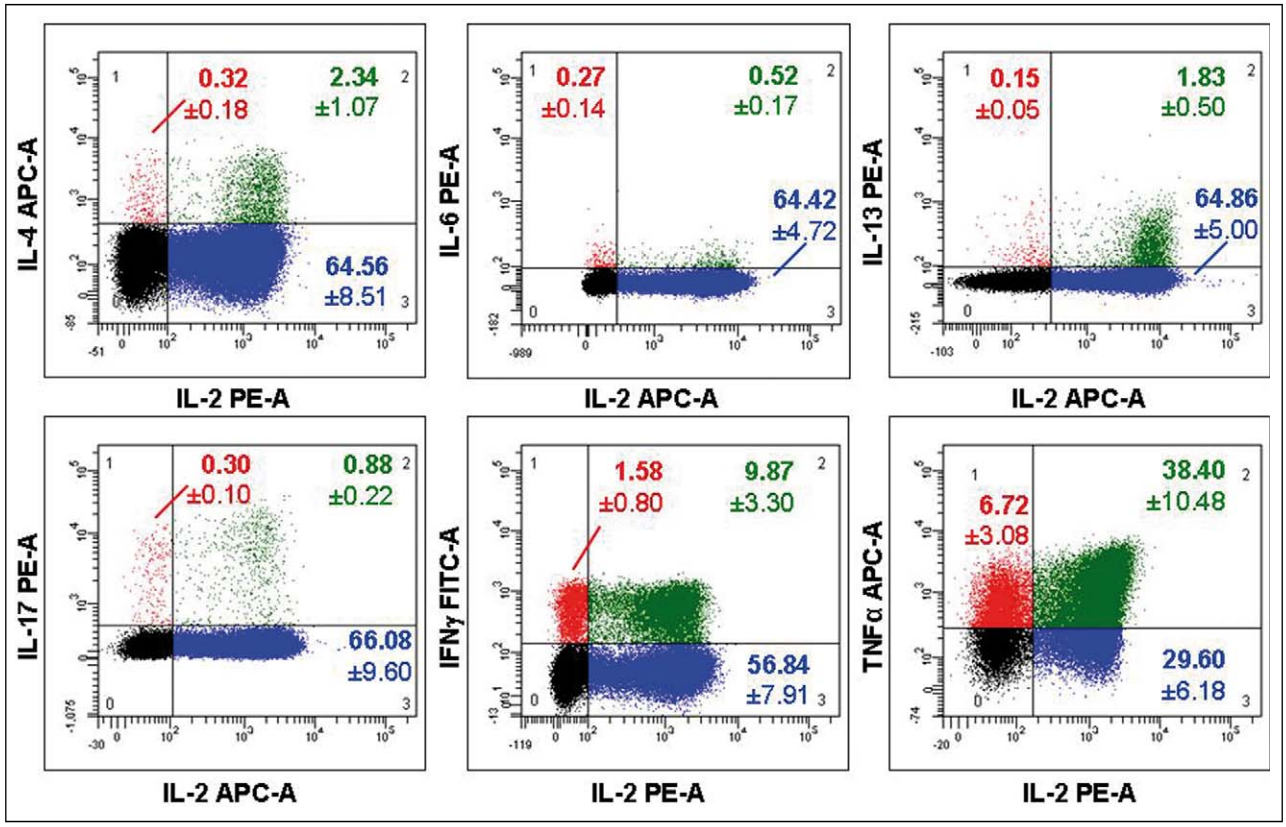


Figure 4

Co-expressions of IL-2 on CD4+ gated T lymphocytes with each cytokine evaluated in this study are illustrated as representative dot plots. Values within quadrants are means of percentages within CD4+ T cells ± standard deviations of six experiments. Surface markers and cytokines are labeled as shown in *table 1*.

