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Early development of cytotoxic T lymphocytes in neonatal foals following oral inoculation with *Rhodococcus equi*

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Abstract

Rhodococcus equi is an important respiratory pathogen of young foals for which a vaccine has long been sought. Two major impediments to effective vaccination are the functionally immature type I immune responses of neonatal foals and early exposure to the bacterium via the environment. Despite these obstacles, it appears that under specific circumstances foals can develop a protective immune response. In this study we investigated the protective mechanisms behind oral inoculation of foals with virulent *R. equi* bacteria. Two foals receiving an oral inoculum demonstrated accelerated development of *R. equi* specific cytotoxic T lymphocytes (CTL) as evidenced by significant lysis of *R. equi* infected, ELA-A mismatched cells at 3 weeks of age. As in a previous study, CTL were not detected until 5–6 weeks of age in two control foals. At each time point the ability of foal peripheral blood mononuclear cells (PBMC) to produce IFN- γ following stimulation with live *R. equi* or extracted cell wall lipids was similar to that of an adult horse control and between foals, regardless of treatment. These results provide a potential mechanism of protection which has previously been shown to occur following oral inoculation, and suggest that the early detection of CTL may be a useful marker for induction of protective immunity.

Keywords

Rhodococcus equi; oral inoculation; neonatal; foal

Introduction

Rhodococcus equi is an important bacterial pathogen of foals between 1–5 months of age. The organism, which produces life threatening pyogranulomatous pneumonia, is ubiquitous in equine environments (Cohen et al., 2008; Hondalus, 1997; Takai, 1997). As a result, foals are exposed and possibly infected in the first weeks of life. In contrast, adult horses are immune and this protection is associated with a type 1 memory response that operates throughout life (Hines et al., 2001; Hines et al., 2003; Patton et al., 2004). The protective response involving both CD4+ and CD8+ T lymphocytes was originally demonstrated in mice (Kanaly et al., 1993, 1996); later experiments suggest a similar mechanism is occurring in adult horses. *R. equi*-specific CD4+ Th1 cells activate macrophages through the production of IFN- γ , while CD8+ cytotoxic T lymphocytes (CTL) recognize and lyse *R.*

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Conflict of Interest Statement:

There are no conflicts of interest.

equi-infected cells in an MHC class I unrestricted fashion (Hines et al., 2001; Hines et al., 2003; Patton et al., 2004). Antibody may also play a role in immunity, although it is likely insufficient without type 1 cellular responses.

The unique age restricted susceptibility of foals to *R. equi* is postulated to reflect their lack of immunologic memory (i.e. their naïve status) and the diminished immunologic capacities that characterize early life in virtually all mammals. In general, neonates and perinates have decreased abilities to mount Th1 responses and to generate CTL compared to adults (Adkins et al., 2004; Boyd et al., 2003; Breathnach et al., 2006; Liu et al., 2009). In some species, there is an initial Th2 bias that is likely an outcome of maternal conditions necessary to maintain pregnancy (Bengt, 1999; Raghupathy, 2001). A number of similar immunologic deficiencies have been identified in young foals, including an apparent decrease in their ability to produce IFN- γ and the absence of *R. equi*-specific CTL during the first 3–6 weeks of life (Breathnach et al., 2006; Liu et al., 2009; Patton et al., 2005). The observations that foals may be infected shortly after birth during a time when the immune system is both naïve and relatively immature has led some veterinarians to propose that active immunization will not be an effective strategy for prevention of rhodococcal pneumonia.

There is, however, evidence to suggest that young foals are capable of mounting protective immune responses to *R. equi*. For example, even on endemic farms where morbidity is high, a majority of foals resist infection and/or clear bacteria to become immune (Chaffin et al., 2003). Likewise, a recent study showed that intrabronchial challenge of neonatal foals with *R. equi* resulted in a strong Th1 response as evidenced by IFN- γ mRNA expression that exceeded that in adults receiving a similar challenge (Jacks et al., 2007). The authors suggested that similar to human neonates immunized with *Mycobacterium bovis* BCG, foals have the ability to mount adult-like type 1 immune responses so long as the stimulus is appropriate. Variables such as the nature of the antigen, antigen dose, route, adjuvant, etc are likely critical. In support of this idea, oral inoculation of foals with virulent *R. equi* during the first 2 weeks of life was shown to provide strong protection against a subsequent intrabronchial challenge (Chirino-Trejo et al., 1987; Hooper-McGrevy et al., 2005). These immunization trials are the most promising to date. Although inoculation of virulent bacteria is not an acceptable real world practice, the studies suggest that the use of an oral route and live bacteria may be the keys. Unfortunately the only immunologic parameter measured was antibody responses.

