

Chemotherapy-induced Changes and Immunosenescence of CD8⁺ T-Cells in Patients with Breast Cancer

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Abstract. *Background:* Changes in sub-populations of cytotoxic (CD8⁺) T-cells, which are observed in aging and in conditions of chronic immune stimulation, are not well-documented in cancer. *Materials and Methods:* Using flow cytometry, CD8⁺ T-cell subsets were analyzed in patients with breast cancer undergoing DNA-damaging chemotherapy and in an older female control group during a six-month longitudinal study, to explore shifts in CD8⁺ T-cells and the effect of DNA-damaging chemotherapy on different T-cell sub-populations. *Results:* As expected, there was a consistent decrease in absolute numbers of leukocytes, lymphocytes, T-cells and CD8⁺ T-cells during chemotherapy in patients with cancer. Among the T-cells, there was a lower CD8⁻/CD8⁺ ratio, persisting over the six months, in patients with cancer compared to controls. The proportion of CD28⁻CD57⁺ cells also remained higher among patients with cancer throughout the sampling duration. The number of CD28⁺CD57⁻ and CD28⁻CD57⁻ cells decreased faster during DNA-damaging chemotherapy than CD28⁺CD57⁺ and CD28⁻CD57⁺ cells, while only CD28⁻CD57⁻ cells showed a significant reconstitutive capacity after six months. *Conclusion:* Immunosenescence appeared to be pronounced in patients with breast cancer, with senescent CD8⁺ T-cells playing a role. The normal condition was not restored after six months of chemotherapy.

Aging results in a decline in immune response and shifts in T-cell sub-populations towards a less functional status, known as immunosenescence (1, 2). Changes associated with

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T-cell immunosenescence, which culminate in an immune risk profile (IRP), include a decreased ratio of the helper (CD4⁺) to the cytotoxic (CD8⁺) T-cells and the accumulation of senescent and terminally-differentiated T-cells (1-3). As immunosenescence-associated changes are exacerbated by persistent immune stimulation, they might play a role in cancer (4). Recently, some reports have emerged on the possible role of immunosenescence in the progression of cancer, which in most cases, when occurring in adults, is an age-associated disorder (5-7).

Although immunosenescence differs from cellular senescence, both phenomena share similarities, including telomere attrition and enhanced expression of some cyclin-dependent kinase (CDK) inhibitors that block cell-cycle progression in T-lymphocytes (8-13). In addition, senescent cells are also recognized by their loss of proliferative capacity and secretion of senescence-associated substances, which include a number of cytokines and chemokines (10). Earlier studies described that the loss of expression of one of the co-receptors required for naïve T-cell activation (CD28) allows the identification of senescent T-cells (14). Later, cells expressing a terminally-sulfated glycan carbohydrate epitope (CD57) (both CD28⁻CD57⁺ and CD28⁺CD57⁺ subpopulations) were shown to have better characteristics of senescent cells based on their loss of proliferative capacity *in vitro*, decreased telomere length, enhanced expression of CDK inhibitors p16 and p21, their cytotoxicity and cytokine secretion profile, differential homing and differentiation characteristics and their higher presence in elderly humans (9, 13, 15-18).

Cellular senescence can be provoked prematurely by DNA-damaging chemotherapy (19, 20). However, this stress-induced premature senescence (21, 22) has largely been established *in vitro*, with limited *in vivo* evidence, especially in humans. Furthermore, the induction of senescence by chemotherapy has mainly been explored in cancer cells and their tumor microenvironment (23). Yet chemotherapy when

Table I. *The cancer diagnoses and the chemotherapy regimens used.*

| Breast cancer subtype | Stage | Chemotherapy | No. of patients (no. of receivers) |
|-----------------------|-------|---------------------|------------------------------------|
| MCA | II | FEC+P (1), EC+P (1) | 2 |
| IDC | I | EC+P (1) | 1 |
| | II | EC+P (1), FEC+P (6) | 7 |
| | III | EC+P (1), FEC+P (7) | 8 |
| | IV | FEC+P (1) | 1 |
| ILC | II | FEC+P (1) | 1 |
| | IV | FEC+P (1) | 1 |

MCA: Medullary carcinoma, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma. EC+P: epirubicin and cyclophosphamide (4 cycles, 1 per 2 weeks), followed by paclitaxel (12 cycles, 1 per week); FEC+P: 5-fluorouracil, epirubicin and cyclophosphamide (4 cycles, 1 per 3 weeks), followed by paclitaxel (12 cycles, 1 per week). 5-Fluorouracil, epirubicin and cyclophosphamide are DNA-damaging drugs, while paclitaxel is an inhibitor of mitosis progression.

administered *in vivo* affects not only cancer cells but also all other cells in the body (24, 25).

