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Research paper

Resistance against *Trichinella spiralis* infection in pups delivered by *T. spiralis*-infected dam

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ABSTRACT

Maternal antibody transmission via placenta and breastmilk are known to confer protection in infants. In this study, we investigated the maternal immunity transmission in pups delivered by rats infected with *Trichinella spiralis* and assessed the resulting resistance against subsequent parasitic infection. Our results revealed that parasite-specific IgG, IgG1 and IgG2a antibodies were present in pups prior to breastmilk ingestion (pre-milk), in which IgG and IgG1 antibodies persisted until week 8 after birth while parasite-specific IgG2a antibodies only lasted until week 4. After weaning on week 3, pups delivered by *T. spiralis*-infected dam and subsequently challenge-infected (immune-challenge) were found to possess higher mucosal IgG antibodies than control groups, whereas mucosal IgA levels were not significantly different across all groups. *T. spiralis* excretory-secretory antigen was discovered to react with pup sera until week 8, correlating with the resistance against parasitic infection which is represented by lessened worm burden. Upon *T. spiralis* infection at weeks 3 and 8, lower levels of eosinophil responses were detected in immune-challenge pups compared to naïve-challenge pups, indicating correlates of resistances in which ADCC may be involved. Findings from the present study demonstrate that resistances against *T. spiralis* infection in pups can be acquired by maternally-derived IgG, IgG1 and IgG2a antibody transmission through the placenta and breastmilk from *T. spiralis*-infected dam, which lasts until week 8.

1. Introduction

Maternal immunity transmission to immunologically naïve neonates occurs in multitudinous ways, such as through direct deposition of antibodies to egg yolk in fishes, reptiles, and birds or through the placenta and breastmilk as in mammals (Grindstaff et al., 2003). Transgenerational effects of maternal immunity are also observable from invertebrates, which use different components of the immune system compared to vertebrates (Huang and Song, 1999; Moret and Schmid-Hempel, 2001). In humans, exclusive breastfeeding during the first six months of the infant's life protected babies from various life-threatening diseases as well as providing nourishment for growth (Alianmoghaddam et al., 2018). Evidently, breastfeeding contributed to the reduction of global mortality rates for children under five years of age by approximately 11.6% (Black et al., 2013). Consolidating evidence for the beneficial effects of breastmilk have been presented, yet the global rate of breastfeeding appears to be declining for various reasons, including socioeconomic factors, physical demand, cultural and psychological issues (Meedya et al., 2010). Regardless of the organism, maternally-derived immunities are of utmost importance in newborns, due to limited endogenous antibody levels and the time required for antibody synthesis (Grindstaff et al., 2003; Hasselquist and Nilsson, 2009). Given the lack of immunity transmission ascribed to declining breastfeeding practices, neonates remain vulnerable to infection by pathogens such as parasites since their rudimentary immune system is functionally inept and requires time for maturation.

Trichinella spiralis is a parasitic nematode responsible for the foodborne zoonotic disease trichinellosis. Although clinical cases of trichinellosis have been on the decline due to improvements in meat inspection and other management policies (Murrell, 2016), more than 65,000 human cases from 41 different countries over the past decades were reported and theses clinical cases continue to occur even in developed nations (Murrell and Pozio, 2011). Transmission of immunity against *T. spirallis* via lactation has been studied using rodents since

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decades ago (Duckett et al., 1971; Perry, 1974; Appleton and McGregor, 1984; Kumar et al., 1989). Additionally, several studies have demonstrated that immunity transfer via mesenteric lymph node cells is conducive to adult *T. spiralis* worm expulsion (Wakelin and Lloyd, 1976; Wakelin and Wilson, 1977a, b). Lymphocyte transfer from *T. spiralis*-immune rats also promoted worm expulsion (Despommier et al., 1977). A few studies have also investigated the larvicidal effect of immune sera against newborn larvae of *T. spiralis* (Moloney and Denham, 1979; Wang and Bell, 1988; Nunez et al., 2002). Although these studies were crucial for understanding the transmission of passive immunity against *T. spiralis* infection, earlier works were predominantly focused on the intestinal worm expulsion and cell-mediated immune responses, which left the humoral aspects such as antibody response profiles and its duration in pups remain to be explored. To this extent, additional immunological responses in offspring contributing to resistance in murine models would also prove to be useful.