In the pilot study reported here, we revisited the oral inoculation model to characterize the immune responses produced. Our first hypothesis was that the protective oral immunization protocol accelerates the appearance of *R. equi*-specific CTL. Recent work in our laboratory has shown that the MHC class I unrestricted CTL which lyse *R. equi* infected cells recognize lipid antigens found in the unique cell wall of *R. equi* (Harris et al., 2010). To further investigate the role of lipid antigens in immunity to *R. equi*, we also examined the ability of *R. equi* cell wall lipids to stimulate transcription of IFN- γ and IL-4. Our second hypothesis was that oral immunization of foals with live bacteria induces adult-like levels of IFN- γ expression in lymphocytes stimulated with *R. equi* lipid and live *R. equi*.

Materials and Methods

Oral inoculation

Four Arabian foals were screened at birth with a physical exam, complete blood count, and IgG level, and were determined to be healthy. Two foals were controls and two were orally infected with virulent *R. equi* using previously published methods (Hooper-McGrevy et al., 2005). Briefly, at 2, 7, and 14 days of age a single inoculum containing 1×10^{10} CFU of *R. equi* ATCC 33701 diluted in 100 ml phosphate buffered saline (PBS) or 100 ml of PBS

alone (negative control) was administered at each time point through a nasogastric tube. The tube was then flushed with 100 ml of distilled water, kinked, and removed. Bacterial concentration was estimated with an optical density reading and confirmed by plating serial dilutions on brain heart infusion agar. All horse use was approved by the Washington State University institutional animal care and use committee.

Cytotoxicity assay

At 1, 3, 5, and 7 weeks of age, 250 ml of blood was collected from alternating jugular veins and the peripheral blood mononuclear cells (PBMC) were harvested for CTL assays using previously published methods (Harris et al., 2010; Patton et al., 2004). Briefly, effector cells were derived by stimulating 10^8 PBMC (average 20% monocytes and 80% lymphocytes) with 6×10^6 *R. equi* 33701 (approximately 0.3 multiplicity of infection in monocytes; MOI) for five days at 37°C with 5% CO₂, followed by resting for 2 days without antigenic stimulation. ELA-A (equine MHC class I) mismatched target cells were obtained from a single Hanovarian horse (H68) by eluting adherent peripheral blood adherent cells (PBAC). The effector cells were then added to target cell wells containing PBAC which were previously labeled with ⁵¹Cr (PerkinElmer, Waltham, MA). Lysis of target cells infected with 5 MOI of virulent *R. equi* in comparison to uninfected control target cells was calculated by chromium release assay using the following formula: $[(E - S)/(T - S)] \times 100$, where E is the mean of three test wells, S is the mean spontaneous release from three target cell wells without effector cells, and T is the mean total release from three target cell wells with 2% Triton X-100 (Sigma-Aldrich, St. Louis, MO). As in previous equine CTL assays, significant lysis was defined as 3 standard errors above the uninfected negative control target cell value, and greater than 10% total lysis (McGuire et al., 1994; Patton et al., 2004). To increase the sample size for statistical analysis between the foals at the 3 week time point, CTL assays from a previous season's foals (Patton et al., 2005) were added and compared in a Fisher's exact test. These foals had no experimental inoculation with *R. equi* during the first 3 weeks of life and so were included as additional controls.

