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Peripheral T lymphocyte cytokine profile (IFNγ, IL-2, IL-4) and CD30 expression/release during measles infection

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ABSTRACT

Background and Objective. **Measles virus infection (MVI) has been reported to be characterized by an imbalanced $Th_{1/2}$-type cytokine profile. CD30 has been proposed as a receptor preferentially associated with the $Th_{0/2}$-type cytokine pattern. The aim of this study was therefore to define the peripheral T lymphocyte cytokine profile and to test which CD30 expression pattern it was associated with in MVI.**

Design and Methods. **The design of the study was a prospective evaluation with comparative analysis. The serum levels of the soluble form of CD30 (sCD30) were determined at diagnosis and at weekly intervals up to 4 weeks, using an ELISA, in 23 males (median age 19), who developed MVI while serving in the Italian army and who were admitted to the Infectious Disease Unit of the Military Hospital in Padua. In 10 of the patients at diagnosis we studied the lymphoid immunophenotype and, after non-specific *ex vivo* stimulation, the expression of IFNγ, IL-2 and IL-4 by peripheral T cells using flow cytometry single cell analysis. In 3 patients such evaluations were also performed 7 weeks later.**

Results. **At diagnosis, we found (i) reduction of $IFN\gamma^+/CD4^+$ T cells (p=0.048 vs controls) in the absence of substantial variation of $IL\text{-}2^+$ and $IL\text{-}4^+$ T cells (p=ns vs controls); (ii) expansion of $CD30^+/CD4^+$ and $CD30^+/CD8^+$ T cell subsets (p<0.01 vs controls); (iii) high sCD30 values (median 61 U/mL; p<0.001 vs controls); (iiii) a context of lymphopenia (0.728±0.292 lymph $\times 10^9$/L). sCD30 remained elevated up to 4 weeks from MVI onset [median values 53, 49, 50, 34 U/mL after 1, 2, 3 and 4 weeks, respectively (p=ns between different time points)]. In 3 patients tested 7 weeks after diagnosis, we still observed decreased IFNγ production by $CD4^+$ and $CD8^+$ T cells (p=0.05 and <0.01, respectively vs controls) and reduction of $CD4^+$ and $CD8^+/IL\text{-}2^+$ T cells (p<0.01).**

Interpretation and Conclusions. **MVI was characterized by features of inadequate Th/Tc_1 activation associated with increased circulating $CD30^+$ T cells and elevated sCD30 levels, supporting a correlation between Th/Tc status and CD30 expression/release pattern *in vivo*.**

Key words: measles, cytokines, CD3O, immune regulation, Th_1/Th_2 response

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Measles virus infection (MVI) and, to some extent, vaccination against measles are accompanied by a number of immune abnormalities, including suppressed delayed-type hypersensitivity, defective mitogenic response of lymphocytes and impaired production of various cytokines *in vivo* and *ex vivo*.[1] The clinical counterpart of such abnormalities *in vivo* is an acquired immunodeficiency, that can be regarded as a paradigm for other similar conditions[1] and is arguably responsible for the secondary infections and autoimmune disorders that some individuals experience during or after MVI.[1,2] Such complications may have serious clinical implications especially in developing countries[3] and in immunocompromised hosts.[4,5]

The immunity of MVI may probably be better understood on the basis of the $Th_{1/2}$ paradigm[6-8] which recognizes two polarized forms of $CD4^+$ or $CD8^+$ T cells, each preferentially associated with the cellular or the humoral arm of the immune system. $CD4^+$ T-helper (Th) and $CD8^+$ T cytotoxic (Tc) cells producing cytokines involved in classic cell-mediated functions, i.e. interferon (IFN)-γ, tumor necrosis factor-β and interleukin (IL)-2, are referred to as Th/Tc_1-type cells.[7] T cells producing cytokines mainly involved in antibody responses, such as IL-4, IL-5, IL-10 and IL-13, are identified as Th/Tc_2-type cells.[7] Th/Tc_0 cells produce both type 1 and 2 cytokines.[7]

