Tc1/Tc2 ratio in the inflammatory process in patients with Behçet's disease

BACKGROUND: Peripheral blood CD8$^+$ T cells expressing interferon gamma and interleukin-4 (IL-4), and lacking CD28 molecules, were responsible for the dynamic interplay between peripheral blood and inflammatory sites.

Introduction: The aim of the current study was to define in Behçet's disease (BD), CD8$^+$ T-cell subsets using CD28 and CD11b monoclonal antibodies, and the characterization of the Tc1/Tc2 ratio and perforin expression.

Methods: Flow cytometry was used for intracytoplasmic cytokines and perforin expression. Effector cells were investigated by adhesion of CD8$^+$ T cells to human microvascular endothelial cells and by chemotaxis using β-chemokine.

Results: Interferon-gamma-producing CD8$^+$ T cells in active and remission BD patients were increased, which induce a significant increase of the Tc1:Tc2 ratio in BD. CD8$^+$CD28$^-$CD11b$^+$ T cells were found to be more expanded in BD patients than in age-matched healthy controls. The expression of CD11b molecules in active BD allowed to CD8$^+$CD28$^+$/CD8$^+$CD28$^-$ subsets to adhere to human microvascular endothelial cells, with more efficiency in BD. Using MIP-1α, we observed that the migratory process of CD28$^-$CD11b$^+$ is more important in BD. CD28$^-$CD11b$^+$ exhibited an increased perforin expression in BD patients.

Conclusion: Taken together these results suggest the presence of immune activation, probably in response to a profound inflammation affecting BD patients. The physiopathological significance of these results were toward autoimmune diseases and/or infectious process.

Key words: Behçet's disease, CD8 T cells, Inflammation, MIP-1α, Human microvascular endothelial cells

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Introduction

Behçet's disease (BD) is an inflammatory multi-systemic disorder with mucocutaneous, ocular, arthritic, vascular and central nervous system involvement.[1] BD is characterized by spontaneous remissions and relapses similar to those of various autoimmune diseases. Neutrophil hyperactivity with increased superoxide production, phagocytosis, release of enzymes and the implication of Heat-shock proteins in BD suggest an activated innate immunity.[2] Investigation of the aetiology of BD has focused predominantly on herpes simplex virus immunopathology, streptococcal infection, and autoimmunity to oral or cross-reactive microbial antigens.[3,4] Only CD4$^+$ cells and Th1/Th2-type immune responses were currently investigated in cell-mediated immunity and inflammation in BD;[5–7] CD8$^+$ T-cell subsets and Tc1/Tc2 cytokine expression were scarcely reported.

In a number of situations ranging from chronic inflammatory conditions, infectious[8,9] to autoimmune diseases,[10] a dysregulation in CD8$^+$ T cells by lacking CD28 molecule (costimulatory molecule) and expression of CD11b marker (β2 integrin α-chain) was reported. CD8$^+$CD28$^-$ T cells are characterized by morphological and functional features of activated/memory T cells.[11] CD11b marker expression can distinguish between memory-type and effector-type T cells in human CD8$^+$CD28$^-$ T subsets.[12] Perforin, a pore-forming protein stored intracellulary that is produced by natural killer cells, gamma/delta (γ/δ) cells and CD8$^+$ T lymphocytes has been reported in elevated concentrations in several chronic inflammatory disorders.[13] Perforin can induce apoptosis in a number of cells and thus might also play a role in the resolution of inflammatory responses by eliminating inflammatory or virally infected cells.[14,15]

The aim of the current study was to define the Tc1/Tc2 ratio, the pattern of expression of CD28,

CD11b monoclonal antibodies on CD8$^+$ T cells, and the function of CD8$^+$ naïve/memory effector cells in peripheral blood from BD.

