AGE-RELATED ALTERATIONS IN THE IMMUNE SYSTEM OF AGING MICE

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Accumulation of senescent cells in the tissues is associated with functional impairment and the development of age-related disorders. The key role in this process is played by the senescence-associated secretory phenotype (SASP) contributing to chronic systemic inflammation, which is associated with the increased risk of autoimmune disorders and cancer, as well as the decreased resistance to infections. Normally, the immune system eliminates senescent cells, but the effectiveness of this process decreases with age, including due to the immune system aging. The study aimed to assess age-related alterations in the main lymphocyte and myelocyte populations in the spleen and bone marrow samples of senile mice. The study involved groups of young (n = 8) and elderly (n = 4) C57BL/6 mice. Populations were tested by flow cytometry using the fluorescence-labeled antibodies. The aging phenotype was assessed based on the β-Gal enzyme activity with pre-treatment with bafilomycin A1, ensuring lysosomal alkalinization and allowing one to detect the increased enzyme activity typical for the aging cells (SA-β-Gal). As a result, the significantly increased levels of myeloid populations, CD11c$^+$ B cells, double-negative T cells, along with the decreased levels of the CD8α$^+$ dendritic cells, were reported in elderly mice. Furthermore, aging was associated with the significant increase in the levels of SA-β-Gal-positive cells, especially in the populations of myeloid cells. The data obtained suggest that the age-related alterations are of systemic nature and reflect the so-called myeloid shift, as well as accumulation of pro-inflammatory populations in the myeloid and lymphoid compartments.

Keywords: aging, senescence, immune system aging, β-galactosidase, SA-β-Gal, lymphocytes, myelocytes, mice

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ВОЗРАСТНЫЕ ИЗМЕНЕНИЯ В ИММУННОЙ СИСТЕМЕ СТАРЕЮЩИХ МЫШЕЙ

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Накопление сенесцентных клеток в тканях связано с функциональным ухудшением и развитием возраст-ассоциированных патологий. Ключевую роль в этом процессе играет сенесцент-ассоциированный секреторный фенотип (SASP), способствующий хроническому вялотекущему системному воспалению, которое ассоциировано с повышенным риском аутоиммунных и онкологических заболеваний, а также снижением устойчивости к инфекциям. В норме иммунная система удаляет сенесцентные клетки, однако с возрастом эффективность этого процесса падает, в том числе по причине старения иммунной системы. Целью исследования было изучить возрастные изменения в основных популяциях лимфоцитов и миелоцитов в образцах селезенки и костного мозга мышей преклонного возраста. Исследование проводили на группах молодых (n = 8) и пожилых (n = 4) мышей линии C57BL/6. Анализ популяций проводили с использованием флуоресцентно-меченых антител методом проточной цитометрии. Фенотип старения оценивали по активности фермента β-Gal с предварительной обработкой бафиломицином A1, который обеспечивает защелачивание лизосом и позволяет выявить повышенную активность фермента, типичную для стареющих клеток (SA-β-Gal). В результате у пожилых мышей было выявлено значимое повышение содержания миелоидных популяций, CD11c$^+$B-клеток, дважды негативных Т-лимфоцитов, а также снижение CD8α$^+$ дендритных клеток. Кроме того, при старении значимо возрастало содержание клеток позитивных по SA-β-Gal, особенно в популяциях миелоидных клеток. Полученные данные указывают, что возрастные изменения носят системный характер и отражают так называемый миелоидный сдвиг, а также накопление провоспалительных популяций в миелоидном и лимфоидном компартментах.

