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

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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.


In adults, the population consists of a subset of memory 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

PMA, ionomycin, monensin, PBS, BSA, saponin, PHA, parafomaldehyde, t-l-lysole monohydrate, sodium azide (NaN3), and Hystoqade density gradient were supplied by Sigma (St. Louis, MO). RPMI 1640 medium, FCS, t-glutamine, penicillin, and streptomycin were purchased from Life Technologies (Paisley, U.K.). Endothelial basal medium (EBM), 3 hep-lial cell; MFI, mean fluorescence intensity.

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 heat-exposed mononuclears and three fetal cords. Mononuclear cells were obtained from heparinized blood by Hystoqade density gradient.

Flow cytometric analysis

Lymphocyte subsets were evaluated on whole fresh blood using different mAb panels. Two- and three-color phenotypic characterizations 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 lysing 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 CD45brightT cells 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-CD28 and CyChrome-conjugated anti-CD3, by flow cytometry (FACS Vantage, Becton Dickinson). Only preparations with purity >98% were used for experiments.

Lymphokine production

Purified CD8+ T cell subsets (3 x 105 cells/well) were cultured in 24-well plates in complete medium (RPMI 1640 supplemented with 2 m M t-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% of heat-inactivated FCS) and stimulated or not with 10 ng/ml PMA plus 1 µM ionomycin in the presence of 1 µ M 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 harvesting (21). Plates were washed twice in PBS, then resuspended to 1 x 109 in 5% CO2 in air, and cells were collected for lymphokine harvesting 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 pore-forming agent. After 15 min at 10°C. After two washes in PBS, the cells were resuspended to 1 x 109 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.

Cytotoxicity assay

The cytotoxic activity of CD8+ T cell subsets was evaluated in an anti-CD3 redirected cytoxicity assay as previously described (23). Briefly, 5 x 103 Fc receptor-bearing P815 target cells were labeled with 50 µCi of Na125I for 2 h at 37°C. Cells were then washed three times and incubated for 30 min at 4°C in the presence of the absence of 2 µg of anti-CD3 mAb. Purified CD8+ T cells were stained for CD11b, CD16, and CD28, but only CD16 cells were gated and sorted according to their CD11b and CD28 phenotypes. Cells were incubated for 4 h at 37°C with 5 x 105 P815 target cells at E:T cell ratios ranging from 20:1 to 1. Supernatants were then collected and counted. Specific cytotoxicity was calculated as follows: cpm of experimental release – cpm of spontaneous release/cpm of maximum release – cpm of spontaneous release x 100. The SEM of the mean percentage lysis never exceeded 5%.

Lymphocyte adhesion to human microvascular endothelial cell cultures

Primary cultures of human adrenal gland capillary endothelial cells (HACECs) were obtained as previously described (24). The endothelial cells were cultured on collagen-coated 96-well plates at a concentration of 5 x 103/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 x 104 lymphocytes in 100 µl EBM containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% of heat-inactivated FCS). After 24 h, the plates were washed with PBS and then fixed in 1% PFA in PBS, pH 7.2, and stained with FITC-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, anti-CD45RO, anti-CD57, anti-perforin, anti-IL-2, PerCP-conjugated anti-CD3, and CyChrome-conjugated anti-CD28 were all supplied by Ortho (Raritan, NJ). FITC- or PE-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, anti-CD45RO, anti-CD57, anti-perforin, anti-IL-2, PerCP-conjugated anti-CD3, 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.

Chemotaxis and migration assays

All migration assays were performed in collagen-coated 24-well Transwell 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 x 105) were placed in the upper compartment. Transwell inserts were maintained 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 x200 magnification. Each test was run in quadruplicate.

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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 and counted as described above. Tests were run in triplicate.

**Long term culture of CD8+ T cells**

Purified CD8+CD28+ T cells (1×10^6) 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 (105 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+CD28+CD11b+ elements in healthy individuals and in patients suffering from various viral infections**

The presence of CD8+CD28+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, CD11b, and CD28 revealed three subsets of CD8+ T lymphocytes: a CD28+CD11b− subset and two CD11b+ subsets (CD28+CD11b+ and CD28−CD11b+). As shown in Table 1, in healthy individuals the CD8+CD28+CD11b+ subset prevailed over CD28−CD11b+, whereas the CD28−CD11b+ subset was only barely present. In patients suffering from acute viral infections (n = 18) we observed a significant increase (p < 0.01) in CD11b+ cells compared with healthy individuals (n = 25; mean, 41.9 ± 14.2 and 29.0 ± 13.9%, respectively). The increase in CD11b+ cells was generally found to be linked to the CD28+ subset. In fact, the percentage of CD28−CD11b+ lymphocytes in patients exhibited an average 3.4-fold increment compared with healthy donors, whereas the percentage of CD28−CD11b+ cells showed no significant difference between the two groups (Table I). The increase in CD28+CD11b+ cells depended on the virus causing the disease (Fig. 1). At the onset of symptoms, EBV consistently elicited the largest increase in the CD28+CD11b+ 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 CD11b+ cells to normal levels as their disease gradually resolved (data not shown).