Cytokine PCR

A total of 3×10^7 PBMC in antibiotic-free complete medium were stimulated with 2×10^6 live *R. equi* 33701, 800 µg of *R. equi* lipid extract, or unstimulated (negative control) using previously published methods (Harris et al., 2010). Following an 18 hour incubation RNA was extracted using the RNeasy plus mini kit (Qiagen, Valencia, CA). Expression of equine IFN-γ, IL-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cells was determined by performing real-time PCR with previously described primers (Lopez et al., 2002). Transcript levels were determined for each sample by comparing the threshold cycle values of IFN-γ, IL-4, and GAPDH to the corresponding plasmid standard curves. Amplification of plasmid DNA also confirmed that the primers and PCR reactions were working. To confirm that no genomic DNA was present, portions of the DNase treated RNA samples were used in cDNA reaction mixtures without reverse transcriptase. In each real-time PCR, reaction mixtures containing no cDNA template were included to control for extraneous DNA within the reagents. As previously published, the transcript levels of IFN-γ and IL-4 were normalized by dividing the copy numbers by the constitutively expressed housekeeping gene GAPDH, and were logarithmically transformed (Harris et al., 2010). For statistical analysis, each assay was run in triplicate; the means of the triplicates were logarithmically transformed and compared across the horses using a one way ANOVA.

Results and Discussion

In a previous study, the age related susceptibility to *R. equi* was found to correlate with the time frame in which foals lack CTL. At 3 weeks of age, all foals lacked *R. equi* specific

CTL. These MHC class I-unrestricted CTL were present in some but not all 6 week old foals (60%), whereas all foals (100%) had CTL activity at 8 weeks of age (Patton et al., 2005). Once present the CTL were able to lyse *R. equi* infected target cells in an MHC class I-unrestricted manner, similar to adult horses. The deficiency in the foals was constrained to the CTL rather than target cells, as adult CTL effectively lyse *R. equi* infected target cells obtained from neonatal foals (Patton et al., 2005). In the current pilot study, two neonatal foals (A2260 and A2263) were orally inoculated with live *R. equi* to test whether oral inoculation would induce the appearance of CTL at an earlier age. This oral inoculation protocol was previously shown to provide complete protection to foals subsequently challenged with *R. equi* (Hooper-McGrevy et al., 2005). When compared against two control foals (A2257 and A2258), there was an accelerated development of *R. equi* specific CTL (Fig. 1). At week 1, no foal had any detectable *R. equi* specific CTL. At week 3, the orally inoculated but not the control foals developed CTL which lysed MHC class I mismatched, *R. equi* infected target cells. At weeks 5 and 7, all foals in the pilot study had developed CTL. A control mare (M186) tested in parallel to confirm the assay was working had statistically significant lysis of *R. equi* infected, but not uninfected, target cells at all time points. In order to provide for statistical power at the key 3-week time point, the CTL results from the two orally inoculated foals were also compared to foals in the previous study (Patton et al., 2005). When the four foals from the previous study were added to the control group a statistically significant association was reached between the treatment group and development of CTL activity using a Fisher's exact test ($P=0.0357$). While combining the two groups of foals has resulted in statistical significance, performing a larger study in the future will be needed to negate the low statistical power inherent within a pilot study utilizing a small sample size.

As the putatively protective type 1 immune responses also encompass cytokine production, IFN- γ , a hallmark Th1 cytokine, and IL-4, a Th2 cytokine, were evaluated. Because of the recent evidence showing that equine CTL recognize *R. equi* lipid antigens, we also measured IFN- γ and IL-4 responses to an *R. equi* lipid extract (Harris et al., 2010). At each time point, PBMC from the two foals orally inoculated with *R. equi* and the two uninoculated controls were stimulated for 18 hours with live *R. equi* or extracted cell wall lipids, and the levels of mRNA produced then quantified using real time RT-PCR (Fig. 2). All foals appeared capable of producing IFN- γ at levels which were similar to an adult mare control, in response to either whole *R. equi* or extracted cell wall lipids. There was no significant difference between the orally inoculated and control foals, and little variation between the different time points for each foal. IL-4 production was low following stimulation at all time points for both the foals and mare.

