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Effector Memory $\alpha\beta$ T Lymphocytes Can Express FcγRIIIa and Mediate Antibody-Dependent Cellular Cytotoxicity

Béatrice Clémenceau,*† Régine Vivien,*‡ Mathilde Berthomé,*‡ Nelly Robillard,‡ Richard Garand,‡ Géraldine Gallot,*† Solène Vollant,*† and Henri Vie‡*†

Human memory T cells are comprised of distinct populations with different homing potential and effector functions: central memory T cells that mount recall responses to Ags in secondary lymphoid organs, and effector memory T cells that confer immediate protection in peripheral tissues. In the present study we demonstrate that a proportion of effector memory T cells express FcγRIIIa (CD16), are perforin positive, and directly mediate Ab-dependent cytotoxicity ex vivo. This particular $\alpha\beta$ T lymphocyte subset has the morphology of large granular lymphocytes, increases proportionately in vivo during reactive lymphocytosis, and can be detected in vitro among EBV-specific T lymphocytes after stimulation with EBV Ags. Consequently, during a normal immune response, amplification of these effector memory T lymphocytes that are capable of Ab-dependent cytotoxicity may have beneficial or harmful consequences depending on the presence of pathogen- or tissue-specific Abs, respectively. The Journal of Immunology, 2008, 180: 5327–5334.

The vast majority of cells that express the IgG FcR responsible for Ab-dependent cellular cytotoxicity (ADCC; FcγRIIIa or CD16) belong to the innate immune system (1). These include monocytes/macrophages and NK cells. Among PBMC, it is assumed that essentially all ADCC activity is mediated by NK cells. Nevertheless, the existence of T lymphocytes bearing FcRs for Ig has long been recognized, although data concerning ADCC mediated by T lymphocytes are rare.

Indeed, in the past, initial attempts to discriminate subsets among T lymphocytes relied on the use of either antiserum or differential FcR binding. Using the latter approach, two human T cell subsets could be separated: those capable of binding the Fc portion of IgM (Tμ) and those capable of binding the Fc portion of IgG (Ty) (2–6). However, at that time, discrimination between different lymphocyte subsets was limited because the separation technique relied on density-gradient centrifugation of the subpopulations of lymphocytes that form rosettes with erythrocytes coated with rabbit IgG or IgM. Using this technique, the so-called “Ty” and “Tμ” populations were found to display different morphological, histochemical, and functional characteristics (7, 8). In particular, Ty cells have been shown to mediate ADCC (9). Later on, when mAb targeting lymphoid populations became available, it became apparent that the Tμ population was similar to the unfractinated T cell population with respect to reactivity with anti-CD3, -CD4, and -CD5 mAb (OKT3, OKT4, and OKT5, respectively) while the Ty-enriched population contained, in fact, only 10–20% of T cells as defined by OKT3 (10–12). The authors of these studies concluded appropriately that the Ty cells included a small population reactive with OKT3 and a large population reactive with OKM1, most likely corresponding to NK cells (10). Based on these findings, it was unclear whether ADCC attributed to the Ty subset was mediated by the CD3- (non-T) NK fraction or the small proportion of CD3+ T cells that express FcR for IgG. Later on, as stated above, essentially all NK activity present among PBMC was attributed to the NK population. In the early 1980s, several mAb were produced (anti-Leu-11, VEP 13, B73.1, and 3G8) that specifically reacted with the IgG FcR responsible for ADCC (FcγRIIIa, CD16) (13–16) and the presence in most individuals of cells with the CD3+CD16+ phenotype was confirmed (14, 17, 18). These cells usually comprise <2% of total PBL, with rare exceptions (18). In addition, it was specified that human T lymphocytes expressing the γδ TCR can express CD16 and mediate ADCC (19–21). In contrast, the link between the expression of CD16 and αβ TCR on T lymphocytes has remained unclear. Although cells from large granular lymphocyte leukemia have been shown to coexpress CD16 and an αβ TCR (22, 23), the existence of “normal” T cells harboring this phenotype has been suggested in only rare instances (24–27) and these cells have not been characterized. The present study was initiated to directly assess the proportion, the phenotype, and the functional capabilities of CD16+ αβ TCR T cells. We demonstrate that CD16+ αβ TCR T cells, which are present in small numbers in all individuals 1) belong to the memory effector subset, 2) are perforin positive, 3) are capable of mediating ADCC immediately ex vivo, 4) increase proportionately in vivo during lymphocytosis, and 5) appear in vitro in all cultures of EBV-specific CTL. Consequently, because of their ADCC potential and their presence among virus-specific T cells during a specific T cell response, amplification of these CD16+ αβ TCR effector memory T lymphocytes during a regular immune response may be involved in beneficial or harmful consequences depending on the presence of pathogen- or tissue-specific Abs.

