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Type 2 immune-inducing helminth vaccination maintains protective efficacy in the setting of repeated parasite exposures

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ABSTRACT

Animal studies have demonstrated that helminth vaccines which induce type 2 immune responses can be protective. To date, however, such vaccines have not been tested against repeated parasite challenges. Since repeated antigenic challenge of patients with allergic disease results in immunologic tolerance, we hypothesized that a helminth vaccine which induces type 2 immune responses may lose its protective efficacy in the setting of repeated parasite exposures (RPEs). To test this hypothesis, we examined whether RPEs induce immunological tolerance and reduce the effectiveness of a type 2 immune-inducing vaccine. BALB/c mice vaccinated against *Litomosoides sigmodontis*, a filarial nematode of rodents, were repeatedly exposed to irradiated larvae for 2 or 8 weeks or to non-irradiated infectious larvae for three months.

Vaccination-induced parasite-specific IgE levels, parasite antigen-driven basophil interleukin 4 (IL-4) release, and Th2 skewing of the cellular immune response remained stable in the face of RPEs. Furthermore, RPEs in vaccinated mice did not augment immunoregulatory responses, as parasite antigen-driven cellular proliferation, production of IL-10, and frequencies of $CD4^+CD25^+FoxP3^+$ regulatory T-cells were not altered by RPEs. Challenge infections with infectious L3-stage larvae resulted in lower worm burdens in vaccinated mice given RPEs than in vaccinated controls. These results demonstrate that vaccines which induce type 2 immune responses can maintain their efficacy in the setting of repeated parasite exposures.

1. Introduction

Helminths infect more than one billion people, primarily in developing regions in Sub-Saharan Africa, Asia and the Americas [1,2]. Because of increasing appreciation for the substantial morbidity caused by these infections, including growth stunting, anemia, malnutrition, and impaired cognition [1,3,4], in the past several years there has been renewed interest in developing vaccines against human helminth infections.

Type 2 immune responses are characterized by the development of antigen-specific IgE, and production of type 2 cytokines such as IL-4 and IL-13. Research efforts have found that induction of type 2 immune responses and antigen-specific IgE may be desirable for the creation of effective helminth vaccines. Protection against helminth challenge after vaccination with irradiated parasites is associated with production of type 2 cytokines in animal models of hookworm and filaria infection [5,6]. Similarly, high titers of parasite-specific IgE antibodies in people are associated with partial protection against reinfection with *Schistosoma japonicum* [7] and hookworm vaccine studies demonstrate that host IgE responses against hookworm antigen correlate with increased protection [8]. Additionally, IgE has been shown to be necessary for vaccine efficacy against *Haemonchus contortus*, a blood-feeding nematode of sheep [9], and for vaccine protection in the *Onchocerca* murine model of filariasis [10]. Because of these findings, consideration has been given towards developing helminth-specific vaccines which induce parasite-specific IgE responses [11].

To date, helminth vaccines that induce type 2 immunity and IgE responses have only been tested against a single challenge infection. A theoretical concern of an IgE-inducing vaccine, however, is that the protective efficacy of an IgE-driven vaccine response could possibly decrease in the setting of repeated parasite exposures in a manner akin to that observed in desensitization protocols of patients with allergen-specific IgE. In allergen-specific immunotherapy (SIT), repeated allergen exposures result in clinical tolerance towards allergen. Immunologic changes associated with SIT include decreases in allergen-specific IgE, increases in allergen-specific IgG4, an allergen-specific T-cell shift from Th2 to Th1, and peripheral T-cell tolerance towards allergen due to increased IL-10 production from antigen-specific and $CD4^+CD25^+$ regulatory T-cells [12,13]. Thus, as Th2 responses and IgE have been shown to be involved in protection to filarial parasites [10,14–19] and as induction of immunotolerance facilitates helminth survival [20,21], we hypothesized that repeated parasite exposures (RPEs) may

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decrease the efficacy of a type 2 immune-inducing vaccine. Such a phenomenon would have important implications for helminth vaccine design since individuals in endemic areas are repeatedly exposed to parasites.

For our studies, we chose to use the *Litomosoides sigmodontis* murine model of filariasis [22,23] in which a series of three vaccinations with irradiated larvae confers protection against challenge infection with infectious-stage L3 larvae [24]. This vaccination regimen induces a type 2 immune response with increases in type 2 cytokines such as IL-4 [5] and, as we show in this study, elevated levels of parasite-specific IgE. Testing of our hypothesis was done by evaluating whether repeated injections of either irradiated infectious-stage (L3) *L. sigmodontis* larvae for 2 or 8 weeks or infectious L3 larvae for 3 months substantially alter the immune responses and protective efficacy of the type 2 immune-inducing vaccination regimen against *L. sigmodontis*.

2. Materials and methods

2.1. Mice and parasites

Female BALB/c and C57BL/6 mice (NCI Mouse Repository, Frederick, MD) were maintained at the Uniformed Services University of the Health Sciences (USU) animal facility. Experiments were performed with 4–6-week-old mice under a protocol approved by the USU Institutional Animal Care and Use Committee. Infectious-stage *L. sigmodontis* L3 larvae were isolated by lavage from the pleural cavity of 4-day infected jirds (*Meriones unguiculatus*, obtained from TRS Laboratory Inc., Athens, GA) as described previously [25].