**Phenotypic characterization of CD8+CD11b+ T cells**

T cell subsets bearing different surface receptors may also display different functions. The dramatic increase in the percentage of CD28+CD11b+ 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+CD11b+ subset using CD45RA and CD45RO isofom expression, respectively, revealed that most of these cells had a memory CD45RO+ phenotype, whereas considerable heterogeneity was observed within CD28−CD11b− and
IL-2 protein expression as described in Materials and Methods was evaluated 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.

CD28^− CD11b^+ populations. The CD28^− CD11b^+ population exhibited features of Ag-primed cells, because CD18 was up-regulated (16, 25) compared with CD28^+ CD11b^− (mean fluorescence intensity [MFI], 99 and 65, respectively) but expressed at less intensity than on CD28^+ CD11b^+ cells (MFI, 120). This population, however, did not show a fully differentiated activated effector phenotype. Indeed, while a high percentage of CD28^+ CD11b^+ cells (71%) expressed the CD57 molecule, an effector cell-associated molecule (26), only 14% of CD28^− CD11b^+ cells were CD57^+$, a percentage almost comparable to that of CD28^+ CD11b^+ lymphocytes (9%). These results suggest that CD28^− CD11b^+ cells may be an intermediate phenotype between CD28^+ CD11b^− and CD28^+ CD11b^+ T cells.

The cytokine-producing capacity of CD8^+ T cell subsets

Unlike naive/memory cells, which mainly synthesize IL-2, the ability to produce IFN-γ is a typical feature of effector T cells (27, 28). CD8^+ lymphocytes were purified from freshly collected PBMCs and stained for CD3, CD28, and CD11b. CD8^+ T cell subsets, namely, CD28^+ CD11b^−, CD28^− CD11b^+, and CD28^− CD11b^+, were then sorted by flow cytometry. The cytokine-producing capacity of CD8^+ cell subsets was measured after stimulation for 12 h with PMA and ionomycin at the single-cell level. As shown in Fig. 2A, the CD28^+ CD11b^− 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 CD28^+ CD11b^+ than in the CD28^+ CD11b^− subset (mean, 81.3 ± 18.0 and 23.9 ± 11.5%, respectively). Notably, CD28^+ CD11b^− 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, CD28^+ CD11b^− CD28^+ CD11b^+ T cells mainly produced IL-2 and IFN-γ, respectively, whereas CD28^+ CD11b^+ 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^+ cell subsets was reflected by their proliferative capacities, the three CD8^+ subpopulations were stimulated with a combination of PHA and IL-2. As expected, CD28^+ CD11b^+ cells exhibited no proliferative activity in a [3H]TdR incorporation assay (6, 11). Instead, both CD28^+ CD11b^− and CD28^+ CD11b^+ 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.

CD28^+ CD11b^+ 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

Table II. Cytokine secretion by CD8^+ T cell subsets^a

<table>
<thead>
<tr>
<th>Cells</th>
<th>IL-2 (U/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28^+ CD11b^−</td>
<td>205.8 ± 152.3</td>
<td>2961 ± 1269</td>
</tr>
<tr>
<td>CD28^+ CD11b^+</td>
<td>523.4 ± 287.4</td>
<td>9604 ± 3281</td>
</tr>
<tr>
<td>CD28^− CD11b^+</td>
<td>30.9 ± 28.6</td>
<td>9612 ± 2818</td>
</tr>
</tbody>
</table>

^a Sorted cells (7.5 × 10^5) 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.

FIGURE 1. Flow cytometric analysis of CD28 and CD11b expression. Blood was obtained from a representative healthy donor (A) and, at the onset of symptoms, from patients suffering from infectious mononucleosis (B), varicella (C), and measles (D). An electronic gate was set on CD8^+ T cells, and the lymphocytes were evaluated for expression of CD28 and CD11b markers. Data were analyzed using CellQuest software and are displayed as bivariate dot plots. The percentage of cells in each quadrant is given in the upper right corner of each panel.

