High numbers of differentiated effector CD4 T cells are found in cancer patients and correlate with clinical response after neo-adjuvant 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 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 2-20% of total CD4+ blood T lymphocytes as compared to only 0.2-2% in healthy individuals. Notably, these CD25–CD127–CD4 T cells expressed effector markers such as CD244 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 neo-adjuvant chemotherapy in breast cancer patients, 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 anti-tumor responses.
Introduction

The immune system does not ignore tumors as tumor antigens (Ag) specific T-cells can be found in the tumor bed, as well as in the blood of cancer patients (1). Most emphasis to date has been put on CD8 T-cells (2), as they can be directly cytotoxic to tumor cells and their abundance inside tumors appears to indicate better prognosis (3). Besides their well-known helper functions (4), in the absence of MHC class II expression on most tumor cells, CD4 T-cell implication in anti-tumor responses has only recently been acknowledged (5). In mouse experimental models, tumor specific CD4 T-cells can reject myelomas by activating macrophages (6). CD4 T-cells can also be more efficient than CD8 T-cells at rejecting solid tumors (7). In fact, CD4 T-cells can either display anti- or pro-tumoral activity (8, 9).

In humans, adoptive transfer of autologous ex vivo expanded tumor specific CD4 T-cells induced a complete remission in one case (10). Tumor specific CD4 T-cells can be induced by vaccination with dendritic cells without strong impact on patient outcome (11, 12). Nevertheless, good clinical responses were observed after long peptide vaccination in HPV related diseases and this was associated with both CD4 and CD8 responses, the former being wider than the latter (13, 14). In the absence of therapeutic manipulation, the presence of IgG isotype antibodies that are specific for tumor antigens indicates that CD4 T-cells spontaneously respond to tumors (15). The number of regulatory CD4 T (T-Reg) is often increased in the blood of cancer patients (16), the tumor (17) and the tumor draining lymph nodes (18), with contradictory data about their prognosis value (19). However, the data on putative anti-tumor responses by conventional CD4 (convCD4s) T-cells in the absence of immunotherapy are scarce in humans.

ConvCD4s 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 Ag
stimulation, before becoming memory (CD45RO⁺RA⁻CD27⁺CCR7⁺). During activation, the IL-2Rα (CD25) chain is upregulated, while IL-7Rα (CD127) levels are transiently down-regulated (20). These two markers also allow the identification of convCD4 T-cells and T-Reg as CD127⁺CD25⁺/- and CD127⁻CD25⁺ CD4⁺ T-cells, respectively. An 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 NK inhibitory receptors increased with age (22). These cells are cycling (Ki67⁺), display low proliferative capacity in vitro in response to 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 metastatic uveal melanoma (mUMs) and breast cancer (BCs) patients 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 (HD). Cells with the same phenotype were also found in HIV and tuberculosis patients, 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 BC patients, the absolute number of CD127⁻CD25⁻ CD4⁺ T-cells is variable according to tumor burden and increases during neo-adjuvant chemotherapy in proportion to tumor regression. This result suggests that these chCD4 T-cells may either be stimulated by the release of Ag induced by neo-adjuvant chemotherapy and/or could be instrumental in tumor regression.
Patients, Material 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 PBMCs were frozen in 10% DMSO, 10% AB serum. Patients for whom enough cells were available for in-depth monitoring were included in the current study. The BC patients belong to a prospective study about the impact of anti-cancer treatments on circulating T-Regs (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, 3 cycles of Epirubicin, cyclophosphamide and 5FU (FEC) and 3 cycles of Docetaxel; in the neo-adjuvant setting, 4 cycles of FEC and Docetaxel.

Other blood samples were leftovers of haematological analysis of patients followed for BC or oesophageal 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 HIV patients were receiving an active anti-viral 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 neo-adjuvant 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 (Ab) on fresh whole blood or thawed PBMCs. Dead cells were excluded from thawed PBMCs by DAPI staining. Intracellular FoxP3 staining was performed according to the manufacturer’s specifications (eBiosciences) and FcR-blocking reagent (Miltenyi) was used prior to Ab 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 (C1.7); PD-1-APC (EH12.2H7); HELIOS-AlexaFluor647 (22F6) (Biolegend); CD127-PE (R34.34); CD8ß-PC5 (2ST8.5H7); CD25-PC7 (cB1.49.9) (Beckman Coulter); CD28-PERCP-Cy5.5 (CD28.2); CD127-APCeFluor780 (eBioRDR5); CD57-eFluor 450 (TB01); FOXP3-PE (236-A/E7) (eBiosciences); CD3-APCAlexa750 (S4.1); CD4-Alexa405 (S3.5); CD4-PE-TexasRed (S3.5) (Invitrogen).

