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

Dossa, Robson G.; Alperin, Debra C.; Garzon, Diana; Mealey, Robert H.; Brown, Wendy C.; Jervis, Peter J.; Besra, Gurdyal S.; Cox, Liam R.; Hines, Stephen A.

DOI:
10.1016/j.dci.2014.11.005

Accepted Manuscript

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

Author: Robson G. Dossa, Debra C. Alperin, Diana Garzon, Robert H. Mealey, Wendy C. Brown, Peter J. Jervis, Gurdyal S. Besra, Liam R. Cox, Stephen A. Hines

PII: S0145-305X(14)00269-9
DOI: http://dx.doi.org/doi: 10.1016/j.dci.2014.11.005
Reference: DCI 2297

To appear in: *Developmental and Comparative Immunology*

Received date: 20-6-2014
Revised date: 6-11-2014
Accepted date: 6-11-2014

Please cite this article as: Robson G. Dossa, Debra C. Alperin, Diana Garzon, Robert H. Mealey, Wendy C. Brown, Peter J. Jervis, Gurdyal S. Besra, Liam R. Cox, Stephen A. Hines, In contrast to other species, α-Galactosylceramide (α-GalCer) is not an immunostimulatory NKT cell agonist in horses, *Developmental and Comparative Immunology* (2014), http://dx.doi.org/doi: 10.1016/j.dci.2014.11.005.

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

Robson G. Dossa[a]
Debra C. Alperin[a]
Diana Garzon [b]
Robert H. Mealey [a]
Wendy C. Brown [a]
Peter J. Jervis [c]
Gurdyal S. Besra [c]
Liam R. Cox [d]
Stephen A. Hines[a,*]

(a) Department of Veterinary Microbiology and Pathology, Washington State University, College of Veterinary Medicine, P.O. Box 647040, Pullman, WA 99164-7040, USA
(b) Unilever Centre for Molecular Science Informatics, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK
(c) School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
(d) School of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

* Corresponding author: Stephen A. Hines

Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, PO Box 647040, Pullman, Washington 99164-7040

Tel.: +1 (509) 335-6030. Fax: +1 (509) 335-8529.

E-mail address: shines@vetmed.wsu.edu (S. A., Hines).

Highlights

- The equine CD1d has a suitable pocket for α-GalCer ligation.
- α-GalCer failed to stimulate equine NKT cells in vitro or in vivo.
- α-GalCer loaded tetramers did not bind equine cells.
- α-GalCer is an unsuitable adjuvant in horses.
- Requirements for equine NKT cells activation is likely unique

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.

***K*eywords**

NKT

α-Galactosylceramide

CD1d

Horse

Rhodococcus equi

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., 2011; Kinjo et al., 2006; Kinjo et al., 2005). 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 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 2-5 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; Patton et al., 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; Lopez et al., 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 (eqCD1) 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. 1).

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. 2A, 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 8h. After the 8h 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., 2011; Jervis et al., 2010).

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 10µg/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) 100ng/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 100ng/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 100ng/ml α-GalCer, (ii) infected with 5×10^5 live *R. equi,* (iii) infected with 5×10^5 live *R. equi* and pulsed with 1μg/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 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). As previously described, significant lysis was defined as 3 standard errors above the negative control value. Cytotoxic assay results shown are a representative example of three animals analyzed in three independent experiments performed in triplicate.

2.6 Lymphoproliferation assays

Equine PBMC were plated in 96-well plates at a density of 2.5×10^5 cells/well in 100μl of complete medium. Each well was either (i) pulsed with 100ng/ml α-GalCer, (ii) infected with 2.5×10^5 live *R. equi*/ml, (iii) infected with 2.5×10^5 live *R. equi*/ml and pulsed with 100ng/ml α-GalCer, (iv) pulsed with 10μg/ml *R. equi* lipid (v) pulsed with 10μg/ml *R. equi* lipid and 100ng/ml α-GalCer, (vi) pulsed with 10U/ml human IL-2 as positive control, or (vii) pulsed with media only containing control vehicle (0.1% DMSO).

One hour *post R. equi* infection, 20 µl of complete medium containing 0.05 mg/ml gentamicin sulfate was added to each culture well to kill extracellular bacteria. The plates were then cultured at 37^{o}C with 5% CO_2 for 72h. [^{3}H] thymidine was added at 0.5µCi/well 18h prior to the termination of culture. Cells were harvested on an automated 96-well plate harvester (TomTec Inc., Orange, CT) and [^{3}H] Thymidine uptake was measured by liquid scintillation spectroscopy (Wallac Inc., Gaithersburg, MD). Splenocytes from Swiss Webster mouse were isolated as described previously (Sakai et al., 1999), plated into 96-well plates and used as described for equine cells to serve as a positive control. All assays were performed in triplicate. Cells from four animals were analyzed in four independent experiments. In subsequent experiments, the assay was repeated using variants of α-GalCer (100ng/ml) with shorter fatty acyl chains (C8, C12, C16, or C20) and with other previously described or putative NKT cell agonists (See Table 1). The α-GalCer analogue (GalNHAc C20:2), where the hydroxyl group at the 2-position of the galactose head-group was removed, was used as an additional negative control. This hydroxyl group is crucial to the activation of murine and human NKT cells (Jervis et al., 2012). *R. equi* lipid antigens (10µg/ml) were used as positive control.

2.7 Intradermal testing

To measure in vivo cell-mediated immune responses, a previously described intradermal injection and biopsy method was used with modifications (Liu et al., 2012). Prior to injections, a rectangular area on the neck of three horses was clipped and

scrubbed with betadine and alcohol. Intradermal injections consisted of 0.2ml PBS containing the following: (i) 400µg α-GalCer, (ii) 10^9 heat-killed *R. equi,* (iii) 10^9 heat-killed *R. equi* with 400µg α-GalCer, or (iv) 400µg *R. equi* lipids. Saline alone containing control vehicle (0.1% DMSO) was also injected as the negative control. After 48h, a 2mm skin biopsy from each injection site was collected and stored in RNALater (Ambion, Austin, TX) at 4°C. An additional 4mm skin biopsy sample was placed in 10% formalin for subsequent histopathology.

2.8 Histopathology of skin biopsies

Serial 6µm sections were cut from 4mm skin biopsy samples using a Leica RM2235 Microtome and placed onto poly-L-lysine-coated glass slides for hematoxylin and eosin (H&E) staining. H&E staining was performed according to established protocols (Fischer et al., 2008). All slides were read by a board certified veterinary pathologist (SAH).