Because of its potential to produce disease and increase shedding of bacteria, inoculation of virulent *R. equi* is not an acceptable method of immunization. However, this study provides further proof of concept for both the oral route and an immunization approach in general. As in previous studies, unimmunized control foals developed *R. equi* specific, MHC class I unrestricted CTL between 3 and 6 weeks of age – likely reflecting normal environmental acquisition of *R. equi* and initiation of the protective responses that operate throughout adult life in horses (Patton et al., 2005). This “natural vaccination” may also occur through an oral route. Foals are normally coprophagic during the first months of life - a practice thought to be important in the establishment of normal intestinal flora. The gut microbial flora, in turn, is a primary stimulus for driving the post-natal maturation of the immune system, notably Th1 responses (Bengt, 1999). As most mares shed virulent *R. equi* in their feces (Buntain et al., 2010), ingestion of feces by foals may target the bacterium to enteric M cells, the unique dendritic cells that populate the intestine, and the common mucosal immune system (Iwasaki, 2007). Therefore, an oral route may also be the key to active immunization of neonates.

Importantly, the induction of CTL activity between 1 and 3 weeks of age in association with a protective immunization protocol also supports the hypothesis that *R. equi* specific CTL play a role in protective immunity. Other evidence includes the progressive appearance of CTL as foals mature and the universal presence of CTL in immune adult horses (Patton et al., 2004; Patton et al., 2005). It will be difficult to determine experimentally whether induction of MHC class I unrestricted CTL is absolutely required for immunity to *R. equi* or simply a “marker” of developing type 1 responses. A larger study is needed, but the ability to induce CTL in the first weeks of life may be a relevant correlate of immunity that also provides a useful way to screen potential vaccines. In other words, a vaccine capable of preventing rhodococcal pneumonia would be expected to effectively stimulate the appearance of CTL in very young foals. Because of early environmental exposure no vaccine is likely to induce sterile immunity. However, it may be very possible to generate the responses that prevent clinical disease –i.e. to drive the response towards the protective type1/Th1 phenotype.

The observation that *R. equi* specific CTL recognize unique bacterial cell wall lipids suggests that candidate vaccines should include *R. equi* lipids (Harris et al., 2010). The effectiveness of live virulent bacteria suggests that a modified live strategy may be the best way to accomplish this. In this experiment the IFN- γ mRNA responses of foals to both *R. equi* lipid antigen and live bacteria was an unexpected finding, especially since other studies have suggested that IFN- γ production is deficient in the equine neonate (Breathnach et al., 2006; Liu et al., 2009). However, another recent study showed significant IFN- γ expression by neonatal foals in response to *R. equi* infection (Jacks et al., 2007). Likewise, there are fundamental differences between studies, specifically related to the antigen utilized and duration of infection, which may account for the differences reported. For example, in some studies PMA and ionomycin are employed to stimulate cells (Breathnach et al., 2006). Our method of using lipid antigen and live, virulent bacteria may have allowed for increased IFN- γ expression by cells of the innate immune system, such as $\gamma\delta$ T-lymphocytes, NKT cells, or NK cells (Chang et al., 2007; Eger et al., 2006; Gibbons et al., 2009; Watkins et al., 2008). Resting NKT cells in particular are known to have a memory or partially activated phenotype and to respond rapidly to produce cytokines, including IFN- γ (Barral and Brenner, 2007). NKT cells represent a pool of CD1d-restricted cells that can be activated *en masse* via their semi-invariant TCR (T cell receptor) and recognition of microbial lipids (Barral and Brenner, 2007). NKT cells can also be activated indirectly via Toll-like receptors (TLR). *R. equi* likely has several ways to activate TLR, including lipids that stimulate antigen presenting cells via TLR-2 (Darrah et al., 2004). These results highlight the fact that *R. equi* lipids may also provide a critical adjuvant effect, thus providing additional support for an immunization strategy that encompass bacterial lipids. Future trials will involve testing the ability of orally delivered, attenuated live bacteria to induce type 1 immune responses, including CTL, during the first weeks of life.

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References

- Adkins B, Leclerc C, Marshall-Clarke S. Neonatal Adaptive Immunity Comes of Age. *Nature Reviews*. 2004; 4:553–564.
- Barral DC, Brenner MB. CD1 antigen presentation: how it works. 2007; 7:929–941.

