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Cytomegalovirus-specific responses of CD38⁺ memory T cells are skewed towards IFN- γ and dissociated from CD154 in HIV-1 infection

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Abstract

Objective—Despite the strong correlation of T-cell CD38 expression with HIV disease progression, evidence linking CD38 expression and dysfunction at the single cell level is scant. Since CD38⁺ memory CD4⁺ T cells, especially those from HIV-infected persons, fail to induce CD154 (CD40L) while responding to a superantigen with interferon (IFN)- γ or interleukin (IL)-2, we aimed to determine if recall responses to cytomegalovirus (CMV) were similarly affected in the CD38⁺ memory CD4⁺ T-cell subpopulation.

Design and methods—Peripheral blood mononuclear cells from HIV+ patients and healthy controls were incubated 14 h with CMV antigens, the superantigen *Staphylococcus aureus* enterotoxin B or medium, and labeled for identification of central memory (T_{CM}) and effector memory (T_{EM}) CD4⁺ T cells, and for the intracellular detection of induced CD154, IFN- γ and/or IL-2 by flow cytometry.

Results—Compared with CD38[−] cells, CD38⁺ T_{CM} cells from patients had less CD40L induction after CMV stimulation, and increased IFN- γ response. Patients' CD38⁺ T_{EM} cells showed a lower IL-2 response, and tended to have a greater IFN- γ response, in which CD154 induction frequently failed. CMV-specific responses of patients' CD38⁺ T_{CM} and T_{EM} cells were dominated by IFN- γ , and almost all IL-2⁺ cells co-expressed IFN- γ . IL-2 responses to the polyclonal activator *S. aureus* enterotoxin B were also significantly less frequent among CD38⁺ T_{CM} and T_{EM} cells than in CD38[−] cells.

Conclusion—Patients' CD38⁺ memory CD4⁺ T-cell responses to CMV favor the effector cytokine IFN- γ over IL-2, in the context of deficient CD154 induction, which may limit co-stimulation, proliferation and survival.

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Conflicts of interest

There are no conflicts of interest.

Keywords

AIDS; CD38 antigen; HIV; immune activation; immunologic memory; T cell

Introduction

CD38 expression on T cells stands out among biomarkers of chronic activation in HIV infection for its robust correlation with HIV disease progression [1], CD4⁺ T-cell loss [2–7], immune dysfunction [8–10] and even non-AIDS-defining illnesses [11–13], sometimes independently of viral control by HAART [5]. There is, however, scant evidence linking this marker or its function to dysfunction of T cells that express it [14,15]. We previously identified a dissociation of CD154 (CD40L) expression and cytokine production in CD38⁺ memory CD4⁺ T cells from HIV-infected persons after super-antigen *Staphylococcus aureus* enterotoxin B (SEB)-induced polyclonal activation [15]. Since SEB-induced activation may not reflect the same physiologic events as cognate peptide responses [16], and may include cells with a more diverse maturation history, we investigated here if CD38 expression on central memory (T_{CM}) or effector memory (T_{EM}) CD4⁺ T cells was associated with impaired expression of interleukin (IL)-2, interferon (IFN)- γ or CD154 in response to antigens of the prevalent opportunistic pathogen cytomegalovirus (CMV).

Methods

The study was approved by the institutional review board for human experimentation at Case Western Reserve University/University Hospitals Case Medical Center. Peripheral blood mononuclear cells were obtained from eight healthy serologically CMV⁺ controls and 19 HIV⁺ patients (14 males, four females and one transgender female, patients' and controls' numbers differing due to availability), with a median 420 CD4⁺ T cells/ μ l blood [interquartile range (IQR) 303–603] and median 17, 293 HIV RNA copies/ml plasma (IQR 8115–113 001). Patients were not receiving antiretroviral therapy and as noted, all were viremic, and most had reduced CD4⁺ T-cell counts. Cells were purified by density sedimentation (Ficoll-Paque, GE Healthcare Bio-Sciences AB, Piscataway, New Jersey, USA), and resuspended in complete RPMI 1640 medium (Lonza, Walkersville, Maryland, USA) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin (penicillin/streptomycin, Lonza), 2 mmol/l L-glutamine (Lonza), 5 mmol/l HEPES buffer (Lonza), and 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah, USA).