Repetoire analysis:

TCR-Vß repertoire was analysed using the IOTest Beta Mark kit (Coulter-Beckman). 24 anti TCR-Vß conjugated antibodies cover about 70% of human TCR-Vß repertoire. The cells were also stained with anti-CD3/CD8ß/CD4/CD25/CD127/CD25RO/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⁺ (T-Reg). 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:**

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

**MGG coloration and immuno-detection of intracytoplasmic GZB and perforin by immunofluorescence:**

FACS-sorted subsets were centrifuged on slides before staining with May-Grünwald-Giemsa (MGG) according to standard technique or being studied by immuno-fluorescence for GZB and perforin expression. For IF analysis, acetone fixed cells were stained with anti-GZB PE-conjugated antibody (351927, R&D), anti-perforin-alexa-700 antibody (DG9, Biolegend) and DAPI. Slides were observed at 1000X 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, CIML) and anti-biotin beads (Miltenyi) coated with biotinylated anti-B7H1 (29E.2A3) and anti-CD3 (OKT3) (Biolegends). FACS sorted subsets were incubated with 10⁴ P815 targets cells at the indicated effector:target ratio together with anti-B7H1/CD3 beads (8x10⁴/well). After a 2h incubation at 37°C, the cells were stained with anti-CD8 or -CD4 and annexin-5 and DAPI according to the manufacturer instruction (Miltenyi).

**Statistical analysis:**
All quantitative data were analysed on Prism software using unpaired or paired non-parametric t-test (U Mann-Whitney) or (Wilcoxon signed rank) where indicated. ***p<0.001.
Results

Expansion of effector CD127\(^{-}\)CD25\(^{-}\)CD4\(^{+}\) T-cells during chronic antigen stimulation.

In the course of monitoring T-Reg 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 (HD) (Fig. 1a, 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 mUM patient (Fig. 1a). In both HD and mUMs (n=12), these particular CD4\(^{+}\) T-cells do not express FoxP3 or Helios transcription factors (Sup. Fig. 1a, b), indicating that these cells are not natural or induced T-Regs. This intriguing CD4 subset was abundant in untreated mUMs and BCs (n=59) (Fig. 1b, c). To assess whether this increased CD127\(^{-}\)CD25\(^{-}\)CD4\(^{+}\) T-cell number was restricted to cancer patients, 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 HIV patients (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 HD. 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 (ref (28) and data not shown). In HD, the convCD4s were mostly naïve with few memory cells and still less effector cells (Fig. 1d, e). Among the few chCD4s found in HD, most (60\%±11)
displayed a memory phenotype (Fig. 1d, 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 was strictly proportional to the absolute number of effector (CD27^−CD45RO^±) chCD4s (Sup. Fig. 1), indicating that the increased number of chCD4s found in cancer patients corresponds to an exclusive expansion of effector cells.

**chCD4 T-cells are highly differentiated effector cells in mUM patients**

To determine whether the expanded effector chCD4s found in untreated mUMs (n=14) were terminally differentiated or still proliferating, we measured Ki-67 expression in convCD4s and chCD4s, and also in T-Reg 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 T-Reg subset (Fig. 2a). 40-50% of the memory subset (CD27^+CD45RO^+) were cycling (Ki67^+), a little higher proportion than in the memory T-Regs. Strikingly, whilst the effector (CD27^−CD45RO^±) T-Regs were mostly Ki67^+, the Ki67 staining of effector (CD45RO^±CD27^−) chCD4s distinguished two groups of patients: one with a majority of Ki67^+ cells and another with few Ki67^+ cells (Fig. 2a, right panel). In HD, the proportion of chCD4s expressing Ki67 was much smaller in all naïve/memory/effector subsets (Sup. Fig. 2a).

The high proportion of non-cycling 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 co-stimulatory 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 in which 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) while CD11b and 2B4 were low on both subsets.

In contrast, CD57, a marker associated with immuno-senescence and chronic Ag-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 PDI 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). Since the increased numbers of chCD4s is due to an increased proportion of effector cells (Fig. 1e), these results show that most of the expanded chCD4s are effector 2B4hiCD11bhiCD57hiCD28loKi67lo CD4+ T-cells, suggesting that these cells are highly differentiated effectors. In contrast, only 2 out of 5 HDs displayed a high proportion of 2B4hiCD11bhiCD57hiCD28Ki67lo in the few effector chCD4s found in these patients (Sup. Fig. 2b, c). Notably, most of the CD57+ chCD4s expressed PDI contrasting with the lower and heterogeneous expressions of this marker by the chCD4s of mUM patients.

