Title: Adjuvant materials that enhance bovine $\gamma\delta$ T cell responses

Jodi F. Hedges^a, Jeff Holderness^{a1} and Mark A. Jutila^a

^aDepartment of Microbiology and Immunology

Montana State University

Bozeman, MT 59717

USA

¹Present Address:

4539 Metropolitan Ct

Frederick, MD 21704

Corresponding author:

Jodi F. Hedges, Ph.D.

960 Technology Blvd

Bozeman, MT 59718

jodi.hedges@gmail.com

Abstract

Despite the availability of vaccines and antibiotics, viral, bacterial and parasite-induced intestinal and pulmonary diseases still cause significant losses to the livestock industry. Excepting improvements in calf survival due to predation, there have been only modest improvements in bovine calf survival since 1991. Strikingly, digestive and respiratory diseases still account for almost half of the non-predator deaths in calves. The innate immune system has evolved to rapidly recognize and respond to invasive microbial threats. Augmentation of innate immunity is a broad-spectrum, potent and non-specific alternative approach to effectively counter a microbial invasion. In recent years we have focused our research efforts on the development of effective and inexpensive adjuvant therapies for cattle that can be used to help mitigate infection. Unique in our approach to the development of the potential new treatments, is our focus on bovine $\gamma\delta$ T cells, which are important lymphocytes of the innate immune system and of particular importance to ruminant immunological health. This review focuses on recent results obtained using two such adjuvant materials.

Keywords: $\gamma\delta$ T cells, innate immunity, adjuvant, Amphotericin B, Acai, Salmonella

Background

Reports from the USDA suggest that only modest improvements have been made in the rates of non-predator death of calves since 1991. Intestinal and respiratory illness still account for 46.3% of non-predator calf mortality (USDA-APHIS-VS-CEAH 2011). Many of these are caused by microbial infection that can be countered by

antibiotic use. However, antibiotic resistance is a current primary concern, and the best approach to addressing this problem is to reduce and refine antibiotic use (Landers, T.F., et al. 2012). Thus, alternative solutions to counter infection are clearly warranted. Stimulation of the innate immune system to overcome microbe-specific mechanisms of evasion represents a broad-spectrum approach to protect animals from disease. Most efforts in this area focus on cells of the myeloid lineage, such as macrophages and neutrophils (Jackson, W.D., et al. 2014). However, other non-myeloid cells, such as certain innate-like lymphocyte subsets are also important in innate immunity. In recent years we have focused our efforts in identifying novel immunomodulatory materials that enhance innate immunity by screening compounds for their effects on $\gamma\delta$ T cells. This lymphocyte subset that localizes to mucosal surfaces is likely critical for protection of ruminant species against intestinal and respiratory infection. Here we review some of our efforts in this area.

$\gamma\delta$ T cells. While the overall importance of $\gamma\delta$ T cells to the immune system is still debated, they clearly have the capacity, if properly stimulated, to mediate a large array of effector functions (Hayday, A.C. 2000, Vantourout, P., et al. 2013). $\gamma\delta$ T cells are potent cytolytic cells and can produce an array of cytokines that enhance the activities of macrophages and neutrophils, as well as other lymphocytes (Born, W., et al. 1999, Ferrick, D.A., et al. 1995, Mak, T.W., et al. 1998). $\gamma\delta$ T cells can present antigen (Brandes, M., et al. 2005, Collins, R.A., et al. 1998), and induce, as well as suppress inflammation (Egan, P.J., et al. 2000, O'Brien, R.L., et al. 2000, Zuany-Amorim, C., et al. 1998). $\gamma\delta$ T cells are found at all mucosal surfaces and are particularly well represented in the intestine, constituting a large fraction of the intraepithelial lymphocyte population

(Boismenu, R., et al. 1998, Komano, H., et al. 1995). Thus, $\gamma\delta$ T cells are one of the first innate immune cell populations to encounter pathogens that invade through the epithelial lining (Ferrick, D.A., et al. 2000). $\gamma\delta$ T cells can be recruited to sites of infection within the gut as they rapidly respond to inflammatory stimuli (Wilson, E., et al. 1999).

Though IFN- γ has historically been defined as a primary cytokine produced by $\gamma\delta$ T cells, it has been shown that $\gamma\delta$ T cells are a major, if not primary, source of IL-17 early in response to infection (Lockhart, E., et al. 2006, Shibata, K., et al. 2007). Both IFN- γ and IL-17 are potent activators of cells of the myeloid lineage. Through these activities, $\gamma\delta$ T cells have been shown to respond to and participate in host defense responses in a variety of pathogen-induced diseases including HSV-1 induced encephalitis, malaria, toxoplasmosis, leishmaniasis, cryptosporidiosis, tuberculosis, listeriosis, salmonellosis, tularemia, brucellosis, erlichiosis, and AIDS (Hayday, A.C. 2000). Because of their possible dual roles within innate and adaptive immunity, these cells have been targeted in the development of new immunotherapeutics to counter infectious and neoplastic disease (Burjanadze, M., et al. 2007).

Bovine $\gamma\delta$ T cells. Several important agricultural species such as cattle, sheep, swine and chickens have high $\gamma\delta$ T cell numbers in circulation (Telfer, J.C., et al. 2015). $\gamma\delta$ T cells comprise up to 70% of circulating T lymphocytes in the blood of bovine calves. These cells are likely critically important to the immunological health of cattle, particularly in newborn calves. Though much work remains to fully understand their importance, studies on bovine $\gamma\delta$ T cells have elucidated 1) antigens that drive their responses (Abrahamsen, M.S. 1998, Baldwin, C.L., et al. 2002, Fikri, Y., et al. 2001,

Fikri, Y., et al. 2002, Mwangi, D.M., et al. 2002, Naiman, B.M., et al. 2001, Rhodes, S.G., et al. 2001, Smyth, A.J., et al. 2001), 2) their potential role in responses against pathogens including: *Mycobacterium* spp.(Rhodes, S.G., et al. 2001, Smyth, A.J., et al. 2001, Telfer, J.C., et al. 2015), *Cryptosporidium parvum*(Abrahamsen, M.S. 1998), *Cowdriaruminantium*(Mwangi, D.M., et al. 2002), *Leptospiraborgpetersenii*(Naiman, B.M., et al. 2001), *Staphylococcus* spp.(Fikri, Y., et al. 2001), *Theileriaparva*(Daubenberger, C.A., et al. 1999) *Anaplasma marginale*(Lahmers, K.K., et al. 2006), and foot and mouth disease virus (Toka, F.N., et al. 2011), 3) ligands and counter-receptors important in their activation (Ahn, J.S., et al. 2002, Fikri, Y., et al. 2002, Sathiyaseelan, T., et al. 2002, Sopp, P., et al. 2001), 4) subset-specific responses (Hedges, J.F., et al. 2003a, Meissner, N., et al. 2003), and 5) the molecular basis for their unique trafficking behavior (Jones, W.M., et al. 1997, Jutila, M.A., et al. 1996, Jutila, M.A., et al. 1997, Walcheck, B., et al. 1994, Wilson, E., et al. 1999, Wilson, E., et al. 2002).

