Cancer Immunology, Immunotherapy (2019) 68:721–729
https://doi.org/10.1007/s00262-019-02312-0

ORIGINAL ARTICLE

Imbalances in cellular immunological parameters in blood predetermine tumor onset in a natural mouse model of breast cancer

Dmitry A. Aronov[1] · **Viacheslav V. Zhukov**[2] · **Svetlana G. Semushina**[1] · **Ekaterina V. Moiseeva**[1]

Received: 17 July 2018 / Accepted: 1 February 2019 / Published online: 11 February 2019

Abstract

The development of new approaches to breast cancer (BC) early diagnosis is an important objective of modern oncology. Although the role of the immune system in cancer initiation process was experimentally well established, the prognostic value of cellular blood immunological parameters (CBIPs) for BC onset prediction was not demonstrated either in clinics or in mouse models. In this study, we focused on revealing informative CBIPs for mammary cancer (MC) onset prediction in the BLRB/BYRB mouse model with a high incidence of natural MC development. Blood samples were collected from 80 aging females of these original mouse strains, 12 basic CBIPs were estimated by flow cytometry. Then mice were followed up for 28 weeks, and the outcome of females (MC diagnosis, death without MC or MC-free survival) was registered. We estimated the patterns of changes in CBIPs with age and in accordance with the outcome. An increasing imbalance in 11 CBIPs during natural aging of females clearly resembled human immunosenescence phenomenon and several patterns corresponded to the results obtained on cancer-free members of BC-affected families. We stratified heterogeneous female population into middle-aged and old subgroups. Low NK-cell levels in middle-aged mice and low B-cell along with high T-helper levels in old mice distinguished females with developed MC from the other groups. We found a reliable correlation of several CBIPs with age at MC diagnosis and survival of cancer-bearing females. Thus, we demonstrated the predictive potential of CBIPs as a basis for the development of prognostic models for BC onset in clinics.

Keywords Breast cancer · Mouse model · Immune system · Cellular blood immunological parameters

Abbreviations

Act.	Activated
BC	Breast cancer (in humans)
CBIPs	Cellular blood immunological parameters
MC	Mammary cancer (in mice)
SPF	Specific-pathogen-free

Introduction

Breast cancer (BC) is the most common oncological disease in women. Early diagnosis of BC is essential for selecting an optimal treatment strategy to increase survival and life quality of a patient [1]. Currently, mammography is the most common procedure for mass screening of population for BC detection. An annual examination is recommended for women over 40 years of age in many countries [2]. However, despite the significant contribution of mammography to reducing mortality from BC, this procedure often leads to overdiagnosis regardless of age, resulting in women receiving excessive treatment with a lot of severe side effects [2, 3]. Moreover, Willem Den Otter with co-authors and other groups have shown that frequent mammographic examinations alone can provoke early tumor manifestation in women, especially those with a predisposition to BC [4, 5]. Therefore, we believe that mammography should only be used to confirm the diagnosis, while early detection of BC should be based on instrumentally measured parameters of a woman's health status, which would allow predicting the

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02312-0) contains supplementary material, which is available to authorized users.

✉ Dmitry A. Aronov
aronov.mml@gmail.com

[1] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, GSP-7, ul. Miklukho-Maklaya, 16/10, Moscow 117997, Russia

[2] Peoples' Friendship University of Russia (RUDN University), ul. Miklukho-Maklaya, 6, Moscow 117198, Russia

early occurrence of BC with sufficient accuracy (at least for women with familial BC predisposition).

The search for approaches to predicting the onset of BC began in the second half of the twentieth century. Collecting and accumulating the *anamnesis vitae* of patients with BC, Mitchell Gail managed to discover risk factors and created a model for individual prognosis of the BC onset probability. Gail model takes into account the time of menarche, the age of first childbirth, family history of BC, and some other factors [6]. Currently, the Gail model is, in fact, the most commonly used model for breast cancer onset; it is widely used in the clinic and regularly improved (discussed in [7]). Nevertheless, the main weakness of all the modern prognostic models is their failure to predict the age of tumor onset in a given woman even approximately. In recent decades, the prognostic value of a number of integral biomarkers at the organism level had already been detected. In particular, hematological and biochemical parameters were shown to predict mammary cancer growth rate [8] and immunotherapy outcome in mice [9]. Some of those biomarkers were proposed for evaluation of cancer risk [10, 11] and the patient's outcome in BC clinic [12].

There is growing evidence that the immune system plays a crucial role in BC development, growth, and response to the therapy. This is not surprising since complex multicomponent interactions of the incipient tumor with various compartments of the host immune system underlie cancer initiation and development [13]. When the prognostic role of the immune system in BC is discussed, the emphasis is usually placed on tumor-related factors; little was published thus far concerning cellular blood immunological parameters (CBIPs) in BC patients. For example, several parameters were shown to be altered depending on the stage of the disease and compared to healthy donors [14]. It was also proposed to use blood immunological characteristics as informative biomarkers to predict the chemotherapy outcome [15, 16]. In view of the above, it is reasonable to assume that some of these parameters could be changed a long time before clinical manifestation of BC.

It is currently problematic to identify BC initiation in humans, so we have to use a model system that adequately describes the basic features of this process. Standard mouse models of BC (both transplanted and genetically engineered) housed in specific-pathogen-free (SPF) environment do not reproduce the natural imbalance of the immune system emerging prior to cancer occurrence in humans [17–19]. Previously, we showed that non-SPF aging BLRB-Rb(8.17)1Iem mouse females [8] were characterized by a high incidence of naturally occurring (spontaneous) mammary cancer (MC); this natural model represented a number of features of BC development in women with a family history of breast cancer [8, 20]. The histopathological analysis of these mouse mammary tumors revealed distinct types of MC pathology during the natural progression of the disease in a mouse female: steps that are similar to progression from hyperplasia and lactating adenoma to carcinoma in situ and invasive lobular, ductal, medullary carcinoma, and comedocarcinoma in humans. Using the flow cytometry analysis, we demonstrated a prognostic role of lymphoid infiltrates within murine mammary carcinoma [8] similarly to that observed in the BC patients [21]. We believe that natural MC-prone mouse models (acquiring MC in a natural way during aging in a natural environment) can mimic the key pathogenetic mechanisms of BC development in women with a family predisposition in general terms, especially regarding the interaction of the immune system and the tumor during the preclinical step of BC. We hypothesized that several cellular parameters of the immune system could be changed before the onset of BC, and these changes might predetermine the course of the disease. Our aim was to discover whether such changes occur and to describe the patterns of these changes in aging precancerous BLRB and BYRB mouse females.