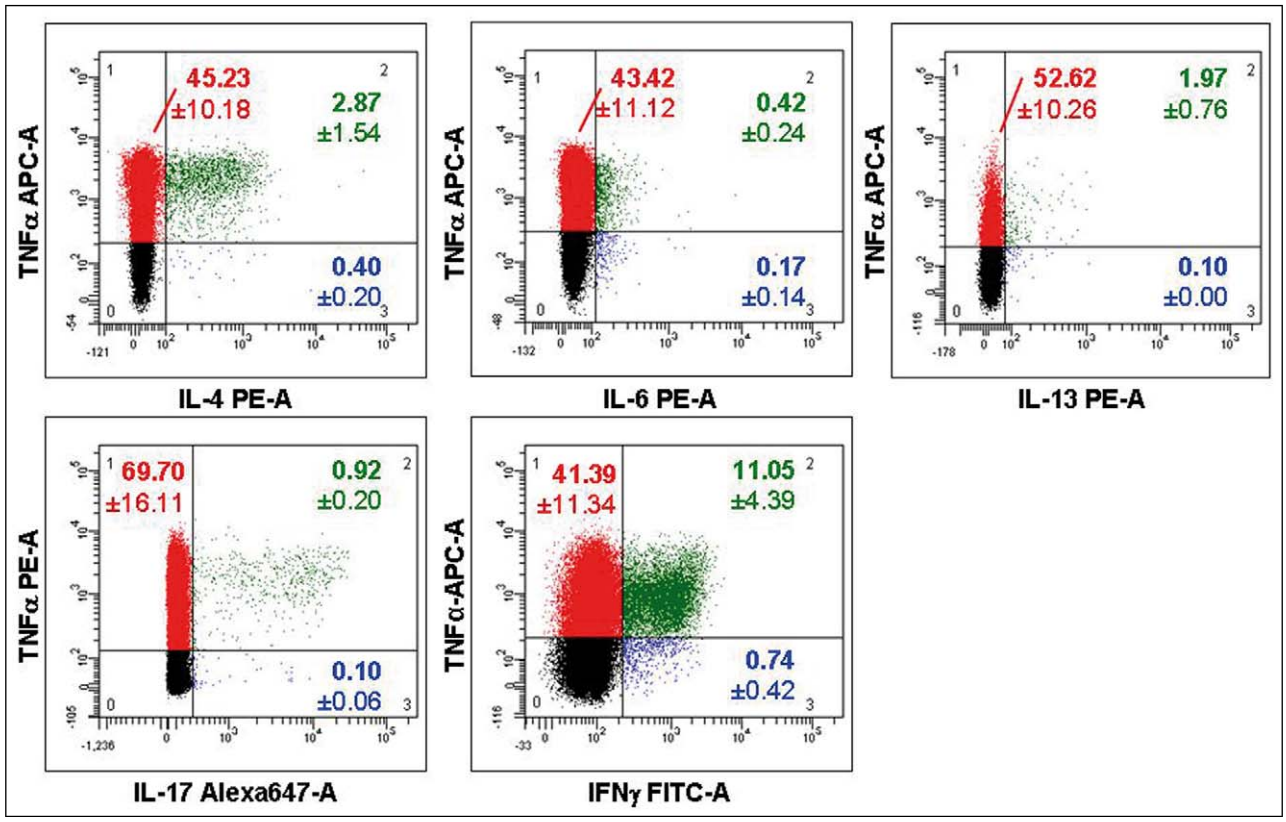


Figure 5

Co-expressions of TNF-α on CD4+ gated T lymphocytes with each cytokine (except IL-2; see *figure 4*) evaluated in this study are illustrated as representative dot plots. Values within quadrants are means of percentages within CD4+ T cells ± standard deviations of six experiments. Surface markers and cytokines are labeled as shown in *table 1*.

