Perforin-low memory CD8$^+$ cells are the predominant T cells in normal humans that synthesize the β-chemokine macrophage inflammatory protein-1β

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The synthesis of antiviral β-chemokines has joined cytolysis as a potential mechanism for the control of HIV-1 infection by CD8$^+$ T cells. Recent evidence suggests that these two effector functions can diverge in some individuals infected with HIV-1; however, little is known about the CD8$^+$ T cell subsets in normal individuals that synthesize antiviral β-chemokines. In this report, we have used mutliparameter flow cytometry to characterize the T cell subsets that secrete the antiviral β-chemokine macrophage inflammatory protein (MIP)-1β. These studies have shown: (*i*) CD8$^+$ cells are the predominant T cell subset that synthesizes MIP-1β; (*ii*) MIP-1β and IFN-γ are synthesized congruently in most CD8$^+$ T cells; however, significant numbers of these cells synthesize only one of these effector molecules; (*iii*) approximately 60% of the CD8$^+$ T cells that synthesize MIP-1β lack perforin; (*iv*) MIP-1β is synthesized with approximately equal frequency by CD28$^+$ and CD28$^-$ subpopulations of CD8$^+$ T cells; (*v*) MIP-1β is synthesized by three distinct CD8$^+$ T cell subsets defined by the expression of CD45R0 and CD62L; and (*vi*) MIP-1β is not synthesized in short-term cultures of naive CD8$^+$ T cells. These results demonstrate substantial subset heterogeneity of MIP-1β synthesis among CD8$^+$ T cells and suggest that these subsets should be evaluated as correlates of protective immunity against HIV-1.

The discovery that β-chemokines inhibit HIV-1 infection *in vitro* (1) raised the possibility that these molecules contribute to protective immunity against HIV-1 *in vivo*. This possibility is supported by three kinds of studies. First, production of β-chemokines by CD8$^+$ T cells correlates with protective immunity elicited by prototype HIV-1 vaccines in rhesus macaques (2–5). Second, β-chemokines produced by peripheral blood mononuclear cells (6, 7), CD8$^+$ T cells (8) activated with phytohemagglutinin A, or specific antigen (9) correlate with favorable prognosis in HIV-1 infection. Third, β-chemokines produced by peripheral blood mononuclear cells activated with phytohemagglutinin (10) or specific antigen (11) correlate with protection in hemophiliacs exposed to contaminated clotting factor and in infants born of HIV-1-infected mothers, respectively. These studies strongly support the hypothesis that antiviral β-chemokines contribute to protective immunity against HIV-1 *in vivo*. More rigorous testing of this hypothesis requires knowing whether the T cell subsets that secrete β-chemokines are the same as those having known effector functions such as cytotoxic T lymphocyte activity.

It is known that HIV-1-specific CD8$^+$ T cell clones that exhibit cytotoxic T lymphocyte (CTL) activity secrete β-chemokines upon encounter with sensitized target cells (12, 13). Those studies suggest that β-chemokines are synthesized by normal CD8$^+$ T cells that have a CTL effector phenotype. Thus, it is possible that β-chemokines contribute to the correlations between CTL and the reduction of viral loads (14–18) or protection against infection with HIV-1 (19–22).

It is also possible that β-chemokines are synthesized by CD8$^+$ T cell subsets that have phenotypes distinct from effector CTL (23–25). If this were so, quantifying these subsets in studies of infected individuals should provide new insights on how CD8$^+$ T cells protect against HIV-1. The need for such studies is illustrated best by example. Most studies reporting an inverse relationship between CTLs and initial HIV-1 viremia used CTL precursor assays as the readout (14, 15, 26), which do not measure effector CTLs directly. These assays quantify the ability of memory CTL to differentiate into effector CTLs *in vitro* and are not direct measures of CTL activity extant at the initiation of culture. It is possible that the frequencies of memory CD8$^+$ T cells that can differentiate into effector CTLs in this assay correlate with the control of HIV-1 viremia; yet these cells do not express CTL effector function without expansion *in vitro*. This scenario would require that such cells inhibit HIV-1 infection by nonlytic mechanisms such as the antiviral β-chemokines (1). Intriguingly, a recent report shows that nonlytic CD8$^+$ T cells specific for HIV-1 can express antiviral β-chemokines (27). Most importantly, in the same HIV-1-infected individuals, most of the CD8$^+$ T cells specific for cytomegalovirus antigens that secrete antiviral β-chemokines had a lytic phenotype (27). This result suggests that under some conditions, the synthesis of antiviral β-chemokines can diverge from the lytic phenotype. Although these studies show that β-chemokine synthesis and the lytic phenotype can diverge in individuals infected with HIV-1, nothing is known about the relationship between these variables in normal individuals. This relationship is investigated below.

