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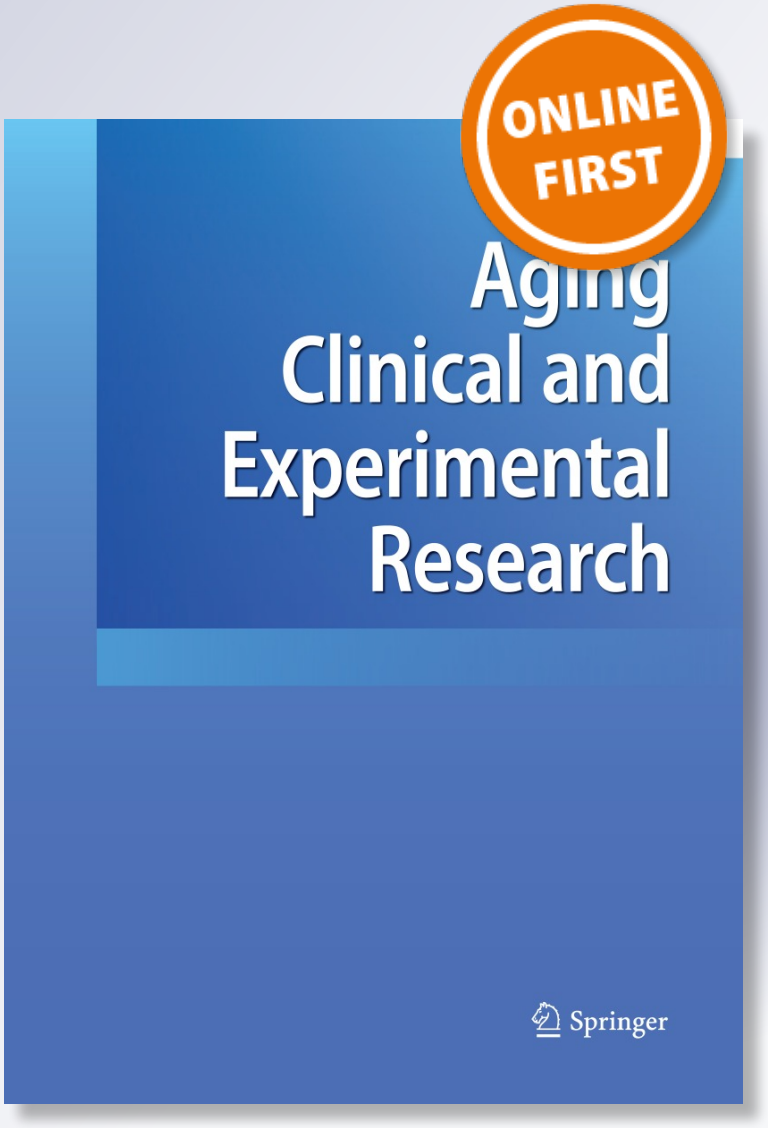

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ORIGINAL ARTICLE

Age-associated aberrations in mouse cellular and humoral immune responses

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Abstract
Background and aims Several contradictions and inconsistent reports regarding nature of dysfunction of immune system with age are known. The lack of multipoint age comparisons in immune functions contributes to the observed ambiguity in understanding immunosenescence. Thus, the present study aimed at a concurrent analysis of different immune cells in an attempt to delineate the nature of dysregulation with progressive aging in mice.
Methods 4, 8, 12 and 16 months old mice were analyzed for various immune parameters involving neutrophils, peripheral blood lymphocytes, peritoneal macrophages, splenocytes, inflamm-aging markers in plasma and humoral immune response in intestine.
Results Neutrophils registered a remarkable decrease in activities of respiratory burst enzymes and phagocytosis, while macrophages recorded a decrease in TLR-2 and TLR-4 expression. MCP-1 and CRP levels increased in plasma, whereas stimulation index and CD28 expression decreased in lymphocytes. Interleukins analysis (IFN-γ, IL-4, IL-10) showed a remarkable shift towards Th_2 response which further resulted in increased IgG1/IgG2a ratio and IgE levels in intestine.
Conclusion A decline in cell-mediated immune response, chronic inflammation and aggravation of humoral immunity was evident which conclusively suggests a skewed Th_2 pathway during aging.

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Keywords Aging · Immunosenescence · Neutrophils · Th_1/Th_2 · Inflamm-aging · Lymphocytes

Introduction

The increase in human life expectancy in the twentieth century has led to a global explosion of aging population in past 50 years. This increase, however, is accompanied by higher rate of infectious diseases and inflammatory disorders in elderly which is largely attributed to gradual waning of the immune system. Indeed, the term immunosenescence has been coined to reflect the complex, multi-faceted dysfunctions that occur in both innate and adaptive arms of the immune system during aging [1, 2]. This general compromised immunity is the foremost reason of increased morbidity and mortality in elderly and poses a major challenge in developing anti-immunosenescence strategies [3]. A vast amount of data aimed at understanding complex interactions of the aging immune system have accumulated in last decade resulting in our present understanding of immunosenescence. It has been established that age-associated immunosenescence not only impedes immune response but may also result in aggravation of at least some of its pathways as observed in inflamm-aging and skewness of cytokine environment in humoral immune response [4, 5].