Table 2
CD4+ T lymphocytes were gated according to their expression of a specific cytokine (= 100%) as indicated in the headings. The proportions of these cells simultaneously producing a specific second cytokine are presented in columns

	IL-2 (100%)	IL-4 (100%)	IL-6 (100%)	IL-13 (100%)	IL-17 (100%)	IFN-γ (100%)	TNF-α (100%)
IL-2	x	86.69	80.93	91.88	72.00	86.83	86.43
±		3.16	9.54	4.25	6.91	3.90	4.14
IL-4	3.46	x	1.11	51.57	0.00	4.95	5.75
±	1.49		2.72	9.06	0.00	3.95	2.29
IL-6	0.80	0.48	x	nd	11.03	1.11	1.10
±	0.27	1.17			7.45	0.89	0.98
IL-13	2.76	43.53	nd	x	1.67	1.93	3.61
±	0.79	7.26	nd		4.08	0.55	1.17
IL-17	1.33	0.00	7.82	0.62	x	0.94	1.44
±	0.40	0.00	4.32	1.51		0.56	0.80
TNF-α	55.83	84.23	71.94	94.84	97.59	93.05	x
±	11.92	11.72	17.31	4.61	12.45	4.12	
IFN-γ	14.87	23.02	12.75	9.78	8.38	x	20.28
±	5.11	17.63	8.38	5.93	4.75		7.46

nd: not determined.