Two million cells were incubated in 1 ml complete medium and human cytomegalovirus (HCMV) lysate (1 : 20 dilution; BioWhittaker, Walkersville, Maryland, USA), or SEB (5 μ g; Sigma, St. Louis, Missouri, USA), and anti-CD28 agonistic monoclonal antibody (1 μ g, azide free, low endotoxin; BD Biosciences, San Jose, California, USA), or medium alone. In some experiments an HCMV-uninfected control lysate (Bio-Whittaker) was used (1 : 20 dilution), with no measurable response.

After 2 h incubation at 37°C, 10 μ g/ml Brefeldin A (Sigma) was added and incubation continued for 12 h. Cells were then stained using fluorochrome-labeled monoclonal antibodies against CD4 (Pacific Blue), CD45RA (APC), CCR7 (PE-Cy7), and CD38

(PerCP-Cy5.5) (BD Biosciences). Cells were fixed and permeabilized with BD FACSPerm solution (BD Biosciences), and stained with antibodies against CD154 (phycoerythrin) [17], and IL-2 [fluorescein isothiocyanate (FITC)], and/or IFN- γ (PE-Cy5 or FITC) (BD Biosciences). Cells were analyzed using an LSR-II flow cytometer (BD Biosciences with blue, violet, yellow-green and red laser configuration). Cytometry results were analyzed with FlowJo 9.3 (Tree Star Inc., Ashland, Oregon, USA). Single lymphocytes were gated according to light scattering, followed by CD4^{high} cells [18–20]. Gates for T_{CM} (CD45RA⁺CCR7⁺) and T_{EM} (CD45RA⁺CCR7⁺) were established using isotype controls (BD Biosciences). Gates for intracellular IL-2, IFN- γ , and CD154 were established with fully stained unstimulated cells (Supplementary Fig. 1, <http://links.lww.com/QAD/A459>). Surface levels of CD38 did not change after culture [15], and were thus representative of ex-vivo expression. Relevant variables were compared between groups using Mann–Whitney's *U* test. Responses of CD38⁺ and CD38[−] cells from the same groups were compared with Wilcoxon's signed-rank test (means and standard errors were used only for data visualization).

Results

HIV+ patients' T_{CM} and T_{EM} expressed CD38 more frequently than controls' cells (50.9 vs. 31.4%, $P=0.008$; and 34.9 vs. 14.2%, $P<0.001$, respectively, not shown). T_{CM} from both patients and controls were more frequently CD38⁺ than T_{EM} cells (50.9 vs. 34.9%, $P<0.001$; and 31.4 vs. 14.2%, $P=0.008$, respectively, not shown). There were no significant group differences in percentage of naive, T_{CM} and T_{EM} subpopulations among CD4⁺ T cells (not shown).

Patients' CD38⁺ T_{CM} expressed CD154 after CMV stimulation less frequently than did CD38[−] T_{CM} ($P=0.044$; Fig. 1a), an effect not found among controls' T_{CM}. In contrast, CMV stimulation induced IFN- γ in a greater percentage of CD38⁺ T_{CM} than in CD38[−] T_{CM}, in both patients and controls ($P=0.007$ and $P=0.025$, respectively; Fig. 1b).

A greater percentage of controls' CD38⁺ T_{CM} produced IL-2 after CMV stimulation than did CD38[−] cells ($P=0.012$; Fig. 1c), and patients' CD38⁺ T_{CM} tended to produce IL-2 more frequently than did CD38[−] T_{CM} ($P=0.052$). In contrast, among patients, CD38⁺ T_{EM} were less often IL-2 producers than were CD38[−] T_{EM} ($P=0.047$; Fig. 1f).

Additionally, after CMV stimulation, a sizable proportion of IFN- γ -producing T_{EM} from patients did not co-express CD154 (Fig. 1g), while virtually all IFN- γ + T_{EM} cells among controls expressed CD154. This proportion was significantly greater among CD38⁺ T_{EM} than among CD38[−] T_{EM} ($P=0.028$; Fig. 1g). (T_{CM} events were not enough for this analysis.)

