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Strain specificities in influence of ageing on germinal centre reaction to inactivated influenza virus antigens in mice: Sex-based differences

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ABSTRACT

Considering variability in vaccine responsiveness across human populations, in respect to magnitude and quality, and importance of vaccines in the elderly, the influence of recipient genetic background on the kinetics of age-related changes in the serum IgG antibody responses to seasonal trivalent inactivated split-virus influenza bulk (TIV) was studied in BALB/c and C57BL/6 mice showing quantitative and qualitative differences in this responses in young adult ages. With ageing the total serum IgG response to influenza viruses declined, in a strain-specific manner, so the strain disparity observed in young adult mice (the greater magnitude of IgG response in BALB/c mice) disappeared in aged mice. However, the sexual dimorphisms in this response (more prominent in females of both strains) remained in aged ones. The strain-specific differences in age-related decline in the magnitude of IgG response to TIV correlated with the number of germinal centre (GC) B splenocytes. The age-related decline in GC B cell number was consistent with the decrease in the proliferation of B cells and CD4+ cells in splenocyte cultures upon restimulation with TIV. Additionally, the age-related decrease in the magnitude of IgG response correlated with the increase in follicular T regulatory (fTreg)/follicular T helper (fTh) and fTreg/GC B splenocyte ratios (reflecting decrease in fTh and GC B numbers without changes in fTreg number), and the frequency of CD4+ splenocytes producing IL-21, a key factor in balancing the B cell and fTreg cell activity. With ageing the avidity of virus influenza-specific antibody increased in females of both strains. Moreover, ageing affected IgG2a/IgG1 and IgG2c/IgG1 ratios (reflecting Th1/Th2 balance) in male BALB/c mice and female C57BL/6 mice, respectively. Consequently, differently from young mice exhibiting the similar ratios in male and female mice, in aged female mice of both strains IgG2a(c)/IgG1 ratios were shifted towards a less effective IgG1 response (stimulated by IL-4 cytokines) compared with males. The age-related alterations in IgG subclass profiles in both strains correlated with those in IFN-γ/IL-4 production level ratio in splenocyte cultures restimulated with TIV. These findings stimulate further research to formulate sex-specific strategies to improve efficacy of influenza vaccine in the elderly.

1. Introduction

According to data from World Population Prospects: the 2015 Revision (United Nations, https://www.un.org/en/development/desa/population/publications/pdf/ageing/WPA2015_Report), the current size of the elderly (over 60 years old) population will be doubled by 2030. Globally, the number of people aged 80 years or over, the "oldest-old" persons, is growing even faster than the number of the elderly overall (United Nations, https://www.un.org/en/development/desa/population/publications/pdf/ageing/WPA2015_Report). This will have a profound effect on public health policy and pharmaceutical innovations as they will need to find the effective strategies to meet the needs of the elderly.

Immunosenescence is believed to be a major contributory factor to the increased frequency of morbidity and mortality among the elderly (Vasto et al., 2010). Generally, immunosenescence is associated with decreased immune responses to pathogens and consequently increased frequencies and severity of infectious diseases in the elderly (Pera et al., 2015). Consistently, the elderly are considered to be at a greater risk of influenza infection and its complications (Haq and McElhaney, 2014).

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In this context, it is noteworthy that influenza virus is shown to be one of the four leading causes of global mortality (Haq and McElhaney, 2014). The highest mortality rate was found in the elderly affected by influenza infection (Haq and McElhaney, 2014). Annual influenza vaccination is the most effective way to prevent influenza and its complications. However, the elderly are insufficiently protected by vaccination due to the immunosenescence which accompanies ageing (Smetana et al., 2018). Specifically, vaccine responses are diminished in the elderly, whereas vaccine-induced protective antibodies, which are crucial for efficacy of most successful vaccines (Lambert et al., 2005), wane faster in the elderly (Zimmermann and Curtis, 2019). Thus, improving preventive measures, primary by enhancing the influenza vaccine efficacy, in the elderly is becoming a significant public health priority (Smetana et al., 2018).

There is an accumulating body of evidence indicating sexual dimorphism in the immune response, and consequently in susceptibility to influenza infection and influenza vaccine efficacy (Bahadoran et al., 2016). Studies in humans and animal models indicate that females generate more robust immune response to viral infections than males, so they exhibit greater viral clearance capacity (Klein et al., 2012). Likewise, women are shown to mount stronger humoral immune response to trivalent inactivated influenza vaccine than males (Bahadoran et al., 2016; Engler et al., 2008; Giefing-Kröll et al., 2015). Additionally, there are data indicating that females experienced slower decline in humoral immunity than males, thereby suggesting that changes in the vaccine efficacy with ageing could be sex-dependent (Gubbels Bupp et al., 2018; Hirokawa et al., 2013; Strindhall et al., 2013). However, studies dedicated to the influence of sex on age-related changes in the vaccine efficacy are still extremely limited.

Genetic of vaccine recipient is another variable that may influence influenza vaccine efficacy (Linnik and Egli, 2016; Poland et al., 2007; Posteraro et al., 2014). To corroborate this notion are findings indicating that different ethnic groups living in the same location exhibit different viral vaccine responses (Haralambieva et al., 2013, 2014; Hsu et al., 1996; Wang et al., 2006). This phenomenon was linked with polymorphisms of genes encoding proteins involved in the immune response (Castrucci, 2018; Poland et al., 2007; Posteraro et al., 2014; Zimmermann and Curtis, 2019). However, data on the influence of genetics on age-related sex-based differences in immune response and, consequently, efficacy of vaccines are extremely limited. Distinct inbred laboratory mouse strains also differ in immune response patterns and responsiveness to influenza vaccine (Asanuma et al., 2001; Petrović et al., 2018; Sellers et al., 2012). It is noteworthy that mouse vaccination models lack the complexity of a clinical situation, such as use of medication affecting the immune response and pre-existing immunity, which may account for the individual variability in immune responses and thereby efficacy of vaccination, so that they have obvious advantage in investigations targeting role of genetic background in the efficacy of vaccination when compared with humans (Trammell and Toth, 2008).

Considering all the aforementioned, the present study was undertaken to: i) examine the influence of genetics and sex on the kinetics of age-related changes in T helper (Th) cells specialized in fundamentally essential B cell help and regulation of antibody response to seasonal trivalent inactivated split-virus influenza bulk (TIV) and ii) correlate these changes with the IgG antibody responses. For this purpose, young and aged BALB/c and C57BL/6 mice of both sexes exhibiting quantitatively and qualitatively different serum IgG antibody responses to TIV in young adult age (Petrović et al., 2018) were immunised with TIV and several parameters of B cell and Th cell immune responses were examined. Specifically, the magnitude, subclass profile and avidity of IgG antibodies to influenza virus antigens from TIV were examined. The IgG response was focused as the most relevant for the efficacy of influenza vaccine (Bahadoran et al., 2016; Plotkin, 2010). Additionally, splenocytes from vaccinated mice were examined for the number of Th cells involved in intricate germinal centre (GC) reaction necessary to ensure sustained a high-affinity antibody-mediate protection against foreign pathogens (Gatto and Brink, 2010). For this purpose, the absolute numbers of follicular Th (fTh) cells, a specialized subset of Th cells helping B cells to differentiate into high-affinity antibody-secreting plasma cells, and follicular T regulatory (fTreg) cells, a subset of T regulatory cells acting either directly or indirectly, in fTh-dependent manner, on GC B cells to restrict their response (Gatto and Brink, 2010; Sage et al., 2014; Wing et al., 2018), were investigated. Additionally, given that IL-21 secretion by fTh cells is suggested to be a key factor in balancing the B cell and fTreg cell activity (Fabrizi et al., 2014; Peluso et al., 2007), CD4+ T splenocytes were examined for IL-21 production. Moreover, given that the ratio in production levels of Th1/Th2 cytokines is suggested to be determinative for IgG subclass profile, and consequently protective efficacy of IgG responses to influenza virus antigens (Cao et al., 2005), the production levels of interferon-γ (IFN-γ, Th1 signature cytokine) and interleukin-4 (IL-4, Th2 signature cytokine) in splenocyte cultures from BALB/c and C57BL/6 mice of both sexes and ages in response to restimulation with TIV antigens were examined.

2. Materials and methods

2.1. Experimental animals

Young (three-month-old) and aged (eighteen-month-old) female and male mice of BALB/c and C57BL/6 strains were used in this study. Mice were bred at the Institute of Virology, Vaccines and Sera “Torlak” under the standard housing conditions, and given rodent chow and tap water ad libitum. The mice were handled and all experimental procedures were performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and with the governmental regulations (Law on Animal Welfare, “Official Gazette of Republic of Serbia”, no. 41/2009). The study protocol evaluated by the Animal Care and Use Committee of Institute of Virology, Vaccines and Sera “Torlak” was approved by The Ministry of Agriculture, Forestry and Water Economy of the Republic of Serbia - Veterinary Directorate (permit no. 323-07-01577/2016-05/1).

