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CD11b Expression Identifies CD8⁺CD28⁺ T Lymphocytes with Phenotype and Function of Both Naive/Memory and Effector Cells¹

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A previously unreported CD8⁺CD28⁺CD11b⁺ T cell subset occurs in healthy individuals and expands in patients suffering from primary viral infections. In functional terms, these cells share the features of naive/memory CD8⁺CD28⁺CD11b⁻ and terminally differentiated effector CD8⁺CD28⁻CD11b⁺ subpopulations. Like CD28⁺ cells, CD28⁺CD11b⁺ lymphocytes have the ability to produce IFN-γ, to express perforin granules in vivo, and to exert a potent cytolytic activity. Moreover, these cells can respond to chemotactic stimuli and can efficiently cross the endothelial barrier. In contrast, like their CD11b⁻ counterpart, they still produce IL-2 and retain the ability to proliferate following mitogenic stimuli. The same CD28⁺CD11b⁺ subpopulation detected in vivo could be generated by culturing naive CD8⁺CD11b⁻ cells in the presence of mitogenic stimuli following the acquisition of a CD45RO⁺ memory phenotype. Considering both phenotypic and functional properties, we argue that this subset may therefore constitute an intermediate phenotype in the process of CD8⁺ T cell differentiation and that the CD11b marker expression can distinguish between memory- and effector-type T cells in the human CD8⁺CD28⁺ T cell subset. The Journal of Immunology, 2001, 166: 900–907.

The CD8 T cell subset can be classified according to CD28 surface marker expression as either CD28⁺ or CD28⁻, with different biological properties and abilities to produce soluble elements such as cytokines. The CD28⁺ T cell population is predominant in healthy individuals and expands during primary viral infection (1), whereas CD28⁻ cells are more common in healthy elderly individuals (2) and increase dramatically in HIV patients (3–5).

CD28⁺ is the only T cell subset present in cord blood (6), which indicates that naive unprimed cells display this phenotype. In adults the population consists of both CD45RA and CD45RO phenotypes, suggesting the presence of both naive and memory cells in the subset (6, 7). Such cells mostly produce IL-2 and exhibit the ability to proliferate in vitro following mitogenic stimulation.

The CD28⁻ population consists of a subset of memory T cells, and in many respects their phenotype and function are highly suggestive of differentiated armed effector T cells, as they produce IFN-γ and TNF-α and express high levels of granzyme A and perforin. They also contain Ag-specific memory CTL (8, 9) and exert a potent cytolytic activity without requiring deliberate in vitro activation (2, 6). CD28⁻ are clonally expanded (10), terminally differentiated lymphocytes with little proliferative response to mitogenic stimulation in vitro (11).

The origin of CD28⁺ cells has long been controversial, but recent data show that they derive from their CD28⁻ counterpart. We were able to generate CD28⁻ cells in vitro from long term IL-2-stimulated CD28⁺ T cells through an intermediate CD28⁻⁰⁻¹⁰⁻ phenotype. The in vitro transformation of CD28⁻ to a stable CD28⁻ phenotype involved the acquisition of various biological functions (12), thus suggesting a link to the normal pattern of functional CD8⁺ T cell maturation. Labalette et al. (13) confirmed our data, highlighting also the possibility of IL-4 to prevent loss of CD28 expression.

It is well known that CD8⁻ CD28⁺ T cells are negative for β₂ integrin α-chain CD11b expression, whereas almost all CD28⁻ cells are CD11b⁺ (14). β₂ integrins mediate the adhesion of lymphocytes to endothelial cells and extravasation (15, 16); they are also required for homing to inflamed tissues (17). Moreover, in the mouse model, the expression of CD11b on CD8⁺ T cells has been associated with acquisition of cytotoxic capacity (18).

We assumed that the functional differentiation of CD8⁻ T cells into mature effector cells, with the disappearance of CD28 and the appearance of CD11b molecules, has to be gradual. It should therefore be possible to observe in vivo, during primary viral infection, the emergence of elements characterized by CD28 and CD11b molecule coexpression. This present article describes the presence of CD8⁻ CD28⁺ CD11b⁻ T cells in freshly collected PBMCs from healthy donors and, to a greater extent, in PBMCs from virus-infected patients. Such cells have all the functions of effector cells but retain the ability to proliferate in vitro. Finally, we observed that CD8⁺CD28⁺CD11b⁻ emerged from both adult and cord blood IL-2-stimulated CD28⁺CD11b⁻ cells during their transformation into a CD28⁻ phenotype.