In the present study, we assessed the level of immunoglobulins over time from pups of rats previously infected with *T. spiralis*, and the contribution of these immune responses to resistance upon *T. spiralis* infection. Our data show that maternal *T. spiralis*-specific antibodies are long-lasting and are associated with increased worm burden reduction. Our findings reveal multiple aspects of the maternal immunity contributing to partial protection against *T. spiralis* in pups and further signifies the importance of maternal immunity.

2. Materials and methods

2.1. Experimental animals, infection, and parasite preparation

Male and female Sprague Dawley (SD) rats (8 weeks old) were purchased from NARA Biotech (Seoul, South Korea). Initially, female SD rats were either uninfected or infected with 3000 *T. spiralis* muscle larvae. At 28 days post-infection (dpi), both uninfected and infected females were mated with the males. After approximately 3 weeks of gestation, the dams gave birth to pups which were weaned and grouped (n = 5) as follows: pups from naïve dam which will not be infected (Uninfected), pups from naïve dam which will be challenge-infected with *T. spiralis* (Naïve-Challenge), and pups from *T. spiralis*-infected dam which will be challenge-infected with *T. spiralis* (Immune-Challenge). Naïve-challenge and immune-challenge pups were infected with 500 *T. spiralis* larvae via orogastric tube at week 3 or week 8 post-birth. *T. spiralis* larvae were maintained through serial passaging in Sprague Dawley (SD) rats. For parasite preparation, infected rats were sacrificed and their muscle tissues were collected. Tissues were cut into small pieces and subsequently digested in pepsin − HCl solution overnight at 37°C. Digested contents were filtered through a metallic mesh to remove debris and serially washed with 0.85% saline solution prior to larvae counting under the microscope. All of the experimental procedures involving animals have been approved and conducted under the guidelines set out by Kyung Hee University IACUC.

2.2. T. spiralis excretory-secretory (ES) antigen preparation

T. spiralis ES antigens were prepared by collecting muscle larvae from *T. spiralis*-infected rat muscle tissues, which were thoroughly washed with autoclaved 0.85% saline solution for debris removal. The larvae were subsequently cultured in serum-free RPMI medium containing penicillin and streptomycin at 37°C, 5% CO_2 for up to 3 days. Harvested culture media were centrifuged at 5000 RPM for 10 min, 4°C and its protein concentration was determined using Bradford protein assay (Thermo Fisher Scientific, Waltham, MA, USA). ES antigens were aliquoted and stored at -20°C until use.

2.3. Serum and intestinal antibody collection

T. spiralis control sera were collected 4 weeks after *T. spiralis* infection from female rats prior to mating through the retro-orbital plexus puncture. Blood from pups was collected at the following time points: immediately after birth, weeks 1, 2, 3, 4, 6, 8, 10, 12, and 14. Blood was acquired from pups through the tail-vein during the first 3 weeks, and by retro-orbital plexus puncture after week 3. Sera were acquired by centrifuging the blood at 6000RPM for 10 min. Small intestines were collected from pups at 4 dpi on weeks 3 and 8. Intestines were incised longitudinally to ensure the release of adult worms in PBS. After incubating at 37°C for 1 h, intestines were resuspended in 1 ml PBS, centrifuged at 3000RPM for 10 min. and supernatants were collected. Collected sera and intestinal supernatants were stored at -20°C and -80°C, respectively, until use.

2.4. T. spiralis-specific antibody responses

Antigen-specific antibody responses from sera and intestines were detected using enzyme-linked immunosorbent assay (ELISA). Each well of the 96-well plates (SPL Life Sciences, Korea) was coated with 4 ug/ml of *T. spiralis* ES antigens in 100 ul of carbonate buffer (pH 9.5) After incubating the plate overnight at 4°C, plates were blocked with gelatin at 37°C for 1 h. Wells were incubated with either rat sera (1:100 dilution in PBS) or undiluted intestinal samples collected at various time points for 1 h at 37°C, which were detected using horseradish peroxide (HRP)-conjugated IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgA (1:2000 dilution in PBS) secondary antibodies purchased from Bio-Rad (Hercules, CA, USA). O-phenylenediamine purchased from Sigma Aldrich (St. Louis, MO, USA) was dissolved in substrate buffer with H_2O_2 following the manufacturer's instructions. One hundred microliters of this mixture were added to each well and reactions were stopped by adding 50ul of diluted H_2SO_4. Colorimetric changes at 490 nm were measured using EZ Read 400 microplate reader (Biochrom Ltd., Cambridge, UK)

2.5. Eosinophil counting in blood

Pups were infected with 500 *T. spiralis* larvae on week 3 or week 8. Peripheral blood from naïve, naïve-challenge, and immune-challenge groups was acquired on 7, 14, and 28 dpi. In clean microcentrifuge tubes, 10ul of blood was mixed with 90ul Discombe's solution and eosinophils were counted under the microscope in a hemocytometer as previously described (Satoh et al., 2000).