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2 Address correspondence and reprint requests to Dr. Henri Vie, Institut National de la Santé et de la Recherche Médicale, Unité 601, 9 Quai Moncousu, 44093 Nantes cedex, France. E-mail address: hvie@nantes.inserm.fr
3 Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; BLCL, B lymphoblastoid cell line; LGL, large granular lymphocyte.
Materials and Methods

Samples and cell lines

PBMCs were prepared by Ficoll (PAA Laboratories) gradient centrifugation of blood obtained from adult volunteers or patients with hyperlymphocytosis that were recruited nonselectively from the Department of Infectious Diseases (Centre Hospitalier de l’Université de Nantes, Nantes, France). All individuals gave informed consent. EBV B lymphoblastoid cell lines (BLCL) were derived from PBMCs by in vitro infection using the EBV-containing culture supernatant from the Marmoset B95.8 cell line purchased from the American Type Culture Collection in the presence of 1 μg/ml cyclosporine A. The K562 cell line was cultured in complete medium consisting of RPMI 1640 (Sigma-Aldrich), 10% heat-inactivated FCS, 2 mM glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 10 μg/ml streptomycin (Sigma-Aldrich).

mAbs and flow cytometric analysis

The following mAbs and their isotype controls were used: anti-αβ-FITC (BMA031; Serotec), anti-CCR7-PE (150503; BD Systems), anti-CD28-PE (BD9.11), anti-CD27-PE (IA4), anti-CD45-RA-FITC (ALB11), anti-CD45-RO-FITC (UCHL1), anti-CD57-PE (NCI), anti-CD62L-PE (DREG56), anti-CD32-PE (2E1), and anti-CD64-PE (2E4) were all obtained from Beckman Coulter. Five hundred thousand (0.5 × 10⁶) PBMC or 50 μl of whole blood were incubated for 12 min at room temperature in 1-ml tubes in the presence of optimal concentrations of Abs diluted with PBS supplemented with 5% human serum. After staining, tubes were centrifuged, the supernatant was discarded, and cells were washed twice with 900 μl of PBS. Data were acquired by a FACSCalibur instrument (BD Biosciences) and analyzed using CellQuest software (BD Immunocytometry Systems). The absolute number of each population was then calculated from the total lymphocyte count within the blood sample determined using an automated cell counter (Sysmex HSTXE 2100; Roche). For intracellular staining of perforin, PBMC were stained with anti-TCR αβ-FITC and anti-CD16-PC5 and incubated in the dark for 15 min. The cells were then washed twice, fixed with 2% paraformaldehyde, permeabilized with 1% Phos Flow (BD Biosciences), and stained with anti-perforin-PE or isotype control-PE. After incubation, washing, and resuspension, the cells were analyzed using a FACSCalibur.

Cell sorting

PBMC (15 × 10⁶) were stained with PE-anti-αβ Ab (BMA031; Beckman Coulter) and PCs-anti-CD16 Ab (3G8; Beckman Coulter). Cell sorting was performed on a FACSVantage or FACSAria flow cytometer (BD Biosciences).

Cell cultures

Sorted CD16-expressing αβ T lymphocytes were cloned using a nonspecific amplification procedure: T cells were seeded at 3.0, 1.0, and 0.3 cells/well in 96-well U-bottom plates together with irradiated (35 Gy) pooled allogeneic feeder cells (1 × 10⁵ PBMC and 1 × 10⁴ B cells from a BLCL), 1 μg/ml leukoagglutinin PHA-L (Sigma-Aldrich), and 300 IU/ml IL-2 (Roussel-Uclaf) in a final volume of 200 μl. After cloning, T cells were further expanded: first, in 24-well culture plates, then in culture flasks using the same culture conditions (feeder cell concentration, IL-2, and PHA concentration) that allow for maximal proliferation of the T cell clones for several weeks.