In the current study, we evaluated the changes in the basic cellular parameters of the immune system in the blood of aging female mice prior to the natural manifestation of MC in the spontaneous mouse model of familial breast cancer. We described the age-related changes in CBIPs resembling the human immunosenescence phenomenon. In addition, we discovered several changes in CBIP patterns preceding the mammary cancer diagnosis in initially middle-aged and old BLRB and BYRB mouse females; and revealed the prognostic potential of some parameters for predicting the outcome and survival of tumor-bearing mice.

Materials and methods

Mice

The females of genetically closely related original mouse inbred strains BLRB-Rb(8.17)1Iem and BYRB-Rb(8.17)1Iem (BLRB and BYRB, respectively) [8, 22] with a high incidence of naturally occurring (spontaneous) mammary cancer (MC) were bred (more than 80 generations of brother–sister littermate mating) and maintained in a thoroughly controlled conventional environment (non-SPF). Each female aged about 4 weeks was labeled at the time of registration in inbred strain documentation and was followed individually lifelong. Bred BLRB and BYRB females formed the initial population of intact aging females ($n = 80$) aged 27–99 weeks (mean age 55 ± 2 weeks).

Flow cytometry

Blood samples in a volume of 200 ± 10 µl were individually taken from the retroorbital sinus of mouse females

($n = 80$) on day 0. Red blood cells were lysed in a solution of ammonium chloride (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 3 min, then the samples were washed with PBS by centrifuging, 100 µl of flow cytometric buffer was added (1% bovine serum albumin, 0.1% sodium azide in PBS), then samples were incubated for 30 min at 4 °C with the following monoclonal antibodies (Biolegend, CA): CD4 (FITC), CD8 (FITC), CD3 (FITC), F4/80 (FITC), CD25 (PE), NK1.1 (PE), CD19 (PE), CD11b (PE), CD45 (PerCP). Flow cytometry was performed using FACScan cytometer (BD Biosciences, CA). Three gates were used for the analysis: (1) first gate was created around the population of total blood leukocytes in a CD45 vs. FSC plot; (2) second gate was defined to exclude debris in an FSC vs. SSC plot; (3) third gate was created around the major leukocyte population of interest in a CD45 vs. SSC plot. Backgating was used for each subset to confirm gating strategies. The level of 12 basic CBIPs was measured. The percentage of lymphocytes ($CD45^{high}SSC^{low}$), monocytes ($CD45^{int}SSC^{int}F4/80^{+}CD11b^{+}$), neutrophils ($CD45^{int}SSC^{int}F4/80^{neg}CD11b^{+}$), and eosinophils ($CD45^{int}SSC^{high}CD11b^{+}F4/80^{+}$) was estimated among total blood leukocytes. The level of the following lymphocyte subpopulations was referred to the percentage of a given cell subset among blood lymphocytes: T-lymphocytes ($CD45^{high}SSC^{low}CD3^{+}NK1.1^{neg}$), T-helpers ($CD45^{high}SSC^{low}CD4^{+}CD25^{neg}$), cytotoxic T-lymphocytes ($CD45^{high}SSC^{low}CD8^{+}$, CTL), B-lymphocytes ($CD45^{high}SSC^{low}CD19^{+}$), NK cells ($CD45^{high}SSC^{low}CD3^{neg}NK1.1^{+}$), NKT-cells ($CD45^{high}SSC^{low}CD3^{+}NK1.1^{+}$), activated (act.) T-helpers ($CD45^{high}SSC^{low}CD4^{+}CD25^{+}$). The $CD4^{+}/CD8^{+}$ ratio was calculated as a ratio of T-helpers to CTL. The analysis of acquired data was performed with Flowing Software 2.5.1 (Turku Center for Biotechnology, Finland).

Experimental design

Health status and survival of mice were evaluated daily for 28 weeks after blood sample collection. Newly appeared mammary tumors were visually detected and palpated weekly, tumor size was measured as described in [20]. Females at the end point (body weight loss more than 15%, and/or refusal of food) were euthanized by cervical dislocation. After 28 weeks of observation, 80 females were stratified into three groups according to the diagnosis and outcome: group 1 ($n = 21$)—females with diagnosed MC (MC^{+}) at the mean age of 71 ± 2 weeks; group 2 ($n = 36$)—females died without MC (died MC^{-}), mean age 66 ± 4 weeks; group 3 ($n = 23$)—females were alive and did not have MC by the end of observation period (alive MC^{-}), mean age 81 ± 3 weeks. Post mortem examination and histopathological analysis were performed for each female to confirm or reject the diagnosis of mammary cancer for females of group 1 and to determine the cause of death for females of group 2. Brown induration of the lung (numerous hemosiderin containing macrophages) and alveolar edema, which commonly caused by congestive heart failure, were found in most cases of premature non-tumor death (data not shown, and will be presented in full elsewhere).

Statistics

Shapiro–Wilk test was used to test for normality, and Levene's test was used to assess the equality of variances. Normally distributed data with equal variances were compared using two-sided Student's t test; non-parametric data were compared using the Mann–Whitney U test. Either one-way ANOVA or Kruskal–Wallis ANOVA was used to compare three independent samples with normally distributed data or non-parametric data, respectively. Correlations between parameters were assessed with Spearman's rank correlation coefficient (r_s). Analysis and artworks were performed with STATISTICA 12 (StatSoft, OK). Data are presented as the mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered significant.

Results

CBIPs failed to distinguish groups due to heterogeneity in age

Blood samples were taken from 80 females on day 0; then females were stratified according to their outcome after 28 weeks of observation (MC^{+}, died MC^{-}, alive MC^{-}); their initial CBIPs were compared.