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Materials and Methods

Human Volunteers. All studies were conducted at the Institute of Human Virology (Baltimore, MD) under informed consent by using normal human volunteers recruited locally.

Reagents. The following fluorochrome-conjugated mAbs were obtained from BD Biosciences (San Jose, CA): allophycocyanin (APC) anti-CD3, phycoerythrin (PE) anti-CD4, PerCP anti-CD8, PE anti-CD69, APC anti-CD45R0, and PE anti-CD28. PE-conjugated mAbs specific for IFN-γ and perforin were obtained from PharMingen, and FITC-conjugated anti-

Abbreviations: CTL, cytotoxic T lymphocyte; MIP, macrophage inflammatory protein; SEB, staphylococcal enterotoxin B; PE, phycoerythrin.

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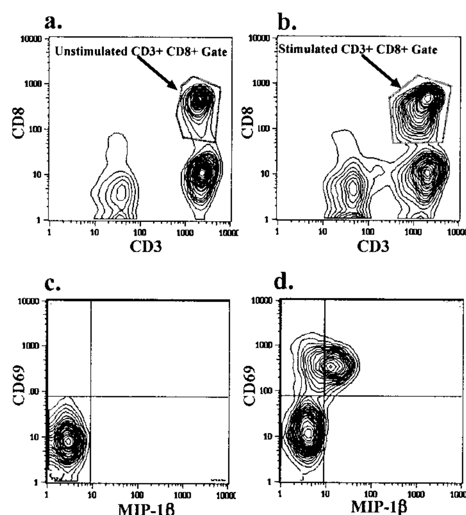

Fig. 1. Coexpression of CD69 and MIP-1β on CD8$^+$ T cells activated with SEB plus anti-CD28. Cultures were either unstimulated (*a* and *c*) or stimulated with SEB plus anti-CD28 (*b* and *d*). Stimulation with SEB plus anti-CD28 causes the concomitant down-regulation of CD3 and CD8 (compare *a* and *b*) and the coordinate up-regulation of CD69 and MIP-1β (compare *c* and *d*).

macrophage inflammatory protein (MIP)-1β was obtained from R & D Systems. Fluorochrome-conjugated isotype controls were obtained from each of the previously listed suppliers as appropriate for the test antibody. Reagents for erythrocyte lysis and fixation (FACS Lysis) and permeabilization (FACS-Perm) were obtained from BD Biosciences and used as described below. Staphylococcal enterotoxin B (SEB) was obtained from Sigma. The metalloproteinase inhibitor, KB8301, was obtained from PharMingen.

Lymphocyte Activation and Flow Cytometry. The method described in Suni *et al.* (28) was followed with minor modifications as noted in the text. Data were acquired by using a FACSCalibur flow cytometer (BD Biosciences) and analyzed by using FlowJo (Tree Star, San Carlos, CA).

Results

CD8$^+$ T Cells Are the Predominant T Cells in Human Peripheral Blood that Synthesize MIP-1β. The development of flow cytometry-based assays to assess antigen-specific lymphocyte function have focused largely on exogenous protein antigens that are presented via class II MHC-restricted CD4$^+$ T cells (28–32). Because our goal was to ask whether there is heterogeneity among CD8$^+$ T cells for MIP-1β synthesis, we used the superantigen, SEB, to activate both CD4$^+$ and CD8$^+$ T cells in the whole blood system (ref. 28 and reviewed in ref. 33).

An example of the data obtained for this system and the gating strategy to identify CD8$^+$ T cells are shown in Fig. 1. In this experiment, whole blood cultures were stimulated by using 10 μg/ml of SEB plus 1 μg/ml of anti-CD28. Control cultures were prepared identically except for the exclusion of SEB. After erythrocyte lysis and fixation in paraformaldehyde, the cells were permeablized, stained, and analyzed for CD3, CD8, CD4, CD69, and MIP-1β as described in *Materials and Methods*. As shown in Fig. 1 *a* and *b*, CD8$^+$ T cells were identified by costaining for CD3 and CD8. Stimulation with SEB plus anti-CD28 caused the down-regulation of both CD3 and CD8 (Fig. 1, compare *a* and *b*). Identical results were obtained when cultures were stimulated with SEB alone (not shown). By using these gates to identify CD8$^+$ T cells, representative bivariate histograms are shown in Fig. 1 *c* and *d* for MIP-1β synthesis