2.2. Immunisation

Experimental animals were immunised with TIV for season 2013/2014 encompassing A/California/7/2009 (H1N1)pdm09-like virus (H1N1), A(H3N2) antigenically like the cell-propagated prototype virus A/Texas/361/2011 (H3N2) and B/Massachusetts/2/2012-like virus (B), which was obtained from the Institute of Virology, Vaccines and Sera “Torlak”. TIV was applied intramuscularly in each caudal tight, in a dose containing 3.33 μg of virus surface protein hemagglutinin (HA) of each of the three virus strains. Immunised animals were monitored daily by researchers and a veterinarian. In none of immunised animals were detected signs of either local or systemic reactions. Non-immunised age-, sex- and strain-matched mice served as controls.

2.3. Reagents and antibodies

For serum antibody titre determination, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a antibodies, biotin-conjugated anti-mouse IgG2c (Jackson ImmunoResearch Laboratories Inc., WestGrove, PA, USA), streptavidin-peroxidase (Abcam, Cambridge, UK) and *o*-phenylenediamine (Sigma Chemie GmbH, Taufkirchen, Germany) were used. For flow cytometric analysis (FCA), fluorescein isothiocyanate (FITC)-conjugated anti-B220 (CD45R, clone RA3-6B2; eBiolegend, Carlsbad, CA, USA), PE-conjugated anti-GL7 (clone GL7; eBiolegend), phycoerythrin (PE)-conjugated anti-CD95 (clone 15A7; eBiolegend), PE-conjugated anti-CD4 (clone RM4-5; eBiolegend), FITC-conjugated anti-Foxp3 (clone FJK-16s; eBiolegend),

peridinin chlorophyll protein cyanine (PerCP-Cy™5.5)-conjugated anti-CXCR5 (Clone 2G8; BD Biosciences Pharmingen, Mountain View, CA, USA), alexa fluor 647-conjugated anti-Bcl6 (K112-91; BD Biosciences Pharmingen), and rabbit polyclonal anti-IL-21 antibody (Merck KGaA, Darmstadt, Germany) were used. As secondary antibody, FITC-conjugated goat anti-rabbit Ig (BD Biosciences Pharmingen) was applied. To determine IL-21 expression, phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Chemie GmbH), ionomycin (Sigma-Aldrich Chemie GmbH) and brefeldin A (eBioscience) were applied. Splenocyte proliferation analyses were performed using PerCP-eFluor 710-conjugated anti-Ki-67 (clone SolA15; eBioscience Inc. San Diego, CA, USA) and 7-aminoactinomicin D (7-AAD), a DNA intercalating dye (BD Biosciences Pharmingen). Medium for cell cultivation consisted of RPMI 1640 (Sigma-Aldrich) with 2 mM L-glutamine (Serva, Heidelberg, Germany), 1 mM sodium pyruvate (Serva), 100 units/ml penicillin (ICN, Costa Mesa, CA, USA), 100 μg/ml streptomycin (ICN) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA).

2.4. Antibody titre determination

Four weeks after the immunisation animals were anaesthetised with 80 mg/kg ketamine (Ketamidor; Richter Pharma AG, Wels, Austria)/8 mg/kg xylazine (Xylased; Bioveta, Ivanovice na Hané, Czech Republic) cocktail and bled from retroorbital sinuses. In order to collect sera, blood samples were left to coagulate for 1 h at the room temperature (RT) and then centrifuged at 1620 ×*g* for 30 min. Sera were heat-inactivated for 30 min at 56 °C and stored at −20 °C for IgG antibody titre determination.

End-point dilution enzyme-linked immunosorbent assay (ELISA) was used to determine the total serum influenza virus-specific IgG antibody titre and the serum titres of IgG1 and IgG2a or IgG2c in mice immunised with TIV. Given that, differently from BALB/c mice, C57BL/6 mice do not have the gene for IgG2a isotype (Martin et al., 1998), in sera from C57BL/6 mice the titres of IgG2c antibody, exerting similar functional properties to IgG2a antibody (Schmitz et al., 2012), were examined.

As described previously (Petrović et al., 2018) mice sera were serially diluted in PBS with 1% BSA, and added to Nunc MaxiSorp 96 well flat bottom plates pre-coated with 50 μl/well (2.5 μg of HA) of inactivated H1N1 or H3N2 or B influenza virus. After one hour incubation at RT plates were washed firstly with 0.05% Tween 20/PBS and then with PBS. HRP- conjugated IgG and IgG subclass specific goat anti-mouse antibodies in appropriate dilutions: IgG (1:10000), IgG1 (1:10000), IgG2a (1:10000), and biotin conjugated IgG2c (1:10000) were added and incubated for 1 h at RT. Plates were washed and for the IgG2c titre determination streptavidin-HRP (1:100) was added and incubated for 30 min. After washing, HRP substrate *o*-phenylalanine with H_2O_2 was added and reaction was stopped after 15 min with 2 M H_2SO_4. Optical density (OD) was measured with Multiscan Ascent (Labsystems) at 490 nm and 620 nm.

2.5. Determination of IgG antibody avidity

To determine avidity of influenza virus-specific antibodies modified ELISA test was used, with potassium thiocyanate (KSCN) as a chaotropic agent (Pullen et al., 1986). Two sets of appropriate sera dilutions in 1% BSA/PBS, which gave the same OD (previously determined by ELISA test), were incubated in H1N1 or H3N2 or B influenza virus HA pre-coated plates for 1 h at 37 °C. Plates were washed three times with 0.05% Tween 20/PBS and once with PBS. One set of sera was incubated for 1 h with PBS while duplicate samples were exposed to 1 M KSCN for 1 h at RT. After washing the plates, secondary HRP-conjugated anti-mouse IgG was added. Procedure continued as described above for antibody titre determination. The percentage of antibodies remained after treating sera with KSCN was calculated according to formula:

$$\text{Relative affinity (\%)} = (A_{492/620\,nm}\ \text{KSCN}/A_{492/620\,nm}\ \text{PBS}) \times 100,$$

$A_{492/620\ nm}$ KSCN represents the absorbance registered for wells incubated with 1 M KSCN and $A_{492/620\ nm}$ PBS the absorbance registered for the corresponding wells incubated with PBS.

2.6. Isolation of splenic mononuclear cells

In order to examine cellular immune response two weeks after the immunisation animals were sacrificed. Spleens were removed from mice and grinded through 60 μm sieve screen in 10% FBS/RPMI 1640 under the sterile conditions. Lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA in H_2O, pH 7.2) was added to remove red blood cells from the suspension. After 10 min of incubation cell suspensions were centrifuged for 10 min at 300 ×*g*, washed once, resuspended in 10% FBS/PBS and counted in 0.2% trypan blue dye using the improved Neubauer haemocytometer. Phenotype, cytokine production and proliferation capability of splenocytes was examined.

2.7. Splenocyte proliferation and cytokine production

Cell suspensions in medium (10% FBS/RPMI 1640) were dispersed (5×10^5 cells/well) in Nunc MaxiSorp 96 well U-bottom plates and cultured in the presence or in the absence of TIV containing 5 μg of each of the three virus strains for 72 h at 37 °C in a 5% CO_2 humidified air atmosphere. To assess B220+ and CD4+ lymphocyte proliferation in vitro, cells were subsequently processed for staining of intracellular Ki-67 expression. For assessment of IL-21 production, harvested cells were cultured for additional 4 h at 37 °C in a 5% CO_2 humidified air atmosphere in 10% FBS/RPMI medium supplemented with 200 ng/ml PMA, 400 ng/ml ionomycin, and 3 μg/ml brefeldin A. Following the incubation, cells were harvested and subjected to intracellular IL-21 immunostaining.

For IFN-γ and IL-4 determination, supernatants from splenocytes cultivated for 72 h at 37 °C in a 5% CO_2 humidified air atmosphere in the presence or in the absence of 5 μg of HA of H1N1 or H3N2 or B influenza virus were collected.

2.8. Cell staining and FCA

2.8.1. Surface antigens immunostaining

Freshly isolated splenocytes (5×10^5 cells) or splenocytes cultivated in the presence or absence of three virus strains were incubated with monoclonal antibodies against CD4, B220, CD95 and GL7 surface antigens for 30 min at 4 °C. After incubation cells were washed and then resuspended in 0.1% NaN_3/PBS for FCA or fixed and permeabilised using fixation/permeabilisation buffers (eBioscience) overnight at 4 °C for intracellular staining.

2.8.2. Intracellular antigen immunostaining

Following surface antigen immunostaining, the cells were fixed/permeabilised overnight, washed and incubated with fluorochrome-conjugated or unconjugated antibodies specific for intracellular antigens (Foxp3, Bcl6, Ki-67 and IL-21) for 30 min at RT. The cells incubated with unconjugated anti-IL-21 antibody were washed and then incubated with FITC-conjugated goat anti-rabbit Ig for further 30 min. Following washing in PBS the cells were acquired on FACS Verse flow cytometer (Becton Dickinson, Mountain View, CA, USA). The data were analysed using FlowJo software, version 7.8. (TreeStar Inc., Ashland, OR, USA).

2.9. Assessment of IFN-γ and IL-4 production

The concentrations of IL-4 and IFN-γ in supernatants of splenocyte cultures were quantified using ELISA kits (Biolegend, San Diego, CA, USA). Both assays were performed according to the manufacturer's

guidelines. Briefly, plates were washed with assay buffer prior to the addition of 50 µl/well of appropriately diluted standards and samples. After 2 h incubation at RT, plates were washed and detection antibody was added and incubated for additional 1 h at RT while shaking. Avidin-HRP solution was added following washing and incubated for 30 min at RT while shaking. After washing, the substrate solution was added, and following 15 min incubation in the dark, the reaction was stopped with the stop solution. Absorbance was read at 450 nm and 570 nm. Standard curve to determine concentrations was calculated for each assay with the limit of detection of 0.5 pg/ml and 8 pg/ml for IL-4 and IFN-γ, respectively.