However, many of the reports regarding immunosenescence, particularly with regard to specific cells and their functions are inconsistent or even contradictory in nature resulting in ambiguous interpretations [6]. While this is generally attributed to different experimental sources (human/mice), culture conditions and methodological approaches; most of these studies are also discrete and lack a multipoint approach for analyzing changes in different branches of the immune system. This lack of a widespread

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analysis of immune behavior during aging finally contributes to the observed inconsistency and vagueness in understanding immunosenescence. As a result, comparisons amongst different studies involving different types of immunological cells and functions often culminate in uncertainty and lack of a consensus. Thus, to gain a conclusive understanding of underlying changes in aging immune system, it is imperative to first profile holistic changes in immune system in a single experimental system. Keeping these observations in view, the present study aims at a simultaneous and multidisciplinary analysis of several functions of the immune system using mice of varying age groups in an attempt to try and articulate an age-associated profile of immunosenescence.

Methods

Animals and feeding procedure

All animal experiments were performed as per guidelines of the Institutional Animal Ethics Committee (IAEC). To eliminate any effect of feeding and infection history on aging study, newborn male swiss albino mice (2 weeks old) were procured from small animal house NDRI, Karnal. The animals were randomized into four groups of six each and were raised and maintained on basal diet (protein 12 % and fat 10 %) till they reached their respective age groups. For analysis of an age-associated profile of immunological functions, the animals were sacrificed by diethyl ether overdose at four specific age groups- 4 months (young), 8 months (adult), 12 months (old) and 16 months (senescent). Subsequently, blood, peritoneal fluid, spleen and intestine of the animals were collected to assess various immunological parameters.

Isolation of immune cells

Lymphocytes and neutrophils were isolated from whole blood by density gradient centrifugation using Histopaque (Sigma, St Louis, USA) solutions 1077 and 1119. Briefly, 3 ml of Histopaque 1077 was carefully layered on top of 3 ml of Histopaque 1119 and 1.5 ml blood was layered on the top of the gradient followed by centrifugation at $800\times g$ for 30 min at room temperature. The upper lymphocyte layer was removed and collected for qRT-PCR analysis while the second layer of neutrophils was carefully removed and subject to lysis of any remaining red blood cells by the method of Costa et al. [7]. The resulting cell suspension contained more than 90 % of neutrophils with overall viability >95 % as determined by Trypan blue exclusion method. Macrophages were collected from the peritoneal cavity of mice with 6 ml of Dulbeco's Modified Eagle Medium (DMEM) Ham's F12 (without phenol red) following gentle massage of the abdomen. The cell suspension was incubated in a humidified atmosphere at 5 % CO_2 in air at 37 °C for 2 h to allow attachment of adherent cells [8]. Non-adherent cells were removed after washing and the adherent macrophages were used to perform qRT-PCR analysis. The isolated lymphocytes and macrophages were immediately lysed in TRI reagent (Sigma, St Louis, USA) and stored at −80 °C till further analysis.

Neutrophil functions

Peripheral blood neutrophils were analyzed to evaluate age-associated functional discrepancies in terms of respiratory burst potential and phagocytic activity. Neutrophil respiratory burst potential was assessed in terms of cytochrome c reductase and Myeloperoxidase (MPO) activity. Neutrophil cytochrome c reductase activity was evaluated using cytochrome c reductase (NADPH) Assay Kit (Sigma, CY0100) as per manufacturer's protocol. Briefly, neutrophil cell suspensions (1×10^6 neutrophils/ml) were sonicated in enzyme dilution buffer (300 mM, pH 7.8) containing 0.05 % Triton X-100. The suspension was centrifuged at $12{,}000\times g$ for 10 min and the supernatant was analyzed for cytochrome c reductase activity. One unit of cytochrome c reductase activity was defined as reduction of 1 μmole of oxidized cytochrome c in the presence of 100 μmoles of NADPH per minute at pH 7.8 at 25 °C.

MPO activity was measured according to Bradley et al. [9] with some modifications. Briefly, neutrophil suspensions were homogenized in 9 volumes of ice-cold potassium phosphate buffer (50 mM, pH 6.0) containing 0.5 % cetyl trimethyl ammonium bromide (CTAB) followed by sonication (10 s) and freeze–thaw (3 times). The suspension was centrifuged at $35{,}000\times g$ for 15 min and the supernatant analyzed for MPO activity by mixing assay buffer (50 mM potassium phosphate buffer, pH 6.0) containing 0.5 mM o-dianisidine dihydrochloride and 0.0005 % H_2O_2 as substrates. The breakdown of H_2O_2 is directly proportional to oxidation of o-dianisidine dihydrochloride which was measured at 460 nm (UV–Visible double beam spectrophotometer, UVD-3500, Labomed Inc., USA). The concentration of oxidized o-dianisidine dihydrochloride was calculated from its molar extinction coefficient (1.13×10^4/cmM). One unit of MPO activity was defined as the degradation of 1 μmole of hydrogen peroxide per min at 25 °C.

Neutrophil suspensions (1×10^6 cells/ml) were further used for assessing the phagocytic activity using yeast cells according to the method of Hay and Westwood [10]. Phagocytosis was observed at 1,000× magnification under

oil immersion (Olympus Optical Co. Ltd, Japan) and following observations were recorded:

Percentage phagocytosis = number of neutrophils with yeast cells internalized per 100 neutrophils.