In the present study, we hypothesized that cancer could be associated with shifts in sub-populations of T-cells associated with immunosenescence that would, at least, be similar to what is seen in the elderly. Since DNA-damaging chemotherapy can induce cell senescence and as proliferating cells should be more responsive to chemotherapy-induced cell death (26, 27), we also expected either less decline or an increase in the frequencies of CD28⁻CD57⁺ and CD28⁺CD57⁺ CD8⁺ T-lymphocytes after DNA-damaging chemotherapy. These hypotheses were tested through a longitudinal observation of patients with breast cancer at the early disease stages undergoing chemotherapy and a control group comprising of older females.

Patients and Methods

Patients. A total of 21 patients with breast cancer (median age=49 years, range=44-56 years) and 17 community dwelling, healthy women (median age 72 years, range=68-74 years) as controls were prospectively recruited into the study and followed-up for six months between November 2011 and July 2013. Previous related studies have shown that the sample size in each participant group was sufficient for the study (13, 28-30). The control group passed a comprehensive medical assessment before they were included in the study. The exclusion criteria for all participants were the presence of hematological disorders, prior immunodeficiency and involvement in strenuous exercise within 24 h prior to sampling (31, 32). The participants were sampled at baseline (T0), after which the patients started receiving chemotherapy; at one month (T1); at three months (T3), corresponding to the end of the DNA-damaging chemotherapy; at six months (T6), corresponding to the end of paclitaxel therapy. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The Institutional Review Board of the Universitair Ziekenhuis

Brussel (OG016) approved the study and all participants provided written informed consent.

Blood cell enumeration and preparation of peripheral blood leukocytes (PBLs). Fresh peripheral venous blood samples, collected in EDTA tubes, were used for the enumeration of blood cells in a Cell Dyn Sapphire[®] Analyzer (Abbot Diagnostics, Wavre, Belgium). Portions of the blood samples were incubated in lysis buffer (154.0 mM NH₄Cl, 0.1mM EDTA and 10.0 mM KHCO₃) for 10 minutes to remove red blood cells and then centrifuged at 800 g for 4 minutes to obtain the PBLs. These were then washed in 1% BSA-PBS and used for analysis of the cell surface markers and delineation of the different subpopulations.

Flow cytometric analysis. PBLs from participants were stained with a panel of antibodies by direct immunofluorescence. The number of cells expressing the different markers was quantified by five-color flow cytometry (Cytomics FC 500) (Beckman Coulter (Analisis), Suarlée, Belgium). The following antibodies were used in appropriate combinations and concentrations: PE-cy5-anti-CD8 (BD Biosciences, Erembodegem, Belgium), FITC-anti-CD28, PE-anti-CD57 and PE-cy7-anti-CD3 (Biolegend (Imtec), Antwerp, Belgium). All antibodies were matched with isotype controls (Santa Cruz Biotech, Heidelberg, Germany). Quality control panels were used in order to exclude autofluorescence, fluorochrome interference and dead cells, including compensation controls based on data collected from single fluorochrome staining, fluorescence-minus-one controls that includes other stains and exclude the stain in a particular channel to define the boundary between positive and negative cells in a given channel and dead cell exclusion control using 7-amino actinomycin-D (7-AAD) staining. Quality controls for the instrument were performed daily by checking the detector voltage values for conformity with initial protocol and running daily verification of the dynamic range of the detectors using standardized quality control compensation beads.

