High Numbers of Differentiated Effector CD4 T Cells Are Found in Patients with Cancer and Correlate with Clinical Response after Neoadjuvant Therapy of Breast Cancer

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Abstract

CD4+ T cells influence tumor immunity in complex ways that are not fully understood. In this study, we characterized a population of human differentiated effector CD4+ T cells that is defined by low levels of the interleukin (IL)-2 and IL-7 receptors (CD25–/CD127+). We found that this cell population expands in patients with various types of cancer, including breast cancer, to represent 2% to 20% of total CD4+ blood T lymphocytes as compared with only 0.2% to 2% in healthy individuals. Notably, these CD25–/CD127+ CD4 T cells expressed effector markers such as CD103 and CD11b with low levels of CD27, contrasting with the memory phenotype dominating this population in healthy individuals. These cells did not cycle in patients, nor did they secrete IL-10 or IL-17, but instead displayed cytotoxic features. Moreover, they encompassed oligoclonal expansions paralleling an expansion of effector CD8+ T cells that included tumor antigen–specific T cells. During neoadjuvant chemotherapy in patients with breast cancer, we found that the increase in CD25–/CD127+ CD4+ T cells correlated with tumor regression. This observation suggested that CD4+ T cells included tumor antigen–specific cells, which may be generated by or participate in tumor regressions during chemotherapy. In summary, our results lend support to the hypothesis that CD4+ T cells are involved in human antitumor responses.

Introduction

The immune system does not ignore tumors as tumor antigen–specific T cells can be found in the tumor bed, as well as in the blood of patients with cancer. Most emphasis to date has been put on CD8 T cells, as they can be directly cytotoxic to tumor cells and their abundance inside tumors seems to indicate better prognosis. Besides their well-known helper functions, in the absence of MHC class II expression on most tumor cells, CD4 T-cell implication in antitumor responses has only recently been acknowledged. In mouse experimental models, tumor-specific CD4 T cells can reject myelomas by activating macrophages. CD4 T cells included tumor antigen–specific cells, which may be generated by or participate in tumor regressions during chemotherapy. In summary, our results lend support to the hypothesis that CD4 T cells are involved in human antitumor responses.

In humans, adoptive transfer of autologous ex vivo expanded tumor-specific CD4 T cells induced a complete remission in one case. Tumor-specific CD4 T cells can be induced by vaccination with dendritic cells without a strong impact on patient outcome. Nevertheless, good clinical responses were observed after long peptide vaccination in human papilloma virus–related diseases and this was associated with both CD4 and CD8 responses, the former being wider than the latter. The presence of immunoglobulin G isotype antibodies that are specific for tumor antigens indicates that CD4 T cells spontaneously respond to tumors. The number of regulatory CD4 T cells is often increased in the blood of patients with cancer, and the tumor draining lymph nodes, with contradictory data about their prognosis value. However, the data on putative antitumor responses by conventional CD4 (conCD4s) T cells in the absence of immunotherapy are scarce in humans.

ConCD4s encompass multiple subsets, with different effector functions, that express specific membrane markers. CD4 T cells exit the thymus with a naïve phenotype (CD45RO−RA−CD27−CCR7+) and acquire an effector (CD45RO+RA+CD27−CCR7−) phenotype after antigen stimulation, before becoming memory (CD45RO+RA−CD27−CCR7−). During activation, the interleukin (IL)-2Rz chain (CD25) levels are upregulated, whereas IL-7Rz (CD127) levels are transiently downregulated. These two markers also allow the identification of conCD4 T cells and regulatory T cell (Treg) as CD127+CD25+ and CD127−CD25−CD4+ T cells, respectively. An

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intriguing CD127−CD25−CD4+ T-cell subset was recently described in the memory CD45RA− compartment (21). These CD127− CD25− CD4+ T cells represent less than 1% of the CD4+ T cells in healthy individuals, although a similar subset expressing natural killer (NK) inhibitory receptors increased with age (22). These cells are cycling (Ki67+), display low proliferative capacity in vitro in response to T-cell receptor (TCR) or cytokine stimulation, do not contain perforin store (22) and secrete significant amounts of IFN-γ and IL-10 (21).

We report here that the blood of patients with metastatic uveal melanomas (mUM) and patients with breast cancer contains an increased proportion of CD127−CD25−CD4+ T cells. These cells bear specific features that are different from the ones found in the rare cells displaying these markers in healthy donors. Cells with the same phenotype were also found in patients with HIV and tuberculosis, suggesting that these cells are chronically stimulated. We will, therefore, call these cells, chCD4 T cells. The frequency of these peculiar chCD4 T cells is correlated with the frequency of effector CD8 T cells, among which tumor antigen–specific T cells are found. In patients with breast cancer, the absolute number of CD127− CD25− CD4+ T cells is variable according to tumor burden and increases during neoadjuvant chemotherapy in proportion to tumor regression. This result suggests that these chCD4 T cells may either be stimulated by the release of antigen induced by neoadjuvant chemotherapy and/or could be instrumental in tumor regression.