The overall pattern of immunologic abnormalities in MVI appears to be consistent with an imbalanced $Th_{1/2}$-type immune polarization leading to a defective Th_1- and to a prevalent Th_2-type immune response.[2,9] This immune pattern may be at least partly related to a down-regulation of IL-12, a major determinant of the switch to Th_1-dominated responses,[10] through virus-mediated inactivation of CD46 membrane protein.[11]

The aim of our study was to characterize the immune response during MVI in a population of young adults by studying the intracellular cytokine profile (IFNγ, IL-2, IL-4) and CD30 expression pattern in circulating T cells as well as levels of soluble CD30 (sCD30) in the blood. CD30 is a TNF receptor family cytokine receptor,[12,13] which is involved in immune maturation[14] and response[15,16] and is up-regulated by at least three signals: TCR triggering,[17,18] IL-4 signaling through IL-4R[19] and CD28 activation.[20] The reason for investigating the CD30 molecule in MVI derives from previous observations suggesting that, following activation, CD30 is preferentially expressed and released by $CD4^+$ and $CD8^+$ Th/Tc_2 clones,[21] probably due to IL-4 activity on such cells.[22] By contrast, Th/Tc_1 clones only poorly and transiently express CD30.[21] This supported a possible *in vivo* association between a $Th/Tc_{0/2}$ shift and an increased number of $CD30^+$ cells and/or elevated serum concentration of sCD30.[16] Such an association, which is strongly suggested in some studies,[23-26] is questioned in others.[27,28] MVI is an additional condition in which to test the possible correlation between Th/Tc status and CD30 expression/release *in vivo*.

Design and Methods

Patients

We studied 23 young males (median age 19) who developed MVI while serving in the Italian army and were admitted to the Infectious Diseases Unit of the Military Hospital in Padua. The diagnosis was based on typical clinical symptoms and signs and supported by specific serologic and hematologic parameters. As a whole, the patients were monitored up to 7 weeks from diagnosis and/or until clinical normalization. Twenty-four healthy age-matched blood donors were used as normal controls.

Detection of serum levels of the sCD30 molecule

Sera were collected at diagnosis and 1, 2, 3 and 4 weeks after diagnosis in 23, 15, 11, 11 and 7 patients, respectively, and stored frozen at -70°C until use. They were investigated using a commercially available enzyme-linked immunosorbent assay for levels of sCD30 molecule [DAKO CD30 (Ki-1 antigen) ELISA, Dako, Glostrup, Denmark], as previously described.[29] The mean (±SEM) serum level of sCD30 in 24 controls was 7.12±1.2, and the median level 5 U/mL.

Cell preparation and culture

Mononuclear cells from 10 patients at diagnosis, from 3 patients 7 weeks after diagnosis and from 11 healthy age-matched blood donors with no history of allergy and normal lymphocyte populations were aseptically separated from PB by Ficoll-Hypaque density gradient centrifugation and washed twice in 1% PBS solution. The mononuclear cells (2×10^6/mL) were then resuspended in RPMI-1640 (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine and antibiotics (penicillin plus streptomycin), and distributed in 24 well flat-bottomed plates for stimulation by PMA.

Cell stimulation

To be evaluated for cytokine production, mononuclear cells were stimulated with PMA (25 ng/mL) plus ION (1 µg/mL) for 4 hours at 37°C, 5% CO_2. After one hour, BFA (10 µg/mL) was added for the remaining three hours to block cytokine transport in Golgi's apparatus. Cellular suspensions were collected, centrifuged, washed twice in 1% PBS and resuspended at the final concentration of 10^7 cells/mL.