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Materials and Methods

Reagents and Abs

PMAs, ionomycins, monensins, PBS, BSA, saponins, PHA, parafomaldehyde, L-lysine monohydrochloride, sodium azide (NaN₃), and Hystopaque density gradient were supplied by Sigma (St. Louis, MO). RPMI 1640 medium, FCS, L-glutamine, penicillin, and streptomycin were purchased from Life Technologies (Paisley, U.K.). Endothelial basal medium (EBM) was supplied by BioWhittaker (Walkersville, MD). Purified anti-CD3 (OKT3) mAb was supplied by Ortho (Raritan, NJ). FITC- or PE-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD16, anti-CD18, anti-CD28, anti-CD45RA, anti-CD45RO, anti-perforin, anti-IL-2, PerCP-conjugated anti-CD3, and CyChrome-conjugated anti-CD28 were all supplied by Becton Dickinson (San Jose, CA). mAb IGM17, an anti-human IFN-γ mAb, was produced in our laboratory (19). Recombinant human TFα and recombinant chemokine macrophage inflammatory protein-1α (MIP-1α) were purchased from PeproTech (London, U.K.). Recombinant IL-2 came from Roche (Basel, Switzerland). Na₅CrO₄ and [3H]TdR aqueous solutions were purchased by ICN Biomedicals (Irvine, CA).

Patients

Blood samples were obtained from 18 patients suffering from infectious mononucleosis (n = 2), CMV (n = 3), varicella (n = 4), herpes-zoster (n = 4), and measles (n = 5). Blood samples were also obtained from 25 healthy volunteers and three fetal cords. Mononuclear cells were obtained from heparinized blood by Histopaque density gradient.

Flow cytometric analysis

Lymphocyte subsets were evaluated on whole fresh blood using different mAb panels. Two- and three-color phenotypic characteristics of lymphocytes were performed as previously described (3). 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 lysis solution, Becton Dickinson) and analyzed by flow cytometry (FACScan, Becton Dickinson). The lymphocyte gate was set using the log fluorescence of a two-color Ab panel (Leukogate (anti-CD45 and anti-CD14 mAbs), Becton Dickinson) with linear 90° side scatter. Live gating was used to collect 10,000 events within the lymphocyte gate defined by Leukogate (Becton Dickinson) staining as CD45+ and with low side scatter (20). The resulting data were analyzed 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 (MiniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). 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, Marseilles, France), PE-conjugated anti-CD28, and PerCP-conjugated anti-CD3, by flow cytometry (FACSVantage, Becton Dickinson). Only preparations with purity >98% were used for experiments.

Lymphokine production

Purified CD8⁺ T cell subsets (3 × 10⁵ cells/well) were cultured in 24-well plates in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% of heat-inactivated FCS) and stimulated with or not with 10 ng/ml PMA plus 1 μM ionomycin in the presence of 1 μM monensin, which inhibits intracellular traffic pathways leading to protein accumulation (21). Plates were incubated at 37°C in 5% CO₂ in air, and cells were collected for lymphokine staining at 6 and 12 h after the addition of stimulants. The cells were then stained for lymphokine production as previously described (22). Briefly, they were washed twice with PBS, pH 7.2, and suspended using ice-cold 5% polyethylene-parafomaldehyde solution for 15 min at 4°C. After two washes in PBS, the cells were resuspended to 1 × 10⁶ in 300 μl of PBS containing 1% BSA (w/v), 0.2% NaN₃ (v/v), and 0.1% saponin (saponin buffer). After 15-min incubation at room temperature, the fixed and permeabilized cells were centrifuged and resuspended in saponin buffer containing 1 μg/ml FITC-conjugated anti-IFN-γ and 1 μg/ml PE-conjugated anti-IL-2 mAb. The cells were then incubated for 30 min at 4°C and washed with saponin buffer. Stained cells were analyzed by flow cytometry as described above. CD8⁺ T cell subsets were also cultured for 48 h in complete medium containing PMA and ionomycin. Cell-free culture supernatants were collected and assayed for the presence of IFN-γ or IL-2 by ELISAs (BioSource, Camarillo, CA).

