T cell cytokine profile during primary Epstein-Barr virus infection (infectious mononucleosis)

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ABSTRACT. Cytokine profiles of $CD4^+$ and $CD8^+$ T-cell subsets were evaluated in 8 patients with infectious mononucleosis (IM). Intracellular detection of cytokines using flow cytometry revealed an expansion of IFN-γ-expressing $CD4^+$ T cells, and particularly $CD8^+$ T cells, while IL-2 expressing cells were less frequently encountered when compared to healthy controls. Single TNF-α-expressing $CD4^+$ and $CD8^+$ T cells were likewise reduced and shifted towards IFN-γ/TNF-α co-production. The predominant pro-inflammatory type 1-biased immune response during IM was emphasized by low frequencies of IL-10 expression in both T cell subsets, although some patients displayed elevated serum levels. Six months later, a decreased, but still elevated IFN-γ expression within the $CD8^+$ T cell subset, and an increased percentage of IL-2-expressing $CD4^+$ and $CD8^+$ T cells, reaching values shown for controls, were noted. Type 2-associated cytokines such as IL-4 and IL-13, as well as IL-6 and TNF-α were not significantly different when compared to controls at study entry and at follow-up. The striking expansion of IFN-γ-producing $CD8^+$ T cells with rather low expression of IL-10, appears to be a key factor for clinically overt disease, but is nevertheless compatible with successful control of the viral infection.

Keywords: EBV, infectious mononucleosis, cytokines, T cells, flow cytometry

INTRODUCTION

Epstein-Barr virus (EBV), a B lymphotropic Herpes virus, is the causative agent of infectious mononucleosis and, in addition, is closely linked to a variety of human cancers, such as B-cell lymphoma, Hodgkin's disease, nasopharyngeal carcinomas, and lymphoblastic lymphomas seen in immune-compromised hosts [1-4].

Primary infection with EBV usually occurs during childhood and adolescence. At an early age, the infection is mostly asymptomatic and more than 90% of adults demonstrate previous exposure to EBV by seropositivity. During adolescence and early adulthood, EBV infection can present as infectious mononucleosis with fever, lymphadenopathy, sore throat, and fatigue, usually resolving within a few weeks [5].

Acute infectious mononucleosis is characterized by an increased virus load and by a vigorous immune response of proliferating αβ $CD8^+$ T cells [6, 7]. This response is believed to play an important role in controlling the virus during acute disease and in suppressing EBV replication in latently infected individuals. There are indications that synthesis of a variety of T cell-derived cytokines such as IFN-γ, IL-2, IL-6, and IL-10 modulate the inflammatory response to EBV, and that a lack of IFN-γ production may cause a severe and more complicated course of disease [8-10]. Most clinical symptoms of IM have been explained by the extent of this host response, although it remains largely unresolved why primary infection results either in asymptomatic seroconversion or acute IM. Differences in viral load, host genetics, and patterns of viral replication have been debated in this context [7, 11, 12]. Understanding the T cell response in several forms of EBV-infection, and that involved in acute IM progression towards clinical recovery, appears to be important to understand the development of EBV-associated malignancies, for which impaired T cell immunity has been implicated [13]. In addition, the design of cytotoxic lymphocytes potentially employed in the treatment of EBV-associated diseases is facilitated by the knowledge of phenotypes and T cell functions involved in successful host responses during a primary infection [14]. Here we characterize the cytokine profile of both $CD4^+$ and $CD8^+$ T cells during acute IM using flow cytometry for the intracellular detection of cytokines.

MATERIALS AND METHODS

Patients

Eight patients (5 male, 3 female) with acute mononucleosis due to Epstein-Barr virus infection were included in

the study. The median age was 25.5 years (range, 18 to 33 years). Diagnosis was made upon clinical presentation and serology (positive IgM-antibodies to viral capsid antigens [IgM-VCA] in combination with negative antibodies to Epstein-Barr nuclear antigen [Ig-EBNA]). Lymphocytosis and elevated aspartate aminotransferase, alanine aminotransferase, and lactic dehydrogenase levels were present in all patients. Courses of the disease were uncomplicated and all 8 patients recovered fully. Five patients were re-assessed for intracellular cytokine expression 6 months after diagnosis when all clinical symptoms were resolved and laboratory values returned to normal. All participants provided informed consent. The study was approved by the Ethics Committee of the University of Vienna.