2.9 Quantitative Real-Time PCR

The 2mm skin biopsies stored in RNALater were submitted to Dr. David W. Horohov, Laboratory of Equine Immunology, Maxwell H. Gluck Equine Research Center, University of Kentucky (Lexington, KY). Relative quantity (RQ) of gene expression for every sample was assessed by real-time PCR and $2^{-\Delta\Delta CT}$ method, following previously established protocols (Liu et al., 2012). RQ data were natural log

transformed to satisfy the assumption of normality. Statistical analyses were performed using SAS software. Results were expressed as log transformed means ± SEM. Mean differences between treatment groups were evaluated using Fisher's least significant difference (LSD) test. A p value < 0.05 was considered significant.

2.10 Flow cytometric analysis – CD1d tetramers

Equine PBMC and murine splenocytes were plated in 75cm^2 flasks at 4 × 10^6 cells/ml, in 25ml of complete media containing 10U/ml of IL-2 and were stimulated for 0 or 5 days with and without 100ng/ml α-GalCer. Before and after stimulation, cells were stained with human or mouse CD1d:Alexa647 tetramers at 1ug/ml, either loaded with α-GalCer or empty (unloaded). The tetramers were generously provided by the National Institutes of Health Tetramer Facility (Atlanta, GA). Briefly, 10^6 equine and murine cells/well were plated in 96-well plates in the respective cocktails containing the species-specific anti-CD3 monoclonal antibodies and CD1d-tetramers diluted in PBS. After 1 hour of incubation, cells were washed and fixed. Data were collected using a FACSort flow cytometer. At least 1 × 10^6 viable cell events were acquired in the peripheral blood lymphocyte gate using Cell Quest software (Becton Dickinson). Results were analyzed using FCS Express software (De Novo Software, Thornton, Ontario, Canada) and FlowJo software (FlowJo, Ashland, OR). Positive staining was determined by comparing loaded and empty CD1d tetramers.

3. Results

3.1 Equine CD1d molecular structure – comparison to other species

Phylogenetic analysis of the eqCD1d binding domain found that eqCD1d was more closely related to huCD1d than any of the other species analyzed (Supplementary Fig. 1), providing evidence of evolutionary proximity and suggesting functional similarities.

The high quality eqCD1d molecular model demonstrated that most structural features of previously described CD1d molecules are conserved in horses. However, some characteristics unique to equine CD1d were also apparent. The total volume of the binding cavity of eqCD1d was 1040 $Å^3$. By comparison, the volume of the binding cavities for the reference CD1d molecules were: huCD1d 1009 $Å^3$, boCD1d 985 $Å^3$ and muCD1d 856 $Å^3$ (Fig. 1A-D). The CD1d antigen/ligand binding cavity is composed of two pockets, F' and A'. The F' pocket of CD1d has been described as rather shallow, resulting in a more closed entrance when compared to the other members of the CD1 family. This shallowness is due to the formation of an F' roof between the carboxy-terminal end of the α1 helix and the amino-terminal end of the α2 helix. In eqCD1d this groove seems to be slightly larger than in previously described CD1d molecules (Supplementary Fig. 3A). The volume calculated for the equine CD1d F' pocket (489 $Å^3$) is 12% greater than in huCD1d, 9% greater than in boCD1d and 15% greater than in muCD1d. The predicted eqCD1d F' pocket entrance is also wider. This is due to the presence of small hydrophobic residues in place of bulkier side chains, such as the Leu/Ile96Val, Leu/Ile98Val, Phe116Ala, and Phe77Val replacements. Amino acids at positions 96, 98 and 114 have been shown to be critical to structure and function because these residues form the floor of the F' pocket (Supplementary Fig. 3B). As a

result of these substitutions, the F' pocket of eqCD1d appears to be deeper than the CD1d F' pocket in other species.

In most members of the CD1 family the A' pocket consists of a rounded "donut-like" tunnel that lies under the amino-terminal side of α1 (and hence carboxy-terminal side of α2). The "donut" is formed by a vertical "pole" produced by two conserved residues, Phe70 on the roof of the pocket and Val12 on the floor. In CD1d isoforms, however, the conserved residue Val12 is replaced by Cys12, resulting in a partially transected tunnel. The model predicted that this feature is conserved in eqCD1d (Supplementary Fig. 3C, D). Similar to other species, the modelled A' pocket of eqCD1d forms on the side of the F' pocket, wraps 360° around the A' pole, and connected back to the F' pocket. Bovine CD1d is unique because a Cys166Phe substitution truncates the pocket under α2. This substitution, which limits the size of ligands that the binding cavity can accommodate, is not present in human, murine, or in equine CD1d.

Finally, "portals" have been proposed to exist in some CD1 isoforms as a means to accommodate the long lipid tails of ligands whose volume would otherwise exceed that of the cavity. For example, in huCD1b, the so-called C' portal exists at the side of the F' pocket, underneath the α2 link. In huCD1c the D' portal opens up the cavity on the side of the A' pocket. No such portals are predicted in eqCD1d. However, our model of eqCD1d predicts the presence of a novel tunnel/portal at the amino-terminal side α1 of the A' pocket. We have putatively named this the "g' tunnel/portal" (Supplementary Fig. 3E). Structurally, the proposed g' portal is an extension of the A' pocket. It appears to result from the replacement of bulky side chains that close the A' pocket in other CD1 isomorphs. In eqCD1, a Cys at position 63 together with the His38Tyr substitution leads

to formation of the putative tunnel. The tunnel is further formed by a Val (huCD1d)/47Ile substitution at the bottom of the pocket and the conserved Leu66 at the top of the pocket. As a result of its strong interaction with 46Ile, Gln67 seems to have a stabilizing effect comparable with the disulfide bridge that maintains the C' portal in CD1b. Stabilization of the tunnel aperture allows for connection to the exterior face of the binding domain (Supplementary Fig. 3F).

3.2 Binding model: eqCD1d in complex with α-GalCer

A primary goal of our modeling work was to make predictions regarding the ability of eqCD1d to bind and present α-GalCer, and to characterize the predicted interactions. Briefly, the different α-GalCer molecules previously co-crystallized with huCD1d, boCD1d and muCD1d were easily placed in the same orientation when inserted into the eqCD1d model (Fig. 1E-G). There were no steric clashes between the acyl chains and the surface of the modeled cavity, further supporting the hypothesis that equine CD1d should be able to effectively bind α-GalCer.