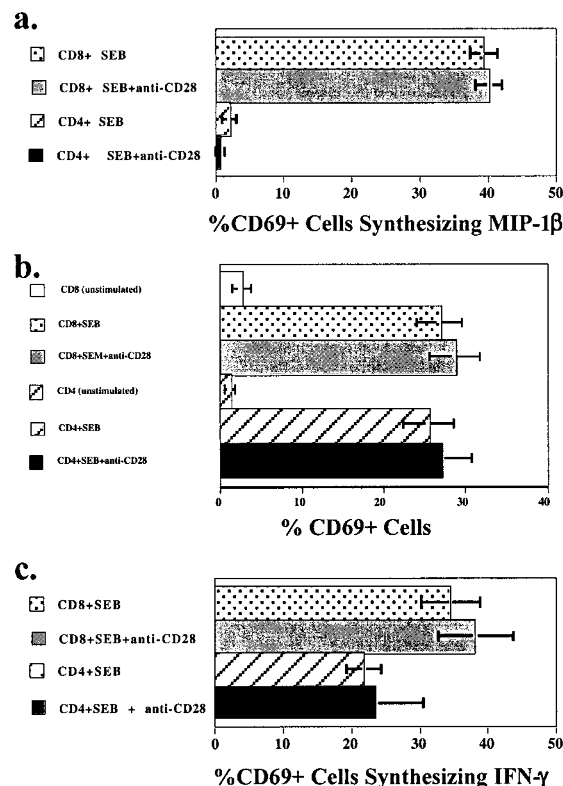

Fig. 2. CD8$^+$ T cells are the major T cell subset synthesizing MIP-1β in response to stimulation with SEB or SEB plus anti-CD28. (*a*) The synthesis of MIP-1β by either CD8$^+$ or CD4$^+$ T cells that are also CD69$^+$. (*b*) The percentage of CD8$^+$ or CD4$^+$ T cells expressing CD69 with no stimulation or stimulation with SEB or SEB plus anti-CD28. (*c*) The synthesis of IFN-γ by either CD8$^+$ or CD4$^+$ T cells that are also CD69$^+$.

(abscissa) vs. the expression of the activation antigen CD69 (ordinate). As shown in Fig. 1*c*, all of the CD8$^+$ T cells that synthesize MIP-1β co-express CD69. Accordingly, we used the frequency of CD69$^+$ cells as the denominator to compare the synthesis of MIP-1β among CD8$^+$ and CD4$^+$ T cells from nine normal volunteers (Fig. 2). In these experiments, the same gating strategy used above for CD8$^+$ T cells was used for CD4$^+$ T cells with the exception that CD4 is not down-regulated in stimulated cells (data not shown).

As shown in Fig. 2, whole blood cultures were left unstimulated or they were stimulated with either SEB or SEB plus anti-CD28, and the percentage of CD69$^+$ cells that are also MIP-1β^+ was determined. In Fig. 2*a*, 34.4% $\pm$ 4.3% (mean $\pm$ SEM, $n = 9$) of the CD69$^+$ cells stimulated with SEB-synthesized MIP-1β. Similarly, 38.2% $\pm$ 5.4% (mean $\pm$ SEM, $n = 9$) of the CD69$^+$ cells stimulated with SEB plus anti-CD28 synthesized MIP-1β. By contrast, as shown in Fig. 2*a*, the frequencies of activated (i.e., CD69$^+$) MIP-1β^+ cells in the CD4 subset were $<$10% of those observed for CD8$^+$ T cells. The values were 1.9% $\pm$ 0.4% (mean $\pm$ SEM, $n = 10$) for CD4$^+$ T cells stimulated with SEB and 2.2% $\pm$ 0.6% (mean $\pm$ SEM, $n = 9$). Taken together, these data show that CD8$^+$ T cells are the predominant T cell subset in peripheral blood that synthesizes MIP-1β in response to SEB or SEB plus anti-CD28. This pattern also is seen when peripheral blood lymphocytes are stimulated with phytohemagglutinin, anti-CD3, phorbol 12-myristate 13-acetate plus ionomycin, and, in preliminary studies, antigen-specific stimulation (data not shown).

