Adaptive Immune Responses Mediated Age-related Plasmodium yoelii 17XL and 17XNL Infections in 4 and 8-week-old BALB/c Mice

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Research article

Keywords: age-related, Plasmodium yoelii 17XL, Plasmodium yoelii 17XNL, adaptive immune responses, 4-week-old BALB/c mice, 8-week-old BALB/c mice

Posted Date: May 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-29497/v1

Version of Record: A version of this preprint was published on January 11th, 2021. See the published version at https://doi.org/10.1186/s12865-020-00391-8.

Abstract

Backgroud: As the quest to eradicate malaria continues, it is important to clarify the opposite clinical outcomes between childhood and adulthood. The relationship between adaptive immune response and age-related malaria infection remains unknown.

Methods: 4 and 8-week-old mice were used to mimic childhood and adulthood, respectively. Parasitemia and the survival rate were monitored. The proportion and function of Th1 and Th2 cells were detected by FACS. The levels of IFN-γ, IL-4, total IgG, IgG1, IgG2a and Plasmodium yoelii MSP-1-special IgG were measured by ELISA.

Results: Our results found that infant mice were more susceptible to P. yoelii 17XNL infection, with lower survival rate and higher parasitemia. The adult group showed greater resistance to P. yoelii 17XL infection, with lower parasitemia. Compared with 4-week-old mice, the percentage of CD4 + T-bet + IFN-γ + Th1 cells as well as IFN-γ production were significantly increased on day 5 p.i. in the 8-week-old mice after P. yoelii 17XNL infection. The percentage of CD4 + GATA3 + IL-4 + Th2 cells and CD4 + CXCR5 + Tfh cells, and IL-4 production in the 8-week-old mice obviously increased on day 5 and day 10 after P. yoelii 17XNL infection. Notably, the levels of total IgG, IgG1, IgG2a and P. yoelii MSP-1-special IgG were also significantly increased in the 8-week-old mice. PD-1, a marker of exhaustion, was up-regulated on CD4 + or activated CD4 + T cells in the 8-week-old mice as compared to the 4-week-old group.

Conclusions: Thus, we consider that enhanced cellular and humoral adaptive immunity might contribute to rapid clearance of malaria among adults, likely in a PD-1-dependent manner due to induction of CD4 + T cells exhaustion.

Background

Malaria is a major cause of mortality and morbidity in tropical countries [1]. According to the recent WHO statistics, 91 countries reported ongoing malaria transmission, with Africa experiencing disproportionately high malaria cases (90% of the total) and accounting for 91% of total malaria deaths worldwide [2]. Notably, children under the age of 5 years are particularly vulnerable to *plasmodium* infection. More than two-thirds of malaria deaths (70%) occur in this age group [3]. Of the five *Plasmodium* species that infect humans, *Plasmodium falciparum* and *Plasmodium vivax* are the most common, and *P. falciparum* is the most virulent and responsible for the majority of deaths [2, 3]. In addition, the multiplicity of infection (MOI) varies depending on the overall prevalence of infection in the population, and the age of the individual [4, 5].The young children are highly susceptible to clinical illness and high parasitemia, whereas the adults are highly resistant [4], resulting in a major difference in the spectrum of disease manifestations between children and adults [6]. Therefore, understanding the immunological mechanisms involved in susceptibility to different virulent *Plasmodium* species during infection in childhood or adulthood could contribute to the development of an immunologically based control strategy to prevent or treat this devastating disease.

Upon infection, anti-parasite immunity plays a central role in removing the parasite from the blood. Innate immunity is first activated, e.g., complement system, innate lymphoid cells and dendritic cells (DCs) act to limit the acute phase of parasitemia, but are insufficient to clear the infection [7, 8, 9]. When DCs present the processed antigen, adaptive immunity is activated. Direct cell cytotoxicity, cytokine secretion as well as anti-malarial antibody work together for effective parasite clearance [10, 11, 12, 13].

Infants and young children are more susceptible to malaria infection than adults worldwide [4]. Age-related changes in immune systems increased prevalence of asthma, nasal polyps and lung injury [14, 15]. However, whether differences in cellular and humoral immunity lead to this age-related infection profile remains unknown. Therefore, we used different virulent *Plasmodium* (lethal *P.y17XL* and non-lethal *P.y17XNL*) strains to infect4-week-old and 8-week-old BALB/c mice to mimic infancy and adulthood, respectively, in order to characterize the relationship between immune cell responses and age-related malaria infection among different age groups, and understand the mechanism of malaria immunity. We propose that the dynamics of MOI can be explained by a model of increasing acquired immunity to blood-stage infection with age.

Results

Comparison of different species of Plasmodium infection course in 4-week-old and 8-week-old BALB/c mice

To investigate the relationship between age-related host immunity against malaria infection, we used BALB/c mice of different age groups to mimic infancy and adulthood, and monitored parasitemia and the survival rate at different time points after lethal *P.y17XL* and non-lethal *P.y17XNL* infections. Within 20 days after *P.y17XNL* infection, 96% of the 8-week-old mice successfully survived whereas only 78% of the 4-week-old mice survived (Fig. 1A). In accordance with the survival rate, the parasitemia peaked at 12% in the 4-week-old mice on day 11 p.i. while it was only 7% in the 8-week-old group, although the onset of parasitemia was similar in both groups on day 3 p.i. (Fig. 1B). Similarly, parasitemia peaked at 80% in the 4-week-old mice on day 8 p.i., and all mice died; however, in the 8-week-old group, parasitemia peaked at 75% on day 8 p.i., subsequently declined, and all mice died on day 11 p.i. (Fig. 1D). Although both groups were sensitive to lethal *P.y17XL* infection and failed to control parasite growth, the 8-week-old mice seemed to be more resistant to lethal infection with slower death rate (Fig. 1C) and lower parasitemia during the early stage of malaria infection (Fig. 1D). Therefore, infants were more susceptible to parasite infection, whereas the adult group seemed to be relatively resistant.