2.10. Statistics

Two-way ANOVA followed by Bonferroni post hoc comparisons test was used to analyse influence of sex and strain on examined parameters in young and in aged mice, unless otherwise indicated. To estimate the influence of age on the values of examined parameters, regression analysis was used, and the slope values (quantifying the steepness) of the lines depicting age-related alterations in values of examined parameters were compared. All statistical analyses were performed using GraphPad Prism 5.0. (GraphPad Software, Inc., La Jolla, CA, USA). Differences are considered significant when $p < 0.05$.

3. Results

3.1. Kinetics of age-dependent decline in the magnitude of influenza virus-specific IgG responses depends on mouse strain

Confirming findings obtained in our previous study (Petrović et al., 2018), irrespective of sex, young BALB/c mice mounted higher serum titres of IgG against H1N1 and H3N2 influenza virus strains compared with sex-matched C57BL/6 ones (Fig. 1). On the other hand, the serum titres of IgG against B influenza virus strain did not statistically significantly differ between these two strains of mice (Fig. 1). Irrespective of mouse strain, in young mice the magnitudes of IgG response to H1N1 and H3N2 influenza vaccine antigens were greater in females than in age-matched males (Fig. 1). Although the serum titres of IgG elicited by influenza virus type B were higher in young female mice than in age- and strain-matched male mice, these differences did not reach statistical significance (Fig. 1). The serum titres of IgG antibodies elicited by all influenza virus strains decreased with age in both BALB/c and C57BL/6 mice, but those generated to H1N1 and H3N2 influenza virus strains to a greater extent in BALB/c mice (Fig. 1). Consequently, in aged mice, their titres, as that generated against influenza virus type B, were similar in BALB/c and in sex-matched C57BL/6 mice (Fig. 1). However, in aged mice of both strains, IgG responses against all influenza virus strains, were greater in females than in males (Fig. 1).

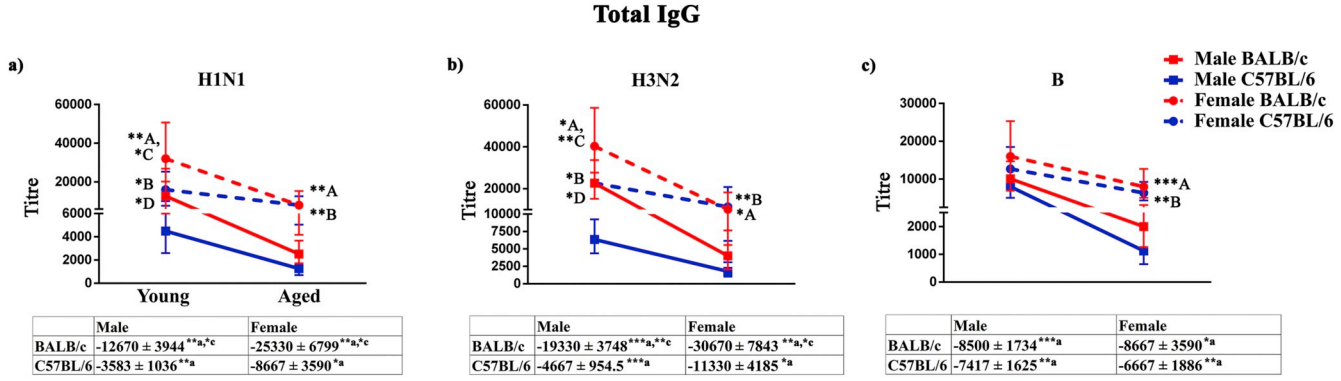
3.2. Ageing influences avidity of influenza virus-specific IgG antibodies only in female mice

Confirming our previous finding (Petrović et al., 2018), in young mice the avidity of influenza virus strain-specific IgG antibodies did not differ across experimental groups (Fig. 2). However, in mice of both strains, their avidity was greater in aged females when compared with age-matched male and sex-matched young mice (Fig. 2).

3.3. Ageing diminishes influenza virus-specific proliferative response of B cells and CD4+ cells in splenocyte cultures in a mouse strain-specific manner

Given that: i) proliferation of antigen-specific B cells is important for the magnitude of antibody response (LeBien and Tedder, 2008; Tan et al., 2014), and ii) specifically in spleen, age-related changes in proliferation of B lymphocytes to T-dependent antigens depend rather on CD4+ cells responsiveness than on B lymphocyte intrinsic defects (Ansell et al., 1980; Haynes et al., 2004), proliferation of both B220+ B-splenocytes and CD4+ T-splenocytes following in vitro restimulation with TIV was examined. Cultures of splenocytes from non-immunised mice were included in analysis for determination of the baseline proliferative B220+ and CD4+ cell responses. In TIV-free cultures of splenocytes from immunised mice of both strains the frequency of Ki-67+ activated/proliferating cells among B220+ cells and CD4+ cells was higher than in TIV-free cultures from strain-, age- and sex-matched non-immunised mice (Fig. 3).

In the presence of TIV, in all splenocyte cultures from immunised mice the frequency of Ki-67+ activated/proliferating cells among both B220+ and CD4+ cells was higher than in control cultures (cultures with medium alone) (Fig. 3). The frequencies of Ki-67+ cells among both B220+ and CD4+ cells were higher in splenocyte cultures from young BALB/c than in those from sex-matched young C57BL/6 mice (Fig. 3). Additionally, in splenocyte cultures from both BALB/c and C57BL/6 young mice the frequencies of Ki67+ cells among both

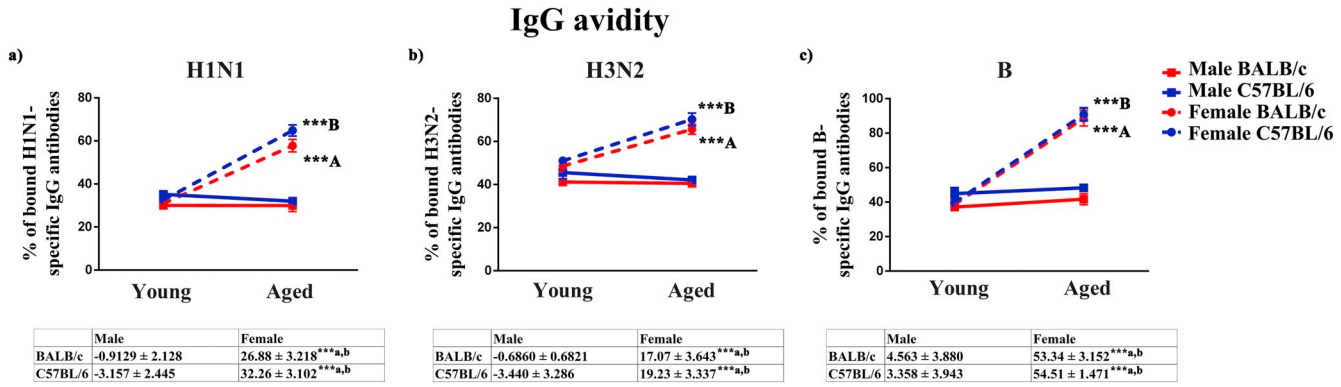


	Male	Female
BALB/c	-12670 ± 3944 **a,*c	-25330 ± 6799 **a,*c
C57BL/6	-3583 ± 1036 **a	-8667 ± 3590 *a

	Male	Female
BALB/c	-19330 ± 3748 ***a,**c	-30670 ± 7843 **a,*c
C57BL/6	-4667 ± 954.5 ***a	-11330 ± 4185 *a

	Male	Female
BALB/c	-8500 ± 1734 ***a	-8667 ± 3590 *a
C57BL/6	-7417 ± 1625 **a	-6667 ± 1886 **a

Fig. 1. Influence of mouse strain on age-related changes in the influenza vaccine antigen-specific IgG responses in females and males. Young and aged BALB/c and C57BL/6 mice of both sexes were immunised with trivalent virus influenza split inactivated bulk contained A/California/7/2009(H1N1)pdm09-like (H1N1) virus, A/Texas/361/2011(H3N2) virus and B/Massachusetts/2/2012-like (B) virus. Serum titres of IgG antibodies against individual influenza virus strains in the bulk were determined four weeks post immunisation by ELISA. The linear diagrams show the geometric mean of IgG titres specific for a) H1N1, b) H3N2 and c) B influenza virus strains. Error bars indicate the 95% confidence interval. $N = 6$ mice/group. Data are from one of three independent experiments. The line slopes depicting age-related changes in the H1N1, H3N2 and B influenza virus strain-specific IgG titres in BALB/c and C57BL/6 mice are shown in the table below each diagram. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. [A]Females vs age-matched males of BALB/c strain; [B]Females vs age-matched males of C57BL/6 strain; [C]BALB/c females vs age-matched C57BL/6 females; [D]BALB/c males vs age-matched C57BL/6 males; [a]significant slope of the regression lines; [c]significant difference in BALB/c mouse vs sex-matched C57BL/6 mouse regression line slopes.