It was important to determine whether the differences in MIP-1β synthesis between CD8$^+$ and CD4$^+$ T cells reflects a

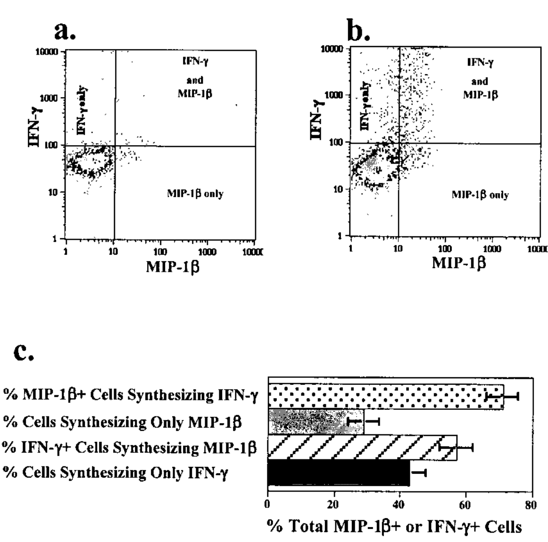

Fig. 3. The synthesis of MIP-1β and IFN-γ by CD8$^+$ T cells activated with SEB plus anti-CD28 is overlapping but not congruent. (*a* and *b*) The synthesis of MIP-1β (abscissa) and IFN-γ (ordinate) by unstimulated and stimulated CD8$^+$ T cells, respectively. (*c*) The fraction of CD8$^+$ T cells that synthesize MIP-1β and IFN-γ or each mediator alone. Each panel represents nine normal volunteers.

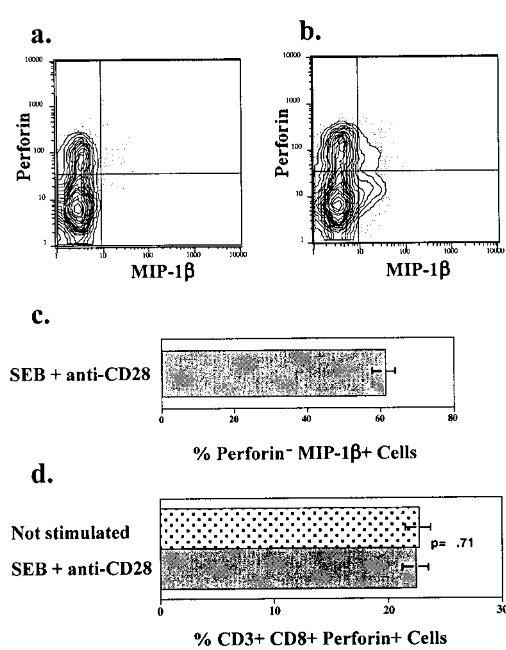

Fig. 4. CD8$^+$ T cells that synthesize MIP-1β in response to SEB plus anti-CD28 are heterogeneous in perforin expression. (*a* and *b*) Representative bivariate histograms comparing MIP-1β synthesis (abscissa) and perforin expression (ordinate) in unstimulated and stimulated cells, respectively. (*c*) The fraction of CD8$^+$ T cells stimulated by SEB plus anti-CD28 that are perforin negative as determined in *a* and *b* for 13 volunteers. (*d*) The percentages of CD8$^+$ T cells that express perforin with and without activation.

true difference between the subsets or simply the poor responsiveness of the CD4$^+$ T cells in this activation system. Two observations favor a true difference between CD8$^+$ and CD4$^+$ T cells. First, as judged by the expression of the activation antigen, CD69, both CD8$^+$ and CD4$^+$ T cells responded equivalently in the culture system (Fig. 2*b*). For CD8$^+$ T cells the frequencies of CD69$^+$ cells were 2.8% ± 0.42% (mean ± SEM, $n = 9$) for unstimulated cells, 27.0% ± 2.7% (mean ± SEM, $n = 9$) for cells stimulated with SEB, and 28.8% ± 2.8% (mean ± SEM, $n = 9$) for cells stimulated with SEB plus anti-CD28. Similar levels of activation were found for CD4$^+$ T cells in which the values were 1.4% ± 0.1% (mean ± SEM, $n = 9$) for unstimulated cells, 25.7% ± 3.4% (mean ± SEM, $n = 9$) for cells stimulated with SEB, and 27.2% ± 3.4% (mean ± SEM, $n = 9$) for cells stimulated with SEB plus anti-CD28.

Second, high frequencies of CD69$^+$ cells synthesized IFN-γ in both the CD8$^+$ and CD4$^+$ T cell subsets (Fig. 2*c*). The frequencies of CD69$^+$ T cells synthesizing IFN-γ were ≈1.6-fold higher for CD8$^+$ T cells than for CD4$^+$ T cells (Fig. 2*c*). For CD8$^+$ T cells, the values were 34.4% ± 4.3% (mean ± SEM, $n = 9$) for cells stimulated with SEB and 38.2% ± 5.4% (mean ± SEM, $n = 9$) for cells stimulated with SEB plus anti-CD28. For CD4$^+$ T cells the values were 21.8% ± 2.6% (mean ± SEM, $n = 9$) for cells stimulated with SEB and 23.4% ± 7.5% (mean ± SEM, $n = 9$) for cells stimulated with SEB plus anti-CD28. Collectively, these data show that the low frequencies of CD4$^+$ T cells that synthesize MIP-1β are not due to poor activation of CD4$^+$ T cells. This finding strongly supports the conclusion that CD8$^+$ T cells are the principal T cell subset in whole blood that synthesizes MIP-1β in response to superantigens.