Comparison of Th1 immune response in different species of Plasmodium-infected 4-week-old and 8-week-old BALB/c mice

Next, the relationship between Th1 cell responses and age during the early stage of malaria infection was determined. The percentage of CD4+ T-bet+ IFN-γ+ Th1 cells was determined by flow cytometry, and the level of IFN-γ in splenocytes was measured by ELISA. Compared with 4-week-old mice, the frequency and absolute number of Th1 cells were significantly increased in *P.y17XNL*-infected 8-week-old mice on day 5

p.i. (Fig. 2A-C) ($p<0.05$). The level of IFN-γ in *P.y17XNL*-infected 8-week-old mice on day 5 p.i. had the same trend as Th1 cells (Fig. 2G). Interestingly, in *P.y17XL*-infected 8-week-old mice, the frequency and absolute number of Th1 cells peaked on day 3 p.i. (Fig. 2D-F) ($p<0.05$), then subsequently decreased, but remained higher than normal control on day 5 p.i. ($p<0.05$). Notably, the level of IFN-γ in lethal *P.y17XL*-infected mice was significantly increased on day 5 p.i. ($p<0.05$), but there was no obvious difference between the 4-week-old mice and 8-week-old mice (Fig 2H). This data suggested that enhanced Th1 cell responses might be associated with age-related non-lethal *P.y17XNL* infection and resistance during the early stage of lethal *P.y17XL* infection.

Comparison of Th2 immune response in different species of Plasmodium-infected 4-week-old and 8-week-old BALB/c mice

To assess the characteristics of Th2 cell responses and their relationship with age during the late stage of malaria infection, we evaluated the percentage and absolute number of $CD4^+GATA3^+IL\text{-}4^+$ Th2 cells and interleukin-4 (IL-4) production. The proportion and absolute number of Th2 cells were elevated in both groups on day 5 p.i. and day 10 p.i. after *P.y17XNL* infection as compared to normal control (Fig. 3A-C) ($p<0.05$). Following, we found that there was obvious difference in the percentage and absolute number of Th2 cells on day 5 and 10 p.i. between the 4-week-old and 8 week-old BALB/c mice (Fig3B) ($p<0.05$). Consistently, the level of IL-4 production in *P.y17XNL*-infected 8-week-old mice was significantly increased as compared to the 4-week-old mice on day 5 p.i. and day 10 p.i. (Fig. 3D) ($p<0.05$). In addition, we detected the percentage and absolute number of $CD4^+CXCR5^+$ Tfh cells, recognized as specialized providers of cognate B cell help. The percentage and absolute number of $CD4^+CXCR5^+$ Tfh cells peaked on day 5 p.i., and then decreased to normal level on day 10 p.i. in the 4-week-old mice. However, in the 8-week-old mice, the percentage and absolute number of $CD4^+CXCR5^+$ Tfh cells were significantly increased on day 10 p.i. as compared to the 4-week-old mice (Fig. 3E, 3F) ($p<0.05$). As expected, the percentage and absolute number of Th2 cells, and $CD4^+CXCR5^+$ Tfh cells, and the level of IL-4 were significantly elevated in *P.y17XNL*-infected 8-week-old group during the late stage of malaria infection. These results indicated that an enhanced Th2 immunity during non-lethal *P.y17XNL* infection might contribute to rapid clearance of *Plasmodium* in adults.

PD-1 expression promotes immune response in different species of Plasmodium-infected 4-week-old and 8-week-old BALB/c mice

PD-1 signaling plays an essential role in regulating immune cell exhaustion. To explore whether PD-1 signaling mediated effector T cell exhaustion and facilitated persistent infection in infancy or adulthood, we detected the expression of PD-1 on $CD4^+$ or activated $CD4^+$ T cells after lethal *P.y17XL* and non-lethal *P.y17XNL* infection by flow cytometry. The expression of PD-1 on $CD4^+$ or activated $CD4^+$ T cells was obviously increased on day 5 and day 10 p.i. after lethal *P.y17XL* and non-lethal *P.y17XNL* infections. Compared with 8-week-old mice, the expression of PD-1 on $CD4^+$ or activated $CD4^+$ T cells after non-lethal *P.y17XNL* infection on day 10 p.i. was significantly raised in the 4-week-old mice (Fig. 4A-C) ($p<0.05$).

Interestingly, in lethal *P.y17XL* infection, the expression of PD-1 on $CD4^+$ or activated $CD4^+$ T cells was obviously higher in the 8-week-old mice than in the 4-week-old mice on day 10 p.i. (Fig. 4D-F).

The levels of total and P.y MSP-1-specific antibody in P.y17XNL-infected 4-week-old and 8-week-old BALB/c mice

Protection from clinical malaria has been reported to be associated with both the breadth and magnitude of the antibody responses to merozoite antigens [18]. ELISA of B cell-related total IgG, IgG1 and IgG2a also showed a significant difference in antibody production in adult mice as compared to infant mice (Fig. 5A, B, C). Interestingly, compared with 4-week-old mice, *P.y* MSP-1-specific IgG antibody production was obviously increased in the 8-week-old mice during malaria infection (Fig 5D) ($p<0.05$).

Discussion

Malaria infection is known to be age-related, with children being more susceptible than adults [19, 20, 21, 22]. This study aimed to investigate whether the susceptibility to malaria infection in childhood and adulthood is associated with cellular and humoral immune responses, using a mouse model of lethal *P.y17XL* and non-lethal *P.y17XNL* infections in different age groups. Infant mice were found to be more susceptible to *P.y17XNL* infection, with lower survival rate and higher parasitemia at various time points. The adult group was more resistant to *P.y17XL* infection with lower parasitemia during the early stage of malaria infection. Importantly, enhanced cellular and humoral immunity, especially MSP-1 special antibody, might contribute to rapid clearance of malaria in the adult group.