Generation and expansion of EBV-specific cytotoxic cell lines

Donor PBMCs were plated in 24-well culture plates at 2 × 10⁶ cells/well in RPMI 1640 with glutamax (Invitrogen Life Technologies) culture medium supplemented with 8% pooled human serum, and stimulated with 5 × 10⁵ 40 Gray-irradiated autologous BLCL (PBMC:BLCL ratio of 40:1). After 10 days, T cells were collected and restimulated at a T:B ratio of 4:1 (5 × 10⁵ T cells and 1.25 × 10⁶ BLCL/well). IL-2 was added 4 days after the second stimulation (40 IU/ml). A third and a fourth stimulation were performed every 7 days in the presence of IL-2 and at the same T:B cell ratio (4:1).

Cytotoxicity assay

Cytotoxic activity was assessed using a standard ⁵¹Cr-release assay. Target cells were labeled with 100 μCi ⁵¹Cr for 1 h at 37°C. washed four times with culture medium, and then plated at the indicated E:T cell ratio in a 96-well plate. An autologous BLCL was used as a model of an autologous tumor and the chimeric anti-CD20 mAb rituximab (Roche) was used (at 2 μg/ml) to induce ADCC. In some experiments, the anti-Her2/neu mAb trastuzumab (Roche) was used (at 10 μg/ml) as a control. For ADCC assays, the indicated mAb was incubated with target cells for 20 min before addition of effector cells. After a 4-h incubation at 37°C, 25 μl of supernatant were removed from each well, mixed with 100 μl of scintillation fluid, and ⁵¹Cr activity was counted in a scintillation counter. Each test was performed in triplicate. The results are expressed as the percentage of lysis, which is calculated according to the following equation: (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100, where experimental release represents the mean cpm for the target cells in the presence of effector cells, spontaneous release represents the mean cpm for target cells incubated without effector cells, and maximal release represents the mean cpm for target cells incubated with 1% Triton X-100. For the assessment of ex vivo ADCC by CD16⁺ αβ T lymphocytes, cells positively sorted by the 3G8-PC5 Ab were washed and incubated overnight in complete medium (without any cytokines) before the functional ADCC assay.

Statistical analysis

Differences between subjects with hyperlymphocytosis and healthy controls were analyzed using the t test. A p value of <0.05 was considered significant.

Results

Frequency and phenotype of CD16⁺ αβ T lymphocytes in the blood of healthy donors

Direct staining of freshly isolated PBMCs with Abs to CD16, αβ TCR, and γδ TCR led to the identification of three subsets of CD16-expressing lymphocytes in all healthy adult donors tested (Fig. 1A): CD16⁺ NK cells, CD16⁺ αβ, and CD16⁺ γδ T lymphocytes. Analysis of absolute cell numbers for each subset within the PBLS revealed that there were 30 CD16⁺ αβ T lymphocytes/μl of blood (range 1–198); 40 CD16⁺ γδ T lymphocytes/μl of blood (range 0–218), and 194 CD16⁺ NK cells/μl of blood (range 50–577) (Fig. 1B, n = 26). Thus, altogether αβ and γδ T cells represented on average 25% of all CD16-expressing lymphocytes. Significant differences were found in terms of CD16 expression between these three populations: the mean fluorescence intensity for CD16 was 20 ± 7 for CD16⁺ αβ T lymphocytes, 56 ± 35 for CD16⁺ γδ T lymphocytes, and 503 ± 120 for CD16⁺ NK cells.

The CD16⁺ αβ T lymphocyte subset was further characterized for CD4 or CD8 expression and with a panel of mAbs that discriminates between different memory T lymphocyte subsets (CD27, CD28, CD45RO, CD45RA, CD57, CD62L, and CCR7). Staining for CD32a/CD32b (anti-FcγRIIa and -FcγRIIb), CD64 (anti-FcγRI), and three killer Ig-like receptors (CD158a,h, CD158b, and CD158e) was performed on a FACSVantage or FACSAria flow cytometer (BD Biosciences). The ADCC activity of CD16⁺ αβ T lymphocytes, and maximal release represents the mean cpm for target cells incubated without effector cells, and maximal release represents the mean cpm for target cells incubated with 1% Triton X-100. For the assessment of ex vivo ADCC by CD16⁺ αβ T lymphocytes, cells positively sorted by the 3G8-PC5 Ab were washed and incubated overnight in complete medium (without any cytokines) before the functional ADCC assay.