MIP-1β and IFN-γ Are Synthesized Congruently in Most, but Not All, CD8$^+$ T Cells. Because CD8$^+$ T cells synthesize both MIP-1β and IFN-γ in response to SEB or SEB plus anti-CD28, we determined whether synthesis of these two mediators is congruent. Cultures were stimulated with SEB or SEB plus anti-CD28 and the frequencies of CD8$^+$ T cells synthesizing MIP-1β, IFN-γ, or both MIP-1β and IFN-γ were determined. Because we found no significant differences between cultures stimulated with SEB or SEB plus anti-CD28 (Figs. 1 and 2, and unpublished studies), the data in subsequent experiments are reported for one stimulus.

A representative bivariate histogram is shown for the synthesis of MIP-1β and IFN-γ by CD8$^+$ T cells in Fig. 3. Fig. 3*a* depicts the synthesis of MIP-1β and IFN-γ by an unstimulated culture, and Fig. 3*b* depicts a culture stimulated with SEB plus anti-CD28. Three populations synthesizing one or both mediators are apparent in Fig. 3*b*. The frequencies of these populations were determined for nine volunteers (Fig. 3*c*). The frequency of MIP-1β^+ cells that also synthesized IFN-γ was 71.1% ± 4.8% (mean ± SEM, $n = 9$) and the frequency of MIP-1β^+ cells that did not synthesize IFN-γ was 27.3% ± 4.5% (mean ± SEM, $n = 9$). The frequency of IFN-γ^+ cells that synthesized MIP-1β was 43.8% ± 5.6% (mean ± SEM, $n = 9$), and the frequency of IFN-γ^+ cells that did not synthesize MIP-1β was 56.2% ± 5.5% (mean ± SEM, $n = 9$). These studies show that although MIP-1β and IFN-γ are frequently synthesized by the same cell, the synthesis of these two mediators also is frequently discordant.

CD8$^+$ T Cells that Synthesize MIP-1β Are Heterogeneous for the Expression of Perforin. The expression of perforin by CD8$^+$ T cells that synthesize MIP-1β was determined as described in *Materials and Methods*. As shown in Fig. 4*a*, no MIP-1β synthesis was observed in either perforin$^+$ or perforin$^-$ CD8$^+$ T cells that were not activated. By contrast, approximately the same frequencies of MIP-1β^+ cells were observed in both perforin$^+$ and perforin$^-$ CD8$^+$ T cells (Fig. 4*b*) activated with SEB. A summary of 13 normal volunteers showed that the frequency of MIP-1β^+ cells that do not coexpress perforin after stimulation was 61.4% ± 7.6% (mean ± SEM, $n = 13$) (Fig. 4*c*). Because activated effector CTL release granules that contain perforin (34, 35), it is possible that the lack of perforin staining in the majority of the cells that synthesize MIP-1β is due to degranulation upon stimulation. This appears not to be the case as activation does not reduce the frequency of cells staining with perforin. There was

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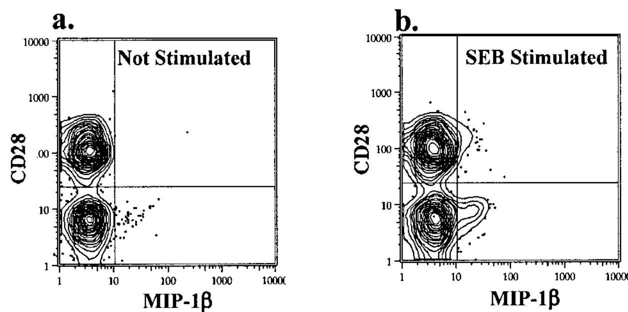

Fig. 5. CD8$^+$ T cells that synthesize MIP-1β in response to stimulation with SEB are both CD28$^+$ and CD28$^-$. (*a* and *b*) The synthesis of MIP-1β by CD8$^+$ T cells that are CD28$^+$ or CD28$^-$ either without stimulation or stimulation with SEB. The frequency of CD28$^+$ cells among CD8$^+$ T cells that synthesize MIP-1β was 50.3% ± 10.0% (mean ± SEM) for five normal volunteers (see text).

no statistically significant difference ($P = 0.71$, *t* test) for perforin staining between unstimulated cells [22.7% ± 4.5% (mean ± SEM, $n = 13$)] and stimulated cells [20.4% ± 4.1% (mean ± SEM, $n = 13$)], suggesting that degranulation leading to the loss of perforin staining is not detectable under the conditions studied here. These data strongly suggest that both lytic and nonlytic CD8$^+$ T cells are able to synthesize MIP-1β.