To assess the effector functions of chCD4s in comparison with those of convCD4s, we analyzed the lymphokines secreted after stimulation by anti-CD3+anti-CD28 beads of purified subsets, convCD4 (CD127+CD25−) and chCD4 (CD127−CD25+), and T-Reg (CD127−CD25+) and CD8 T-cells as controls in 3 HD 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 3 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 HD's (Fig. 3a). While the chCD4s from HD 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 T-Reg from patients and HD (Fig. 3a and data not shown).

The expanded chCD4s from mUMs also secreted cytotoxic molecules such as GZA and FASL in larger amount than the cells from HD. Accordingly, contrary to convCD4s and T-Regs, 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, d) and the chCD4s were cytotoxic towards P815 targets in a redirected cytotoxic assay (Fig. 3e, 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 HD (40). Importantly, the effector chCD4s of the one HD we studied also harbored cytotoxic granules (Sup. Fig. 2e).

Altogether, this phenotype is compatible with the expansion of chronically Ag 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, 3 distinctive features when compared to the similar subset found in HD: 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 Ag 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-b) or CD8 (Sup. Fig. 3a) 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 30-50% of the repertoire (for instance Vβ2 and 13.6 in patient C and A, respectively). An increase of at least one particular Vβ 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 T-Regs. The much smaller expansions found in the convCD4s (Vβ13.6 and Vβ17 in patient A, or Vβ2 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 Ag 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⁺CD27⁻) CD8 T-cells in mUM, BC patients as well as in the chronic viral (HIV) infected patients seen above. The number of effector CD8 T-cells was increased (Sup. Fig. 3c) and the proportions of effectors in chCD4 and CD8 T-cells were strongly correlated in all pathological settings (Fig. 4c and Sup. Fig. 3c). This suggests that the expansion of these 2 effector subsets corresponds to a coordinated immune response. Similarly, in a longitudinal analysis of oesophageal cancer patients treated by radio-chemotherapy-surgery, we observed a progressive increase over time of the effector compartment
in both CD8 and chCD4 subsets in several instances (Sup. Fig. 3c). The chCD4s of these patients displayed the cytoplasmic granules and multi-lobed nucleus (Sup. Fig. 3d) observed in cancer patients (Fig. 3b, Sup. Fig. 3d, Sup. Fig. 4f) and the very minor subset of HD (Sup. Fig. 2e).

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 (Ag) as the specificity of the CD8 T-cells can be assessed using tetramer (Tet). A control Tet staining with an EBV epitope showed the presence of memory (CD45RO⁺CD27⁺) CD8 T-cells (Fig. 4d) in similar frequency in patients and HD. The CD8 T-cells specific for the melanocyte differentiation Ag, MART-1, displayed a naïve phenotype in HD but were more numerous and had an effector/memory phenotype in mUMs as previously observed (42) (Fig. 4d,e). Similarly, the T-cells specific for the tumor specific Ag, Na-17, were more numerous and displayed an effector phenotype in 8 out 9 mUM as compared with HD. This frequency could reached values (patient B: 1/667 and patient C: 1/1136) generally observed in anti-viral 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 towards the tumor in mUMs.

**Effector 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 (b) and before neo-adjuvant chemotherapy (c). In group (c), 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 2 samplings (one month) supports a putative impact of the tumor on 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 HD, 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 neo-adjuvant 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 neo-adjuvant setting. Thus, the amount of tumor antigen release induced by the chemotherapy is probably much higher in a neo-adjuvant than in an adjuvant setting. Although the neo-adjuvant chemotherapy is slightly stronger, these 2 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. While the total number of chCD4s was not modified by adjuvant therapy, it significantly increased during neo-adjuvant chemotherapy (Fig. 5b). However, the absolute number 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 neo-adjuvant chemotherapy (Fig. 5d). These results suggest that the tumor burden and/or chemotherapy have a differential impact on chCD4 vs 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 upper panels). In contrast, the correlation was very weak before but increased after neo-adjuvant chemotherapy (Fig. 5e bottom panels) suggesting that tumor Ag 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 impacts the proportion of effector chCD4s and that the release of antigen, decrease in tumor burden, T-Reg 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 BC patients undergoing neo-adjuvant chemotherapy, (Sup. Fig. 4). In the 2 patients with the highest proportion of effectors in the chCD4s, these cells were Ki67lo2B4+CD28-CD11b+CD57+ and displayed cytotoxic granules. These results indicate that the effector chCD4s found in mUM and BC patients are similar suggesting that the chCD4s of BC patients may encompass tumor antigen specific T-cells.