Flow cytometry analysis

The following monoclonal antibodies (mAbs) were added to heparinized whole blood samples (100 µL) according to standard, previously described, procedures:[30] fluorescein isothiocyanate (FITC)-conjugated anti-CD45, –CD3, –CD19, –CD8, –CD2, –CD45RA (Becton Dickinson, S. José, CA, USA) and –CD30 (Dako); phycoerythrin (PE)-conjugated anti-CD14, –CD25, –CD4, –CD8, –CD16, –CD56, –HLA-DR, and -CD122 (Becton Dickinson); tricolor-conjugated anti-CD3 (Caltag, South S. Francisco, CA, USA). Intracellular cytokine (IC) production was evaluated using tricolor-conjugated anti-CD4 and -CD8 (Caltag). After permeabilization (FACS solution, Becton Dickinson, 0.5 mL/sample for 10 minutes, room temperature), washing with 1% PBS/5% BSA/0.5% sodium azide, and incubation for 15 min with unconjugated mouse immunoglobulins (10 µg/10^6 cells), FITC-conjugated anti-IFNγ (Pharmingen, San Diego, CA, USA) and PE-conjugated anti-IL-2 (Pharmingen) and anti-IL-4 (Becton-Dickinson) mAbs were added to cells. After 30-minute incubation at 4°C, cells were washed and resuspended at 10^7 cells/mL and analyzed with an argon-ion laser flow cytometer (FACScan, Becton Dickinson), using Cell Quest software. Adequate controls were performed. Lymphocyte surface markers and IC were evaluated acquiring at least 3×10^4 and 5×10^4 events, respectively. CD30 and IC were evaluated on the entire $CD4^+$ and $CD8^{bright+}$ lymphoid subsets.

Statistical analysis

Statistical comparisons included Kruskall-Wallis ANOVA by ranks, Mann-Whitney U-test or Student's t-test, according to the specific requisites of each set of data. Differences were considered statistically significant when the p value was <0.05.

Results

sCD30 values

We evaluated whether the serum levels of sCD30 were elevated or not during MVI. The results are shown in Table 1. Increased sCD30 levels were found in all cases but two in serum samples collected as soon as MVI was clinically recognizable (mean±SEM: 96±20; median 61 U/mL) and sustained increased

Table 1. Serum sCD30 values in measles virus infection.

	Cases	Median age (yrs)	sCD30 U/mL (mean±SEM)	median
Measles				
at diagnosis	23	19	96±20	61
1 week	15		67±18	53
2 weeks	11		55±18	49
3 weeks	11		52±10	50
4 weeks	7		61±30	34
Controls	24	22	7.12±1.2	5

Statistics: median sCD30 at different serum collection times: p=ns; MVI at diagnosis vs controls: p<0.001.

values were observed up to one month from diagnosis. No significant difference was found when comparing sCD30 values at different time points. However, in 8 out of 11 patients who had a sequential evaluation up to 3 weeks from diagnosis, elevated sCD30 levels persisted throughout the observation period, whereas 3 patients had sCD30 concentrations which decreased progressively towards the range of normal values (Figure 1).

Circulating lymphocyte immunophenotyping

We analyzed the absolute numbers and flow cytometry pattern of lymphoid subsets at the time of diagnosis. Lymphopenia was present in all patients (0.728±0.292 lymph $\times 10^9$/L). Figure 2, panel A, shows that the numbers of both $CD4^+$ and $CD8^+$ lymphocytes were reduced as compared to those in normal controls (0.199± 0.110 and 0.144±0.090 vs 0.779±0.091 and 0.309± 0.079 cells $\times 10^9$/L, respectively, $p<0.05$), with a ratio of 1.38±0.3. The immunophenotype patterns of circulating lymphocytes in MVI at diagnosis are listed in Table 2. B cells and NK cells were not significantly increased. Among the $CD3^+$ lymphocytes, $CD45RA^+$ cells (31.23±9.09%, median 29.85%) were prevalent on $CD45RO^+$ subset (23.01±6.53%, median 23.90%). No significant increase in CD25 (7.07±1 .61%, median 7.00%) and HLA-DR (13.71±8.77%, median 10.05%) expression was detected as compared to controls (7.09±6.45% and 10.46±4.06%, respectively), while CD122 was expressed in a larger proportion of T cells in MVI than in controls (8.95±6.99%, median 6.82% vs 2.0±1.3%).

