Interleukin-7-receptor-signaling plays a pivotal role in T-cell development and maintenance of T-cell memory. We studied IL-7Rα (CD127) expression in PBMCs obtained from patients with breast cancer and cancer- and inflammation-mediated downstream effects defined by STAT5 phosphorylation (p-STAT5). Reduced numbers of IL-7Rα-positive cells were identified in CD4+ T-cells as well as in a CD8+ T-cell subset defined by CD8α/β homodimer expression in patients with breast cancer. PBMCs obtained from healthy donors (n = 19) and from patients with breast cancer (n = 19) exhibited constitutive p-STAT5 expression in the range of 0–6.4% in CD4+ T-cells and 0–4% in CD8+ T-cells. Stimulation with recombinant human IL-7 for 15 min increased p-STAT5 expression up to 36–97% in CD4+ T-cells and to 26–90% in CD8+ T-cells obtained from healthy control donors (n = 19). In contrast, PBMCs obtained from 13/19 patients with breast cancer did not respond to IL-7 as defined by STAT5 phosphorylation, despite expression of IL-7Rα on T-lymphocytes. T-cells were further characterized for IL-2 and IFN-γ production induced by PMA/Ionomycin. PBMCs from 9/19 patients with breast cancer showed decreased IL-2 and IFN-γ production combined with IL-7-signaling defects; PBMCs from 4 patients with breast cancer exhibited deficient IL-7-signaling, yet intact cytokine production. Reduced numbers of IL-7Rα-positive cells and nonresponsiveness to IL-7, defined by lack of STAT5 phosphorylation, characterizes the immunological profile in T-cells from patients with breast cancer.

**Material and methods**

**Subjects**

PBMC samples from age-matched healthy volunteers were obtained from the Blood Bank, Karolinska Hospital, Stockholm, Sweden and PBMCs from patients with breast cancer were provided from the Department of Obstetrics and Gynecology, Johannes Gutenberg University, Mainz, Germany. Samples have been obtained after informed consent of the patients, the study has been approved by the local ethics committee (filed 837.287.05 (4945)). PBMCs were obtained at the day of the diagnostic procedure to test for malignancy, prior to initiation of therapy.

**Flow cytometric analysis of PBMCs**

PBMCs from both healthy donors (n = 19) and patients with breast cancer were thawed, washed in RPMI standard medium (Invitrogen Corporation, Carlsbad, USA), and analyzed for interleukin-7 receptor (IL-7Rα) expression. Flow cytometric analysis was performed using the monoclonal antibodies anti-CD127 PE (clone 2ST8.5H7), anti-CD8α PC7 (clone SFC121Thy2D3), obtained from Beckman Coulter Inc. (BCI), Fullerton, USA. Anti-CD107a FITC (clone H4A3) was obtained from BD Pharmingen, San Diego, USA.

Binding of IL-7 to IL-7Rα leads to receptor heterodimerization with γc and to a series of intracellular phosphorylation events mediated by Jak1 and Jak3, Src kinases and STATs (signal transducer and activators of transcription), mainly STAT5A/B. Jak3 and Jak1 phosphorylate tyrosine residues in the cytoplasmic region of IL-7Rα, which leads to the recruitment of intracellular signaling molecules.

**Key words:** T-cells; cytokines; memory; signal transduction; tumor immunity


Reduced numbers of IL-7 receptor (CD127) expressing immune cells and IL-7-signaling defects in peripheral blood from patients with breast cancer

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Constitutive and IL-7-induced phosphorylated STAT5 (p-STAT5) expression was evaluated in PBMCs from healthy donors and patients with breast cancer in CD4+ and in CD8+ T-cells. PBMCs were starved overnight in serum-free medium (AIM-V from Invitrogen, Carlsbad, USA) followed by incubation with recombinant human IL-7 (rh IL-7-100 ng for 10⁵ cells provided from Dr. Adrian Minty, Sanofi, France) for 15 min at 37°C. Cell surface markers were stained with anti-CD3 ECD (clone UCHT1), anti-CD4 PC5 (clone 13B8.2) and anti-CD8α PC7 (clone SFC121Thy2D3) obtained from BCI. Cells were incubated for 15 min at 4°C and immediately fixed with 2% PFA at 37°C for 10 min followed by washing with staining buffer (BD Biosciences) and centrifugation at 800 rpm without break. PBMCs were permeabilized with 90% methanol for 30 min on ice, followed by washing twice with staining buffer. The anti-p-STAT5 antibody (y694)-Alexa 488 (BD Biosciences) was added for 1 h at room temperature in the dark and p-STAT5 was analyzed by flow cytometry using a FACSAria (BD Biosciences). Optimal conditions for IL-7 concentrations ranging from 1 ng up to 100 ng, as well as the optimal time frame (5, 10, 15, 30 and 120 min) were evaluated prior to testing (data not shown). A 15-min stimulation
with 100 ng IL-7 turned out to be the optimal condition to gauge IL-7-mediated effects; IL-7-mediated STAT-5 phosphorylation could effectively be blocked by preincubation of immune cells with the anti-IL-7Rα monoclonal antibody R34.34 (data not shown).