Patients and Methods

Patients

The mUMs were part of a vaccine clinical trial (IC-2004-01) in which a leukapheresis was performed before and after serial peptide vaccination as previously described (23). Ficoll isolated peripheral blood mononuclear cells (PBMC) were frozen in 10% dimethyl sulfoxide, 10% AB serum. Patients for whom enough cells were available for in-depth monitoring were included in this study. The patients with breast cancer belong to a prospective study about the impact of anticancer treatments on circulating Treg (IC-2008-05). These studies were approved by the regional ethics board, and all patients signed an informed consent form. The chemotherapy regimens were: in an adjuvant setting, three cycles of epirubicin, cyclophosphamide, and 5-fluorouracil (FEC) and three cycles of docetaxel; in the neoadjuvant setting, four cycles of FEC and docetaxel.

Other blood samples were leftovers of hematologic analysis of patients followed for breast cancer or esophageal cancer in our institute. Following the French regulations, all patients are informed that leftovers of specimens obtained for diagnosis or through therapeutic procedures may be used for research purposes.

The patients with tuberculosis and HIV infection were part of a study described elsewhere (24). At the time of sampling, all the patients with HIV were receiving an active antiviral treatment leading to low viremia and high CD4 counts.

Healthy donor blood samples were obtained from the blood bank in accordance with institutional regulations.

Tumor regression assessment

Tumor regression after neoadjuvant chemotherapy was assessed according to the proportion of tumor cells remaining on the surgical resection specimen.

Flow cytometry

Cell surface antigens were detected with labeled antibodies on fresh whole blood or thawed PBMCs. Dead cells were excluded from thawed PBMCs by 4,6-diamidino-2-phenylindole (DAPI) staining. Intracellular FoxP3 staining was performed according to the manufacturer’s specifications (eBioscience) and FcR-blocking reagent (Miltenyi Biotec) was used before antibody staining.

We used CD45RA-APC (clone HI100); CD45 RO-Alexa700 (UCHL1); Ki67-PE (B56; all from BD Biosciences); CD27-BV605 (O323); CD3-BV570 (UCHT-1); CD11b-Alexa488 (ICRF44); 2B4-PE (CL7); PD-1-APC (EH12.2H7); HELIOS-AlexaFluor647 (22F6; BioLegend); CD127-PE (R34.34); CD8β-PC5 (2ST8.5H7); CD25-PCT (cB1.49.9; Beckman Coulter); CD28-PerCP-Cy5.5 (CD28.2); CD127-APCFluor780 (eBioRDR5); CD57-eFluor450 (TB01; FOXP3-PE (236-A/E7; eBioscience); CD3-APCALexa750 (S4.1); CD4-Alexa405 (S3.5); and CD4-PE-TexasRed (S3.5; Invitrogen).

Repertoire analysis

TCR-Vβ repertoire was analyzed using the IOTest Beta Mark Kit (Beckman-Coulter). Twenty-four anti–TCR-Vβ-conjugated antibodies cover about 70% of human TCR-Vβ repertoire. The cells were also stained with anti-CD3/CD8/CD4/CD25/CD127/CD45RO/CD27 antibodies to allow for subset discrimination.

Cell sorting

The cells were sorted on a FACSAria sorter (BD Biosciences) according to CD127/CD25 expression: CD127− CD25− CD4+ (convCD4), CD127+ CD25+ CD4+ (chCD4), and CD127− CD25− CD4+ (Treg). Post-sort purity was >99%. In patients, convCD4 and chCD4 were sorted according to their effector differentiation status (CD45RO−CD27+) to prevent a biased comparison between convCD4 and chCD4 T cells.

Lymphokine production

Of note, 105 convCD4, chCD4, and Treg T cells were cultured in 200 μL SYN-H medium (Interchim) alone or with anti-CD3/anti-CD28 beads (Invitrogen) at 1:1 ratio in flat 96-well plates. After a 24-hour culture, lymphokine secretion was measured using the CBA Kit (BD Biosciences).