For identification of cell surface antigens, approximately 5×10⁵ lymphocytes in 50 µl of 1% BSA -PBS were incubated with 20 µl of appropriate combination of antibodies for 20 minutes at room temperature in the dark. After staining, the cells were washed with PBS and were resuspended in 500 µl of PBS for flow cytometric analysis. For all samples, 100.000 PBL events were acquired for analysis. The gating procedure for the delineation of the different subpopulations is as we previously reported (13). Firstly, the lymphocyte sub-population was gated according to granularity and size in the forward scatter *versus* side scatter plot. To obtain a pure T-lymphocyte cluster, eliminate duplexes and avoid contamination with monocytes, neutrophils, natural killer cells and cell debris, we combined CD3 positivity and side scatter properties to gate pure CD3⁺ lymphocytes (T-cells). Next, CD3⁺CD8⁺ lymphocytes were gated within the CD3⁺ cells on the CD3 *versus* CD8 dot plot. Flow cytometric dot plots and histograms were used to separate and identify different subpopulations of CD3⁺CD8⁺ lymphocytes based on their expression or non-expression of a combination of markers.

Statistical analysis. Statistical analysis was performed using IBM[®] SPSS[®] (version 22) (Armonk, NY, USA). Data on immunological parameters are presented in box plots, with the bottom and top of the boxes representing the lower (Q1) and upper (Q3) quartiles respectively, the line inside the box representing the median and the whiskers representing the highest

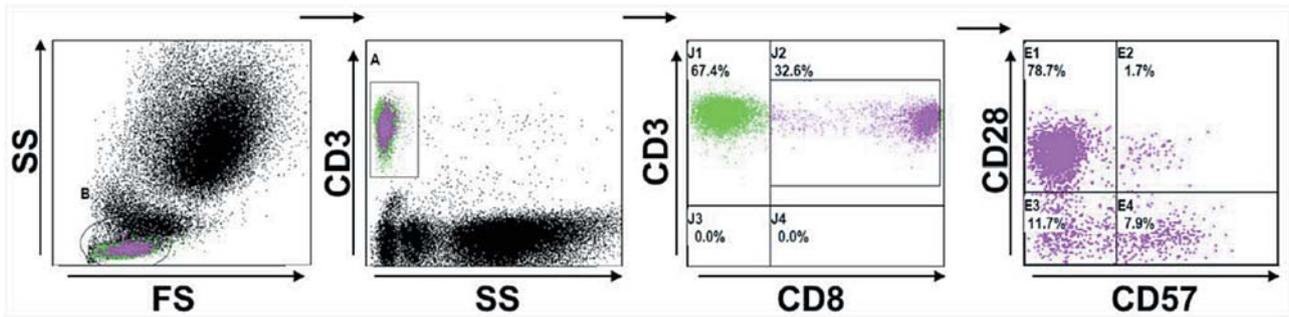


Figure 1. Representative dot plots for the delineation of the different sub-populations by flow cytometry. By combining side scatter (SS) versus forward scatter (FS) and anti-CD3 conjugated fluorochrome fluorescence versus SS plots, CD3⁺ cells were identified as cells that were both in gates A and B. CD8⁺ cells were obtained from the pure CD3⁺ population and were further sub-divided based on the expression of CD28 and CD57.

and lowest observed values that were not outliers. The baseline ages are reported as median and range as mentioned in “Patients” section. Analysis of differences between two independent groups was performed with the Mann-Whitney *U*-test. Evolution of outcome measures over time among related groups of variables was analyzed using Friedman’s test. When the evolution over six months was significant, differences between the baseline and subsequent time-points were analyzed with the Wilcoxon Rank test. Changes between two points in the test group were compared with those of the control group using the Mann-Whitney *U*-test. Exact statistical testing was used in the estimation of significant differences. Differences were considered to be significant at a two-sided *p*-value of less than 0.05. Significant differences are shown in the figures.

Results

The diagnoses and chemotherapy regimens of the patients are given in Table I. The gating procedure for the delineation of the different subpopulations is shown in Figure 1. Figure 2 shows the absolute numbers of leukocytes, lymphocytes, T-lymphocytes and CD8⁺ T-lymphocytes at the various sampling stages for all participants. At baseline, the leukocyte, lymphocyte, T-lymphocyte and CD8⁺ T-cell densities were significantly higher in the patients with cancer than in the controls. As expected, during follow-up, leukocyte, lymphocyte, T-lymphocyte and CD8⁺ T-lymphocyte counts declined among the patients and remained stable among the controls.

