The Influence of Adjuvant Radiotherapy and Single Nucleotide Polymorphisms on Circulating Immune Response Cell Numbers and Phenotypes of Patients With Breast Cancer

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Abstract. *Background/Aim: Adjuvant radiotherapy (RT) damages multiple layers of skin, muscle, blood vessels and blood cells that are included within the RT area. Indirect, bystander systemic effects could also develop in cells not directly hit by radiation. Materials and Methods: Ninety-three female patients recovering from breast cancer surgery and 82 female healthy blood donors were analyzed. For identification of systemic adaptive and innate immune response, rapid and low-cost blood-based biomarkers were assayed. Results: Post-operated breast cancer patients had a decreased number of circulating adaptive immune response cells but increased number of circulating immunosuppressive myeloid subpopulations. RT decreased the number of T-cells and platelets without influencing the number of immunosuppressive myeloid subpopulations. Alterations in the number and phenotypes of T-cell subpopulations were associated with SNPs. Conclusion: The combination of RT and immunotherapy might provide optimal treatment for cancer patients.*

Adjuvant radiotherapy (RT) is an essential treatment of breast cancer and various other solid tumours after surgery. The anti-neoplastic properties of RT are primarily related to DNA damage and cell death. Direct damage in multiple layers of skin, muscle, blood vessels and blood cells that are

included within the RT area could occur. Indirect, bystander systemic effects could also develop in cells not directly hit by radiation. Direct and indirect RT processes could lead to alterations of host immune response and inflammation (1-4).

Monocytes, neutrophils, natural killer (NK) cells and platelets are innate immune response cells that interact with lymphocytes, the adaptive immune response cells (5). Such interactions could result in either the activation or suppression of host immune response (6). Dysregulated numbers and ratios of circulating monocytes to lymphocytes (MLR), neutrophils to lymphocytes (NLR) or platelet to lymphocytes (PLR) suggests poor survival of cancer patients (7).

CD8^{+} cells in the T-lymphocyte group could be divided into cells expressing high levels of CD8 (CD8bright) with α/β heterodimer receptors and cells expressing low levels of CD8 (CD8dim) with α/α homodimer receptors (8-10). Surface CD8 substantially contributes to CD8^{+} cell-mediated functions such as cytokine production and cytolytic activity (9). Increased number of CD8^{dim} cells has been associated with immunosuppressed state (10) and cytotoxic T-lymphocyte (CTL) impairment in patients and healthy individuals (11-13).

Granzyme B (GZB), a cytolytic enzyme and perforin (PRF), a pore-forming protein, are the major mediators of granule-exocytosis activity (14). Variation in GZB expression in CD8^{dim} subpopulations due to single nucleotide polymorphisms (SNPs) has been detected in healthy individuals (15). Over-expression of GZB in CD8^{+} cells is a putative biomarker of impaired immunity in systemic lupus erythematosus patients (16).

Blood flow to normal breast tissue is highly variable between subjects, approximated 22±13 ml/min (17, 18). With this flow rate, innate and adaptive immune response cells in 1,100 ml to 1,750 ml circulating blood could be influenced directly by adjuvant RT.

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The patient immune response status plays an important role in the outcome of patients with cancer (19). The present study aimed to determine systemic immune status of patients after recovery from surgery for breast cancer and the influence of adjuvant RT. The role of certain SNPs was also investigated. Rapid and low-cost methods analysing circulating biomarkers concerning innate and adaptive immune response were applied.

Materials and Methods

Patients and controls. Ninety-three female breast cancer patients aged over 18 years and scheduled for adjuvant RT after surgery were invited to participate. If they accepted the invitation, peripheral blood was drawn twice. The first baseline sample was obtained after recovery from surgery and before adjuvant RT (R_0). The second follow-up sample was obtained directly, after completion of adjuvant RT (R_1). Eighty two anonymous female healthy blood donors with no history of cancer or use of any immunomodulation agents were included as controls. One peripheral blood sample was obtained from controls.

Informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki and the Ethical Board at Linköping approved this investigation.

Adjuvant RT. Adjuvant RT was delivered at the Department of Oncology, Ryhov hospital Sweden, using Varian True beam machines (Varian Lina 2100 CD, Paulo Alto, CA, USA). According to size of the remaining breast after recover from partial mastectomy, two parallel opposing tangential fields was used. They were prescribed to the 95 % isodose according to International Commission on Radiation Units (ICRU), a 3-dimensional treatment planning system (Oncentra masterplan v 4.3, Elekta AB, Stockholm, Sweden). The absorbed dose was 50 Gy in 25 fractions given as 2 Gy per fraction. This treatment required approximately 2 min per day, 5 days per week for a total of 5 weeks.

Haematology and flow cytometry analysis. The levels of circulating platelets, total white blood cells (WBCs) and their subpopulations were analysed from whole blood samples using System XE5000 (Sysmex Corporation, Kobe, Japan).

Phenotypes of *ex vivo*, peripheral WBCs were analyzed using Becton Dickinson FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) within three hours. All monoclonal antibodies were purchased from BD Biosciences. These cells were directly stained for surface marker expression of CD3, CD4, CD8, CD11, CD13, CD14, CD33 and CD56 or intracellularly stained for GZB and PRF expression, according to BD's protocol. Phenotypes and the real numbers of circulating blood lymphocyte, monocyte and neutrophil subpopulations were analyzed after gating according to their location.

SNP analysis. High-molecular weight DNA was extracted from *ex vivo* peripheral blood samples using QIAGEN Bio Robot M48 with MagAttract DNA Blood M48 kits (Qiagen, Valencia, CA, USA). The quantity and quality of DNA was determined by the Pico-green method using Quant-iT™ Pico Green™ dsDNA Assay Kit (ThermoFischer Scientific, MA, USA). The Pico Green fluorescence intensity was determined by Hidex Sense Microplate reader (Hidex Oy, Turku, Finland).

Table I. *Characteristics of 93 female breast cancer patients.*

Patients	
Age (years), median (range)	65 (41-86)
Tumour	**n**
Stage	
≤T1	59
>T1	24
Tis	10
Lymph node status	
N0	72
>N0	21
Histology	
DCIS	11
Ductal	63
Lobular	14
Others	5
Estrogen receptor positive	77
Progesteron receptor positive	67
Her 2 receptor positive	3
Triple negative	3

Based on our previous investigation, the following SNPs in GZB rs8192917, PRF rs10999426 and PRF rs3758562 were analyzed (15). These SNP sequences were HapMap-validated with Illumina design ability score according to the manufacturer's protocol (20). The genotyping of SNPs was performed at the SNP & SEQ Technology Platform, Uppsala University, Sweden (www.genotyping.se).

Statistical analysis. Mann-Whitney *U*-test was used for comparisons of immune response parameters between controls and patients before adjuvant RT, patient R_0. To examine the influence of adjuvant radiotherapy, Wilcoxon test was used for comparisons between the patients paired blood sample, before adjuvant RT (Patient R_0) and after adjuvant RT (Patient R_1), respectively. All comparisons were two-sided and $p \leq 0.05$ were considered statistically significant.

Results

Characteristics of patients and controls. A total of 93 female breast cancer patients were prospectively included after recovering from partial mastectomy. Their median age was 65 years (range=41-86 years). They were heterogeneous regarding TNM stage, histology and hormone receptor expression in the tumour (Table I).

Eighty-two female healthy blood donors, median age of 56 years (range=41-70 years), were included as controls. The median age of patients was higher than the controls, but this did not reach statistical significance.