To evaluate the conservation of key residues of eqCD1d that are not part of the binding cavity but are likely to interact with the α-GalCer head-group or the NTK cell receptor, we aligned eqCD1d with huCD1d, boCD1d and muCD1d (Supplementary Fig. 4). These amino acids are likely essential for the correct presentation of α-GalCer and recognition of the CD1d/α-GalCer complex by NKT cells. Among the residues in the CD1d side chains that interact with the sugar head-group of the α-GalCer ligand (huCD1d: Asp80, Asp151, and Trp153 and muCD1d: Asp80, Asp153, and Trp156), all were conserved in eqCD1d (Borg et al., 2007; Koch et al., 2005). Among the 11 CD1d

residues known to mediate direct contact of CD1d/α-GalCer with the NTK T cell receptor, two amino acids (huCD1d: Arg89 Gln150 and muCD1d: Ser89 Asn150) are replaced (with His89 and Asp150) in eqCD1d (Borg et al., 2007).

3.3 The effects of α-GalCer on equine CTL

Previous work in our lab demonstrated that the age-associated acquisition of protective immune responses against *R. equi* in horses correlates with the development of *R. equi*-specific CTL. In other species, α-GalCer can act as a potent adjuvant for priming and boosting CTL response. In this study, we first investigated the ability of α-GalCer alone to stimulate equine CTL. As expected, effector cells stimulated with *R. equi* produced significant killing of *R. equi*-infected cells but did not kill uninfected cells or cells pulsed with α-GalCer. In contrast, cells stimulated with α-GalCer failed to kill *R. equi*-infected or uninfected cells, and they also failed to kill cells pulsed with α-GalCer (Fig. 2A). MHC-class I matched target cells and target cells pulsed with higher and lower concentrations of α-GalCer (1ng/ml to 5μg/ml) were also tested and demonstrated similar results (data not shown).

To investigate if α-GalCer could enhance the cytotoxic activity of *R. equi*-specific CTL, effector cells were concomitantly stimulated with *R. equi* and α-GalCer and compared with cells stimulated with *R. equi* only. *R. equi*-stimulated cells were able to kill infected targets, and this specific killing increased with an increase in the effector:target cell (E:T) ratio. The effector cells that were concomitantly stimulated with *R. equi* and α-GalCer were also able to kill infected targets, and the specific killing increased with an increase in the E:T ratio. However α-GalCer did not enhance *R. equi*-

specific CTL activity. There was no significant α-GalCer-associated increase in the killing of infected cells at any E:T ratio (Fig. 2B). Additional stimulation time points (from 24h up to 10 days) and higher and lower concentrations of α-GalCer (1ng/ml to 5μg/ml) were also tested and demonstrated similar results (data not shown).

3.4 The effects of α-GalCer on proliferation of equine PBMC

In other species, even small amounts of α-GalCer are able to activate the entire NKT cell population. To investigate the capacity of α-GalCer to stimulate lymphocyte proliferation in horses, equine PBMC were pulsed with α-GalCer and cell proliferation measured after 72 hours. Murine cells served as positive controls. After the incubation period, murine cells (which are known to be activated by α-GalCer), demonstrated a significant proliferative response (data not shown, but this response is shown in Fig. 5, Section 3.7 – below). In contrast to murine cells, α-GalCer had no significant effect on equine cells (Fig. 2C). Additional stimulation time points (from 24h to 7 days) and higher and lower concentrations of α-GalCer (1ng/ml to 5μg/ml) were also tested and demonstrated similar results (data not shown).

To assess the capacity of α-GalCer to act as an immunostimulatory molecule that could enhance the proliferation of equine lymphocyte when co-administrated with *R. equi* antigen, equine cells were concomitantly pulsed with α-GalCer and *R. equi* lipid antigens or concomitantly pulsed with α-GalCer and infected with live *R. equi*. These were compared with cells infected with live *R. equi* or stimulated with *R. equi* lipid antigen alone. After 72h of incubation, proliferation was measured and demonstrated

that both live *R. equi* and *R. equi* lipids were able to stimulate equine cells; however, α-GalCer did not significantly increase the lymphoproliferative effect when co-administrated with either live *R. equi* or *R. equi* lipid antigens (Fig. 2C).

3.5 *In vivo* equine immune responses to α-GalCer

Because of α-GalCer's capacity to trigger a strong immune response in other species, this glycolipid has been tested as an adjuvant for several vaccines. Perhaps our *in vitro* systems are lacking a key component that is nonetheless present *in vivo*. To investigate if α-GalCer would also have an adjuvant/immunostimulatory effect in horses, a group of three animals were randomly selected to test the effects of α-GalCer *in vivo*. As expected, intradermal injection of heat-killed *R. equi* bacteria induced intense dermatitis that was characterized by perivascular lymphocyte accumulations and multifocal accumulation of neutrophils. Injection of *R. equi* lipid antigen induced a similar but less severe inflammatory response. The skin from sites injected with saline (negative control) were within normal limits. Biopsies from skin injected with α-GalCer alone were mostly indistinguishable from the negative control except for some occasional very mild perivascular accumulations of lymphocytes in the deep dermis. The addition of α-GalCer to *R. equi* antigen did not produce detectable increases in the local tissue response (Supplementary Fig. 5).

Quantitative real-time PCR performed on mRNA extracted from skin biopsies demonstrated that heat-killed *R. equi* was able to significantly increase the recruitment of CD4, CD8 and CD86 positive cells to the site of injection (Fig. 3). Likewise, there was

an increase in mRNA encoding perforin and granzyme. Heat-killed *R. equi* also increased local transcription of the cytokines IFN-γ, IL-12, and IL-4. No increase in the transcript of IL-6 was detected. Although not as potent as heat-killed bacteria, *R. equi* lipid antigens were also able to significantly increase the recruitment of CD4, CD8 and CD86 positive cells to the site of injection and increase the number of transcripts for IFN-γ, granzyme, and perforin. However, there was no significant increase in transcription of IL-12, IL-4 or IL-6. Under the conditions of our experiment, α-GalCer alone was unable to significantly increase the recruitment of any cell marker, nor did it increase the production of any cytokine investigated. Additionally, there was no significant increase in any cell surface marker or cytokine production when α-GalCer was co-injected with heat-killed *R. equi*. Small changes in some cytokines and markers were not statistically significant. A small increase in the production of IL-12 was observed when α-GalCer was co-injected, but was not statistically significant (Fig. 3).