- Bengt B. The intrauterine and postnatal environments. *The Journal of allergy and clinical immunology*. 1999; 104:1119–1127. [PubMed: 10588990]
- Boyd NK, Cohen ND, Lim WS, Martens RJ, Chaffin MK, Ball JM. Temporal changes in cytokine expression of foals during the first month of life. *Veterinary Immunology and Immunopathology*. 2003; 92:75–85. [PubMed: 12628765]
- Breathnach CC, Sturgill-Wright T, Stiltner JL, Adams AA, Lunn DP, Horohov DW. Foals are interferon gamma-deficient at birth. *Veterinary Immunology and Immunopathology*. 2006; 112:199–209. [PubMed: 16621024]
- Buntain S, Carter C, Kuskie K, Smith J, Stepusin R, Chaffin MK, Takai S, Cohen N. Frequency of *Rhodococcus equi* in Feces of Mares in Central Kentucky. *Journal of Equine Veterinary Science*. 2010; 30:191–195.
- Chaffin MK, Cohen ND, Martens RJ, Edwards RF, Nevill M. Foal-related risk factors associated with development of *Rhodococcus equi* pneumonia on farms with endemic infection. *Journal of the American Veterinary Medical Association*. 2003; 223:1791–1799. [PubMed: 14690209]
- Chang SR, Wang KJ, Lu YF, Yang LJ, Chen WJ, Lin YH, Chang HH, Wang SL. Characterization of Early Gamma Interferon (IFN- γ) Expression during Murine Listeriosis: Identification of NK1.1+ CD11c+ Cells as the Primary IFN- γ -Expressing Cells. *Infect Immun*. 2007; 75:1167–1176. [PubMed: 17158904]
- Chirino-Trejo JM, Prescott JF, Yager JA. Protection of Foals Against Experimental *Rhodococcus equi* Pneumonia by Oral Immunization. *Canadian Journal of Veterinary Research*. 1987; 51:444–447. [PubMed: 3453264]
- Cohen ND, Carter CN, Scott HM, Chaffin MK, Smith JL, Grimm MB, Kuskie KR, Takai S, Martens RJ. Association of soil concentrations of *Rhodococcus equi* and incidence of pneumonia attributable to *Rhodococcus equi* in foals on farms in central Kentucky. *American Journal of Veterinary Research*. 2008; 69:385–395. [PubMed: 18312138]
- Darrah PA, Monaco MCG, Jain S, Hondalus MK, Golenbock DT, Mosser DM. Innate Immune Responses to *Rhodococcus equi*. *J Immunol*. 2004; 173:1914–1924. [PubMed: 15265925]
- Eger KA, Sundrud MS, Motsinger AA, Tseng M, Kaer LV, Unutmaz D. Human Natural Killer T Cells Are Heterogeneous in Their Capacity to Reprogram Their Effector Functions. *PLoS ONE*. 2006; 1:e50. [PubMed: 17183680]
- Gibbons DL, Haque SFY, Silberzahn T, Hamilton K, Langford C, Ellis P, Carr R, Hayday AC. Neonates harbour highly active $\gamma\delta$ T cells with selective impairments in preterm infants. *European Journal of Immunology*. 2009; 39:1794–1806. [PubMed: 19544311]
- Harris SP, Fujiwara N, Mealey RH, Alperin DC, Naka T, Goda R, Hines SA. Identification of *Rhodococcus equi* lipids recognized by host cytotoxic T-lymphocytes. *Microbiology*. 2010;mic. 0.035915–035910.
- Hines MT, Paasch KM, Alperin DC, Palmer GH, Westhoff NC, Hines SA. Immunity to *Rhodococcus equi*: antigen-specific recall responses in the lungs of adult horses. *Vet Immunol Immunopathol*. 2001; 79:101–113. [PubMed: 11356253]
- Hines SA, Stone DM, Hines MT, Alperin DC, Knowles DP, Norton LK, Hamilton MJ, Davis WC, McGuire TC. Clearance of virulent but not avirulent *Rhodococcus equi* from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4+ and CD8+ T lymphocytes. *Clin Diagn Lab Immunol*. 2003; 10:208–215. [PubMed: 12626444]
- Hondalus MK. Pathogenesis and virulence of *Rhodococcus equi*. *Vet Microbiol*. 1997; 56:257–268. [PubMed: 9226840]
- Hooper-McGrevy KE, Wilkie BN, Prescott JF. Virulence-associated protein-specific serum immunoglobulin G-isotype expression in young foals protected against *Rhodococcus equi* pneumonia by oral immunization with virulent R. equi. *Vaccine*. 2005; 23:5760–5767. [PubMed: 16112256]
- Iwasaki A. Mucosal Dendritic Cells. *Annual Review of Immunology*. 2007; 25:381–418.
- Jacks S, Giguere S, Crawford PC, Castleman WL. Experimental Infection of Neonatal Foals with *Rhodococcus equi* Triggers Adult-Like Gamma Interferon Induction. *Clin Vaccine Immunol*. 2007; 14:669–677. [PubMed: 17409222]