	Male	Female
BALB/c	-0.9129 ± 2.128	26.88 ± 3.218***a,b
C57BL/6	-3.157 ± 2.445	32.26 ± 3.102***a,b

	Male	Female
BALB/c	-0.6860 ± 0.6821	17.07 ± 3.643***a,b
C57BL/6	-3.440 ± 3.286	19.23 ± 3.337***a,b

	Male	Female
BALB/c	4.563 ± 3.880	53.34 ± 3.152***a,b
C57BL/6	3.358 ± 3.943	54.51 ± 1.471***a,b

Fig. 2. Ageing increases avidity of influenza virus-specific IgG antibody in female mice in both BALB/c and C57BL/6 mice. Young and aged BALB/c and C57BL/6 mice of both sexes were immunised with trivalent inactivated virus influenza bulk contained A/California/7/2009 (H1N1)pdm09-like (H1N1) virus, A/Texas/361/2011 (H3N2) virus and B/Massachusetts/2/2012-like (B) virus. Avidity of IgG antibodies against individual influenza virus strains in the bulk were determined four weeks post immunisation in sera by avidity ELISA assay. Appropriately diluted sera were treated with 1 M KSCN and the percentage of a) H1N1, b) H3N2 and c) B influenza strain-specific IgG antibodies remaining bound was measured by dividing the absorbance after 1 M KSCN treatment with that of untreated samples × 100%. Data are presented as means ± SEM. N = 6 mice/group. Data are from one of three independent experiments. $^{***}p < 0.001$. The line slopes depicting age-related changes in the avidity of H1N1, H3N2 and B influenza virus strain-specific IgG antibodies in BALB/c and C57BL/6 mice are shown in the table below each diagram. [A]Females vs age-matched males of BALB/c strain; [B]Females vs age-matched males of C57BL/6 strain; [a]significant slope of the regression lines; [b]significant difference in female vs strain-matched male regression line slopes.

lymphocyte subpopulations were higher in cultures from female young mice than in those from age-matched male mice (Fig. 3). The frequencies of Ki-67+ cells among TIV-restimulated B220+ and CD4+ cells were lower in all splenocyte cultures from aged mice compared with those from strain- and sex-matched young ones (Fig. 3). This age-related decrease in the frequency of Ki-67+ cells among of B220+ and CD4+ lymphocytes was more prominent in splenocyte cultures from BALB/c mice when compared with sex-matched C57BL/6 mice (Fig. 3). Consequently, there was no significant difference in the frequency of Ki-67+ cells among either B220+ or CD4+ cells in splenocyte cultures from aged BALB/c mice compared with age- and sex-matched C57BL/6 ones (Fig. 3). On the other hand, in splenocyte cultures from aged female mice of both strains their frequency was higher than in splenocyte cultures from strain- and age-matched male mice (Fig. 3). It is

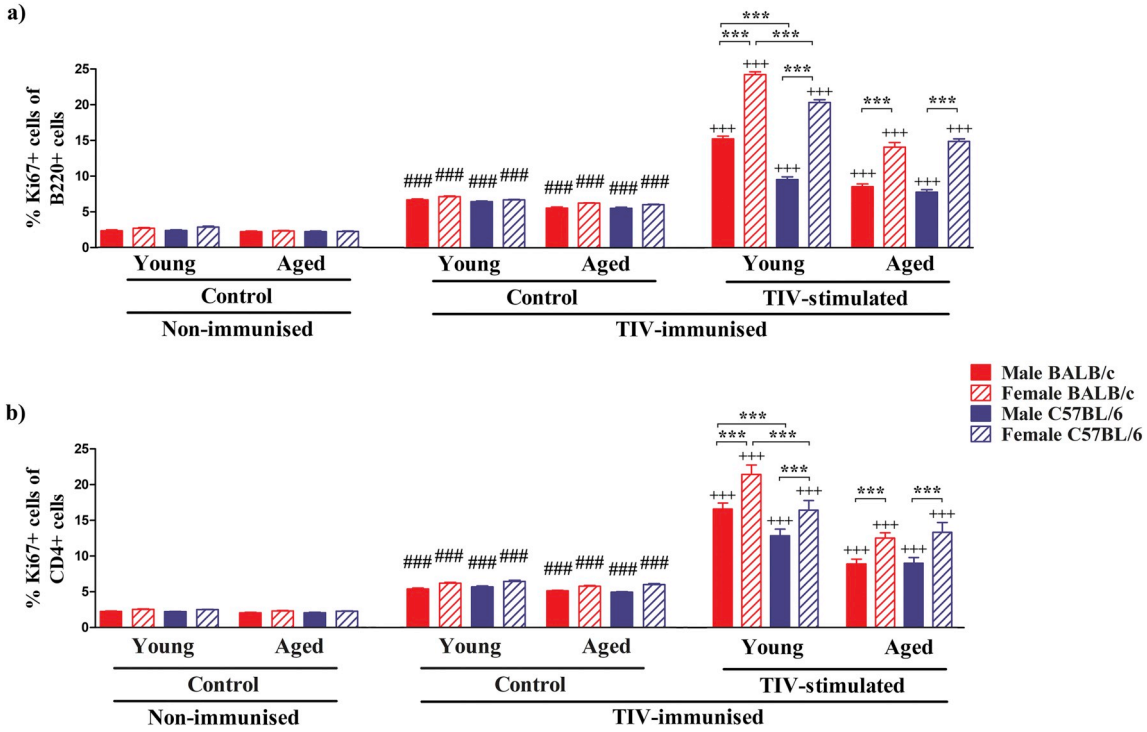


Fig. 3. Ageing diminishes influenza virus antigen-specific B- and T-cell proliferative responses in splenocyte culture in a mouse strain-specific way. Young and aged BALB/c and C57BL/6 mice of both sexes were immunised with trivalent influenza virus split inactivated (TIV) bulk composed of A/California/7/2009 (H1N1)pdm09-like (H1N1) virus, A/Texas/361/2011 (H3N2) virus and B/Massachusetts/2/2012-like (B) virus. Two weeks post immunisation, splenocytes were isolated and cultivated for 72 h in RPMI medium alone (Control) or in the presence of TIV bulk (TIV-stimulated). Bar graphs represent the percentage of Ki-67+ cells among a) B220+ and b) CD4+ splenocytes in cultures from TIV- immunised and non-immunised young and aged BALB/c and C57BL/6 mice of both sexes stimulated with TIV or cultured in medium alone (Control). Gating strategy for flow cytometry analysis of Ki-67+ cells within B220+ and CD4+ cells is displayed in Suppl. Fig. 1. Data are presented as means ± SEM. N = 6 mice/group. Data are from one of two independent experiments. $^{***}p < 0.001$. + TIV-stimulated cultures vs. Control cultures from TIV-immunised mice by Student *t*-test; $^{+++}$ p < 0.001. # TIV- immunised Control vs. Non-immunised Control by Student *t*-test; $^{###}$ p < 0.001.

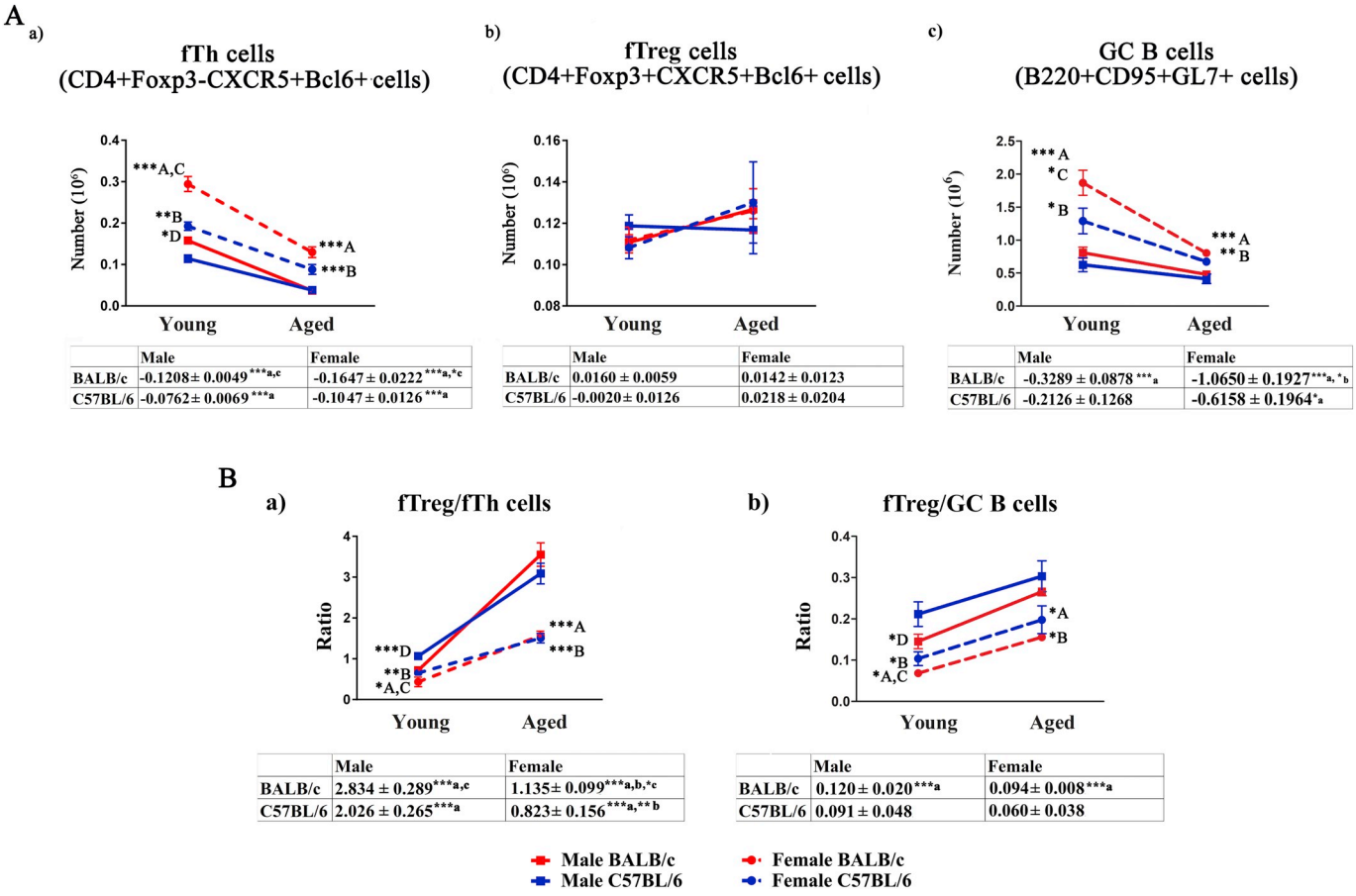
noteworthy that the similar pattern of mouse strain-, sex- specific and age-dependent differences in CD4+ and B220+ lymphocyte proliferation was observed upon restimulation with any of three monovalent inactivated split-virus influenza bulks (Suppl. Fig. 2).