The division of the whole cohort into three groups regardless of female age allowed finding the only one parameter, which enabled to distinguish alive MC^{-} females from the others. Precisely, the initial level of lymphocytes was the highest in alive MC^{-} females comparing to died MC^{-} ($63.3 \pm 1.3\%$ vs. $57.7 \pm 1.8\%$; $p = 0.016$) and MC^{+} females ($63.3 \pm 1.3\%$ vs. $58.5 \pm 2.1\%$; $p = 0.043$). Thus, we found no one immunological parameter to distinguish between the females with developed MC and those, which died without MC.

Cellular blood immunological parameters correlated with age of intact females

Further, we assumed that some CBIPs might have been changed in the different ways during natural aging of mice. We used the Spearman's rank correlation coefficient (r_s) to estimate the relationship between the initial CBIP values and the initial age of the females. We found a correlation of 11

Table 1 Correlations between levels of cellular blood immunological parameters (%) and age (weeks) of intact BLRB and BYRB females using Spearman's rank correlation coefficient (r_s)

Parameter	Direction of change with age	r_s value	p value
White blood cell populations			
Lymphocytes	Decreased	− 0.32	0.003
Neutrophils	Increased	0.33	0.002
Monocytes	Not changed	− 0.05	0.65
Eosinophils	Decreased	− 0.24	0.036
Lymphocyte subpopulations			
T-lymphocytes	Decreased	− 0.47	0.00001
B-lymphocytes	Increased	0.51	0.000001
T-helpers	Decreased	− 0.81	0.000000
CTL	Increased	0.54	0.000000
Act. T-helpers	Decreased	− 0.25	0.024
NK cells	Decreased	− 0.63	0.000000
NKT-cells	Decreased	− 0.26	0.019
$CD4^+/CD8^+$ ratio	Decreased	− 0.78	0.000000

out of 12 estimated parameters of various strength with the age of the females at day 0: the levels of lymphocytes, eosinophils, T-lymphocytes, T-helpers, act. T-helpers, NK cells, NKT-lymphocytes, and $CD4^+/CD8^+$ ratio were decreased, and the levels of neutrophils, CTL, and B-lymphocytes were increased with age (Table 1). Scatter plots of CBIPs against age are presented in Supplementary Fig. 1 (a-l).

Therefore, we revealed an increasing immunological imbalance in the intact BLRB and BYRB females as they aged and sometimes long before they acquired MC or died without MC.

Different immunological parameters changed prior to the mammary cancer diagnosis in mouse females stratified by age

The discovered immunological imbalance associated with natural aging of females served as a basis for dividing the whole female population ($n = 80$) into two subgroups, and the age of 61 weeks (14 months) was used as a threshold to distinguish "*middle-aged*" and "*old*" subgroups. The age of 61 weeks was considered as a borderline between these two age periods in line with the rules that determine mouse age according to the human age [23]. Further, all interrelations between the initial values of CBIPs and the outcome of mice were estimated in these two subgroups independently.

In a subgroup of middle-aged mice ($n = 49$), MC was diagnosed in 11 females (MC^+); the mean age at tumor detection was 65 ± 3 weeks. Females died without MC at the mean age of 47 ± 3 weeks (died MC^-, $n = 21$). Females without symptoms of MC were alive at the end of the experiment at the mean age of 74 ± 2 weeks (alive MC^-, $n = 17$). In this subgroup, the initial level of T-helpers ($37.9 \pm 1.7\%$ vs. $43.8 \pm 2.0\%$; $p = 0.025$) and $CD4^+/CD8^+$ ratio (1.7 ± 0.1 vs. 2.1 ± 0.1; $p = 0.010$) were lower in MC^+ than in died MC^- females. In addition, females with diagnosed MC were characterized by the lowest initial level of NK cells compared to died MC^- and alive MC^- mice ($1.9 \pm 0.3\%$ vs. $2.9 \pm 0.2\%$ vs. $2.4 \pm 0.2\%$; $p = 0.0007$ and $p = 0.047$, respectively, Fig. 1). Thus, in the middle-aged female population low initial NK-cell level allowed distinguishing MC^+ females from the other two groups.

In a subgroup of initially old females ($n = 31$), MC was diagnosed in 10 mice at the mean age of 77 ± 2 weeks (MC^+); 15 females died without MC at the mean age of 90 ± 5 weeks (died MC^-); 6 females remained alive without MC symptoms after 28 weeks of observation at the mean age of 99 ± 2 weeks (alive MC^-). The level of T-helpers was higher in the MC^+ females than in died MC^- females ($31.8 \pm 1.9\%$ vs. $22.9 \pm 2.6\%$; $p = 0.018$). At the same time, the initial level of B-lymphocytes was lower in MC^+ females than in alive MC^- females ($36.0 \pm 1.2\%$ vs. $44.7 \pm 1.7\%$; $p = 0.002$). Thus, the combination of the initially elevated level of T-helpers and the lowered level of B-lymphocytes defined the subgroup of old females with subsequently diagnosed MC (Fig. 2).

We would like to highlight the fact that without stratification of the initial female population by age, the prognostic value of CBIPs was undetectable.

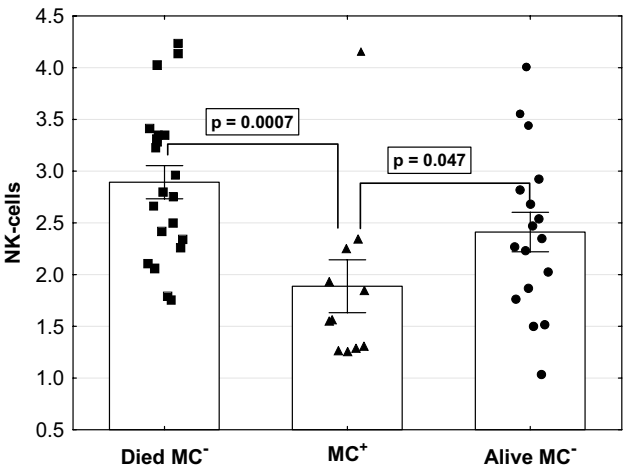

Fig. 1 The lowest initial level of NK cells was observed in a subgroup of middle-aged females with diagnosed MC (MC^+, triangles) relative to those dying without MC (died MC^-, squares), and females alive after 28 weeks of observation without MC symptoms (alive MC^-, circles). Mean values are shown as columns, SEM—as whiskers