MGG coloration and immunodetection of intracytoplasmic granzyme B and perforin by immunofluorescence

Fluorescence-activated cell sorter (FACS)-sorted subsets were centrifuged on slides before staining with May–Grunwald–Giemsa (MGG) according to standard technique or being studied by immunofluorescence for granzyme B (GZB) and perforin expression. For immunofluorescence analysis, acetone-fixed cells were stained with anti–GZB PE-conjugated antibody (351927; R&D Systems), anti–perforin-alexa-700.
antibody (DG9; BioLegend), and DAPI. Slides were observed at \( \times 1,000 \) magnification (LEICA DM 4000B). Images were analyzed with ProgRes Capture software (Jenoptik) and MetaMorph (Molecular Device).

**Cytotoxic assay**

A redirected cytotoxic assay was performed according to (25) using B7H1-transfected P815 target cells (from E. Vivier, Centre d’Immunologie de Marseille-Luminy, Marseille, France) and antibiotin beads (Miltenyi) coated with biotinylated anti-B7H1 (29E.2A3) and anti-CD3 (OKT3; BioLegends). FACS-sorted subsets were incubated with \( 10^5 \) P815 targets cells at the indicated effector:target ratio together with anti-B7H1/CD3 beads (\( 8 \times 10^3 \)/well). After a 2-hour incubation at 37°C, the cells were stained with anti-CD8 or -CD4 and Annexin V and DAPI according to the manufacturer’s instruction (Miltenyi).

**Statistical analysis**

All quantitative data were analyzed on Prism software using the unpaired or paired nonparametric tests (\( U \) Mann–Whitney) or (Wilcoxon-signed rank), where indicated; \( * * * \), \( P \) < 0.001; the Mann–Whitney test.
Results

Expansion of effector CD127^-CD25^-CD4^+ T cells during chronic antigen stimulation

In the course of monitoring Treg numbers in patients with various cancers using a CD25/CD127 staining of CD4^+ T cells (26), we noticed the expansion of CD127^-CD25^-CD4^+ T cells in peripheral blood lymphocytes (PBL; Fig. 1). These cells were a small proportion (0.97% ± 0.41; n = 39) of CD4^+ T cells in healthy donors (Fig. 1A and B), as previously described (21). The expanded CD127^-CD25^-CD4^+ T cells represented up to 20% of the CD4^+ T cells as shown for a patient with mUM (Fig. 1A). In both healthy donors and mUMs (n = 12), these particular CD4^+ T cells do not express FoxP3 or Helios transcription factors (Supplementary Fig. S1A and S1B), indicating that these cells are not natural or induced Tregs. This intriguing CD4 subset was abundant in untreated mUMs and breast cancers (n = 59; Fig. 1B and C). To assess whether this increased CD127^-CD25^-CD4^+ T-cell number was restricted to patients with cancer, we studied patients with bacterial (tuberculosis, n = 9) or viral (HIV, n = 30) chronic infections, as CD127^-CD4^+ T cells have been previously reported increased in patients with HIV (27). The proportion (Fig. 1B) and the absolute number (Fig. 1C) of CD127^-CD25^-CD4^+ T cells in all disease groups were higher (P<0.0001) than in healthy donors. These results suggest that chronic immune stimulation is correlated with increased number of CD127^-CD25^-CD4^+ T cells. Hereafter, we will name this population, "chronically stimulated CD4^+" (chCD4s) T cells.

To define the nature of the chCD4s, we explored their naïve/effector/memory differentiation stage. CD4^+ T cells can be divided into naïve (CD27^+CD45RO^-), memory (CD27^-CD45RO-), and effector (CD27^-CD45RO^+) subsets (Fig. 1D). These subsets correspond to those identified in other reports using combinations of CCR7 with CD45RA or CD45RO (data not shown; ref. 28). In healthy donors, the convCD4s were mostly naïve with few memory cells and still less effector cells (Fig. 1D and E). Among the few chCD4s found in healthy donors, most (60% ± 11%) displayed a memory phenotype (Fig. 1D and E). In contrast, in the cancer groups, the proportion of chCD4s with an effector (CD27^-CD45RO^+) phenotype was largely increased in about half of the patients (Fig. 1F). In fact, the absolute numbers of chCD4s were strictly proportional to the absolute number of effector (CD27^-CD45RO^+) chCD4s (Supplementary Fig. S1), indicating that the increased number of chCD4s found in patients with cancer corresponds to an exclusive expansion of effector cells.

chCD4^+ T cells are highly differentiated effector cells in patients with mUM

To determine whether the expanded effector chCD4s found in untreated mUMs (n = 14) were terminally differentiated or still proliferating, we measured Ki67 expression in convCD4s and chCD4s, and also in Treg and CD8 T cells as controls. Surprisingly, among the chCD4s, a notable proportion of the few naïve (CD27^+CD45RO^-) cells found in these patients expressed the proliferation marker Ki67, more than in the naïve Treg subset (Fig. 2A). Of note, 40% to 50% of the memory subset (CD27^-CD45RO^-) were cycling (Ki67^+), a little higher proportion than in the memory Tregs. Strikingly, although the effector (CD27^-CD45RO^+) Tregs were mostly Ki67^-, the Ki67 expression in the indicated T-cell subsets according to their naïve/memory/effector status.