Phagocytic index = (% phagocytic neutrophils containing ≥1 yeast) × (mean number of yeasts/phagocytic neutrophils containing yeasts).

Splenocytes stimulation index (SSI)

Splenocytes were isolated and cultured from spleen tissue as described previously [11]. SSI in response to concanavalin A (ConA) and lipopolysaccharide (LPS) was assessed using colorimetric MTT assay [12]. Briefly, 10 µl fresh filtered MTT [3-(4,5-dimethlythiazol-2yl)-2,5-diphenyl tetrazolium bromide; 5 mg/ml dissolved in RPMI-1640] was added in cultured splenocytes and the plate was further incubated for 4 h at 37 °C in humidified CO_2 incubator. Spent media along with suspension of cultured cells was pipetted out, and 100 µl of acid isopropanol (0.1 N HCl in anhydrous isopropanol) was mixed to dissolve formazan crystals and absorbance was read at 540 nm.

Cytokine response

One fraction of the splenocytes was used to estimate interleukins (IFN-γ, IL-4 and IL-10) in supernatants using commercially available ELISA kits (eBiosciences, San Diego, CA, USA) according to manufacturer's protocol. Briefly, interleukin levels in culture supernatants were determined using quantitative sandwich ELISA. To detect these proteins, NUNC Maxisorp 96-well plates were coated with 100 µl of 1× capture antibody (goat anti-mouse IFN-γ/IL-4/IL-10) and incubated overnight at 4 °C. The samples were diluted two times before adding in the experimental wells followed by the addition of detection antibody and 100 µl of avidin horseradish peroxidase (HRP). Plates were allowed to develop with the TMB substrate (3,3,5,5-tetramethyl diamine benzidine containing 0.03 % H_2O_2) and reaction was finally stopped with 50 µl of 2 M H_2SO_4. Plates were read at 450 nm.

The basal level of circulatory inflammation was analyzed by estimation of monocyte chemotactic protein -1 (MCP-1) and C-reactive protein (CRP) in blood plasma. In brief, MCP-1 levels in plasma were determined using quantitative sandwich ELISA (eBiosciences, San Diego, CA, USA). NUNC Maxisorp 96-well plates were coated with 100 µl of 1× capture antibody (goat anti-mouse MCP) and incubated overnight at 4 °C. The samples were added into the experimental wells followed by the addition of detection antibody and 100 µl of avidin horseradish peroxidase (HRP). Plates were allowed to develop with the TMB substrate (3,3,5,5-tetramethyl diamine benzidine containing 0.03 % H_2O_2) and reaction was finally stopped with 50 µl of 2 M H_2SO_4. Plates were read at 450 nm. CRP levels were also estimated by quantitative sandwich ELISA in plasma using commercially available kit (Uscnk Life science, China) and manufacturer's protocol. Briefly, samples were added on a pre-coated 96-well strip followed by addition of detection reagent and TMB substrate solution. The reaction was stopped using 50 µl of stop solution and absorbance was read at 450 nm.

Table 1 Primer sequences of CD28, TLRs and GAPDH for real-time RT-PCR

Gene	Primer sequence (5′–3′)	Fragment size
CD28	5′CTGGCCCTCATCAGAACAAT3′	101
	5′GGCGACTGCTTTACCAAAATC3′	
TLR-2	5′AAGAGGAAGCCCAAGAAAGC3′	199
	5′CGATGGAATCGATGATGTTG3′	
TLR-4	5′ACCTGGCTGGTTTACACGTC3′	201
	5′CTGCCAGAGACATTGCAGAA3′	
GAPDH	5′TCAAGAAGGTGGTGAAGCAG3′	201
	5′AAAGATGGAAGCTAAGACCC3′	

qRT-PCR of TLRs and CD28

Total RNA content in isolated lymphocytes and macrophages was extracted by Tri Reagent (Sigma, St Louis, USA) according to the manufacturer's instructions. RNA concentration was quantified by NanoQuant (Tecan, Germany) and RNA integrity was checked on 1.4 % agarose. RNA concentration was adjusted to 1 µg/µl for the synthesis of cDNA. Two micrograms of total RNA was reverse transcribed by initiation at 65 °C for 5 min followed by incubation at 42 °C for 1 h in a 25 µl mixture consisting 200 U RevertAid M-MuLV reverse transcriptase, 20 U RiboLock RNase inhibitor, 0.5 µg oligo $(dT)_{18}$, 4 µl 5× reaction buffer, 0.8 mM dNTP mixture. The reaction was terminated at 70 °C for 5 min.

qPCR was performed to amplify target genes TLR-2 and TLR-4 in macrophages and CD28 in lymphocytes with GAPDH as reference gene using Light Cycler 480 Real-time PCR system (Roche) with Maxima SYBER Green/Fluorescein qPCR master mix (Thermo Scientific, Lithuania). The sequence of forward and reverse primers used is given in Table 1. One microliter of cDNA was used for PCR in a final volume of 20 µl, containing 10 µl 2× SYBER Green master mix, 0.2 µM of each forward and reverse primers and 8 µl nuclease free water. The PCR amplification was carried out using 1 cycle of initial denaturation at 94 °C for 5 min, 35 cycles of denaturation (94 °C for 30 s), annealing (53 °C for 30 s), extension

(72 °C for 45 s) and a final extension cycle at 72 °C for 4 min. After amplification, thresh hold (Ct) values of both control and experimental groups with reference genes were taken for calculating fold change in target gene expression.