2.6. Western blot and antigen detection

T. spiralis ES antigens were resolved using 10% polyacrylamide gel and proteins were transferred onto a nitrocellulose membrane. After blocking with 5% skim milk for 1 h at RT, membranes were incubated with polyclonal rat antibodies collected from naïve dam, infected dam, and pups nurtured by the infected dam on weeks 3, 8, and 12 overnight at 4°C. Next day, membranes were washed three times, 5 min per wash using tris-buffered saline with Tween 20 (TBST) and incubated with HRP-conjugated anti-rat IgG antibody for 1 h at RT. After subsequent washing steps, proteins were detected with enhanced chemoluminence kit purchased from Thermo Fisher and manually developed using x-ray films in the darkroom.

2.7. Worm burden reduction and counting

At 3 or 8 weeks after birth, naïve-challenge pups and immune-challenge pups were infected with 500 *T. spiralis* larvae. Pups in both groups were sacrificed at 4 and 28 dpi to determine worm burden reduction of adult worms and muscle larvae, respectively. Adult worm burdens from pups were quantified by counting the number of worms released from the small intestines on day 4 post-infection (pi). At 28 dpi, diaphragm from rats was collected, weighed, and digested in pepsin − HCl solution overnight at 37°C. Muscle larvae from the diaphragm were counted the next day and counts were normalized to per

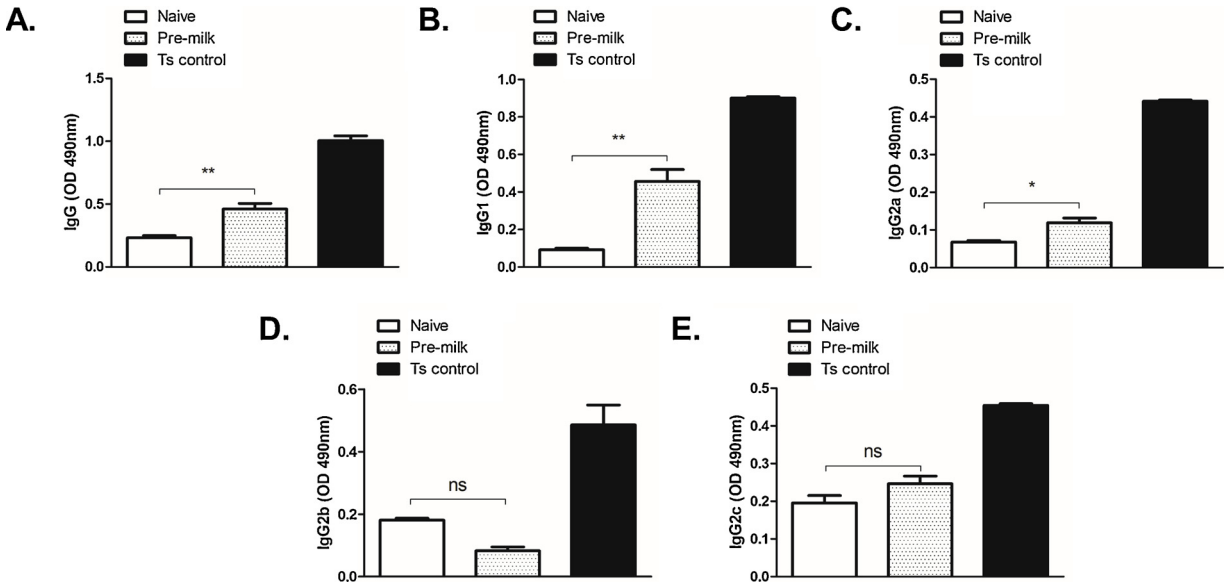

Fig. 1. Placental transmission of IgG and its subclasses in pups delivered by *T. spiralis*-infected dam. IgG (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgG2c (E) levels were assessed from sera collected from pups immediately after birth by ELISA. All experiments were conducted in triplicates and data are expressed as mean ± SEM. *$P < 0.05$ and **$P <$ 0.01 represents statistical significance, whereas no statistical significance were denoted by "ns".

gram of tissue.