3.6 The recognition of equine NKT cells by CD1d-tetramers

The capacity of human CD1d (huCD1d) to present α-GalCer to murine NKT cells and *vice versa* demonstrates the strong conservation of the NKT cell – CD1d interaction between species. Likewise, human and murine CD1d (muCD1d) tetramers loaded with α-GalCer bind NKT cells from both species, whereas empty (unloaded) tetramers bind neither. Investigators have successfully used murine CD1d-αGalCer tetramers as a tool to unequivocally identify an NKT cell population in pigs (Thierry et al., 2012). Therefore, we tested human and muCD1d tetramers binding to equine cells to look for a similar

cross-reactive NKT cell population in horses. Briefly, equine PBMC were stained with tetramers and species-specific anti-CD3 monoclonal antibody at the day of cell collection (Day 0). Murine cells were used as positive controls. A second population of PMBC from the same cell collection was studied after being stimulated for 5 days with α-GalCer to expand NKT cells. Gating on viable leukocytes demonstrated that both human and murine α-GalCer loaded-CD1d-tetramers bound a subpopulation of CD3 positive murine cells. As previously demonstrated, empty tetramers (no α-GalCer) did not bind. After 5 days of stimulation with α-GalCer, the number of murine cells positive for α-GalCer loaded-tetramers significantly increased, when compared with cells from Day 0 or with unstimulated cells. However, we were unable to detect tetramer-positive cells among the equine CD3 population. Neither α-GalCer-loaded-human CD1d-tetramers nor α-GalCer-loaded-mouse CD1d- tetramers reacted with equine cells. There was no significant staining of equine cells after 5 days of stimulation with α-GalCer, i.e. no tetramer-positive cells were detected among the equine CD3 positive cells after an attempt to expand α-GalCer-reactive cells, as previously demonstrated with murine cells (Fig. 4).

3.7 The effects of α-GalCer analogues and other NKT cell agonists on equine PBMC

The bovine CD1d homologue has a shallow antigen-binding pocket compared to other species and consequently cannot accommodate the 26-carbon fatty acyl chain of α-GalCer. Thus full-length α-GalCer does not bind bovine CD1d and does not activate bovine NKT cells (Wang et al., 2012). However, bovine CD1d is able to bind and present shorter α-GalCer analogues that contain a C12 or C16 fatty acyl chain (Nguyen

et al., 2013). To explore whether pocket size or conformation might also affect α-GalCer presentation in horses, α-GalCer with shorter acyl chains containing C8, C12, C16 and C20 were produced and their capacity to induce the proliferation of equine lymphocytes was tested. Murine cells, which are known to be activated by α-GalCer, were used as a control – i.e. to confirm that the synthetic lipids were biologically active. After 72h of incubation, all α-GalCer variants were able to stimulate the proliferation of murine lymphocytes. In contrast, none of the short chain analogues induced proliferation of equine cells (Fig 5A).

To determine whether other human or murine NKT cell agonists might bind to equine CD1d and activate equine NKT cells, 9 additional lipid compounds were synthesized and tested (Table 1). The specific chemical structures of these lipids are shown in Supplementary Fig. 6. Murine cells demonstrated a significant proliferative response to all nine NKT cell agonists tested (Fig. 5B). Murine cells did not proliferate in response to the negative control that lacked a hydroxyl group at the 2-position of the galactose head group, demonstrating the NKT cell specificity of the assay. As expected, *R. equi* lipids stimulated strong proliferative responses when added to equine PBMC. However, none of the NKT cell agonists were able to induce proliferation of equine cells.

4. Discussion

R. equi is a soil-borne organism that is ubiquitous in equine environments and an important cause of morbidity and mortality in young horses. Virtually all foals are exposed to *R. equi* shortly after birth. The vast majority of foals develop protective immune responses that operate throughout adult life, whereas affected foals develop pyogranulomatous pneumonia resembling caseous tuberculosis. Attempts to induce or accelerate protective responses in neonatal foals by vaccination have thus far been unsuccessful. We hypothesized that the immunostimulatory glycolipid α-GalCer could be used as an adjuvant to expand equine NKT cells and thereby overcome the relatively poor immune responses that are typical of newborns in virtually all species (Chappuis, 1998; Flaminio et al., 2009). Importantly, a successful vaccine to prevent equine rhodococcal pneumonia will likely need to induce type 1 cellular immune responses, including IFN-γ-producing Th1 cells and *R. equi*-specific CTL (Breathnach et al., 2006; Harris et al., 2011; Patton et al., 2005). The CTL have been shown in our laboratory to be MHC-unrestricted and to recognize *R. equi* lipids, likely via the CD1 antigen-presenting system.

Using data from other species, we built a detailed model of the equine CD1d molecule. Modeling showed strong homology to previously described CD1d molecules (notably human) and predicted that eqCD1d would bind α-GalCer and present it to NKT cells. Nevertheless, multiple studies, including intradermal injection of α-GalCer *in vivo* and *in vitro* CTL assays, convincingly showed no immunostimulatory effects. Moreover, α-GalCer-loaded CD1d tetramers (which have been shown to be cross-reactive in other

species and were used to identify NKT cells in various species) did not bind equine cells, whereas they bound readily to the murine controls.

There are several possible explanations for the failure of α-GalCer, α-GalCer short carbon-chain variants, and multiple α-GalCer analogues (all potent NKT cell activators in other species) to stimulate equine cells. One possibility is that none of these molecules is bound by eqCD1d. This explanation seems unlikely considering that the molecular model shows a highly conserved lipid binding groove that should be suitable for loading and presentation of α-GalCer and its variants. Nevertheless, we attempted to test this explanation using biotin-labeled α-GalCer and FACS (data not shown). In our hands, this reagent bound a broad range of equine and non-equine cells non-specifically (binding could not be blocked with unlabeled α-GalCer), raising questions about previous reports where such controls were not in place. Ultimately, we were unable to directly address this unlikely explanation.