Ключевые слова: старение, сенесцентность, старение иммунной системы, β-галактозидаза, SA-β-Gal, лимфоциты, миелоциты, мыши

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Cellular senescence is a complex, multifactorial process triggered by diverse stressors, including DNA damage, telomere attrition, retrotransposon activation, oxidative and mechanical stress, as well as adverse physical, chemical, and biological factors [1, 2]. The accumulation of mutations and various types of molecular damage in aging cells increases the risk of neoplastic transformation. Currently, entry into a senescent state is recognized as one of the key tumor-suppressive mechanisms [3, 4]. Senescence is orchestrated through the convergence of several signaling cascades, primarily the $p53/p21^{CIP1}$ and $p16^{INK4a/RB}$ pathways [5]. These pathways are activated in response to telomere shortening and DNA damage (DNA damage response, DDR), oncogene activation, epigenetic alterations, chromatin architecture disruption, excessive reactive oxygen species (ROS) production due to organelle dysfunction — particularly mitochondrial dysfunction — as well as specific inflammatory and paracrine signals [6, 7]. Stress-induced activation of the NF-κB and mTOR pathways drives senescent cells to secrete a broad spectrum of pro-inflammatory mediators (the senescence-associated secretory phenotype, SASP) and impairs autophagy [8, 9]. Concurrently, upregulation of anti-apoptotic BCL-2 family proteins — BCL-2, BCL-XL, and MCL-1 — inhibits apoptosis [10]. Collectively, these alterations define the hallmark features of senescent cells: irreversible cell cycle arrest, apoptosis resistance, a pro-inflammatory SASP, mitochondrial dysfunction, and proteostasis impairment [11]. A morphofunctional manifestation of the metabolic imbalance and lysosomal dysfunction characteristic of senescent cells is the hypertrophy of the lysosomal compartment and elevated activity of the lysosomal enzyme β-galactosidase (β-Gal) [12]. Consequently, the high concentration of this enzyme within enlarged lysosomes results in detectable enzymatic activity at a suboptimal pH of 6.0, enabling its use as a biomarker for senescent cells – termed senescence-associated β-galactosidase (SA-β-Gal) [13, 14]. Traditionally, chromogenic substrates have been employed to assess β-Gal activity; however, these are incompatible with multiparametric phenotypic analysis of senescent cells by fluorescence-based techniques, including flow cytometry. The recent development of fluorogenic β-Gal substrates has substantially expanded the utility of this marker, allowing simultaneous quantification of SA-β-Gal activity across distinct immune cell populations via flow cytometry [15]. To date, limited data exist regarding age-related changes in SA-β-Gal activity across different immune cell subsets. Evaluating SA-β-Gal activity in lymphoid and myeloid populations from both central (bone marrow) and peripheral (spleen) compartments of the immune system is therefore of considerable interest for understanding immunosenescence. Given that the immune system is continuously exposed throughout life to stressors of varying nature and intensity, distinct lymphoid and myeloid subsets are expected to exhibit heterogeneous trajectories and rates of aging. An increased senescent burden within the immune system exacerbates "inflammaging," heightens the risk of autoimmune and neoplastic disorders, and enhances susceptibility to infections [16]. Consequently, a detailed characterization of age-associated alterations in the immune system provides a critical foundation for developing targeted strategies to restore immune competence in the elderly. Thus, the aim of this study was to perform a comparative analysis of SA-β-Gal activity in major immune cell populations isolated from the spleen and bone marrow of young (3-month-old) and very old (26-month-old) C57BL/6 mice. This approach enabled us to map the distribution of senescent-like cells within the aged immune system and to directly compare central and peripheral immune compartments with respect to their senescent cell content.

METHODS

Mice

The study involved 12 C57BL/6 mice: 8 mice aged 3 months and 4 elderly mice aged 26 months. The animals were kept in the vivarium with the 12-h ligh/dark cycle, unlimited access to water and balanced laboratory feed. Euthanasia compliant with the principles of animal welfare was performed under deep isoflurane anesthesia by cervical dislocation. Appropriate biomaterial was collected immediately after euthanasia.

Splenocyte isolation

After euthanasia the spleen was retrieved, put it in the glass homogenizer with cold PBS (1% FCS, 0.02% EDTA), and gently grinded with the glass pestle to obtain the homogenous suspension. The resulting cell suspension was twice filtered though the nylon filter (70 μm) with PBS washing. The filtered material was centrifuged for 5 min at 300 g and 8 °C. Precipitate was resuspended in 5 mL of buffer for 2 min to lyse red blood cells. Then it was supplemented with 10 mL of PBS with 1% FBS and centrifuged again. After elimination of supernatant, the cells were resuspended to the desired concentration in the RPMI-1640 complete medium or PBS, depending on the goal.