Intracellular cytokine detection in PBMC by flow cytometry

Flow cytometric assessment of T cell cytokine production was performed, essentially according to the technique described recently [15]. PBMC were isolated from heparinized blood by Ficoll-diatrizoate centrifugation. The cells were then cultured in Ultra Culture medium (Bio-Whittaker, Walkersville, MD, USA) supplemented with L-glutamine (2 mM/L; Sigma, St. Louis, MO, USA), gentamicine (170 mg/L; Sigma), and 2-mercaptoethanol (3.5 µL/L; Merck, Darmstadt, Germany), and were stimulated with phorbol 12-myristate 13-acetate (10 ng/mL; Sigma), in the presence of ionomycin (1.25 µM; Sigma) and brefeldin A (1 µM; Sigma) for 4 hours at 37 °C in 5% CO_2. Cells were then harvested on ice, washed twice in phosphate-buffered saline (PBS) and fixed with 2% formaldehyde (1 mL per 2×10^6 cells; Merck) for 20 min. After two additional washes in PBS, the cells were resuspended in Hank's balanced salt solution supplemented with 0.3% bovine serum albumin and 0.1% sodium azide and stored at 4 °C in the dark until staining.

The fixed cells were washed twice with PBS and made permeable with saponin (0.1%; Sigma), resuspended with 50 µL of saponin buffer-diluted antibodies, and incubated for 25 min. at room temperature in the dark. The following monoclonal antibodies (mAbs) were used: cytokine-specific mouse anti-human mAb (IFNγ [clone B27], fluorescein isothiocyanate [FITC]-conjugated); rat anti-human mAb (IL-2 [MQ1-17H12], IL-4 [MP4-25D2], IL-6 [MQ2-13A5], IL-10 [JES3-9D7], IL-13 [JES10-5A2], TNF-α [Mab11], all phycoerythrin [PE]-conjugated); the anti-CD4 mAb, was allophycocyanin-conjugated, the anti-CD8 mAb was peridinin chlorophyll-conjugated; all cytokine-specific mAbs were purchased from Pharmingen (San Diego, CA, USA), the surface marker-specific mAbs from Becton Dickinson (Mountain View, CA, USA). Four-color staining was performed, and at least 10^4 cells were analysed by flow cytometry on a FACSCalibur (Becton Dickinson) equipped with a two-laser system (488 nm and 633 nm wavelength, respectively). All cytokine combinations were stained in conjunction with CD4 and CD8. Data were analysed with CELLQuest software (Becton Dickinson). Samples were gated on lymphocytes according to their light scatter characteristics and the results were expressed as the percentage of cytokine-producing cells in the $CD4^+$ or $CD8^+$ cell population, respectively.

Detection of circulating cytokines by ELISA

Serum was separated by centrifugation, aliquoted, and frozen at – 20 °C until assayed. Circulating IL-6, IL-10, IL-18, TNF-α (R&D Systems, Minneapolis, MN, USA) and IFN-γ (Bender Medsystems, Vienna, Austria) were quantitated using specific, commercially available ELISAs. All tests were performed following the manufacturer's instructions.

Statistical methods

Statistical analysis was performed using a standard statistical package (SPSS 10.0 for Windows; SPSS Inc., Chicago, Ill, USA). The Mann-Whitney U-test was applied for group differences, the Wilcoxon signed rank test was used to compare dependent variables within the IM group. A p value of ≤ 0.05 was considered significant.

RESULTS

Percentages of $CD4^+$ and $CD8^+$ T cells peripheral blood mononuclear cells within the lymphocyte scatter gate

At admission, an inverse CD4/CD8 ratio typical for EBV-infection was noted (mean percentage of $CD4^+$ 22.5%; mean percentage of $CD8^+$ 49%). In the patients studied again after 6 months, this ratio was significantly reversed (mean percentage of $CD4^+$ 45.7%; mean percentage of $CD8^+$ 27%, $p < 0.05$, respectively).

