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In contrast to other species, α-Galactosylceramide (α-GalCer) is not an immunostimulatory NKT cell agonist in horses

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ABSTRACT

α-GalCer is a potent immunomodulatory molecule that is presented to NKT cells via the CD1 antigen-presenting system. We hypothesized that when used as an adjuvant α-GalCer would induce protective immune responses against *Rhodococcus equi*, an important pathogen of young horses. Here we demonstrate that the equine CD1d molecule shares most features found in CD1d from other species and has a suitable lipid-binding groove for presenting glycolipids to NKT cells. However, equine CTL stimulated with α-GalCer failed to kill cells infected with *R. equi*, and α-GalCer did not increase killing by CTL co-stimulated with *R. equi* antigen. Likewise, α-GalCer did not induce the lymphoproliferation of equine PBMC or increase the proliferation of *R. equi*-stimulated cells. Intradermal injection of α-GalCer in horses did not increase the recruitment of lymphocytes or cytokine production. Furthermore, α-GalCer–loaded CD1d tetramers, which have been shown to be broadly cross-reactive, did not bind equine lymphocytes. Altogether, our results demonstrate that in contrast to previously described species, horses are unable to respond to α-GalCer. This raises questions about the capabilities and function of NKT cells and other lipid-specific T lymphocytes in horses.

1. Introduction

Natural killer T (NKT) cells are a subset of novel T lymphocytes that have characteristics of classic activated or memory T cells and express the NK 1.1 marker (Makino et al., 1995). These cells share features of both the innate and adaptive immune systems (Brennan et al., 2013). NKT cells are found in the circulation and tissues in a primed stage (memory phenotype) that does not require prior contact with foreign antigen (de Lalla et al., 2008). Upon activation, NKT cells rapidly release significant amounts of both T helper type 1 (Th1) and Th2 cytokines. Although NKT cells are mostly $CD4^+$ or $CD4^-CD8^-$ (double negative) and can serve as helper cells, they can also exert potent cytotoxic effects (Gapin et al., 2001; Kawano et al., 1998; Metelitsa et al., 2001).

In contrast to classical T lymphocytes, which recognize antigens presented via MHC molecules, NKT cells are CD1d-restricted (Bendelac et al., 1995). CD1d is a member of the CD1 family, a group of non-polymorphic MHC class I-like surface glycoproteins that contain a hydrophobic binding groove and are specialized in their ability to present lipid-based antigens to T cells (Moody et al., 2005). The CD1d gene is broadly conserved across species and is found in all mammals studied so far with the exception of marsupials (Baker and Miller, 2007).

A wide variety of natural exogenous ligands have been shown to bind CD1d and then activate NKT cells (Fischer et al., 2004; Kinjo et al., 2005, 2006, 2011). However, most studies with NKT cells are performed with a synthetic analogue of a glycolipid originally derived from the marine sponge *Agelas mauritianus*, α-galactosylceramide (α-GalCer). This molecule contains a saturated C26 fatty acyl chain and a C18 phytosphingosine base (Kawano et al., 1997). In numerous species, including humans (Spada et al., 1998), pigs (Thierry et al., 2012), rats (Monzon-Casanova et al., 2010), and mice (Kawano et al., 1997), α-GalCer binds CD1d molecules and is presented to specialized invariant NKT cells (iNKT cells, also called Type 1 NKT cells) that recognize the glycolipid via a highly conserved T cell receptor (TCR) α-chain (Borg et al., 2007). As a result, α-GalCer acts as a strong NKT cell agonist. In mice, a single injection of α-GalCer can activate the NKT cell population, stimulating the immediate release of

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cytokines including IFN-γ, IL-4, TNF, IL-2, and IL-10 (Fujii et al., 2003; Nishimura et al., 2000; Reilly et al., 2012). These cytokines simultaneously activate other cells including classic T cells, NK cells, monocytes, and B cells. This leads to the production of more cytokines and chemokines, giving α-GalCer/NKT cells a potent immunomodulatory capacity (Subrahmanyam and Webb, 2012).

The capacity of NKT cells to regulate the immune response has been shown to improve the outcome of numerous vaccines (Chackerian et al., 2002; Gonzalez-Aseguinolaza et al., 2002; Huang et al., 2008; Sada-Ovalle et al., 2010). In these experiments, α-GalCer acts as an effective vaccine adjuvant by modulating cytokine levels, boosting cytotoxic T lymphocyte (CTL) and humoral responses, as well as enhancing antigen presentation by dendritic cells. A recent study in mice showed that immunization with *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) with α-GalCer incorporated into its membrane increased protection against challenge with virulent *M. tuberculosis* when compared to immunization with unmodified BCG (no α-GalCer) (Venkataswamy et al., 2009).

The presence of NKT cells in horses and their ability to recognize α-GalCer remains unknown. However, recent work in our laboratory identified an equine CD1 cluster that is composed of 13 genes (Dossa et al., 2014). This cluster includes a CD1d homologue that is expressed in several antigen-presenting cells (APC), including macrophages and dendritic cells. Additionally, previous work in another lab indicated that horses possess a TCR α-chain that is homologous to the highly conserved TCR used by human and murine iNKT cells (Looringh van Beeck et al., 2009). This equine TCR was predicted to interact with equine CD1d. Together these findings strongly suggest that the CD1d/NKT cell system is present in horses.