Bone marrow cell isolation

Bone marrow cells were isolated from the mouse femur and tibia by washing the bone marrow out of the bone cavity with the PBS solution using a syringe (27 G). The resulting suspension was twice filtered though the 70 μm nylon filter, washed in PBS (0.02% EDTA) by centrifugation for 5 min at 300 g and 8 °C. Then red blood cells were lysed (see above) and resuspended in the complete medium or PBS.

The splenocyte and bone marrow cell viability was assessed by the fluorescent method using acridine orange and propidium iodide; the average viability was 98%.

SA-β-Gal staining

To estimate SA-β-Gal activity in living cells, the SPiDER-βGal vital dye was used (Cellular Senescence Detection Kit, Dojindo Laboratories, Japan), which represents a fluorogenic substrate specific for β-Gal. The bone marrow cells or splenocytes, 2×10^5 cells per well, were incubated in the flat-bottom 96-well plate (NEST Biotechnologies, China), in 200 μL of the RPMI-1640 complete medium supplemented with bafilomycin A1 (Sigma Aldrich, USA) to the final concentration of 100 nM as a lizosome alkalinizing agent, for 1 h in the CO2 incubator at 37°. Then the cells were added the substrate to the final concentration of 1 μmol/L and incubated under the same conditions for 1 h. Then the cells were washed by centrifugation for 5 min at 300 g, 20 °C, stained with the FVS780 dye (BD Biosciences, USA) in accordance with the manufacturer's protocol to eliminate the dead cells from the further analysis, and antibody-labeled. As a positive control, the cells were simultaneously incubated with the substrate not supplemented with bafilomycin. As a negative control, the cells were incubated with added bafilomycin without the substrate.