Since CD38 expression was related to increased IFN- γ and decreased IL-2 responses to CMV, we investigated if the two effects were occurring at the single-cell level. We analyzed the co-expression of IFN- γ and IL-2 after stimulating with CMV or SEB in samples from 10 HIV+ patients. After CMV stimulation, the proportion of IFN- γ single positive CD38⁺ T_{EM} was 6.7 times greater than that of CD38[−] T_{EM} ($P=0.017$; Fig. 2a). And although IFN- γ -producing cells were 1/10 as frequent among T_{CM} than among T_{EM}, the frequency of

CD38⁺ T_{CM} responding to CMV with IFN- γ only (IFN- γ ⁺ IL-2⁻) was significantly greater than that of CD38⁻ T_{CM} ($P=0.007$; Fig. 2a). Cells responding to CMV by expressing IL-2 alone were almost undetectable, with almost all IL-2 production found in cells co-expressing IFN- γ . Even though there were no significant differences in percentage of IL-2⁺IFN- γ ⁺ between CD38⁺ and CD38⁻ cells, IFN- γ clearly dominated the response to CMV mainly among CD38⁺ cells (same bias as when comparing cytokine patterns as percentage of total cytokine-producing cells).

In contrast to the response to CMV, polyclonal stimulation with SEB did elicit sizeable frequencies of IL-2 single positive T_{CM} and T_{EM} (Fig. 2b). Here, a higher proportion of CD38⁺ T_{CM} were IFN- γ ⁺IL-2⁻, compared with CD38⁻ T_{CM} cells ($P=0.022$; Fig. 2b). This also happened in T_{EM} cells ($P=0.047$; Fig. 2b). Of note, fewer CD38⁺ T_{CM} were IL-2 single positive than were CD38⁻ T_{CM} ($P=0.050$). The same difference in the frequency of SEB-stimulated IL-2 production in the absence of IFN- γ was seen in CD38⁺ and CD38⁻ T_{EM} ($P=0.005$). CD38⁺ and CD38⁻ memory subsets did not differ significantly in their percentage of IFN- γ ⁺IL-2⁺ (double positive) cells after SEB stimulation.

Discussion

In an earlier study, we described a dissociation between CD154 expression and expression of IFN- γ and IL-2 in CD38⁺ T_{CM} and T_{EM} after stimulation with SEB [15]. Lymphocyte responses to SEB may not accurately reflect antigen-specific responses, since SEB induces polyclonal activation via nonspecific human leukocyte antigen–T-cell receptor (TCR) interaction [16], and can activate naive T cells, presumably more as a consequence of TCR structure than as a result of specific memory [21]. Therefore, in this study, we wanted to ascertain if the character of memory T-cell responses to cognate peptides derived from CMV was altered among CD38⁺ memory CD4⁺ T subpopulations.

Central memory T cells are characterized by an IL-2 response that typically exceeds the IFN- γ response [22,23], and is associated with the capacity to both expand and survive [24]. Unexpectedly, we found a predominant IFN- γ response to CMV in CD38⁺ T_{CM}, particularly those from patients, which was significantly greater than in CD38⁻ cells (Fig. 1b). Additionally, IFN- γ was co-expressed by nearly all IL-2-producing cells in response to CMV (Fig. 2a), and whereas the response to polyclonal activation by SEB was also dominated by IFN- γ (and was increased in CD38⁺ cells), there was still a sizable proportion of cells expressing only IL-2. Among HIV⁺ patients' T_{EM}, the CD38⁺ subset also had an imbalanced predominant IFN- γ response to both CMV and SEB (Fig. 1e and Fig. 2), and a less frequent IL-2 response than CD38⁻ T_{EM} cells (Fig. 1f and Fig. 2). A predominant IFN- γ response has been amply described in CMV-reactive cells [25,26], and in the CMV response in the setting of HIV infection [27–29]. Our original finding is that, in HIV⁺ patients, this IFN- γ -predominance occurs mainly among CD38⁺ cells.

Production of effector cytokines [IFN- γ and tumor necrosis factor (TNF)] has been found in T_{CM} responding to CMV, a persistent antigen, but not in T_{CM} responding to cleared antigens (tetanus toxoid and hepatitis B virus surface antigen) [30]. IFN- γ production has also been found in T_{CM} with a lower capacity to expand that differentiates from naive cells

primed with a strong antigenic stimulus [20]. Indeed, both CMV antigen persistence and abundance [31–33] can be expected in the setting of uncontrolled HIV infection, as can exposure to microbial products that also drive CD38 expression [34–36], thus contributing to this altered functionality of CD38⁺ memory CD4⁺ T cells. Additionally, IFN- γ production has been reported in a distinct T_{CM} subset committed to differentiate to Th1 effectors upon stimulation via TCR or with the homeostatic cytokines IL-7 and IL-15, but not in T_{CM} subsets that proliferate and self-renew [37]. In light of these studies, our findings raise the possibility that this CD38-related alteration in cytokine profile underlies the recognized alterations in memory CD4⁺ T-cell homeostasis in HIV infection, via a lowered proliferative capacity [38–40], and might suggest a greater degree of differentiation of CD38⁺CD4⁺ T_{CM} cells.