Humoral immune response in intestine

The small intestine of animals was flushed with 5 ml of phosphate buffer saline (PBS) followed by gentle teasing with sterile needles in the same medium to separate the cells, and then centrifuged at 2,000×*g* for 20 min at 4 °C. The resultant supernatant, i.e., intestinal fluid was recovered and stored at −80 °C until used for estimation of IgG1, IgG2a and IgE antibodies. IgG1 and IgG2a in intestinal fluid were determined using quantitative sandwich ELISA kit (eBiosciences, San Diego, CA, USA). In brief, plates were coated with 100 µl of 1× capture antibodies (goat anti-mouse IgG1 and IgG2a) and incubated overnight at 4 °C. Samples were added in the experimental wells followed by the addition of 50 µl horseradish peroxidase (HRP) conjugated antibodies (detection antibodies). After 3 h of incubation at room temperature, plates were allowed to develop with the TMB substrate (3,3,5,5-tetramethyl diamine benzidine containing 0.03 % H_2O_2) and reaction was finally stopped with 50 µl of 2 M H_2SO_4. Plates were read at 450 nm. To detect IgE, plates were coated with 1 µg/ml of goat anti-mouse IgE as per manufacturer's protocol (Komabiotech, Korea). The samples were added in the experimental wells and incubated followed by the addition of 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE. Plates were allowed to develop with the TMB substrate (3,3,5,5-tetramethyl diamine benzidine containing 0.03 % H_2O_2) and reaction was finally stopped with 100 µl of 2 M H_2SO_4. Plates were read at 450 nm. Results are expressed as per mg of total protein.

Statistical analysis

Data were analyzed using GraphPad Prism (Version 5.01) software. Experimental results are presented as mean ± S.E.M (standard error mean). Data were subjected to analysis of variance (ANOVA) and the Tukey test was used to separate the means ($P < 0.05$), which were considered statistically significant.

Results

Neutrophil functions

The enzymes cytochrome c reductase and MPO in neutrophils together constitute a robust reactive oxygen species (ROS) generating machinery in neutrophils and thus are critical for proper functioning of these cells. A significant ($P < 0.01$) decrease in cytochrome c reductase activity was observed in old group animals which continued to decrease in senescent mice. The MPO activity also started declining in old age and a remarkable decrease ($P < 0.001$) was observed in senescent mice. Similar to neutrophil enzymes, the percentage phagocytosis and phagocytic index of neutrophils also recorded a considerable decrease ($P < 0.01$) in old and senescent mice as compared to young and adults (Fig. 1).

Splenocytes stimulation index

Stimulation of lymphocytes marks a key event in generating adaptive immune response. The stimulatory index of splenocytes was estimated using ConA and LPS as stimulants and the results are depicted in Fig. 2. Regardless of nature of the stimulant, SSI was invariably maximum and significantly higher in adult mice as compared to other aging groups ($P < 0.05$). No significant variations amongst other aging groups could be observed.

Cytokine response

The age-related profile of interleukins in splenocytes on stimulation with ConA is represented in Fig. 3. IFN-γ is a major pro-inflammatory molecule driving the Th_1 pathway, while IL-4 is major regulator of Th_2 pathway. In the present study, IFN-γ production registered a remarkable decrease while IL-4 recorded a gradual significant increase in senescent mice as compared to young group ($P < 0.001$). IL-10 plays an important role in orchestring Th1/Th2 pathway. In our study, unlike IFN-γ and IL-4 production, IL-10 secretion did not result a parallel pattern with age. However, an increasing trend in IL-10 levels with age was observed and both old and senescent mice recorded significantly higher levels of IL-10 as compared to young animals ($P < 0.01$). The levels of circulatory inflammatory cytokines in plasma were estimated to determine inflamm-aging (Fig. 4). The concentration of MCP-1 showed a remarkable increase ($P < 0.001$) in old animals which continued to persist in senescent mice. Another inflammatory marker CRP was assessed in plasma but unlike MCP-1 it did not show any dramatic variations with age. Nonetheless, a subtle but non-significant increase in CRP levels was observed in senescent mice as compared to young.

qRT-PCR of TLRs and CD28

Activation of TLRs and CD28 constitute major event in initiation of innate and adaptive immune response. In the