A more plausible explanation for the lack of an immunostimulatory effect in horses is that the TCR from equine NKT cells are incapable of interacting with eqCD1d and/or recognizing α-GalCer presented by eqCD1d. Although the lipid binding groove of eqCD1d has a very high homology with the huCD1d binding groove, there are substitutions that could affect the interaction of CD1d or α-GalCer-loaded CD1d with equine NKT cells. Among the 11 CD1d residues known to mediate direct contact of CD1d/α-GalCer with the NTK T cell receptor, two amino acids (huCD1d: Arg89 Gln150 and muCD1d: Ser89 Asn150) were replaced (with His89 and Asp150) in eqCD1d (Borg et al., 2007). In humans, the CD1d Arg89 residue forms van der Waals contacts with the NKT-TCR CDR2 β loop and Gln150 forms a hydrogen bond with the Thr98 of the CDR3

α loop. A previous study demonstrated that replacement of huCD1d Arg89 with His89, as found in horses, did not significantly affect the activation of human NKT cells (Zhang et al., 2009). The replacement of the CD1d Gln150 with Asp has not been tested, but aspartic acid could potentially conserve the ability to interact with the NKT-TCR loop since this residue is strongly negatively charged and typically behaves like Gln.

Other residues towards the surface of the CD1d molecule near the binding groove might also influence the glycolipid head-orientation and the recognition of lipids by NKT cells (Kamada et al., 2001). Among these residues the only replacement found in eqCD1d is Met157 (huCD1d Thr157, muCD1d Thr159). Although this residue does not directly interact with α-GalCer, muCD1d Thr159 forms a conserved extra hydrogen bond with the carbonyl oxygen of galactose-modified α-GalCer analogues (Aspeslagh et al., 2011). Therefore a substitution at this site might affect the orientation of the α-GalCer head and thereby alter presentation.

Another possible explanation for the lack of activation of equine cells by α-GalCer is that horses may lack a T cell population homologous to human and murine NKT cells. This explanation seems unlikely considering the strong cross-species conservation of both CD1d and the NKT cell TCR α-chain. However, it is consistent with our failure to find a population of equine PBMC that bound α-GalCer loaded tetramers. An alternative interpretation of this result is that human and murine CD1d-αGalCer tetramers simply do not cross-react with equine NKT cells. Therefore, our current data do not directly address the question of whether horses have NKT cells with similar biological and functional abilities as NKT cells in other species. Likewise, it does not address the potential for expanding this population with an appropriate NKT cell agonist as part of a

novel vaccination strategy. A different agonist may be required to broadly active equine NKT cells. Alternatively, it is possible that CD1d-restricted T cells in horses are more antigen-specific (similar to CD1a, CD1b, and CD1c restricted T cells) and that horses lack a broadly reactive iNKT (Type 1 NKT cell) sub-population equivalent to what has been described.

In conclusion, further studies are required to confirm the presence of an equine NKT cell and the roles played by lipid antigens in the protection against equine infectious diseases. However, this work suggests that horses are unique among previously described species and provides strong evidence that α-GalCer is unsuitable as an equine vaccine adjuvant in this species.

Acknowledgments

We thank Emma Karel and Casey Lawson for their animal handling expertise. Special thanks to Dr. Marc Evans for statistical support. This work was funded by Morris Animal Foundation Grant D10EQ-046 and by United States Department of Agriculture-Agricultural Research Service 5348-32000-034-00D.

References

Aspeslagh, S., Li, Y., Yu, E.D., Pauwels, N., Trappeniers, M., Girardi, E., Decruy, T., Van Beneden, K., Venken, K., Drennan, M., Leybaert, L., Wang, J., Franck, R.W., Van Calenbergh, S., Zajonc, D.M., Elewaut, D., 2011. Galactose-modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumour metastasis. EMBO J 30, 2294-2305.

Baker, M.L., Miller, R.D., 2007. Evolution of mammalian CD1: marsupial CD1 is not orthologous to the eutherian isoforms and is a pseudogene in the opossum Monodelphis domestica. Immunology 121, 113-121.

Bendelac, A., Lantz, O., Quimby, M.E., Yewdell, J.W., Bennink, J.R., Brutkiewicz, R.R., 1995. CD1 recognition by mouse NK1+ T lymphocytes. Science 268, 863-865.

Borg, N., Wun, K., Kjer-Nielsen, L., Wilce, M., Pellicci, D., Koh, R., Besra, G., Bharadwaj, M., Godfrey, D., McCluskey, J., Rossjohn, J., 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature 448, 44-49.

Breathnach, C.C., Sturgill-Wright, T., Stiltner, J.L., Adams, A.A., Lunn, D.P., Horohov, D.W., 2006. Foals are interferon gamma-deficient at birth. Vet Immunol Immunopathol 112, 199-209.
Brennan, P.J., Brigl, M., Brenner, M.B., 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol 13, 101-117.

Chackerian, A., Alt, J., Perera, V., Behar, S.M., 2002. Activation of NKT cells protects mice from tuberculosis. Infect Immun 70, 6302-6309.

Chang, Y.J., Huang, J.R., Tsai, Y.C., Hung, J.T., Wu, D., Fujio, M., Wong, C.H., Yu, A.L., 2007. Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids. Proc Natl Acad Sci U S A 104, 10299-10304.

Chappuis, G., 1998. Neonatal immunity and immunisation in early age: lessons from veterinary medicine. Vaccine 16, 1468-1472.

de Lalla, C., Festuccia, N., Albrecht, I., Chang, H.D., Andolfi, G., Benninghoff, U., Bombelli, F., Borsellino, G., Aiuti, A., Radbruch, A., Dellabona, P., Casorati, G., 2008. Innate-like effector differentiation of human invariant NKT cells driven by IL-7. J Immunol 180, 4415-4424.

Dossa, R.G., Alperin, D.C., Hines, M.T., Hines, S.A., 2014. The equine CD1 gene family is the largest and most diverse yet identified. Immunogenetics 66, 33-42.

Fischer, A.H., Jacobson, K.A., Rose, J., Zeller, R., 2008. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc 2008, pdb.prot4986.

Fischer, K., Scotet, E., Niemeyer, M., Koebernick, H., Zerrahn, J., Maillet, S., Hurwitz, R., Kursar, M., Bonneville, M., Kaufmann, S.H., Schaible, U.E., 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. Proc Natl Acad Sci U S A 101, 10685-10690.