Cell proliferation

Purified CD8⁺ T cell subsets were seeded in triplicate at different concentrations (ranging from 2 × 10⁴ to 6 × 10⁴) in 96-well culture plates in complete medium and stimulated with PHA (5 μg/ml) and IL-2 (10 U/ml). On day 3 the cells were pulsed with 1 μCi/ml of [3H]TdR and harvested after 18 h. The SD of the three replicate samples was <10% of the mean in all experiments.

Intracellular perforin detection

Purified CD8⁺ cells were washed twice with PBS, pH 7.2, and fixed in suspension using a 4% parafomaldehyde solution for 5 min at room temperature. After two washes in PBS, cells were resuspended to 1 × 10⁶ in 1 ml of PBS containing 1% BSA (w/v), 0.2% NaN₃ (v/v), and 0.2% saponin (saponin buffer). After a 15-min incubation at room temperature, fixed and permeabilized cells were centrifuged and resuspended in saponin buffer containing FITC-conjugated anti-CD11b, PE-conjugated anti-perforin, and CyChrome-conjugated anti-CD28 mAbs. Cells were then incubated for 30 min at 4°C and washed with saponin buffer. Stained cells were analyzed by flow cytometry as described above.

Cytotoxicity assay

The cytotoxic activity of CD8⁺ T cell subsets was evaluated in an anti-CD3-redirected cytotoxicity assay as described previously (23). Briefly, 5 × 10⁵ Fe receptor-bearing P815 target cells were labeled with 50 μCi of Na₂CrO₄ for 2 h at 37°C. Cells were then washed three times and incubated for 30 min at 4°C in the presence or the absence of 2 μg of anti-CD3 mAb. Purified CD8⁺ T cell subsets were cultured for 72 h in 100 μl of EBM containing 10% FCS, 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 TNF-α (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 × 10⁵ lymphocytes in 100 μl of EBM, 1:100, 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 ×200 magnification. Each test was run in quadruplicate.

Lymphocyte adhesion to human microvascular endothelial cell cultures

Primary cultures of human adrenal gland capillary endothelial cells (HAECs) were obtained as previously described (24). The endothelial cells were plated onto collagenated 96-well plates at a concentration of 5 × 10⁴/well in 100 μl of EBM containing 10% FCS, 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 TNF-α (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 × 10⁵ lymphocytes in 100 μl of EBM, 1:100, 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 ×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). 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 × 10⁵) were placed in the upper chamber in 200 μl of modified EBM containing, for not MIP-1α was added in the lower well. The optimal chemotactic dose for MIP-1α was 100 ng/ml.

Transendothelial migration experiments were performed simultaneously with chemotaxis. HAECs (5 × 10⁴) were seeded on collagen-coated 24-well plate Trans-well culture inserts and cultured in EBM complete medium until confluence was reached. Under these conditions, HAECs did not cross the membrane and formed a complete monolayer usually after 2 days of culture and only on the upper surface of the filter, as confirmed by staining with Diff-Quick of a batch of Trans-well inserts.
before use. The HACEC monolayer was treated for 6 h with 10 ng/ml of TNF-α. Cells were washed three times, and CD₈⁺ T cell subpopulations were added to the insert before Trans-well immersion. The Trans-well inserts were then removed, and migrated cells were collected by centrifugation. Live gating was defined as previously described (5). Cord blood lymphocytes were long term cultured and phenotypically characterized as described above. Tests were run in triplicate.

**Long term culture of CD₈⁺ T cells**

Purified CD₈⁺CD₁₀⁺ T cells (1×10⁶) were seeded in 96-well microtiter wells (Nunc, Roskilde, Denmark) in 200 µl of RPMI 1640 complete medium supplemented with PHA (5 µg/ml), IL-2 (100 U/ml), and irradiated (3000 rad) autologous PBMCs (10⁴ cells/well). For cellular expansion, growing cells were split twice a week, and 100 µl of medium was replaced with a fresh aliquot containing 100 U/ml of IL-2. Flow cytometric analysis of growing cells was performed once a week. The viability of the cells at any time of harvesting always exceeded 80% as determined by flow cytometry. Live gating was defined as previously described (5). Cord blood lymphocytes were long term cultured and phenotypically characterized as described above.