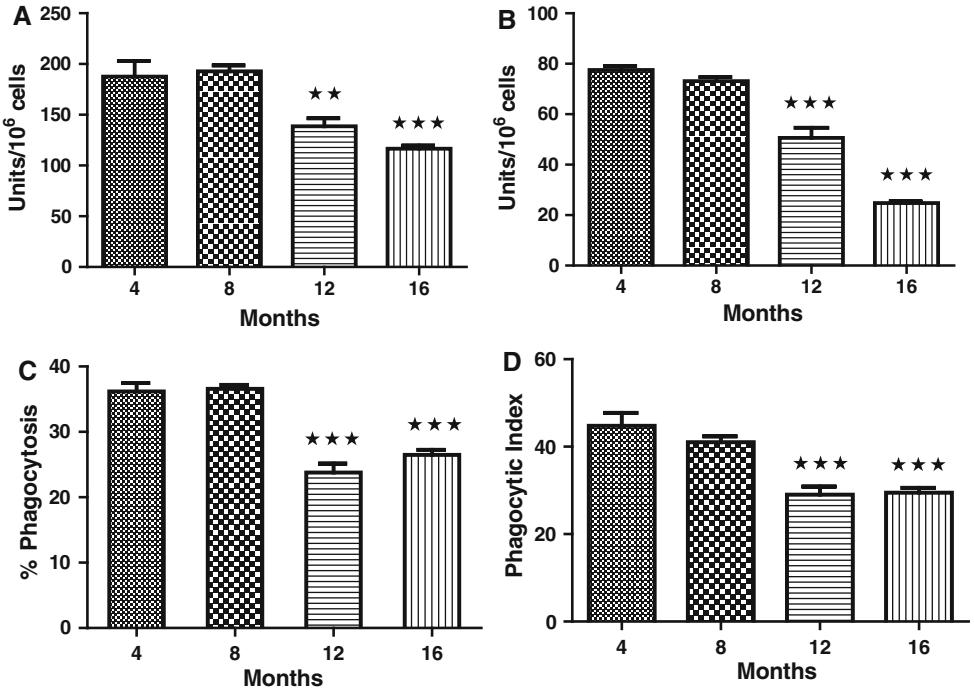

Fig. 1 Neutrophils isolated from peripheral blood of the age shown and assessed for **a** cytochrome c reductase activity **b** MPO activity **c** percent phagocytosis and **d** phagocytic index. Values are mean ± SEM; $***P < 0.001$, $**P < 0.01$ represents significant difference as compared to 4 months old group

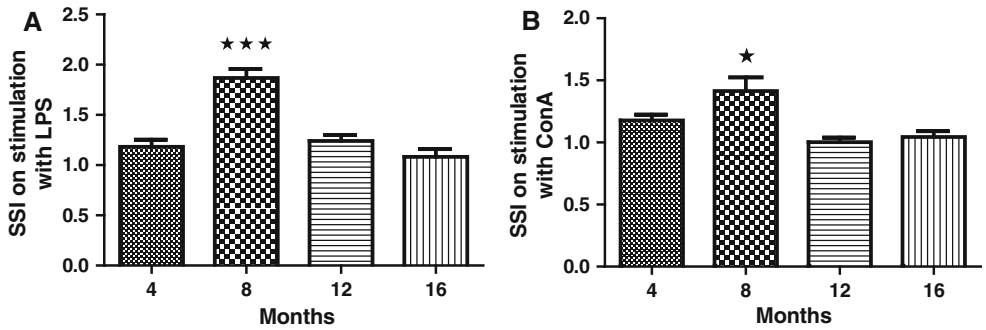

Fig. 2 Effect of age on splenocytes stimulation index in response to **a** LPS and **b** ConA. Values are mean ± SEM; $***P < 0.001$, $*P < 0.05$ represents significant difference as compared to 4 months old group

present study, a remarkable decrease in TLR-2 ($P < 0.001$) and TLR-4 ($P < 0.01$) expression was observed in old and senescent mice as compared young group. However, CD28 expression did not follow a regular pattern. Instead a significant and highest increase ($P < 0.05$) was observed in adults as compared to remaining groups (Fig. 5).

Humoral immune response in intestine

The aging profile of humoral immune response in intestinal fluid is depicted in Fig. 6. The levels of IgG1 registered an increasing trend with aging and a significant increase ($P < 0.05$) was observed in senescent mice. IgG2a concentration in intestinal fluid was highest in adult mice and recorded a significant decrease in senescent mice ($P < 0.05$). The ratio of IgG1/IgG2a indicated an increasing trend and recorded a remarkable 101.96 % increase in senescent mice. IgE levels in intestinal fluid also recorded significant increase ($P < 0.05$) in senescent mice as compared to young group. No statistically significant variations could be observed amongst old and senescent mice.

Discussion

Analysis of various immune parameters in the present study indicated profound differences in innate and adaptive immune functions with aging. The first line of immune defense, i.e., innate immunity depends heavily on neutrophils and macrophages for protection against invading pathogens. On stimulation, neutrophils can aggressively