2.2. Repeated parasite exposures (RPEs) with irradiated, cryopreserved L3 larvae

BALB/c mice were vaccinated with three weekly subcutaneous injections of either 25 irradiated L3s (450 Gy, cobalt 60 irradiator) in media (RPMI-1640, Mediatech, Herndon, VA) or media alone. Two weeks after the third vaccination, mice were given RPEs by subcutaneous injection of five irradiated, cryopreserved L3s or media as control every other day for 2 weeks or three times a week for 8 weeks. L3s were cryopreserved in RPMI-1640 containing 0.27 M sucrose (Sigma, St. Louis, MO) and 6% DMSO (Sigma). Use of cryopreserved larvae for RPEs ensured a regular supply of worms available for injection every 2–3 days. Because irradiated larvae migrate through the tissues but do not develop into adult worms, use of irradiated, cryporeserved L3 larvae enabled us to easily distinguish worms acquired during RPEs from adult worms acquired by challenge infection.

Three days after the last RPE mice were sacrificed for immunological studies. Additionally, some BALB/c mice that received 2 or 8 weeks of RPEs were challenged with 40 infectious L3s and euthanized 56 days later for enumeration of adult worms by pleural lavage.

2.3. Repeated parasite exposures with infectious L3 larvae

In an additional experiment BALB/c mice were vaccinated as described above and 2 weeks after the final vaccination repeatedly infected with five live, fully infectious L3 larvae ($n = 10$) that were isolated from the pleural cavity of recently infected jirds or RPMI as control ($n = 9$). Mice were repeatedly parasite exposed with infectious L3 larvae three times per month for 3 months with a total of nine repeated parasite exposures. Two weeks after the last repeated parasite exposure mice were challenged with 40 infectious L3 larvae and euthanized 20 days after challenge. Numbers of fourth stage (L4) larvae in the pleural space at that timepoint represented worms from the final challenge infection, whereas any adult (L5) worms recovered at that timepoint were acquired by RPEs (34 days after the last RPE).

In a single experiment naïve C57BL/6 mice were infected with a total of 40 infectious *L. sigmodontis* L3 larvae by either a single challenge with 40 L3s ($n = 4$) or eight challenges of five L3s every other week ($n = 4$). At day 62 after the last/single challenge, mice were sacrificed, worm burdens determined, and immunological studies performed.

2.4. Repeated administration of parasite antigen

In addition to testing RPEs with living worms, in a separate experiment we analyzed development of immunologic tolerance in BALB/c mice that were vaccinated with three weekly intraperitoneal injections of 100 μg of *L. sigmodontis* adult worm antigen (LsAg, prepared as described in Section 2.5) adsorbed to alum (Thermo Fisher Scientific Inc., Waltham, MA). Two weeks after the last vaccination mice were injected three times per week with 5 μg of LsAg ($n = 10$) or PBS as control ($n = 10$) for a total of 8 weeks. Blood was collected from mice 2 weeks into the course of repeated LsAg/PBS injections and mice were euthanized after 8 weeks of LsAg/PBS injections to obtain blood and splenocytes for immunological studies.

2.5. L. sigmodontis adult worm antigen (LsAg)

Frozen adult *L. sigmodontis* worms were lyophilized, resuspended in PBS and stirred overnight at 4 °C. After centrifugation (750 × *g*, 10 min, 4 °C) the supernatant was collected. The pellet was stirred again overnight, centrifuged, and the supernatant combined with the first supernatant. After a final centrifugation at 5300 × *g* for 30 min at 4 °C, supernatant was collected, sterile filtered, and the protein content measured with the BCA Protein Assay kit (Pierce, Rockford, IL).

2.6. Cellular proliferation

Three days after the last RPE, spleen, brachial and axillary lymph node cells were isolated and single cell suspensions processed. Red blood cell lysis was performed with spleen cells (ACK Lysing Buffer, Invitrogen Inc., Carlsbad, CA). Cells were plated as triplicates at 2×10^5 cells in 100 μl enriched media (Iscove's Dulbecco modified medium (Mediatech) including 10% fetal calf serum (Valley Biomedical, Winchester, VA), 1% L-glutamine (Mediatech), 1% insulin-transferrin-selenium (Invitrogen Inc.) and 80 μg/ml gentamicin (Invitrogen Inc.)). Cells were stimulated with 20 μg/ml LsAg or 5 μg/ml anti-CD3 (eBioscience, San Diego, CA) and 2 μg/ml anti-CD28 (eBioscience) and cultured at 37 °C, 5% CO_2. After 2 days, BrdU was added and cells were cultured for an additional 16 h. Cell proliferation was assessed using a BrdU chemiluminescent assay per the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

2.7. Flow cytometric detection of regulatory T-cells and intracellular cytokine production by T-cells and basophils

Three days after the last RPE, spleen cells were isolated and stimulated as described above, at a total of 1×10^7 cells in 5 ml enriched media. After two hours of incubation, BD GolgiStop (BD Biosciences, San Jose, CA) was added and cells were incubated for an additional four hours. Cells were prepared for flow as described previously [26]. Collected cells were blocked for one hour with PBS/1% BSA (Sigma), followed by fixation and permeabilization overnight in fix/perm-buffer (eBioscience). After two washing steps, cells were stained for flow cytometry with rat-anti-mouse CD4 PerCP (BD Biosciences), rat-anti-mouse FoxP3 FITC (eBioscience), rat-anti-mouse