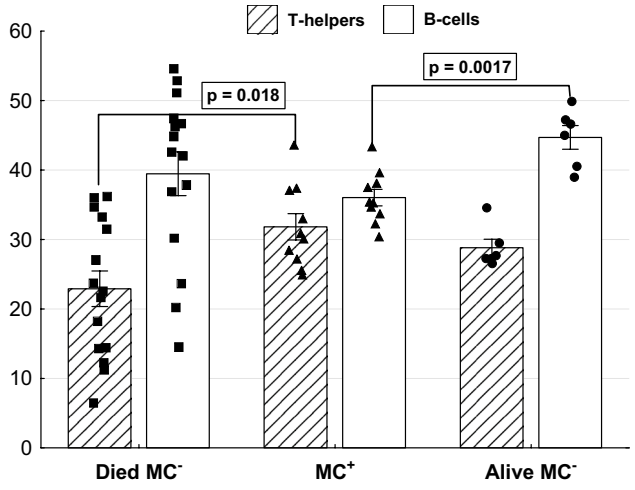

Fig. 2 Initial levels of B cells (open columns) and T-helpers (striped columns) in a subgroup of old mice. Mice with diagnosed MC (MC^+, triangles) were characterized by a combination of reduced B cells relative to the group of surviving MC^- females (circles) and increased T-helpers relative to deceased MC^- females (squares). Mean values are shown as columns, SEM—as whiskers

The initial level of several parameters related to age at the diagnosis and survival of MC-bearing mice

During the 28 weeks of the observation period, mammary tumors were detected in 21 females of BLRB and BYRB mouse strains ($n = 17/57$ and $n = 4/23$, respectively). All the tumors were identified as mammary adenocarcinomas by histopathological analysis according to [8] (data not shown). In this chapter, we examined the relationships between the initially measured CBIPs and age at the diagnosis and specific survival time (from the MC diagnosis to the death of MC-bearing female).

The mean age at MC diagnosis was 71 ± 2 weeks; and this value served as a threshold to stratify females depending on their age of MC onset into subgroups of mice with earlier ($n = 10$), and *later* ($n = 11$) MC onset. Mice with earlier MC onset developed MC faster after blood collection than mice with later onset ($p = 0.0008$, Supplementary Fig. 2). Females with earlier onset were characterized by lowered initial B-lymphocyte level ($33.8 \pm 1.0\%$ vs. $36.9 \pm 1.6\%$; $p = 0.036$, Fig. 3a) and elevated NK-cell level ($2.4 \pm 0.3\%$ vs. $1.7 \pm 0.2\%$; $p = 0.043$, Fig. 3b) in the blood comparing to mice with later onset. We observed a moderate direct correlation between the age of MC onset and B-lymphocytes ($r_s = 0.48$, $p = 0.026$), and CTL ($r_s = 0.44$, $p = 0.047$), and a moderate negative correlation between age of MC onset and T-helpers ($r_s = -0.59$, $p = 0.005$) and the $CD4^+/CD8^+$ ratio ($r_s = -0.50$, $p = 0.021$). Scatter plots are presented in Supplementary Fig. 3 (a-d).

The mean survival time of MC-bearing females was 8.2 weeks; and this value served as a threshold to stratify females depending on the individual survival time into short survivors (5.4 ± 0.4 weeks, $n = 11$) and long survivors (11.3 ± 0.8 weeks, $n = 10$, Fig. 4a). Short survivors had initially higher T-lymphocyte level ($66.4 \pm 1.5\%$ vs. $59.4 \pm 1.2\%$; $p = 0.002$, Fig. 5a), T-helper level ($37.8 \pm 1.8\%$ vs. $31.9 \pm 1.8\%$; $p = 0.033$, Fig. 5b), and act. T-helper level ($3.0 \pm 0.2\%$ vs. $2.4 \pm 0.1\%$; $p = 0.010$, Fig. 5c); while the level of B-lymphocytes was lower than in long survivors ($33.0 \pm 1.3\%$ vs. $38.1 \pm 1.1\%$, $p = 0.008$, Fig. 5d). There was no link between survival time and either the age of MC onset (Fig. 4b) or tumor growth rate (data not shown). However, we found a reliable moderate direct correlation between

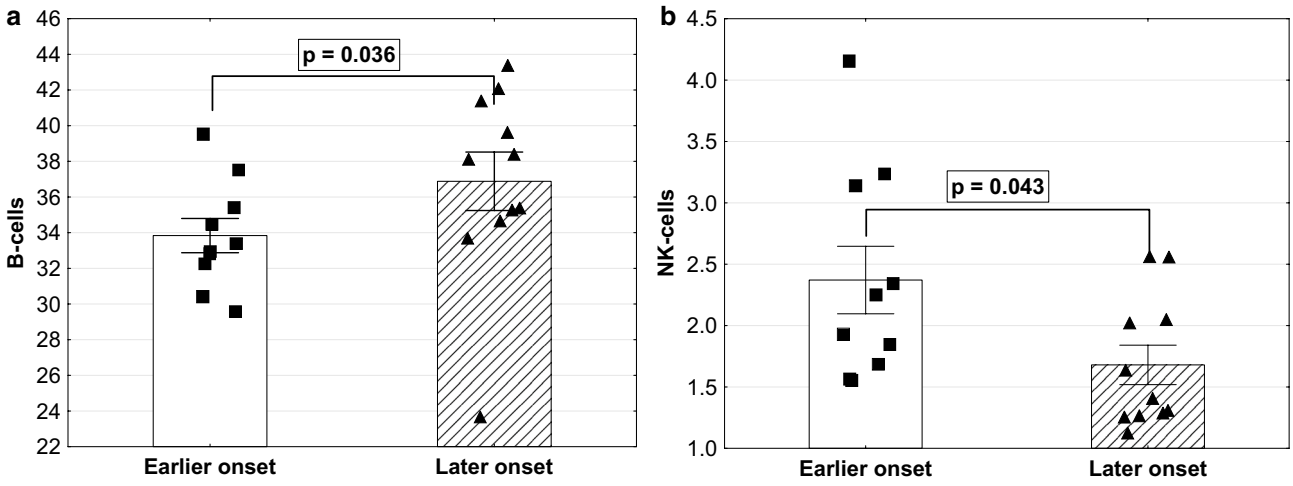

Fig. 3 Initial levels of B cells (**a**) and NK cells (**b**) in females diagnosed with MC at different ages. MC-bearing females with earlier onset were characterized by a lower initial level of B cells and a higher initial level of NK cells relative to MC-bearing mice with later onset. Mean values are shown as columns, SEM—as whiskers