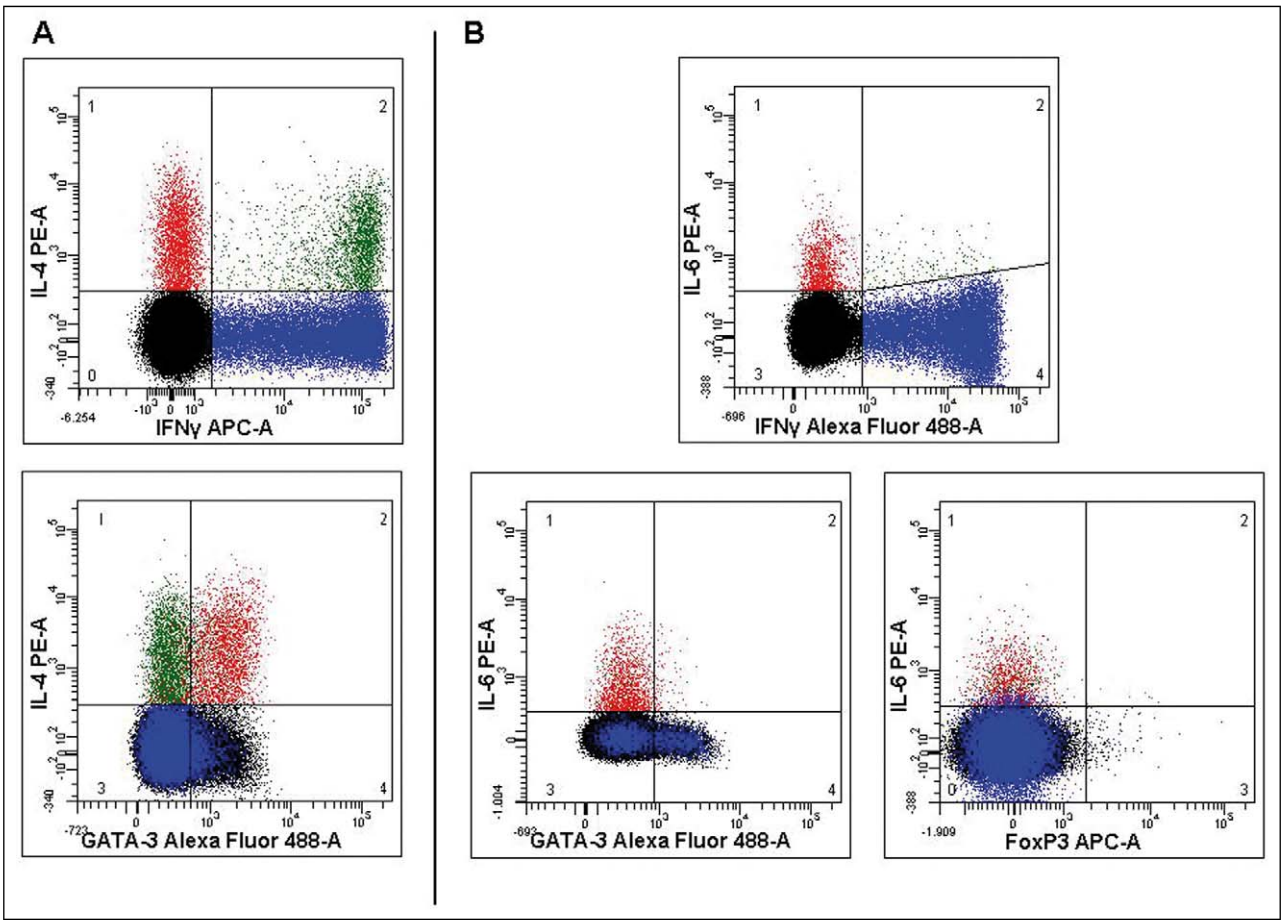


Figure 6
Th2 cells (IFN-γ-IL-4+) and Th0 (IFN-γ+IL-4+) cells were distinguished according to their IFN-γ expression. The subsets appear as red (Th2) and blue (Th0) dots in the dot plot showing GATA-3/IL-4 expression (**A**). IL-6-producing T cells with (red) or without (green) co-expression of IFN-γ are displayed as representative dot plots showing GATA-3/IL-6 and FoxP3/IL-6 (**B**).

the transcription factor *(figure 6A)*. In cells expressing IL-6, GATA-3 was not detectable *(figure 6B)*. Similarly, no co-expression of IL-6 and the Treg-related transcription factor FoxP3 could be detected. In addition, there was no co-expression of IL-10 and IL-6 *(figure 7)*.

DISCUSSION

The era of studies on functional subpopulations of CD4+ T lymphocytes, distinguishable and characterizable by their cytokine production, was heralded by the description

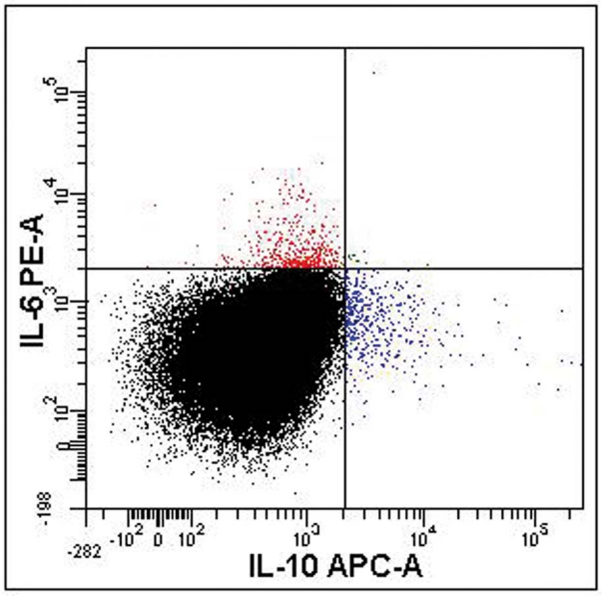


Figure 7
Expression of IL-6 and IL-10 in CD4+ gated T lymphocytes after stimulation as described in "methods and materials".

of Th1 and Th2 subsets by Mosmann *et al.* in 1986 [1]. The distinction, originally based on function, has focused on the expression of IL-4 or IFN-γ. Meanwhile, this classification has been extended by the description of a significant proportion of T lymphocytes co-expressing both cytokines (termed Th0), and by cells characterized by expression of different cytokines such as TGF-β or IL-10 (hallmarks for subsets of regulatory T cells). Recently, expression of IL-17 has been used to classify a new functional subset as Th17. A further subset, characterized by IL-6 as the signature cytokine, has been described in a culture system.

However, in no case, is a single cytokine sufficient for the definition of a functional subset, and indeed various patterns of co-expression of the signature cytokines have been described. Other cytokines have been assigned to functional subsets, although with some controversy, *e.g.* IL-2 to Th1 cells [14].

