LYMPHOCYTE RESPONSES TO THE POLYPHENOL, OENOTHEIN B, AND THE
INFLUENCE OF AGE ON THESE RESPONSES

by

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For the family, friends, and teachers who encouraged me to pursue this goal.
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ABSTRACT

Innate lymphocytes, such as NK cells, play an important role in a number of human diseases. Therefore, they have become popular target cells for new therapeutics. Studies to identify materials that stimulate these cells have identified a number of molecules from nutritional supplements which can activate and/or inhibit their activity. While the use of nutritional supplements has potential to enhance the activity of innate lymphocytes to help treat and prevent disease, there is still much which is currently unknown about the activity of these compounds. Previous work has identified oenothein B, an ellagitannin isolated from *Epilobium angustifolium*, to be a stimulant for myeloid cells. In this study, we further examined the immunomodulatory effects of oenothein B and identified that it also stimulates lymphocytes. We found that oenothein B induces the production of IFNγ by NK cells and both αβ and γδ T cells. In addition to inducing the production of IFNγ by NK cells alone, oenothein B also enhances IFNγ production in response to secondary stimuli, such as IL-18 or a tumor cell line, in a synergistic manner. We also identified a novel effect of aging on the lymphocyte response to oenothein B. The induction of IFNγ, as well as GM-CSF, production by T cells in response to oenothein B increases in adults compared to young individuals. The predominant producers of IFNγ in response to oenothein B also shift from the NK cell population in young individuals to the T cell population in adults. The increase in cytokine production in adult T cells correlates with increasing numbers of CD45RO+ T cells, which produce more cytokines in response to oenothein B than CD45RO- T cells. This influence of aging on the immune response to oenothein B may be applicable to numerous nutritional supplements which stimulate innate immunity and could be an important variable in the effectiveness of these compounds in improving human health. In this dissertation, we identify age as an important factor which should be taken into account when examining the effect of polyphenols on innate immunity and could have important implications for optimizing the benefit of immunomodulatory polyphenols.
A significant portion of research in immunology is directed towards the identification of potential prophylactic treatments, which can enhance host defense against infection. Many of these types of studies focus on vaccines, which expose the host’s immune system to an antigen or antigens, stimulating a specific, long-lived immune protection to a specific pathogen expressing those antigens. Vaccination is very effective, but there are downsides to this type of approach for countering infectious disease. Even in the best case, identification, testing, and applying a vaccine in humans takes many years and cost 100s of millions of dollars\textsuperscript{1,2}. Vaccines tend to be monospecific, thus the time, effort, and funding has to be repeated for each pathogen that new countermeasures are needed. Pathogens also mutate, which results in strains that are resistant to previous vaccinations, such as with influenza and Hepatitis B\textsuperscript{3,4}. Therefore, new host strategies are needed for largely unforeseen and unknown pathogens at this time, but ones that could be problematic in the future. The innate immune system provides a broad spectrum of response to help counter a wide array of pathogens, and has been targeted for the development of novel countermeasures against infectious disease. Both prophylactic and post-exposure strategies involving innate immune stimulation have been shown to be plausible to prevent or ameliorate infections in animal models\textsuperscript{5-7}. 
The innate immune system is the immune system’s first line of defense against pathogens. It is sufficient for the clearance of many infections and its importance can be seen with individuals who have deficiencies in specific components of their innate immune system, as they become highly susceptible to infections. An advantage of the innate immune system is that it has multiple means of detecting pathogens that are far less specific to any given pathogen when compared to the adaptive immune response. These include pattern recognition receptors (PRRs), receptors that detect molecules unique to microbes, as well as methods to detect cellular damage caused by pathogens. This allows the innate immune system to rapidly respond to a broad range of pathogens. By enhancing innate immune responses, protection against a broad spectrum of pathogens could be strengthened. Hence, this strategy could potentially be used to protect individuals from more than one pathogen for which there are no effective vaccines.

One way innate immunity can be enhanced is through nutrition and numerous plant-derived products are believed to enhance immunological health. Many studies have investigated the role of nutritional supplements on immune function and a significant percentage of this research has focused on plant polyphenols. Polyphenols are plant metabolites important in plant defense against insects and other stresses. Many nutritional supplements contain large amounts of polyphenols, and pre-clinical and clinical studies suggest an overall health benefit for the consumption of these
The major benefit ascribed to these compounds is anti-oxidant activity, in which polyphenols bind to free reactive oxygen species (ROS). ROS molecules are produced by numerous cells in the body to help combat infections; however, free ROS has been described to promote cellular and DNA damage, leading to inflammation and carcinogenesis. Therefore, the consumption of polyphenols and other anti-oxidants are believed to reduce inflammation and inhibit carcinogenesis.