B, representative staining for the indicated markers of chCD4s (CD127^-CD25^-) according to naïve/memory/effector status.

C, heterogeneous expression of CD57 in effector chCD4s without correlation with PD1 expression.

D, the proportion of effectors in chCD4s is correlated with the proportion of cells expressing the indicated markers in effector chCD4s.
**A**

Graphs showing cytokine production by Treg and convCD4 and chCD4 cells.

**B**

Images of Treg, Effector convCD4, chCD4, and Effector CD8 cells.

**C**

Confocal microscopy images of DAPI, GRANZYM B, PERFORIN, and MERGE stains on Treg, convCD4, chCD4, and CD8 cells.

**D**

Graph showing cell count of Granzym B and Perforin.

**E**

Flow cytometry analysis showing the percentage of Annexin V on CD8, chCD4, and convCD4 cells.

**F**

Graph showing the ratio of E:T and the percentage of Annexin V on Effector CD8, Effector chCD4, and Effector convCD4 cells.
staining of effector (CD45RO^hi^CD27 ^− ^) chCD4s distinguished two groups of patients: one with a majority of Ki67^+^ cells and another with few Ki67^+^ cells (Fig. 2A, right). In healthy donors, the proportion of chCD4s expressing Ki67 was much smaller in all naïve/memory/effector subsets (Supplementary Fig. S2A).

The high proportion of noncycling effector chCD4s found in half of the patients led us to measure other markers commonly associated with long-lived effector cells, such as low expression of the costimulatory molecule, CD28, and increased expression of the activation marker CD11b or of the NK marker, 2B4 (CD244, Slamf4). Indeed, these features have been observed in diseases where chronic antigen stimulation is obvious, such as HIV infection (27, 29, 30), or hypothesized, such as rheumatoid arthritis and multiple sclerosis (31–35). In mUMs, CD28 expression was low on effector chCD4s (Fig. 2B), but 2B4 and CD11b were high on effector chCD4s. naïve and memory chCD4s expressed homogenous levels of CD28 (intermediary and high, respectively), whereas CD11b and 2B4 were low on both subsets.

In contrast, CD57, a marker associated with immunosenescence and chronic antigen stimulation (30, 34, 36), but also expressed by germinal center (GC) helper T cells (37, 38) was highly expressed only on a fraction of the effector chCD4s, suggesting some heterogeneity of this subset. However, the expression of PD1 as an inhibitory receptor found on chronically stimulated CD4 T cells and on GC CD4 T cells, and whose expression often parallels CD57 (39) was heterogeneous and not correlated with CD57 expression in mUMs (Fig. 2C). A large proportion of effectors among the chCD4s was correlated with low Ki67 or CD28 expression and with a large proportion of cells expressing 2B4, CD11b, and CD57, indicating a coordinated expression of these markers and thereby a specific differentiation program (Fig. 2D). Because the increased number of chCD4s is due to an increased proportion of effector cells (Fig. 1E), these results show that most of the expanded chCD4s are effector 2B4^hi^CD11b^hi^CD57^hi^CD28^−^Ki67^hi^CD4^+^ T cells, suggesting that these cells are highly differentiated effectors. In contrast, only 2 of 5 healthy donors displayed a high proportion of 2B4^hi^CD11b^hi^CD57^hi^CD28^−^Ki67^hi^ T cells in the few effector chCD4s found in these patients (Supplementary Fig. S2B and S2C). Notably, most of the CD57^+^ chCD4s expressed PD1 contrasting with the lower and heterogeneous expressions of this marker by the chCD4s of patients with mUM.