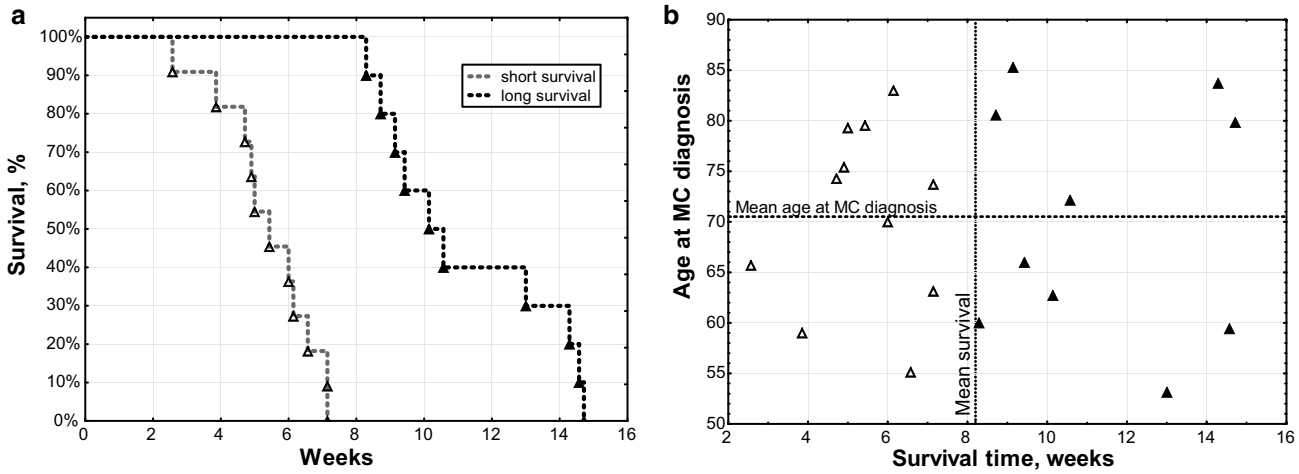

Fig. 4 Survival of MC-bearing mice (**a**) and scatter plot of age at MC diagnosis against survival time (**b**). Both short survivors (open triangles) and long survivors (closed triangles) could be found among females with earlier and later tumor manifestation