The CD16⁺ αβ T lymphocyte subset is capable of mediating ADCC ex vivo

The ADCC activity of CD16⁺ αβ T lymphocytes from three healthy donors was tested and compared with that of autologous NK cells. CD16⁺ αβ T lymphocytes, CD16⁺ NK cells, and CD16-negative αβ T lymphocytes from the same donor were
CD16+ αβ T lymphocytes from two healthy donors were sorted and cloned by limiting dilution using nonspecific stimulation (lectin plus feeder plus IL-2). Cloning efficiency was 0.75 and 0.30 according to the Poisson distribution (Fig. 3A). Nineteen days after cloning, 47 of 52 clones from donor 1 and 18 of 19 clones from donor 2 were CD16 positive. Next, 4 clones from donor 1 and 6 from donor 2 were selected according to their CD16 expression levels and tested for changes in CD16 expression and ADCC activity over a 3-mo culture period. One CD16-negative αβ T cell clone from each donor was included in these experiments as a negative control for ADCC assays as well as a control for assessment of CD16 expression during culture. After stimulation (lectin plus feeder plus IL-2) CD16 on CD16-positive clones was downregulated for 10–15 days but was eventually re-expressed and maintained for several weeks. Fig. 3B shows one example of CD16 expression and ADCC activity monitoring for clone 2 from donor 1. CD16 expression is shown at day 28 after cloning (Fig. 3Ba), at days 27 (Fig. 3Bb) and 52 (Fig. 3Bc) after the first restimulation and then after freezing and thawing, 38 days after stimulation. Under the same conditions, the control CD16-negative αβ T cell clone never expressed CD16. These results indicate that T cells programmed to express CD16 maintained CD16 after TCR stimulation at a level depending on the state of activation. ADCC activity of CD16+ αβ T cell clones was also assessed using a 4-h 51Cr-release assay performed against the autologous target BLCL. Fig. 3B shows the results obtained at different time points with the CD8+ CD16- αβ T cell clone 2 that was derived by limiting dilution from the CD16+ αβ T cell fraction of a healthy donor and whose TCR specificity was unknown. In the absence of mAb, sorted by FACS and their cytotoxic activity assessed using a 4-h 51Cr-release assay against the NK-sensitive K562 cell line and the autologous BLCL in the presence of absence of anti-CD20 or anti-Her2/neu humanized mAb (BLCL were all positive for CD20 and negative for Her2/neu Ags). Fig. 2 shows representative data obtained from one healthy donor. Only NK cells were able to kill the K562 cell line. In the absence of mAb, NK cells, CD16+ αβ, and CD16-negative αβ T lymphocytes did not recognize the autologous BLCL. In contrast, both CD16− NK cells and CD16+ αβ T lymphocytes killed the BLCL incubated with anti-CD20 mAb. This cytotoxicity was not observed in the presence of anti-Her2/neu mAb. Together, these results demonstrate that despite expressing low levels of CD16, CD16+ αβ T lymphocytes express perforin (one example of six donors tested is presented in Fig. 2B) and are able to mediate ADCC ex vivo.