Intracellular cytokine staining

Interleukin-2 (IL-2) and Interferon-γ (IFN-γ) production were measured in unstimulated PBMCs (medium only), after crosslinking the TCR (plate bound OKT-3 1 μg/well) or PMA (25 ng/ml)/Ionomycin (1 μg/ml) stimulation for 6 h in the presence of Brefeldin-A (10 μg/ml) by intracellular cytokine staining (ICS). The Intraprep permeabilization reagent kit was purchased from BCI. Anti-IL-2 PE (Clone N7-48A) was obtained from Holzel Diagnostics, Cologne, Germany and anti-IFN-γ (clone 45.15) was obtained from BCI. Samples were analyzed using a FACSAria and FACSDiva software (BD Biosciences).

Statistical analysis

Flow cytometry results are presented as median (percentile) and mean fluorescence intensity (MFI). Differences between expression of IL-7Rα, coexpression of CD107a and CD127, IL-2 and IFN-γ production in CD8αβ+/- T-cells, or CD4+ T-cells obtained from patients with breast cancer and healthy controls were evaluated using the Mann–Whitney test. The proportion of PBMCs responding to IL-7 defined by p-STAT5 expression (comparison of healthy donors and patients with breast cancer) was tested with the χ² test. Responding PBMCs to PMA/Ionomycin or anti-CD3 stimulation for cytokine production from healthy donors and patients with breast cancer was tested by the Fishers exact test. All tests were considered significant if the p value was less than 0.05.

Results

IL-7Rα (CD127) analysis in T-cells from patients with breast cancer

PBMCs obtained from age-matched healthy blood donors (n = 19) and patients with breast cancer (n = 19) were analyzed for IL-7Rα (CD127) and CD107a (LAMP-1) expression shown in Figure 1a. In PBMCs obtained from healthy blood donors, the majority of CD8αβ+ and CD8αβ+ T-cells stained positive for IL-7Rα (17–100% for CD8αβ with a median of 72% and 24–100% for CD8αβ with a median of 70%). PBMCs obtained from patients with breast cancer showed a reduced number of IL-7Rα-positive CD4+ T-cells (44–88% with a median of 77%, p < 0.01, Table I). The percentage of LAMP-1 (CD107a) in CD4+ T-cells from PBMCs obtained from healthy controls ranged from 0 to 0.2% with a median of 0% and in CD8αβ+ from 0 to 1%, with a median of 0.1%. In contrast, PBMCs obtained from patients with breast cancer showed higher numbers of CD107a+ T-cells and CD4+ T-cells (0–0.8% with a median of 0.2%, Table I) and decreased numbers of CD107+ CD8αβ+ T-cells, i.e. 0–1% (median 0%), analyzed with the Mann–Whitney Test (p < 0.01). Increased numbers of coexpressing CD107α and CD127 immune cells was found in CD8αβ+ T-cells and CD4+ T-cells in PBMCs from patients with breast cancer in the range of 0–15% with a median of 3% in CD8αβ+ T-cells (p = 0.02) and 0–5.2% with a median of 0.5% (p = 0.03) in CD4+ T-cells. Differences were statistically significant, tested by the Mann–Whitney test (Table I). Isotype-matched control antibodies were included in the experiment as shown in Figure 1a.

These data address the percentage of CD127 positive cells in immune cell subsets, they do not reflect the density of CD127 or CD107 expression on T-cells subsets. Thus, a higher percentage of CD107a/CD127+ T-cells in PBMCs from patients with breast cancer patients, particularly in CD8αβ T-cells as compared to cells from healthy donors (Fig. 1b). Thus, a higher percentage of CD107a/CD127+ T-cells in PBMCs (Table I) is not necessarily associated with increased IL-7 receptor protein expression in these T-cell subsets reflected by reduced MFI values (Fig. 1b).