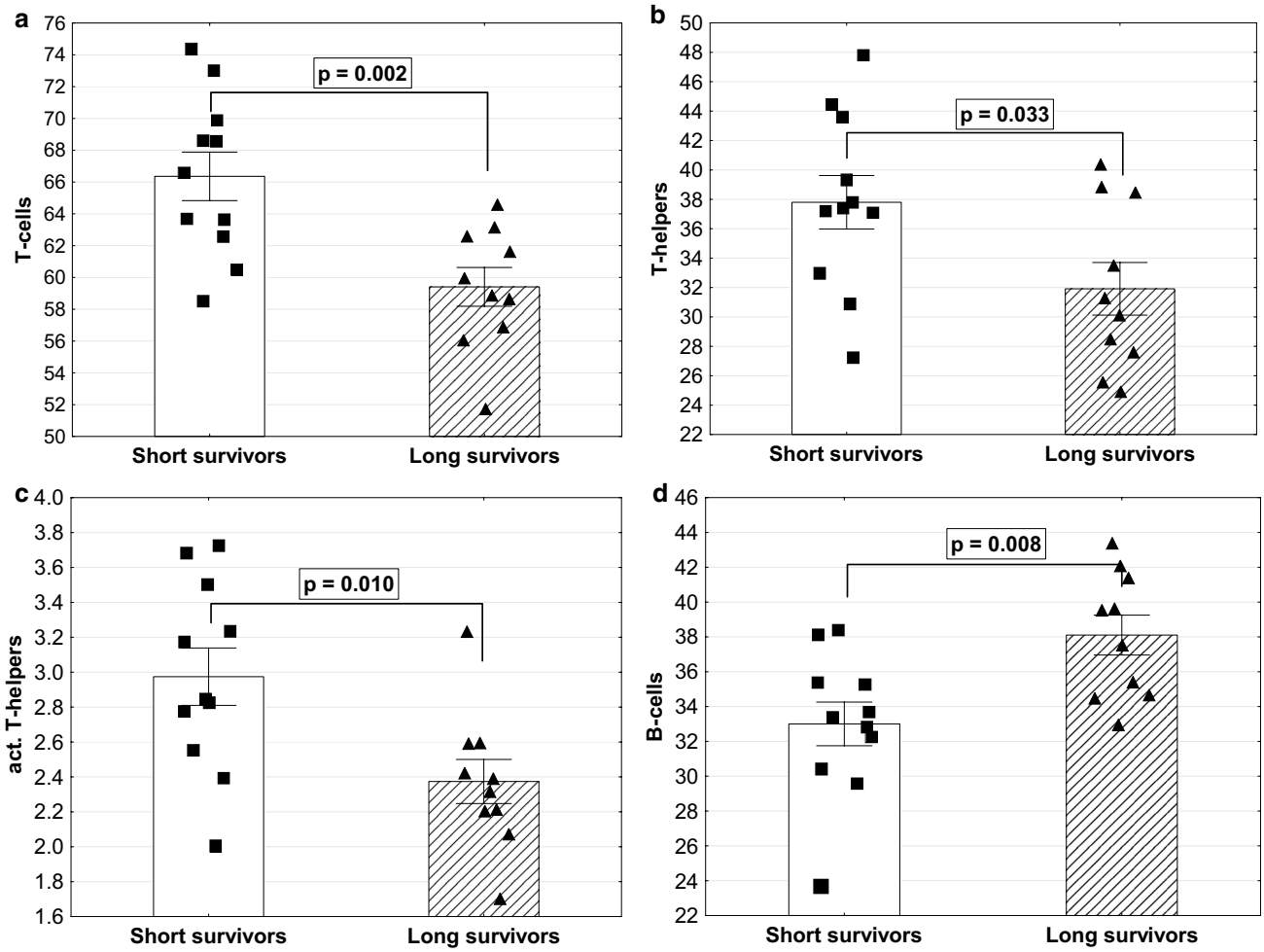

Fig. 5 The level of several initial blood immunological parameters of MC-bearing mice with short and long survival. Short survivors were characterized by higher levels of T cells (**a**), T-helpers (**b**), activated T-helpers (**c**), and by diminished levels of B cells (**d**) relative to long survivors. Mean values are shown as columns, SEM—as whiskers

survival time and initial level of B cells ($r_s = 0.54$, $p = 0.011$) as well as moderate negative correlation between survival time and level of T-lymphocytes ($r_s = -0.52$, $p = 0.015$), and act. T-helpers ($r_s = -0.45$, $p = 0.040$). Scatter plots are presented in Supplementary Fig. 4 (a-c).

Thus, initially measured CBIPs in intact females turned out to be informative biomarkers for determining the age of MC diagnosis and survival time of MC-bearing mice.

Discussion

The mean age of women at BC diagnosis worldwide varies significantly. In the US population, it was 62 years according to SEER statistics [24]. Women from the familial set of BC (when two or more first- or second-degree relatives are affected in a family) usually have earlier onset (discussed in [25]); e.g., the mean age of 49–52 years was reported for Swedish patients [26]. Mouse females of the BLRB/BYRB strains are characterized by the high incidence of naturally occurring MC during aging in the non-SPF environment. In the current study, the mean age of MC diagnosis in the studied cohort of 80 females of various ages was about 70 weeks; this value corresponds to 50–55 human years [23, 27]. Thus, we suggest that the presented natural mouse model of human BC reproduced the main age-related intrinsic trait of an organism predisposed to mammary cancer. Earlier, distinct histopathological characteristics of the spontaneous mammary carcinoma and leukocyte tumor infiltrates in BLRB/BYRB females were shown to resemble those characteristics of human BC [8]. In the current study, we dealt with another salient attribute contributing to the predisposition to this disease—immunosenescence, or aging of the immune system. We and others consider the phenomenon of immunosenescence as a process characterized by the accumulation of multiple disorders in a host that might serve as a mechanism for an incipient tumor to escape the immunosurveillance [28]. We found significant multidirectional changes in 11 CBIPs with age in intact MC-prone mouse females. Immunosenescence was well documented for humans. For example, Sansoni and co-authors reported data on the decrease in the number of $CD3^+$ T-lymphocytes and $CD4^+$ T-helpers in elderly people (discussed in [29]), this corresponds to the results we obtained in intact aging BLRB/BYRB mice. In elderly people (general population of both sexes), the level of $CD56^+CD3^{neg}$ NK-lymphocytes was increased, and the level of $CD8^+$ CTL and $CD19^+$ B-lymphocytes was decreased after 70 years. However, in aging MC-prone mouse females, we observed the opposite dependences. Presumably, these differences were due to the high predisposition of BLRB and BYRB mouse females to the natural MC development. The antitumor immunity in mouse females, which may be attributable to CTL and B-lymphocytes, remained active throughout the latent period before the onset of MC. Importantly, in accordance with our data, the decrease of NK-cell level was observed in unaffected members of breast cancer families [30]. Natural NK-cell reducing before MC onset shows one more similarity between mouse model used and familial set of BC. Recently, the total level of B cells was reported to be elevated in BC patients comparing to healthy donors [31], which also corresponds to our data obtained in precancerous mouse females. Taken together, this shows the natural BLRB/BYRB model as a proper tool to study basic immunological disorders preceding MC clinical manifestation.

Previously, we proposed a personalized 3S-paradigm for biomedical research [9, 32]. According to 1 of the 3 components of our concept, the stratification of recipients into two subgroups by the parameter that influences the studied process allows revealing initially undetectable regularities in more homogeneous subgroups. In the present study, we found multidirectional changes in CBIPs depending on the age of the MC-prone mouse females. This served as a basis for dividing the initial population of mice into "middle-aged" and "old" subgroups. The observed decrease of natural killers in middle-aged mice with subsequently diagnosed MC could indicate the deterioration of at least one of the innate antitumor immunity elements in females of the MC-prone strains of mice. In accordance with our data, the decrease in the number of NK cells was shown not only in the peripheral blood of unaffected members of BC families [30, 33] but also in BC patients as compared to healthy donors [34].

In the subgroup of old mice, we revealed that the combination of the initially lowered level of B-lymphocytes and elevated level of T-helpers preceded MC diagnosis. No differences in the initial level of NK cells between MC^+ and MC^- groups were observed, presumably because of the natural reduction of NK cells with age in BLRB and BYRB mouse females. Currently, both experimental and clinical data demonstrate either pro- or antitumor activity of B-lymphocytes (discussed in [35]). Our findings suggest that B-cell mediated antitumor immunity prevailed in older females with a decreased function of the NK-mediated innate immune response since only females with high initial B-lymphocyte level (as well as high T-helper level) remained alive without any sign of MC at the end of the 28-week observation period.

The lowered initial level of B-lymphocytes and the elevated level of NK cells in aging BLRB/BYRB mouse females with diagnosed MC were associated with earlier onset comparing to later onset. The lower frequency of earlier MC onset in the subgroup of old females with initially lowered values of NK cells may serve to explain this. Circulating B cells were shown to be a useful marker for calculating BC risk using flow-variant assays [36]. Prognostic value

of NK cells for BC risk remains uncertain while a few data concerning NK-cell cytotoxicity in unaffected members of BC families are available [33, 37].