Figure 3 shows the absolute numbers of the four CD8⁺ T-cell subpopulations CD28⁺CD57⁻, CD28⁺CD57⁺, CD28⁻CD57⁻ and CD28⁻CD57⁺ among the various participants over the six-month period. In order to better relate the changes occurring among the four subpopulations, Figure 4 shows their proportion relative to the total number of CD8⁺ cells. At baseline, the absolute number of CD28⁺CD57⁻, CD28⁺CD57⁺ and CD28⁻CD57⁺ cells for the patients with cancer were significantly higher than in the control group. Only the CD28⁻CD57⁺ cells were proportionally higher in the patient

group than in the controls (Figure 4b), while the proportion of CD28⁻CD57⁻ cells was lower (Figure 4c).

During follow-up, there was no change in the numbers or in the proportions of the four subpopulations among the control participants. Among the patients with cancer, there was a significant decline in the absolute numbers of the four sub-populations. The decline appeared to be faster in the CD57⁻ cells (significant decline after one month) than in the CD57⁺ cells (significant decline only after three months). This appeared to be reflected, after one and three months of treatment, in significantly higher numbers (Figure 3b) and proportions (Figure 4b) of CD28⁺CD57⁺ cells among the patients with cancer compared to the controls. The percentage change between baseline and three months was significantly higher among the patients than among the controls (*p*=0.043; data not shown). On the other hand, the number and proportion of CD28⁻CD57⁻ cells at the first and third months were significantly higher among the control group than among patients with cancer.

At the sixth month, no significant differences between the patients and the controls were noted for the absolute cell numbers of the subpopulations. At the sixth month, the proportions of the subpopulations were similar in patients and controls, except for CD28⁻CD57⁺ cells, which proportionally remained higher in the patients with cancer throughout the whole observation period.

The ratio of CD8⁻/CD8⁺ T-cells is shown in Figure 5. At all time-points, patients with cancer had a significantly lower CD8⁻/CD8⁺ ratio than did the control group.

Discussion

We performed a six-month longitudinal analysis of subpopulations of CD8⁺ T-cells, focusing on those that have been described as senescent cells, in breast cancer patients, before and during chemotherapy and in an the older female control group.