Expression of surface markers has been correlated with the enrichment of functional subsets. Transcription factors have been shown to play a key role in the differentiation and maintenance of specific subsets. In polarized forms, these factors have been described as being expressed in a mutually exclusive way [7]. Finally, it is the actual profile of cytokines produced that defines function and thereby assignment to one of these functional subset. Cytokines mainly exert their effect in a paracrine way, acting over very short distances or, in an extreme form, as an immunological synapse [15]. The actual cytokine (co-)production by a single T lymphocyte may therefore be decisive for the mode of immunological response regulated by this type of cell. For these reasons we applied a technique that directly elucidated the cytokine production capacity at the single cell level.

Nevertheless, the choice of activators used in a study is a critical one, since an *in vitro* imitation of a biological micro-environment can naturally only approximate *in vivo* conditions. Depending on various pathways of T lymphocyte activation and on the choice of *in vitro* stimulators respectively, different biases in the cytokine production have been described [16]. The method for intracellular cytokine detection applied in this study is mainly based on polyclonal stimulation with phorbol ester acetate PMA and the calcium ionophore ionomycin, together with the secretion blocker Brefeldin A. This combination of stimulators represents a strong, non-specific stimulation, with high signals for intracellular cytokine detection with best possible standardization, and thus reproducibility. However, results from such a stimulation reflect the the competence of cells to produce cytokines *in vitro* but not necessarily *in vivo*.

Stimulation of T cells by antibodies to the T cell receptor and to the co-stimulatory molecules CD2/CD3/CD28 imitates the physiological pathway of T cell activation, and is therefore regarded as simulating *in vivo* conditions better than stimulation with PMA and ionomycin acting directly within second messenger signalling. Nevertheless, the CD2/CD3/CD28 stimulation appeared to be less efficient, leading to comparable distribution patterns of cytokines, but at much lower frequencies, thus reducing the very small subsets of co-expressing T cells below the detection limit.

The low frequency of IL-2- and IL-4-producing CD4+ T-cells could additionally be explained by the long period needed for stimulation with CD2/CD3/CD28. Owing to fast but temporary production kinetics [17, 18], along with a rapid secretion process, this protocol may lead to lower signals, since Brefeldin A can only be added for the last few hours. Evaluation of regulatory T-cell subsets by CD25 and/or FoxP3 was also hampered by the long period needed for the CD2/CD3/CD28 stimulation, since distinction from human T cells expressing these markers in course of their activation was no longer possible [19].

On examination of the extended cytokine profile of Th1, Th2 and Th0 cells, no substantial difference in their IL-2 or TNF-α expression was detected, no matter which kind of stimulation was applied in this study. Noticeably, IL-13 shows a strong association with IL-4 expression, with approximately 50% co-expression with the other cytokine (see *table 2*). Nevertheless, cells expressing IL-4 and, simultaneously IFN-γ, demonstrate a significantly lower frequency of IL-13 than IL-4+IFN-γ− cells. This supports the concept of IL4+IFN-γ− as the signature cytokine pattern of Th2, leading to significantly different patterns for Th0 (IL-4+IFN-γ+) in respect to the other cytokines. IL-17 shows a substantial co-expression with IL-6 or IFN-γ. In contrast, a strict separation from Th2-type cytokines IL-4 or IL-13 seems to be a hallmark of all cells expressing IL-17. Thus, the total lack of a specific cytokine may be as relevant for the function of a cell as the expression of another.

IL-6 has been proposed to be essential for the differentiation of human Th17. Expression of IL-6 itself by Th17 has been discussed controversially [6, 20, 21]. About 10% of Th17 stained positive for IL-6 in our study. In freshly isolated CD4+ T lymphocytes stimulated for 4 hours, IL-6 only showed a weak signal. However, the presence of IL-6+ cells and co-expression with IL-17 was confirmed after three weeks of culture following initial