The phenotypes and the numbers of circulating innate and adaptive immune response cells in controls and patients. After recovery from removal of their breast cancer mass, the patients had lower number of circulating lymphocytes

Table II. *Circulating innate and adaptive immune response parameters in controls and patients before (R_0) or after (R_1) adjuvant radiotherapy.*

	Controls Median	Patient R_0 Median	Patient R_1 median	*p*-Value* Control *vs.* Patient R_0	*p*-Value** Patient R0 vs R_1
A. Real median number of Platelets, WBCs and its subpopulations					
WBC (10^6/l)	6510	5970	5005	ns	<0.0001
Lymphocytes	1965	1650	1070	0.0014	<0.0001
Monocytes	530	520	470	ns	0.018
Neutrophils	3425	3550	3265	ns	<0.0001
Eosinophils	145	120	130	ns	ns
Basophils	30	30	25	ns	0.001
Platelets (10^6/l)	229000	247000	223000	ns	<0.0001
MLR	0.28	0.31	0.45	0.0067	<0.0001
NLR	1.86	2.27	2.91	0.0014	<0.0001
PLR	126	156.30	202	<0.0001	<0.0001
B. Real median number and phenotypes of lymphocytes (10^6/l)					
CD3$^+$	1336	1078	730	<0.0001	<0.0001
CD56$^+$	299	141	71	<0.0001	<0.0001
CD3$^+$CD56$^+$	66	54	35	ns	0.0014
CD4$^+$	897	719	531	0.0002	<0.0001
CD8$^+$	459	460	286	ns	<0.0001
CD8^{bright}	334	316	175	ns	<0.0001
CD8^{dim}	140	136	83	ns	<0.0001
CD4/CD8 Ratio	1.97	1,61	1.86	0.001	<0.0001
C. Number and phenotype of myelocytes (10^6/l)					
Monocytes					
CD13$^+$CD56$^+$	30	93	83	<0.0001	ns
Neutrophils					
CD13$^+$CD56$^+$	80	404	371	<0.0001	ns

MLR: Monocyte/lymphocyte ratio; NLR: neutrophils/lymphocyte ratio; PLR: platelet/lymphocyte ratio; ns: non-significant value. *Mann-Whitney *U*-test; ** Wilcoxon test.

compared to controls (*p*=0.0014) but similar number of monocytes or neutrophils (Table II). As a consequence, the monocyte to lymphocyte ratio (MLR), neutrophils to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) were statistically significantly increased in the patients R_0 (Table II).

Alterations in the phenotypes of lymphocytes (Figure 1), monocytes and neutrophils (Figure 2) were detected in the patients compared to controls. The number of CD3$^+$, CD4$^+$ and CD56$^+$ cells in lymphocyte population were significantly lower in patients R_0 compared to controls (Table II and Figure 1). Number of CD13$^+$CD56$^+$ cells in monocyte population or neutrophil population were significantly higher (*p*=0.0001) in patients R_0 compared to controls (Table II and Figure 2). Decreased ratio of CD4$^+$ to CD8$^+$ cells (CD4/CD8 ratio) was also detected in the patients R_0 (*p*=0.001).

Adjuvant RT significantly decreased real numbers of all investigated adaptive and innate immune response subpopulations, except eosinophils (Table II). Despite decreasing real numbers of cells, increased MLR, NLR and PLR were observed in patients R_1 compared to R_0 (*p*=0.0001). At lymphocytes gate, lowest levels of CD3$^+$, CD56$^+$, CD4$^+$, CD8$^+$, CD8^{dim} and CD8^{bright} cells were also detected in the patients R_1 compared to patients R_0 (Table II). A higher CD4/CD8 ratio was also detected after adjuvant RT (*p*≤0.0001). The adjuvant RT had no influence on circulating numbers of CD13$^+$CD56$^+$ expressing cell in monocyte or neutrophil gate.