2.8. Statistics

Data sets were presented as mean ± SEM. A one-way analysis of variance (ANOVA) with Tukey's *post hoc* analysis and two-tailed, unpaired student's *t*-test were used to assess statistical significance between the groups. Analyses were performed using GraphPad Prism 5 software (San Diego, CA, USA). ***P < 0.001, **P < 0.01, and *$P < 0.05$ were used to represent statistical significance.

3. Results

3.1. Serum immunoglobulin response against T. spiralis antigen

To address the types of maternal antibodies being transferred to the pups via placenta or breastmilk from *T. spiralis*-infected dam, sera collected at various time points were used to detect *T. spiralis*-specific antibody responses via ELISA. *T. spiralis* antigen-specific IgG, IgG1, and IgG2a levels were detected from pups even before breastfeeding (Fig. 1A–C, *P < 0.05, **P < 0.01). On the contrary, IgG2b and IgG2c subtypes were not found at significant levels (Fig. 1D, E), suggesting placental transmission of *T. spiralis* antigen-specific IgG, IgG1 and IgG2a. After 1 week, IgG and IgG1 antibody responses were detected at high levels, which were similar to that of *T. spiralis*-infected control sera. The antibody responses remained consistently high until week 3, which marks the beginning of the weaning period. This was followed by gradual decline in antibody levels until week 14 (Fig. 2A, **P < 0.01, ***P < 0.001). Similar patterns were observed for IgG1 and IgG2a (Fig. 2B, C, *P < 0.05, ***P < 0.001), where drastic reduction in antibody responses became noticeable after week 3. In IgG2b and IgG2c, changes in antibody levels over time were marginal and no significant differences were observed at all time points (Fig. 2D, E).

3.2. Mucosal antibody response against T. spiralis and eosinophil count

Significantly higher IgG responses were detected at day 4 pi for immune-challenge pups infected at week 3 compared to naïve-challenge pups, whereas no significant differences were observed between the two groups at day 4 pi for pups challenged at week 8 (Fig. 3A, *P < 0.05). Mucosal IgA responses were not detected from both time points (Fig. 3B). Week 3 eosinophil count differences were noticeable starting from 14 dpi, where significantly lesser eosinophils were found in the immune-challenge pups. This trend continued onwards until 28 dpi, albeit with reduced eosinophilia for both naïve-challenge and immune-challenge pups (Fig. 3C, *P < 0.05). An identical pattern was observed for week 8 blood eosinophil counts as the number of eosinophils detected from naïve-challenge pups and immune-challenge pups were drastically different (Fig. 3D, *P < 0.05). As with week 3 eosinophils, noticeable differences in eosinophils were observed starting from 14 dpi which further waned by 28 dpi. Overall, lower levels of eosinophil responses were detected from pups delivered by *T. spiralis*-infected dams compared to naïve-challenge pups on both 14 and 28 dpi.

3.3. T. spiralis ES antigen reacted with sera from pups

T. spiralis ES antigens were probed with polyclonal antibodies of pups acquired at different time points at weeks 3, 8 and 12 after birth. As expected, naïve rat sera could not react with the antigens whereas sera from infected dam identified multiple protein components of the ES antigen. Sera from week 3 pups successfully detected multiple proteins, although these antigen components were detected to a lesser extent compared to *T. spiralis*-infected dam sera. Detection levels were much weaker in week 8 as only a single protein was detected around 45 kDa, and virtually non-existent in week 12 sera (Fig. 4).

3.4. Worm burden reduction

To investigate the effect of these humoral factors on resistance to parasite infection, worm burden reductions were assessed. Adult and muscle larvae worm burdens were measured by counting the worms from intestine at 4 dpi and from the diaphragm at 28 dpi, respectively. Compared to the control group, adult worm burdens were significantly less in immune-challenge pups at both weeks 3 and 8 (Fig. 5A, B, *P < 0.05). Similarly, muscle larvae worm burden reductions were also present at weeks 3 and 8, 28 dpi (Fig. 6A, B, *P < 0.05).