IL-7-signaling defects in PBMCs from patients with breast cancer

Next, we evaluated potential defects in IL-7-receptor-mediated-signaling using a technique, which measures phosphorylated STAT5 (p-STAT5) in PBMCs upon stimulation with recombinant human IL-7 as shown in Figure 2a. Intracellular p-STAT5 levels are IL-7 dose-dependent and can be blocked by preincubation of immune cells with the monoclonal antibody R34.34 (data not shown). PBMCs obtained from blood donors (n = 19) expressed p-STAT5 constitutively in the range of 0–4% (median 0.7%) in CD8+ T-cells and 0–6% (median 2.3%) in CD4+ T-cells. In con-
Contrast, PBMCs from patients with breast cancer exhibited comparably lower numbers of constitutive p-STAT5 expressing cells, i.e. 0–0.9% (median 0.2%) in CD8+ T-cells and 0–6.4%, with a median of 0.2%, in CD4+ T-cells tested by the Mann–Whitney test with a p-value <0.001 in CD4+ T-cells and 0.002 in CD8+ T-cells.

p-STAT5 was readily detectable after IL-7 stimulation in PBMCs from all healthy control donors (26–83% with a median of 67% in CD8+ T-cells and 36–97% with a median of 87% in CD4+ T-cells). In PBMCs obtained from patients with breast cancer, only 6/19 PBMC samples were able to respond to IL-7 stimulation in the range of 65–90% with a median of 84% in CD8+ T-cells and 69–87% (median 87%) in CD4+ T-cells. PBMCs obtained from 13/19 patients did not respond to IL-7 stimulation despite of IL-7 receptor expression (Fig. 2b). The proportion of PBMCs responding to IL-7 is lower in patients with breast cancer as compared to healthy donors (p-value < 0.001) tested with the χ²-test. Constitutive expression of p-STAT5 was comparably lower in patients with breast cancer calculated by using Mann–Whitney test (p-value < 0.001 in CD4+ T-cells and 0.002 in CD8+ T-cells). C = healthy control blood donor, Pat = patient with breast cancer. *p-value < 0.001 with the χ²-test. **p-value < 0.001 and ***p-value ≤ 0.002 tested with the Mann–Whitney test.

Cytokine production is impaired in PBMCs from patients with breast cancer.

Thirteen of nineteen PBMC samples from patients with breast cancer showed significant defects in IL-7-mediated-signaling.
Next, we tested if other immune effector functions are impaired in CD4+ or CD8+ T-cells from patients with cancer. Intracellular cytokine production, i.e. Interleukin-2 (IL-2) and Interferon-γ (IFN-γ) was tested after stimulating PBMCs with PMA/Ionomycin or by T-cell receptor crosslinking using ICS. Stimulation of PBMCs obtained from blood donors with PMA/Ionomycin leads to IFN-γ production in CD8αβ+ T-cells (9.5–80% with a median of 31%) and 5.4–66% in CD8αβ+ T-cells respond with IFNγ production. PBMCs obtained from 9/19 patients with breast cancer were not able to respond either to PMA/Ionomycin or to OKT-3 stimulation (Figs. 3b and 3c was calculated using the Fishers exact-test (< 0.001)).

**FIGURE 3** – Impaired cytokine production in PBMCs obtained from patients with breast cancer. (a) Gating strategy: PBMCs were first gated on CD3+ T-cells and further differentiated into CD8αα+ and CD8αβ+ T-cells, followed by measuring IFN-γ and IL-2 production in CD8αα+, CD8αβ+ T-cells and CD4+ T-cells. The data set is representative for PBMCs from a healthy blood donor and PBMCs from a patient with breast cancer who did not respond to PMA/Ionomycin stimulation. (b,c) PMA/Ionomycin or TCR crosslinking. Stimulation of PBMCs with PMA/Ionomycin or with OKT-3 for 6 h leads to increased numbers of IFN-γ and IL-2 producing CD4+, CD8αα+ and CD8αβ+ T-cells in PBMCs from all healthy controls, but PBMCs from 9 out of 19 patients did not respond to any stimuli, e.g. OKT-3 or PMA/Ionomycin (p-value < 0.001 tested with Fishers exact-test) and these patients are also defective in the IL-7 receptor-mediated-signal pathway. Higher numbers of IFN-γ producing T-cells in CD8αα+ T-cells as compared to CD8αβ+ T-cells except in PBMCs from a single patient. C = healthy control blood donor, Pat = patient with breast cancer. The proportion of responders versus nonresponders to PMA/Ionomycin (Fig. 3b) or TCR crosslinking (Fig. 3c) was calculated using the Fishers exact-test (< 0.001).
PBMCs from the same patients showed also defects in IL-7-signaling. The proportion of ‘nonresponders’ (9/19 individuals) to PMA/Ionomycin or to TCR-crosslinking is higher in the group of patients with breast cancer, with a p-value <0.001 tested with the Fisher’s exact-test as compared to healthy, age-matched blood donors. PBMCs from 4/19 patients with breast cancer exhibited exclusively defects in IL-7-signaling, but they were still able to produce cytokines in response to PMA/Ionomycin or TCR-crosslinking (Table II). PBMCs from 6/19 patients produced IL-2 and IFN-γ and also responded to IL-7-signaling. The percentage of IFN-γ-producing cells is higher and IL-2 production lower in CD8αβ T-cells as compared to CD8αβ+ T-cells. This is true for PBMCs obtained from healthy blood donors as well as for patients with breast cancer.