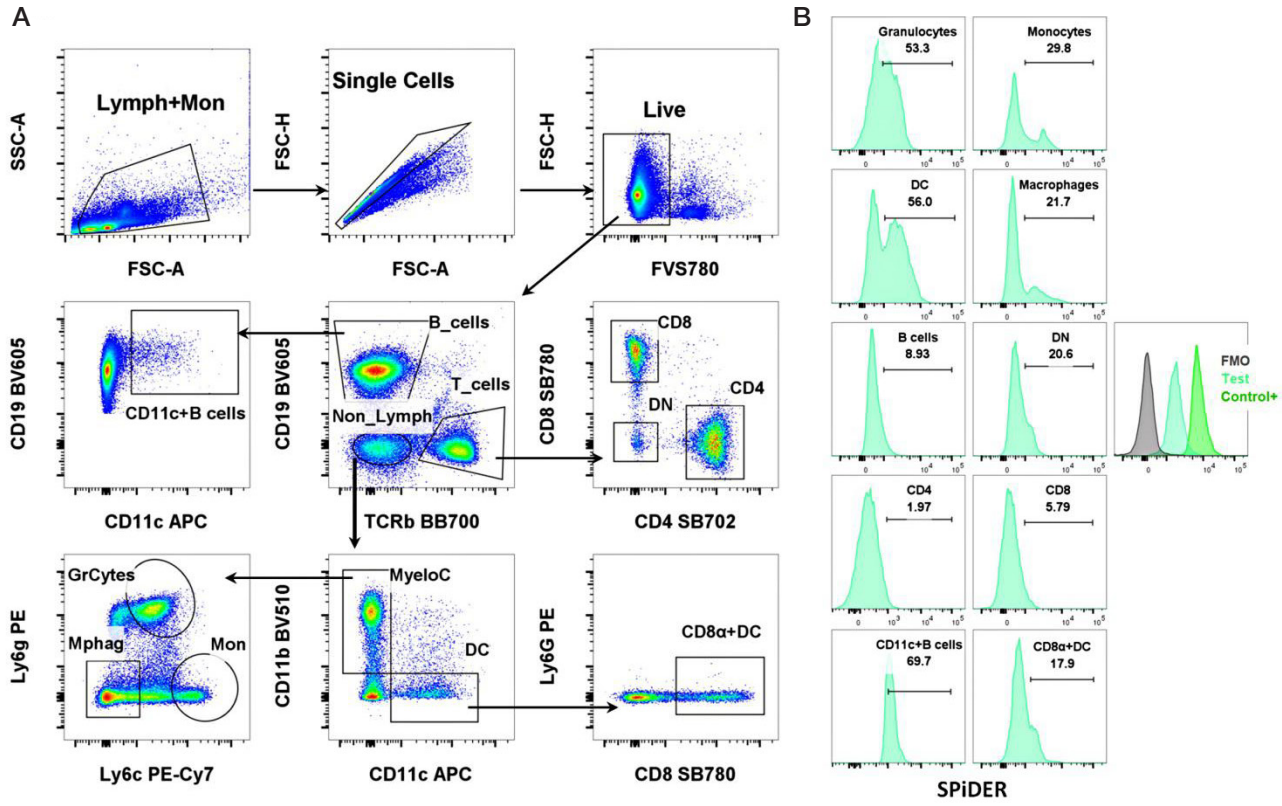

Fig. 1. Gating strategies for lymphoid and myeloid populations in spleen and bone marrow samples from mice **(A)** and quantification of the proportion of SA-β-Gal-positive cells within each analyzed population **(B)**. The histogram on the right shows SPiDER fluorescence intensity in the 488-530/30 nm detection channel for the FMO control (without fluorogenic substrate) and the positive control (Control+, without bafilomycin A1)

Phenotyping

For antibody staining a total of 2×10^5 cells were resuspended in 200 µL of the FACS buffer, added 50 µL of the antibody mixture, mixed thoroughly by pipetting, and incubated at 4 °C for 30 min in the dark. The anti-mouse antibodies used were as follows: TCRβ BB700 (#745846, BD Biosciences, USA), CD19 BV605 (#563148, BD Biosciences, USA), CD11c APC (#550261, BD Biosciences, USA), CD11b BV510 (#562950, BD Biosciences, USA), Ly6G PE (#12-9668-82, ThermoFisher, USA), Ly6C PE-Cy7 (#560593, BD Biosciences, USA), CD4 SB702 (#67-0041-82, ThermoFisher, USA), CD8 SB780 (#78-0081-82, ThermoFisher, USA). Then the cells were twice washed for 5 min at 300 g with the FACS buffer and resuspended in 300 µL. Then these were analyzed using the BD LSRFortessa flow cytometer (BD Biosciences, USA).

Statistical analysis

Flow cytometry data were analyzed using FlowJo software (version 10.8.1; BD Biosciences, USA). Statistical analyses were performed with GraphPad Prism (version 9.3.1; GraphPad Software, USA). The normality of data distributions was assessed using the Shapiro–Wilk test. Comparisons between young and aged mouse groups were carried out using the non-parametric Mann–Whitney U test. Data are presented as medians with interquartile ranges.

RESULTS

Multiparameter flow cytometry enabled the assessment of SA-β-Gal activity across ten distinct immune cell populations. These populations were broadly categorized by lineage into lymphoid (T and B cells) and myeloid subsets (conventional dendritic cells, monocytes, macrophages, and granulocytes). The gating strategy is illustrated in Fig. 1A. Within each defined population, the proportion of cells exhibiting elevated SA-β-Gal activity was quantified. The gating threshold was established using fluorescence-minus-one (FMO) controls, in which cells were treated with bafilomycin A1 but without the fluorogenic substrate SPiDER-βGal (Fig. 1B). This control is particularly critical, as bafilomycin A1 itself can alter cellular autofluorescence levels, thereby influencing background signal in the absence of the substrate.