FIGURE 2. A, Flow cytometric detection of cytokine production. Purified CD8^+ lymphocyte subsets from a representative healthy donor were stimulated with PMA (10 ng/ml) plus ionomycin (1 μM) in the presence of monensin (1 μM). Cells were then fixed, permeabilized, and stained for IFN-γ and IL-2 protein expression as described in Materials and Methods. Histograms show the staining for the intracellular IL-2 (solid line) and IFN-γ (dotted line). B, Proliferation of CD8^+ T cell subsets. CD28^+ CD11b^− (●), CD28^+ CD11b^+ (■), and CD28^− CD11b^+ (▲) T cells from five healthy donors were purified and activated with PHA (5 μg/ml) in the presence of IL-2 (10 U/ml). The proliferative response was measured by [3H]TdR incorporation. Results are the mean ± SD of all the experiments performed.
CD8\(^+\)CD28\(^-\)CD11b\(^+\) T cells with effector features

IFN-γ and develop in vivo the enzymatic machinery necessary for the exocytic pathways of cytolysis (29, 30). Because the three CD8\(^+\) subpopulations differed for cytokine production, we investigated whether there were also differences in intracellular molecules involved in cytolyis, 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\(^+\) subpopulations were evaluated using freshly purified lymphocytes as effector cells and the mouse cell line P815 as a nonspecific target in a CD3-redirected cytotoxicity 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).

**FIGURE 3.** A, Expression of perforin in CD8\(^+\) T cell subsets. After purification from freshly collected PBMCs, CD8\(^+\) cells were stained with anti-CD28 and anti-CD11b mAbs. Cells were then fixed, permeabilized, and stained for intracellular perforin. The histogram shows the staining for the intracellular protein. The solid line indicates perforin-expressing CD28\(^-\)CD11b\(^-\) cells, whereas the bold and dotted lines show perforin-expressing CD28\(^+\)CD11b\(^-\) and CD28\(^+\)CD11b\(^+\) cells, respectively. B, Cytotoxic capacity of CD8\(^+\) subpopulations. Purified CD8\(^+\) T cell subsets, namely CD28\(^+\)CD11b\(^-\) (□), CD28\(^+\)CD11b\(^+\) (●), and CD28\(^-\)CD11b\(^+\) (▲), were directly analyzed for cytotoxicity against P815 target cells in the presence of anti-CD3 mAb in a 4-h ⁵¹Cr release assay. Results are the mean of at least three independent experiments.

**FIGURE 4.** Binding of purified CD8\(^+\) T cell subsets to HACECs. CD8\(^+\) T cell subsets were purified from the blood samples of five healthy donors and allowed to interact with a monolayer of 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 quadruplicate. Bars represent the mean number of bound CD28\(^+\)CD11b\(^-\) (□), CD28\(^+\)CD11b\(^+\) (●), and CD28\(^-\)CD11b\(^+\) (▲) lymphocytes per microscopic field ± SD.

**FIGURE 5.** A, Migration of freshly isolated and purified CD8\(^+\) T cell subsets. The number of cells migrating into the bottom well in the absence or presence of MIP-1α 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 chemotactic agent from the number of cells migrating in response to MIP-1α. Each experiment is representative of results obtained with cells from three independent healthy donors. B, Transendothelial migration of CD8\(^+\) T cell subsets. The number of cells migrated through TNF-α-activated HACECs in the absence or the presence of MIP-1α was evaluated as described above. The experiment is representative of results obtained with cells from three independent healthy donors.
tracted by the chemokine, whereas CD28\(^{+}\)CD11b\(^{-}\) cells were unable to respond to chemotactic stimulus.

Finally, we looked at the ability of CD8\(^{+}\) subsets to perform transendothelial migration. Being unable to interact efficiently with HACECs or to migrate at optimal chemotactic concentrations of MIP-1\(\alpha\), CD28\(^{+}\)CD11b\(^{-}\) T cells expectedly failed to perform active transendothelial migration (Fig. 5B). Instead, the two CD11b\(^{+}\) 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\(^{+}\)CD11b\(^{+}\) and CD28\(^{-}\)CD11b\(^{+}\) cells after MIP-1\(\alpha\) 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\(^{+}\) and CD28\(^{-}\) phenotypes from CD8\(^{+}\)CD28\(^{+}\) T lymphocytes**