Number of circulating CD4$^+$ and CD8$^+$ cells expressing GZB and PRF in relation to SNPs and adjuvant RT. Lower numbers of GZB and PRF expressing in CD4$^+$ cells were detected in patient R_0 compared to controls (Table III). The PRF rs375862 AG+GG sequence correlated to increasing real numbers of PRF$^+$ cells in CD8$^+$ dim population of patients compared to controls (*p*=0.046).

Adjuvant RT decreased the real numbers of lymphocytes expressing GZB and PRF in CD4$^+$ cells and CD8$^+$ cells. Based on the GZB rs8192917 sequences, the number of GZB expressing CD4$^+$, CD8^{bright} and CD8^{dim} cells was lower in

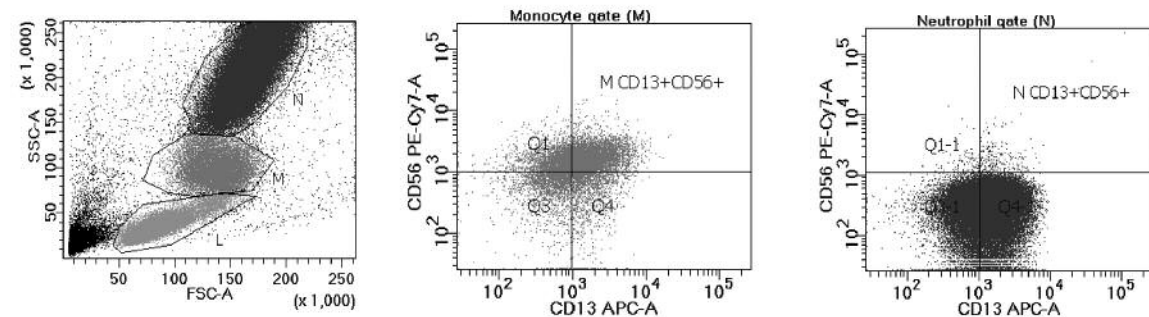## A. Control
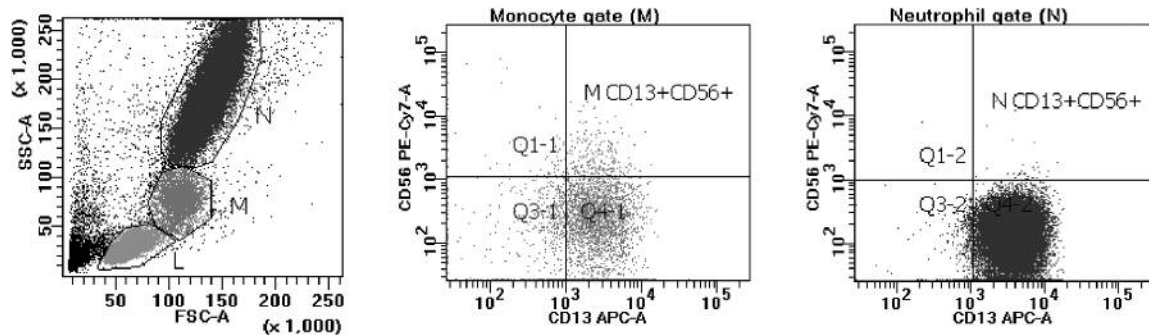
B. Patient R_0
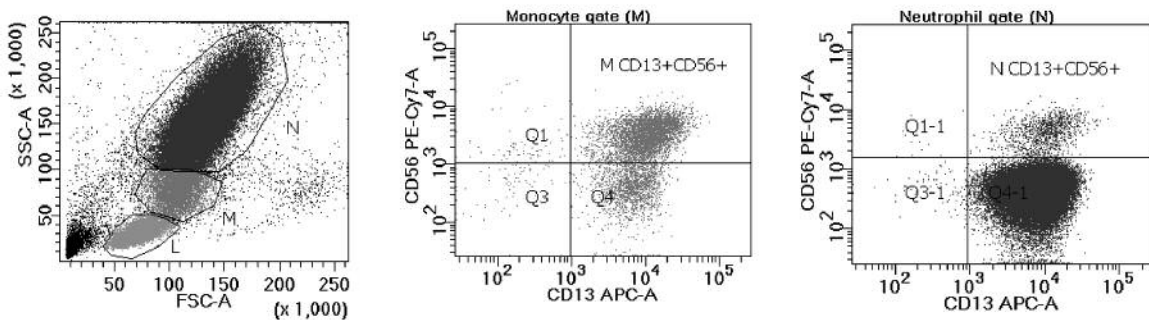
C. Patient R_1