4. Discussion

Passive transfer of immunity against *T. spiralis* via breastmilk have

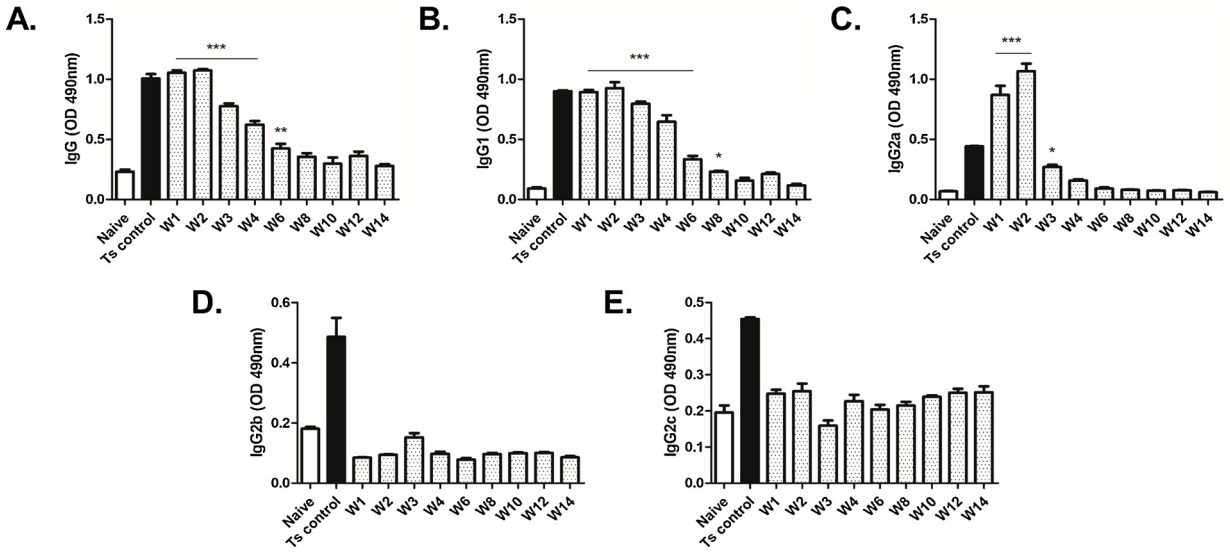

Fig. 2. Antibody transmission from *T. spiralis*-infected dam through placenta and breastmilk. IgG (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgG2c (E) levels were assessed from sera collected from pups over the span of 14 weeks. Pups were weaned 3 weeks after birth and the antibody responses were measured by ELISA. All experiments were conducted in triplicates and data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ represents statistical significance compared to naïve control.

been documented (Duckett et al., 1971), yet the persistence of immunity over time and factors contributing to resistance in pups breastfed by immunized dams require more elucidation. In this study, we investigated the humoral immune response factors contributing to parasitic resistance in pups delivered and nurtured by *T. spiralis*-infected dams.

IgG response from pups was strongest within the first two weeks and began to fade once the pups began to ingest solid food products, which signifies antibody transmission through the breastmilk. Of the immunoglobulin subtypes, IgG1 and IgG2a appeared to contribute more to parasitic resistance compared to IgG2b and IgG2c. In rats, IgG1 and IgG2a were reported to be correlated with Th2 response whereas IgG2b and IgG2c subtypes were associated with the Th1 responses (Gracie and Bradley, 1996; Cetre et al., 1999). The immune response induced upon