**Statistical analysis**

The data were analyzed by (multivariate) variance analysis. Student’s t test was used to determine significant differences between group averages. When multiple individual groups were compared, p values were corrected with the Bonferroni correction. Significance was defined as p ≤ 0.05.

**Results**

**CD₈⁺CD₁₀⁺CD₁₁ᵇ⁺ T cells with effector features**

The presence of CD₈⁺CD₁₀⁺CD₁₁ᵇ⁺ T lymphocytes was evaluated in blood samples from healthy individuals and patients suffering from primary viral infections, characterized by activated cell-mediated immunity. Staining of PBMCs with mAbs to CD₈, CD₁₁ᵢ, and CD₂₈ revealed three subsets of CD₈⁺ T lymphocytes: a CD₂₈⁺CD₁₁ᵢᵇ⁻ subset and two CD₁₁ᵢᵇ⁺ subsets (CD₂₈⁺CD₁₁ᵢᵇ⁺ and CD₂₈⁻CD₁₁ᵢᵇ⁺). As shown in Table I, in healthy individuals the CD₂₈⁺CD₁₁ᵢᵇ⁺ subset prevailed over CD₂₈⁻CD₁₁ᵢᵇ⁻, whereas the CD₂₈⁺CD₁₁ᵢᵇ⁻ subset was only barely present. In patients suffering from acute viral infections (n=18) we observed a significant increase (p<0.01) in CD₁₁ᵢᵇ⁺ cells compared with healthy individuals (n=25; mean, 41.9±14.2 and 29.0±13.9%, respectively). The increase in CD₁₁ᵢᵇ⁺ cells was generally found to be linked to the CD₂₈⁺ subset. In fact, the percentage of CD₂₈⁺CD₁₁ᵢᵇ⁻ lymphocytes in patients exhibited an average 3.4-fold increment compared with healthy donors, whereas the percentage of CD₂₈⁻CD₁₁ᵢᵇ⁺ cells showed no significant difference between the two groups (Table I). The increase in CD₂₈⁻CD₁₁ᵢᵇ⁺ cells depended on the virus causing the disease (Fig. 1). At the onset of symptoms, EBV consistently elicited the largest increase in the CD₂₈⁺CD₁₁ᵢᵇ⁻ subset (~60% of all CD₈⁺ T cells) compared with the other viruses considered (range, 9.8–49.7%). All the patients showed a decline in the percentage of CD₁₁ᵢᵇ⁺ cells to normal levels as their disease gradually resolved (data not shown).

**Phenotypic characterization of CD₈⁺CD₁₁ᵢᵇ⁺ T cells**

T cell subsets bearing different surface receptors may also display different functions. The dramatic increase in the percentage of CD₂₈⁻CD₁₁ᵢᵇ⁺ T cells during primary viral infections suggests that in humans these may function as primed cells that expand in response to Ag stimulation. Indeed, evaluation of naive and memory cells within the CD₂₈⁻CD₁₁ᵢᵇ⁺ subset using CD₄₅RA and CD₄₅RO isoform expression, respectively, revealed that most of these cells had a memory CD₄₅RO⁺ phenotype, whereas considerable heterogeneity was observed within CD₂₈⁺CD₁₁ᵢᵇ⁻ and
IL-2 protein expression as described in Materials and Methods.

The cytokine-producing capacity of CD8 T cell subsets. CD28CD11b−, CD28CD11b+, and CD28 CD11b−, were then sorted by flow cytometry. The cytokine-producing capacity of CD8 T cell subsets was measured after stimulation for 12 h with PMA and ionomycin at the single-cell level. As shown in Fig. 2A, the CD28CD11b− subset had a higher percentage of IL-2+ cells (mean, 18.9 ± 5.7%) than the CD28− subset (mean, 2.1 ± 1.8%). Conversely, the percentage of IFN-γ-expressing cells was higher in the CD28CD11b− than in the CD28CD11b+ subset (mean, 81.3 ± 18.0 and 23.9 ± 11.5%, respectively). Notably, CD28CD11b+ cells showed an intermediate pattern of IL-2 and IFN-γ expression (mean, 6.3 ± 3.1 and 40.6 ± 17.6%, respectively). Results obtained by flow cytometry were confirmed by measurements of cytokines in culture supernatants by ELISA. Again, CD28CD11b− CD28CD11b+ T cells mainly produced IL-2 and IFN-γ, respectively, whereas CD28CD11b+ cells were capable of secreting both cytokines (Table II).