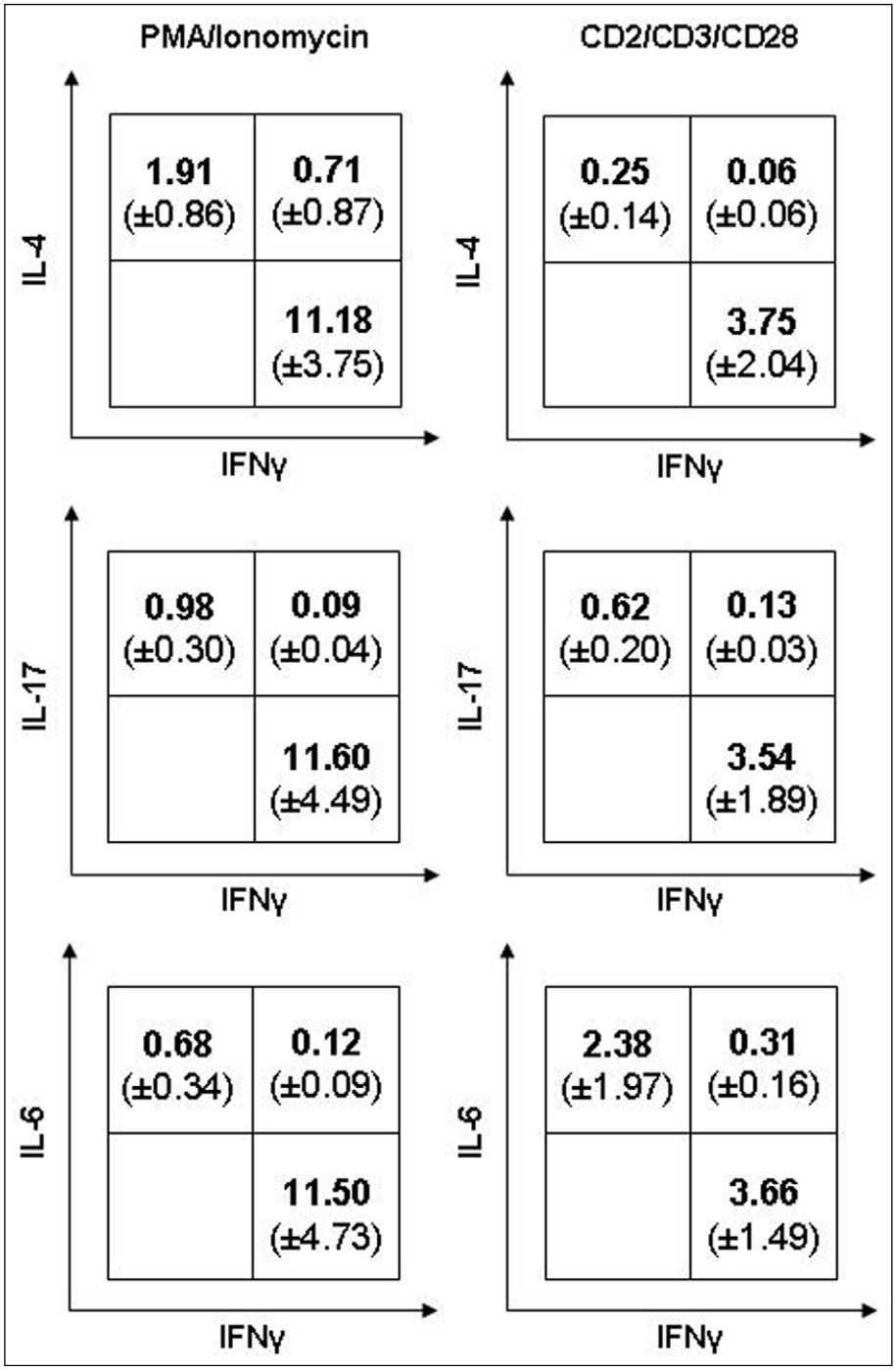


Figure 8

Cytokine production patterns in CD4+ T-lymphocytes after stimulation with either PMA/ionomycin or CD2/CD3/CD28: values within quadrants are means of percentages within CD4+ T cells ± standard deviations of six (PMA/ionomycin) or three (CD2/CD3/CD28) experiments, respectively.

activation with PHA. In this case, not only frequency, but also signal strength increased.