Discussion

IL-7Rα expression plays an important role in T-cell survival and immunological memory formation. In general, IL-7-mediated-signals are needed to maintain effective immune functions in animals, as well as in humans. Several reports highlighted the functional impact of decreased IL-7Rα expression on immune cells: IL-7Rα-negative, CMV-specific CD8αβ T-cells show decreased proliferative capacity as compared to IL-7Rα-positive CMV-reactive T-cells. Age-associated decrease of IL-7Rα expression leads to impaired STAT-5-signaling and chronic infection has been shown to decrease IL-7Rα expression on memory T-cells, which respond poorly to exogenously added IL-7. These data suggest that IL-7-mediated-signals are crucial to induce and maintain functional memory formation in antigen-specific T-cells. We report here that the percentage of IL-7Rα-expressing T-cells is reduced in a subpopulation of memory CD8αβ+ T-cells and CD4+ T-cells in patients with breast cancer, but not in CD8αβ+ T-cells: memory T-cell formation may be particularly impaired in the CD8αβ+ T-cell subset. It is unlikely that the reduced IL-7 R expression and impaired IL-7 response in patients with cancer reflects the age-distribution of the patients with cancer (and therefore the risk to respond poorly to IL-7), since the control group is age-matched.
The CD107a (lysosomal associated membrane protein-1 LAMP-1) molecule has been described as a cytotoxic cell marker. Enhanced LAMP-1 expression was observed in T-cells obtained from seminoma lesions as compared to peripheral T-cells, which suggested enhanced cytotoxic potential of T-cells infiltrating into human tumors. We observed an increased percentage of CD107a-positive cells and decreased numbers of IL-7Rα-positive cells in CD8α+ T-cells and CD4+ T-cells, but not in CD8β+ T-cells in PBMCs from patients with breast cancer in the current study (Table 1). Enhanced expression of CD107a (11/19 in PBMCs obtained from patients with breast cancer) coincides with unresponsiveness to IL-7 defined by p-STAT5 phosphorylation except in a single patient. CD107a expression on freshly isolated CD8+ T-cells may reflect an active cellular immune response associated with the disappearance, i.e. breast cancer. Up to now, it is not clear whether nonresponsiveness to IL-7 in the CD8αα+ T-cell compartment represents a potential defect in memory T-cell formation (despite detectable IL-7Rα expression) or if reduced IL-7-responsiveness represents a physiological feature of CD107aα+ T-lymphocytes.

STAT5 plays a significant role in T-cell survival and proliferation: STAT5-mediated-signaling sustains TCR induced gene expression via IL-2 and stabilizes the gene expression program in CD8+ T-cell effector cells in murine models. In this study, we observed profound IL-7-signaling defects in patients with breast cancer, despite the expression of IL-7Rα on the cell surface. It is not clear whether the failure to phosphorylate STAT-5 represents a “dysfunctional” immune competence or, not mutually exclusive, if decreased STAT-5 phosphorylation reflects the status of chronically stimulated T-cells. Stimulation of PBMCs from healthy donors for 6 h either with anti-CD3 (TCR-crosslinking) or anti-CD28 (costimulation) led to enhanced LAMP-1 expression in T-cells obtained from seminoma lesions as compared to peripheral T-cells,32 which may improve the design of biological therapy in patients with cancer and aid to monitor cellular immune-competence.

To summarize, PBMCs from a majority of patients with breast cancer show defects in STAT5 phosphorylation and altered expression of IL-7 in CD8αα+ T-cells and CD4+ T-cell subsets, important immune cells for memory T-cell development. CD107α is highly expressed on CD8αα+ T-cells as compared to CD8β+ T-cells, which underlines the biological role of this T-cell subset in mediating immune-effector mechanisms. A detailed analysis of T-cell function, including the analysis of IL-7-mediated-signaling, may improve the design of biological therapy in patients with cancer and aid to monitor cellular immune-competence.

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