Differences in the frequency of cytokine expressing $CD4^+$ T cells between mononucleosis patients and healthy individuals

The frequency of exclusively IFN-γ-expressing $CD4^+$ T cells was significantly increased in patients when compared to the healthy individuals (Figures 1, 2). In addition, significant differences for $CD4^+$ T cells co-expressing IFN-γ with other cytokines were shown for the IFN-γ/IL-4 (Figures 1, 2), IFN-γ/IL-10, and IFN-γ/IL-13 staining combinations ($p < 0.05$). The percentage of overall IFN-γ-expressing $CD4^+$ T cells was much higher in mononucleosis patients than in controls (Table 1, Figure 2). In contrast, a significant decrease of IL-2-expressing $CD4^+$ T cells was detected in mononucleosis patients (Table 1), which was attributed to the decrease of IL-2 positive cells without co-expression of IFN-γ (Figure 2). Single TNF-α-expressing $CD4^+$ T cells were also more frequent in controls than in patients (Figures 1, 2), yet the frequency of overall TNF-α-expressing $CD4^+$ T cells was not significantly different between groups (Table 1, Figure 2). Due to the pronounced increase in IFN-γ-and IL-4-co-expressing $CD4^+$ T cells in patients, an overall increased percentage of IL-4-producing cells was noted, whereas differences for IL-6, IL-10, and IL-13 were not significant (Table 1).

Differences in the frequency of cytokine-expressing $CD8^+$ T cells between mononucleosis patients and healthy individuals

$CD8^+$ T cell cytokine expression was heavily skewed towards IFN-γ production, again associated with a decrease of single IL-2-(IFN-γ negative) and overall IL-2-

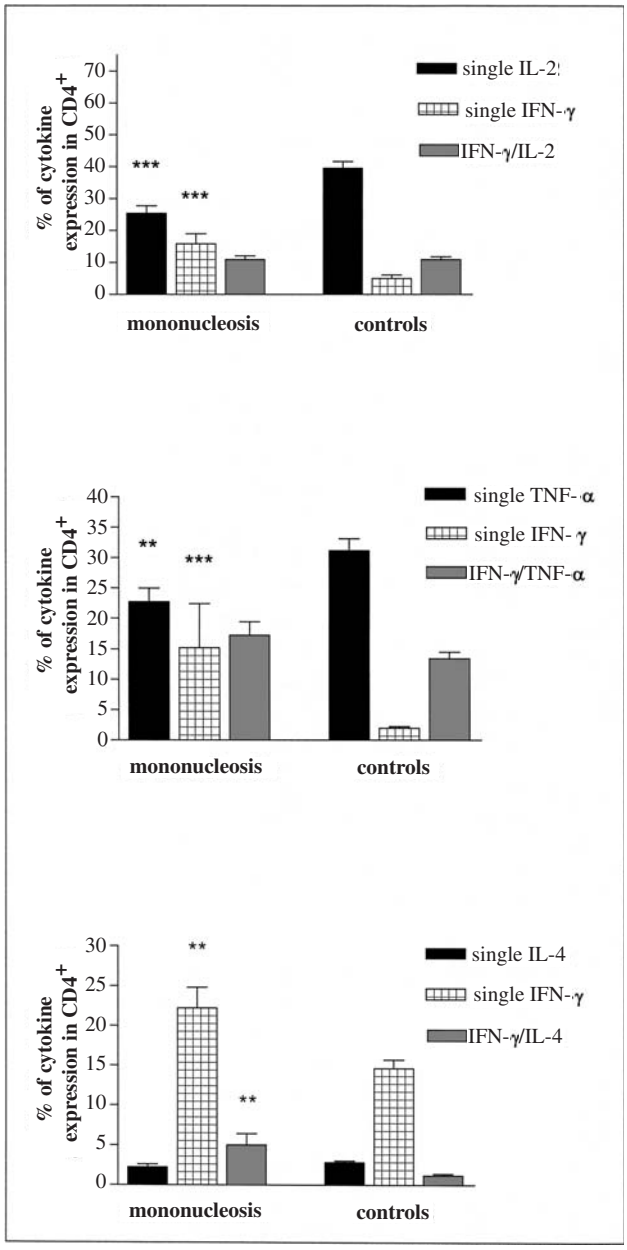

Figure 1
Frequency of cytokine expressing CD4+ cells in mononucleosis patients compared to healthy controls.
Each bar indicates the mean percentage and standard error of mean. Values that are statistically different between groups are denoted by either two asterisks ($p < 0.01$) or by three asterisks ($p < 0.001$). The varying pattern of exclusive expression (e.g. single IFN-γ) and co-expression of cytokines (IFN/IL-2, IFN/TNF-α, IFN/IL-4) explain different bar appearances of individual cytokines. Values of the overall expression of cytokines are shown in Table 1.