Malaria infections have various clinical phenotypes, ranging from a mild febrile illness to life-threatening severe anemia and acidosis, as well as end-organ failure among individuals with little or no acquired anti-malarial immunity. In part, this is explained by heritable differences in susceptibility to malaria infections, including different parasite proliferation rates governed by erythrocyte and hemoglobin polymorphisms [23]. Both the strain and host were thought to be important determinants of the disease profile [24]. In this study, 4-week-old and 8-week-old mice were used to mimic infancy and adulthood, respectively. We successfully established the age-related malaria infection mouse model to study the age-related anti-malaria immunity. The survival rate and parasitemia at different time points indicated that the 4-week-old group was more susceptible to both lethal and non-lethal parasite infections. After non-lethal *P.y17XNL* infection, parasitemia was significantly higher in the 4-week-old mice than the 8-week-old mice during the acute and chronic stages of infection. After lethal *P.y17XL* infection, a significant difference in parasitemia was observed in the early stage of infection. In accordance with the parasitemia, enhanced Th1 immune responses were only observed in the early stage (day 3) in adult mice after lethal *P.y17XL* infection and enhanced adaptive immune responses were detected in adult mice during both the early and late stages of non-lethal *P.y17XNL* infection. These data suggested that the difference in response to non-lethal and lethal *Plasmodium* infections was associated with the pattern of immune cell responses in the host.

Similar to other infectious diseases, accumulating evidences have indicated that $CD4^+$ T cells are essential to control malaria infection [25, 26, 27, 28]. Numerous studies have highlighted the role of Th1/Th2 cells or related signaling mechanisms in controlling malaria infection [29, 30, 31, 32, 33]. In this study, enhanced Th1 and Th2 responses were displayed in 8-week-old mice after malaria infection. Significantly higher percentage of Th1/Th2 cells and level of IFN-γ/IL-4 were observed in the 8-week-old mice as compared to the 4-week-old mice. *In vitro* studies also showed an enhanced Th1 cell response, which indicated an important role of Th1/Th2 cell-mediated age-related anti-malarial response. However, many studies suggested a shift from Th2 to Th1 cell responses with age. Li et al. found that IFN-γ level increased with age but not Th-related transcription factors, while IL-4 expression in plasma and $CD4^+$ splenocytes declined with age [34]. A shift from Th2 towards Th1 immune responses was also observed in children with tertian or tropical malaria infection [35]. These studies partly supported our conclusion that enhanced Th1 cells might contribute to malaria clearance during the early stage of malaria infection. However, we observed enhanced Th2 cells during the late stage/chronic stage of malaria infection. Further studies are needed to investigate if any shift exists during the early stage of malaria infection. In addition, follicular T helper (Tfh) cells are essential for *Plasmodium* infection clearance by activating germinal center B cell responses [36, 37, 38, 39]. In this study, the percentage of $CD4^+CXCR5^+$ Tfh cells was also up-regulated in adult mice as compared to the infant group. Moreover, Crompton PD et al. found that the activation of PD-1^+CXCR5^+ Tfh cells in malaria-infected children did not correlate with antibody responses indicating that Tfh cells may exhibit impaired B cells in children, which is different from Tfh cells in adults [40]. These studies supported our findings that the impaired function of antibody-secreting B cells and Tfh cells in infants and children may account for their susceptibility to malaria infection.

We also observed a dampening of PD-1 signaling on activated $CD4^+$ T cells after non-lethal *P.y17XNL* infection but not lethal *P.y17XL* infection in the 8-week-old mice. PD-1 co-inhibitory signaling was reported to regulate helper T cell differentiation and anti-*Plasmodium* humoral immunity [41], and PD-1 deficiency could enhance humoral immunity during malaria infection [42]. PD-1 was also a marker of T-cell exhaustion [43]. Several studies have also proven that chronic malaria infection drives T cell exhaustion through PD-1 signaling [44, 45]. Therefore, we speculated that during non-lethal infection, humoral immunity plays an essential role in the late stage of malaria clearance, perhaps correlated with enhanced PD-1 signaling on activated $CD4^+$ T cells, which may help to drive $CD4^+$ effector T cell exhaustion and promote persistent infection in infants. Therefore, differences in PD-1 signaling could be observed in different age groups after non-lethal but not lethal malaria infection.

Several studies have confirmed that immune effector mechanisms are required to eliminate malarial parasites, and B cells secrete specific antibodies supported by Th2 cells, which can effectively remove the parasites to prevent the recidivation and recrudescence [46, 47]. Similarly, infusion of malaria hyperimmune serum resulted in rapid clearance of parasitized erythrocytes [47]. Anti-*Plasmodium* antibodies can prevent merozoites of infected red blood cells (RBCs), block cytoadherence to endotheliar capillary of infected RBCs (iRBCs), and promote phagocytosis by mononuclear cells [48, 49, 50]. However, persistence of significant levels of antimalarial antibodies relies on the continuous challenge resulting

from chronic exposure to infection [51], probably as a consequence of impaired establishment of B cell memory [52]. This might explain the short-lived antibody responses [53, 54], mainly in young children [55, 56]. In this study, we detected the levels of B cell-related total IgG, IgG1 and IgG2a in *P.y17XNL*-infected BALB/c mice. The results showed a difference in antibody production between adult and infant mice, and the levels of total antibody might contribute to rapid clearance of malarial parasites in the adult group during the chronic stage of non-lethal *P.y17XNL* infection. Moreover, IgG1 and IgG3 antibodies against merozoite surface proteins (MSPs) are thought to be instrumental in protection, which is considered as a major vaccine candidate [57]. Therefore, we detected the levels of *P.y* MSP-1 special antibody. Consistently, the dynamics of *P.y* MSP-1 special antibody was the same as total antibody. These data implied that an enhanced antibody response during chronic stage of non-lethal *P.y17XNL* infection might contribute to rapid clearance of malaria in the adult group.

Conclusion

Taken together, the findings of this study revealed that in non-lethal *P.y17XNL* infection, higher burden of parasitemia and lower survival rate in infant mice were associated with weakened Th1 cellular immune responses, down-regulated humoral immunity with decreased percentage and number of Th2 and Tfh cells as well as lower level of antibody secretion and enhanced PD-1 signaling on activated $CD4^+$ T cells. Higher resistance to lethal *P.y17XL* infection in the early stage in adult mice was associated with enhanced Th1 cellular immune responses and weakened PD-1 signaling on activated $CD4^+$ T cells. These results provide a new insight on immune responses in malaria infection.