Materials and methods

Patients and control subjects

Patients with BD (five females and 31 males) were studied during the clinically active stage. The mean age of active BD was 32 years (range, 27–52 years) and the mean duration of disease was 74 months (range, 10–168 months). All active BD patients were showing at least three of the four major symptoms, including recurrent aphthous stomatitis, uveitis, genital ulcers, and skin lesions (erythema nodosum, folliculitis or subcutaneous thrombophlebitis). Peripheral blood was obtained from BD in the active stage before treatment, and clinical activity was assessed at the time of venipuncture for activity signs and symptoms (clinical criteria defined by the International Study Group).[16] Nine BD patients have pulmonary manifestations (chronic cough, pulmonary aneurysms) and 12 other BD patients were diagnosed as having progressive neuro-Behçet's (persistent and progressive central nervous system manifestations for at least 1 year). After venipuncture, active BD patients received treatments, including steroids and colchicine. Remission BD group was composed of asymptomatic patients (mean duration of remission, 2 months). Their mean age was 39 years (range, 36–50 years). No patient was studied in both stages. Twenty healthy volunteers, composed of laboratory personnel (mean age, 42 years; range, 22–50 years), participated after giving informed consent. At the time of venipuncture, none of the blood donors had been receiving any medication or had an overt infection. The design of the study was approved by our National Ethics Committee.

Cell preparation

Mononuclear cells were separated from heparinized venous blood that was mixed with 20 ml of Hanks's balanced salt solution (Gibco, Grand Island, NY, USA), using Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. The cells were washed twice in phosphate-buffered saline (PBS), and then resuspended in PBS supplemented with 5% foetal calf serum. The former cell suspensions were finally adjusted to a concentration of 2×10^6 cells/ml, and were processed further for intracellular staining studies.

Flow cytometric analysis

Lymphocyte subsets were evaluated on whole fresh blood using different monoclonal antibodies (mAbs) panels. Two-colour and three-colour phenotypic characterizations of lymphocytes were performed as previously described.[12] Briefly, 100 µl of heparinized blood was incubated for 30 min on ice with the appropriate amounts of mAb. Cells were then lysed with buffer (FACS lysing solution; Becton Dickinson, Stockholm, Sweden) and analysed by flow cytometry (FACScan; Becton Dickinson). The lymphocyte gate was set using the log fluorescence of a two-colour mAb panel (Leukogate (anti-CD28 and anti-CD11 mAbs); Becton Dickinson) with linear 90° side scatter. The resulting data were analysed with CellQuest software (Becton Dickinson).

Purification of CD8$^+$ T-cell subsets

CD8$^+$ cells were purified from lymphocytes by positive selection using anti-CD8 magnetic beads (Miltenyi Biotec, Sunnyvale, CA, USA) as we have recently reported.[6] Sorting of CD8$^+$CD28$^+$CD11b$^-$, CD8$^+$CD28$^+$CD11b$^+$, and CD8$^+$CD28$^-$CD11b$^+$ T cells was performed from purified CD8$^+$ lymphocytes stained with FITC-conjugated anti-CD11b (Immunotech, Marseille, France), PE-conjugated anti-CD28, and PerCP-conjugated anti-CD3, by flow cytometry (EPICS XL; Coulter Electronics, Hialeah, FL, USA). Only preparations with purity >98% were used for experiments.

Flow cytometric analysis of interferon gamma and interleukin-4 intracellular cytokine synthesis

Intracellular cytokine detection was performed as previously described.[6] Purified CD8$^+$ T cells were incubated in a medium containing phorbol myristate acetate (50 ng/ml), ionomycin (100 ng/ml) and monencin (20 µM) (Sigma Chemical Co., St Louis, MO, USA) for 6 h. Then CD8$^+$ T cells were collected and intracellular staining of interferon gamma (IFN-γ) and interleukin (IL)-4 was performed by flow cytometry as previously described.[6] Briefly, CD8$^+$ T cells were washed with PBS, supplemented with 0.5% bovine serum albumin (BSA) (GIBCO, Grand Island, NY, USA). After washing, cells were fixed with 4% formaldehyde for 15 min at room temperature, washed twice with PBS containing 0.5% BSA and then permeabilized with PBS containing 0.5% BSA and 0.5% saponin. Cells were incubated with PE-conjugated anti-IL-4 or with FlTC-conjugated anti-IFN-γ MoAb. Cells were then washed with PBS containing 0.5% BSA and 0.5% saponin and next washed with PBS. Finally, cells were stained with biotin-labelled anti-CD8 MoAb, followed by RED670-conjugated streptavidin. After washing with PBS, the

samples were finally analysed with a flow cytometer and the percentage of cells expressing the cytokine were recorded.