Our lab has spent nearly 20 years studying the immunobiology of bovine $\gamma\delta$ T cells, with the ultimate goal of identifying approaches to control the activity of these cells for the benefit of the animal. A recurring theme in much of our work, as well as in other published reports, is the similarity of $\gamma\delta$ T cells to cells of the myeloid lineage. Their tissue tropism, the inflammatory nature of a subset of $\gamma\delta$ T cells, and their capacity to rapidly respond to different stimuli are similar to myeloid cells. In global gene expression studies with sorted bovine $\gamma\delta$ T cells we found evidence for transcripts encoding myeloid surface antigens, such as CD14, CD11b, scavenger receptor 1, cytokines characteristic of myeloid cells, NADPH oxidase, super oxide dismutase

(SOD), nitric oxide, toll-like receptors (TLRs), and myeloid cell transcription factors (Hedges, J.F., et al. 2003a, Meissner, N., et al. 2003). B-lymphocyte-induced maturation protein 1 (BLIMP-1) is one of these transcription factors that was definitively expressed in $\gamma\delta$ T cells (Meissner, N., et al. 2003). At the time of our publication, BLIMP-1 was known to be expressed in B cells and cells of the myeloid lineage (Chang, D.H., et al. 2000, Shaffer, A.L., et al. 2002), but not in T cells. Since then, the function of BLIMP-1 in B cells has been extensively characterized (Martins, G., et al. 2008). Its expression and function as a transcriptional repressor in T cells has since been characterized in T cells (Kallies, A., et al. 2006, Martins, G.A., et al. 2006).

We demonstrated that NRAMP1 (or SLC11A1), a myeloid-associated gene is differentially expressed in resting and activated bovine $\gamma\delta$ T cells (Hedges, J.F., et al. 2003a, Meissner, N., et al. 2003). This selective regulation by $\gamma\delta$ T cells of NRAMP1 is particularly intriguing, since NRAMP1 function has previously been thought to be restricted to monocytes and dendritic cells (Stober, C.B., et al. 2007). NRAMP1, a metal ion transporter localized to phagosomal membranes in macrophages, enhances killing of intracellular pathogens (Barthel, R., et al. 2001, Nevo, Y., et al. 2006). NRAMP1, via transport of iron, augments cellular signaling in monocytes by reducing phosphotyrosine phosphatase activity in myeloid cells (Gomez, M.A., et al. 2007), suggesting that NRAMP1 is part of the monocyte/macrophage program for pathogen responses. We recently confirmed expression of NRAMP1 in bovine and human $\gamma\delta$ T cells and demonstrated that its expression is associated with enhanced activation of these cells (Hedges, J.F., et al. 2013). Collectively, our findings underscore similarities of $\gamma\delta$ T cells to cells of the myeloid lineage.

We have also studied the expression and function of innate immune cell receptors in $\gamma\delta$ T cells. In earlier studies, we found that bovine $\gamma\delta$ T cells express transcripts for many pathogen associated molecular pattern (PAMP) receptors (Hedges, J.F., et al. 2003a, Hedges, J.F., et al. 2003b, Meissner, N., et al. 2003), and respond to a number of PAMPs (Hedges, J.F., et al. 2005, Kerns, H.M.M., et al. 2009, Lubick, K., et al. 2006). Rapid, innate responses of bovine $\gamma\delta$ T cells are detected early during *Salmonella* infection of the intestinal mucosa (Hedges, J.F., et al. 2007). Together these data indicate that use of materials that activate these innate receptors on bovine $\gamma\delta$ T cells could enhance their function early in infection. To this end, using high throughput cell-based screening assays, we identified a number of agonists that specifically prime bovine $\gamma\delta$ T cells for enhanced innate immune cell function (Graff, J.C., et al. 2009, Holderness, J., et al. 2007, Holderness, J., et al. 2008, Holderness, J., et al. 2011, Jutila, M.A., et al. 2007, Skyberg, J.A., et al. 2012). One of the identified agonists is a clinically approved drug, amphotericin B (AmB), a polyene produced by *Streptococcus* spp. used to treat fungal infections (Hedges, J.F., et al. 2015). In these screens we also found that Nystatin, another polyene, was also an agonist for bovine $\gamma\delta$ T cells. AmB showed superior activity over nystatin, thus we focused most of our studies on various clinical and research grade preparations of AmB. Other potent agonists are plant-derived polysaccharides (Graff, J.C., et al. 2009, Holderness, J., et al. 2011). One uniquely effective polysaccharide material is isolated from Acai berries (Acai PS), a widely used nutritional supplement with purported immune enhancing and activities (Holderness, J., et al. 2011). Below we review some of our most recent work

and present new data demonstrating the immunostimulatory properties of AmB and Acai PS.

Materials and Methods

AmB treatment *in vivo*

BALB/c mice (6 animals/group, aged between 6-12 weeks) were lightly anaesthetized with isoflurane and either received no treatment or were treated intratracheally with 10 µg of AmB (Sigma). Twenty four hours later, lung cells were isolated from all 12 animals and plated in serum-free X-VIVO 15 medium (Lonza). Phase II *Coxiella burnetii* (1×10^8 bacteria per well) was added to half the cells from of control or AmB-treated mice (3 wells/group), and the other half was uninfected. The plates were then incubated for 48 hours at 37°C in a humidified incubator with 5% CO₂. Supernatant fluids were collected and IFN-γ levels in were measured by ELISA (Mabtech).

To determine if AmB enhances antibody responses, C57BL/6 mice (5 per group) were intraperitoneally injected with 10µg OVA antigen alone or in combination with AmB (10µg). Blood was collected on day 0 and at 7 day intervals post-injection, up to day 42. Serum was separated by centrifugation, and assessed by ELISA for anti-OVA antibody responses.

Calf Infection Model

Salmonella challenge infection was performed as described elsewhere (Hedges, J.F., et al. 2015). Calves were treated with a subcutaneous dose of 1.7mg AmB (212.5µg/ml in 8 ml, 4ml per site x 2 sites) or saline only 24 hours prior to infection, then

infected with *Salmonella* by the oral route (n=6 per group in 3 separate experiments). Briefly, calves were housed in ABSL2 SPF conditions from 1-6 weeks of age to ensure equivalent health status prior to ST infection studies. At 6 weeks, calves were orally infected with 1×10^6 - 1×10^8 CFUs of a natural calf isolate of *Salmonella* serovar Typhimurium (ST, Type O, Group B). This isolate has been utilized in prior studies (Hedges, J.F., et al. 2007) and causes a predictable and severe infection in calves. Prior to infection, 1 μ l of ST stock was added to 4ml of LB media and shaken for 18 hours at 37°. Bacteria were then subcultured 1:40 then again shaken for 2 hours at 37°C. CFU counts were verified by plating serial dilutions of bacterial suspensions on LB agar plates. Fecal samples were collected at infection and at 24 hour intervals post infection. Calves were monitored twice daily, and hourly when disease symptoms became apparent. Calves were scored on a scale of 1-5 for fevers, attitude, appetite and fecal status. Enterocolitis was defined as fever ($>105^\circ$ F) and diarrhea between 24 and 48 hours post infection. Fecal samples were collected daily and submitted to the Montana State Veterinary Diagnostic Service. Those from experimentally infected calves tested positive for the input strain of ST and negative for other infectious agents. All animal protocols were reviewed and approved by the MSU Institutional Animal Care and Use Committee.