The altered cytokine responses occurred concurrently with a decreased induction of CD154 (CD40L) by CD38⁺ T_{CM} from HIV⁺ patients. Whereas this effect was not found among CD38⁺ T_{EM} from patients, this subset had an increased frequency of cells producing IFN γ but not co-expressing CD154 (IFN- γ ⁺CD154⁻). An impaired expression of CD154 can be expected to hamper CD154-CD40 signaling to and from antigen-presenting cells [41,42], a requirement for optimal CD4⁺ T-cell priming [43,44], APC activation and maturation, type 1 cytokine production [45,46], long-term immune memory [47], and cytotoxic memory cell expansion [48]. As an extreme example, the absence of functional CD154 in X-linked hyper-IgM syndrome causes defects in both humoral and cellular immune responses predisposing to opportunistic infections [49]. Plausibly, this also plays a role in the impaired T_{CM} response to CMV and other opportunistic pathogens in HIV infection.

A decreased expression of CD28 [23,50,51] does not explain the differential responsiveness of CD38⁺ cells as analysis of data from our earlier study [15] shows that whereas the proportion of CD28⁺ memory cells was diminished in HIV infection, the expression of CD28 was comparable in patients' CD38⁺ and CD38⁻ CD4⁺ memory populations (not shown). In summary, specific responses to CMV by CD38⁺ memory CD4⁺ T-cell subsets is biased towards IFN- γ expression, a diminished IL-2 response and is, in some instances, linked to a failure of CD154 induction. This could limit the co-stimulation, proliferation, and survival [52] of memory CD4⁺ T cells, and could contribute to a failure to control opportunistic pathogens like CMV in HIV⁺ patients. A bias towards IFN- γ responses to CMV and SEB characterizes CD38⁺ memory subpopulations in HIV infection and may contribute to the inflammatory environment characteristic of HIV disease.

Acknowledgments

Description of the role of each of the authors in this study:

G.O.-G.: Experimental design, experimental work, data collection, analysis and interpretation, manuscript writing; E.E.: Conception and design, statistical analysis, interpretation, manuscript writing; S.F.S.: Conception and design, critical review of the article; M.M.L.: Conception and design, analysis and interpretation, manuscript writing, patient recruitment.

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Fig. 1. Mean percentage expression of CD154 (a and d), IFN-γ (b and e) and IL-2 (c and f) in TCM (a to c) and TEM (d to f) after CMV stimulation. (g) Percentage of TEM cells producing IFN-γ⁺ without producing CD154 after CMV stimulation

PBMCs from 19 HIV+ and eight HIV- controls were cultured 12 h in complete medium with Brefeldin A and then stained for flow cytometry analysis. The CD38⁻ cells are on the left of each graph and CD38⁺ cells on the right. CMV, cytomegalovirus; IFN, interferon; IL, interleukin; PBMCs, peripheral blood mononuclear cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells. Error bars are ±1 SEM. White bars represent healthy donors, and grey bars represent HIV+ patients. (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$.

Fig. 2. Simultaneous or single production of IFN- γ and/or IL-2 after CMV stimulation (a) or SEB stimulation (b)

PBMCs from 10 HIV+ patients were cultured 12 h in complete medium with Brefeldin A with stimulation and agonistic anti-CD28 antibody, and then stained for flow cytometry analysis. Data correspond to an independent staining for IFN- γ /IL-2 co-expression, and was performed on cells from 10 out of the 19 patients whose data are shown in Fig. 1. Bar level corresponds to mean percentage. Bars with vertical lines depict IFN- γ ⁺IL-2⁻ cells, empty bars IFN- γ ⁻IL-2⁺ cells, and gridded bars show IFN- γ ⁺ IL-2⁺ (co-expressing) cells. CMV, cytomegalovirus; IFN, interferon; IL, interleukin; PBMCs, peripheral blood mononuclear cells; SEB, *Staphylococcus aureus* enterotoxin B; T_{CM}, central memory CD4⁺ T cells; T_{EM}, effector memory CD4⁺ T cells. (*) $P < 0.05$, (**) $P < 0.01$ significant differences in the cytokine pattern indicated by dotted lines.