Lymphocyte adhesion to human microvascular endothelial cell cultures

Primary cultures of human adrenal gland capillary endothelial cells (HACECs) were obtained as previously described.[12] The endothelial cells were plated onto collagenated 96-well plates at a concentration of 5×10^3/well in 100 µl of endothelial basal medium (EBM) containing 10% fœtal calf serum, heparin (100 µg/ml), epidermal growth factor (10 ng/ml), and bovine brain extract (15 µg/ml; EBM complete medium). The plates were incubated for 4−5 days to obtain a monolayer. Endothelial cells were activated by adding tumour necrosis factor alpha (10 ng/ml) for 6 h at 37°C. Cells were then washed with PBS and allowed to interact with purified CD8$^+$ T-cell subsets (2×10^4 lymphocytes/well in RPMI 1640, containing 0.2% BSA). The plates were incubated for 2 h at 37°C, and unbound lymphocytes were removed by three washes with warm PBS. The lymphocytes attached to endothelial cells were fixed for 5 min with 100 µl of cold methanol, and the cells were stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland) for 30 min at room temperature. Plates were then washed several times with deionized water, and the lymphocytes bound to endothelial cells were counted with a calibrated eyepiece in 15 different fields at $\times 200$ magnification. Each test was run in quadruplicate.

Chemotaxis and migration assays

All migration assays were performed in collagen-coated 24-well Trans-well culture inserts (6.5 mm diameter clear polycarbonate membrane with 3-µm pores; Costar, Cambridge, MA, USA). The medium used was RPMI 1640 containing 0.2% BSA. All migration assays were conducted for 4 h at 37°C. Purified CD8$^+$ T-cell subsets (2×10^5) were placed in the upper chamber in 200 µl, and then 500 µl of medium containing, or not, MIP-1α was added in the lower well. The optimal chemotactic dose for MIP-1α was 100 ng/ml.

Intracellular perforin staining

After incubation of CD8 T cells with anti-CD28, and anti-CD11b, cells were fixed in paraformaldhehyde (4% in PBS) for 15 min on ice, washed twice and permeabilized with saponin (0.1% in PBS). Subsequently, cells were incubated with FITC-conjugated anti-perforin antibodies for 30 min, washed twice again, and then analysed by flow cytometry.

Statistical analysis

Mann−Whitney U-tests were used to compare variables between two groups. Bonferroni's test was used for multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

Intracellular cytokine analysis in CD8$^+$ T cells

Cytokine-producing CD8$^+$ T cells were assessed in BD patients, compared with healthy controls, after stimulation for 12 h with phorbol myristate acetate and ionomycin at the single cell level, using intracellular cytokine staining and flow cytometry with two-colour analysis (Fig. 1). Samples obtained from unstimulated culture were negative for any of these
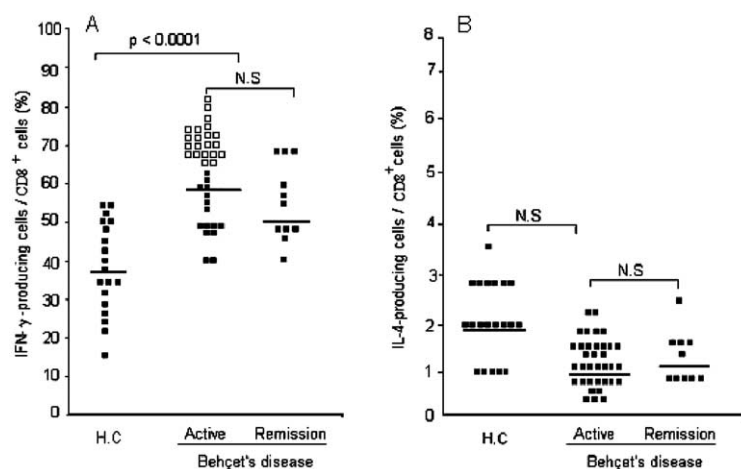