CD30 expression on T cell subsets

We examined the expression of CD30 on T cell subsets in detail. Figure 2, panel B shows that $CD4^+$/$CD30^+$ cells were similar to controls in terms of absolute numbers (p=0.66), while $CD8^+$/$CD30^+$ cells tended to be increased, though not to a statistically significant extent (p=0.11). In percentage terms, however, $CD30^+$ cells were clearly expanded among both $CD4^+$ (5.88±2.26%, median 4.85% vs 1.6±0.6% in controls) and $CD8^+$ T cells (11.10±5.81%, median

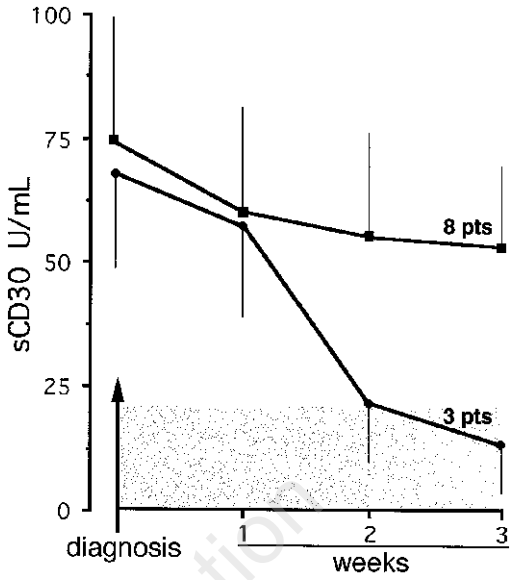

Figure 1. Time course evaluation of sCD30 serum concentration in 11 patients with measles virus infection. Three weeks after diagnosis, 8 patients had consistently high sCD30 values, while 3 patients reverted to normal sCD30 values. Data are expressed as mean values (±SD) at each given time. The shaded area corresponds to the range of sCD30 values in normal controls.

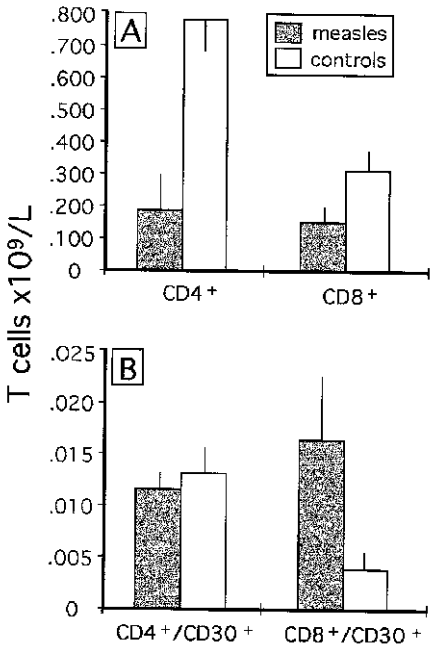

Figure 2. Absolute number (mean±SD) of circulating $CD4^+$, $CD8^+$ and $CD30^+$ T cells in 10 patients with measles virus infection at diagnosis and in 11 controls. Panel A: $CD4^+$ and $CD8^+$ T cells were significantly reduced as compared to controls ($p<0.05$). Panel B: $CD8^+$/$CD30^+$ T cells showed a tendency to increase without any statistical significance.

Table 2. Immunophenotype analysis of circulating lymphocytes in measles virus infection at diagnosis

	Measles virus infection		*Controls*	
	mean±SD n=10	*median n=10*	*mean±SD n=11*	*median n=11*
Lymph x10⁹/L	0.728±0.292	0.705	2.300±0.195	2.100
	%	%	%	%
CD3⁺	52.61±10.68	54.45	72.27±8.78	80.00
CD3⁺/CD4⁺	27.43±9.42	30.15	48.14±8.37	48.90
CD3⁺/CD8⁺	19.81±7.52	21.15	23.52±6.25	27.30
CD3⁺/CD45RA⁺	31.23±9.09	29.85	6.31±6.23	2.50
CD3⁺/CD45R0⁺	23.01±6.53	23.90	28.39±6.59	24.30
CD3⁺/HLA-DR⁺	13.71±8.77	10.05	10.46±4.06	9.60
CD3⁺/CD25⁺	7.07±1.61	7.00	7.09±6.45	7.00
CD3⁺/CD122⁺	8.95±6.99	6.82	2.0±1.3	1.9
CD4⁺/CD30⁺	5.88±2.26	4.85	1.6±0.6	1.2
CD8⁺/CD30⁺	11.10±5.81	10.20	1.3±0.7	1.3
CD3⁺/CD(16⁺56)⁺	4.5±4.34	3.00	4.55±1.99	5.00
CD19⁺	19.47±15.73	12.75	10.32±3.44	7.80
CD19⁺/CD25⁺	2.34±1.65	2.50	<1	-
CD(16⁺56)⁺	19.93±9.08	18.90	12.45±4.93	6.10
CD4⁺/CD8⁺ ratio	1.38±0.3	1.42	2.04±0.4	2