Limiting dilution analysis of the CD16+ αβ T lymphocytes

CD16+ αβ T lymphocytes from two healthy donors were sorted and cloned by limiting dilution using nonspecific stimulation (lectin plus feeder plus IL-2). Cloning efficiency was 0.75 and 0.30 according to the Poisson distribution (Fig. 3A). Nineteen days after cloning, 47 of 52 clones from donor 1 and 18 of 19 clones from donor 2 were CD16 positive. Next, 4 clones from donor 1 and 6 from donor 2 were selected according to their CD16 expression levels and tested for changes in CD16 expression and ADCC activity over a 3-mo culture period. One CD16-negative αβ T cell clone from each donor was included in these experiments as a negative control for ADCC assays as well as a control for assessment of CD16 expression during culture. After stimulation (lectin plus feeder plus IL-2) CD16 on CD16-positive clones was downregulated for 10–15 days but was eventually re-expressed and maintained for several weeks. Fig. 3B shows one example of CD16 expression and ADCC activity monitoring for clone 2 from donor 1. CD16 expression is shown at day 28 after cloning (Fig. 3Ba), at days 27 (Fig. 3Bb) and 52 (Fig. 3Bc) after the first restimulation and then after freezing and thawing, 38 days after stimulation. Under the same conditions, the control CD16-negative αβ T cell clone never expressed CD16. These results indicate that T cells programmed to express CD16 maintained CD16 after TCR stimulation at a level depending on the state of activation. ADCC activity of CD16+ αβ T cell clones was also assessed using a 4-h 51Cr-release assay performed against the autologous target BLCL. Fig. 3B shows the results obtained at different time points with the CD8+ CD16- αβ T cell clone 2 that was derived by limiting dilution from the CD16+ αβ T cell fraction of a healthy donor and whose TCR specificity was unknown. In the absence of mAb, sorted by FACS and their cytotoxic activity assessed using a 4-h 51Cr-release assay against the NK-sensitive K562 cell line and the autologous BLCL in the presence of absence of anti-CD20 or anti-Her2/neu humanized mAb (BLCL were all positive for CD20 and negative for Her2/neu Ags). Fig. 2 shows representative data obtained from one healthy donor. Only NK cells were able to kill the K562 cell line. In the absence of mAb, NK cells, CD16+ αβ, and CD16-negative αβ T lymphocytes did not recognize the autologous BLCL. In contrast, both CD16− NK cells and CD16+ αβ T lymphocytes killed the BLCL incubated with anti-CD20 mAb. This cytotoxicity was not observed in the presence of anti-Her2/neu mAb. Together, these results demonstrate that despite expressing low levels of CD16, CD16+ αβ T lymphocytes express perforin (one example of six donors tested is presented in Fig. 2B) and are able to mediate ADCC ex vivo.

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CD16+ αβ T lymphocytes appear in vivo during hyperlymphocytosis

Because CD16 was found to be expressed by memory αβ T lymphocytes in normal, healthy donors, we anticipated that this particular subset would appear and be amplified during the T cell response. To test whether this is indeed the case, we analyzed CD16 expression by αβ T cells in blood samples from patients with hyperlymphocytosis that were retrieved nonselectively from the Department of Infectious Diseases (above 4000 lymphocytes/μl). Twenty-six healthy controls and 15 subjects with hyperlymphocytosis were tested. The results shown in Fig. 4 demonstrate a significant increase (p = 0.0002) in the proportion of CD16+ cells among the αβ T lymphocyte subset in the patients (range 2.13–26.38%) compared with the healthy volunteer control group (range 0.04–15.0%). These results demonstrate a systematic increase in the proportion of CD16+ cells among αβ T lymphocytes during these T cell responses.

CD16+ αβ T lymphocytes appear in cultures of EBV-specific CTLs (EBV-CTL)

The data presented above demonstrate that reactive T lymphocytes can express CD16. Nevertheless, neither the specificity of these T cells nor the time delay between their encounter with Ag and CD16 expression was known. To directly test CD16 levels and its kinetics of expression by memory T cells of known specificity, we analyzed CD16 expression in EBV-specific T cell lines. In vitro stimulation of PBMC from an EBV-seropositive donor with the

FIGURE 2. CD16-expressing lymphocytes are able to mediate ADCC ex vivo. A. The cytotoxicity activity of FACS-sorted CD16− NK, CD16+ αβ T cells, and CD16-negative αβ T cells from the same donor was evaluated against a 51Cr-labeled K562 cell line and 51Cr-labeled autologous BLCL in the absence or presence of either rituximab (anti-CD20, 2 μg/ml) or herceptin (anti-Her2/neu, 10 μg/ml) as a negative control. Results are expressed as percentage of specific lysis (E:T ratio = 10:1, mean of triplicate wells). Similar results were obtained with two other donors. B. Intracellular expression of perforin by CD16+ αβ T lymphocytes ex vivo. Similar results were obtained with six other donors.