Methods

Mice, Parasite and experimental infection

The 4-week-old and 8-week-old female BALB/c mice were purchased from Beijing Animal Institute. *P.y*17XL and *P.y*17XNL strains were provided by Dr. Motomi Torii (Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Infections were initiated by intraperitoneal (i.p.) injection of 1×10^6 *P.y* 17XL or 1×10^6 *P.y* 17XNL parasitized erythrocytes in BALB/c mice. All animal procedures were conducted in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1), and humanely treated. The experimental mice were matched for age and sex. Parasitemia was examined by light microscopy of Giemsa-stained, tail blood smears. Mortality was monitored daily. All experiments were performed in compliance with local animal ethics committee requirements. The animals were not submitted to euthanasia during the process of *plasmodium* infection. Other mice were submitted to euthanasia during detecting the relative index in indicated time points the way to do it is posterior cervical dislocation after eyeball blood extraction.

Spleen cell culture

Spleen cell culture was prepared as previously described [16]. Briefly, spleen was aseptically removed from each mouse and passed through a sterile fine-wire mesh with 10 ml of RPMI1640 (Life Technologies, Burlington, Ontario, Canada) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Inc.), 25mM Hepes (Life Technologies), 0.12% gentamicin (Schering, Montreal, Quebec, Canada) and 2mM glutamine (Life Technologies). Cell suspensions were centrifuged at 350×*g* for 10 min at room temperature (RT). Erythrocytes were lysed with cold 0.17M NH_4Cl. The cells were washed twice with fresh medium. Viability of the spleen cells was determined by trypan blue exclusion, and was always >90%. Spleen cells were adjusted to a final concentration of 10^7cells/ml in RPMI1640 supplemented with 10% heat-inactivated FCS. Aliquots (500μl/well) of the cell suspension were incubated in 24-well flat-bottom culture plates (FALCON) in triplicate for 48 hours at 37°C in a humidified 5% CO_2 incubator. Then, the plates were centrifuged at 350×*g* for 10 min at RT, supernatants were collected and stored at -80°C until they were assayed for the levels of IFN-g, IL-4, IgG, IgG1, IgG2a and *P. y* MSP-1-special IgG.

Cytokine analysis

Levels of IFN-γ and IL-4 were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (R&D Systems, Minneapolis, MN). The OD values were read in a microplate reader at 450 nm. The concentrations of cytokines in samples were calculated against the standard curve generated using recombinant IFN-g and IL-4, respectively.

Multiplex assay for antibody determination

Levels of total serum IgG, IgG1, IgG2a and *P.y* MSP-1-special IgG were measured by ELISA as previously described with some modifications [17]. Briefly, Maxisorp flat-bottomed, 96-well microplates were coated overnight at 4°C with 50 μg of *P.y* MSP-1 antigens in a carbonate-bicarbonate buffer (pH 9.6). The plates were washed with PBS-Tween (PBS-T) and blocked with 0.05% bovine serum albumin (BSA)-PBS-T. Next, 100 μl of plasma dilutions in 0.05% BSA-PBS-T (1:50 for *P.y* MSP-1 IgG) were added in duplicate and incubated at RT for 2 h. After washing with PBS-T, the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, USA) at a dilution of 1:5000. The OD values were read in a microplate reader at 490 nm.

Cell surface/intracytoplasmicstaining and flow cytometry

To assess the function of $CD4^+$ T cells, spleen cells from BALB/c mice infected with *P.y17XL/P.y17XNL* at different time points were double-stained with FITC-conjugated anti-CD4 (clone GK1.5, BD), BV421-conjugated anti-PD-1 (clone J43, BD), PE-conjugated anti-CXCR-5 (clone 2G8, BD) and APC-conjugated anti-CD62L (MEL-14, BD), followed by two washes, staining and analysis by flow cytometry.

To assess dynamics of Th1 and Th2 cells, spleen cells from BALB/c mice infected with *P.y17XL/P.y17XNL* at different time points were triple-stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-T-bet (clone eBio4B10, eBioscience), APC-

conjugated anti-IFN-γ (XMG1.2, BD) for Th1 cells, and FITC-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-GATA-3 (clone L50-823, BD), APC-conjugated anti-IL-4 (clone 11B11, BD) for Th2 cells. After stimulation for 2 hours with PMA and ionomycin at 37°C, Golgi Stop (BD Bioscience) was added to each reaction (1:500, vol/vol). After co-culture for 4 hours at 37°C, the cells were washed with 3% FCS and then resuspended in 100μl of 3% FCS. FITC-anti-CD4, PE-anti-T-bet and PE-anti-GATA3were added for surface staining. Then, the cells were fixed and permeabilized, and intracytoplasmic staining was performed using allophycocyanin (APC)-anti-IFN-γ/IL-10 (JES5-16E3). FITC rat IgG2b was used as the isotype control. All antibodies were purchased from BD Pharmingen.

Statistical analysis

All analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA). Data are presented as mean ± standard error of the mean (SEM). Survival analysis was performed using the Kaplan-Meier log-rank test. Statistical significance of differences between the two groups was assessed by unpaired Student's t-tests. P-values were calibrated using Bonferroni correction, and were considered statistically significant if they were less than 0.05.

Abbreviations

P.y17XL: Plasmodium yoelii 17XL; *P.y17XNL*: *Plasmodium yoelii 17XNL;* DCs: dendritic cells; IL-4: interleukin-4; Tfh: follicular T helper cells; RBCs: red blood cells; iRBCs: infected RBCs; MSPs: merozoite surface proteins; i.p.: intraperitoneal; FCS: fetal calf serum.