Flaminio, M.J., Nydam, D.V., Marquis, H., Matychak, M.B., Giguère, S., 2009. Foal monocyte-derived dendritic cells become activated upon Rhodococcus equi infection. Clin Vaccine Immunol 16, 176-183.

Fujii, S., Shimizu, K., Smith, C., Bonifaz, L., Steinman, R.M., 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 198, 267-279.

Gapin, L., Matsuda, J.L., Surh, C.D., Kronenberg, M., 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. Nat Immunol 2, 971-978.

Garzón, D., Bond, P.J., Faraldo-Gómez, J.D., 2009. Predicted structural basis for CD1c presentation of mycobacterial branched polyketides and long lipopeptide antigens. Mol Immunol 47, 253-260.

Gonzalez-Aseguinolaza, G., Van Kaer, L., Bergmann, C.C., Wilson, J.M., Schmieg, J., Kronenberg, M., Nakayama, T., Taniguchi, M., Koezuka, Y., Tsuji, M., 2002. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. J Exp Med 195, 617-624.

Harris, S., Fujiwara, N., Mealey, R., Alperin, D., Naka, T., Goda, R., Hines, S., 2010. Identification of Rhodococcus equi lipids recognized by host cytotoxic T lymphocytes. Microbiology 156, 1836-1847.

Harris, S.P., Hines, M.T., Mealey, R.H., Alperin, D.C., Hines, S.A., 2011. Early development of cytotoxic T lymphocytes in neonatal foals following oral inoculation with Rhodococcus equi. Vet Immunol Immunopathol 141, 312-316.

Huang, Y., Chen, A., Li, X., Chen, Z., Zhang, W., Song, Y., Gurner, D., Gardiner, D., Basu, S., Ho, D.D., Tsuji, M., 2008. Enhancement of HIV DNA vaccine immunogenicity by the NKT cell ligand, alpha-galactosylceramide. Vaccine 26, 1807-1816.

Jervis, P.J., Cox, L.R., Besra, G.S., 2011. Synthesis of a versatile building block for the preparation of 6-N-derivatized α-galactosyl ceramides: rapid access to biologically active glycolipids. J Org Chem 76, 320-323.

Jervis, P.J., Moulis, M., Jukes, J.P., Ghadbane, H., Cox, L.R., Cerundolo, V., Besra, G.S., 2012. Towards multivalent CD1d ligands: synthesis and biological activity of homodimeric α-galactosyl ceramide analogues. Carbohydr Res 356, 152-162.

Jervis, P.J., Veerapen, N., Bricard, G., Cox, L.R., Porcelli, S.A., Besra, G.S., 2010. Synthesis and biological activity of alpha-glucosyl C24:0 and C20:2 ceramides. Bioorg Med Chem Lett 20, 3475-3478.

Kamada, N., Iijima, H., Kimura, K., Harada, M., Shimizu, E., Motohashi Si, Kawano, T., Shinkai, H., Nakayama, T., Sakai, T., Brossay, L., Kronenberg, M., Taniguchi, M., 2001. Crucial amino acid residues of mouse CD1d for glycolipid ligand presentation to V(alpha)14 NKT cells. Int Immunol 13, 853-861.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H., Taniguchi, M., 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 278, 1626-1629.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Sato, H., Kondo, E., Harada, M., Koseki, H., Nakayama, T., Tanaka, Y., Taniguchi, M., 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. Proc Natl Acad Sci U S A 95, 5690-5693.

Kinjo, Y., Illarionov, P., Vela, J.L., Pei, B., Girardi, E., Li, X., Li, Y., Imamura, M., Kaneko, Y., Okawara, A., Miyazaki, Y., Gómez-Velasco, A., Rogers, P., Dahesh, S., Uchiyama, S., Khurana, A., Kawahara, K., Yesilkaya, H., Andrew, P.W., Wong, C.H., Kawakami, K., Nizet, V., Besra, G.S., Tsuji, M., Zajonc, D.M., Kronenberg, M., 2011. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. Nat Immunol 12, 966-974.

Kinjo, Y., Tupin, E., Wu, D., Fujio, M., Garcia-Navarro, R., Benhnia, M.R., Zajonc, D.M., Ben-Menachem, G., Ainge, G.D., Painter, G.F., Khurana, A., Hoebe, K., Behar, S.M., Beutler, B., Wilson, I.A., Tsuji, M., Sellati, T.J., Wong, C.H., Kronenberg, M., 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat Immunol 7, 978-986.

Kinjo, Y., Wu, D., Kim, G., Xing, G.W., Poles, M.A., Ho, D.D., Tsuji, M., Kawahara, K., Wong, C.H., Kronenberg, M., 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. Nature 434, 520-525.

Koch, M., Stronge, V.S., Shepherd, D., Gadola, S.D., Mathew, B., Ritter, G., Fersht, A.R., Besra, G.S., Schmidt, R.R., Jones, E.Y., Cerundolo, V., 2005. The crystal structure of human CD1d with and without alpha-galactosylceramide. Nat Immunol 6, 819-826.

Li, X., Fujio, M., Imamura, M., Wu, D., Vasan, S., Wong, C.H., Ho, D.D., Tsuji, M., 2010. Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant. Proc Natl Acad Sci U S A 107, 13010-13015.

Liu, C., Cook, F.R., Cook, S.J., Craigo, J.K., Even, D.L., Issel, C.J., Montelaro, R.C., Horohov, D.W., 2012. The determination of in vivo envelope-specific cell-mediated immune responses in equine infectious anemia virus-infected ponies. Vet Immunol Immunopathol 148, 302-310.

Liu, Y., Goff, R.D., Zhou, D., Mattner, J., Sullivan, B.A., Khurana, A., Cantu, C., Ravkov, E.V., Ibegbu, C.C., Altman, J.D., Teyton, L., Bendelac, A., Savage, P.B., 2006. A modified alpha-galactosyl ceramide for staining and stimulating natural killer T cells. J Immunol Methods 312, 34-39.

Looringh van Beeck, F.A., Reinink, P., Hermsen, R., Zajonc, D.M., Laven, M.J., Fun, A., Troskie, M., Schoemaker, N.J., Morar, D., Lenstra, J.A., Vervelde, L., Rutten, V.P., van Eden, W., Van Rhijn, I., 2009. Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants. Mol Immunol 46, 1424-1431.