To assess the effector functions of chCD4s in comparison with those of convCD4s, we analyzed the lymphokine secreted after stimulation by anti-CD3/anti-CD28 beads of purified subsets, convCD4 (CD127^hi^CD27^−^) and chCD4 (CD127^+^CD27^−^), and Treg (CD127^+^CD25^+^) and CD8 T cells as controls in 3 healthy donors and 3 mUMs (Fig. 3A). Because the great majority of the chCD4s are effectors in mUMs, we analyzed sorted effector convCD4s to prevent a biased comparison. The purity of the sorted subsets was confirmed by the pattern of lymphokine secretion: for instance, in the three mUMs, no IL-17 secretion was detected in the chCD4 T-cell fraction, whereas significant amount of this cytokine was measured in the convCD4 culture. The convCD4s of mUMs displayed a tendency to a Th2 pattern with more IL-4, IL-5, and IL-13 secretion than healthy donors (Fig. 3A). Although the chCD4s from healthy donors secreted some TNF-α, IFN-γ, IL-2, and IL-17, and notable amount of IL-10, the expanded chCD4s from mUMs secreted smaller amount of IL-2 and no IL-17, nor IL-10 (Fig. 3A). No consistent difference was observed between the lymphokine secretion pattern of the Treg from patients and healthy donors (Fig. 3A and data not shown). The expanded chCD4s from mUMs also secreted cytotoxic molecules such as GZB and Fas ligand (FASL) in larger amount than the cells from healthy donors. Accordingly, contrary to convCD4s and Tregs, the chCD4 T-cell cytoplasm contained granules very similar to those found in effector CD8 T cells (Fig. 3B). These granules contained GZB and perforin (Fig. 3C and D) and the chCD4s were cytotoxic toward P815 targets in a redirected cytotoxic assay (Fig. 3E and F). Moreover, the nucleus of the effector chCD4s displayed an atypical morphology, similar to the one found in neutrophils as previously described for a CD28^−^CD4^+^ T-cell subset found in few healthy donors (40). Importantly, the effector chCD4s of the one healthy donor, we also studied harbored cytotoxic granules (Supplementary Fig. S2E).

Altogether, this phenotype is compatible with the expansion of chronically antigen-stimulated highly differentiated effector CD4s in untreated mUMs, similar to what has been described in other settings such as chronic viral infection (21, 27, 29) or rheumatoid arthritis (32–34), with however, three distinctive features when compared with the similar subset found in healthy donors: an effector phenotype associated with a cytotoxic potential instead of a memory phenotype, few cycling cells, and no IL-10 secretion.

The effector T-cell subsets expanded in mUMs represent oligoclonal expansions harboring tumor-specific T cells

A feature of chronic antigen stimulation is restriction of the TCR repertoire, which can be detected by the presence of oligoclonal expansions (41). Using a panel of anti-TCRVβ segment antibodies, we looked for amplifications of clones bearing particular Vβ segments in chCD4 (Fig. 4A and B) or CD8 (Supplementary Fig. S3A) T cells of 14 mUMs. We found such amplifications of cells using particular Vβ segments in both chCD4 and CD8 T cells with some Vβ making up to 30% to

Figure 3. Lymphokine secretion and cytotoxic potential of chCD4s in patients with mUM. A, lymphokine production after anti-CD3/anti-CD28 bead stimulation of FACS-sorted convCD4s (CD127^hi^CD25^−^CD4^+^) or chCD4s (CD127^+^CD25^−^) from three healthy donors and three mUMs (open and filled circles, respectively). As control, IL-10 secretion by Treg (CD127^+^CD25^+^CD4^+^) is shown in a box. B, MGG staining of the indicated FACS-sorted subsets. Arrows, cytoplasmic granules. Magnification ×1,000. C and D, the granules found in chCD4s contain GZB and perforin as do those of effector CD8 T cells. C, immunofluorescence staining of the indicated FACS-sorted subsets (the blurred red staining in convCD4 and Treg is probably nonspecific). D, cytometric analysis of intracellular expression of GZB and perforin. E and F, FACS effector chCD4s kill P815 in a redirected cytotoxic assay as do effector CD8 T cells. E, representative FACS dot plots of B7H1/P815 target cells after incubation with the indicated effector T cells for 2 hours and anti-CD3-B7H1 beads at a 5:1 E:T. F, the percentage of apoptotic/dying cells according to the E:T ratio with the indicated effector T cells.
50% of the repertoire (for instance, Vb2 and 13.6 in patient C and A, respectively). An increase of at least one particular Vb segment above normal values was found in the 6 patients harboring an increased proportion of effectors in the chCD4s (Fig. 2). As exemplified in Fig. 4B, the oligoclonal expansions were exclusively found in the chCD4 subset and absent from the Tregs. The much smaller expansions found in the convCD4s (Vb13.6 and Vb17 in patient A, or Vb2 in patient C, for instance) are probably related to the difficulty in separating the convCD4s from chCD4s according to CD127 expression alone (data not shown). Thus, chCD4s of mUMs harbor oligoclonal expansions, suggesting chronic antigen stimulation.