Proliferative response to mitogenic stimulation

To test whether the distinction in cytokine production profiles among the three CD8+ T cell subsets was reflected by their proliferative capacities, the three CD8+ subpopulations were stimulated with a combination of PHA and IL-2. As expected, CD28CD11b+ cells exhibited no proliferative activity in a [3H]Tdr incorporation assay (6, 11). Instead, both CD28CD11b− and CD28CD11b+ subsets showed a proliferative response to mitogenic stimulation (Fig. 2B), thus indicating that despite IFN-γ production the latter group maintains a replicative capacity, which shows that they are nonterminally differentiated effector cells.

CD28CD11b+ exhibit perforin expression and cytolytic activity without prior in vitro activation

The protective response in tissues is mediated by mature effector T cells. These lymphocytes are able to produce cytokines such as

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<th>CD28+CD11b−</th>
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<th>CD28−CD11b+</th>
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<tr>
<td>205.8 ± 152.3</td>
<td>2961 ± 1269</td>
<td>287.4 ± 604</td>
</tr>
<tr>
<td>523.4 ± 287.4</td>
<td>9604 ± 3281</td>
<td>287.3 ± 612</td>
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<tr>
<td>30.9 ± 28.6</td>
<td>9612 ± 2818</td>
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* Sorted cells (7.5 × 10⁵) from a representative healthy donor were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 48 h. Cytokine content of the supernatants was tested at the same time for the presence of IL-2 and IFN-γ by ELISAs. Values represented the mean ± SD of the cytokine released in triplicate cultures.
IFN-γ and develop in vivo the enzymatic machinery necessary for the exocytic pathways of cytolysis (29, 30). Because the three CD8+ subsets differed for cytokine production, we investigated whether there were also differences in intracellular molecules involved in cytolysis, such as perforin. Freshly isolated CD8+ cells were stained with anti-CD28, anti-CD11b, and anti-perforin mAbs and were analyzed by flow cytometry. As shown in Fig. 3A, CD28+CD11b+ cells did not contain perforin, while high staining was observed in the CD28− population. As earlier remarked for the analysis of cytokine production, cells with a CD28+CD11b+ phenotype exhibited an intermediate pattern of perforin expression.

We next investigated whether CD28+CD11b+ cells could exert cytotoxic activity even without previous in vitro activation. The cytotoxic activities of the three CD8+ subsets were evaluated using freshly purified lymphocytes as effector cells and the mouse cell line P815 as a nonspecific target in a CD3 redirected cytolysis assay. CD28−CD11b+ cells exhibited an efficient cytotoxic response, with >50% specific lysis at an E:T cell ratio of 20:1, whereas CD28+CD11b− cells were unable to efficiently lyse target cells (<10% lysis) at the same E:T cell ratio. Remarkably, the CTL activity of CD28+CD11b+ was approximately half the cytotoxicity of CD28− cells. The percentage of lysis reached by CD28− cells at an E:T cell ratio of 5:1 or 10:1 was achieved with CD28+CD11b+ cells at E:T cell ratios of 10:1 and 20:1, respectively (Fig. 3B).

Adhesion of CD8+ T cell subsets to HACECs, chemotaxis, and transendothelial migration

Effector cells are characterized by further unique biological properties, such as the ability to home to peripheral tissue and secondary lymphoid organs by adhesion to the capillary endothelium and by transendothelial migration (31–33). All these functions are important in allowing effector cells to exert their protective response in tissues. Integrins mediate the adhesion of lymphocytes to endothelial cells and, accordingly, lymphocyte extravasation (34, 35). We therefore investigated whether the integrin α-chain CD11b expression on the surface of CD8+ cells renders them capable of adhering to HACECs. As shown in Fig. 4, a high number of CD28+CD11b+ cells adhered to HACECs, whereas only a few CD28−CD11b+ cells bound to them. The expression of CD11b molecules on the surface of CD28− cells allowed them to adhere to HACECs with almost the same efficiency showed by CD28+CD11b+ cells.