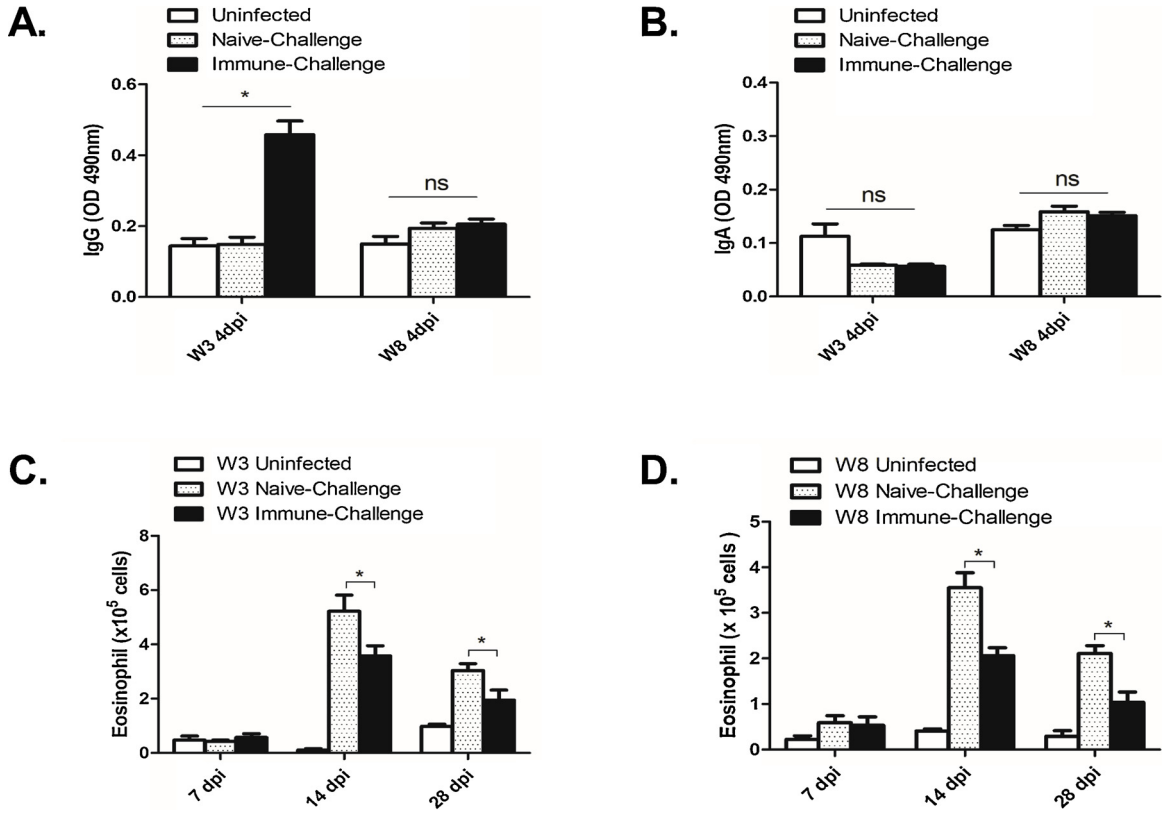

Fig. 3. Mucosal antibody responses and eosinophilia in pups following *T. spiralis* infection. Intestinal IgG (A) and IgA (B) responses were observed from week 3 and week 8 pups at 4 dpi using ELISA. Eosinophils were counted from peripheral blood on 7, 14, and 28 dpi from week 3 and 8 pups (C, D). All experiments were conducted in triplicates and data are expressed as mean ± SEM. *$P < 0.05$ were considered significant, whereas no statistical significance was denoted by "ns".

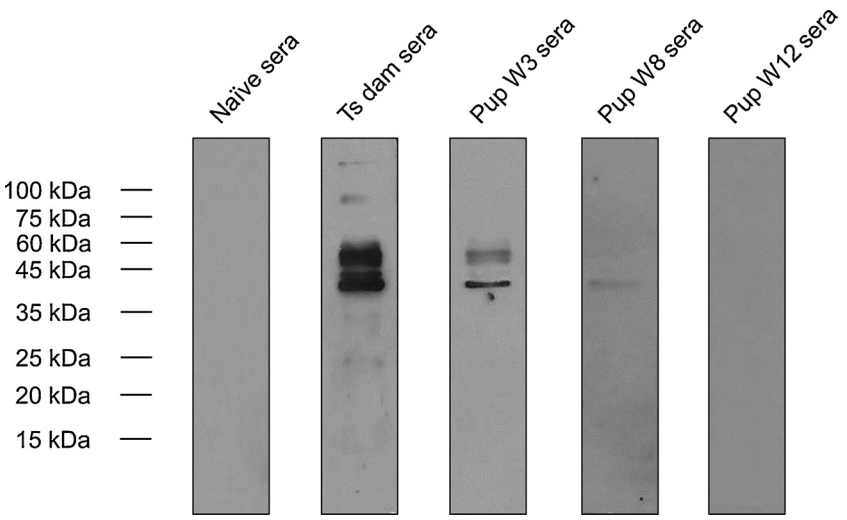

Fig. 4. Polyclonal antibodies from sera successfully detect *T. spiralis* ES antigen at various time points. Polyclonal antibodies from naïve rat, *T. spiralis*-infected dam, week 3 pup, week 8 pup, and week 12 pups were used to probe the ES antigens. Successful detection of ES antigen components was observed from the dam, week 3 pup, and week 8 pup sera whereas naïve rat and week 12 rat sera failed to demonstrate antigen-antibody binding.