**The expansion of chCD4s is correlated to clinical response to neo-adjuvant chemotherapy for breast cancers**

The increased chCD4 T-cell numbers found after neo-adjuvant chemotherapy led us to examine whether other immunological 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 neo-adjuvant chemotherapy (Fig. 6a). Among CD4 T-cells, T-Reg and convCD4s were also strongly decreased after treatment (Fig. 6b) as previously reported (43, 44). Notably, chCD4 T-cell numbers increased in 17/22 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 re-circulated to the blood after chemotherapy treatment with anti-mitotic drugs.

Neo-adjuvant chemotherapy is followed by surgery allowing the assessment of tumor regression, which is certainly correlated with the amount of tumor-Ag 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 Ag 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 pathological situations in which chronic antigenic stimulation is suspected: cancers and chronic bacterial or viral infections. This subset increases in number in the blood during neo-adjuvant chemotherapy for BC and the magnitude of this expansion is correlated with clinical response.

It is not clear whether the chCD4s found in cancer patients are similar to the cells previously described in HD (21, 22). Most of the abundant chCD4s found in cancer patients are effectors, Ki67neg 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 HD (Sup. Fig. 2). 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 Ag stimulations, without complete characterization of their phenotype or effector functions (30-35). The chCD4s present in cancer patients 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 neo-adjuvant 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 BC (47), another hypothesis could explain the dynamics of chCD4s in BC patients requiring neo-adjuvant therapy, in comparison with primary surgery treated patients: the chCD4s would be in lower numbers in the blood of the former patients because there would be recruited into the more abundant TLOs of these larger tumors. These LTOs 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 intra-tumoral presence of a Tfh related transcriptomic signature indicates a good response to neo-adjuvant therapy in breast cancer.

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

In malignant diseases, the number of chCD4s was correlated with progression of the disease in hepato-cellular carcinoma (HCC) patients and decreased after treatment of liver lesions with radio-frequency (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 neo-adjuvant 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 HCC and breast studies.
The study of immune parameters in cancer patients 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 BC patients studied here were not lymphopenic at inclusion. Although the number of lymphocytes decreased during neo-adjuvant 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 cancer patients. The oligoclonal expansions observed in both subsets and the anti-tumor Ag specificity found in the effector CD8 T-cells suggest that both chCD4 and effector CD8 T-cells represent a coordinated anti-tumor 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 anti-tumor 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 neo-adjuvant 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 towards tumors. On-going studies will determine whether monitoring chCD4 numbers and characteristics could also become a predictive bio-marker for prognosis and staging before chemotherapy.
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Figure legends

Figure 1: Increased number of effector CD127+CD25−CD4+CD3+ (chCD4s) T-cells in the blood of patients with chronic antigen stimulation.

(a) Expression of CD127 and CD25 defines three subsets among CD4+ T-cells in human PBL. Representative staining in a healthy donor (HD), an untreated metastatic uveal melanoma (mUM) patient and a breast cancer (BC) patient. (b-c) Proportion (b) and absolute numbers (c) of CD127−CD25−CD4+ T-cells in HD, untreated mUM or BC patients, HIV-treated patients and patients with recently diagnosed tuberculosis. (d) Expression of CD45RO and CD27 define naïve (N:CD45RO−C27+), memory (M:CD45RO+C27+) and effector (E:CD45RO±C27-) subsets in conventional (convCD4) and chCD4 lymphocytes. (e) Distribution of N/M/E subsets in convCD4s (CD127+CD25±) and chCD4s (CD127−CD25+) in HD and (f) in chCD4s of mUM and BC patients. *** p<0.0001 Mann-Whitney test.

Figure 2: Phenotypic and functional characterization of chCD4s from mUM patients:

(a) 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 N/M/E 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.

Figure 3: Lymphokine secretion and cytotoxic potential of chCD4s in mUM patients

(a) Lymphokine production after anti-CD3/anti-CD28 bead stimulation of FACS-sorted convCD4s (CD127+CD25+CD4+) or chCD4s (CD127−CD25+) from 3 HDs and 3 mUMs (open and filled circles, respectively). As control, IL-10 secretion by T-Reg (CD127−CD25+CD4+) is
shown in a box. (b) MGG staining of the indicated FACS-sorted subsets. Arrows indicate cytoplasmic granules. 1000X magnification. (c, d) The granules found in chCD4s contain GZB and perforin as do those of effector CD8 T-cells. (e) IF staining of the indicated FACS-sorted subsets (the blurred red staining in convCD4 and T-Regs is probably non-specific) (d) Cytometric analysis of intra-cellular expression of GZB and Perforin. (e, f) FAC-sorted 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 2h and anti-CD3-B7H1 beads at a 5:1 E/T. (f) Percentage of apoptotic/dying cells according to E:T ratio with the indicated effector T-cells.