β -glucan Inhibition ELISA

β -(1-3)glucan was measured using a capture ELISA similar to that described by Meikle et al (Meikle, P.J., et al. 1991). 96-well MaxiSorp plates (Nunc, Thermo Scientific) were coated overnight with laminarin (Sigma) freshly reconstituted to 2 μ g/mL in PBS. Plates were then blocked for 90min. with 1% BSA in PBS. Next, 50 μ L

polysaccharides or PBS were added, followed immediately by the addition of 50 μ L of 1 μ g/mL anti- β (1-3)-glucan (Biosupplies; Melbourne, Australia). After 90m, plates were washed twice, anti-mouse-HRP was added for 60min., and the plates were washed thrice. The HRP substrate ABTS was added and color change was visualized in a microplate reader.

Measurement of Dectin-1 surface receptor binding by flow cytometry

All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For primary cells, whole blood was collected from healthy human adult donors with ACT tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Histopaque 1077 (Sigma-Aldrich) as per the manufacturer's instructions. Freshly collected human PBMCs were either fixed (2% PFA) prior to or immediately treated with Acai, laminarin, zymosan, curdlan, or pullulan polysaccharides for 1-2h in 100 μ L PBS. After this incubation period, 100 μ L Dectin-1 mAb directed to the polysaccharide-binding pocket of the receptor (Clone 259931, R&D Systems) or mAbs to CD14, MHCII, CD11b, or PBS was added and incubated for an additional 10minutes. Cells were washed with cold PBS containing 1% horse serum and analyzed for mAb binding by flow cytometry.

Mouse Treatments

Cytokine induction following ingestion of a water extract of Acai was assessed in dysbiotic mice. In this experiment, we wished to subtly perturb the digestive tract by induction of dysbiosis, to determine if ingestion of Acai could reverse this non-infectious damage. Dysbiosis was induced following the procedure described elsewhere, in which

mice are fed a cocktail of 3 antibiotics for 4 days, then injected with a 4th (Chen, X., et al. 2008). Following this treatment, mice were fed 3 doses of 5mg Acai or vehicle (water) only at intervals of about 12 hours. Four hours after the final dose, mice were euthanized and MLNs, spleens and serum were collected. The cells in MLNs and spleens were isolated by homogenization and cultured at 2×10^6 cells per ml for 48 hours in XVIVO serum-free media (Lonza). Then culture supernatant fluids were collected and assessed for cytokine expression along with the collected serum samples. The cytokines KC, IL-22, IL-12 and IFN- γ were measured by commercial ELISA (MabTech). There was minimal detection of IL-22, and no differences in expression of KC or IFN- γ between Acai and vehicle-treated mice (data not shown).

Acai ingestion was also assessed in mice with colitis, induced by ingestion of Dextran sulfate sodium (DSS). DSS was delivered as a 3% solution in the drinking water. Mice were fed 5mg of a water extract of Acai or vehicle only by oral gavage on day -2 of DSS treatment and daily thereafter. Mice were weighed daily for 5 days then euthanized. Colon length was assessed, and colons embedded in 10% formalin for histology, performed as described elsewhere (Ramstead, A.G., et al. 2016). MLN were also collected and the cells were placed in culture at 2×10^6 cells per ml in XVIVO 15 serum free media (Lonza). After 48 hours supernatant fluids were collected and assessed for cytokine (IFN- γ , IL-22 and IL-12) expression by ELISA (Mabtech) according to the manufacturer's instructions. Changes to IFN- γ and IL-22 induced by Acai treatment were not significant (data not shown).

Statistical Analyses

Studies in mice involved groups of 3 to 5 whereas calf studies show combined experiments with n=6 calves per treatment group. Groups were compared using the Student's t test, or 2-way ANOVA as specified in the figure legends. Comparisons that yielded a p value <0.05 were considered significant.

Results

Immunomodulatory activity of AmB. AmB is a commonly used anti-fungal drug that has documented innate immunostimulatory capacity (Hauser, W.E., et al. 1983, Little, J.R., et al. 1983, Lohr, K.M., et al. 1982). AmB induces expression of inflammatory cytokines upon direct interaction with macrophages (Arning, M., et al. 1995, Cleary, J.D., et al. 1992, Rogers, P.D., et al. 1998). Toll-like receptors (TLRs) are implicated in the immune stimulatory effects of AmB. However, TLR recognition of AmB may be concentration, formulation, and tissue dependent (Bellocchio, S., et al. 2005, Sau, K., et al. 2003). Thus, AmB is not only an effective antifungal agent, it also affects innate macrophage responses through TLR stimulation.

AmB also has both stimulatory and inhibitory effects on lymphocytes. Specifically, AmB enhances proliferation of lymphocyte subsets and is a B cell mitogen (Hammarstrom, L., et al. 1976, Mehta, R.T., et al. 1985, Sarthou, P., et al. 1986, Shirley, S.F., et al. 1979), consistent with its TLR4 activity. AmB is stimulatory to T cells, as well (Hauser, W.E., et al. 1983, Kumar, S., et al. 2000, Mehta, R.T., et al. 1985). However, AmB suppresses T- and B-cell activity at high doses, in particular (Boggs, J.M., et al. 1991, Ferrante, A., et al. 1979, Hauser, W.E., et al. 1983, Kretschmar, M., et al. 2001, Schindler, J.J., et al. 1993, Walls, E.V., et al. 1982).

AmB treatment of bovine PBMCs leads to specific stimulatory effects on $\gamma\delta$ T cells, as reflected by increased expression of IL-2R(Hedges, J.F., et al. 2015). AmB-treated cells proliferated directly in response to addition of IL-2, thus functioning as an antigen-independent priming agent(Hedges, J.F., et al. 2015). AmB induced increased expression of activation markers and proliferation of $\gamma\delta$ T cells from cattle, humans and mice *in vitro*(Hedges, J.F., et al. 2015), thus, the response is highly conserved. Of cells from these three species, bovine $\gamma\delta$ T cells responded most robustly to AmB, even at low nanomolar concentrations. Activating effects of AmB on macrophages were confirmed in our studies as well. We found that robust activation of $\gamma\delta$ T cells required the presence of macrophages, showing a cooperative effect in response to AmB. In addition to increased responsiveness to IL-2, AmB treatment of PBMCs increased downstream production of IFN- γ following IL-18 treatment. The source of IFN- γ *in vitro* was NK cells, rather than $\gamma\delta$ T cells(Hedges, J.F., et al. 2015). AmB also increased IFN- γ production in mouse lung cells following *in vitro* infection/co-stimulation by avirulent *C. burnetii* bacteria (Figure 1). The adjuvant activity of AmB was also reflected in enhanced antibody responses against protein antigens (Figure 2). Thus, AmB has potential both to enhance innate responses to infection and function as a vaccine adjuvant.