CD8$^+$ T Cells that Synthesize MIP-1β Are Heterogeneous for CD28. Because a significant proportion of the CD8$^+$ T cells that synthesize MIP-1β do not express perforin, we determined whether there is heterogeneity of CD28 that is differentially expressed between effector CTL and memory cells. Although both naive and certain memory cells express CD28, it is known that effector CTL do not express this marker (24). These experiments used SEB without anti-CD28 to avoid masking CD28. Fig. 5 *a* and *b* shows that approximately one-half of CD8$^+$ T cells that synthesize MIP-1β are CD28$^+$. The frequency of CD28$^+$ cells among CD8$^+$ T cells that synthesize MIP-1β was 50.3% ± 10.0% (mean ± SEM, $n = 5$). This result is consistent with the hypothesis that MIP-1β synthesis can occur in CD8$^+$ T cells that do not have an effector CTL phenotype (23, 24).

Synthesis of MIP-1β by CD8$^+$ Memory Subsets Defined by CD45R0 and CD62L. The synthesis of MIP-1β by naive and memory subsets was investigated by using CD45R0 and CD62L to define naive (CD45R0$^-$ CD62L$^+$), M1 (CD45R0$^+$ CD62L$^-$), M2 (CD45R0$^+$ CD62L$^+$), and M3 (CD45R0$^-$ CD62L$^-$) subsets similar to those described by Roederer and coworkers (36, 37) (Fig. 6*a*). Because CD62L is cleaved from the cell surface by metalloproteinases (38–41) shortly after activation, we included the metalloproteinase inhibitor KB8301 (42) at 100 μM in cultures stimulated with SEB plus anti-CD28. Inclusion of this inhibitor prevented the loss of CD62L$^+$ cells as compared to cultures stimulated in the absence of the inhibitor. Otherwise, the inhibitor did not affect activation by SEB plus anti-CD28 or SEB alone (data not shown).

Before measuring the synthesis of MIP-1β by the subsets defined by CD45R0 and CD62L, it was important to establish that each subset responds to SEB plus anti-CD28. As shown in Fig. 6 *b* and *c*, each subset responded to stimulation by the up-regulation of CD69. The synthesis of CD69 is depicted for each of the subsets by plotting it versus CD45R0 after gating on CD8 and the relevant subset. In the absence of stimulation, only the M1 subset exhibited some constitutive expression of CD69 (Fig. 6*b*). Poststimulation, each subset (including the naive subset) responded by up-regulating CD69 (Fig. 6*c*). As shown in Fig. 6*f*, a survey of five normal volunteers showed that each subset responded to approximately the same degree as judged by the up-regulation of CD69. The means ± SEMs for the subsets