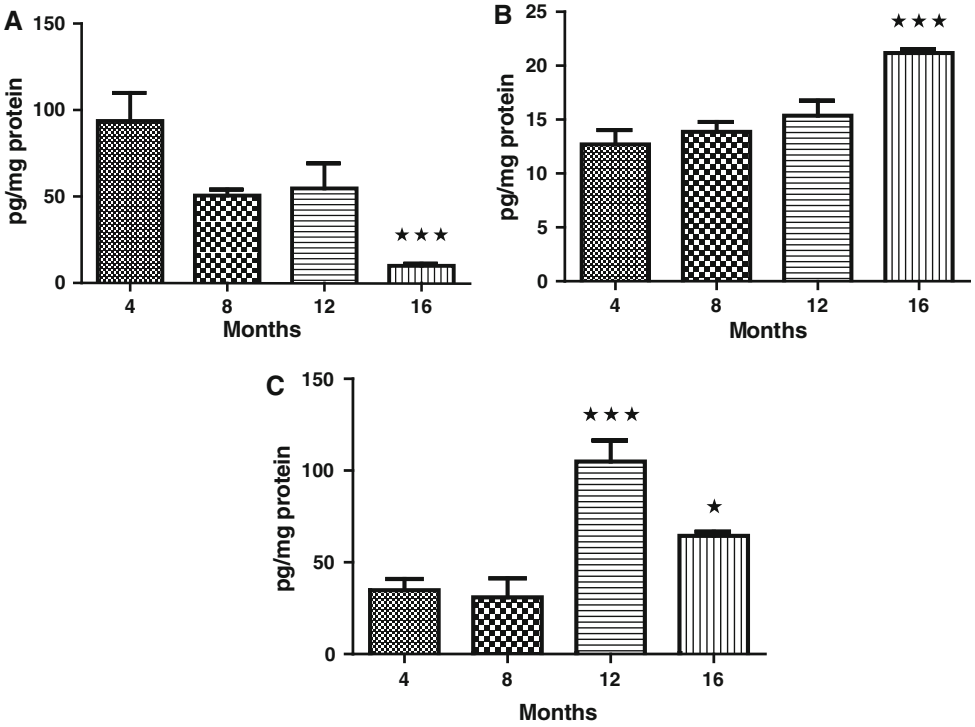

Fig. 3 Age-related changes in interleukins production in splenic lymphocytes **a** IFN-γ **b** IL-4 **c** IL-10. Values are mean ± SEM; $***P < 0.001$, $*P < 0.05$ represents significant difference as compared to 4 months old group

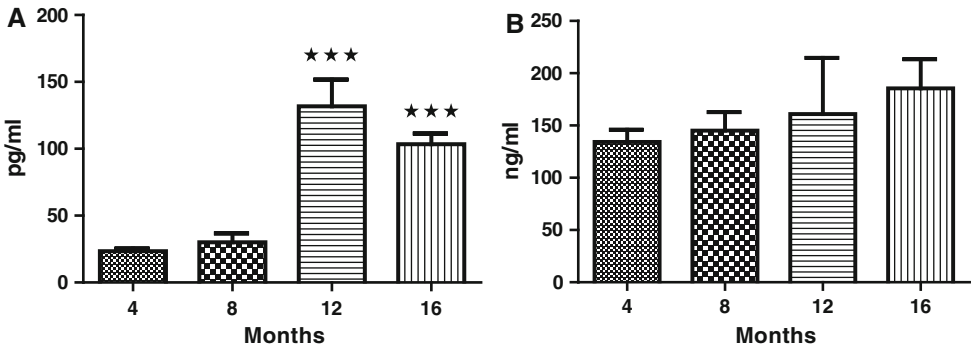

Fig. 4 Age-related changes in inflammatory markers in plasma **a** MCP-1 **b** CRP. Values are mean ± SEM; $***P < 0.001$ represents significant difference as compared to 4 months old group

increase uptake of molecular oxygen (respiratory burst) to produce superoxides and other ROS using enzymes cytochrome c reductase and MPO. The effect of aging on intracellular and extracellular superoxide production in neutrophils is controversial. While some studies claim a decreased ROS production in old age, others have also reported increased superoxide production [13–18]. In the present study, we directly assessed activities of enzymes (cytochrome c reductase and MPO) involved in neutrophil ROS production and found that aging strongly inhibited their activities which suggests impaired ROS production in neutrophils. Further, a corresponding gradual decrease in neutrophil phagocytic activity was also observed which was at par with decreasing enzyme activities. This is the first report describing age dependent changes in activities of neutrophil respiratory burst enzymes and their simultaneous effect on phagocytosis. In a recent study, a decrease in MPO activity has been directly implicated for impaired phagocytic response of neutrophils [19]. Thus, it may be concluded that a decline in activities of enzymes cytochrome c reductase and MPO in neutrophils during aging could be responsible for diminished functional capacity of these cells in terms of phagocytic potential. Macrophages are also potent phagocytes and antigen presenting cells of the innate immune system which are on constant guard against pathogens. The evolutionarily conserved set of molecules called TLRs play a critical role in recognition of specific molecular patterns on surface of microbes and subsequent downstream activation of various signaling pathways. Aging has been linked with decreased TLR expression and function in previous studies [20–23]. Our study results also corroborated the same notion and found a profound decrease in expression of TLR-2 and TLR-4 in peritoneal macrophages. A lack of TLR expression can