infection with encapsulating *Trichinella* species such as *T. spiralis* has been documented to be a mixed Th1/Th2 response with Th2 dominance (Dvoroznakova et al., 2011), which reflects the immunoglobulin profile observed from the current study. In this regard, antibody transmission to the pups of *T. spiralis*-infected dams via breastmilk and its persistence until week 8 conferred the pups much-required resistance against subsequent parasitic infection. One possible explanation for the lack of IgG2 levels may arise from the fact that IgG2 immunoglobulins generally possess a short hinge region as well as a having a distinct structural isoform, which ultimately results in inefficient placental transmission (Einarsdottir et al., 2014). As such, breastmilk ingestion remains the predominant source of IgG2 subclasses, which drastically faded post-weaning on week 3. Our findings herein are supported by the results of several clinical trichinellosis reports, which documented the presence of *T. spiralis* antigen-specific antibodies in newborns delivered by pregnant women (Nunez et al., 2008; Saracino et al., 2016).

Previous study by Kumar et al (1990) stated that maternal immunity transfer to its progeny is the result of breastfeeding process and not due to placental antibody transfer, whereas others have documented that immunity transfer via the placenta might occur to a small extent (Duckett et al., 1972; Perry, 1974). Our serum immunoglobulin profiles have revealed that sera from pups that have not been breastfed yet retained *T. spiralis*-specific antibodies reacting with *T. spiralis* ES antigen, indicating that resistances against *T. spiralis* infection in pups can be acquired partially by *T. specific* antibody transmission through the placenta from *T. spiralis*-infected dam. The results are further supported by the strikingly similar hemochorial structure of placenta found in rodents and humans, both of which enables the transplacental transfer of maternal antibody (Niewiesk, 2014). Combined, these results indicate that significant portions of anti-*T. spiralis* antibody transmitted to pups are of transplacental origin and these persist in the pup's circulatory system to provide resistance against *T. spiralis* infection.

Intestinal IgG results were strikingly similar to those of serum IgG data, indicated by heightened intestinal IgG response at week 3, 4 dpi that waned to normal levels by week 8. On the contrary, no significant differences were noticed between the groups for intestinal IgA, which may signify that mucosal IgA may not contribute much to passive immunity against *T. spiralis* infection. In this regard, maternal antibodies acquired from the placenta and/or breastmilk contributed to enhanced intestinal worm expulsion during the early stages of infection, which faded upon cessation of breastfeeding. It is noteworthy that the rapid intestinal worm expulsion characteristic of rodents may not be applicable to other mammals. For instance, swine models demonstrated a more delayed and gradual worm expulsion (Murrell, 1985). Compared to swine models, rodent models were more suitable for our purposes primarily due to extremely short gestation period and ease of handling. Although *T. spiralis* rodent model may pale in comparison to other models such as *Sus scrofa* or *Vulpes vulpes*, the authors feel that studies done in rodents should not be neglected as they contribute substantially to the persistence of trichinellosis, whether it be domestic or sylvatic.

Eosinophil profiles from *T. spiralis* infections were consistent with the previous findings, which reported the highest eosinophil levels between weeks 2 and 3 (Lee and Best, 1983; Wakelin and Donachie, 1983). Re-evaluation of the role of eosinophils during *T. spiralis* infections has revealed that eosinophils promote parasitic survival rather than protecting the host from parasitic infection, at least during primary infection in murine models (Fabre et al., 2009; Gebreselassie et al., 2012). The level of eosinophils observed from pups were significantly less than those of naïve-challenge pups at both week 3 and week 8, which correlates with host resistance against *T. spiralis* infection. One possible explanation for these results may be antibody-dependent cell-mediated cytotoxicity (ADCC) involving eosinophils. Earlier works have documented eosinophil-mediated *T. spiralis* destruction (Kazura and Grove, 1978) and in line with this notion, we hypothesize that the partial reduction of eosinophil could have resulted from initial immune response exerted by the host, which perished after