Lopez, A.M., Hines, M.T., Palmer, G.H., Knowles, D.P., Alperin, D.C., Hines, S.A., 2003. Analysis of anamnestic immune responses in adult horses and priming in neonates induced by a DNA vaccine expressing the vapA gene of Rhodococcus equi. Vaccine 21, 3815-3825.

Lopez, A.M., Townsend, H.G., Allen, A.L., Hondalus, M.K., 2008. Safety and immunogenicity of a live-attenuated auxotrophic candidate vaccine against the intracellular pathogen Rhodococcus equi. Vaccine 26, 998-1009.

Makino, Y., Kanno, R., Ito, T., Higashino, K., Taniguchi, M., 1995. Predominant expression of invariant V alpha 14+ TCR alpha chain in NK1.1+ T cell populations. Int Immunol 7, 1157-1161.

Mealey, R.H., Stone, D.M., Hines, M.T., Alperin, D.C., Littke, M.H., Leib, S.R., Leach, S.E., Hines, S.A., 2007. Experimental Rhodococcus equi and equine infectious anemia virus DNA vaccination in adult and neonatal horses: effect of IL-12, dose, and route. Vaccine 25, 7582-7597.

Metelitsa, L.S., Naidenko, O.V., Kant, A., Wu, H.W., Loza, M.J., Perussia, B., Kronenberg, M., Seeger, R.C., 2001. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J Immunol 167, 3114-3122.

Miyamoto, K., Miyake, S., Yamamura, T., 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 413, 531-534.

Monzon-Casanova, E., Steiniger, B., Schweigle, S., Clemen, H., Zdzieblo, D., Starick, L., Müller, I., Wang, C.R., Rhost, S., Cardell, S., Pyz, E., Herrmann, T., 2010. CD1d expression in

paneth cells and rat exocrine pancreas revealed by novel monoclonal antibodies which differentially affect NKT cell activation. PLoS One 5.

Moody, D.B., Zajonc, D.M., Wilson, I.A., 2005. Anatomy of CD1-lipid antigen complexes. Nat Rev Immunol 5, 387-399.

Nguyen, T.K., Koets, A.P., Vordermeier, M., Jervis, P.J., Cox, L.R., Graham, S.P., Santema, W.J., Moody, D.B., van Calenbergh, S., Zajonc, D.M., Besra, G.S., Van Rhijn, I., 2013. The bovine CD1D gene has an unusual gene structure and is expressed but cannot present α-galactosylceramide with a C26 fatty acid. Int Immunol 25, 91-98.

Nishimura, T., Kitamura, H., Iwakabe, K., Yahata, T., Ohta, A., Sato, M., Takeda, K., Okumura, K., Van Kaer, L., Kawano, T., Taniguchi, M., Nakui, M., Sekimoto, M., Koda, T., 2000. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. Int Immunol 12, 987-994.

Patton, K., McGuire, T.C., Fraser, D.G., Hines, S.A., 2004. *Rhodococcus equi*-infected macrophages are recognized and killed by CD8+ T lymphocytes in a MHC class I- unrestricted fashion. Infect Immun 72, 7073-7083.

Patton, K.M., McGuire, T.C., Hines, M.T., Mealey, R.H., Hines, S.A., 2005. *Rhodococcus equi*-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. Infect Immun 73, 2083-2093.

Rahman, M., Herron, L., Kapur, V., Meijer, W., Byrne, B., Ren, J., Nicholson, V., Prescott, J., 2003. Partial genome sequencing of Rhodococcus equi ATCC 33701. Vet Microbiol 94, 143-158.

Reilly, E.C., Thompson, E.A., Aspeslagh, S., Wands, J.R., Elewaut, D., Brossay, L., 2012. Activated iNKT cells promote memory CD8+ T cell differentiation during viral infection. PLoS One 7, e37991.

Sada-Ovalle, I., Sköld, M., Tian, T., Besra, G.S., Behar, S.M., 2010. Alpha-galactosylceramide as a therapeutic agent for pulmonary Mycobacterium tuberculosis infection. Am J Respir Crit Care Med 182, 841-847.

Sakai, T., Naidenko, O.V., Iijima, H., Kronenberg, M., Koezuka, Y., 1999. Syntheses of biotinylated alpha-galactosylceramides and their effects on the immune system and CD1 molecules. J Med Chem 42, 1836-1841.

Silk, J.D., Salio, M., Reddy, B.G., Shepherd, D., Gileadi, U., Brown, J., Masri, S.H., Polzella, P., Ritter, G., Besra, G.S., Jones, E.Y., Schmidt, R.R., Cerundolo, V., 2008. Cutting edge: nonglycosidic CD1d lipid ligands activate human and murine invariant NKT cells. J Immunol 180, 6452-6456.

Spada, F.M., Koezuka, Y., Porcelli, S.A., 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J Exp Med 188, 1529-1534.

Subrahmanyam, P., Webb, T.J., 2012. Boosting the Immune Response: The Use of iNKT cell ligands as vaccine adjuvants. Front Biol (Beijing) 7, 436-444.

Thierry, A., Robin, A., Giraud, S., Minouflet, S., Barra, A., Bridoux, F., Hauet, T., Touchard, G., Herbelin, A., Gombert, J.M., 2012. Identification of invariant natural killer T cells in porcine peripheral blood. Vet Immunol Immunopathol 149, 272-279.

Venkataswamy, M., Baena, A., Goldberg, M., Bricard, G., Im, J., Chan, J., Reddington, F., Besra, G., Jacobs, W.J., Porcelli, S., 2009. Incorporation of NKT cell-activating glycolipids enhances immunogenicity and vaccine efficacy of Mycobacterium bovis bacillus Calmette-Guerin. J Immunol 183, 1644-1656.

Vázquez-Boland, J.A., Giguère, S., Hapeshi, A., MacArthur, I., Anastasi, E., Valero-Rello, A., 2013. Rhodococcus equi: the many facets of a pathogenic actinomycete. Vet Microbiol 167, 9-33.

Wang, J., Guillaume, J., Pauwels, N., Van Calenbergh, S., Van Rhijn, I., Zajonc, D.M., 2012. Crystal structures of bovine CD1d reveal altered αGalCer presentation and a restricted A' pocket unable to bind long-chain glycolipids. PLoS One 7, e47989.