Because of its profound effect on multiple responses of the innate immune system, AmB was applied in a non-fungal infectious challenge model. Bovine calves are highly prone to infection in early life and *Salmonella* is a major problem for ranchers and farmers(USDA-APHIS-VS-CEAH 2011). We reasoned that, since bovine $\gamma\delta$ T cells and NK cells respond to very low doses of AmB *in vitro*, a very low dose *in vivo* would not induce reported toxic side effects, yet might stimulate these important cell subsets to

contribute to protection from infection. Six week old calves were given one intravenous injection of approximately 0.029-0.031mg/kg AmB (1.7mg in 10ml of saline), or saline only 24 hours prior to infection with *Salmonella* by the oral route. This dose of AmB is approximately 10-fold lower than the recommended dose delivered daily to human patients for treatment of fungal infections and did not induce fever or other outwardly apparent detrimental effects in the animals. The calves that received AmB developed significantly lessened disease states (as measured by lower fevers, decreased fecal *Salmonella* burden, and other parameters) than did saline injected calves (Hedges, J.F., et al. 2015). Thus, not only were calves injected with a single low-dose of AmB protected from disease symptoms and severity, but they also subsequently shed less bacteria into the environment than did vehicle-treated calves which would likely improve individual as well as herd protection.

Recently, we have modified the treatment approach and tested delivery of AmB by subcutaneous (s.c.) injection. This route is easier and more commonly used in practice than i.v. delivery. In this case, calves injected with AmB were significantly protected from *Salmonella*-induced diarrhea (Figure 3A) and similarly fared better overall compared to saline treated calves (Figure 3B). Interestingly, s.c. injection did not protect calves against *Salmonella*-induced fever responses (Figure 3C), in contrast to i.v. treatment (Hedges, J.F., et al. 2015). Following s.c. treatment of calves with AmB there was also a trend toward decreased detection of bacteria in fecal samples in each of 3 separate experiments, although the variation between experiments precluded the conclusion of significance. These results suggest that AmB may be a safe, well

characterized, and readily available drug for use in enhancing innate immunity in bovine calves that are highly susceptible to infection.

Immunomodulatory activity of Acai. Polysaccharides from the Acai berry extract (Acai PS) have potent stimulatory activity for $\gamma\delta$ T cells and NK cells in a mixed cell preparation (Holderness, J., et al. 2011, Skyberg, J.A., et al. 2012). Acai PS activity is similar in most respects to the responses induced by AmB. Although Acai berries contain anthocyanidins, we demonstrated that the innate immune enhancement in Acai berries is associated with the polysaccharide fraction (Holderness, J., et al. 2011). Acai PS is specifically stimulatory to $\gamma\delta$ T cells in a mixed population of blood cells, and also increases IL-12 expression in monocytes treated *in vitro* (Holderness, J., et al. 2011). Like AmB, Acai PS had immunostimulatory effects on human and mouse $\gamma\delta$ T cells and monocytes/macrophages. Some of the responses were dependent on TLR4 and the innate adaptor protein MyD88 in mice, however neutrophils were recruited to the peritoneum through an additional pathway that is MyD88-independent (Graff, J.C., et al. 2009, Holderness, J., et al. 2011).

Because of TLR4- and MyD88- independent activities, the potential for Acai PS to signal through Dectin-1 was determined. Dectin-1 is important for sensing β -glucan, derived from food sources (eg. oats, barley, and mushrooms), infectious fungi, and bacteria (Brown, G.D., et al. 2003, Gordon, S. 2002). Dectin-1 is involved in activation of enterocytes, and in particular, expression of IL-12 by immune cells in this region following ingestion (Volman, J.J., et al. 2010). We tested whether Acai PS contained the appropriate linkages for recognition by Dectin-1 using an inhibition ELISA against β -glucan (Figure 4). Acai PS, and the polysaccharides zymosan, pullulan, and laminarin,

were assayed for the ability to block binding of an anti- β -glucan antibody to laminarin-coated plates. Pullulan, which does not contain β -glucan linkages, served as a negative control. Acai PS, zymosan, and laminarin all decreased antibody binding, indicating the presence of β -glucan residues in each of these preparations (Figure 4A). To determine if Acai PS could also bind the Dectin-1 receptor, human PBMCs were cultured with the same polysaccharides and the binding affinities of a Dectin-1 antibody, as well as other myeloid cell receptors, were analyzed by flow cytometry. Laminarin, zymosan, and AcaiPS inhibited binding of the Dectin-1 antibody, but not the binding of other monocyte receptors (Figure 4B, C). This unique capacity of a plant-derived compound to bind multiple innate receptors likely contributes to its specific immune-protective effects following ingestion.

Working with Jerod Skyberg and David Pascual, we also showed that Acai PS enhances innate protection in mice from *Francisella tularensis* and *Burkholderiapseudomallei* infections following intranasal and, though less effectively, oral administration (Skyberg, J.A., et al. 2012). The primary outcome of this treatment is enhanced IFN- γ production by both $\gamma\delta$ T cells and NK cells (Skyberg, J.A., et al. 2012). Inhibition of IFN- γ by blocking using an antibody *in vivo* eliminated the protection in both infection models. Thus, Acai PS has a strong likelihood of providing innate protection by augmenting IL-12 and IFN- γ expression that is applicable in a wide range of inflammatory and infectious conditions.

Since Acai supplement is ingested and appears safe(Skyberg, J.A., et al. 2012)we were interested in its effects following oral delivery. We further tested Acai PS

in two intestinal models. In the first, Acai PS was fed to mice pretreated with an antibiotic cocktail to induce dysbiosis (disruption of normal flora), which is associated with increased susceptibility to infection and colitis (Chen, X., et al. 2008). Some mice were fed 3 doses of Acai PS, 5mg each, comparable to a 1g dose in humans, every 12 hours for 2 days, and euthanized 4 hours after the final dose. This is a much lower dose than doses of other supplement extracts we have used in previous studies (Skyberg, J.A., et al. 2011), but also more relevant to supplement use in humans. MLNs and spleen cells were collected and cultured for 24 hours. Consistent with *in vitro* findings (Holderness, J., et al. 2011), feeding Acai PS induced expression of IL-12 in culture supernatant fluids from MLNs, spleens, and in serum (Figure 5). IFN- γ was also increased in spleens from Acai PS-fed mice (data not shown). Thus, ingestion of Acai PS appears to have a substantial effect on immune cells that underlay or traffic from the mucosa. The animals showed no adverse inflammatory effects following Acai PS treatment, thus, the treatment was well tolerated. These data indicate that following antibiotic treatment, Acai PS can induce innate responses in mucosal and associated immune cells.