Thymidine incorporation and TNF-α production by CD16+ αβ T lymphocyte clones

To test whether T cell responses other than cytotoxic activity could be initiated in CD16+ αβ T lymphocytes, several T cell clones were tested for their ability to incorporate thymidine and to produce TNF-α after exposure to Ab-coated cells. To exclude the possibility that the clones were activated by soluble Ab, mAb concentrations of up to 1000 μg/ml were tested in the absence of target cells. Examples of the results are presented in Fig. 3, C and D. In Fig. 3C, the CD16+ αβ T cell clones 3 and 21 and the CD16-negative αβ T cell clone 16 obtained by limiting dilution of FACS-sorted CD16+ αβ T cells from donor 2 were tested in a 72-h proliferation assay in the presence of IL-2 (40 IU/ml). The assays were performed in the presence of either soluble anti-CD20 mAb (1–1000 μg/ml) or against the autologous BLCL in the absence or presence of rituximab (0.02 and 2 μg/ml). As shown, thymidine incorporation was observed in the presence of rituximab and the target cell only. In the presence of rituximab only, no thymidine incorporation was detected at any concentration tested, demonstrating that cross-linking was required to induce proliferation. The same conclusion could be drawn for cytokine production, as presented in Fig. 3D. Essentially all cells from CD16+/CD8− T cell clones 14 (Fig. 3D, D1) and 21 (Fig. 3D, D2) were able to produce TNF-α when stimulated with PMA and calcium ionophore. Following cross-linking to target BLCL, 14.4 and 14.6% of cells from clones 14 and 21 became positive for TNF-α, respectively. In contrast, in the absence of target cells, the soluble mAb was unable to induce significant TNF-α production by the clones, even at concentrations of up to 1000 μg/ml.

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FIGURE 3. Limiting dilution analysis and functional characterization of CD16⁺ αβ T lymphocytes. A, PBMC were stained with PE-anti-CD16 Abs. Next, CD16⁺ αβ T lymphocytes were isolated using a FACSVantage and cloned by limiting dilution (see Materials and Methods). Cloning efficiency was 0.75 and 0.30 (according to the Poisson distribution). B, Upper panel, Maintenance of CD16 expression by a CD16⁺ αβ T cell clone. T cell clones were analyzed by flow cytometry for CD16 expression over a 2.5-mo period. a, Day 28 after cloning; b and c, days 27 and 52 after the first restimulation and d, after freezing and thawing, 38 days after stimulation. B, Lower panel, The same T cell clone was tested for ADCC against ⁵¹Cr-labeled autologous BLCL, in the presence of either rituximab (anti-CD20, 0.02 or 2 μg/ml) or herceptin (anti-HER-2, 10 μg/ml) as a negative control. Results are expressed as percentage of specific lysis (E:T ratio = 30:1, mean of triplicate). C, CD16⁺ αβ T cell clones can proliferate only when the CD16 molecule is cross-linked in the presence of mAbs and target cells. Two CD16⁺ αβ T cell clones and one CD16-negative αβ T cell clone were tested in a 72-h proliferation assay in the presence of IL-2 (40 IU/ml). Assays were performed in the presence of soluble anti-CD20 mAb alone or against the autologous BLCL in the absence or the presence of rituximab. D, CD16⁺ αβ T cell clone produce cytokines only when the CD16 molecule is cross-linked in the presence of mAbs and target cells. D1, CD16⁺/CD8⁺ T cell clones 14 and 21 (D1 and D2, respectively) (which do not recognize the autologous BLCL through their TCR) produced TNF-α after PMA/ ionomycin stimulation (a). The CD16⁺/CD8⁺ T cell clones 14 and 21 (D1 and D2, respectively) (which do not recognize the autologous BLCL through their TCR) produced TNF-α after PMA/ ionomycin stimulation (a) were activated only after CD16 cross-linking in the presence of the BLCL and 0.02 or 2 μg/ml anti-CD20 (b–d) but remained unstimulated by the soluble mAb at concentrations up to 1000 μg/ml (e–g).
autologous EBV B lymphoblastoid cell line allowed for the amplification of the EBV-specific memory T cell repertoire. In this situation, it becomes possible to analyze Ag expression directly on virus-specific T cells and at a documented time point after Ag exposure. We analyzed EBV-specific cytotoxic T cell lines from six healthy donors. The results shown in Fig. 5 demonstrate that CD16+ αβ T lymphocytes were detected in all EBV-specific cytotoxic T cell lines. CD16 expression became detectable from days 15 to 20 after restimulation with the autologous BLCL. The frequency of CD16+ EBV-CTL ranged from 1 to 27% (Fig. 5A). Finally, the ADCC activity of these EBV-CTL containing significant numbers of CD16+ T cells was revealed by their cytotoxic activity against an allogeneic BLCL in the presence of an anti-CD20 mAb (Fig. 5B).