Yu, K.O., Im, J.S., Molano, A., Dutronc, Y., Illarionov, P.A., Forestier, C., Fujiwara, N., Arias, I., Miyake, S., Yamamura, T., Chang, Y.T., Besra, G.S., Porcelli, S.A., 2005. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. Proc Natl Acad Sci U S A 102, 3383-3388.

Zhang, P., Li, D., Stewart-Jones, G., Shao, X., Zhang, Y., Chen, Q., Li, Y., He, Y.W., Xu, X.N., Zhang, H.T., 2009. A single amino acid defines cross-species reactivity of tree shrew (Tupaia belangeri) CD1d to human invariant natural killer T (iNKT) cells. Immunology 128, 500-510.

Zhang, W., Lonning, S.M., McGuire, T.C., 1998. Gag protein epitopes recognized by ELA-A-restricted cytotoxic T lymphocytes from horses with long-term equine infectious anemia virus infection. J Virol 72, 9612-9620.

Fig. 1. Structural comparison of the binding domains of CD1d across species. (A-D) In each image, the protein fold is shown in transparent grey cartoon format, whilst the binding groove is shown as a green wireframe structure. Each CD1d molecule is viewed on the lateral α1 side. (A) eqCD1d, (B) huCD1d, (C) boCD1d, and (D) muCD1d. **(E-G)** Snapshots showing the eqCD1d cavity bound to (E) huCD1d α-GalCer (C18;C26), (F) boCD1d α-GalCer (C18;C16) and (G) muCD1d OCH (α-GalCer analogue) (C9;C26). The α-GalCer (CPK wireframe format) are shown bound within the cavity (white wireframe surface). Each binding domain is viewed from the α1 side. These models predict that the eqC1d binding groove will accommodate all three α-GalCer molecules.

Fig. 2. Effects of α-GalCer on equine immune cells. (A, B) CTL Assays: **(A)** Effector cells from equine PBMCs were stimulated for 7 days with α-GalCer (grey bars) or *R. equi* (horizontally striped bars). Effector cells were then added to MHC mismatched target cells at a 9:1 E:T cell ratio. Target cells were either pulsed with α-GalCer (100ng/ml), infected with *R. equi*, or untreated (negative control). **(B)** Effector cells stimulated for 7 days with either *R. equi* (horizontally striped bars), *R. equi* + α-GalCer @ 100ng/ml (vertically striped bars), or media containing control vehicle (no stimulation - open bars) were added to *R. equi*-infected target cells at E:T ratios of 1:1, 3:1 or 9:1. In all CTL experiments, the percentage specific lysis was measured using a chromium release assay. Data shown is a representative of 3 independent experiments performed in 3 horses. An asterisk indicates significant lysis, defined as 3 standard errors above the negative control value. **(C) Lymphoproliferation:** Equine cells were pulsed with

media only (no stimulation - open bars), α-GalCer (grey bars), live *R. equi* (horizontally striped bars), *R. equi* lipids (ascendant striped bars) or IL-2 (black bars - positive control). Cells were also concomitantly pulsed with live *R. equi* plus α-GalCer (vertically striped bars) or *R. equi* lipids plus α-GalCer (descendent striped bars). The proliferation of equine cells using H^3-Thymidine uptake was assessed after 72 hours of stimulation. Data shown (Horse #181, Horse #189, and Horse #187) are from 3 independent experiments; each value is the mean of triplicates plus standard deviation. An asterisk represents significant difference when compared with media control (*p* value < 0.05).

Fig. 3. *In vivo* effects of α-GalCer in horses: Recruitment of immune cells and transcription of cytokines and cytolytic molecules as measured by quantitative RT-PCR. Adult horses were injected intradermally with saline only (open bars), α-GalCer alone (grey bars), heat-killed *R. equi* (horizontally striped bars), heat-killed *R. equi* plus α-GalCer (vertically striped bars) or *R. equi* lipids (ascendant striped bars). mRNA expression for **(A)** cell markers and **(B)** cytokines and cytolytic molecules were detected by Real-Time PCR in biopsy samples from the injection site. Relative increase/decrease was compared to injection with saline only and results were natural log transformed to satisfy the statistic assumption. Data are from 3 animals are expressed as means plus standard error. An asterisk represents significant difference when compared with saline control (*p* value < 0.05).

Fig. 4. Failure of CD1d-tetramers to bind an equine NKT population. Equine PBMC and murine cells were membrane-labeled with anti-CD3-Alexa488 species-specific

monoclonal antibodies (x-axis), and stained with human or murine CD1d tetramers-Alexa647 (y-axis) that were either loaded with α-GalCer or empty, before (day 0) or after (day 5) stimulation with α-GalCer (100ng/ml) + IL-2. Number in gates indicates percentage of live cells that were concomitantly positive for CD3 and the tetramers. *One* representative case showing 1×10^6 viable cells events is shown. An asterisk indicates a significant difference when compared with the respective matching empty tetramers.

Fig. 5. Effects of α-GalCer analogues and other NKT cell agonists on equine PBMC. (A) Equine PBMC and murine cells, as a positive control, were pulsed with media alone (open bars), α-GalCer (100ng/ml) containing alkyl chains ranging between eight carbons to 26 carbons (grey bars) or with IL-2 (black bars – positive control). **(B)** Equine PBMC and murine cells, were pulsed with media alone (open bars), α-GalCer (grey bar), the additional nine NKT cell agonists (100ng/ml), lipid control, *R. equi* lipids (ascendant striped bars) or IL-2 (black bars - positive control). The proliferation of cells using thymidine uptake was assessed after 72 hours of stimulation. Data shown (Horse #184, #185 and #188) are representative of 4 independent experiments performed in 4 horses, and each value shows the mean of triplicates plus standard deviation. An asterisk represents significant difference when compared with media control (*p* value < 0.05).

Table 1 NKT cell agonists tested

Glycolipids	Reference
OCH	(Miyamoto et al., 2001)
α-GalCer C20:2	(Yu et al., 2005)
7DW8-5	(Li et al., 2010)

α-GalCer C4Ph	(Chang et al., 2007)
α-GlcCer C26	(Kinjo et al., 2006)
α-GalDAG	(Jervis et al., 2010)
α-ThrCer	(Silk et al., 2008)
PBS-57	(Liu et al., 2006)
Dimer	(Jervis et al., 2012)
GalNHAc C20:2	PJJ

1

2

1