Using MIP-1α, a β-chemokine that regulates T lymphocyte migration from vessels to tissues (35), we observed that both CD28−CD11b+ and CD28+CD11b+ (Fig. 5A) were efficiently attracted by TNF-α-stimulated HACECs. Lymphocytes bound to HACECs were counted using a calibrated eyepiece in 15 different fields at ×200 magnification. Each test was run in duplicate. Bars represent the mean number of bound CD28−CD11b+ (□), CD28+CD11b+ (●), and CD28+CD11b+ (■) lymphocytes per microscopic field ± SD.
tracted by the chemokine, whereas CD28<sup>+</sup>CD11b<sup>-</sup> cells were unable to respond to chemotactic stimulus.

Finally, we looked at the ability of CD8<sup>+</sup> subsets to perform transendothelial migration. Being unable to interact efficiently with HACECs or to migrate at optimal chemotactic concentrations of MIP-1α, CD28<sup>-</sup>CD11b<sup>-</sup> T cells expectedly failed to perform active transendothelial migration (Fig. 5B). Instead, the two CD11b<sup>+</sup> subsets efficiently crossed the endothelial barrier (Fig. 5B).

As evaluated by flow cytometric analysis, no modulation of CD11b or CD28 marker expression was observed on CD28<sup>-</sup>CD11b<sup>+</sup> and CD28<sup>-</sup>CD11b<sup>+</sup> cells after MIP-1α chemotraction, whereas a slight decrease in the MFI of CD11b marker expression was observed on both cell subsets after transendothelial migration (data not shown).

**Development of CD11b<sup>+</sup> and CD28<sup>-</sup> phenotypes from CD8<sup>-</sup>CD28<sup>+</sup> T lymphocytes**

The stability of the CD28<sup>-</sup> phenotype and the acquisition of CD11b molecule expression were evaluated in long term cultures of CD8<sup>-</sup>CD28<sup>-</sup>CD11b<sup>-</sup> T lymphocytes. When CD28<sup>-</sup>CD11b<sup>-</sup> cells were purified and stimulated in vitro, almost all the cells recovered after 10 days were CD45RO<sup>+</sup>. At the same time, we observed a consistent percentage of CD28<sup>-</sup>CD11b<sup>+</sup> cells, which expanded over time, usually reaching a peak at 3–4 wk after mitogenic stimulation. Following prolonged culture (5–6 wk), the percentage of CD28<sup>-</sup>CD11b<sup>-</sup> cells decreased concomitantly with an increase in the percentage of CD28<sup>-</sup>CD11b<sup>+</sup> cells. To investigate the likelihood of naive CD8<sup>+</sup> cells giving rise to CD28<sup>-</sup>CD11b<sup>-</sup> cells, cord blood T lymphocytes were polyclonally stimulated; all CD8<sup>-</sup> cord blood lymphocytes showed a CD28<sup>-</sup>CD11b<sup>-</sup> phenotype when freshly analyzed but, similarly to peripheral blood CD28<sup>-</sup> lymphocytes, they acquired a CD45RO<sup>+</sup> phenotype after 10 days of mitogenic stimulation. Flow cytometric analysis showed that a high percentage of CD28<sup>-</sup> cells (usually >20%) acquired a CD11b<sup>+</sup> phenotype after 2-wk stimulation. The expansion of CD28<sup>-</sup>CD11b<sup>+</sup> cells usually peaked at 3–5 wk and later declined, concomitantly to the emergence of CD28<sup>-</sup>CD11b<sup>-</sup> elements. Data are representatively shown in Fig. 6 and are summarized in Table III.