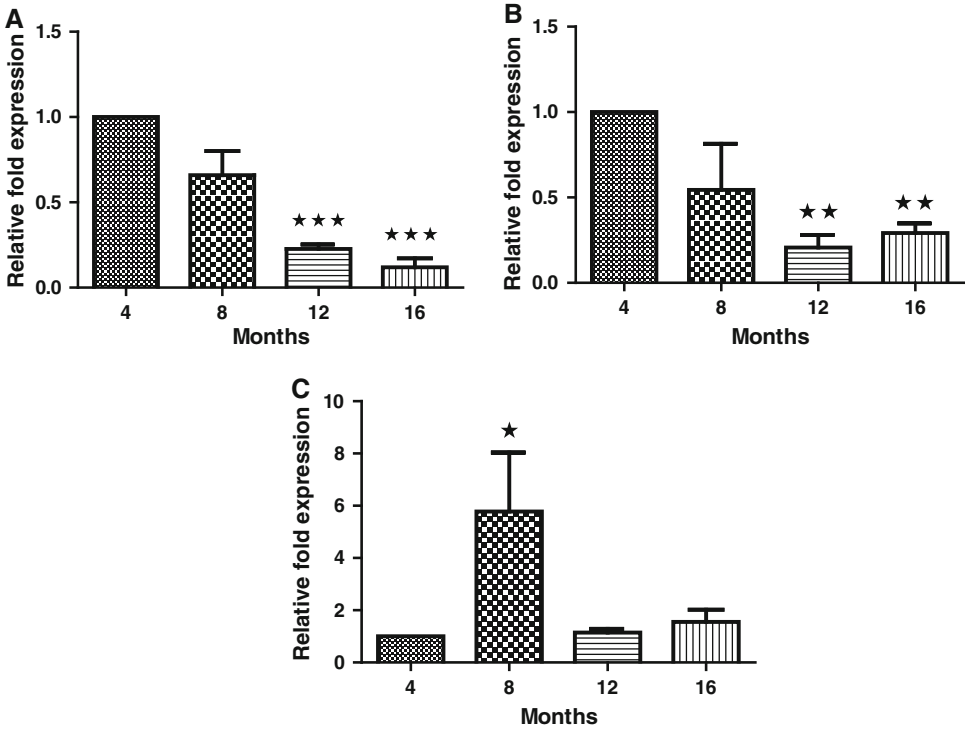

Fig. 5 Peritoneal macrophages showing age-related changes in expression of **a** TLR-2 and **b** TLR-4. Peripheral blood lymphocytes assessed for age-related changes in expression of **c** CD28. Values are mean ± SEM; $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ represents significant difference as compared to 4 months old group

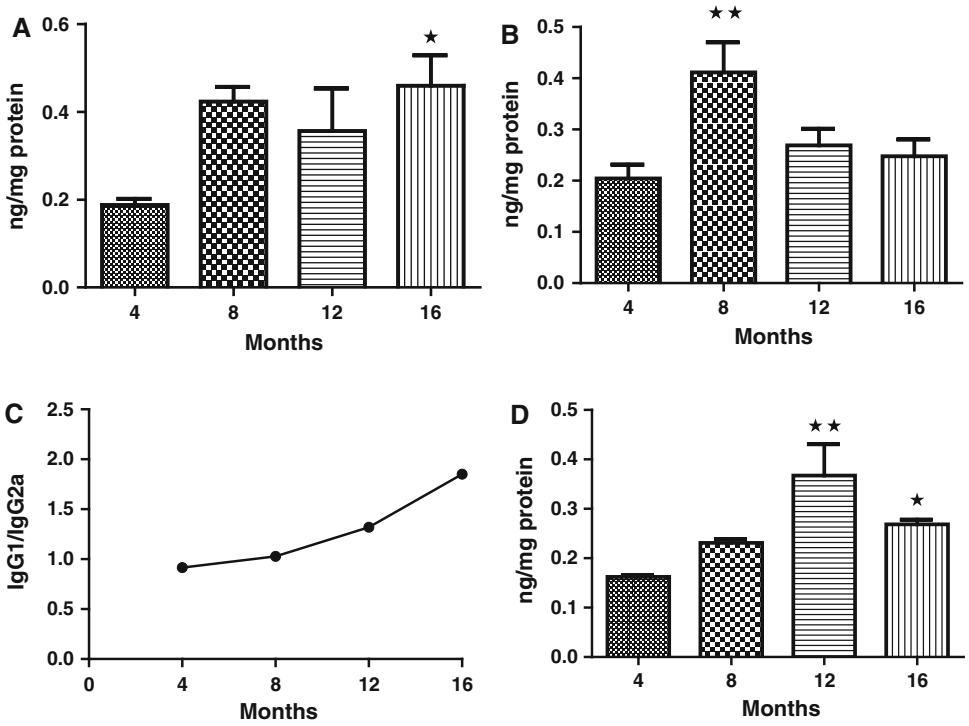

Fig. 6 Age-related changes in humoral response in intestinal fluid **a** IgG1 **b** IgG2a **c** IgG1/IgG2a and **d** IgE. Values are mean ± SEM; $**P < 0.01$, $*P < 0.05$ represents significant difference as compared to 4 months old group

inhibit macrophage activation thereby deterring a quick and effective immune response.

The phenomenon of increased systemic inflammation amidst decreased innate immune response is termed as inflamm-aging and is widely considered as an immunological paradox. While this idea is suggested in humans, no such data exists in murine models. MCP-1, a potent chemotactic and pro-inflammatory protein showed a remarkable increase in old and senescent animals as compared to nominal levels in young and adults. The increased levels of MCP-1 have been related with increased memory T-cells population [24] and present findings suggest a possible

redirection in trafficking of monocytes in systemic immunity of elderly. The other inflammatory marker CRP is an acute phase protein whose concentrations rise remarkably in case of an infection and has also been reported to increase in human aging studies [18, 25]. In current study, although no statistically significant variations in CRP levels could be observed; a subtle and gradual increase in CRP concentration with age was apparent. Given the fact that inflamm-aging is representative of a low grade and chronic inflammatory state; the apparent low level increase in CRP levels cannot be neglected. CRP has been reported to induce interleukin-8 (IL-8) secretion through peroxynitrite signaling in human neutrophils [26]. IL-8 itself is a potent chemokine and an autocrine inflammatory mediator which helps trafficking of neutrophils during host defense systems. The subtle increase in CRP levels with age in the present study could be sufficient enough to influence the systemic levels of IL-8 thereby contributing to inflammation. While this effect can currently only be hypothesized, nevertheless increased MCP-1 and delicate CRP levels in aging mice indicate chronic inflammatory state which is a hallmark of inflamm-aging.