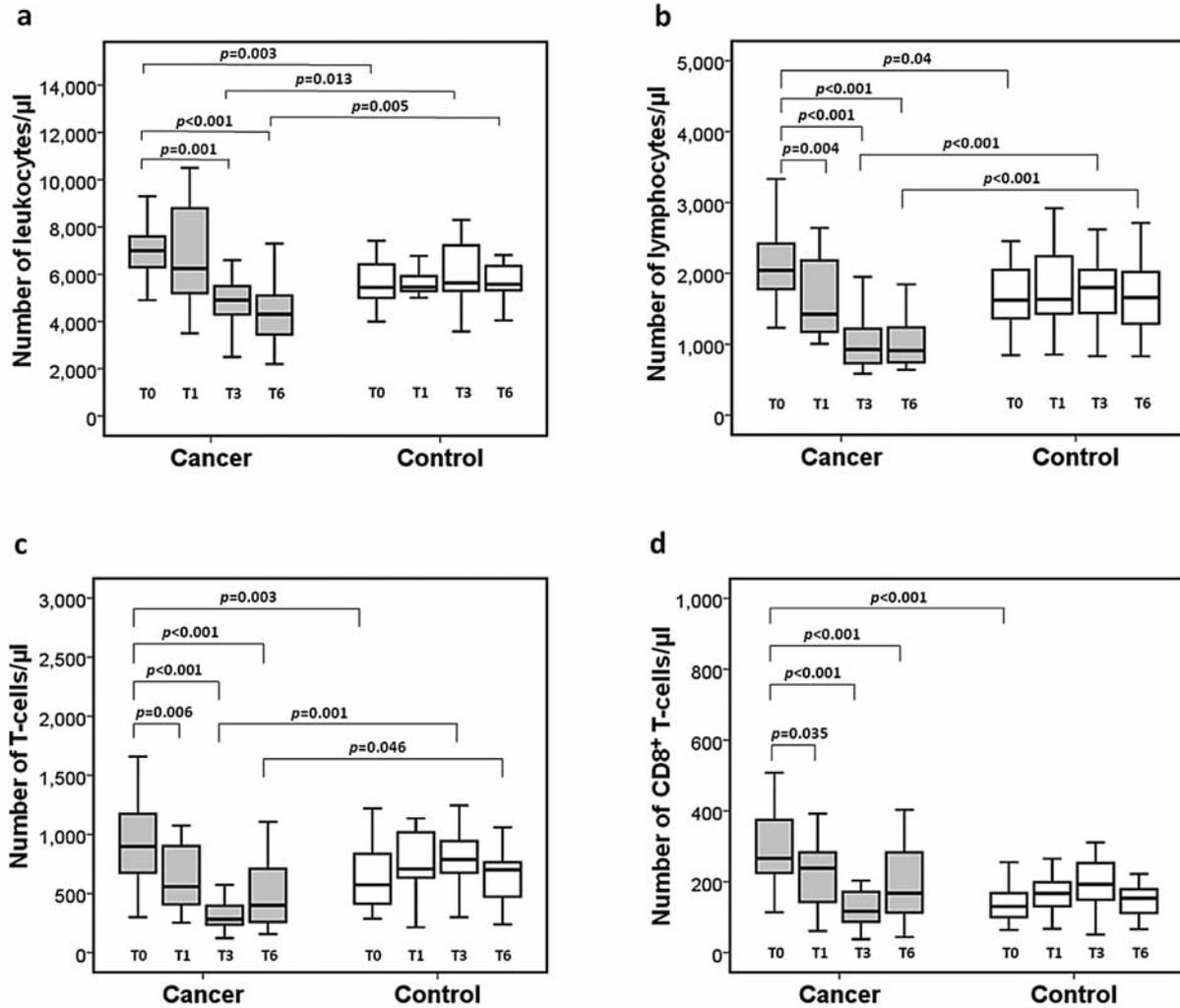


Figure 2. Absolute numbers of leukocytes (a), lymphocytes (b), T-lymphocytes (c) and CD8⁺ T-lymphocytes (d) among patients with cancer and controls at baseline (T0), 1 month (T1), 3 months (T3) and 6 months (T6).

The group of patients with cancer presented a sub-population profile of CD8⁺ T-cells that might refer to an IRP. Firstly, the terminally-differentiated effector memory and senescent-enriched CD28⁻CD57⁺ cells were more predominant in patients with cancer than in the controls. Of note is that the CD28⁻CD57⁺ cells remained proportionally at the same high level during the six-month follow-up in patients with breast cancer. Secondly, an increase in the proportion of CD8⁺ cells, which results in a decreased ratio of CD4⁺/CD8⁺ cells, has been identified as an immunosenescence marker and is part of the IRP (33, 34). Similar observations have recently been made in some groups of patients with cancer (5). Since at least 95% of CD8⁻ T-cells are CD4⁺ (13, 35), CD8⁻ T-cells are a suitable approximation of the proportion of CD4⁺ T-cells. The decreased CD8⁻/CD8⁺ ratio that we found among the patients with cancer provides

further support for the presence of enhanced immunosenescence and IRP. As our control group was older than the cancer group, the inverse observations were to be expected if only age had been a factor (13, 16). Previous reports have shown that individuals with unfavorable IRP maintain the same state for a long period, which in most cases results in a shortened life expectancy (34, 36). A study in patients with advanced gastric cancer demonstrated an association between CD57⁺ cells and worsened survival (37).

Besides age, cytomegalovirus (CMV) infection has been associated with changes in immunosenescence-related parameters in the elderly (33, 36, 38). A recent report showed no influence of CMV seropositivity on parameters of immunosenescence between patients with cancer and healthy controls (6). Therefore, even though we did not determine the CMV status of our participants, CMV