Declarations

Acknowlegements

We thank Dr. Motomi Torii (Ehime University Graduate School of Medicine, Ehime, Japan) for his guidance in this research and providing malaria parasite strains of *P.y17XL and P.y17XNL*.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81101278; 81429004); Outstanding youth program of Taizhou university (Z2020080); and doctor launching fund project of Liaoning province (20180540019). The funders had no role in the design of the study or in the collection analysis or interpretation of the data.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Author's contributions

YM.C authored the manuscript. G.C and W.P designed the experiments. QB.W, YT.D F.L, XD.S and S.X performed all experiments. YT.D provided critical support for all data analysis. G.C critically reviewed the manuscript. All authors have read and approved the manuscript

Ethics approval and consent to participate

The 4-week-old and 8-week-old female BALB/c mice were used for all experiments and protocols complied with the China medical University Animal Ethics Committee requirements. This study does not involve the use of human data or tissue. All authors have read and approved the manuscript.

Consent for publication

Not applicable.

Competing Interests

No potential conflict of interest was reported by the authors.

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Figures

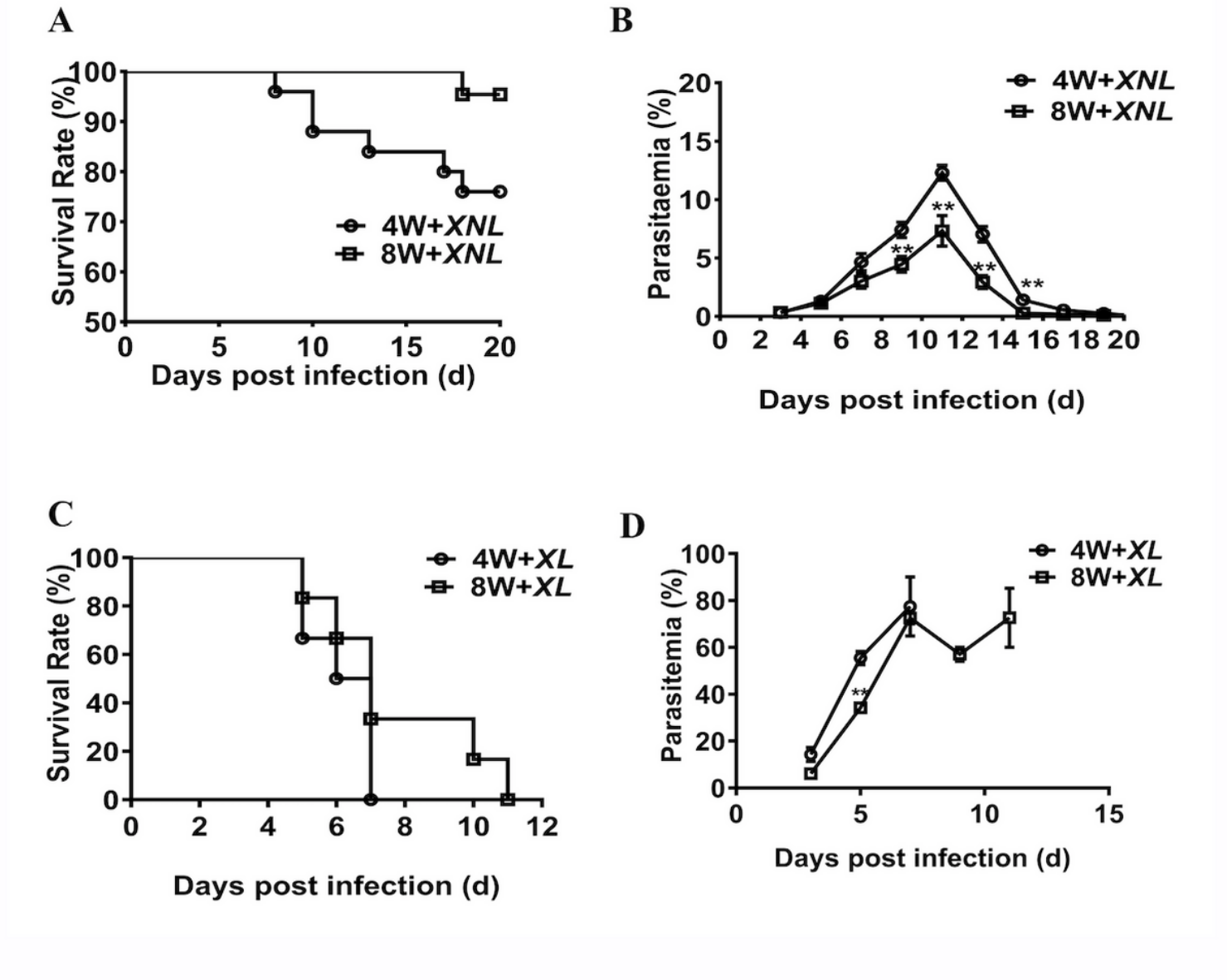

Figure 1

Parasitemia (A, C) and survival rate (B, D) of Py17.XNL or Py17.XL infection in 4-week-old and 8-week-old BALB/c mice. Parasitemia was calculated by counting the number of parasite-infected erythrocytes per 1000 erythrocytes. Mortality was checked daily. The data are representative of two separate experiments.