3.4. Ageing increases the fTreg predominance over fTh and GC B cells in a mouse strain-specific way

Given that the magnitude of antibody response to T-dependent antigens depends on GC reaction (Sage et al., 2014) involving fTh, fTreg and GC B cells, their numbers in spleen have been examined using FCA (Suppl. Fig. 4). More fTh (CD4 + Foxp3-CXCR5 + Bcl6+) cells and GC B (B220 + CD95 + GL7+) cells were recovered from spleens of young BALB/c mice compared with sex-matched young C57BL/6 mice (Fig. 4). Additionally, more fTh and GC B cells were recovered from young female mice of both strains compared with age-matched male mice (Fig. 4). The number of fTh and GC B splenocytes decreased with ageing in female and male mice of both strains due to age-related decline in the total splenocyte yield (Fig. 4). However, this decrease was more prominent in BALB/c mice than in sex-matched C57BL/6 mice (Fig. 4). Consequently, in aged mice of both sexes, strain differences in the number of fTh and GC B cells observed in young mice disappeared (Fig. 4). However, in aged mice their numbers remained greater in females compared with strain-matched males (Fig. 4).

On the other hand, the number of fTreg (CD4 + Foxp3 + Bcl6 + CXCR5+) cells was comparable across all examined groups of mice (Fig. 4).

Given that efficacy of humoral immune response depends rather on delicate balance between fTh and fTreg cells than on absolute number of any of them alone (Sage et al., 2014), fTreg/fTh cell ratio was calculated. In young mice, this ratio was higher in C57BL/6 mice than in sex-matched BALB/c mice, reflecting fewer fTh cells in C57BL/6 mice (Fig. 4). However, in both strains of young mice this ratio was shifted towards fTh cells in females compared with males (Fig. 4). In mice of both strains, irrespective of sex, this ratio increased with ageing (Fig. 4). This increase was more prominent in BALB/c mice of both sexes when compared with sex-matched C57BL/6 mice, leading to the loss of strain differences in this ratio in aged mice (Fig. 4). However, in aged mice of both strains fTreg/fTh cell ratio remained higher in male mice reflecting greater number of fTh cells in females compared with males (Fig. 4). Given that fTreg cells may regulate GC reaction acting directly on GC B cells (Miles and Connick, 2018), fTreg/GC B cell ratio was also calculated. In young mice of both sexes it was shifted towards GC B cells in BALB/c mice compared with C57BL/6 mice (Fig. 4).



A	Male	Female
BALB/c	-0.1208± 0.0049 ***a,c	-0.1647 ± 0.0222 ***a,*c
C57BL/6	-0.0762± 0.0069 ***a	-0.1047 ± 0.0126 ***a

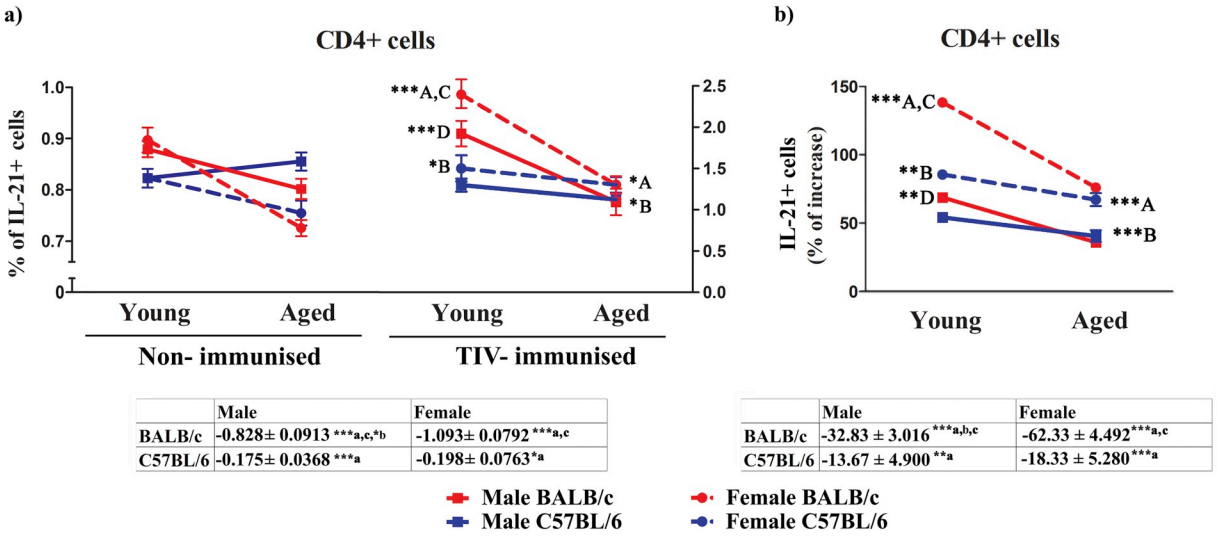
b	Male	Female
BALB/c	0.0160 ± 0.0059	0.0142 ± 0.0123
C57BL/6	-0.0020± 0.0126	0.0218 ± 0.0204

c	Male	Female
BALB/c	-0.3289 ± 0.0878 ***a	-1.0650 ± 0.1927 ***a, *b
C57BL/6	-0.2126 ± 0.1268	-0.6158 ± 0.1964 *a

B a)	Male	Female
BALB/c	2.834 ± 0.289 ***a,c	1.135± 0.099 ***a,b,*c
C57BL/6	2.026 ± 0.265 ***a	0.823± 0.156 ***a,** b

B b)	Male	Female
BALB/c	0.120± 0.020 ***a	0.094± 0.008 ***a
C57BL/6	0.091 ± 0.048	0.060± 0.038

Fig. 4. Ageing influences absolute number of fTh and GC B cells and fTreg/fTh and fTreg/GC B cell ratio in a mouse strain-specific manner. Young and aged BALB/c and C57BL/6 mice of both sexes were immunised with trivalent inactivated virus influenza bulk contained A/California/7/2009 (H1N1)pdm09-like (H1N1) virus, A/Texas/361/2011 (H3N2) virus and B/Massachusetts/2/2012-like (B) virus. Two weeks post immunisation spleens were analysed by flow cytometry for fTh, fTreg and GC B cells. A The linear diagrams represent the number of a) fTh cells (CD4 + CXCR5 + Bcl6 + Foxp3- cells), b) fTreg cells (CD4 + CXCR5 + Bcl6 + Foxp3+ cells) and c) GC B cells (B220 + CD95 + GL7+ cells). The frequency of fTh, fTreg and GC B cells and gating strategy for flow cytometry analysis of these cell subpopulations are displayed in Suppl. Fig. 4. B The linear diagrams represent the ratio of a) fTreg/fTh cells and b) fTreg/GC B cells. Data are presented as means ± SEM. N = 6 mice/group. Data are from one of three independent experiments, except for GC B cell number and fTreg/GC B cell ratio where data from one of two experiments were presented. The line slopes depicting age-related changes in the examined parameters in BALB/c and C57BL/6 mice are shown in the table below each diagram. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. [A]Females vs age-matched males of BALB/c strain; [B]Females vs age-matched males of C57BL/6 strain; [C]BALB/c females vs age-matched C57BL/6 females; [D]BALB/c males vs age-matched C57BL/6 males; [a]significant slope of the regression lines; [b]significant difference in female vs strain-matched male regression line slopes; [c]significant difference in BALB/c mouse vs sex-matched C57BL/6 mouse regression line slopes.