**FIGURE 4.** Increase in CD16+ αβ T lymphocytes during hyperlymphocytosis in vivo. Frequency of CD16+ αβ T lymphocytes in healthy control donors and patients with hyperlymphocytosis (superior to 4000 lymphocytes/μl). PBMC were stained with Abs to αβ TCR and CD16. The percentage of CD16+ αβ double-positive cells as a fraction of all αβ T lymphocytes is shown for each sample. Bars indicate means.

**Discussion**

In this study, we demonstrated that T lymphocytes coexpressing the αβ TCR and FcγRIIIa (CD16) are present in significant numbers in all individuals tested. The mean numbers of these cells were similar to those of the previously described “innate-like” CD16+ γδ TCR T cells. Together with the latter cells, these CD16+ αβ TCR T cells represent a pool of 70 lymphocytes/ml of blood, while ~194 CD3+ CD16+ NK cells/ml were detected in the same population. Thus, these two T cell subsets make up one-fourth of all peripheral lymphocytes expressing CD16 and capable of mediating ADCC. Phenotypic analysis revealed that the vast majority of CD16+ αβ TCR T cells do not express CCR7 but do express CD45RA and belong to the T effector memory CD45RA+ or TEMRA lymphocyte subset (28, 29). We additionally showed here that CD16+ αβ TCR T cells are perforin positive, directly mediate ADCC ex vivo, and increase in vivo during hyperlymphocytosis. Finally, we showed that CD16+ αβ TCR T cells appeared in vitro in all cultures of EBV-specific CTL.

Because the main effector cell type involved in ADCC is of a non-T and non-B phenotype the finding that a significant portion of Ag-specific T lymphocytes from the adaptive immune system can have two specificities, one through their TCR and the other through FcR recognition, may have as yet unrecognized physiopathological consequences. For example, given that gp350 has been shown to be a target Ag for EBV-specific ADCC during EBV infection (30), our results suggest that the same T lymphocytes able to recognize an EBV-infected target cell after TCR mediated recognition of EBV-peptide/HLA-complex could potentially recognize the same EBV-infected target cell after CD16 mediated ADCC in the presence of gp350 Abs. In the case of HIV-1-seropositive individuals, the gp120-specific cell-mediated cytotoxicity was attributed to the presence of circulating CD16+ effector cells armed in vivo with cytotoxic Abs. In this latter work, the cells responsible for this activity were identified as NK cells. Nevertheless, it remains possible that under certain circumstances, such as EBV or CMV reactivation in HIV-infected patients, the effector memory cells that become CD16+ engage this mechanism against HIV-infected target cells (31). In this case, the mechanism would be of benefit to the host. In contrast, CD16 has also been involved as a route of virus entry into mononuclear cells and if this mechanism is also efficient for T cells then in the presence of anti-HIV Abs, the CD16+ fraction of T lymphocytes reacting against other pathogens may also become infected (32).

If Abs are directed against healthy tissues, in some circumstances the CD16+ fraction of a pathogen-reactive T cell population might be involved in tissue damage. Because the ADCC process requires three components (target cells expressing Ags on their surface, Abs of the IgG1 isotype directed against the target Ag and effector cell bearing FcγR), its occurrence in vivo will depend on the local concentration of Ags, Abs, and CD16+ effector cells. Accordingly, large variations in the concentration of each of these components may have an impact on the risk of autoimmune manifestation. Because autoantibodies, autoantigens, and CD16+ effector cells capable of ADCC are present in all healthy individuals, only future longitudinal exploration of the quantitative variation in specific CD16+ αβ TCR T cells will enable an exploration into the potential participation of these cells in autoimmune processes. Nevertheless, an already well-known clinical condition exists where an abnormal proliferation of αβ TCR T lymphocytes expressing CD16 is observed. This condition is referred to as LGL leukemia (22, 23) and one of the unique features of this malignancy is its frequent association with autoimmune disease.