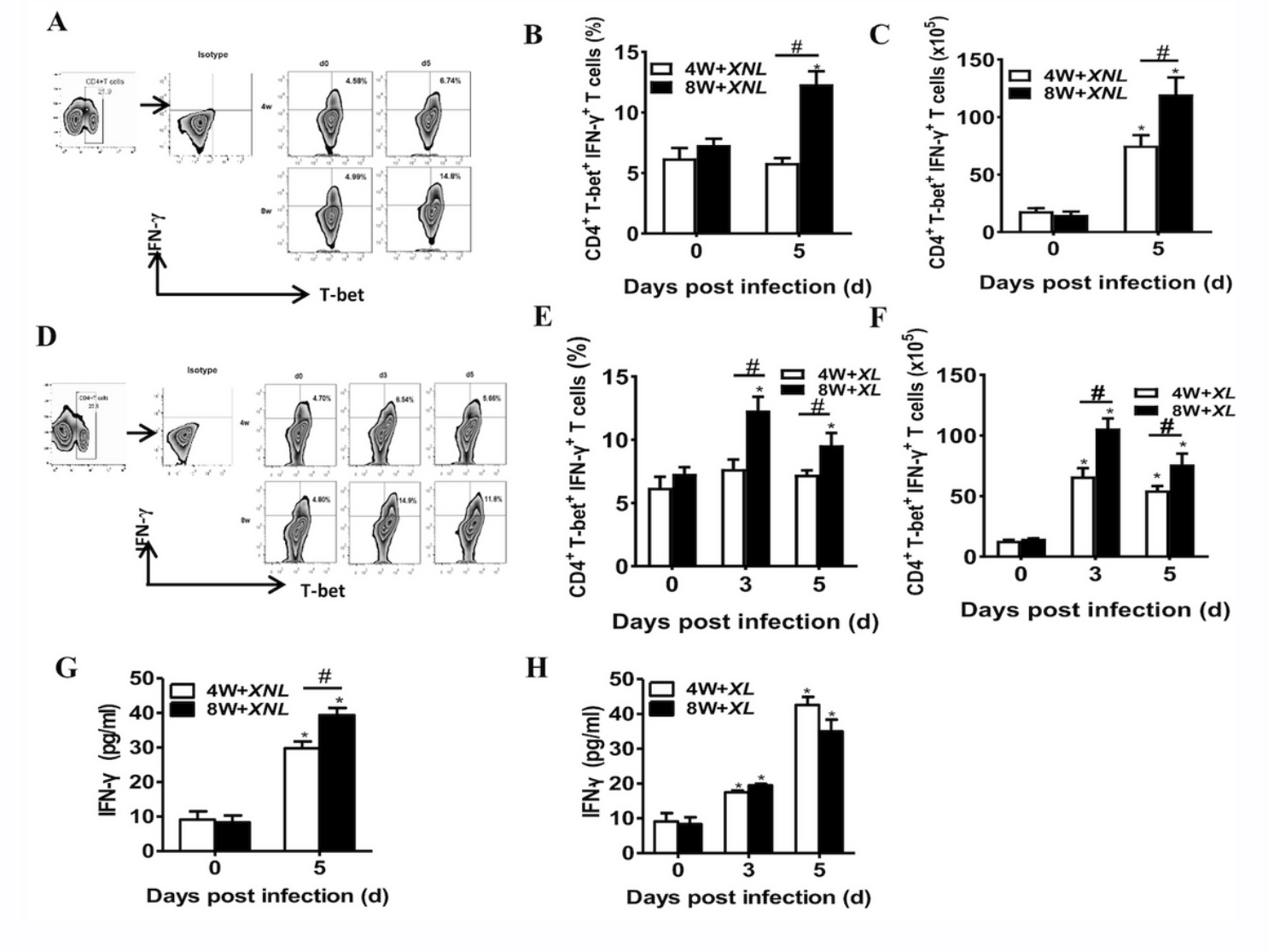

Figure 2

Flow cytometric and ELISA analysis demonstrated Th1 immune response in different species of Plasmodium-infected 4-week-old and 8-week-old BALB/c mice. Two-dimensional contour map (left-upper panel) (A), colu mn diagram: left-upper panel, the proportion of Th1 cells in CD4+ T cells (B) and absolute cell number (C) of CD4+T-bet+IFN-γ+ Th1 cells in 4-week-old and 8-week-old BALB/c mice after Py17XL infection are displayed; Representative dot plots (left-lower panel) (D), column diagram:left-lower panel, the proportion of Th1 cells in CD4+ T cells (E) and absolute cell number (F) of CD4+T-bet+IFN-γ+ Th1 cells in 4-week-old and 8-week-old BALB/c mice after Py17XNL infection are displayed. Level of IFN-γ (G, H) in spleen cell supernatants in Py17XNL/Py17XL-infected BALB/c mice were measured. Results are presented as arithmetic mean of 9 mice per group ± SE. Single asterisk (*) and double asterisks (**) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to control mice. A single pound sign (#) and a double pound sign (##) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to 8-week-old mice.