To assess whether the expansion of effector chCD4 was correlated with an expansion of effector CD8 T cells, we measured the proportion of effector (CD45RO+CD8+) CD8 T cells in mUM, patients with breast cancer as well as in the chronic viral (HIV) infected patients seen above. The number of effector CD8 T cells was increased (Supplementary Fig. S3C) and the proportions of effectors in chCD4 and CD8 T cells were strongly correlated in all pathologic settings (Fig. 4C and Supplementary Fig. S3C). This suggests that the expansion of these two effector subsets corresponds to a coordinated immune response. Similarly, in a longitudinal analysis of patients with esophageal cancer treated by radiochemotherapy surgery, we observed a progressive increase over time of the effector compartment in both CD8 and chCD4 subsets in several instances (Supplementary Fig. S3C). The chCD4s of these patients displayed the cytoplasmic granules and multilobed nucleus (Supplementary Fig. S3D) observed in patients with cancer (Fig. 3B, Supplementary Figs. S3D and S4F) and the very minor subset of healthy donors (Supplementary Fig. S2E).

The increased frequency of effector CD8 T cells in mUM allowed us to examine whether these cells may represent an immune response toward tumor antigens as the specificity of the CD8 T cells can be assessed using tetramer (Tet). A control Tet staining with an Epstein-Barr virus (EBV) epitope showed the presence of memory (CD45RO+CD8+) CD8 T cells (Fig. 4D) in similar frequency in patients and healthy donors. The CD8 T cells specific for the melanocyte differentiation antigen, MART-1, displayed a naïve phenotype in healthy donors but were more numerous and had an effector/memory phenotype in mUMs as previously observed (Fig. 4D and E; ref. 42). Similarly, the T cells specific for the tumor-specific antigen, Na-17, were more numerous and displayed an effector phenotype in 8 of 9 patients with mUM as compared with healthy donors. This frequency could reach values (patient B, 1/667 and patient C, 1/1,136) generally observed in antiviral responses. Altogether, the presence of oligoclonal expansions in the effector compartment of both CD4 and CD8 T cells, and the high frequency of tumor-specific T cells in this latter subset is highly suggestive of a coordinated immune response toward the tumor in mUMs.

**Effectors chCD4s expand during chemotherapy for breast cancers**

One way to determine whether the chCD4s are specific for the tumor is to assess the influence of tumor burden and dynamics on their number. We, therefore, studied chCD4 T-cell numbers in patients with breast cancer at different stages of their treatment: before primary surgery (group a), after surgery but before adjuvant chemotherapy (group b), and before neoadjuvant chemotherapy (group c). In group a, the tumor is present and only removed after chemotherapy and the disease is usually more severe than in group a (Fig. 5A). chCD4 T-cell numbers were lower (P = 0.017) in group b than in group a, suggesting that tumor removal may induce a decrease of the chCD4s. The short-time interval between the two samplings (one month) supports a putative impact of the tumor on the chCD4 T-cell numbers, through the release of either antigen or inflammatory mediators. In contrast, although chCD4 T-cell numbers in group c were largely increased (P < 0.0001) in comparison with healthy donors, they were lower (P = 0.013) than in group a despite poorer prognosis or bigger tumors. The number of chCD4s was also somewhat lower in group c in comparison with group b without, however, reaching statistical significance (P = 0.06). These results indicate that, in addition to tumor burden, other characteristics of the tumor may determine the expansion or blood recirculation of the chCD4s.

To better assess the relationship between a putative antigenic load and expansion of effector chCD4 or CD8 T cells, we measured their frequency before and after treatment in patients, undergoing either adjuvant or neoadjuvant chemotherapy. Adjuvant chemotherapy is given after surgery to eradicate potential disseminated tumor cells and the tumor burden is much smaller in most cases than in a neoadjuvant setting. Thus, the amount of tumor antigen release induced by the chemotherapy is probably much higher in a neoadjuvant than in an adjuvant setting. Although the neoadjuvant chemotherapy is slightly stronger, these two clinical contexts allowed us to estimate a potential impact of tumor lysis on the immune system, independently of the direct effect of chemotherapy on the immune cells. Although the total number of chCD4s was not modified by adjuvant therapy, it significantly increased during neoadjuvant chemotherapy (Fig. 5B). However, the absolute number...