	Male	Female
BALB/c	-0.828± 0.0913 ***a,c,*b	-1.093± 0.0792 ***a,c
C57BL/6	-0.175± 0.0368 ***a	-0.198± 0.0763*a

	Male	Female
BALB/c	-32.83 ± 3.016 ***a,b,c	-62.33 ± 4.492***a,c
C57BL/6	-13.67 ± 4.900 **a	-18.33 ± 5.280***a

Fig. 5. Ageing influences the frequency of IL-21-producing CD4 + cells in a mouse strain specific way. Young and aged BALB/c and C57BL/6 mice of both sexes were immunised with trivalent influenza virus split inactivated (TIV) bulk composed of A/California/7/2009 (H1N1)pdm09-like (H1N1) virus, A/Texas/361/2011 (H3N2) virus and B/Massachusetts/2/2012-like (B) virus. Splenocytes were isolated from mice two weeks post immunisation. a) The linear diagrams present the frequency of IL-21 + cells among CD4 + freshly isolated splenocytes from non-immunised and TIV-immunised mice. b) The linear diagram present the percentage of increase of IL-21 + cells among CD4 + splenocytes in TIV-stimulated cultures over the corresponding cultures of splenocytes in medium alone from TIV-immunised young and aged BALB/c and C57BL/6 mice of both sexes. Gating strategy for flow cytometric analysis of IL-21 + cells within CD4 + cells is displayed in Suppl. Fig. 5. Data are presented as means ± SEM. N = 6 mice/group. Data are from one experiment. The line slopes depicting age-related changes in the frequency of IL-21 + among CD4 + cells in BALB/c and C57BL/6 mice are shown in the table below each diagram. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. [A]Females vs age-matched males of BALB/c strain; [B]Females vs age-matched males of C57BL/6 strain; [C]BALB/c females vs age-matched C57BL/6 females; [D]BALB/c males vs age-matched C57BL/6 males; [a]significant slope of the regression lines; [b]significant difference in female vs strain-matched male regression line slopes; [c]significant difference in BALB/c mouse vs sex-matched C57BL/6 mouse regression line slopes.

Additionally, in young mice of both strains fTreg/GC B cell ratio was lower in females than in males (Fig. 4). In aged mice, strain differences disappeared (Fig. 4). However, in mouse of both strains this ratio remained lower in females compared with males (Fig. 4).

3.5. Ageing decreases the frequency of IL-21-producing CD4 + cells in a mouse strain-specific way

The frequency of IL-21-producing (IL-21 +) cells among splenocytes from TIV-immunised young BALB/c mice was higher when compared with their sex-matched C57BL/6 counterparts (Fig. 5). In young mice of both strains their frequency among CD4 + splenocytes was higher in females compared with males (Fig. 5). With ageing, the frequency of IL-21 + cells among CD4 + splenocytes decreased in both BALB/c and C57BL/6 mice, but to a greater extent in BALB/c mice (Fig. 5). Consequently, in aged mice, the frequency of IL-21 + cells among CD4 + cells was similar in BALB/c mice and sex-matched C57BL/6 mice. However, in aged mice of both strains it was greater in females than in males (Fig. 5). It is noteworthy that their frequency was higher in immunised mice compared with strain-, age- and sex-matched non-immunised mice (Fig. 5).

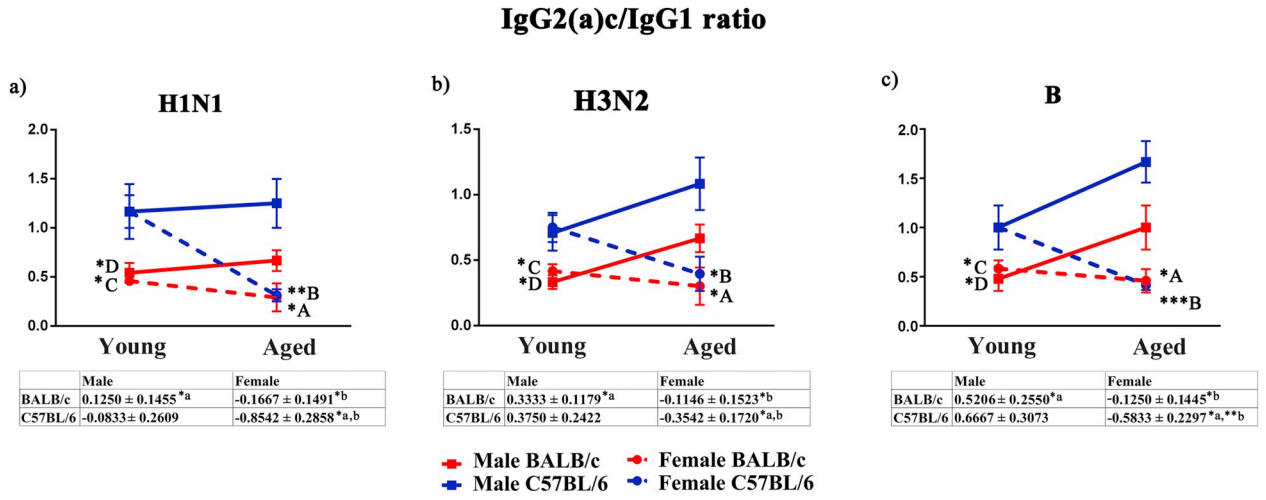
To assess antigen specificity of IL-21 + CD4 + cells, splenocytes from TIV-immunised mice were restimulated with TIV in vitro, and the percentage of increase in the frequency of IL-21 + cells among restimulated CD4 + cells over that among non-stimulated CD4 + cells (in control cultures with medium alone) was determined. The magnitude of the increase in frequency of IL-21 + cells was greater among CD4 + cells in TIV-restimulated splenocyte cultures from young BALB/c mice than in those from sex- and age-matched C57BL/6 mice (Fig. 5). In TIV-restimulated splenocyte cultures from mice of both strains and sexes this increase declined with ageing, so it was comparable in splenocyte cultures from aged BALB/c and age- and sex-matched C57BL/6 mice (Fig. 5). In splenocyte cultures from young and aged female mice of both strains the percentage of the increase was greater than in the corresponding cultures from male ones (Fig. 5).

3.6. Ageing affects IgG2a(c)/IgG1 ratio in TIV-immunised mice in a mouse strain- and sex-specific way

Given that IgG subclass responses differ between mouse strains (Hocart et al., 1989) affecting the overall IgG antibody protective capacity (DiLillo et al., 2014), serum IgG2a(c)/IgG1 ratios were calculated. As expected (Petrović et al., 2018), irrespective to antibody specificity, IgG2a/IgG1 antibody ratio in young BALB/c mice, as that in age-matched C57BL/6 mice, was comparable between sexes (Fig. 6). With ageing, in BALB/c mice, IgG2a/IgG1 antibody ratio did not change in females, whereas in male mice it shifted towards an IgG2a antibody response reflecting a less prominent decrease in IgG2a antibody titres (Fig. 6; Suppl. Fig. 6). Consequently, compared with aged male BALB/c mice, in aged female mice of the same strain this ratio was shifted towards an IgG1 antibody response (Fig. 6; Suppl. Fig. 6). On the other hand, in male C57BL/6 mice we failed to find significant age-related changes in IgG2c/IgG1 antibody ratio with ageing, whereas in female mice of this strain, IgG2c/IgG1 antibody ratio markedly decreased with ageing due to a less prominent decline in the IgG1 antibody titres (Fig. 6; Suppl. Fig. 6). Thus, compared with male aged C57BL/6 mice, in female age- and strain-matched mice, irrespective of antibody specificity to virus influenza antigens, this ratio was also shifted to the side of IgG1 antibody response (Fig. 6; Suppl. Fig. 6).

3.7. Ageing affects influenza virus-stimulated Th1/Th2 cytokine production level ratio in splenocyte cultures in a mouse strain- and sex-specific way

Given that the balance between Th1 and Th2 signature cytokines regulates IgG2a(c)/IgG1 production ratio and consequently serum titres (Marshall et al., 1999), the production levels of Th1 signature cytokine (IFN-γ) and Th2 signature cytokine (IL-4) were measured in supernatants from splenocyte cultures restimulated with monovalent inactivated split-virus influenza bulks. It is noteworthy that in splenocyte cultures from young mice IFN-γ/IL-4 production level ratio was lower in splenocyte cultures from BALB/c mice compared with those with sex-



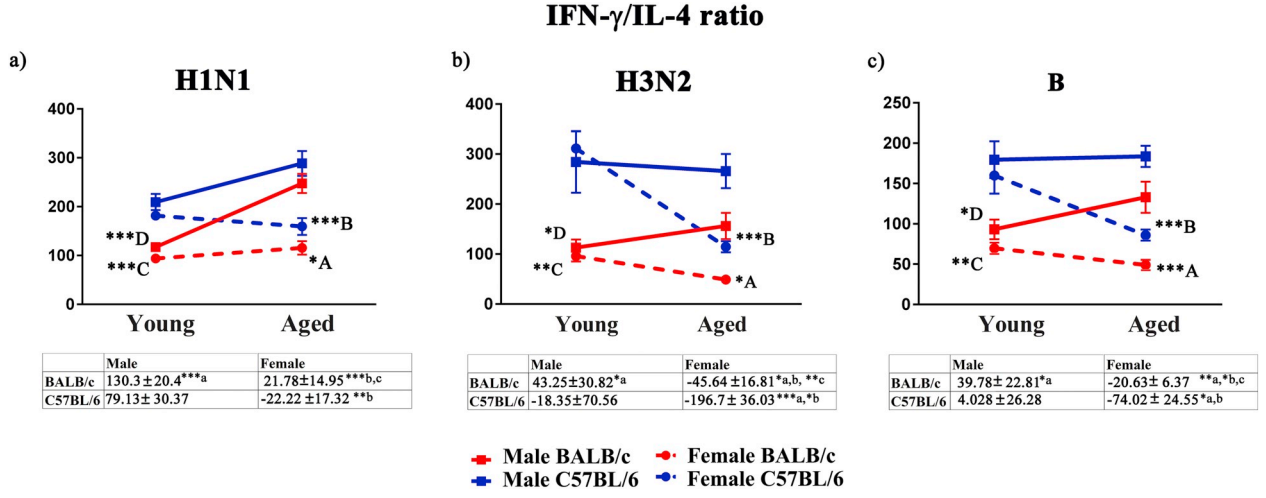
	Male	Female
BALB/c	0.1250 ± 0.1455 *a	-0.1667 ± 0.1491 *b
C57BL/6	-0.0833± 0.2609	-0.8542 ± 0.2858 *a,b