**Discussion**

We have demonstrated the occurrence of an unreported CD8<sup>-</sup>CD28<sup>-</sup>CD11b<sup>+</sup> T cell subset in healthy individuals and its enlargement in patients suffering from primary viral infections. The increase in such cells was transient, as their percentage fell to normal values when the disease gradually resolved. The finding suggests the hypothesis that the presence of these cells during the viremic phase plays an important role in defense against viruses. In functional terms, such cells share features of both CD28<sup>-</sup> and CD28<sup>-</sup> cells. IFN-γ production during a short term assay (25, 28) and the presence of intracellular perforin granules ex vivo both point to their effector potential (36, 37). Indeed, they exert a potent cytolytic activity in a CD3–redirected assay, which mimics Ag-specific cytotoxicity in vitro (38). Our results show that these cells can respond to chemotactic stimuli and efficiently penetrate the endothelial barrier. In contrast, a very low percentage of these cells expressed the CD57 molecule, which is described as a marker for late or terminal CD8 differentiation (26, 39), and, remarkably, they still produce IL-2 and retain the ability to proliferate following mitogenic stimuli. Our data agree with the finding by Callan et al. (40) that during primary EBV infection virus-specific effector cells vary considerably in CD28 expression, thus indicating that some Ag-reactive cells still express a CD28 marker.

We found that during the acute phase of a primary viral infection such as infectious mononucleosis most CD8<sup>-</sup> T cells were CD28<sup>-</sup>CD11b<sup>+</sup>, with a massive presence of CD45RO<sup>+</sup> cells within the subset. Long term cultures of purified CD8<sup>-</sup>CD28<sup>-</sup>CD11b<sup>+</sup> cells, originally containing both CD45RA<sup>+</sup>
and CD45RO+ phenotypes, gave rise to an entirely CD45RO+ population, which gradually acquired CD11b marker expression. To establish whether CD11b- cells also originate from unprimed naive CD8+ T cells, we cultured cord blood lymphocytes and found again that all the cells switched to a CD45RO+ phenotype before acquiring the CD11b molecule. These findings suggest that all CD8+CD28+ T cells regardless of their CD45 isotype expression may acquire an activated CD11b+ phenotype, and that acquisition of the CD45RO+ phenotype is necessary, at least in vitro, for further development into CD11b+ and eventually into CD28- terminal effector cells. With respect to the parameter analyzed, it appears therefore that the same subsets of memory/effector T cells detected in vivo can also be generated by stimulating cultured naive cells. Hamman et al. (41) have recently suggested a model of T cell differentiation in which effector cells may arise from a proliferating memory pool (CD45RA-CD28+) and acquire, during the process of down-regulation of CD28, the features of mature effectors, but the difficulty with this model is that both memory and effector cells, within the CD28- subset, express CD45RO, so there was no way to distinguish between the two. Our data demonstrate that the early stages of CD28+ memory differentiation into effector cells are characterized by acquisition of a CD11b+ phenotype. Indeed, CD28+CD11b- cells are more similar in behavior to true memory cells, being incapable of cytotoxicity and transendothelial migration, whereas their CD11b+ counterpart has all the properties of fully competent effector cells. CD11b expression and CD45RO+CD28+ phenotype therefore distinguish nonterminally differentiated effector cells from the memory pool. As suggested above, the CD28-CD11b- subset provides a model for a critical step in the development of functional CTL, which precedes the process of CD28 down-regulation.

A recent study highlights the relationship between the functional activities of lymphocytes and their migration properties. Cells migrating to lymph nodes lack inflammatory and cytotoxic function, whereas cells migrating to peripheral tissues are endowed with various effector functions (25). CD11b has 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+ subset with tissue-homing properties. Indeed, it was reported that CD11b+ cells are present in blood, liver, and spleen, but are absent from tonsil, lymph node, and thymus (43).

In conclusion, the present study demonstrates that the expression level of CD28 and CD11b can discriminate among three subsets of circulating CD8+ T cells with different functional properties. The acquisition of CD11b molecules identifies CD28- lymphocytes with effector cell features, which may form an intermediate phenotype in the process of CD8+ T cell differentiation. In this respect, it is remarkable that CD28-CD11b+ cells, unlike their CD28+ counterpart, retain the capacity to proliferate, thus enabling the population to expand greatly both in vivo and in vitro. A better understanding of the mechanisms that govern transition from early (proliferating) to mature (nonproliferating) effector cells will allow researchers to manipulate immunological memory for vaccination and adoptive immunotherapy purposes.

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**References**