Little is also known about the specific roles played by CD1, the lipid antigen presentation system, or NKT cells in horses. Evidence suggests that immune responses to microbial lipids are essential in the protection against *Rhodococcus equi*, an important equine pathogen (Vázquez-Boland et al., 2013). *R. equi* is a nocardioform actinomycete bacterium that is closely related and structurally similar to *M. tuberculosis* (Rahman et al., 2003). Whereas *M. tuberculosis* causes tuberculosis in humans, *R. equi* causes pyogranulomatous pneumonia in horses between 2 and 5 months of age. Rhodococcal pneumonia is a common cause of morbidity and mortality in young horses worldwide. As a result of exposure early in life, adult horses are almost invariably immune. Furthermore, immune adult horses have CTL that lyse *R. equi* infected cells in an MHC class-I unrestricted fashion (Patton et al., 2004, 2005). These CTL recognize unique *R. equi* lipids, presumably presented by the CD1 system (Harris et al., 2010).

Despite the observation that naturally developing adaptive immune responses strongly protect adult horses, efforts to develop a vaccine to prevent rhodococcal pneumonia in foals have been unsuccessful (Lopez et al., 2003, 2008; Mealey et al., 2007). The need to stimulate protective cell-mediated responses in the first weeks of life is likely a critical barrier. Considering that horses express a CD1d molecule and carry at least one apparent NKT cell TCR homologue, we hypothesized that the glycolipid α-GalCer would stimulate equine NKT cells and consequently enhance the immunogenicity of an *R. equi vaccine*. In this study, we demonstrate that molecular models predict that the equine CD1d (eqCD1d) binding groove site will accommodate and bind α-GalCer. However, we were unable to demonstrate an immunomodulatory effect *in vitro* or *in vivo*, suggesting important differences between horses and previously studied mammals.

2. Material and methods

2.1. Construction of an eqCD1d binding domain homology model

To determine the evolutionary relationship between equine CD1d (eqCD1d) and CD1d from other species, an alignment of the CD1d binding domain was performed using ClustalW. Subsequently, a phylogenetic analysis based on a neighbor joining tree was created using MEGAv5.2 software (http://www.megasoftware.net) (Supplementary Fig. S1).

A high quality model of the binding domain of the eqCD1d molecule was generated using a previously described multiple template homology model protocol and X-ray crystal structure data available for different species of CD1d bound to α-GalCer (Garzón et al., 2009) (Supplementary Fig. S2A and B). The detailed methodology used is provided in Supplementary Material and Methods.

2.2. Horses

Adult horses of various breeds were used in accordance with the Washington State University institutional animal care and use committee. Venous blood was collected from the jugular vein of each horse using evacuated containers (Baxter, Deerfield, IL) containing 16% anticoagulant citrate dextrose A (ACD; Baxter). Peripheral blood mononuclear cells (PBMC) were isolated from venous blood using a Ficoll-Hypaque technique (Zhang et al., 1998).

2.3. Bacteria

The plasmid-bearing virulent *R. equi* strain ATCC 33701 bacteria were grown in brain heart infusion broth (BHI; Becton Dickinson, Franklin Lakes, NJ), overnight at 37 °C with shaking for 8 h. After the 8-h growth, the number of bacteria per ml was estimated with an optical density reading of 0.050 Å at 600 nm (Beckman DU-64) equaling 1.5×10^8 *R. equi* per ml. The bacterial concentration was confirmed by plating serial dilutions on BHI plates and calculating the colony-forming units per ml.

2.4. Lipids

The synthetic glycolipids α-GalCer (KRN7000) and 7DW8-5 were purchased from Avanti Polar Lipids, Alabaster, AL and Diagnocine, Hackensack, NJ. The remaining synthetic lipids were synthesized by one of the investigators (PJJ) at the School of Biosciences, University of Birmingham (Birmingham, UK), using modified procedures to those described previously (Jervis et al., 2010, 2011).

R. equi lipids were isolated using a previously described chloroform/methanol extraction method (Harris et al., 2010). After overnight separation in a separatory funnel, the organic layer was collected and dried on a rotary evaporator at 37 °C. The sample was then resuspended in PBS, with sonication to a concentration of 10 μg/ml.

2.5. Cytotoxicity assay

CTL assays were performed using previously published methods (Harris et al., 2010; Patton et al., 2004). Briefly, effector cells were derived by stimulating equine PBMC with one of the following for 5 days at 37 °C with 5% CO_2: (i) 100 ng/ml of α-GalCer, (ii) 6×10^6 *R. equi* ATCC 33701/ml (multiplicity of infection in monocytes approximately 0.3), or (iii) 6×10^6 *R. equi* ATCC 33701/ml plus 100 ng/ml of α-GalCer. Effectors were then rested for 2 days without antigenic stimulation prior to testing in the CTL assay. Target cells were obtained by eluting adherent peripheral blood adherent cells (PBAC) from MHC class I matched or mismatched horse. The effector cells were then added to target cells previously labeled with ^{51}Cr per ml (PerkinElmer, Waltham, MA). Target cells had been (i) pulsed with 100 ng/ml α-GalCer, (ii) infected with 5×10^5 live *R. equi*, (iii) infected with 5×10^5 live *R. equi* and pulsed with 1 μg/ml α-GalCer, or (iv) pulsed with media containing only control vehicle (0.1% DMSO). Specific lysis was calculated using the formula $[(E - S)/(T - S)] \times 100$, where E is the mean of three test wells, S is the mean