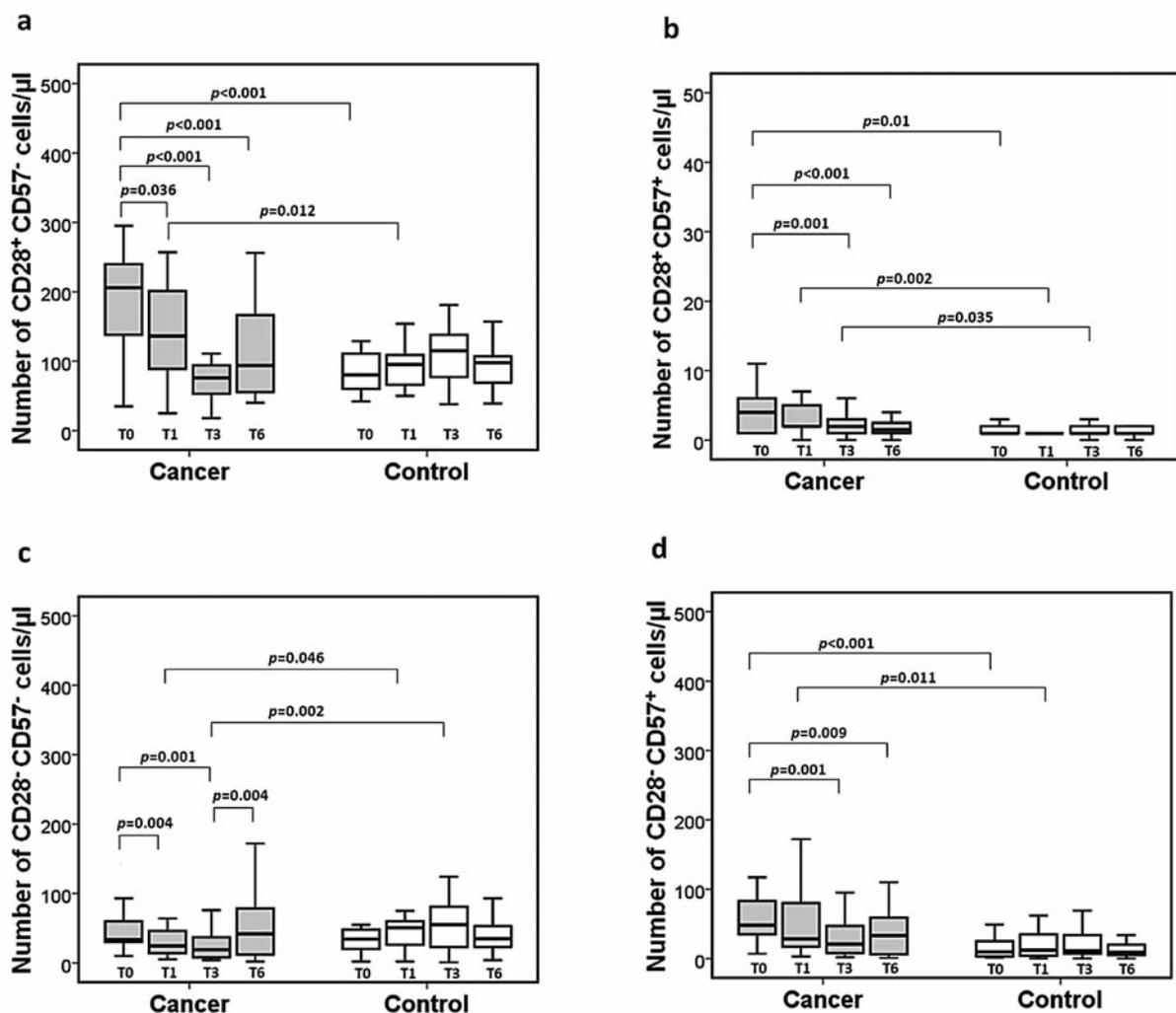


Figure 3. Absolute numbers of CD28⁺CD57⁻ (a), CD28⁺CD57⁺ (b), CD28⁻CD57⁻ (c) and CD28⁻CD57⁺ (d) cells from CD8⁺ T-cells among patients with cancer and controls, at baseline (T0), 1 month (T1), 3 months (T3) and 6 months (T6).

seropositivity might not have played a significant role in the outcomes of the present study. Furthermore, the observation of a higher degree of immunosenescence in patients with cancer than in the older control group, which are more prone to CMV infection (33, 36, 38), strengthens our view on the importance of immunosenescence in cancer.

Differences in CD8⁺ T-cell sub-populations might be attributed to different degrees of T-cell stimulation by various tumour antigens (6, 39). Analogous to the occurrence of IRP, our observations might point to enhanced antigen exposure and persistent immune stimulation in determining the level of CD28⁻CD57⁺ cells. Notably, our data indicate that these changes are already present, even at the early stages of breast cancer, thereby corroborating a previous report (40). However, it is unclear from our study whether the differences in CD8⁺ T-cell sub-populations among the patients occurred

prior to the occurrence of the cancer, or were a consequence of their presence.