	Male	Female
BALB/c	0.3333 ± 0.1179 *a	-0.1146 ± 0.1523 *b
C57BL/6	0.3750 ± 0.2422	-0.3542 ± 0.1720 *a,b

	Male	Female
BALB/c	0.5206± 0.2550 *a	-0.1250 ± 0.1445 *b
C57BL/6	0.6667 ± 0.3073	-0.5833 ± 0.2297 *a,**b

Fig. 6. Ageing affects the ratio between IgG subclasses relevant for protection against viruses incorporated in TIV in strain- and sex-specific manner. Young and aged BALB/c and C57BL/6 mice of both sexes were immunised with trivalent inactivated virus influenza bulk composed of A/California/7/2009 (H1N1)pdm09-like (H1N1) virus, A/Texas/361/2011 (H3N2) virus and B/Massachusetts/2/2012-like (B) virus. Four weeks post immunisation blood samples were taken and serum titres of IgG1 and IgG2a(c) subclass antibodies specific for individual influenza virus strains in the bulk were determined by ELISA (Suppl. Fig. 6). The linear diagrams show a) H1N1, b) H3N2 and c) B influenza virus strain-specific serum IgG2a(c)/IgG1 subclass ratio in young and aged BALB/c and C57BL/6 mice of both sexes. Data are presented as means ± SEM. N = 6 mice/group. Data are from one of three independent experiments. The line slopes depicting age-related changes in the influenza virus strain-specific IgG2a(c)/IgG1 subclass ratio in BALB/c and C57BL/6 mice are shown in the table below each diagram. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. [A]Females vs age-matched males of BALB/c strain; [B]Females vs age-matched males of C57BL/6 strain; [C]BALB/c females vs age-matched C57BL/6 females; [D]BALB/c males vs age-matched C57BL/6 males; [a]significant slope of the regression lines; [b]significant difference in female vs strain-matched male regression line slopes.

matched C57BL/6 ones (Fig. 7), reflecting the greater production level of IL-4 in splenocyte cultures from BALB/c mice (Suppl. Fig. 7). However, there were no significant sex differences in IFN-γ/IL-4 production level ratio between splenocyte cultures from young females and age- and strain-matched males (Fig. 7). The age-related patterns of changes in IFN-γ/IL-4 production level ratio in splenocyte cultures from both BALB/c and C57BL/6 mice alike followed those in the serum IgG2a(c)/IgG1 ratios (Fig. 6). Consequently, in splenocyte cultures from aged mice, the strain differences in IFN-γ/IL-4 production level ratio observed in young mice disappeared, but sex differences in this ratio in splenocyte cultures from mice of both strains were registered (Fig. 7). Specifically, in splenocyte cultures from female aged mice of both strains IFN-γ/IL-4 production level ratio was shifted towards IL-4 compared with the corresponding cultures from strain-matched male aged mice (Fig. 7; Suppl. Fig. 7). This shift in splenocyte cultures from aged BALB/c mice was associated with the age-related increase in IFN-γ/IL-4 production level ratio in cultures from male mice, as this ratio did not significantly change in splenocyte cultures from strain-matched female mice (Fig. 7; Suppl. Fig. 7). On the contrary, IFN-γ/IL-4 production level ratio was decreased in splenocyte cultures from aged



	Male	Female
BALB/c	130.3±20.4***a	21.78±14.95***b,c
C57BL/6	79.13± 30.37	-22.22 ±17.32 **b

	Male	Female
BALB/c	43.25±30.82*a	-45.64 ±16.81*a,b, **c
C57BL/6	-18.35±70.56	-196.7± 36.03***a,*b

	Male	Female
BALB/c	39.78± 22.81*a	-20.63± 6.37 **a,*b,c
C57BL/6	4.028 ±26.28	-74.02± 24.55*a,b

Fig. 7. Ageing affects IFN-γ/IL-4 concentration ratio in splenocyte cultures in a mouse strain- and sex-specific manner. The linear diagrams show IFN-γ/IL-4 ratio in supernatants of cultures of splenocytes retrieved from young and aged BALB/c and C57BL/6 mice of both sexes two weeks post immunisation with trivalent inactivated virus influenza bulk and cultivated 72 h with a) H1N1, b) H3N2 or c) B influenza virus strain antigens, as determined by ELISA (Suppl. Fig. 7). Data are presented as means ± SEM. N = 6 mice/group. Data are from one of three independent experiments. The line slopes depicting age-related changes in the influenza virus strain-specific IFN-γ/IL-4 ratio in BALB/c and C57BL/6 mice are shown in the table below each diagram. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. [A]Females vs age-matched males of BALB/c strain; [B]Females vs age-matched males of C57BL/6 strain; [C]BALB/c females vs age-matched C57BL/6 females; [D]BALB/c males vs age-matched C57BL/6 males; [a]significant slope of the regression lines; [b]significant difference in female vs strain-matched male regression line slopes; [c]significant difference in BALB/c mouse vs sex-matched C57BL/6 mouse regression line slopes.

female C57BL/6 mice compared with strain-matched aged males, whereas it was comparable between splenocyte cultures from young and aged C57BL/6 males (Fig. 7).

4. Discussion

The current study extended our previous findings indicating Th cell-dependent age-related changes in the serum IgG antibody responses to immunisation with TIV in BALB/c mice (Arsenović-Ranin et al., 2019) by side-by-side comparison of these responses in C57BL/6 mice, who are shown to mount weaker and qualitatively different IgG responses in young adult age (Hirabayashi et al., 1991; Petrović et al., 2018), to mimic immunologically based individual variations in response to influenza vaccination observed in adult humans (Castrucci, 2018).

4.1. Ageing diminishes the magnitude of mouse IgG responses to TIV leading to loss of strain-specific difference, but not sex-specific differences

In accordance with previous studies in humans (Del Giudice et al., 2015; Sasaki et al., 2011), in mice of both sexes and strains, ageing led to significant declines in the total serum IgG antibody responses to TIV. Differently from young BALB/c mice who mounted the higher serum titres of IgG against the influenza virus antigens than their C57BL/6 counterparts, aged BALB/c mice did not differ from age-and sex-matched C57BL/6 mice in the ability to mount IgG antibody responses to TIV. The loss of strain differences in the serum titres of IgG with ageing was associated with more prominent decline in their titres in BALB/c mice compared with sex-matched C57BL/6 mice. Thus, it may be assumed that the kinetics of age-associated decline in the magnitude of IgG responses to TIV depends on mouse recipient genetic make-up. In favour of influence of genetics on age-related changes in mouse IgG response are data showing that degree of age-related down-regulation in the serum IgG response to the adjuvant-combined A/PR/8/34 (PR8; H1N1) virus vaccine in mice varies from strain to strain (Asanuma et al., 2001). In the same line are findings obtained by examining age-dependent changes in the magnitude of antibody response to some bacterial antigens in BALB/c and C57BL/6 mice (Nicoletti and Cerny, 1991).

In accordance with data obtained in humans (Engler et al., 2008; Falsey et al., 2009; Furman et al., 2014), ageing did not influence sexual dimorphism in IgG response to TIV in either BALB/c or C57BL/6 mice, so in aged mice, as in their young counterparts, the magnitude of serum IgG responses to each of three influenza virus strains from TIV was greater in females than in males. However, a recent study was shown that differently from young adult female C57BL/6 J mice exhibiting greater IgG response to mouse-adapted (ma)2009 H1N1 vaccine compared with age-matched males, female and male aged C57BL/6J mice mounted IgG response of a similar magnitude (Potluri et al., 2019). This discrepancy between the studies could be ascribed not only to genetic (substrain) disparities between mice used in these studies, but also to the local environmental cage differences, as they are shown to drive the composition of the faecal bacteria and to be more deterministically important than the host genotype and age for microbiota, and consequently vaccine-induced humoral immunity (Fischinger et al., 2019; Lees et al., 2014). This was in keeping with data indicating the greater IgG response to pandemic influenza vaccine in young female compared with age-matched male mice was at least partly associated with the greater expression of Toll-like receptor 7 caused by epigenetic mechanisms in B cells from females (Fink et al., 2018), as environmental factors have been shown to modify epigenetic states (Bollati and Baccarelli, 2010).