FIG. 1. Frequencies of CD8$^+$ T cells producing IFN-γ and IL-4 in peripheral blood from BD in active and remission stage and from normal controls (HC). Intracellular expression of IL-4 and IFN-γ were determined by flow cytometry. Bars indicate the medians. Open squares, with central nervous system and pulmonary manifestations. (A) IFN-γ-producing cells. (B) IL-4-producing cells. NS, not significant.

cytokines. IFN-γ-producing $CD8^+$ T cells in active and remission BD patients were respectively more increased (median, 60%; range, 40–79%; median, 52.5%; range, 40–70%) than normal controls (median, 37%; range, 16–54%; $p < 0.001$) (Fig. 1A).

When BD patients were studied according to their clinical manifestations, the frequencies of IFN-γ-producing $CD8^+$ T cells were significantly higher in patients with pulmonary manifestations (median, 73%; range, 70–79%; $p < 0.001$), and in patients with central nervous system manifestations (median, 76%; range, 68–82%), when compared with BD patients without these manifestations (median, 57%; range, 40–63%; $p < 0.05$).

No significant difference was found in the proportion of IL4-producing cells among $CD8^+$ T cells between BD groups and normal controls (Fig. 1B).

The Tc1:Tc2 ratio was evaluated from IFN-γ to IL-4-producing cells. The Tc1:Tc2 ratios of $CD8^+$ T cells in active (median, 14.5; range, 25.3–8) and remission BD patients (median, 12.9; range, 23.3–9.8; $p < 0.05$) exhibited a drastic increase, when compared with control subjects (median, 8; range, 11.6–4). The Tc1:Tc2 were similar in BD patients in active and remission phases.

CD28 and CD11b expression on $CD8^+$ T lymphocytes in BD

Three subsets of $CD8^+$ T lymphocytes ($CD28^+CD11b^-$, $CD28^+CD11b^+$ and $CD28^-CD11b^+$) were revealed as presented in Table 1. In healthy controls, the $CD11b^-$ prevailed over $CD11b^+$ subsets. The $CD28^+CD11b^+$ subset was barely present in healthy controls (median, 7.4; range, 5–8), when compared with active and remission BD ($p < 0.05$). In patients suffering from active and remission BD, we observed a significant increase in $CD28^-CD11b^+$ cells ($p < 0.01$), when compared with the same subset in healthy controls. $CD28^+CD11b$ subset was highly expressed in healthy controls compared with active and remission BD patients ($p < 0.01$).

Table 1. CD28 and CD11b expression on $CD8^+$ T lymphocytes from BD, compared with healthy controls

	Healthy controls	Active BD	Remission BD
$CD28^+CD11b^-$	72 (59–78)	37.2 (32–40)[†]	39 (22–47)[†]
$CD28^+CD11b^+$	7.4 (5–8)	22 (15–35)*	20.5 (29–46)*
$CD28^-CD11b^+$	19 (12–24)	36 (27–44)[†]	38 (25–34)[†]

Staining with monoclonal antibodies to CD8, CD11b and CD28 with three-colour analysis. * Significant differences ($p < 0.05$) compared with the same subset of healthy controls. [†] Significant differences ($p < 0.01$) compared with the same subset of healthy controls.

Adhesion of $CD8^+$ T-cell subsets to HACECs, chemotaxis

We investigated the adhesion of $CD28^+CD11b^+$ and $CD28^-CD11b^+$ subsets to HACECs, both in healthy controls and in active BD. In active BD, $CD28^-CD11b^+$ cells adhere to HACECs at higher levels than the same subset in healthy controls ($p < 0.05$) (Fig. 2A). Both in BD patients and in healthy controls, the expression of CD11b molecules allowed $CD8^+CD28^+/CD8^+CD28^-$ subsets to adhere to HACECs; the efficiency of the adhesion