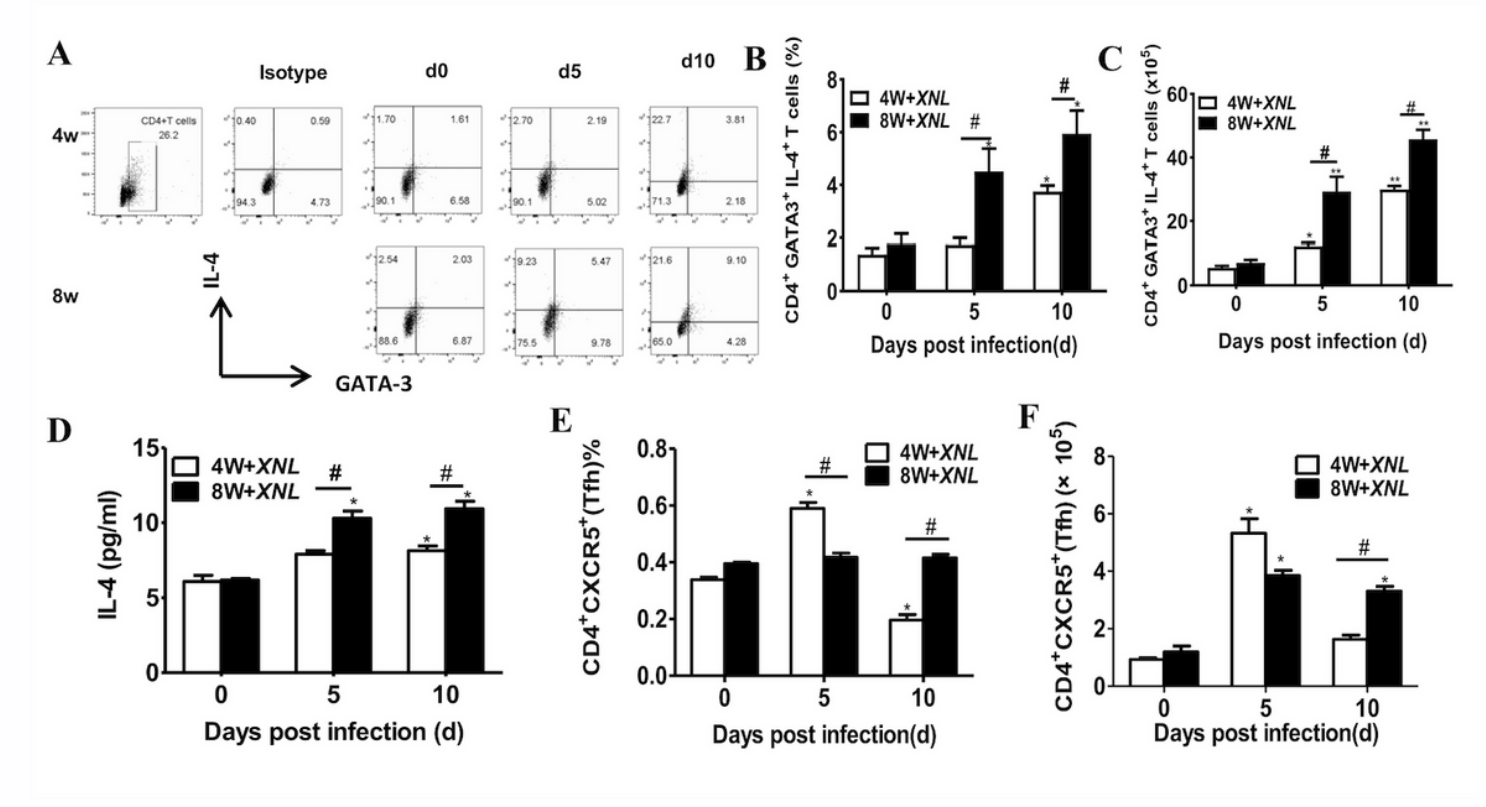

Figure 3

Flow cytometric and ELISA analysis demonstrated Th2 immune response in different species of Plasmodium-infected 4-week-old and 8-week-old BALB/c mice. Representative dot plots (left-upper panel) (A), column diagram:left-upper panel, the proportion of Th2 cells in CD4+ T cells (B) and absolute cell number (C) of CD4+GATA3+IL-4+ Th2 cells in 4-week-old and 8-week-old BALB/c mice after P.y17XNL infection are displayed. Level of IL-4 (D) in spleen cell supernatants in P.y17XNL-infected BALB/c mice were measured. The proportion (E) and absolute cell number (F) of CD4+CXCR5+Tfh cells were measured by flow cytometry in 4-week-old and 8-week-old BALB/c mice after Py17XNL infection. Results are presented as arithmetic mean of 9 mice per group ± SE. Single asterisk (*) and double asterisks (**) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to control mice. A single pound sign (#) and a double pound sign (##) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to 8-week-old mice.