As expected from previous studies (Lefebvre et al., 2016), the decrease in serum influenza virus-specific IgG titres in mice of both strains and sexes correlated with the decline in the number of GC B splenocytes. This decline in the number of GC B splenocytes correlated with the age-associated decrease in B-cell activation/proliferation in response to TIV in splenocyte cultures, as shown by Ki-67 staining. This age-related decrease in B-cell activation/proliferation could reflect not only the B cell intrinsic changes occurring with ageing (Frasca et al., 2003), but also, as previously suggested (Kirschmann and Murasko, 1992; Swain et al., 2005), the decline in the capacity of CD4+ T cells to proliferate in response to antigenic stimulation (as shown in TIV-restimulated splenocyte cultures) and thereby to provide optimal help to the B cells. Additionally, the age-related decrease in B cell proliferation may reflect downregulation of CD154 (CD40L), the key molecule providing direct CD4+ T cell-to-B cell communication, due to chronic energy stress in CD4+ T cells occurring with ageing (Eaton et al., 2004; Weyand and Goronzy, 2016; Yu et al., 2012). Noteworthy, this phenomenon was registered after either CD4+ T cell activation in vitro or immunisation with influenza vaccine in vivo (Weyand and Goronzy, 2016; Yu et al., 2012). Furthermore, considering the key role of IL-21 in maintaining GC B cell proliferation (Zotos et al., 2010), the age-related decline in their proliferation could be linked with the decrease in the frequency of IL-21-producing cells among CD4+ splenocytes with ageing. To the best of our knowledge, there is no data on influence of ageing on the differentiation of IL-21-producing cells. Given that fTreg cells suppress genes involved in fTh cell effector functions such as IL-21 production (Fazilleau and Aloulou, 2018), and that for their suppressive action is critical fTreg/fTh cell ratio (Sage et al., 2013), but not their absolute numbers, it may be assumed that the age-related decline in IL-21-producing cells reflected the shift in fTreg/fTh splenocyte ratio towards fTh splenocytes. The age-dependent shift in fTreg/fTh cell ratio has been shown by other researches (Sage et al., 2015). On the other hand, given that IL-21 might alter fTreg cell metabolism and thereby reduces suppressive activity (Fazilleau and Aloulou, 2018), fTreg cells from aged mice are expected to be also more suppressive. It is noteworthy that IL-21 could modulate the magnitude of influenza virus-specific antibody responses to the protein antigens not only through control of GC B cell proliferation, but also their differentiation in plasma cells (Zotos et al., 2010). To add to complexity, the age-related decline in mouse ability to mount IgG responses to TIV could reflect the greater direct suppressive action of fTreg cells on GC B cells, as suggested by the shift in fTreg/GC B cell balance towards fTreg cells (Gong et al., 2017; Miles and Connick, 2018).

Additionally, although it was shown that B splenocytes from immunised adult BALB/c mice are more prone to proliferate in response to antigens and polyclonal activators than those from adult C57BL/6 mice (Pellegrini et al., 2007), so far the influence of the genetics on age-related changes in B-cell proliferative capacity has not been studied. However, it was shown that ageing has differential effects on polyclonal and antigen-specific proliferation of T cells isolated from different strains of mice (Kirschmann and Murasko, 1992). This is relevant as age-dependent changes in B-splenocyte proliferation were shown to mostly reflect not intrinsic defects, but alterations in the compartment including T cells (Ansell et al., 1980). Additionally, the more prominent decreases in the magnitudes of TIV-antigen specific IgG responses in BALB/c mice compared with their C57BL/6 counterparts with ageing could be linked with the sharper increase in fTreg/GC B cell ratio in BALB/c mice (reflecting the sharper decrease in the number of GC B cells), as it suggests a more robust GC B suppression by fTreg cells (Gong et al., 2017; Miles and Connick, 2018).

4.2. Age-related changes in qualitative characteristics of influenza virus-specific IgG responses

4.2.1. Ageing increases avidity of influenza virus-specific IgG antibody in female mice of both strains

With ageing the avidity of serum IgG antibodies specific to all virus strains in TIV increased not only in female BALB/c mice (Arsenović-Ranin et al., 2019), but also in their sex-matched C57BL/6 counterparts. However, the avidity of serum antibody specific to ma2009 H1N1 influenza virus strain diminished with ageing in female C57BL/6J mice

immunised with ma2009 H1N1 influenza vaccine (Potluri et al., 2019). Similarly, in humans, ageing differently affected the avidity of antibodies produced in response to vaccination with H1N1pdm09 influenza vaccine (increased) (Khurana et al., 2012) and either the 2007 or the 2009 seasonal TIV vaccine (did not affected) (Sasaki et al., 2011). These inconsistences may reflect strain/substrain (individual) differences, as well as differences in antigens used for immunisation, vaccine formulations, protocols for immunisation and methods for avidity estimation (Cremers et al., 2014).

Given that antibody avidity correlates with long term immunity (Ahmed and Gray, 1996), the previous findings suggest qualitatively superior IgG response to TIV in aged females of both strains.

4.2.2. Ageing influences Th1/Th2 cytokine ratio and influenza virus-specific IgG subclass profile in a mouse strain- and sex-specific way

Ageing, irrespective of IgG antibody virus influenza strain specificity, affected IgG2a/IgG1 and IgG2c/IgG1 ratio in male BALB/c and female C57BL/6 mice, respectively. This led to the shift in IgG2a/IgG1 and IgG2c/IgG1 ratio towards an IgG1 response in aged females from both strains compared with strain-matched males. It is noteworthy that strain differences in the influence of ageing on splenocyte production of IgG1 and IgG2 antibodies were also observed in female BALB/c and CBA mice (Harris and Harris, 1979). Additionally, age-related changes in IgG subclass profile to pathogen antigens were reported in humans (Potluri et al., 2019; Tongren et al., 2006). As expected (Finkelman et al., 1990; Reinhardt et al., 2009), the age-related changes in IgG2c/IgG1 ratio in our study fully correlated with alterations in the production level ratio of IFN-γ (Th1 signature cytokine) and IL-4 (Th2 signature cytokine) in splenocyte cultures restimulated with the influenza virus antigens. Strain-specificities in age-related changes in Th1/Th2 cytokine network were also observed following *Leishmania major* infection in female BALB/c and C57BL/6 mice (Ehrchen et al., 2004). The mouse strain-specific age-related changes in Th1/Th2 cytokine network could be associated with strain-dependent changes in microbiota with ageing (Kemis et al., 2019; Magrone and Jirillo, 2013).

To support sex specificities in (sub)strain-dependent age-related changes in IgG subclass profile are recently reported findings indicating that with ageing IgG2c/IgG1 ratio in male C57BL/6J mice immunised with inactivated ma2009 H1N1 vaccine shifted towards an IgG1 response, while in strain-matched females it remained unaltered (Potluri et al., 2019). The discrepancy between these findings and those obtained in our study could be related to differences in microbiota of mice due to the local environmental cage differences (Fischinger et al., 2019; Lees et al., 2014; Magrone and Jirillo, 2013). Alternatively, but not mutually exclusive, this discrepancy may be ascribed to different antigen specificity (seasonal influenza virus vs pandemic influenza virus) of IgG antibodies, as IgG subclass distribution is shown to depend on the nature of the target antigen (Stavnezer, 1996). To the best of our knowledge, there is no data on the influence of strain on age-related sex-specific alterations in pathogen-specific IgG subclass profile. To underscore significance of these findings are data indicating that IgG1 and IgG2a(c) exhibit differential functional characteristics (DiLillo et al., 2014; Huber et al., 2006; Schmitz et al., 2012). Namely, IgG2a antibodies are more effective at clearing infections than antibodies of the IgG1 isotype, even when each was specific for the same epitope (Huber et al., 2006), so efficacy of IgG antibody response does not depend only on magnitude, but also on IgG subclass profile (Potluri et al., 2019). Thus, although aged females of both strains are shown to mount the IgG response of greater magnitude to TIV than age-matched males, this response seems to be qualitatively (considering subclass profile) more favourable in males.

In conclusion, the study showed that ageing, in mouse of both strains and sexes, leads to the decline in the magnitude of the total serum IgG response to TIV, so that in aged mice, as in young ones, this magnitude was greater in females. However, this age-related decrease was sharper in BALB/c than in C57BL/6 mice, so, differently from young mice, in aged mice the magnitude of IgG response was comparable between sex-matched mice of these two strains. Additionally, the study revealed that in both BALB/c and C57BL/6 mice, ageing affected qualitative characteristics of the serum IgG responses to TIV, in a sex-specific manner. Furthermore, it pointed out a number CD4+ cell-dependent mechanisms underlying age-related alterations in IgG responses to TIV to be targeted in the further research aimed to improve response to influenza vaccination in elderly. Moreover, the study suggests that in tailoring medical approaches to improve efficacy of protection against influenza virus in the elderly, a sex-based Th cell-dependent differences in quantitative and qualitative characteristics of IgG response should be considered.

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CRediT authorship contribution statement

Biljana Bufan: Conceptualization, Methodology. **Nevena Arsenović-Ranin:** Investigation, Writing - original draft. **Raisa Petrović:** Visualization, Investigation. **Irena Živković:** Investigation. **Vera Stoiljković:** Resources. **Gordana Leposavić:** Conceptualization, Writing - review & editing, Supervision.

Declaration of competing interest

None.

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