At the third month, we observed higher numbers and proportions of CD28⁺CD57⁺ cells among patients with cancer compared with controls. Notably, the chemotherapy combinations used for the patients during the first three months were all DNA-damaging and have been identified to precipitate cellular senescence *in vitro* (19, 20, 23, 41). As the third-month sampling corresponded with the end of the DNA-damaging chemotherapy regimen, this difference might point to a possible effect of DNA-damaging chemotherapy in enhancing the accumulation of senescent CD28⁺CD57⁺ cells. However, other explanations for this observation are possible. Apart from the induction of cellular senescence, the acute lymphopenic effect of DNA-damaging chemotherapy is well documented (27, 42-44), with proliferating T-cells as

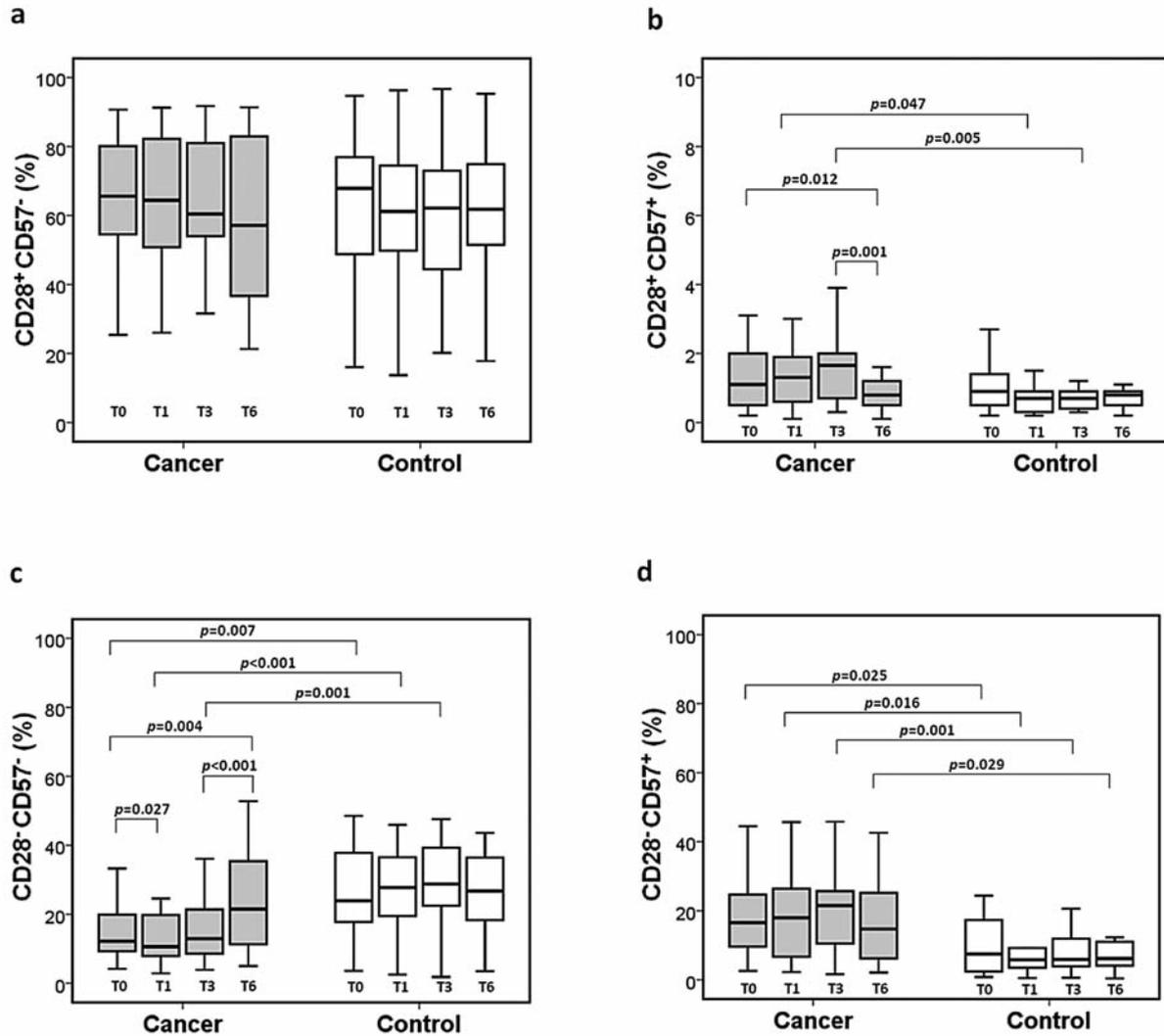


Figure 4. Proportions of CD28⁺CD57⁻ (a), CD28⁺CD57⁺ (b), CD28⁻CD57⁻, (c) and CD28⁻CD57⁺ (D) cells from CD8⁺ T-cells among patients with cancer and controls at baseline (T0), 1 month (T1), 3 months (T3) and 6 months (T6).