10.2% vs 1.3±0.7%, $p<0.01$) in acute MVI (Table 2) and returned to normal values during the follow-up: circulating CD30⁺ cells were around 1% in all 3 cases evaluated at 7 weeks. A representative case is illustrated in Figure 3.

IFNγ, IL-2, IL-4 expression in CD4⁺ and CD8⁺ T cells

In order to define the cytokine profile of T subsets in MVI, we evaluated the expression of IFNγ, IL-2 and IL-4 by CD4⁺ and CD8⁺ T cells at single-cell level using flow cytometry. As shown in Table 3, 10 patients could be evaluated soon after MVI diagnosis. The percentage of IFNγ⁺/CD4⁺ T cells tended to be lower than in controls (10.79±6.01%, median 8.10% vs 16.90±6.58%, median 17.40, $p=0.048$), whereas the percentage of IL-2- or IL-4-expressing cells among CD8⁺ and CD4⁺ T cells was similar to that in controls (Table 3 and Figure 4). Three patients who were tested 7 weeks after diagnosis showed a significant decrease in the percentage of CD4⁺/IFNγ⁺ (5.60±1.80%, median 5.70% vs 15.9±5.2%, 15% in controls, $p=0.05$), CD8⁺/IFNγ⁺ (5.90±2.40%, median 4.80% vs 47.70±8.80%, 45%, $p<0.01$), CD4⁺/IL-2⁺ (3.20±1.90%, median 2.00% vs 9.2±3.7%, 9.5%, $p<0.01$) and CD8⁺/IL-2⁺ T cells (0.48±0.12%, median 0.43% vs 5.3±3.4%, 5%, $p<0.01$), with normal peripheral lymphocyte counts (Figure 5).

Discussion

The aim of our study was to investigate the possible relation between patterns of T cell cytokine production and CD30 expression as well as sCD30 serum concentrations in young adults with MVI. We found that

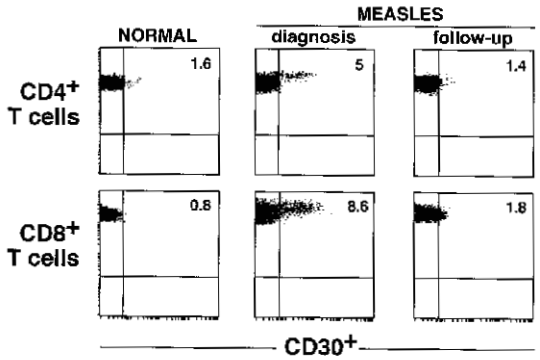

Figure 3. Flow cytometry plots showing the percentage of CD30⁺ cells among circulating CD4⁺ and CD8⁺ lymphocytes in a representative patient with measles virus infection at diagnosis. Seven weeks later, CD30⁺ T cells returned to a percentage similar to that observed in controls.

Table 3. Percentages of IFNγ⁺, IL-2⁺ and IL-4⁺ cells among circulating CD4⁺ and CD8⁺ lymphocytes in measles virus infection at diagnosis.