expressing cells (Table 1, Figures 2, 3). As seen for the $CD4^+$ T cell subset, single TNF-α-expressing $CD8^+$ T cells were less frequently encountered, yet cells co-expressing TNF-α-with IFN-γ were increased in mononucleosis patients (Figures 2, 3), resulting in an increased percentage of overall TNF-α producing $CD8^+$ T cells (Table 1, Figure 2). Frequencies of the type 2 cytokines IL-4- and IL-13-expressing $CD8^+$ T cells were generally low in patients and controls and displayed no significant differences (Table 1, Figure 2). The same was true for IL-6 and IL-10 expression within the $CD8^+$ T cell subset (Table 1, Figure 2).

Frequency of cytokine expressing CD4+ and CD8+ T cells at admission and during follow-up

The cytokine profile of the $CD4^+$ and $CD8^+$ T cell subsets were studied in 5 patients at admission and 6 months later. At this time point, all clinical symptoms were resolved and laboratory values had returned to normal. There was a clear decrease in IFN-γ production both in $CD4^+$ and $CD8^+$ T cells (Figure 4), which, however, was only significant for the T cells exclusively expressing this cytokine. Interestingly, IFN-γ-expressing $CD8^+$ T cells were still much more frequent at the time of follow-up in IM patients (mean 49%, range 41.4 to 54.7) than in controls (Table 1), whereas after 6 months, IFN-γ-expressing $CD4^+$ cells were similar to those of controls. Again, an inverse relationship of IFN-γ to IL-2 production was shown, as both single IL-2 as well as the overall IL-2 expression within the $CD4^+$ and $CD8^+$ T cells increased during follow-up leading to values comparable to controls (Figure 4).

IL-4, IL-6, IL-10, IL-13, as well as TNF-α expression were not significantly different when compared at study entry and at follow-up.

Serum levels of cytokines

IL-6 levels were detectable in 4 out of 8 mononucleosis patients (median 0.75, range 0-124 pg/ml), IFN-γ and TNF-α levels were detectable in 7 patients (IFN-γ: median 1.15, range 0-19.3 pg/ml; TNF-α: median 28, range 0-56.6 pg/ml). Levels of anti-inflammatory IL-10 were detectable in 4 out of 8 mononucleosis patients during acute disease (median 0.55, range 0-49.5 pg/ml). Differences found when compared to controls were too small to be of statistical significance for all the cytokines assayed. IL-18 levels were detectable in all study participants and were not significantly different between groups (EBV: median 222, range 15-373 pg/ml; controls: median 208.6, range 87-312 pg/ml). In those patients with elevated serum cytokine levels at admission, values had returned to normal during convalescence.

DISCUSSION

In the present study, cytokine profiles of T-cell subsets were evaluated in patients with primary EBV infection. As expected, an inverse CD4/CD8 ratio with the expansion of $CD8^+$ cells was noted. Both T cell subsets were highly committed to IFN-γ production, with a concomitant marked decrease in IL-2 expression, indicating a switch to an effector cell-biased response in acute infectious mononucleosis. Even a shift of exclusively TNF-α-expressing $CD4^+$ and $CD8^+$ cells to IFN-γ/TNF-α co-producing cells could be noted, emphasizing the role of IFN-γ as a major cytokine in IM. These data exemplify the usefulness of the evaluation of cytokine profiles, which may not be obvious when only total cytokine levels are measured. However, this impressive type-1 response parallels the acute phase of the disease and is probably involved in the generation of IM-associated symptoms, as the clinical presentation of IM is related to the cytokine production by EBV-reactive cells. We suggest that in order to overcome disease, an efficient immune response is at least partly dependent on IFN-γ,