Production of IL-6 by peripheral blood T lymphocytes had already been described at the single cell level two decades ago [22]. A tremendous expansion of IL-6-expressing T lymphocytes has been shown in cultures incubated with 1α,25-dihydroxyvitamin D3 [8-10]. Although IL-6 was originally assigned to the Th2 subset [14], the cells generated in culture match neither Th1 nor Th2 profiles, and in the last few years several studies have suggested the induction of T cells with regulatory properties [11-13]. We therefore focused on the distinction between Th6 and Th2 and regulatory T cell subsets. The lack of any substantial overlap of IL-6 with the transcription factors GATA-3 or FoxP3 and the completely distinct expression of IL-6 and IL-10, a cytokine attributed to a subset of regulatory T cells, contradicts a designation to the Th2 or the regulatory subsets.

In freshly isolated, peripheral blood CD4+ T lymphocytes, a substantial proportion of IL-6+ cells co-express IFN-γ (approximately 10%). Then again, only minimal co-expression can be detected between IL-6 and IL-4,

as shown in *table 2*. Therefore, IL-6-producing CD4+ T lymphocytes negative for IFN-γ are, at the same time, almost negative for IL-4. Cells with this unique cytokine production profile were considered to be a functional subset we hereby termed "Th6". This subset can not only be detected in freshly isolated PBMC stimulated with PMA and ionomycin, but also after overnight stimulation with CD2/CD3/CD28. Remarkably, the frequency of Th6 was at least as high as under PMA/ionomycin conditions, despite the reduced frequencies of all other T cell subsets *(figure 8)*. In line with results from long-term culture, it could be assumed that development of Th6 differs in respect to kinetics and stimulatory pathways from Th1 and Th2 differentiation.

The considerable overlap of IL-6-producing T-cells with the expression of IL-17 raises the question of the relation between Th6 and Th17. However, the extent of co-expression is similar between IL-17 and IL-6 or IL-17 and IFN-γ, respectively. In both cases, this is by no means as strong an association observed between IL-4 and IL-13 (see above).

We attempted to integrate the existing paradigms of particular Th subsets to a general concept of Th cytokine expression: all of the functional subsets of CD4+ T lymphocytes described and their relation to each other are illustrated in *figure 9*. The co-expression patterns described in our study have resurrect the question of Th-subset definition; *e.g.* Th1 may be defined as IL-4 negative-/IFN-γ positive, or, more precisely, as IFN-γ positive cells negative for all other signature cytokines (IL-4, IL-6, IL-17). However, this may be simply semantics, since in all cases the actual cytokine production of a single cell defines its function, independent of any subset assignment by scientists.

CD4+ T lymphocytes co-expressing IL-4 and IFN-γ are established as Th0. Another subset co-expressing IL-17 and IFN-γ has recently been termed Th17/Th1 [25]. Our data demonstrate that there is, in fact, no overlap between these two subpopulations. Similarly, cells defined by the co-expression of IL-6 and IFN-γ (Th6/Th1) are distinct from Th0. Also, Th17/Th1 and Th17/Th6 appear as distinct subsets. Moreover, Th6/Th17 and Th6/Th1 depicted in *figure 9* are distinct cytokine production patterns with minimal overlap.

Signature cytokines used for the definition of functional subsets seem to be inducible in a mutually dependent manner. Co-expression of two of them may be observed, but nearly excludes the production of a third (or fourth) signature cytokine. In contrast, cells expressing *e.g.* IFN-γ, TNF-α and IL-4, or IFN-γ, TNF-α and IL-2 simultaneously, can be detected rather frequently.

IL-2 and TNF-α are not considered signature cytokines. IL-2 was originally assigned to Th1, but no clear evidence has ever been provided for the human system. In this study, we have demonstrate that a substantial proportion of cells positive for any of the other cytokines are completely negative for IL-2. This proportion is significantly higher in the Th17 and the Th6 subsets. The lack of co-expression of IL-2 may correlate with the differentiation of cells into specialized effector cells. TNF-α has been associated with Th1, but is also described as being expressed in Th2 cells [4]. We show that TNF-α is not restricted to the Th1 phenotype. A significantly lower proportion of TNF-α positive cells can be detected in the Th6 type. Nevertheless, it should be borne in mind that expression or lack of expression of non-signature cytokines, such as IL-2 or TNF-α, may be of crucial importance for the function, independent of the role in any Th-classification.