Acai PS was also tested in a chemically-induced model of colitis using dextran sulfate sodium (DSS). We had previously found that another plant derived $\gamma\delta$ T cell agonist, oligomeric procyanidins, reduced DSS-induced colitis, following oral delivery (Skyberg, J.A., et al. 2011). Ingestion of Acai PS similarly provided benefit in DSS-induced colitis in mice as evidenced by increased colon lengths (Figure 6A) and again, increased expression of IL-12 derived from cultured MLN cells (Figure 6B). Finally, histological changes were apparent in Acai PS- and DSS-fed mice. Histological slides

from these mice were stained for mucin as described elsewhere (Lindin, S.K., et al. 2008). In the ceca, the Acai PS-treated mice appeared to be secreting less mucin, and less was produced at the bases of the villi compared to water-treated mice with DSS (Figure 6C). Also apparent in this figure is the relatively diminished influx of neutrophils in the sub-mucosal space in both treated mice, compared to the water controls. These data indicate a diminished inflammatory response at the mucosa and suggest a tissue-specific response following ingestion of Acai PS. A safe and effective orally delivered plant product with the capacity to enhance innate protection and dampen inflammation in colitis could be used to alleviate disease severity and potentially decrease the dose or duration of antibiotic use.

Based on the striking results with Acai PS in mice, we are now focusing our efforts on developing treatment protocols in calves, using the same models as described above for AmB. Results following oral delivery in mice, suggest that a similar oral delivery approach could be developed for Acai PS in at risk ruminant groups, thus simplifying its use.

In summary, our studies show that $\gamma\delta$ T cells are an effective target cell in the screening of potential new therapeutics to serve as immunomodulatory agents. Emergence of pathogens that are resistant to current antibiotic therapies is of major concern, thus the use of antibiotics should be restricted. Therapies that enhance innate responses could be used to complement antibiotic use and thus allow for reduction in dose and duration of antibiotics.

Figure Legends

Figure 1. AmB treatment *in vivo* results in expression of IFN- γ following *ex vivo* bacterial infection/stimulation. BALB/c mice (3 animals/group) either received no treatment or were treated intratracheally with 10 μ g of AmB. Twenty four hours later, lung cells were isolated from all 12 animals and plated in serum-free X-VIVO 15 medium. Phase II *Coxiella burnetii* (1×10^8 bacteria per well) was added to some of the wells containing control or AmB-treated cells. The plates were then incubated for 48 hours and IFN- γ levels in the supernatant fluids were measured by ELISA. Error bars represent means $\pm$ standard error. ***Difference between control plus *C. burnetii* and AmB-treated plus *C. burnetii* was significant at a p value of <0.0001 using the Student's t test.

Figure 2. AmB enhances antibody responses against ovalbumin (OVA). Mice (5 per group) were immunized i.p. with 10 μ g OVA alone or in combination with 10 μ g AmB. At different time points after the primary immunization (x axis), serum was collected and anti-OVA antibody responses were quantified. Error bars represent standard deviation. Data sets were compared using 2-way ANOVA and found to be significantly different with **** $p < 0.0001$.

Figure 3. Subcutaneous delivery of AmB protects calves from *Salmonella*-induced diarrhea. Calves were treated with a s.c. dose of 1.7mg AmB or saline only 24 hours prior to infection, then infected with *Salmonella* by the oral route (n=6 per group in 3 separate experiments). A. Calves injected with AmB had significantly reduced diarrhea compared to controls. B. When attitude, appetite, fevers and fecals were combined, the AmB-treated calves were still significantly improved compared to controls. C. In

contrast to findings following i.v. injection, calves injected s.c. did not show improvements in fever responses to *Salmonella* infection. (Two-way ANOVA, *** $p < 0.001$, * $p < 0.05$).

Figure 4. Acai-PS contains β -glucan structures that bind to the Dectin-1 receptor. A. $\beta(1,3)$ -glucan content was measured by inhibition ELISA. Increasing concentrations of Acai PS blocked binding of an anti- β -glucans antibody to laminarin-coated plates, similar to compounds known to contain β -glucans, laminarin and zymosan. Pullulan, which does not contain β -glucan, did not block antibody binding at any concentration. Results are representative of three similar experiments. B,C) Human PBMCs were treated with polysaccharides or PBS for 1h and then stained with anti-Dectin-1 or other antibodies prior to analysis. B) mAb staining for four surface markers and four polysaccharides indicate that Acai PS, laminarin and zymosan specifically block anti-Dectin-1 antibody staining. C. Acai-PS-treatment decreases Dectin-1 mAb staining compared to PBS in cells from 4 different donors. (p-value calculated using one-tailed paired t test).

Figure 5. Expression of IL-12 was increased in cells and serum from dysbiotic mice fed Acai PS. Mice were fed 3 doses of Acai PS then 4 hours after the final dose MLNs, spleens and serum were collected. MLNs and spleens were cultured for an additional 24 hours before supernatant fluids were collected. IL-12 was measured in culture supernatant fluids or serum by ELISA. (Error bars represent means $\pm$ standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as calculated by one-tailed unpaired t test).

Figure 6. Acai PS ingestion alleviates DSS colitis. A. Treatment with Acai PS resulted in significantly increased colon lengths. B. Expression of IL-12 was increased in MLN cell culture supernatant fluids derived from Acai PS-fed mice, relative to those fed water. C. Histological slides were assessed for mucin staining (pink). Acai PS-treated mice had diminished neutrophil influx into the ceca (arrow), and mucin production more similar to healthy than to vehicle-treated mice with DSS-induced colitis. (Error bars represent means $\pm$ standard error. * $p < 0.05$, ** $p < 0.01$ as calculated by one-tailed unpaired t test)

Acknowledgements

This project is supported by the Agriculture and Food Research Initiative competitive grant no. 2014-67016-21552 of the USDA National Institute of Food and Agriculture, with partial funding through NIH-NCCAM (AT0004986-01), NIHIDeA Program grant GM110732, M.J. Murdock Charitable Trust and The Montana State University Agricultural Experimental Station.

Reference List

Abrahamsen, M. S., 1998. Bovine T cell responses to *Cryptosporidium parvum* infection. *Int J Parasitol* 28, 1083-1088.

Ahn, J. S., Konno, A., Gebe, J. A., Aruffo, A., Hamilton, M. J., Park, Y. H., Davis, W. C., 2002. Scavenger receptor cysteine-rich domains 9 and 11 of WC1 are receptors for the WC1 counter receptor. *J. Leukoc. Biol.* 72, 382-390.

Arning, M., Kliche, K. O., Heer-Sonderhoff, A. H., Wehmeier, A., 1995. Infusion-related toxicity of three different amphotericin B formulations and its relation to cytokine plasma levels. *Mycoses* 38, 459-465.

Baldwin, C. L., Sathiyaseelan, T., Naiman, B., White, A. M., Brown, R., Blumerman, S., Rogers, A., Black, S. J., 2002. Activation of bovine peripheral blood gammadelta T cells for cell division and IFN-gamma production. *Vet. Immunol Immunopathol.* 87, 251-259.

Barthel, R., Feng, J., Piedrahita, J. A., McMurray, D. N., Templeton, J. W., Adams, L. G., 2001. Stable transfection of the bovine NRAMP1 gene into murine RAW264.7 cells: effect on *Brucella abortus* survival. *Infect. Immun.* 69, 3110-3119.

Bellocchio, S., Gaziano, R., Bozza, S., Rossi, G., Montagnoli, C., Perruccio, K., Calvitti, M., Pitzurra, L., Romani, L., 2005. Liposomal amphotericin B activates antifungal resistance with reduced toxicity by diverting Toll-like receptor signalling from TLR-2 to TLR-4. *J Antimicrob Chemother.* 55, 214-222.