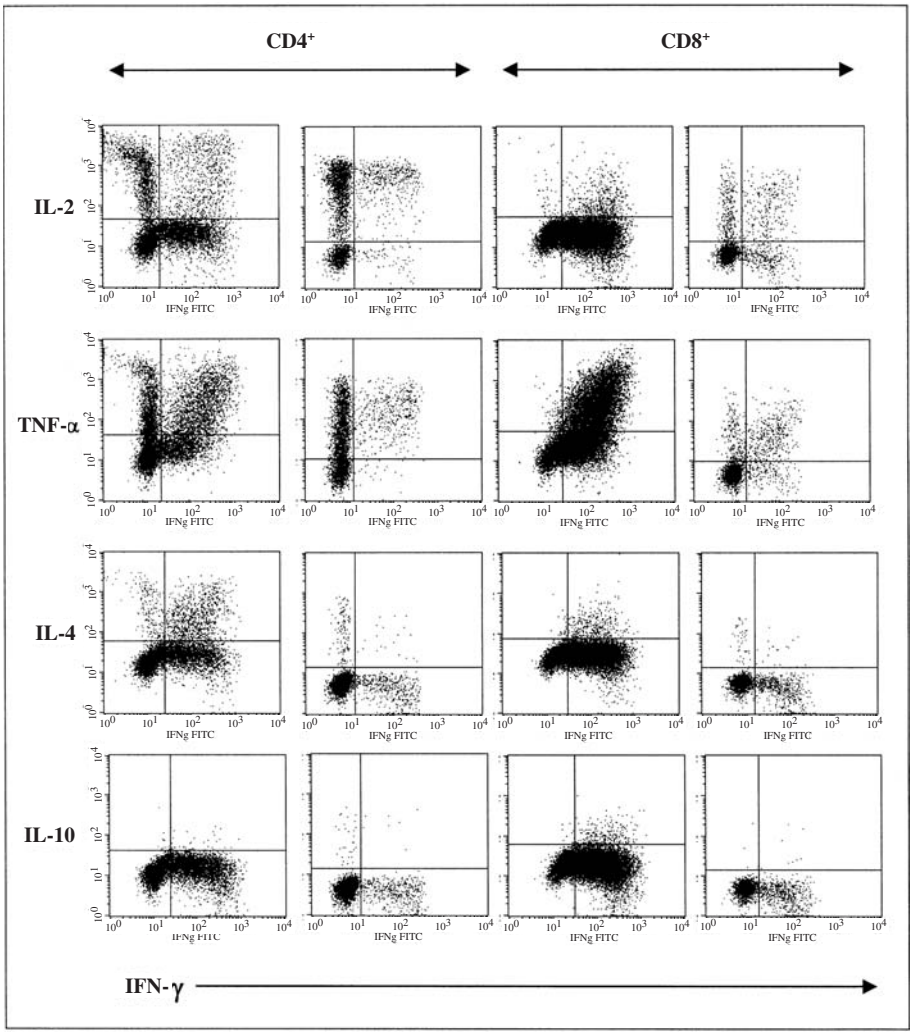

Figure 2
Representative two-parameter dot plots of cytokine expressing CD4+ and CD8+ T cells.
The first (CD4+ subset) and the third (CD8+ subset) vertical row depict the results of cytometric analyses performed for one representative mononucleosis patient. The second (CD4+ subset) and the fourth (CD8+ subset) vertical row indicate the results of one representative healthy control. IFN-γ (x axis) was stained in combination with IL-2, TNF-α, IL-4, and IL-10 (y axis). Corresponding data and statistics are displayed in the text and Table 1 as well as in Figures 1 and 3.

which is not only generated by CD8+ but also by CD4+ cells, as indicated by our results. Recently, CD4+ T cells have been shown to be essential for the inhibition of EBV-induced early B lymphocyte proliferation [16]. The vast majority of IFN-γ-producing cells in our patients lie within the CD8+ cell population, and it has been shown previously that most of these cells are virus-specific and express activation markers such as HLA-DR and CD45RO [6, 7, 17, 18]. The role of IFN-γ as the key antiviral molecule in IM was further substantiated by a case report of a patient with primary EBV-infection complicated by granulomatous pneumonitis, encephalitis and genital ulceration [10]. A selective deficiency of IFN-γ synthesis by lymphocytes was evident. The patient

Table 1
Frequency of overall cytokine expression within the CD4+ and CD8+ subsets in mononucleosis patients and controls