Figure 4. Presence of chCD4 oligoclonal expansions and tumor-specific CD8 T cells in 14 patients with mUM. A, TCR-Vb repertoire of mUM chCD4s. Bars, the percentage of chCD4s expressing the indicated Vb segment in one patient. Minima/maxima/mean values in 85 healthy donors (HD; provided by the manufacturer) are indicated in black. B, oligoclonal expansions are only found in chCD4s. TCR-Vb repertoire in the indicated CD4 subsets of three patients displaying large oligoclonal expansions (patient A square, B circle, and C diamond). C, correlation between the proportion of effectors (CD27+c) in chCD4 and CD8+ T cells in the indicated pathologic groups. D and E, phenotype and frequency of tumor (MART-1, NA-17) or viral (EBV)-specific T cells assessed by tetramer staining of CD8+CD3+ cells in mUMs (unique black symbols) and healthy donors (red circles). D, representative MART-1 or NA.17 versus EBV Tet, staining (top). Naive/memory/effector status of tet+ cells (bottom). E, frequency of MART-1, NA.17, and EBV-specific CD8 T cells in mUMs and healthy donors.
of effector chCD4s increased in both settings (Fig. 5C). The number of effector CD8 T cells decreased during adjuvant chemotherapy, but was not modified by neoadjuvant chemotherapy (Fig. 5D). These results suggest that the tumor burden and/or chemotherapy have a differential impact on chCD4 versus effector CD8 T cells.
Interestingly, the proportions of effectors in the chCD4 and CD8 T cells were correlated both before and after adjuvant chemotherapy (Fig. 5E, top). In contrast, the correlation was very weak before but increased after neoadjuvant chemotherapy (Fig. 5E, bottom), suggesting that tumor antigen release or change in tumor burden may have more impact on chCD4 blood levels than on effector CD8 T cells in this clinical setting. Altogether, these results suggest that the presence of the tumor affects the proportion of effector chCD4s and that the release of antigen, decrease in tumor burden, Treg depletion or inflammation lead to expansion, or change in the recirculation pattern of chCD4s.

Finally, we characterized the phenotype of the chCD4s in a few patients with breast cancer undergoing neoadjuvant chemotherapy (Supplementary Fig. S4). In the two patients with the highest proportion of effectors in the chCD4s, these cells were Ki67+/CD28−/CD11b−/CD57+ and displayed cytotoxic granules. These results indicate that the effector chCD4s found in patients with mUM and breast cancer are similar, suggesting that the chCD4s of patients with breast cancer may encompass tumor antigen–specific T cells.

The expansion of chCD4s is correlated with clinical response to neoadjuvant chemotherapy for breast cancers

The increased chCD4 T-cell numbers found after neoadjuvant chemotherapy led us to examine whether other immunologic parameters would be modified. We also looked for a correlation between the magnitude of the chCD4 T-cell increase and tumor regression. As expected, the number of total lymphocytes, CD4 and CD8 T cells were lower in most patients after neoadjuvant chemotherapy (Fig. 6A). Among CD4 T cells, Treg and convCD4s were also strongly decreased after treatment (Fig. 6B) as previously reported (43, 44). Notably, chCD4 T-cell numbers increased in 17 of 22 patients after treatment (Fig. 6B) contrasting with the absence of significant variation of effector CD8 numbers (Fig. 5D). These results suggest that chCD4s have either proliferated and/or recirculated to the blood after chemotherapy treatment with antimitotic drugs.

Neoadjuvant chemotherapy is followed by surgery allowing the assessment of tumor regression, which is certainly correlated with the amount of tumor antigen released during chemotherapy. We, therefore, examined whether tumor regression would be correlated with the variation in the numbers of effector CD8 or chCD4 T cells during treatment. We found no significant correlation between the variation in the numbers of effector CD8 T cells and tumor regression (Fig. 6C). Most strikingly, we found a strong correlation ($r = 0.7, P = 0.0003$) between the increased number of chCD4 and tumor regression (Fig. 6D): The more the tumor regressed, the more the absolute number of chCD4s increased. It is difficult to ascertain a causal relationship between these two parameters: either the antigen release induced by the chemotherapy stimulated a proliferation of the chCD4s or the increased number of effector chCD4s was instrumental in tumor regression as proposed by some authors (45). Alternatively, chemotherapy may have induced a redistribution of the chCD4 from the tumor to the blood.

Discussion

We characterize a new population of effector CD4 T cells, chCD4s, whose number is increased in pathologic situations in which chronic antigenic stimulation is suspected: cancers and chronic bacterial or viral infections. This subset increases in number in the blood during neoadjuvant chemotherapy for breast cancer and the magnitude of this expansion is correlated with clinical response.