(A): binding of cells to HACEC

(B): Migration of cells in the presence of MIP-1α
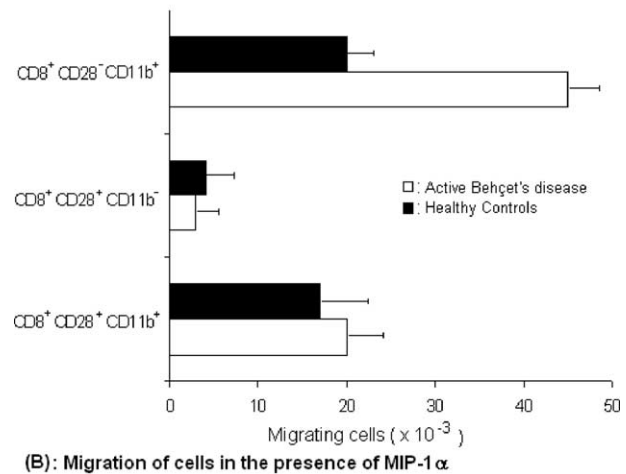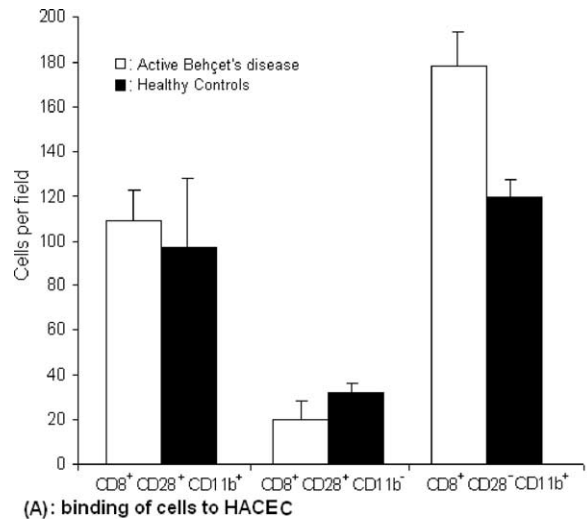
FIG. 2. Function of $CD8^+CD28^{+/-}CD11b^{+/-}$ T-cell subsets. (A) Binding to HACECs. Cells allowed to interact with a monolayer of tumour necrosis factor alpha-stimulated HACECs. Lymphocytes bound to HACECs were counted using a calibrated eyepiece in 10 different fields at ($\times200$) magnification. (B) Migration in active BD. The number of cells migrating into the bottom well in the absence or presence of MIP-α was evaluated by counting cells from duplicate wells. The net migration of cells was calculated by subtracting the number of cells migrating in the absence of a chemoattractant from the number of cells migrating in response to MIP-1α. Each test was run in quadruplicate. Bars represent the mean number of $CD8^+CD28^+CD11b^+$, $CD8^+CD28^+CD11b^-$, $CD8^+CD28^-CD11b^+$ T cells per microscopic field ± standard deviation. The experiments are representative of results obtained with cells from four healthy controls and seven active BD patients.

was more important in CD8$^+$ cells that have lacked CD28 antigen, particularly in active BD.

Using MIP-1α, we observed that both CD28$^+$CD11b$^+$ and CD28$^-$CD11b$^+$ from healthy controls and BD patients were efficiently attracted by the chemokine, whereas CD28$^+$CD11b$^-$ cells were unable to respond to chemotactic stimulus (Fig. 2B). MIP-1α is a β-chemokine that regulates T-lymphocyte migration from vessels to tissues. The migratory process of CD28$^-$CD11b$^+$ T cells is more important in active BD compared with healthy controls ($p < 0.001$).