The analysis of the data obtained for the mouse spleen revealed a significant increase in the counts of monocytes (8.2% (5.6–12.4) vs. 23.7% (17.3–29.2), $p < 0.05$), dendritic cells (12.9% (12–14.2) vs. 27.9% (18.2–29.5), $p < 0.05$), and B cells (53.5% (49.8–57.5) vs. 66.4% (64.9–69.5), $p < 0.05$) in the group of elderly mice (Fig. 2A). It is interesting to note that the counts of $CD11c^+$ B cells increased significantly with age (0.39% (0.32–0.47) vs. 2.18% (1.33–2.58), $p < 0.01$). This is a fairly recently described population of B cells associated with aging. Furthermore, despite the increase in the general dendritic cell population, the counts of $CD8\alpha^+$DC significantly decreased (31.9% (29–33) vs. 23.8% (19.3–31.2), $p < 0.01$). The bone marrow samples also showed the increase in the counts of monocytes (18.2% (14–20) vs. 23.7% (22–28.2), $p < 0.05$) and decrease in B cell counts (32.9% (29.9–36.3) vs. 26.2% (22.8–27.5), $p < 0.05$), while the counts of $CD11c^+$ B cells increased (0.03% (0.025–0.048) vs. 0.26% (0.18–0.4), $p < 0.01$) and that of $CD8\alpha^+$DC decreased (21.6% (19.1–24) vs. 6.5% (4.9–7.1), $p < 0.01$), like in the splenic samples (Fig. 2B).

In the next phase, we assessed the distribution of cells showing the increased SA-β-Gal activity across lymphoid and myeloid populations. Samples of the spleen showed a considerable increase in the counts of SA-β-Gal-positive