It is not clear whether the chCD4s found in patients with cancer are similar to the cells previously described in healthy donors (21, 22). Most of the abundant chCD4s found in patients with cancer are effectors, Ki67+ and do not secrete IL-10, contrary to the described subsets, which are Ki67−, memory and secrete IL-10 (21). However, a very small number of effector cells with similar features are found among the chCD4s in healthy donors (Supplementary Fig. S2). The high number of effector chCD4s found in patients may correspond to an expansion of this very minor subset. The expansion of CD127−/CD25−/CD4 T cells has already been described in HIV (27), but their functional activities were not characterized. Similarly, expansions of CD28−, 2B4+, or CD57+ CD4 T cells were observed in other chronic antigen stimulations, without complete characterization of their phenotype or effector functions (30–35). The chCD4s present in patients with cancer are probably related to these previously described subsets. In fact, they are very similar to a CD4 subset expressing NK inhibitory receptors and containing specificities for recall antigens that increases with age (22). During chronic inflammatory diseases, CD57 expression was interpreted as a sign of "exhaustion," as it was difficult to induce in vitro proliferation of CD57+ T cells (29, 33, 36). Whether this is also true in vivo is unknown, although the increasing frequency observed after neoadjuvant chemotherapy argues against this hypothesis.

CD57 expression is also found on germinal CD4 T cells in the tonsils (37) and B-cell helper functions have been attributed to CD57+ or CD127−/CD4 T cells (37, 46). Although many of the follicular helper T-cell (Tfh) features are missing in chCD4s, expression of CD57 could indicate a tropism for tertiary lymphoid organs (TLO). Indeed, as TLO are found in breast cancer (47), another hypothesis could explain the dynamics of chCD4s in patients with breast cancer requiring neoadjuvant therapy, in comparison with primary surgery–treated patients: the chCD4s would be in lower numbers in the blood of the former patients because these cells would be recruited into the more abundant TLOs of these larger tumors. These TLOs could trap the CD57+ chCD4 T cells within the tumor, and chemotherapy could release the cells, resulting in accumulation in the blood. Although the chCD4s do not have all the features of Tfh, this hypothesis is supported by a recent study (48) showing that the intratumoral presence of a Tfh related transcriptomic signature indicates a good response to neoadjuvant therapy in breast cancer.

Contrary to the chCD4 found in healthy donors, the chCD4s from patients with cancer secreted barely detectable IL-17 or IL-10 and higher amount of FASL and granzymes than the chCD4s from healthy donors. This would indicate different functional activities by the effector chCD4s in patients with...
cancer as compared with healthy donors. The presence of granules in the cytoplasm of the chCD4s as well as their cytotoxic activity in a redirected cytotoxic assay observed in a patient with mUM further suggests a cytotoxic potential, which could play a role in the antitumor response.

In malignant diseases, the number of chCD4s was correlated with progression of the disease in patients with hepatocellular carcinoma and decreased after treatment of liver lesions with radiofrequency (49). In our study, we found no strong correlation between chCD4 numbers and tumor burden in the absence of treatment (Fig. 5A). On the contrary, the number of chCD4s was lower in the group that requires neoadjuvant chemotherapy and increased in proportion with tumor regression (Fig. 6D). The timing of sampling and the mechanisms of tumor destruction may explain this difference between the hepatocellular carcinoma and breast studies.

The study of immune parameters in patients with cancer at different stages of their treatment is important to assess a putative role of the immune response during the natural history of cancer and also in the prospect of associating immunotherapy to conventional treatments. The patients with breast cancer studied here were not lymphopenic at inclusion. Although the number of lymphocytes decreased during
neoadjuvant chemotherapy (Fig. 6), it did not reach the values found in metastatic patients that have been shown to indicate poor prognosis (50). The chCD4 T-cell numbers were correlated with effector CD8 T-cell expansion in patients with cancer. The oligoclonal expansions observed in both subsets and the antitumor antigen specificity found in the effector CD8 T cells suggest that both chCD4 and effector CD8 T cells represent a coordinated antitumor immune response. The identification of the MHC class II epitopes recognized by the effector chCD4s is an important issue to address in future works.

Finally, we assessed the impact of tumor burden on CD4 subsets and observed that surgical tumor resection leads to a decrease in the number of effector chCD4s. This suggests that these cells may represent a specific antitumor response as removal of the antigen stimulation led to a decrease in their number. An important role for CD4 T cells in cancer is further suggested by the correlation during neoadjuvant chemotherapy of tumor regression with chCD4 cell increase and not with CD8 subset modifications. Although the causal relationship between chCD4 expansion and tumor regression cannot be ascertained at this stage, the measure of chCD4 numbers could be a useful biomarker for monitoring the presence of an immune response toward tumors. Ongoing studies will determine whether monitoring chCD4 numbers and characteristics could also become a predictive biomarker for prognosis and staging before chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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