Perforin expression in CD8$^+$ subsets

We investigated intracellular perforin expression in the three CD8$^+$ subsets. Freshly isolated CD8$^+$ cells were stained with anti-CD28, anti-CD11b, and anti-perforin mAbs, and were analysed by flow cytometry. CD28$^+$CD11b$^-$ did not contain perforin both in healthy controls and patients with BD (median, 4%; range, 0–6%), while high staining was observed in the CD28$^-$ population. CD28$^-$CD11b$^+$ exhibited an increased perforin expression in BD patients (median, 57%; range, 33–62%) compared with healthy subjects (median, 22%; range, 12–34%) ($p < 0.01$). In patients with BD and in healthy controls, CD28$^+$CD11b$^+$ cells expressed similar levels of perforin (BD: median, 23%; range, 11.5–27%; healthy controls: median, 25%; range, 9–24%).

Discussion

In this study, intracellular cytokine analysis at the single cell level showed that the numbers of IFN-γ-producing CD8 T cells in active BD were increased compared with normal controls. In patients with BD, and in healthy controls, CD8$^+$ cells were exclusively producer of IFN-γ, they express insignificant IL-4 cytokine level. The main source of IL-4 were CD4$^+$ cells, as reported by Matsui *et al.*[17] Evidence suggested that BD was characterized by an increased Tc1/Tc2 ratio, in the same way as it has been reported in Th1/Th2 ratio.[5,6] Th1(IFN-γ) cells are involved in the protection against intracellular parasites and delayed-type hypersensitivity, but they can cause autoimmune diseases.[18] We have also demonstrated the occurrence of an unreported CD8$^+$CD28$^-$ T-cell subset in BD. The increase in such cells was not transient, as their percentage was highly expressed both in active and remission stages. The finding suggests the hypothesis that the presence of such cells during BD plays an important role in defence against a hypothetic aetiological agent.[2–4] Recently, it has been reported that neuro-BD has a cytokine and chemokine pattern in resemblance with non-

specific inflammations such as neuro-infections compared with autoimmune disorders.[19] Circulating CD8$^+$CD28$^-$ T cells are increased not only in various infectious diseases,[20] but also in patients with autoimmune diseases.[10] In rheumatoid arthritis patients, cytomegalovirus seropositivity has been directly correlated with increased levels of CD8$^+$CD28$^-$ T cells.[21] Weeks *et al.*[22] reported that functional virus-specific memory CTL clones are present in CD8$^+$CD28$^-$ T cells expressing or no CD57 marker (HNK-1). CD8$^+$CD28$^-$ T cells have been reported to be effector cells, producing perforin, granzyme B, tumour necrosis factor alpha and IFN-γ.[23,24]

Although this is the first study to report an increase in the percentage of perforin-expressing CD8$^+$CD28$^-$ in patients with BD, our study cannot provide conclusive evidence of perforin-cytotoxicity in BD. Their relative cytotoxic potential must remain unclear since to date there are no studies that have satisfactorily addressed this question in BD. However, we have previously reported that patients with BD have elevated cytolytic CD8$^+$ T lymphocytes in peripheral blood against HSV1 target cells.[25] Increased perforin expression has been reported in several other chronic inflammatory disorders with autoimmune phenomena such as multiple sclerosis,[26] Takaysu's arthritis[27] and Crohn's disease.[28] In an animal model of lupus erythematosus, perforin-deficient animals had more severe disease, suggesting that cytolytic lymphoid regulation plays a critical role in the immune homeostasis of these animals.[29]

One of the effects of IFN-γ is to suppress the development of Th2 cytokines[18,30] and point to the effector potential of CD8$^+$ T cells.[31] IL-4 prompts CD8$^+$ T cells to maintain CD28 expression in long-term cultures.[32] The addition of IL-4 led both the neonatal and the adult lymphocytes to keep their expression of CD28.[32,33] Besides, delayed addition of IL-4 to CD8$^+$ T cell cultures did not re-induce CD28 on cells that have already lost the marker, indicating that IL-4 can prevent, but cannot reverse, the switch in CD28 expression