Cytokines	CD4+		CD8+	
	Mononucleosis (n = 8)	*Controls* (n = 26)	*Mononucleosis* (n = 8)	*Controls* (n = 26)
IL-2+	36.3 ± 2.9 (21.5-46.3)**	50.5 ± 2.7 (13.4-73.4)	4.8 ± 0.9 (0.9-9.1)***	17.9 ± 1.9 (4.1-50.8)
IFN-γ+	26.7 ± 3.8 (6.1-43.7)**	15.9 ± 1.5 (4.6-41.1)	70.5 ± 4.4 (49.9-89.1)***	28.1 ± 3.4 (5.2-86.8)
TNF-α+	39.9 ± 3.3 (28.5-50.5)	44.5 ± 2.8 (21.3-71.6)	34.8 ± 3.2 (25-46.6)*	21.5 ± 2.4 (2.9-45.6)
IL-4+	7.3 ± 1.6 (1-17.4)*	4.0 ± 0.4 (0.6-10)	2 ± 0.6 (0.3-6)	2.8 ± 0.4 (0.5-6.8)
IL-6+	0.5 ± 0.2 (0.1-1.9)	0.7 ± 0.1 (0.1-1.6)	0.5 ± 0.1 (0.1-1.3)	0.5 ± 0.1 (0-1.3)
IL-10+	1.8 ± 0.4 (0.3-4)	1.0 ± 0.1 (0.2-2.3)	0.8 ± 0.2 (0.2-1.7)	0.5 ± 0.1 (0.1-1.2)
IL-13+	3 ± 0.6 (0.9-5.9)	2.6 ± 0.3 (0.4-7.8)	0.8 ± 0.2 (0.1-2)	1.6 ± 0.3 (0.1-6)

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when mononucleosis patients were compared to healthy controls, Mann-Whitney U-test

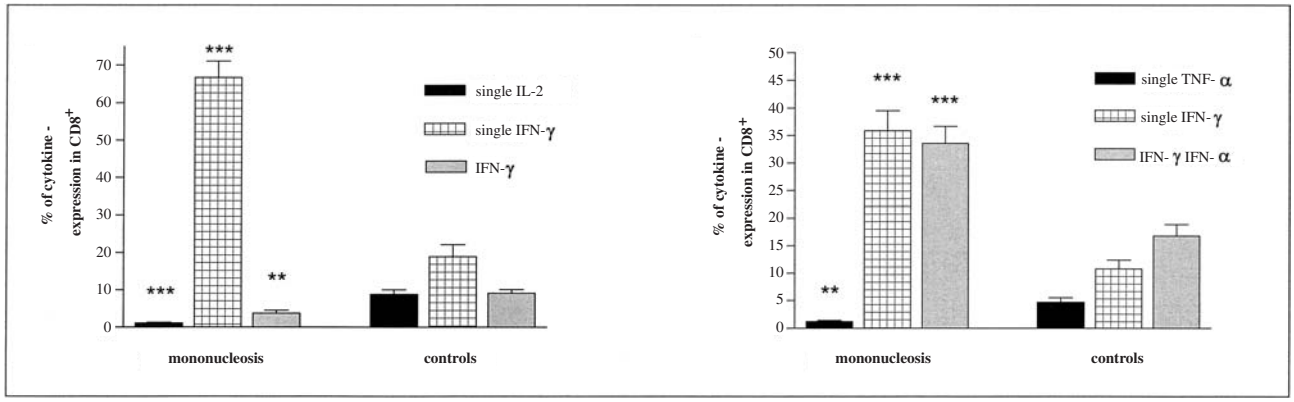

Figure 3

Frequency of cytokine expressing CD8⁺ cells in mononucleosis patients compared to healthy controls.

Each bar indicates the mean percentage and standard error of mean. Values that are statistically different between groups are denoted by either two asterisks ($p < 0.01$) or by three asterisks ($p < 0.001$). The varying pattern of exclusive expression (e.g. single IFN-γ) and co-expression of cytokines (IFN/IL-2, IFN/TNF-α) explain different bar appearances of individual cytokines. Values of the overall expression of cytokines are depicted in Table 1.

lacked lymphocytes cytotoxic to autologous EBV-transformed B-lymphocytes and no spontaneous or *in vitro* EBV-induced IFN-γ production was evident. Full clinical recovery was achieved only with administration of IFN-γ, which was paralleled by the development of EBV-specific cytotoxic T-lymphocytes.