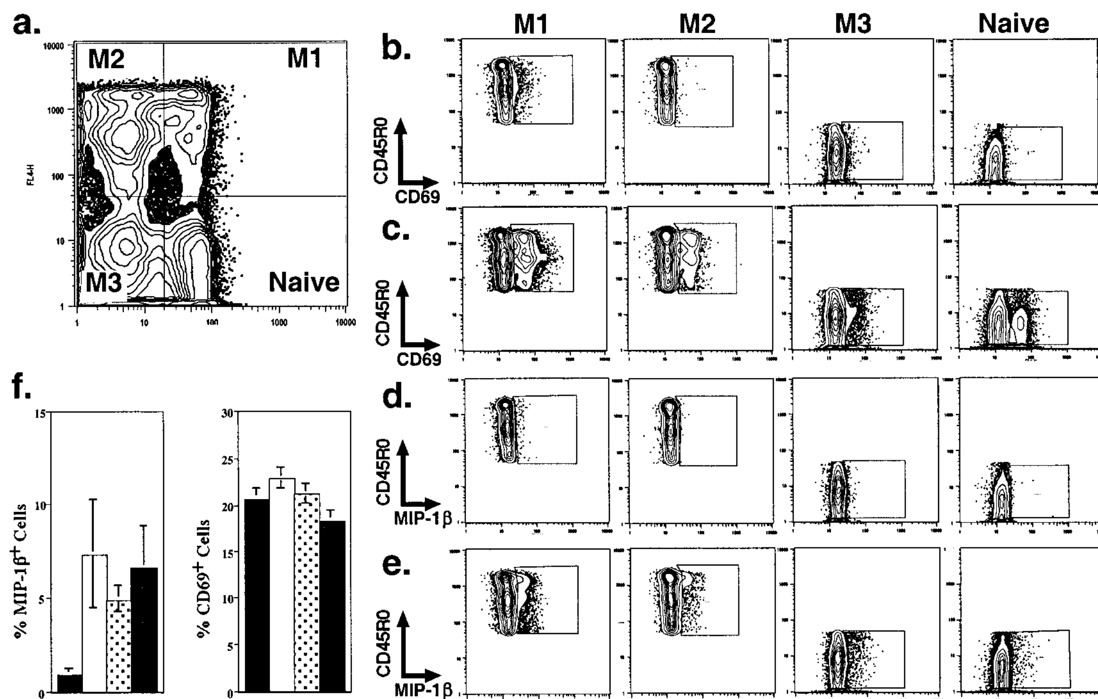

Fig. 6. CD8$^+$ memory subsets synthesize MIP-1β. (*a*) Memory subsets of CD8$^+$ T cells defined by CD45R0 and CD62L. (*b* and *d*) Depiction of the expression of CD69 or MIP-1β, respectively, by each of these subsets without stimulation. (*c* and *e*) Depiction of the expression of CD69 or MIP-1β, respectively, by each of these subsets after stimulation with SEB plus anti-CD28. (*f*) The expression of MIP-1β and CD69 for the M1, M2, M3, and naive CD8$^+$ T cell subsets activated with SEB plus anti-CD28. The means ± SEM are shown for five normal volunteers.

responding are as follows: naive = 20.6% ± 2.7%; M1 = 22.8% ± 4.2%; M2 = 21.1% ± 2.8%; and M3 = 18.3% ± 4.0%. Thus, naive $CD8^+$ T cells as well as the three memory subsets respond equivalently to SEB plus anti-CD28.

By contrast, only the memory $CD8^+$ T cell subsets synthesized MIP-1β upon activation. Representative bivariate histograms are shown in Fig. 6 *d* and *e* for unstimulated $CD8^+$ T cells and $CD8^+$ T cells stimulated with SEB plus anti-CD28, respectively. Analysis of the responses for the five normal volunteers showed that each memory subset responds to approximately the same degree as judged by MIP-1β synthesis (Fig. 6*e*). The means ± SEMs for the subsets synthesizing MIP-1β are as follows: naive = 0.86% ± 0.3%; M1 = 7.3% ± 2.9%; M2 = 4.9% ± 0.7%; and M3 = 6.6% ± 2.2%. Thus, multiple "activated/memory" $CD8^+$ T cell subsets synthesize MIP-1β in response to stimulation with SEB. Naïve $CD8^+$ T cells were marginally responsive, whether at all. Collectively, these data show that the naive subset responds as well as the memory subsets as judged by CD69 up-regulation and that only the memory subsets synthesize MIP-1β. Also, these data show that two of the three MIP-1β^+ populations express high levels of CD45R0, which is incompatible with them being effector CTL.

Discussion

The principal conclusion from the data presented above is that the antiviral β-chemokine, MIP-1β, is synthesized selectively by activated/memory $CD8^+$ T cells. This observation is significant in that the synthesis of MIP-1β has been reported for only $CD8^+$ T cell clones that exhibit effector CTL activity *in vitro* (12, 13). Three aspects of our study suggest that MIP-1β synthesis by $CD8^+$ T cells is not restricted to effector CTL. First, ≈60% of the cells that secrete MIP-1β have very low levels of perforin, if any at all. Second, approximately one-half of the cells that secrete MIP-1β express CD28 and the majority (≈70%) express CD45R0. These markers are expressed poorly by effector CTL (cf. ref. 24). Third, analysis of MIP-1β secretion by $CD8^+$ memory subsets defined by CD45R0 and CD62L showed that MIP-1β^+ cells are equally distributed among the M1, M2, and M3 subsets. By contrast, naive $CD8^+$ T cells synthesize MIP-1β poorly, if at all. The small numbers of naive $CD8^+$ T cells that synthesize MIP-1β are probably more apparent than real in that this subset contains small numbers of cells that have a surface phenotype that is inconsistent with their being truly naive (e.g., $CD11a^+$, $CCR5^+$, unpublished work). For this reason, the few "naive" cells that secrete MIP-1β probably belong to a memory subset that awaits definition.