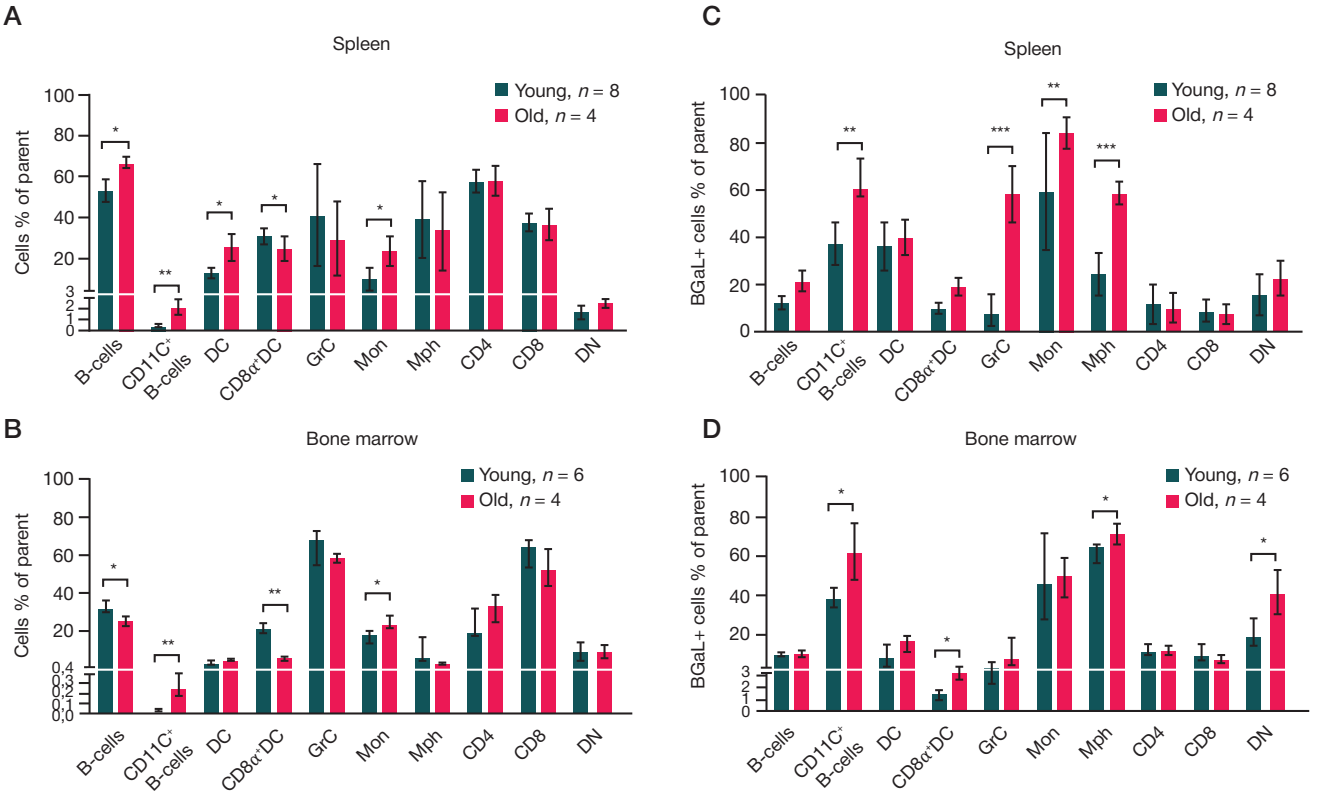

Fig. 2. Proportions of lymphoid and myeloid populations in spleen **(A)** and bone marrow **(B)** samples. Additionally, the frequency of senescent-like cells positive for the SA-β-Gal marker in spleen **(C)** and bone marrow **(D)** is shown. Abbreviations: DC — dendritic cells; GrC — granulocytes; Mon — monocytes; Mph — macrophages; DN – double-negative T cells. Group comparisons were performed usin the Mann–Whitney U-test. Data are presented as Me ± interquartile range (IQR); * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$

granulocytes (7.2% (2.4–15.5) vs. 62.5% (45.8–66.1), $p < 0.001$), macrophages (23.8% (16.8–29.1) vs. 57.2% (55.1–63.5), $p < 0.001$), and monocytes (50.5% (43.8–86) vs. 85.1% (77.7–90.2), $p < 0.01$), as well as CD11c⁺ B cells (39.2% (35.4–43) vs. 60.5% (57.5–73.3), $p < 0.01$) in the group of elderly mice (Fig. 2C). The bone marrow showed the age-related increase in the counts of SA-β-Gal-positive macrophages (64.4% (56.8–65.8) vs. 70.9% (66.1–76.5), $p < 0.05$), CD8α⁺DC (1.5% (0.96–1.7) vs. 3.25% (2.71–4.26), $p < 0.05$), double negative lymphocytes (19.5% (15.2–28.3) vs. 41% (30.9–53.3), $p < 0.05$), and CD11c⁺ B cells (38.4% (34.2–43.6) vs. 62% (47.8–76.8), $p < 0.05$) (Fig. 2D).

Thus, the study revealed age-related alterations in the major populations of lymphoid and myeloid cells, associated with the larger share of cells showing the increased SA-β-Gal activity. In the bone marrow, as a primary lymphoid organ, these alterations were less prominent.

DISCUSSION

The findings showed that physiological aging of the immune system is uneven [17] and is accompanied by significant quantitative changes in the populations of B cells, dendritic cells, and monocytes. Furthermore, the cells showing the increased SA-β-Gal activity are accumulated faster in myeloid populations, than in the lymphoid compartment.

The increase in B-cell counts in the spleen of elderly mice can reflect the life-long history of antigenic challenges, and accumulation of the age-associated CD11c⁺ B cells is considered to be associated with aging and the increased risk of autoimmune disorders, as well as the inflammaging phenomenon [18, 19]. These cells with impaired functions, which contribute to acquisition of pro-inflammatory phenotype by macrophages, show the increased counts in various autoimmune disorders and can constitute a large proportion of the mature B-cell population in the elderly body [20].