Figure 1. *Circulating $CD13^+CD56^+$ cells in monocyte (M) and neutrophil (N) populations. Flow cytometry dot plot results presented ex vivo fresh blood cells of one control (Control) and one breast patient before (R_0) and after (R_1) adjuvant radiotherapy, respectively.*

patients R_1 than patients R_0 (Table III). The influence of adjuvant RT on PRF expression in $CD4^+$, $CD8^{bright}$ and $CD8^{dim}$ differed. Decreased real numbers of $CD8^{dim}$ cells expressing PRF after adjuvant RT were associated with the SNPs in PRF rs10999426 and PRF rs375862 ($p<0.0001$) of these patients. The influence of adjuvant RT and SNPs on PRF expression in circulated $CD8^{bright}$ cells were not detected in these patients.

Discussion

Real numbers and distribution of innate and adaptive immune response subpopulations play an important role in cancer patients (7). Elevated levels of circulating innate and adaptive immune response cells in patients indicate systemic inflammation and poor survival (7). Decreased numbers of circulating helper T-cells, NK cells and increased number of
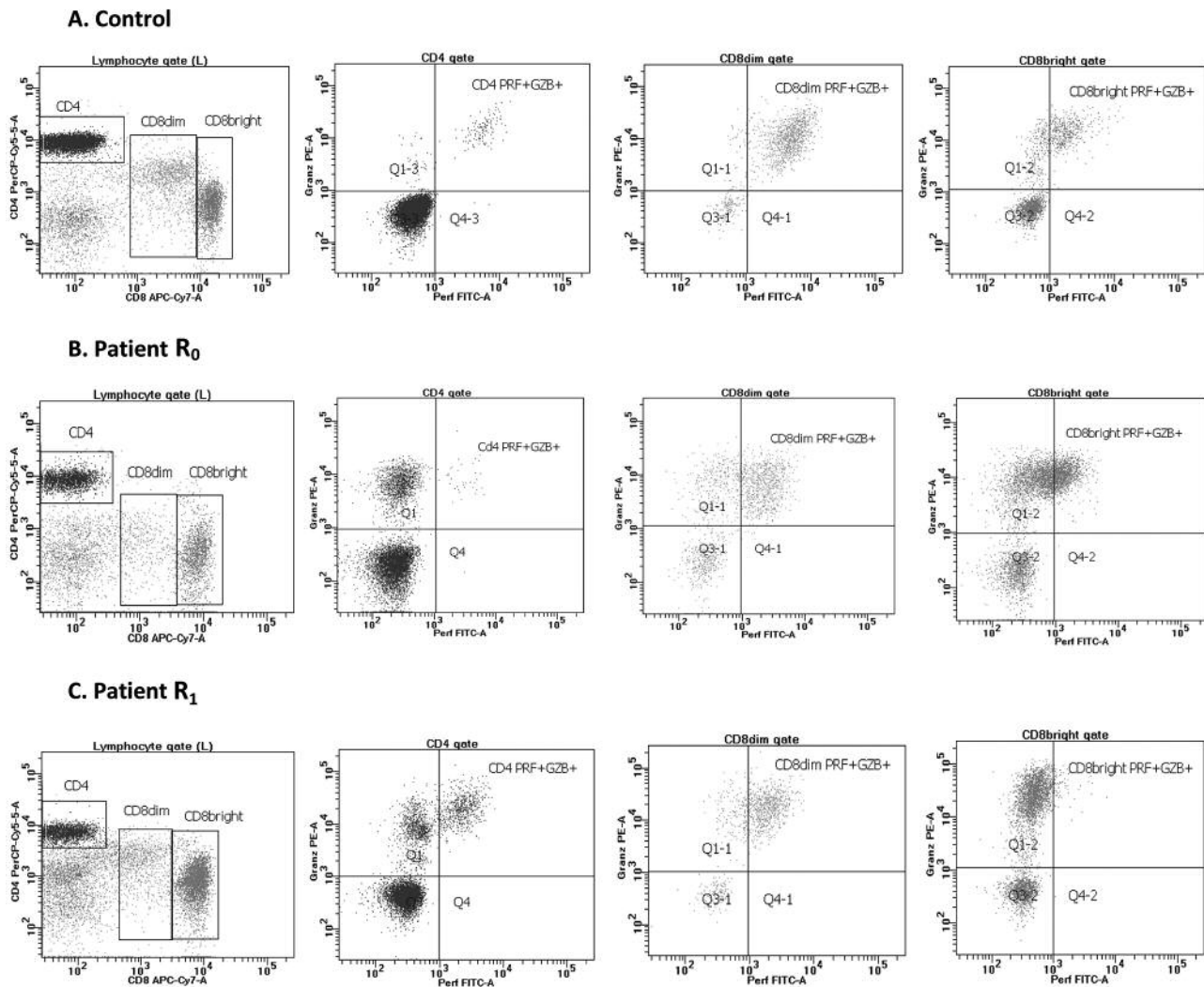
Figure 2. *Circulating perforin (PRF⁺) and granzyme B (GZB⁺) expressing CD4⁺, CD8^{dim} and CD8^{bright} cells populations. Flow cytometry dot plot results represented ex vivo fresh blood cells of one control (Control) and one breast cancer patient before (R0) and after (R1) adjuvant radiotherapy, respectively.*