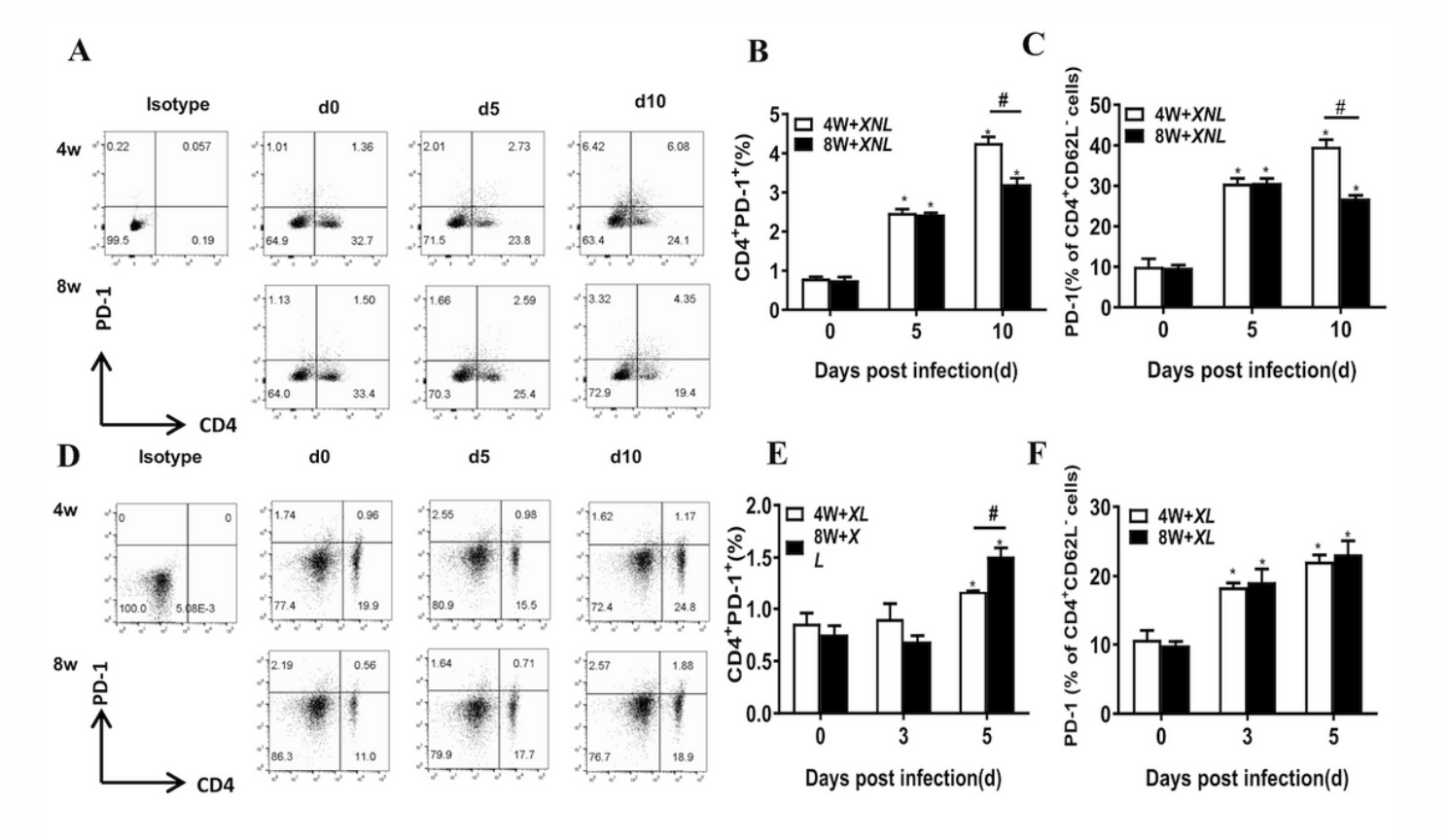

Figure 4

Flow cytometric analysis demonstrated PD-1 signaling promoted immune response in different species of Plasmodium-infected 4-week-old and 8-week-old BALB/c mice. Representative dot plots (left-upper panel) (A) and column diagram:left-upper panel, the proportion of CD4+PD-1+ cells in splenocytes (B) and absolute cell number (C) of CD4+PD-1+ cells in 4-week-old and 8-week-old BALB/c mice after P.y17XNL infection are displayed. Representative dot plots (left-lower panel) (D) and column diagram:left-lower panel, the proportion of CD4+PD-1+ cells in splenocytes (E) and absolute cell number) (F) of CD4+PD-1+ cells in 4-week-old and 8-week-old BALB/c mice after P.y17XL infection are displayed. Results are presented as arithmetic mean of 9 mice per group ± SE. Single asterisk (*) and double asterisks (**) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to control mice. A single pound sign (#) and a double pound sign (##) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to 8-week-old mice.

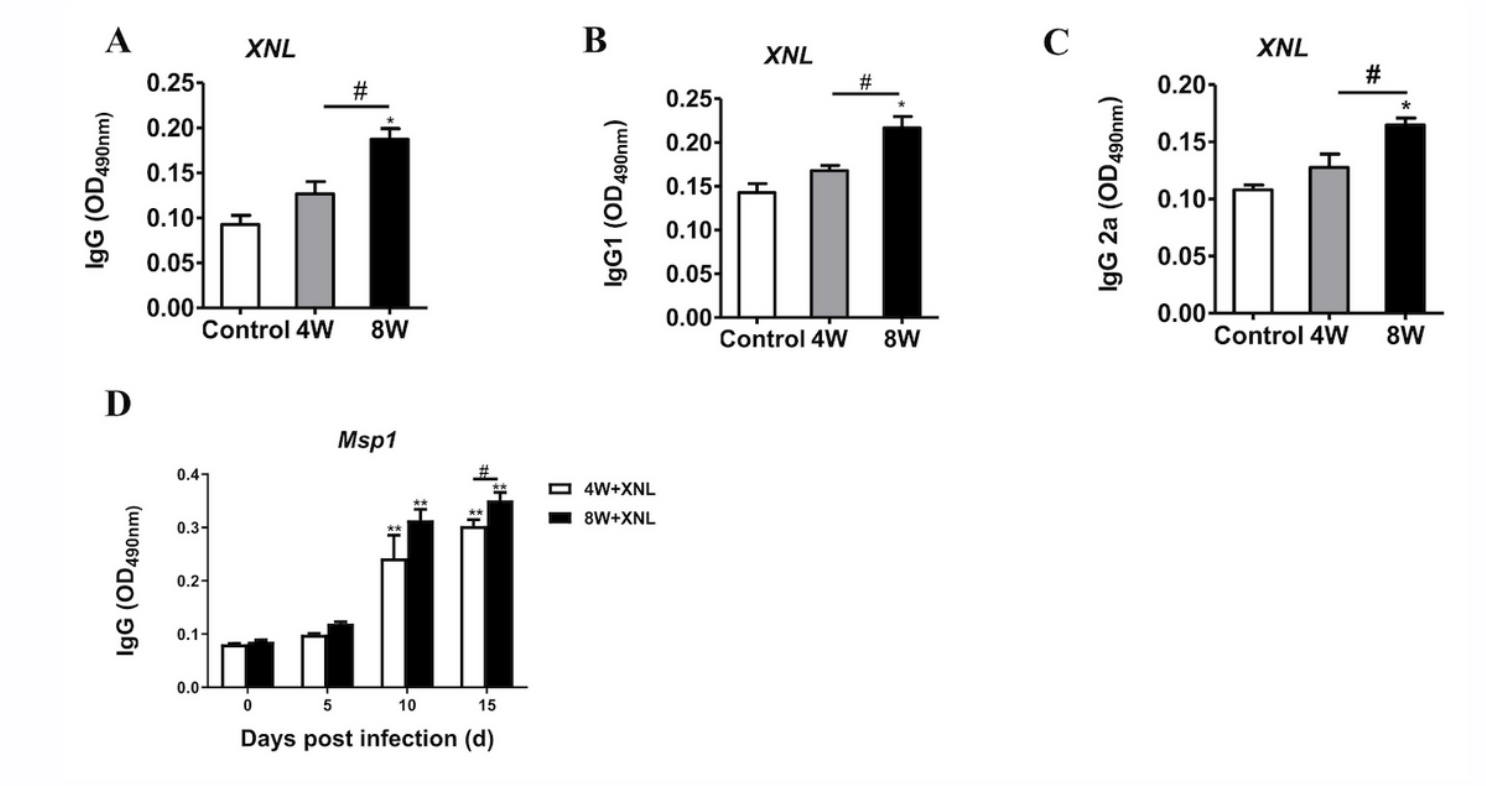

Figure 5

ELISA analysis demonstrated the levels of total and P.y MSP-1-specific antibodies in spleen supernatants of P.y17XNL-infected 4-week-old and 8-week-old BALB/c mice. IgG (A), IgG1(B), IgG2a (C) and P.y MSP-1-specific IgG (D) were measured in supernatants of P.y17XNL-infected 4-week-old and 8-week-old BALB/c mice by ELISA. Results are presented as arithmetic mean of 9 mice per group ± SE. Single asterisk (*) and double asterisks (**) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to control mice. A single pound sign (#) and a double pound sign (##) indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to 8-week-old mice.

Supplementary Files

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