Boggs, J. M., Chang, N. H., Goundalkar, A., 1991. Liposomal amphotericin B inhibits in vitro T-lymphocyte response to antigen. *Antimicrob. Agents Chemother.* 35, 879-885.

Boismenu, R., Havran, W. L., 1998. Gammadelta T cells in host defense and epithelial cell biology. *Clin Immunol Immunopathol* 86, 121-133.

Born, W., Cady, C., Jones-Carson, J., Mukasa, A., Lahn, M., O'Brien, R., 1999. Immunoregulatory functions of gamma delta T cells. *Adv Immunol* 71, 77-144.

Brandes, M., Willimann, K., Moser, B., 2005. Professional antigen-presentation function by human gammadelta T Cells. *Science* 309, 264-268.

Brown, G. D., Gordon, S., 2003. Fungal [beta]-glucans and mammalian immunity. *Immunity* 19, 311-315.

Burjanadze, M., Condomines, M., Reme, T., Quittet, P., Latry, P., Lugagne, C., Romagne, F., Morel, Y., Rossi, J. F., Klein, B., Lu, Z. Y., 2007. In vitro expansion of gamma delta T cells with anti-myeloma cell activity by Phosphostim and IL-2 in patients with multiple myeloma. *British Journal of Haematology* 139, 206-216.

Chang, D. H., Angelin-Duclos, C., Calame, K., 2000. BLIMP-1: trigger for differentiation of myeloid lineage. *Nat. Immunol.* 1, 169-176.

Chen, X., Katchar, K., Goldsmith, J. D., Nanthakumar, N., Cheknis, A., Gerding, D. N., Kelly, C. P., 2008. A Mouse Model of *Clostridium difficile*–Associated Disease. *Gastroenterology* 135, 1984-1992.

Cleary, J. D., Chapman, S. W., Nolan, R. L., 1992. Pharmacologic modulation of interleukin-1 expression by amphotericin B-stimulated human mononuclear cells. *Antimicrob. Agents Chemother.* 36, 977-981.

Collins, R. A., Werling, D., Duggan, S. E., Bland, A. P., Parsons, K. R., Howard, C. J., 1998. Gammadelta T cells present antigen to CD4+ alphabeta T cells. *J. Leukoc. Biol.* 63, 707-714.

Daubenberger, C. A., Taracha, E. L., Gaidulis, L., Davis, W. C., McKeever, D. J., 1999. Bovine gammadelta T-cell responses to the intracellular protozoan parasite *Theileria parva*. *Infect. Immun.* 67, 2241-2249.

Egan, P. J., Carding, S. R., 2000. Downmodulation of the inflammatory response to bacterial infection by gammadelta T cells cytotoxic for activated macrophages. *J Exp Med* 191, 2145-2158.

Ferrante, A., Rowan-Kelly, B., Thong, Y. H., 1979. Suppression of immunological responses in mice by treatment with amphotericin B. *Clin Exp. Immunol* 38, 70-76.

Ferrick, D. A., King, D. P., Jackson, K. A., Braun, R. K., Tam, S., Hyde, D. M., Beaman, B. L., 2000. Intraepithelial gamma delta T lymphocytes: sentinel cells at mucosal barriers. *Springer Semin. Immunopathol.* 22, 283-296.

Ferrick, D. A., Schrenzel, M. D., Mulvania, T., Hsieh, B., Ferlin, W. G., Lepper, H., 1995. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* 373, 255-257.

Fikri, Y., Denis, O., Pastoret, P., Nyabenda, J., 2001. Purified bovine WC1+ gamma delta T lymphocytes are activated by staphylococcal enterotoxins and toxic shock syndrome toxin-1 superantigens: proliferation response, TCR V gamma profile and cytokines expression. *Immunol Lett* 77, 87-95.

Fikri, Y., Pastoret, P. P., Nyabenda, J., 2002. Costimulatory molecule requirement for bovine WC1+gammadelta T cells' proliferative response to bacterial superantigens. *Scand. J Immunol* 55, 373-381.

Gomez, M. A., Li, S., Tremblay, M. L., Olivier, M., 2007. NRAMP-1 Expression Modulates Protein-tyrosine Phosphatase Activity in Macrophages: Impact on host cell signalling and functions. *J Biol Chem.* 282, 36190-36198.

Gordon, S., 2002. Pattern recognition receptors: Doubling up for the innate immune response. *Cell* 111, 927-930.

Graff, J. C., Kimmel, E. M., Schepetkin, I. A., Freedman, B., Lubick, K. J., Holderness, J., Quinn, M. T., Jutila, M. A., Hedges, J. F., 2009. Polysaccharides derived from YamoTM (*Funtumia elastica*) affect innate immunity in part by priming $\gamma\delta$ T cells. *International Immunopharmacology* 9, 1313-1322.

Hammarstrom, L., Smith, E., 1976. Mitogenic properties of polyene antibiotics for murine B cells. *Scand J Immunol* 5, 37-43.

Hauser, W. E., Remington, J. S., 1983. Effect of amphotericin B on natural killer cell activity in vitro. *J Antimicrob Chemother.* 11, 257-262.

Hayday, A. C., 2000. $\gamma\delta$ cells: a right time and a right place for a conserved third way of protection. *Ann Rev Immunol* 18, 975-1026.

Hedges, J. F., Buckner, D. L., Rask, K. M., Kerns, H. M. M., Jackiw, L. O., Trunkle, T. C., Pascual, D. W., Jutila, M. A., 2007. Mucosal lymphatic-derived $\gamma\delta$ T cells respond early to experimental *Salmonella* enterocolitis by increasing expression of IL-2R α . *Cell Immunol* 246, 8-16.

Hedges, J. F., Cockrell, D., Jackiw, L., Meissner, N., Jutila, M. A., 2003a. Differential mRNA expression in circulating $\gamma\delta$ T lymphocyte subsets defines unique tissue-specific functions. *J. Leukoc. Biol.* 73, 306-314.

Hedges, J. F., Graff, J. C., Jutila, M. A., 2003b. Transcriptional profiling of gamma delta T cells. *J Immunol* 171, 4959-4964.

Hedges, J. F., Kimmel, E. M., Snyder, D. T., Jerome, M., Jutila, M. A., 2013. SLC11A1 is expressed by innate lymphocytes and augments their activation. *J. Immunol.* 190, 4263-4273.

Hedges, J. F., Lubick, K. J., Jutila, M. A., 2005. $\gamma\delta$ T cells respond directly to pathogen associated molecular patterns. *J Immunol* 174, 6045-6053.

Hedges, J. F., Mitchell, A. M., Jones, K., Kimmel, E., Ramstead, A. G., Snyder, D. T., Jutila, M. A., 2015. Amphotericin B stimulates $\gamma\delta$ T and NK cells, and enhances protection from *Salmonella* infection. *Innate Immun.* 6, 598-608.

Holderness, J., Hedges, J. F., Daughenbaugh, K. F., Kimmel, E., Graff, J. C., Freedman, B., Jutila, M. A., 2008. Response of $\gamma\delta$ T cells to plant-derived tannins. *Crit Rev Immunol* 28, 377-402.