**Figure 4: Presence of chCD4 oligoclonal expansions and tumor specific CD8 T-cells in 14 mUM patients.**

(a) TCR-Vß repertoire of mUM chCD4s. Each bar represents the percentage of chCD4s expressing the indicated Vß segment in one patient. Minima/maxima/mean values in 85 HD (provided by the manufacturer) are indicated in black. (b) Oligoclonal expansions are only found in chCD4s. TCR-Vß 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+) in chCD4 and CD8+ T-cells in the indicated pathological groups. (d-e) Phenotype and frequency of tumor (MART-1, NA-17) or viral (EBV) specific T-cells assessed by tetramer staining of CD8+CD5+ cells in HLA-A2+ mUMs (unique black symbols) and HD (red circles). (d) Representative MART-1 or NA.17 versus EBV Tet. staining (upper panels). Naïve/memory/effector status of tet+ cells (bottom panels). (e) Frequency of MART-1, NA.17 and EBV specific CD8 T-cells in mUMs and HDs.
Figure 5: Effector chCD4 numbers vary according to tumor burden and chemotherapy in breast cancer patients:

(a) chCD4 numbers in BC patients before (group (a)) and after primary surgery (b) in patients who then received adjuvant chemotherapy, or before neo-adjuvant chemotherapy (c). The shaded area indicates the values found in HD. p values according to Mann-Whitney U tests. (b-d) Numbers of total (b) or effector (c) chCD4s and effector CD8 (d) T-cells in BC patients before and after the indicated chemotherapy. p values: Wilcoxon signed-rank test. (e) Correlation between the proportion of effectors in chCD4 and CD8 T-cells before (left panels) and after (right panels) adjuvant (upper panels) or neo-adjuvant (lower panels) chemotherapy. p values: Spearman’s rank test.

Figure 6: The expansion of chCD4s during neo-adjuvant chemotherapy is correlated with tumor regression in breast cancer:

(a-b) Variation of the indicated parameters before and after neo-adjuvant chemotherapy in 22 patients. The data are expressed as a percentage of the difference between the pre and post treatment absolute numbers normalized to the initial value (c) Absence of correlation between the variation in the number of effector CD8 T-cells and tumor regression. (d) Correlation between the variation in the absolute number of chCD4s and tumor regression after neo-adjuvant chemotherapy.
Figure 1

HD

T-Reg

CD4conv

1.05

CD127 CD25

mUM

BC

HD

n=39 12 59 30 8

HIV Tuberculosis

naive memory effector

CD127 CD25
d

e

convCD4

chCD4

CD45RO

CD27

CD4 convCD4 (CD127^-CD25^-CD4^+CD3^+)

chCD4 (CD127^+CD25^-CD4^+CD3^+/

percentage

naive memory effector

HD

mUM

BC

naive memory effector

BC

mUM

CD4 conv

chCD4

CD45RO

CD27

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Figure 3

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Figure 4

<table>
<thead>
<tr>
<th>a</th>
<th>Reference percentage of TCR Vß expression in CD4+CD3+ (min; mean and max)</th>
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<tbody>
<tr>
<td>b</td>
<td>% of TCR Vß in chCD4 (%)</td>
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<tr>
<td>c</td>
<td>% of effectors in CD8+CD3+ (%)</td>
</tr>
<tr>
<td>d</td>
<td>Frequency of Tetramer in CD8+CD3+ (%)</td>
</tr>
<tr>
<td>e</td>
<td>Frequency of Tetramer in CD8+CD3+ (%)</td>
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Legend:
- CTRL
- MART-1
- NA.17
- mUM
- HD
- EBV
- Memory
- Naive
- CD45RO
- CD27

Patient A
Patient B
Patient C

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
% of effectors in CD8+CD3+

% of effectors in chCD4 (CD127-CD25-CD4+CD3+)

r/two.superior=0.41
r/two.superior=0.40
r/two.superior=0.24
r/two.superior=0.64

p=0.025
p<0.0001

10/three.superior
10/two.superior
10/one.superior

Figure 5
Figure 6

∆ of absolute number of T cells from initial value (%)

Spearman r= 0.70
p=0.0003

Delta of Effector CD8(+CD3(-)

Spearman r=0.12
p=0.60

∆ of absolute number from initial value (%)

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