the major targets (27) and increasing proportions of non-dividing cells as a result. A significant decrease in the number of CD28⁺CD57⁻ (mainly naïve and central memory T-cells) and CD28⁻CD57⁻ cells (richly populated with effector memory cells) (18), was already observed after one cycle of chemotherapy, while it took longer for CD28⁺CD57⁺ and CD28⁻CD57⁺ cells to decrease significantly. This observation, therefore, would be compatible with the non- or low proliferative capacity of CD28⁺CD57⁺ and CD28⁻CD57⁺ cells previously observed *in vitro* (9). Apoptosis has been described as a mechanism for chemotherapy-induced T-cell death (45, 46). A better resistance to chemotherapy-induced lymphopenia of CD28⁺CD57⁺ and CD28⁻CD57⁺ cells might also indicate

resistance to apoptosis of CD57⁺ cells *in vivo*. Both resistance to apoptosis and loss of proliferative capacity are characteristics of senescent cells (47, 48).

A previous study demonstrated an increased expansion and a faster rate of reconstitution of CD28⁻ cells than naïve and memory cells after DNA-damaging chemotherapy (42). Three months after the last cycle of DNA-damaging chemotherapy, which also corresponded with the end of paclitaxel therapy, a significant subpopulation reconstitution was observed only among CD28⁻CD57⁻ cells. This observation would indicate that CD28⁻CD57⁻ cells, more than CD28⁻CD57⁺ cells, account for the increased expansion of CD28⁻ cells observed after chemotherapy (42). As paclitaxel is known to have a reduced effect on modulating immune

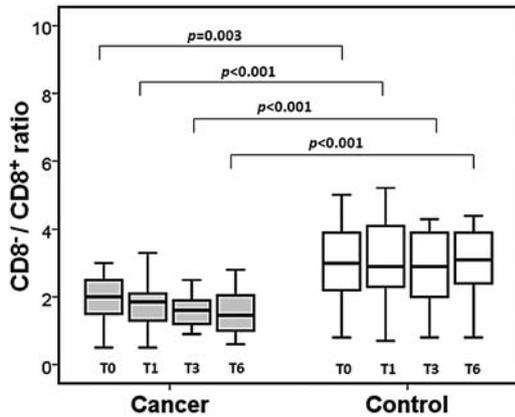


Figure 5. The ratio of CD8⁻/CD8⁺ T-cells among cancer patients and controls, at baseline (T0), 1 month (T1), 3 months (T3) and 6 months (T6).

parameters (49), this provides *in vivo* evidence for the proliferation of CD28⁻CD57⁻ cells, distinguishing them from non- (or slowly) proliferating CD28⁻CD57⁺ cells.

In conclusion, the present study shows that immunosenescence and immune risk parameters are more pronounced in patients with breast cancer than in controls. Together, the observed shifts in CD8⁺ T-cell subpopulations tend to aggravate the senescence and exhaustion of T-cells, subverting the immune system in favor of cancer progression. An important component of this shift is the filling of the immunological space with less cancer-reactive subpopulations of T-cells, at the detriment of the naïve and central memory cells that are more potent against cancer (39). This situation resembles the higher degree of immunosenescence and subpopulation variations seen in patients with chronic antigen challenge and immune activation, as during persistent viral infections (50) and in rheumatoid arthritis (51). Immunosenescence might, therefore, negatively contribute to outcomes in patients with breast cancer and could be a target for therapy. Our results also indicate that CD57⁺ cells (both CD28⁺CD57⁺ and CD28⁻CD57⁺ cells) exhibit better resistance to DNA-damaging chemotherapeutic agents than do the other subpopulations, providing further evidence for their senescent properties and the existence of senescent cells *in vivo*. A longer observation period will be necessary to determine if reconstitution of the immune cells after stopping chemotherapy can neutralize the immunosenescence profile.

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