Holderness, J., Jackiw, L., Kimmel, E., Kerns, H. M. M., Radke, M., Hedges, J. F., Petrie, C., McCurley, P., Glee, P. M., Palecanda, A., Jutila, M. A., 2007. Select plant tannins induce IL-2R α up-regulation and augment cell division in $\gamma\delta$ T cells. *J. Immunol.* 179, 6468-6478.

Holderness, J., Schepetkin, I. A., Freedman, B., Kirpotina, L. N., Quinn, M. T., Hedges, J. F., Jutila, M. A., 2011. Polysaccharides isolated from Acai fruit induce innate immune responses. *PLoS ONE* 6, e17301-

Jackson, W. D., Woollard, K. J., 2014. Targeting monocyte and macrophage subpopulations for immunotherapy: a patent review (2009-2013). *Expert Opin. Ther. Patents* 24, 779-790.

Jones, W. M., Watts, G. M., Robinson, M. K., Vestweber, D., Jutila, M. A., 1997. Comparison of E-selectin-binding glycoprotein ligands on human lymphocytes, neutrophils, and bovine gamma delta T cells. *J Immunol* 159, 3574-3583.

Jutila, M. A., Holderness, J., Graff, J. C., Hedges, J. F., 2007. Antigen Independent priming: A transitional response of bovine $\gamma\delta$ T cells to infection. *Anim Health Res Rev* 9, 47-57.

Jutila, M. A., Kurk, S., 1996. Analysis of bovine gamma delta T cell interactions with E-, P-, and L-selectin. Characterization of lymphocyte on lymphocyte rolling and the effects of O-glycoprotease. *J Immunol* 156, 289-296.

Jutila, M. A., Wilson, E., Kurk, S., 1997. Characterization of an adhesion molecule that mediates leukocyte rolling on 24 h cytokine- or lipopolysaccharide-stimulated bovine endothelial cells under flow conditions. *J Exp Med* 186, 1701-1711.

Kallies, A., Hawkins, E. D., Belz, G. T., Metcalf, D., Hommel, M., Corcoran, L. M., Hodgkin, P. D., Nutt, S. L., 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 7, 466-474.

Kerns, H. M. M., Jutila, M. A., Hedges, J. F., 2009. The distinct response of $\gamma\delta$ T cells to the Nod2 agonist, muramyl dipeptide. *Cellular Immunology* 257, 38-43.

Komano, H., Fujiura, Y., Kawaguchi, M., Matsumoto, S., Hashimoto, Y., Obana, S., Mombaerts, P., Tonegawa, S., Yammamoto, H., Itohara, S., Nanno, M., Ishikawa, H., 1995. Homeostatic regulation of intestinal epithelia by intraepithelial $\gamma\delta$ T cells. PNAS 92, 6147-6151.

Kretschmar, M., Geginat, G., Bertsch, T., Walter, S., Hof, H., Nichterlein, T., 2001. Influence of Liposomal Amphotericin B on CD8 T-Cell Function. Antimicrob. Agents Chemother. 45, 2383-2385.

Kumar, S., Chakrabarti, R., 2000. Amphotericin B both inhibits and enhances T-cell proliferation: Inhibitory effect is mediated through H₂O₂ production via cyclooxygenase pathway by macrophages. J. Cell. Biochem. 77, 361-371.

Lahmers, K. K., Hedges, J. F., Jutila, M. A., Deng, M., Abrahamsen, M. S., Brown, W. C., 2006. Comparative gene expression by WC1+ $\gamma\delta$ and CD4+ $\alpha\beta$ T lymphocytes, which respond to Anaplasma marginale, demonstrates higher expression of chemokines and other myeloid cell-associated genes by WC1+ $\gamma\delta$ T cells. J. Leukoc. Biol. 80, 939-952.

Landers, T. F., Cohen, B., Wittum, T. E., Larson, E. L., 2012. A Review of Antibiotic Use in Food Animals: Perspective, Policy, and Potential. Public Health Rep 127, 4-22.

Lindin, S. K., Iorin, T. H. J., cGuckin, M. A., 2008. Mucin Dynamics in Intestinal Bacterial Infection. PLoS ONE 3, e3952-

Little, J. R., Abegg, A., Plut, E., 1983. The relationship between adjuvant and mitogenic effects of amphotericin methyl ester. *Cell Immunol* 78, 224-235.

Lockhart, E., Green, A. M., Flynn, J. L., 2006. IL-17 Production Is Dominated by $\gamma\delta$ T Cells rather than CD4 T Cells during *Mycobacterium tuberculosis* Infection. *J Immunol* 177, 4662-4669.

Lohr, K. M., Snyderman, R., 1982. Amphotericin B alters the affinity and functional activity of the oligopeptide chemotactic factor receptor on human polymorphonuclear leukocytes. *J. Immunol.* 129, 1594-1599.

Lubick, K., Jutila, M. A., 2006. LTA recognition by bovine $\gamma\delta$ T cells involves CD36. *J. Leukoc. Biol.* 79, 1268-1270.

Mak, T. W., Ferrick, D. A., 1998. The gammadelta T-cell bridge: linking innate and acquired immunity. *Nat Med* 4, 764-765.

Martins, G., Calame, K., 2008. Regulation and Functions of Blimp-1 in T and B Lymphocytes. *Annu. Rev. Immunol.* 26, 133-169.

Martins, G. A., Cimmino, L., Shapiro-Shelef, M., Szabolcs, M., Herron, A., Magnusdottir, E., Calame, K., 2006. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 7, 457-465.

Mehta, R. T., Mehta, K., Lopez-Berestein, G., Juliano, R. L., 1985. Effect of liposomal amphotericin B on murine macrophages and lymphocytes. *Infect. Immun.* 47, 429-433.

Meikle, P. J., Bonig, I., Hoogenraad, N. J., Clarke, A. E., Stone, B. A., 1991. The location of (1 $\rightarrow$ 3)-beta-glucans in the walls of pollen tubes of *Nicotiana glauca* using a (1 $\rightarrow$ 3)-beta-glucan-specific monoclonal antibody. *Planta* 185, 1-8.

Meissner, N., Radke, J., Hedges, J. F., White, M., Behnke, M., Bertolino, S., Abrahamsen, M. S., Jutila, M. A., 2003. Serial analysis of gene expression in circulating $\gamma\delta$ T cell subsets defines distinct immunoregulatory phenotypes and unexpected gene expression profiles. *J Immunol* 170, 356-364.

Mwangi, D. M., McKeever, D. J., Nyanjui, J. K., Barbet, A. F., Mahan, S. M., 2002. Immunisation of cattle against heartwater by infection with *Cowdria ruminantium* elicits T lymphocytes that recognise major antigenic proteins 1 and 2 of the agent. *Vet. Immunol. Immunopathol.* 85, 23-32.

Naiman, B. M., Alt, D., Bolin, C. A., Zuerner, R., Baldwin, C. L., 2001. Protective killed *Leptospira borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and $\gamma\delta$ T lymphocytes. *Infect. Immun.* 69, 7550-7558.