In this study, we present reference cytokine profiles of healthy individuals, applicable to further studies in various disease states. Accurate and stringent analysis

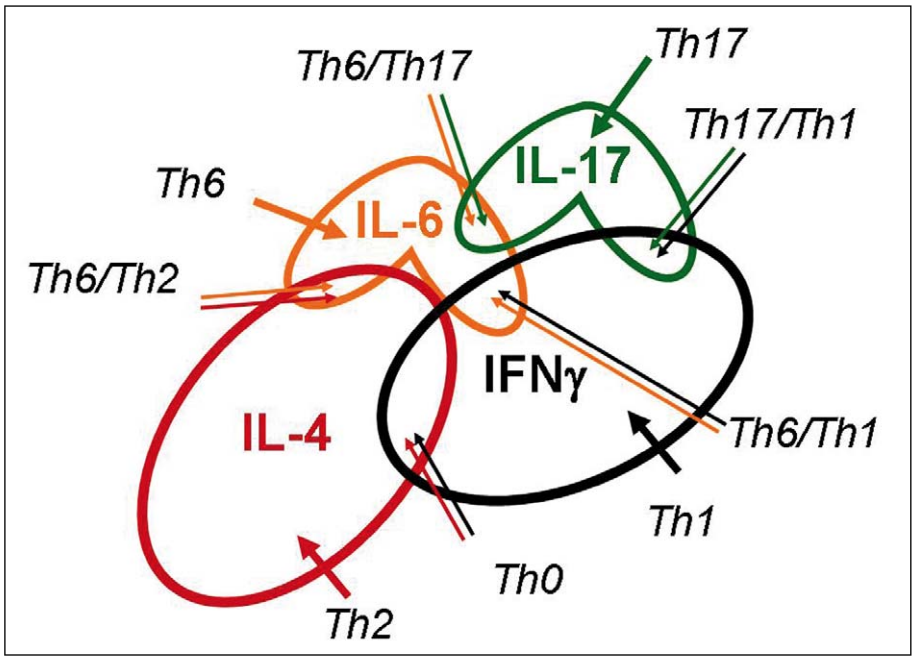


Figure 9
Proposed functional subsets of CD4+ T lymphocytes emerging from the analyses of co-expression patterns for signature cytokines are visualized schematically.

of cytokine co-expression has revealed distinct subsets with limited overlaps of signature cytokines. In this context, T cell-derived IL-6 fulfills the criteria for a signature cytokine of a new CD4+ T cell subset, which we termed Th6.

IL-6 is produced in large quantities by a variety of cells including monocytes and fibroblasts [23], and a specific function of a Th6 subset remains to be elucidated. IL-6 is a cytokine with a wide range of immunological activities: besides its role as a major, systemic, pro-inflammatory cytokine, it is considered to be a factor involved in the differentiation of various immune cells, as well as a B cell-stimulatory factor inducing antibody production. IL-6 has been discussed as being necessary for Th17 differentiation, and the reciprocal relationship between Th17 and Tregs can also be influenced by IL-6: while TGF β induces T cell differentiation to i(inducible) Tregs, it promotes differentiation to Th17 in the presence of IL-6 [24].

Preliminary data show no significant differences in Th6 frequencies in the peripheral blood of patients with allergy or autoimmune diseases, compared to healthy individuals. However, Th cell subsets exert their impact mainly via local cytokine production, creating a pivotal microenvironment or, in an extreme form, an immune synapsis [15]. Therefore, independent of significant changes in the peripheral blood, the local recruitment of Th6 cells to sites of inflammation may be critical in autoimmune diseases and chronic inflammatory conditions.

Acknowledgments. *All flow cytometric analyses were performed at the Core Cell Sorting Unit of the Medical University of Vienna. We are grateful to Ursula Luschin as a native speaker for proofreading and editing the manuscript for style and English language.*

Disclosure*. None of the authors has any conflict of interest to disclose.*

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