Stimulation, differentiation and proliferation of lymphocytes are the key events in generation of an adaptive immune response. In the present study, SSI was assessed in splenocytes in response to ConA and LPS in the aging groups. The adult animals showed a significantly higher response to stimulants as compared to rest of the groups. It thus appears that adult mice were most active in terms of stimulation of lymphocytes which decreased with progressive aging. Many previous studies have corroborated the same notion and lack of stimulation of lymphocytes is considered as a hallmark of aging immune system [27–31]. Co-stimulatory molecule CD28 plays a critical role in stimulation of T-cells in response to pathogens. Previous studies in humans have suggested a decrease in CD28 expression with aging while no similar data are present on murine models [32, 33]. In the present study, the expression of CD28 on lymphocytes was maximum in adult animals and a significant difference was observed with young and aging groups. It has been postulated that repeated antigen stimulation throughout aging could be responsible for diminished CD28 expression with age while the decrease in young animals could be a result of insufficient antigenic exposure [34]. Since CD28 plays a key role in stimulation of lymphocytes; the enhanced CD28 expression in adult mice could also explain the observed increase in SSI in adult animals as compared to other aging groups.

Depending on the type of antigen, naïve T cells (T_0) can differentiate to generate cellular (Th_1 pathway) or humoral (Th_2 pathway) immune response. Any skewness in this differentiation process can render the elderly more prone to either autoimmune conditions (Th_1) or allergic disorders (Th_2). While it is generally agreed that differentiation of T_0 cells is skewed in aging; the nature of this skewness has been controversial and warrants further validation [35–38]. By measuring interleukins responsible for specific pathways, we found that aging in mice is accompanied by a shift from Th_1 to Th_2 pathway. The level of IFN-γ, a classic Th_1 cytokine decreased during aging while levels of IL-4, a Th_2 cytokine progressively increased in senescent mice. IL-4 acts as agonist to IFN-γ and might be responsible for its decreased levels and finally the observed skewness towards Th_2 pathway. The age-related changes in regulatory cytokine IL-10 showed a significant increase in 12–16 months groups as compared to young animals. IL-10 is a functionally pleiotropic cytokine with regulatory effects on Th_1/Th_2 pathway. It is known to suppress chronic secretion of Th_1 pro-inflammatory cytokines and enhance polarization of Th_2 response [39–42]. Hence, the suppression of Th_1 pathway and aggravation of Th_2 response could be mediated by an enhanced IL-10 production. A previous report by Dayan et al. [43] on profiling of interleukins in mice of varying age groups also supported our results on Th_1/Th_2 imbalance and role of IL-10.

The levels of secretory immunoglobulins in intestinal fluid were determined to assess non-specific humoral immune response. All immunoglobulins registered a general tendency of increased production with age. While IgG1 and IgG2a both increased significantly in aging groups; the IgG1/IgG2a ratio registered a remarkable twofold increase in senescent mice indicating robust changes in IgG1 as compared to IgG2a. Cytokines of Th_2 pathway, IL-4 in particular is considered main driving force of favoring IgG1 production while Th_1 cytokine IFN-γ is responsible for IgG2a synthesis in mice. The evident decreased IFN-γ production and concomitant increased IL-4 and IL-10 production in the present study might have influenced the final IgG1/IgG2a ratio in 12–16 months old animals. Similarly, increased IgE levels in the present study could also be attributed to skewed Th_2 response as increased IL-4 levels favor IgE class switching. Previous studies have reported contradictory results regarding ratios of different immunoglobulin subtypes in aging. Many groups claim increased levels, while some ascertain decreased non-specific humoral immune response with advancing age [44–47]. The present study, however, clearly demonstrates that increased IL-4 production due to enhanced Th_2 response during aging could be responsible for increased class switching of IgG1 and IgE subtypes. The augmentation of Th_2 mediated B cell response during aging could also increase the production of auto-reactive antibodies resulting in autoimmune diseases and increased IgE production can directly influence allergic disorders.

The contradictions and anomalies in behavior of different cells of immune system with progressive aging have a profound effect in rationalizing the aging immune system and development of subsequent mitigating strategies. In this study, we argued that one way of achieving a substantial scenario in understanding functional immunosenescence could be possible by simultaneous analysis of diverse immune cells and phenomenon in a single experimental system. While no single study can claim complete exhaustive analysis of immunosenescence, we selected some of the most basic and essential parameters of innate and adaptive immune system governing immune response in mammals. In conclusion, this aging profile of immune system suggests a strong decline in cellular innate immune response, prevalence of chronic inflamm-aging, a robust skewness towards Th_2 response and subsequent altered immunoglobulins class switching, thereby effectively indicating the deleterious effects of age on mouse immune system.

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Conflict of interest The authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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