Nevo, Y., Nelson, N., 2006. The NRAMP family of metal-ion transporters. *Biochim. Biophys. Acta* 1763, 609-620.

O'Brien, R. L., Yin, X., Huber, S. A., Ikuta, K., Born, W. K., 2000. Depletion of a gamma delta T cell subset can increase host resistance to a bacterial infection. *J Immunol* 165, 6472-6479.

Ramstead, A. G., Robison, A., Blackwell, A., Jerome, M., Freedman, B., Lubick, K. J., Hedges, J. F., Jutila, M. A., 2016. Roles of TLR2, TLR4, and MyD88 during pulmonary *Coxiella burnetii* infection. *Infect. Immun.*

Rhodes, S. G., Hewinson, R. G., Vordermeier, H. M., 2001. Antigen recognition and immunomodulation by gamma delta T cells in bovine tuberculosis. *J Immunol* 166, 5604-5610.

Rogers, P. D., Jenkins, J. K., Chapman, S. W., Ndebele, K., Chapman, B. A., Cleary, J. D., 1998. Amphotericin B Activation of Human Genes Encoding for Cytokines. *J Infect Dis* 178, 1726-1733.

Sarthou, P., Primi, D., Cazenave, P. A., 1986. B cell triggering properties of a nontoxic derivative of amphotericin B. *J. Immunol.* 137, 2156-2161.

Sathiyaseelan, T., Naiman, B., Welte, S., Machugh, N., Black, S. J., Baldwin, C. L., 2002. Immunological characterization of a gammadelta T-cell stimulatory ligand on autologous monocytes. *Immunology* 105, 181-189.

Sau, K., Mambula, S. S., Latz, E., Henneke, P., Golenbock, D. T., Levitz, S. M., 2003. The Antifungal Drug Amphotericin B Promotes Inflammatory Cytokine Release by a Toll-like Receptor- and CD14-dependent Mechanism. *J. Biol. Chem.* 278, 37561-37568.

Schindler, J. J., Warren, R. P., Allen, S. D., Jackson, M. K., 1993. Immunological effects of amphotericin B and liposomal amphotericin B on splenocytes from immune-normal and immune-compromised mice. *Antimicrob. Agents Chemother.* 37, 2716-2721.

Shaffer, A. L., Lin, K. I., Kuo, T. C., Yu, X., Hurt, E. M., Rosenwald, A., Giltnane, J. M., Yang, L., Zhao, H., Calame, K., Staudt, L. M., 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17, 51-62.

Shibata, K., Yamada, H., Hara, H., Kishihara, K., Yoshikai, Y., 2007. Resident $V\{\delta\}1+ \{\gamma\}\{\delta\}$ T Cells Control Early Infiltration of Neutrophils after *Escherichia coli* Infection via IL-17 Production. *J Immunol* 178, 4466-4472.

Shirley, S. F., Little, J. R., 1979. Immunopotentiating Effects of Amphotericin B: II. Enhanced in Vitro Proliferative Responses of Murine Lymphocytes. *J. Immunol.* 123, 2883-2889.

Skyberg, J. A., Robison, A., Golden, S., Rollins, M. F., Callis, G., Huarte, E., Kochetkova, I., Jutila, M. A., Pascual, D. W., 2011. Apple polyphenols require T cells to

ameliorate dextran sulfate sodium-induced colitis and dampen proinflammatory cytokine expression. *J. Leukoc. Biol.* 90, 1043-1054.

Skyberg, J. A., Rollins, M. F., Holderness, J. S., Marlenee, N. L., Schepetkin, I. A., Goodyear, A., Dow, S. W., Jutila, M. A., Pascual, D. W., 2012. Nasal Acai polysaccharides potentiate innate immunity to protect against pulmonary *Francisella tularensis* and *Burkholderia pseudomallei* infections. *PLoS Pathog* 8, e1002587-

Smyth, A. J., Welsh, M. D., Girvin, R. M., Pollock, J. M., 2001. In vitro responsiveness of gammadelta T cells from *Mycobacterium bovis*-infected cattle to mycobacterial antigens: predominant involvement of WC1(+) cells. *Infect. Immun.* 69, 89-96.

Sopp, P., Howard, C. J., 2001. IFN gamma and IL-4 production by CD4, CD8 and WC1 gamma delta TCR(+) T cells from cattle lymph nodes and blood. *Vet. Immunol. Immunopathol.* 81, 85-96.

Stober, C. B., Brode, S., White, J. K., Popoff, J. F., Blackwell, J. M., 2007. Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function. *Infect. Immun.* 75, 5059-5067.

Telfer, J. C., Baldwin, C. L., 2015. Bovine gamma delta T cells and the function of gamma delta T cell specific WC1 co-receptors. *Cellular Immunology* 296, 76-86.

Toka, F. N., Kenney, M. A., Golde, W. T., 2011. Rapid and Transient Activation of $\gamma\delta$ T Cells to IFN- γ Production, NK Cell-Like Killing, and Antigen Processing during Acute Virus Infection. *J. Immunol.* 186, 4853-4861.

USDA-APHIS-VS-CEAH, 2011. Cattle and Calves Nonpredator Death Loss in the United States, 2010
2010. #631.1111,

Vantourout, P., Hayday, A., 2013. Six-of-the-best: unique contributions of $\gamma\delta$ T cells to immunology. *Nat Rev Immunol* 13, 88-100.

Volman, J. J., Mensink, R. P., Ramakers, J. D., de Winther, M. P., Carlsen, H., Blomhoff, R., Buurman, W. A., Plat, J., 2010. Dietary (1–3), (1–4)- β -D-glucans from oat activate nuclear factor- κ B in intestinal leukocytes and enterocytes from mice. *Nutrition Research* 30, 40-48.

Walcheck, B., Jutila, M. A., 1994. Bovine gamma delta T cells express high levels of functional peripheral lymph node homing receptor (L-selectin). *Int. Immunol.* 6, 81-91.

Walls, E. V., Kay, J. E., 1982. Inhibition of proliferation of a murine myeloma cell line and mitogen-stimulated B lymphocytes by the antibiotic amphotericin B (Fungizone). *Immunology* 47, 115-121.

Wilson, E., Aydintug, M. K., Jutila, M. A., 1999. A circulating bovine gamma delta T cell subset, which is found in large numbers in the spleen, accumulates inefficiently in an artificial site of inflammation: correlation with lack of expression of E-selectin ligands and L-selectin. *J Immunol* 162, 4914-4919.

Wilson, E., Hedges, J. F., Butcher, E. C., Jutila, M. A., 2002. Bovine gammadelta T cell subsets express different patterns of chemokine responsiveness and adhesion molecules: a mechanism for tissue specific gammadelta T cell subset accumulation. *J Immunol* 169, 4970-4975.

Zuany-Amorim, C., Ruffie, C., Haile, S., Vargaftig, B. B., Pereira, P., Pretolani, M., 1998. Requirement for gammadelta T cells in allergic airway inflammation. *Science* 280, 1265-1267.

Figure 1

Figure 2

Figure 4

A.

B.

C.

Figure 5

Figure 6