**FIGURE 5.** EBV-specific cytotoxic T cell lines (CTL) contain CD16+ αβ T cells that mediate ADCC. A, EBV-specific CTLs were selected against the autologous BLCL and stained with PE-anti-αβ Ab and PC5-anti-CD16 Ab. The ADCC activity of two EBV-specific CTLs was evaluated against 51Cr-labeled allogeneic BLCL (not recognized by the TCR of the CTL) in the presence of either rituximab (anti-CD20, 2 μg/ml) or herceptin (anti-HER-2, 10 μg/ml) as a negative control. Results are expressed as percentage of specific lysis (E:T ratio = 30:1, mean of triplicate wells).
Although the etiology of LGL leukemia is unknown, chronic activation of T cells with auto or viral Ag has been suggested as an initial stimulus leading to an expansion of LGL (33). Moreover, Baesso et al. (34) recently demonstrated that LGL cells display a phenotypic pattern typical of effector cytotoxic cells and the central hypothesis put forward by Loughran, Jr. and colleagues (33) is that leukemic LGL are Ag-driven CTL. Indeed, the usual immunophenotype of the malignant T cells in LGL leukemia resembles that of typical effector memory T cells (CD3+CD4+CD8−CD16+CD27+CD45RO+CD57−) and in the vast majority of cases, the TCR is of the αβ subtype and the cells are capable of ADCC (35–37). Although the exact mechanism involved in LGL-associated autoimmunity disease is not completely understood, the efficacy of immunosuppression or depletion in improving this condition strongly suggests a direct implication of the leukemic LGL in the clinical symptoms (33). Data presented in this study fit well with the hypothesis of Loughran, Jr. and colleagues (33) because the characteristics of the T cell subset we describe correspond exactly to those of LGL leukemia cells. We reasoned that if the chronic amplification of LGL leukemia cells is directly involved in autoimmune diseases, then the transient amplification of their normal counterparts may also be directly responsible for transient autoimmunity. This phenomenon would rely on an increase in the ADCC potential of a patient through amplification of this particular memory subset above the threshold that determines the appearance of autoimmune symptoms. Such an increase may be either transitory as is the case during a normal immune response, or abnormal, as suggested in many examples of autoimmune disease where activation-induced cell death has been found to be disturbed (38–42).

Along these lines, in the presence of tissue-specific Abs, the possible influence of CD16αβ TCR T cells may theoretically provide a simple link between microbial infections and autoimmune exacerbation. This could explain how a single autoimmune disease can be exacerbated by many different infections, or alternatively, how different infections can be associated with exacerbation of the same autoimmune disease (see the recent review by Fujinami et al. (43)).

Beyond the analysis of CD16αβ TCR T lymphocyte participation in different pathophysiopathological processes, manipulation of a patient’s “ADCC potential” through amplification of this specific memory subset could be worth considering in the context of Ab-based immunotherapy. Although CD16 expression by CD16αβ TCR T lymphocytes appeared lower than that observed in NK lymphocytes, this T lymphocyte subset does demonstrate ADCC that can be amplified in vitro, as shown in the present work. Because amplification, freezing, and thawing is far easier with T than with NK lymphocytes, CD16αβ TCR T lymphocytes are good candidates for ex vivo amplification before reinfusion into a patient with a view to improving their ADCC potential. One can also note that if EBV-CTL can express CD16 some time after Ag exposure, then patients treated with EBV-CTL for post-transplant lymphoproliferative disease after a first line of treatment involving anti-CD20 injection may also benefit from the ADCC activity of the EBV-CTL they receive.

Inclusion of T cells can express CD16 and mediate ADCC will likely stimulate many investigations in different fields of immunology 1) to precisely assess the potential role of these populations during a normal immune response; 2) to directly confirm their relationship with LGL leukemia cells; 3) to test their precise implication in different autoimmune diseases where a role for autoantibodies has already been established; and 4) to consider their potential in the context of immunotherapy.

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