	Measles virus infection		*Controls*	
	CD4+ n=10	*CD8+ n=10*	*CD4+ n=11*	*CD8+ n=11*
	%	%	%	%
IFNγ⁺				
mean±SD	10.79±6.01	42.40±12.70	16.90±6.58	45.90±10.20
median	8.10	43.80	17.40	44.3
IL-2⁺				
mean±SD	9.66±2.50	3.81±1.80	11.30±4.72	5.69±3.31
median	9.30	3.25	11.00	6.10
IL-4⁺				
mean±SD	1.02±0.67	1.24±0.96	1.82±1.33	2.15±1.21
median	0.82	1.00	1.50	2.61

Statistics: IFNγ+/CD4+ T cells in MVI vs controls, p=0.048; other percentages were not significantly different.

acute MVI at diagnosis was characterized by: (i) low percentages of IFNγ⁺/CD4⁺ T cells; (ii) increased percentages of circulating CD4⁺/ and CD8⁺/CD30⁺ T cells; (iii) increased levels of serum sCD30; (iiii) a context of lymphopenia, low expression of activation markers, and absence of a consistent switch from a naive (CD45RA⁺) to a memory (CD45R0) phenotype. During the follow-up, while circulating CD30⁺ T cells returned to within the normal range, sCD30 values were persistently elevated up to 4 weeks after the acute phase of MVI, except in a minority of patients who reverted to normal sCD30 levels (Figure 1). In addition, IFNγ and IL-2 expression by T cells remained significantly decreased in all 3 patients evaluated 7 weeks after diagnosis.

These findings are consistent with a defective

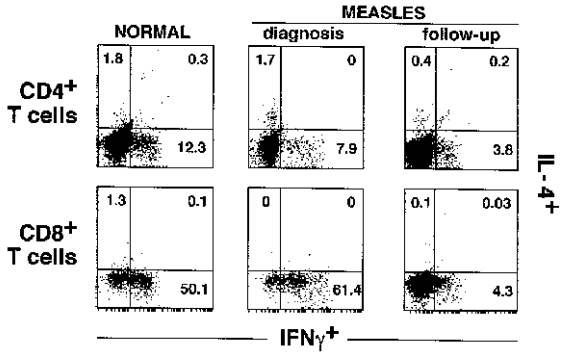

Figure 4. Flow cytometry plots showing the percentage of IFNγ+ and IL-4+ cells among circulating CD4+ and CD8+ lymphocytes in a representative MVI patient at diagnosis. Seven weeks later, a clear-cut decrease in IFNγ+ and IL-4+ T cells was evident as compared to percentages at diagnosis and in normal controls.

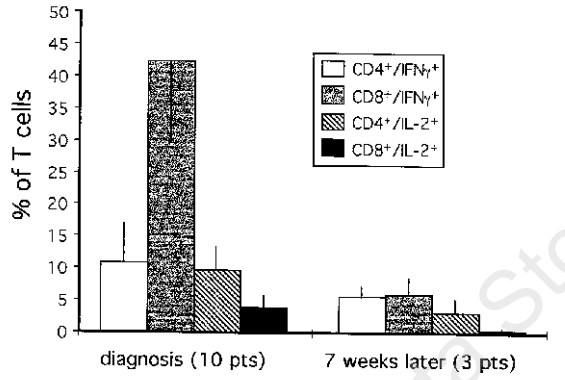

Figure 5. Percentages (mean±SD) of IFNγ+ and IL-2+ cells among circulating CD4+ and CD8+ T cells in measles virus infection at diagnosis (10 patients) and 7 weeks later (3 patients). A statistically significant decrease in Th/Tc1-type cells (IFNγ+, IL-2+) was detected (from $p=0.05$ for CD4+/IFNγ+ cells to $p<0.01$ for each other subset).

Th/Tc$_1$-type response present at the beginning and full-blown in the late phases of MVI[1,2] in association with increased sCD30 levels/circulating CD30+ T cells. Such a scenario is partly at variance with previous reports, suggesting a Th/Tc$_1$-type cytokine pattern characterizing at least the acute phase of MVI.[2,31] This diversity might be explained on technical grounds, such as the different experimental approach and/or different evaluation of normal controls, or on biological grounds, considering that we were evaluating a population consisting entirely of young adults.