During the follow-up of our patients, a gradual decrease in IFN-γ expression, with an opposite shift of IL-2 expression, was noted. The lack of IL-2 during acute IM renders activated $CD8^+$ cells susceptible to activation-induced cell death [18, 19], which might contribute to both the decrease of IFN-γ-expressing T cells and to the resolution of clinical symptoms and abnormal laboratory parameters in our patients. Interestingly, while IFN-γ expression in $CD4^+$ cells returned to values seen in the healthy control group, it was still about two-fold higher within the $CD8^+$ subset at follow-up. It is still unclear why primary EBV infection manifests differently between patients. Recently it has been shown that both asymptomatic infected and IM patients displayed high levels of EBV DNA in blood, but only the IM group showed T cell perturbations [11]. The authors have speculated that clinically overt disease might be associated with a more lytic type of infection rather than latent B cell invasion, and/or might be associated with different host genetic factors. Various polymorphisms of cytokine genes have been linked to susceptibility to EBV infection [12, 20, 21]. In the case of IL-10, the presence of specific haplotypes conferred protection to primary infection and was associated with higher IL-10 levels in plasma, whereas low IL-10-producing capacity made individuals more susceptible to a severe EBV infection [12, 21]. Although elevated serum levels of IL-10 have been detected in some of our patients at admission, we were not able to demonstrate increased expression of this cytokine in $CD4^+$ and $CD8^+$ T cells. Moreover, IL-10 expression was not altered during follow-up. Most IL-10-expressing cells also co-produced IFN-γ, identifying a subset in IM that has recently been shown to exert immune-regulatory roles in other infectious diseases as well [22]. However, the relatively low expression of IL-10 in our uncomplicated IM patients must be seen in the light of the pronounced IFN-γ production, skewing the host response to a pro-inflammatory, type 1-biased one. Relative down-regulation of IL-10 might be necessary to control the initial virus load, as IL-10 abrogates the inhibitory activity of T cells on EBV-induced B-cell transformation [23]. The balance between IL-10 and IFN-γ might play a critical role in the development of EBV-associated malignancies as malignant transforma-

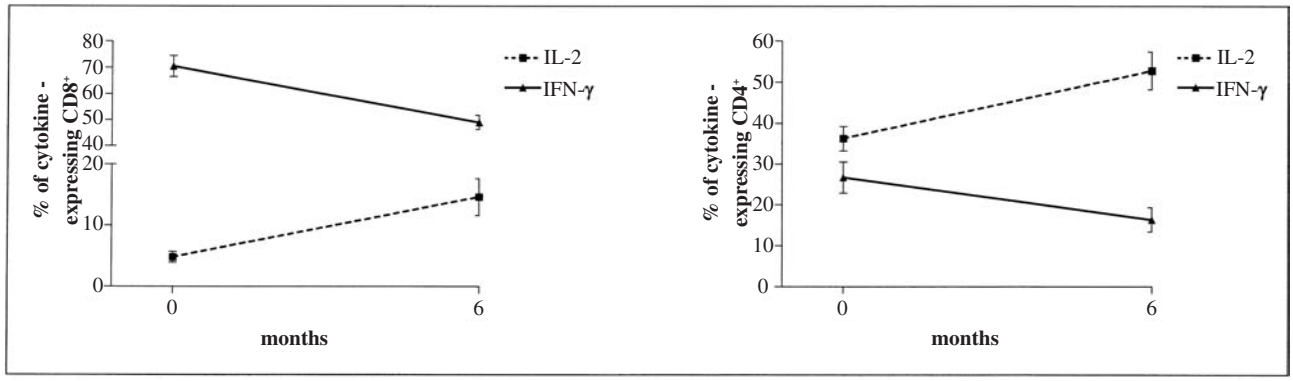

Figure 4

Frequency of overall IL-2 and IFN-γ expressing $CD8^+$ and $CD4^+$ T cells in 5 mononucleosis patients at admission and after follow-up (6 months).

The data points indicate the mean percentages and standard error of mean. Differences were significant for IL-2 within both subsets at a $p < 0.05$ as calculated by the Wilcoxon signed rank test.

tion and lymphomagenesis are linked to high production of IL-10 in immune-suppressed individuals [13, 14, 24].

Although the T cell repertoire perturbations seen in IM might be an overreaction to EBV-infection as suggested recently [11], it is obvious that the host response in uncomplicated disease remains successful in controlling viral infection. The cytokine profile described in this study, with its significant shift to IFN-γ production in $CD4^+$ and $CD8^+$ T cells, exemplifies such a successful host response and its evaluation may help us to understand the patho-mechanisms of EBV infections with their different clinical manifestations.

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