- Kanally ST, Hines SA, Palmer GH. Failure of pulmonary clearance of *Rhodococcus equi* infection in CD4+ T-lymphocyte-deficient transgenic mice. *Infect Immun*. 1993; 61:4929–4932. [PubMed: 8104903]
- Kanally ST, Hines SA, Palmer GH. Transfer of a CD4+ Th1 cell line to nude mice effects clearance of *Rhodococcus equi* from the lung. *Infect Immun*. 1996; 64:1126–1132. [PubMed: 8606068]
- Liu T, Nerren J, Liu M, Martens R, Cohen N. Basal and stimulus-induced cytokine expression is selectively impaired in peripheral blood mononuclear cells of newborn foals. *Vaccine*. 2009; 27:674–683. [PubMed: 19056444]
- Lopez MA, Hines MT, Palmer GH, Alperin DC, Hines SA. Identification of pulmonary T-lymphocyte and serum antibody isotype responses associated with protection against *Rhodococcus equi*. *Clin Diagn Lab Immunol*. 2002; 9:1270–1276. [PubMed: 12414760]
- McGuire TC, Tumas DB, Byrne KM, Hines MT, Leib SR, Brassfield AL, O'Rourke KI, Perryman LE. Major histocompatibility complex-restricted CD8+ cytotoxic T lymphocytes from horses with equine infectious anemia virus recognize Env and Gag/PR proteins. *J Virol*. 1994; 68:1459–1467. [PubMed: 8107209]
- Patton K, McGuire TC, Fraser DG, Hines SA. *Rhodococcus equi*-infected macrophages are recognized and killed by CD8+ T lymphocytes in a MHC class I- unrestricted fashion. *Infect Immun*. 2004; 72:7073–7083. [PubMed: 15557631]
- Patton KM, McGuire TC, Hines MT, Mealey RH, Hines SA. *Rhodococcus equi*-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. *Infect Immun*. 2005; 73:2083–2093. [PubMed: 15784549]
- Raghupathy R. Pregnancy: success and failure within the Th1/Th2/Th3 paradigm. *Seminars in Immunology*. 2001; 13:219–227. [PubMed: 11437629]
- Takai S. Epidemiology of *Rhodococcus equi* infections: a review. *Vet Microbiol*. 1997; 56:167–176. [PubMed: 9226831]
- Watkins MLV, Semple PL, Abel B, Hanekom WA, Kaplan G, Ress SR. Exposure of Cord Blood to *Mycobacterium bovis* BCG Induces an Innate Response but Not a T-Cell Cytokine Response. *Clin Vaccine Immunol*. 2008; 15:1666–1673. [PubMed: 18815231]

Figure 1. Development of *R. equi* specific, MHC class I-unrestricted CTL in foals PBMC from two control foals (Cont-1, Cont-2) and two orally infected foals (Inf-1, Inf-2) were stimulated for 5 days with 0.3 MOI *R. equi*, rested for 2 days, and then tested for CTL activity. MHC mismatched target cells (PBAC) were infected with 5 MOI *R. equi* 33701 for 9 h prior to the addition of effector cells at an E:T ratio of 9:1. An asterisk indicates statistically significant lysis of target cells (>3 standard errors compared to the corresponding uninfected control). Mare 186 is a positive control. Time points are 1, 3, 5, and 7 weeks of age.

Figure 2. Expression of IFN- γ and IL-4 mRNA in response to *R. equi* lipid antigen and live bacteria

PBMC from two control foals and two orally infected (vaccinated) foals were stimulated for 18 hours with live *R. equi* or cell wall lipids. Following stimulation the copy number of IFN- γ and IL-4 was normalized against GAPDH and the fold increase was measured, compared to an unstimulated negative control. Mare M186 is included as a positive control.