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

**Discussion**

We have demonstrated the occurrence of an unreported CD8\(^{-}\)CD28\(^{+}\)CD11b\(^{+}\) 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\(^{+}\) and CD28\(^{-}\) cells. IFN-\(\gamma\) 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\(^{-}\) T cells were CD28\(^{+}\)CD11b\(^{-}\), with a massive presence of CD45RO\(^{+}\) cells within the subset. Long term cultures of purified CD8\(^{+}\)CD28\(^{+}\)CD11b\(^{-}\) cells, originally containing both CD45RA\(^{-}\)

![FIGURE 6. Modulation of CD11b and CD28 marker expression on CD8\(^{+}\)CD28\(^{+}\)CD11b\(^{-}\) T cells. Flow cytometrically sorted CD8\(^{+}\)CD28\(^{+}\)CD11b\(^{-}\) T cells from a representative healthy donor (A) were harvested at 14 (A), 21 (B), and 35 (C) days from the beginning of culture and stained for CD3, CD11b, and CD28. Samples were analyzed by three-color flow cytometry. All cells showed a CD3\(^{+}\) phenotype at all test times. Data are given as bivariate dot plots, and the percentages of CD28\(^{+}\)CD11b\(^{-}\), CD28\(^{+}\)CD11b\(^{-}\), and CD28\(^{+}\)CD11b\(^{-}\) T cells are shown in the upper right corner of each panel. Lymphocytes of a representative cord blood sample were harvested at 14 (D), 35 (E), and 52 (F) days from the beginning of culture and stained for CD8, CD11b, and CD28. An electronic gate was set on CD8\(^{bright}\) T cells, and lymphocytes were then evaluated for the expression of CD28 and CD11b markers. Data are given as described above.](http://www.jimmunol.org/)

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**FIGURE 6.** Modulation of CD11b and CD28 marker expression on CD8\(^{+}\)CD28\(^{+}\)CD11b\(^{-}\) T cells. Flow cytometrically sorted CD8\(^{+}\)CD28\(^{+}\)CD11b\(^{-}\) T cells from a representative healthy donor (A) were harvested at 14 (A), 21 (B), and 35 (C) days from the beginning of culture and stained for CD3, CD11b, and CD28. Samples were analyzed by three-color flow cytometry. All cells showed a CD3\(^{+}\) phenotype at all test times. Data are given as bivariate dot plots, and the percentages of CD28\(^{+}\)CD11b\(^{-}\), CD28\(^{+}\)CD11b\(^{-}\), and CD28\(^{+}\)CD11b\(^{-}\) T cells are shown in the upper right corner of each panel. Lymphocytes of a representative cord blood sample were harvested at 14 (D), 35 (E), and 52 (F) days from the beginning of culture and stained for CD8, CD11b, and CD28. An electronic gate was set on CD8\(^{bright}\) T cells, and lymphocytes were then evaluated for the expression of CD28 and CD11b markers. Data are given as described above.
CD45RO, so there was no way to distinguish between the two. Our suggestion may acquire an activated CD11b molecule in both memory and effector cells, within the CD28 T cell population, which gradually acquired CD11b expression and CD45RO phenotype. Indeed, CD28 T cells regardless of their CD45 isotype expression level of CD28 and CD11b can discriminate among three subsets: naive, central memory, and effector memory T cells. The acquisition of CD11b molecules identifies CD28 T cells with distinct functional properties. The acquisition of CD11b molecules identifies CD28 T 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 T 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.

Acknowledgments
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References

Table III. Modulation of CD11b and CD28 marker expression on CD8+CD28+CD11b+ T cells

<table>
<thead>
<tr>
<th>CD28+CD11b+</th>
<th>CD28+CD11b+</th>
<th>CD28+CD11b+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy donors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CD28+CD11b+</td>
<td>74.1</td>
<td>60.6</td>
</tr>
<tr>
<td>CD28+CD11b+</td>
<td>43.6</td>
<td>28.3</td>
</tr>
<tr>
<td>CD28+CD11b+</td>
<td>55.2</td>
<td>36.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>69.8 ± 10.7</td>
<td>53.0 ± 16.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cells were harvested at different weeks from beginning of culture. Cells were stained and analyzed as in Fig. 6. Data are shown as a percentage of positive cells within the total CD8+ T cells.

**Significant differences compared to the other groups within the same subset.

**Significant differences compared to the first group within the same subset.

**Significant differences compared to the second group within the same subset.

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 could 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 human CD8+ 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 effector cells, but the difficulty with this model is that both memory and effector cells, within the CD8+ subset, express CD45RO, so there was no way to distinguish between the two. Our data demonstrate that the early stages of CD8+ memory differentiation into effector cells are characterized by acquisition of a CD11b+ phenotype. Indeed, CD8+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.