immunosuppressive monocytes and neutrophils in cancer patients has been documented (21, 22). After the patient's recovery from removal of visible tumour, our investigation suggests that a general immunosuppression persists.

The impact of adjuvant RT on human body is more complex than in *in vitro* or *in vivo* models. Blood vessels and blood flow under the treatment area will be affected during adjuvant RT. Given the general blood flow rate, immune response cells in more than 1000 ml blood are expected to be directly in contact with radiation (17, 18).

Decreasing platelet numbers in blood circulation after adjuvant RT might be a consequence of platelet activation and consumption due to blood vessel destruction. Adjuvant RT did not influence the real number of circulating $CD13^+CD56^+$

monocytes and neutrophils. The impaired immune response in patients R_1 might associate to decreased numbers of lymphocytes but stable number of immunosuppressive $CD13^+CD56^+$ monocytes and neutrophils (21, 22) .

In healthy individuals, the CD4/CD8 ratio declines with age indicating immunological changes due to the ageing process or viral infection (23, 24). The decreasing CD4/CD8 ratio is correlated to increased mortality in the elderly (23). Increased circulating $CD8^{dim}$ cells are associated with peripheral exhaustion or impairment of effective CTL (12, 13).

SNPs in GZB and PRF genes influence numbers of GZB and PRF expressing cells in blood circulation (15). SNPs in GZB and PRF genes seem to have a higher impact on the numbers of GZB and PFR expressing cells in the $CD4^+$

Table III. *SNP genotypes and real numbers of circulating granzyme B and perforin expressing cells in controls and patients before (R_0) or after (R_1) adjuvant radiotherapy.*