The mean survival time for untreated BC patients was 3–4 years, and only 5–10% of patients lived longer than 10 years [38, 39]. In the used BLRB/BYRB natural (spontaneous) mouse model for human BC, the mean survival time for MC-bearing females was 8 weeks (similar values were obtained earlier [8]) with minimum and maximum survival of 3 and 15 weeks, respectively. 8 weeks of a mouse lifespan corresponds to about 6 human years [23, 27]; thus, the mean survival of untreated mice was a little bit higher, but still very close to the parameter value in untreated BC patients. Revealing the factors affecting BC patient's survival rate and predicting its longevity in a given patient is imperative both in personalized BC clinics and in pharmacology. Very few papers considering the survival of untreated BC patients are available, while the therapy impact has been regularly reviewed [40, 41]. In the current study, we found several regularities between immune status of cancer-prone females prior to MC diagnosis and lifespan of untreated MC-bearing mice: elevated initial level of B cells and lowered initial level of T-helpers and activated T-helpers were observed in BLRB/BYRB females with long survival. Interestingly, the total number of tumor-infiltrating B-lymphocytes irrespective of localization was also associated with a good prognosis in BC patients [42]. Moreover, in accordance with our data on the murine blood, low expression of $CD4^+$ T-helpers in tissue samples obtained from BC patients was related to better specific overall survival [43]. Finally, although we have not yet presented a model for an individual prognosis, CBIP patterns described here for MC-prone females along with the correlations of some CBIPs with age of MC diagnosis and survival time give a basis for developing such models for the needs of biomedical research and BC clinics.

In conclusion, this study reveals evident imbalance in cellular immunological parameters of the blood before MC detection in the BLRB/BYRB natural mouse model of human BC and distinct subpopulation disproportions influencing the survival of MC-bearing mice. Besides, similar cellular changes were found in the blood of unaffected BC family members and BC patients; this shows some general similarities in immune system parameter changes in precancerous mouse females and BC-prone women and BC human patients. This implies that similar prognostic markers in cancer-prone individuals of both species appear to be feasible.

Author contributions DAA contributed in study design, flow cytometry, in vivo studies, data analysis, interpretation of results, and manuscript preparation. VVZ contributed in data analysis, interpretation of results, and manuscript drafting. SGS contributed in study design, histopathological analysis of mammary tumors, and manuscript drafting. EVM contributed in study design, in vivo studies, histopathological analysis to confirm or reject mammary cancer diagnosis, data analysis, interpretation of results, and manuscript preparation. All authors approved the final version of the manuscript.

Funding No relevant funding.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the animal experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (US Department of Health and Human Services, National Institute of Health Publication No 93–23, revised 1985) and were approved by the Institutional Animal Care and Use Committee (http://www.ibch.ru/downloads/documents/553/Institutional_Policy_on_the_Use_of_Laboratory_Animals.pdf). Animal research approval number: 155/2014.

Animal source Original mouse strains BLRB-Rb(8.17)1Iem and BYRB-Rb(8.17)1Iem were developed by Ekaterina Moiseeva [22] and have been bred and maintained at Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia since 1993.

References

1. Walters S, Maringe C, Butler J et al (2013) Breast cancer survival and stage at diagnosis in Australia, Canada, Denmark, Norway, Sweden and the UK, 2000–2007: a population-based study. Br J Cancer 108:1195–1208. https://doi.org/10.1038/bjc.2013.6
2. Løberg M, Lousdal ML, Bretthauer M, Kalager M (2015) Benefits and harms of mammography screening. Breast Cancer Res 17:63. https://doi.org/10.1186/s13058-015-0525-z
3. Gøtzsche PC, Nielsen M (2011) Screening for breast cancer with mammography. Cochrane Database Syst Rev. https://doi.org/10.1002/14651858.CD001877.pub4
4. Den Otter W, Merchant TE, Beijerinck D, Koten JW (1996) Breast cancer induction due to mammographic screening in hereditarily affected women. Anticancer Res 16:3173–3175
5. Jansen-van der Weide MC, Greuter MJW, Jansen L et al (2010) Exposure to low-dose radiation and the risk of breast cancer among women with a familial or genetic predisposition: a meta-analysis. Eur Radiol 20:2547–2556. https://doi.org/10.1007/s00330-010-1839-y
6. Gail MH, Brinton LA, Byar DP et al (1989) Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 81:1879–1886. https://doi.org/10.1093/jnci/81.24.1879
7. Gail MH (2015) Twenty-five years of breast cancer risk models and their applications. J Natl Cancer Inst 107. https://doi.org/10.1093/jnci/djv042
8. Moiseeva E (2009) Anti-breast cancer drug testing. Original approaches. novel set of mouse models. Lambert Academic Publishing, Saarbrücken
9. Moiseeva EV, Aronov DA, Semushina SG, Bozhenko VK (2014) Advantages of a new paradigm of experimental oncology: individual prognosis of immunotherapy outcome based on surrogate marker analysis. Mol Med (2):47–50
10. Okuturlar Y, Gunaldi M, Tiken EE et al (2015) Utility of peripheral blood parameters in predicting breast cancer risk. Asian Pac