The synthesis of MIP-1β equally among the M1, M2, and M3 subsets is of particular importance in that the M1 and M2 subsets are not effector CTL as defined by expression of CD45R0. Furthermore, the M2 subset is almost entirely $CD28^+$ (unpublished data). In other studies (unpublished data), we have found that the perforin$^+$ cells are distributed in a 40:60 ratio between the M1 and M3 subsets with very few being found in the M2 subset. Because MIP-1β^+ cells are found equally among these subsets, it is clear that both $CD8^+$ CTL effectors and nonlytic $CD8^+$ T cells can synthesize MIP-1β. This result strongly suggests that the frequencies of these subsets and their abilities to synthesize MIP-1β should be examined over the course of HIV infection using both antigen-specific and polyclonal stimuli. In preliminary studies, we have observed MIP-1β synthesis in the M1, M2, and M3 subsets in normal individuals responding to cytomegalovirus antigens and in HIV-1-infected individuals responding to HIV-1 antigens. In those studies there are clear differences among individuals with respect to the distribution of the subsets synthesizing MIP-1β. In the case of HIV-1-infected individuals, we predict that the ability to maintain strong β-chemokine synthesis by nonlytic $CD8^+$ T cells is a correlate of protective immunity against HIV-1. Taken together the data presented above show that at least one antiviral β-chemokine, MIP-1β, is synthesized by three memory subsets of $CD8^+$ T cells and not by naive $CD8^+$ T cells. Three additional aspects of this study warrant further discussion.

First, it is important to view the synthesis of MIP-1β in the context of the differentiation of $CD8^+$ T cells in response to antigen. Several recent studies (43, 44) suggest a linear model of differentiation for $CD8^+$ T cells in which memory cells derive from effector CTL that arise early in the immune response through an undefined pathway. This model provides a useful framework for understanding the role of β-chemokine synthesis in the normal functions of $CD8^+$ T cells and in immunity against HIV-1. In this model there are two major phases, the differentiation of naive $CD8^+$ T cells to effector CTLs and the differentiation of effector CTLs to memory $CD8^+$ T cells. The cell surface markers commonly used to define "memory" cells, including CD28, CD62L, and CD45R0, also define activation states of $CD8^+$ T cells. For this reason, we cannot discern which of the two phases harbors the perforin$^-$ $CD8^+$ T cells that secrete MIP-1β. Our data show clearly that ≈60% of the $CD8^+$ T cells that secrete MIP-1β lack perforin. Our studies also show that ≈70% of the $CD8^+$ T cells that secrete MIP-1β express CD45R0 and that ≈50% of these cells express CD28, indicating that these cells are not likely to be effector CTL. However, these markers do not allow us to place this subset in the two phases of $CD8^+$ T cell differentiation indicated above. It will be important to make this placement. Knowing how the synthesis of antiviral β-chemokines is controlled during the differentiation of nonlytic subpopulations of $CD8^+$ T cells can offer a new window on potential correlates of protective immunity against HIV-1 and on new strategies for vaccination against this virus.

Second, our studies show that $CD8^+$ T cells are the predominant T cell subset in normal human peripheral blood that synthesizes MIP-1β in response to SEB and other stimuli such as anti-CD3 plus anti-CD28 (unpublished data). In the studies described above, both $CD4^+$ and $CD8^+$ T cells were activated to the same degree as judged by the up-regulation of CD69 but the frequencies of $CD4^+$ T cells that secrete MIP-1β were less than 10% of those for $CD8^+$ T cells. In other studies, we have found that the frequencies of $CD4^+$ T cells that secrete MIP-1β can be increased by stimulation with anti-CD3 plus anti-CD28 as described (45). Even in that system the frequencies of $CD8^+$ T cells that secrete this β-chemokine are always substantially higher (unpublished data), indicating this relationship is intrinsic to the cells and not due to the stimulus used to activate them. Taken together, these studies show that although both $CD4^+$ and $CD8^+$ T cells can synthesize MIP-1β, the latter subset is programmed to do so more readily.

Third, we found that the synthesis of MIP-1β and IFN-γ often overlap but are not congruent. This observation is important in that IFN-γ enzyme-linked immunospot (ELISPOT) assays are rapidly replacing conventional CTL assays as measures of $CD8^+$ T cell effector function (46). Although our results in no way should temper this trend away from the cumbersome, poorly quantitative CTL assay, they do provide a strong indication that significant responses might be missed in confining routine ELISPOT assays to one cytokine. In addition to this technical caveat, it is very likely that the $CD8^+$ T cells that synthesize either MIP-1β or IFN-γ will differ in their biological potential from those that secrete both mediators.

In summary, our study shows that there is considerable heterogeneity among $CD8^+$ T cell subsets with respect to their abilities to synthesize the antiviral β-chemokine MIP-1β. Most importantly, we show that MIP-1β is clearly synthesized by a major population of $CD8^+$ T cells that has a phenotype that is not consistent with CTL effector function. This observation strongly suggests that nonlytic $CD8^+$ T cell subsets should be examined as correlates of protective immunity against HIV-1.

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