Our BD data demonstrate that CD8$^+$ T-cell differentiation into effector cells is characterized by acquisition of a CD11b$^+$ phenotype. Indeed, CD28$^+$CD11b$^-$ cells are more similar in behaviour to true memory cells, being incapable of chemotaxis and adhesion to human microvascular endothelial cells, whereas their CD11b$^+$ counterpart has all the properties of fully competent effector cells. As suggested, the CD28$^+$CD11b$^+$ subset provides a model for a critical step in the development of functional CTL, which precedes the process of CD28 downregulation.[12] In functional terms, CD8$^+$CD28$^-$ cells share features of both CD28$^+$ and CD28$^-$ T cells. CD8$^+$CD28$^-$ cells are relatively resistant to apoptosis compared with their CD8$^+$CD28$^+$ precursors.[34] CD8$^+$CD28$^-$ have shorter telomers than CD8$^+$CD28$^+$ cells, indicating a

history of increased cell divisions.[35,36] The persistence of the expanded *in vivo* CD8^{+}CD28^{−} subset may be explained by antigen-driven differentiation from CD8^{+}CD28^{+} memory precursors, with relative resistance to apoptosis as the clones become perforin-positive effector cells. We have reported that patients with BD expressed high levels of soluble Fas/APO-1, which may be a useful marker in evaluating the extent of injury in vasculitis conditions.[37] Patients with active BD expressed high levels of the bcl-2 in inflammatory sites,[38] and the chronic inflammation in BD was confirmed by the increased amount of bcl-2 messenger RNA.[39]

Cells migrating to lymph nodes lack inflammatory and cytotoxic function, whereas cells migrating to peripheral tissues are endowed with various effector functions.[40] CD11b^{+} cells are present in blood;[41] they have been described as an important molecule for the extravasation of neutrophils and monocytes to the site of inflammation — it is also involved in adhesion, chemotaxis, and diapedesis.[42] Our report demonstrates an increase in the capacity of CD28^{+} cells to migrate in response to MIP-1α at the time that they acquire expression of CD11b, thus supporting the prospect of an effector CD28^{+}/CD28^{−} subset with tissue-homing properties. Our BD patients, having pulmonary or central nervous system manifestations were characterized by expanded CD8^{+}CD28^{−}CD11b^{+} cells, which govern transition to the inflammatory sites. Such investigations allow us to manipulate cells for immunotherapy purposes. In our patients, the level of CD8^{+}CD28^{−}CD11b^{−} was scarcely represented. The biologic properties of CD8^{+}CD28^{−}CD11b^{−} T cells suggest that these cells might be end stage or aberrant differentiated effector cells. It has been reported that if CD8^{+}CD28^{−}CD11b^{−} T cells lacked cell−cell adhesion and impaired cytolytic functions, this would favour the hypothesis of a role for the development of immunodeficiency.[43] Several observations suggest that the phenotypic evolutions detected in long-term cultures from healthy adult donors also occur *in vivo*: (i) CD8^{+} T cells with high, moderate and low CD28 density can be identified in normal adult blood;[33] (ii) progressive emergence of CD8^{+}CD28^{−} T cells has been observed in healthy human adults, particularly but not exclusively in elderly subjects, and considerably more during many clinical situations associated with inflammation, chronic immune responses,[44−46] where some pathogens, such as herpes simplex virus,[47] cytomegalovirus and Epstein−Barr virus,[34,48] are never eliminated, and maintenance of a high frequency of virus-specific CD8^{+} T cells is essential to prevent viral reactivation from becoming symptomatic.

Our report suggests that the BD immune system reflects an autoimmune and/or viral situation, and CD8^{+} T cells acquire or lose markers according to the extended inflammatory process. We have shown a strong shift to Tc1 cells among CD8^{+} T cells in peripheral blood from BD patients, the persistence of the expanded *in vivo* CD8^{+}CD28^{−} subset, which may be explained by antigen-driven differentiation from CD8^{+}CD28^{+} memory precursors, with relative resistance to apoptosis as these cells become perforin-positive effector cells. In general, it would be important for CD8 effector cells to avoid apoptosis. It undergoes repetitive antigen exposure when it serially kills one target after another.[49] Perhaps this is the reason of the loss of CD28 and acquisition of CD11b expression in perforin-positive cells. Expansion of CD28^{−} T cells are not a distinctive feature of some special antigenic stimulation, but instead would merely reflect repeated stimulations of the BD immune system by any persisting antigen.

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