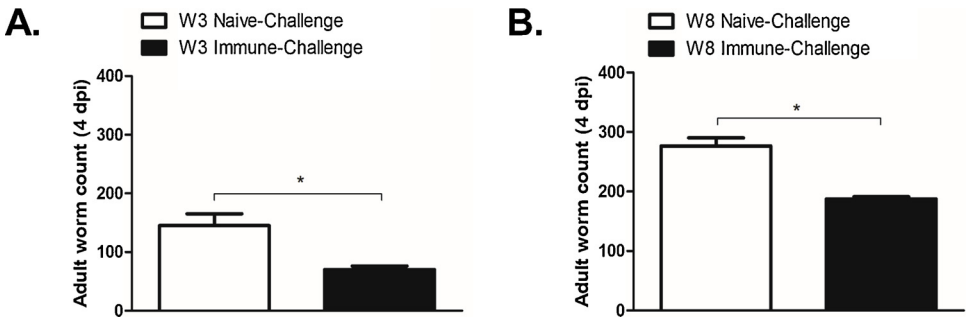

Fig. 5. Adult worm burden reduction post-*T. spiralis* infection. Rat pups were orally infected with 500 *T. spiralis* muscle larvae on weeks 3 and 8. After 4 dpi, adult worms from the small intestines were counted under the microscope. Data are expressed as mean ± SEM from three independent experiments. P value was determined by unpaired Student's *t*-test (*$P <$ 0.05) for statistical significance.

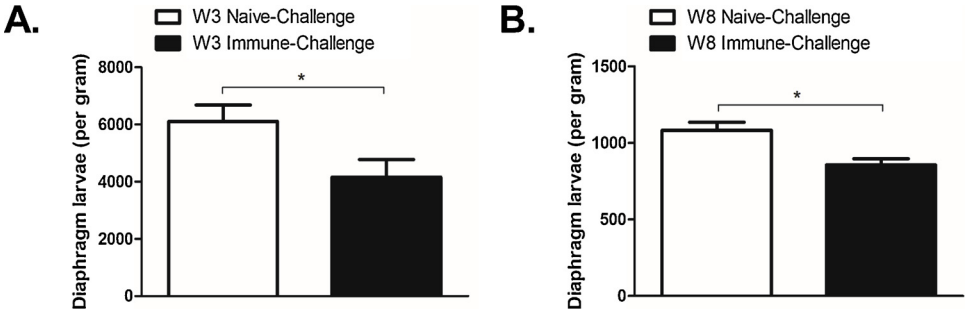

Fig. 6. Muscle larvae burden reduction following *T. spiralis* infection. Pups were infected with 500 *T. spiralis* muscle larvae through the oral route at weeks 3 and 8. On day 28, pups were sacrificed and muscle larvae encysted in the rat diaphragm were counted. Larvae were normalized to per gram of tissue and data are expressed as mean ± SEM from three independent experiments. P value was determined by unpaired Student's *t*-test (*$P < 0.05$) for statistical significance.

degranulation process. These results, indicated by the weakened eosinophilia in pups at various time points post-infection, suggests that lesser eosinophilia in pups nurtured by *T. spiralis*-infected dams hindered parasite survival, thereby enabling pups to cope better against *T. spiralis* infection.

Western blot results of *T. spiralis* ES antigen revealed interesting results, of which proteins near 40 to 60 kDa appeared to react strongly with pup sera. The membrane probed with the infected dam sera detected multiple bands, similar to the results displayed in the study by Appleton et al. (1988), whereas pup sera were only capable of detecting the bands within 40 to 60 kDa range. The pup sera at week 3 and week 8 were capable of detecting *T. spiralis* ES antigen components, whereas the week 12 sera were unable to do so. The weak band detected near 45 kDa from week 8 indicates persisting resistance against *T. spiralis*, which would diminish further and completely disappear by week 12. Previous investigations by Duckett et al. (1971, 1972) have reported that by six weeks after birth, no significant difference in immunity between pups from immunized dams and its counterparts were present. In contrast, immunity in pups persisted even after week 8 as demonstrated by the worm burden reductions in our study. This can be attributed to differences in infection dose since immune responses to *T. spiralis* infection is correlated with infection in a dose-dependent manner (deVos et al., 1992; Franssen et al., 2011). The current study used 500 larvae for infection, whereas 100 larvae were used in the aforementioned study, indicating the persistence of higher antibody levels that contributed to host defense against parasitic infection.

5. Conclusion

In summary, the present study has demonstrated that the immunoglobulin profiles from pups nurtured by *T. spiralis*-infected dams are effective up to approximately 8 weeks for conferring resistance against *T. spiralis*. ES antigen protein detected near 45 kDa, which were detectable up to week 8 in pups, may have significantly contributed to resistance. Future works involving identification of this ES antigen component and its role in immunity would be interesting and valuable to broadening our current understanding of *T. spiralis* immunity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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