J Cancer Prev 16:2409–2412. https://doi.org/10.7314/APJCP.2015.16.6.2409
11. Choudhari A, Desai P, Indumati V, Kadi S (2013) Activities of serum Ada, GGT and alp in carcinoma breast-a case control study for diagnostic and prognostic significance. Indian J Med Sci 67:123–129. https://doi.org/10.4103/0019-5359.122740
12. Dezayee ZMI, Al-Nimer MSM (2016) The clinical importance of measurement of hematological indices in the breast cancer survivals: a comparison between premenopausal and postmenopausal women. World J Oncol 7:1–4. https://doi.org/10.14740/wjon956e
13. Standish LJ, Sweet ES, Novack J et al (2008) Breast cancer and the immune system. J Soc Integr Oncol 6:158–168
14. Verronèse E, Delgado A, Valladeau-Guilemond J et al (2016) Immune cell dysfunctions in breast cancer patients detected through whole blood multi-parametric flow cytometry assay. Oncoimmunology. https://doi.org/10.1080/2162402X.2015.1100791
15. Song G, Wang X, Jia J et al (2013) Elevated level of peripheral CD8 + CD28—T lymphocytes are an independent predictor of progression-free survival in patients with metastatic breast cancer during the course of chemotherapy. Cancer Immunol Immunother 62:1123–1130. https://doi.org/10.1007/s00262-013-1424-8
16. Shao B, Li HP, DI LJ et al (2016) Predictive and prognostic value of monitoring lymphocyte subsets in peripheral blood before and after chemotherapy in patients with metastatic breast cancer. Beijing Da Xue Xue Bao 48:304–309
17. Murphy WJ (2016) Being "penny-wise but pound foolish" in cancer immunotherapy research: the urgent need for mouse cancer models to reflect human modifying factors. J Immunother Cancer 4:88. https://doi.org/10.1186/s40425-016-0195-0
18. Beura LK, Hamilton SE, Bi K et al (2016) Normalizing the environment recapitulates adult human immune traits in laboratory mice. Nature 532:512–516. https://doi.org/10.1038/nature17655
19. Masopust D, Sivula CP, Jameson SC (2017) Of mice, dirty mice, and men: using mice to understand human immunology. J Immunol 199:383–388. https://doi.org/10.4049/jimmunol.1700453
20. Semushina SG, Aronov DA, Moiseeva EV (2018) Local interleukin-2 immunotherapy of breast cancer: benefit and risk in a spontaneous mouse model. Pathol Oncol Res. https://doi.org/10.1007/s12253-018-0396-6
21. Mao Y, Qu Q, Chen X et al (2016) The prognostic value of tumor-infiltrating lymphocytes in breast cancer: a systematic review and meta-analysis. PLoS One 11:e0152500. https://doi.org/10.1371/journal.pone.0152500
22. Festing M (1998) Inbred strains of mice: BLRB. In: Mouse genome informatics. http://www.informatics.jax.org/external/festing/mouse/docs/BLRB.shtml. Accessed 16 Jul 2018
23. Flurkey K, Currer JM, Harrison DE (2007) Mouse models in aging research. In: The mouse in biomedical research, 2nd edn, pp 637–672
24. Howlader N, Noone AM, Krapcho M et al (2016) SEER Cancer Statistics Review, 1975–2014, National Cancer Institute. Bethesda, MD, based on November 2016 SEER data submission, posted to the SEER web site, April 2017. https://seer.cancer.gov/csr/1975_2014/
25. Collaborative Group on Hormonal Factors in Breast Cancer (2001) Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease. Lancet 358:1389–1399. https://doi.org/10.1016/S0140-6736(01)06524-2
26. Brandt A, Bermejo JL, Sundquist J, Hemminki K (2010) Age of onset in familial breast cancer as background data for medical surveillance. Br J Cancer 102:42–47. https://doi.org/10.1038/sj.bjc.6605421
27. Dutta S, Sengupta P (2016) Men and mice: relating their ages. Life Sci 152:244–248
28. Poschke I, De Boniface J, Mao Y, Kiessling R (2012) Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early stage breast cancer patients. Int J Cancer. https://doi.org/10.1002/ijc.27410
29. Sansoni P, Vescovini R, Fagnoni F et al (2008) The immune system in extreme longevity. Exp Gerontol 43:61–65
30. Shevde LA, Joshi NN, Shinde SR, Nadkarni JJ (1998) Studies on functional status of circulating lymphocytes in unaffected members from cancer families. Hum Immunol 59:373–381. https://doi.org/10.1016/S0198-8859(98)00027-5
31. Tsuda B, Miyamoto A, Yokoyama K et al (2018) B-cell populations are expanded in breast cancer patients compared with healthy controls. Breast Cancer 25:284–291. https://doi.org/10.1007/s12282-017-0824-6
32. Moiseeva E (2011) Towards personalized paradigm of experimental anticancer research with "3S" concept. EPMA J 2:114–115. https://doi.org/10.1007/s13167-011-0119-5
33. Shevde LA, Rao NM, Joshi NN et al (1998) Natural killer cell function and genetic instability in unaffected individuals from breast cancer families. Eur J Cancer Prev 7:141–148
34. Muraro E, Comaro E, Talamini R et al (2015) Improved Natural Killer cell activity and retained anti-tumor CD8 + T cell responses contribute to the induction of a pathological complete response in HER2-positive breast cancer patients undergoing neoadjuvant chemotherapy. J Transl Med 13:204. https://doi.org/10.1186/s12967-015-0567-0
35. Fremd C, Schuetz F, Sohn C et al (2013) B cell-regulated immune responses in tumor models and cancer patients. Oncoimmunology 2:e25443. https://doi.org/10.4161/onci.25443
36. Syeda MM, Upadhyay K, Loke J et al (2017) Prediction of breast cancer risk based on flow-variant analysis of circulating peripheral blood B cells. Genet Med. https://doi.org/10.1038/gim.2016.222
37. Strayer DR, Carter W, Brodsky I (1986) Familial occurrence of breast cancer is associated with reduced natural killer cytotoxicity. Breast Cancer Res Treat 7:187–192
38. Johnstone PA, Norton MS, Riffenburgh RH (2000) Survival of patients with untreated breast cancer. J Surg Oncol 73:273–277
39. Galmarini CM, Tredan O, Galmarini FC (2015) Survivorship in untreated breast cancer patients. Med Oncol 32:466
40. Bodai BI, Tuso P (2015) Breast cancer survivorship: a comprehensive review of long-term medical issues and lifestyle recommendations. Perm J 19:48–79. https://doi.org/10.7812/TPP/14-241
41. Cedolini C, Bertozzi S, Londero AP et al (2014) Type of breast cancer diagnosis, screening, and survival. Clin Breast Cancer 14:235–240. https://doi.org/10.1016/j.clbc.2014.02.004
42. Mahmoud SMA, Lee AHS, Paish EC et al (2012) The prognostic significance of B lymphocytes in invasive carcinoma of the breast. Breast Cancer Res Treat 132:545–553. https://doi.org/10.1007/s10549-011-1620-1
43. Matkowski R, Gisterek I, Halon A et al (2009) The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. Anticancer Res 29:2445–2451