In contrast, the bone marrow showed the decrease in B-cell counts reflecting the age-related decline in B-cell production, which is likely to negatively affect the immune system capability of responding to new antigenic challenges [21]. Furthermore, despite reduction of the general B-cell population, the CD11c⁺ B-cell counts were increased, like in the spleen. The age-related increase in the counts of the CD11c⁺ dendritic cells in the spleen and monocytes in the bone marrow is likely to reflect the so-called “myeloid shift” representing the typical feature of the immune system aging described in detail in the recent reports [22–24]. The detected increase in the share of SA-β-Gal-positive granulocytes, monocytes, and macrophages in the spleen of elderly mice is of special interest. The age-related accumulation of SA-β-Gal-positive cells in myeloid populations is likely to contribute to chronic low-grade inflammation, i.e. inflammaging resulting primarily from production of SASP factors by senescent myeloid cells [25, 26]. The increased counts of SA-β-Gal-positive macrophages, DN T cells, and especially CD11c⁺ B cells in the bone marrow suggest involvement of the central immune system departments in the aging processes. In this context it should be noted, that the close relationship between the aging macrophages/CD11c⁺ B cells and hematopoietic stem cells (HSCs) can have a negative effect on the microenvironment in the niches due to SASP production and result in the HSC functional depletion and lymphopoietic potential reduction [27]. This can create a vicious circle, when accumulation of the cells showing signs of senescence in the bone marrow negatively affects hematopoiesis, which, in turn, enhances accumulation of dysfunctional and aging cells [28].

We observed a significant increase in SA-β-Gal activity across multiple lymphoid and myeloid cell populations in aged mice, which – taken together with existing evidence – supports the utility of this marker for investigating immunosenescence. However, certain limitations of our study should be noted. The data obtained are based on the assessment of SA-β-Gal activity as the main cellular senescence marker, but this marker is not absolutely specific and can increase with activation and alteration of metabolism in some types of cells, as well as in the phase of the cell transition to the senescent state [29, 30]. Moreover, we did not assess the functional potential of the studied populations and did not use additional senescence markers, such as p16INK4a, p21CIP1, HMGB1 [5] or SASP components [31], which limits interpretation of the phenomena observed exclusively in the context of the cellular senescence. However, aging is a complex, multifaceted process that is not limited to the cell transition to the senescent state. Thus, the reported quantitative changes in the counts of lymphoid and myeloid subpopulations in the group of elderly mice, along with the changes in SA-β-Gal activity, are likely to reflect the most prominent age-related alterations in the immune system. That is why further comprehensive research is required including transcriptome and proteome assessment and functional tests aimed at investigation of various aspects of the immune system aging. Such an approach will contribute to better understanding of the immune aging mechanisms and the development of strategies aimed at restoring the immune system competence in the elderly.

CONCLUSIONS

Our findings support the hypothesis of heterogeneous aging across different compartments of the immune system and highlight myeloid skewing as a hallmark feature of immunosenescence. Importantly, age-related alterations were observed not only in the peripheral immune compartment but also in the bone marrow. Specifically, the decline in B-cell frequency reflects age-associated suppression of B-lymphopoiesis, while the marked increase in pro-inflammatory $CD11c^+$ B cells and SA-β-Gal-positive double-negative (DN) T lymphocytes, macrophages, and $CD8\alpha^+$ dendritic cells indicates active involvement of central immune organs in the aging process [27]. Notably, the accumulation of SA-β-Gal-positive cells occurs in the bone marrow in close proximity to hematopoietic stem cells (HSCs). In this microenvironment, SASP factor secretion may disrupt the functional integrity of HSC niches, impair lymphopoietic potential, and thereby perpetuate a vicious cycle of age-related immune dysfunction [27, 28]. These observations expand current understanding of the dynamic remodeling of the immune system during aging. Consequently, further investigation of age-associated immune alterations — using a multimodal approach that includes SA-β-Gal assessment — holds significant translational potential. Such research could guide the development of targeted strategies for the selective elimination of pro-inflammatory senescent cells, restoration of lymphopoietic capacity, and enhancement of overall immune competence in the elderly.

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