Lymphocyte gate	Gene	rs	Sequence	Controls (n) Median positive cells	Patients R_0 (n) Median positive cells	Patients R_1 (n) Median positive cells	*Controls vs. Patients R_0 p-Value	**Patients R_0 vs. R_1 p-Value
CD4$^+$ (10^6 cells/l)	GZB	8192917	AA	90 (37)	59 (49)	40 (45)	0.034	<0.0001
			AG+GG	98 (12)	39 (25)	33 (24)	ns	0.013
CD8^{bright} (10^6 cells/l)	GZB	8192917	AA	125 (38)	175 (51)	91 (45)	ns	<0.0001
			AG+GG	133 (12)	146 (27)	60 (24)	ns	0.004
CD8^{dim} (10^6 cells/l)	GZB	8192917	AA	100 (38)	113 (50)	54 (45)	ns	<0.0001
			AG+GG	90 (12)	97 (27)	56 (24)	ns	0.003
CD4$^+$ (10^6 cells/l)	PRF	10999426	AA+AG	27 (25)	0 (47)	3 (45)	<0.0001	0.046
			GG	42 (14)	3 (28)	6 (25)	<0.0001	ns
CD8^{bright} (10^6 cells/l)	PRF	10999426	AA+AG	22 (31)	21 (47)	17 (45)	ns	ns
			GG	26 (17)	26 (29)	17 (25)	ns	ns
CD8^{dim} (10^6 cells/l)	PRF	10999426	AA+AG	62 (31)	85 (47)	36 (45)	ns	<0.0001
			GG	74 (18)	82 (29)	40 (25)	ns	<0.0001
CD4$^+$ (10^6 cells/l)	PRF	375862	AA+AG	37 (13)	7 (32)	6 (28)	<0.0001	ns
			GG	27 (26)	0 (44)	1 (42)	<0.0001	ns
CD8^{bright} (10^6 cells/l)	PRF	375862	AA	33 (16)	25 (33)	14 (28)	ns	ns
			AG+GG	21 (32)	21 (44)	17 (42)	ns	ns
CD8^{dim} (10^6 cells/l)	PRF	375862	AA	74 (17)	81 (33)	40 (28)	ns	<0.0001
			AG+GG	56 (32)	85 (44)	37 (42)	0.046	<0.0001

GZB: Granzyme B; PRF: perforin; ns: non significant value. *Mann-Whitney *U*-test; **Wilcoxon test.

population than in the CD8+population. Adjuvant RT and GZB rs8192917 influenced the number of circulating GZB expressing CD4$^+$, CD8^{bright} and CD8^{dim} cell populations. The impact of adjuvant RT and SNPs in the PRF gene on the number of cells expressing PRF seem to be pronounced in CD4$^+$ and CD8dim cells but not in the CD8^{bright} population. Thus, decreasing numbers of GZB expressing CD8^{dim} cells, stable numbers of GZB expressing CD8^{bright} and increasing CD4/CD8 ratio might be the beneficial effects of RT on adaptive immunological functions (9, 25).

Conclusion

A state of immunosuppression persists in breast cancer patients after recovery from removal of visible tumor by surgery. This suppression is manifested by a decreasing number of circulating adaptive immune response, CD4$^+$ cells and increased numbers of immunosuppressive, CD13$^+$CD56$^+$ myeloid cells. Alteration in this adaptive immune response was associated with genotype variations in GZB and PRF genes. Adjuvant RT decreased the real numbers of platelet and adaptive immune response subpopulations without influencing the number of immunosuppressive cells in myelocytes. Our investigation suggests that the combination of RT and immunotherapy may provide an optimized treatment for these patients. The use of these rapid and low-cost blood-based biomarkers for the identification of patient's immune status and the influence of RT needs further investigation.

Conflicts of Interest

The Authors declare that they have no conflicts of interest to disclose in regard to this study.

Authors' Contributions

NL, LER, SL and FL designed the studies. TL, LS, DO and B-ÅA included the blood samples and carried out the experiments. All Authors analyzed data, wrote, read and approved the final manuscript.

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