In point of fact, the evidence argues against a Th/Tc$_1$-type cytokine profile of circulating lymphocytes during the acute phase of MVI in our patients. We failed, however, to demonstrate any increase in IL-4+ T cells as compared to controls. By contrast, Ward and Griffin reported a predominant production of IL-4 after measles virus vaccination, suggesting induction of a Th$_2$ response.[9] Actually, IL-4, which, as a rule, is rapidly transported and does not accumulate to be easily demonstrated,[24] is detectable in peripheral blood lymphocytes only following repeated T-cell stimulation *in vitro* or under conditions of long-lasting abnormal production *in vivo*. Thus, the lack of evidence of increased IL-4 expression by circulating lymphocytes in our MVI cases can not be regarded as a definite argument against the possibility of a Th/Tc$_2$-shift in MVI. Conversely, the clear-cut expression of both surface and soluble CD30 in MVI might suggest the production of amounts of IL-4, which is a major determinant of CD30+ lymphocyte appearance.[19]

The deficient number of circulating cells producing Th/Tc$_1$-type cytokines might actually reflect a Th/Tc$_{0/2}$-type immune prevalence in lymphoid tissues, where viral replication is controlled mostly by the production of specific antibodies. This is even more likely because MV, through its interaction with CD46 membrane protein on infected cells, is directly responsible for down-regulating IL-12, a stimulatory factor inducing Th$_1$-specific immune responses.[7,8,10] In this context, the lack of the acquisition of a memory phenotype, as described above, suggests inadequate stimulation through IL-2,[32] and, in fact, the percentage of IL-2+ cells in our MVI patients was similar to that observed in controls. On the whole, this would result in a state of anergy, explaining the suppressed delayed-type hypersensitivity, the defective mitogenic response of lymphocytes and the impaired production of various cytokines *in vivo* and *ex vivo*[1,2,9,11] typically observed in MVI.

It may be of interest to remark that we found high percentages of circulating CD30+ T cells during the acute phase of MVI. This is not a usual finding in infectious or reactive diseases, even those characterized by high sCD30 values in the serum.[29,33-36] Though there is agreement on the preferential expression of CD30 by Th/Tc$_2$ clones, as previous evidence has suggested,[21] the possibility that CD30 expression may discriminate between Th/Tc$_1$- and $_{2/0}$-type cells has been questioned.[27,28] As a matter of fact, CD30 knockout mice, which present a defect in negative selection,[14] have normal Th$_2$ differentiation and effector responses, indicating that CD30 is not required for Th$_2$ development.[26]

Nevertheless, as mentioned above, expansion of CD30+ subsets may be regarded as a hallmark of concomitant production of IL-4,[19] even independently of its direct demonstration.[24] Indeed, we have found high numbers of CD30+ T cells in the lymph nodes and skin in Omenn's syndrome, a Th$_2$-dominated condition[23] and similar results have been reported in other diseases characterized by strong, persistent activation of Th/Tc$_2$ cells.[24,25,36,37-39] By contrast, diseases dominated by activated T cells showing a

Th/Tc_1 cytokine profile have been usually reported to present no or rare $CD30^+$ T cells,[24,39-42] though a proportion of patients with diseases in which Th1 responses should be predominant have been demonstrated to present increased $CD30^+$ cells and/or sCD30 levels.[43-46] At least in some patients suffering from these diseases, however, production of both IL-4 and IFNγ, i.e. a Th/Tc_0 pattern, can be postulated on the basis of preliminary experiments.

The present study characterizes MVI as an additional *in vivo* condition in which increased circulating $CD30^+$ T cells and high sCD30 serum levels are detectable in association with a clear-cut impairment of Th/Tc_1-type of cytokine pattern production. Clearly, only the demonstration that Th/Tc_2-type cytokines are preferentially produced by $CD30^+$ T cells in blood or tissues would definitely link CD30 expression and Th/$Tc_{0/2}$-type immune response in MVI.

Contributions and Acknowledgments

FV had main responsibility for all aspects of this study and for writing the paper. MK, LM, AR contributed to analysis of data and commented on the draft. SR and GP discussed core ideas and contributed to conception, design and critical revision of the paper. The order of authorship reflects these contributions.

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Disclosures

Conflict of interest: none.

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