In addition to their anti-oxidant properties, polyphenols are also thought to modulate immune responses. Surveying the many studies on polyphenols, an interesting contradiction in the data has emerged. Numerous studies describe an anti-inflammatory property of polyphenols, demonstrating their benefit in multiple inflammatory disease models. In contrast, other studies describe an immune enhancing property for polyphenols and these data suggest great potential for enhancing human and domestic animal resistance to a variety of diseases through nutritional supplementation. Interestingly, some compounds that are described as anti-inflammatory in some studies are described as proinflammatory in others. For example, oligomeric procyanidins isolated from unripe apple peels (APP) are capable of suppressing inflammation and reducing pathology in a model of colitis, but are also capable of activating lymphocytes and enhancing their proliferation and cytokine production. Even within the colitis model there is a dichotomy, as APP suppressed inflammatory cytokine production in wild-type mice with colitis, but enhanced cytokine production in $\alpha\beta$ T cell-deficient mice.
with colitis. Additional studies are needed which investigate factors that influence the pro- and anti-inflammatory properties of these compounds, possibly skewing their behavior toward one or the other. Results from such studies may help guide the development of proper dosing regimens for polyphenols, maximizing their benefit while minimizing potential deleterious effects.

In Chapter Two, background information relevant for this dissertation is provided. This information describes several innate lymphocyte cell types and their role in a variety of diseases in humans, mice, and cattle, focusing on γδ T cells, NK cells, and the innate-like activity of memory αβ T cells. The effects of aging on the distribution and activity of these innate lymphocytes are also described. Finally, stimulation of these cells by plant polyphenols, specifically oenothein B, a polyphenol extracted from the *Epilobium angustifolium* plant, is characterized.

In Chapter Three, published data describing the influence of oenothein B on IFNγ production by innate lymphocytes is presented. Previous work found that oenothein B could stimulate macrophages, inducing the production of several cytokines. However, the effect of oenothein B on lymphocytes had not been investigated. Certain polyphenols, such as APP, promote lymphocyte proliferation and the production of certain cytokines, such as Granulocyte macrophage colony-stimulating factor (GM-CSF). However, little work has examined the ability for polyphenols to induce the production of interferon-γ (IFNγ), an important cytokine produced by several innate
lymphocytes and critical for their ability to enhance host defense to a variety of different pathogens and tumors. In this chapter, it is shown that, in addition to myeloid cells, oenothein B also stimulates lymphocytes and primes bovine and human NK cells to produce increased amounts of IFNγ in response to a cytokine, IL-18, or a tumor cell line. Data is also provided which shows that oenothein B also stimulates human γδ T cells and αβ T cells to produce IFNγ. These data adds to the collective knowledge of the proinflammatory properties of oenothein B and provides an additional possible mechanism for the proposed antitumor activity of this compound.

In Chapter Four, in a paper being submitted for publication, the influence of age on immune cell responses to oenothein B is described. During studies of lymphocyte stimulation by plant-derived polyphenols, including oenothein B, considerable variability in responses from different human and bovine donors was observed. While this variability could provide insights into which individuals could receive the most immunological benefit from polyphenol-rich supplementation, the potential factors which contribute to this variability are largely unknown. One possible variable which was hypothesized to influence the response of innate immune cells to polyphenols is the age of the individual.

Aging has a profound effect on the composition and effectiveness of the immune system. This can be observed clinically, as the very young and very old are usually most susceptible to infections. While aging is known to influence innate lymphocyte
distribution and responsiveness, the possible influence of age on immune cell responsiveness to nutritional supplements is largely unknown. In this chapter, age is shown to be an important variable in the response of human and bovine T cells to stimulation by oenothein B. These data adds to the collective knowledge of the immunostimulatory properties of polyphenols for individuals in different age groups and suggest that age should be taken into account when examining the capabilities for certain polyphenols to enhance immunity.

In Chapter Five, a review written on the complex role γδ T cells play in antitumor responses is provided. This review focuses on the different cytokines and other soluble factors produced by γδ T cells during antitumor responses and how these factors can have conflicting roles in promoting or inhibiting tumor growth. This variable activity by γδ T cells in tumors could influence the effectiveness of γδ T cell-stimulating polyphenols to enhance antitumor responses and should be taken into account when examining the effects of supplements on the antitumor activity of γδ T cells.
CHAPTER TWO

BACKGROUND

Innate Lymphocytes in Innate Immunity

Innate immunity encompasses a variety of phagocytic cells of the granulocytic and myeloid lineages, such as neutrophils and macrophages. In contrast, cells of the lymphoid lineage, such as T and B cells, are most often associated with adaptive immunity. However, there are subsets of lymphocytes that respond rapidly to a variety of pathogen-associated molecular patterns (PAMPs) and danger signals, participating as first-line defenders of the immune system and are considered part of the innate immune system.

The innate lymphocyte pool includes innate lymphoid cells (ILCs), innate T cells, and innate B cells. Innate T cells include γδ T cells, NKT cells, and some memory αβ T cells. Despite this diversity in cell types, many of these cells exhibit common behaviors. These include the early, antigen-independent secretion of cytokines important for the recruitment and activation of cells involved in both innate and adaptive immunity, as well as the ability to lyse infected or malignant cells in an antigen-independent manner. The roles of innate B cells during innate immunity have been reviewed elsewhere and are beyond the scope of this dissertation. For this chapter, we will focus on two broad categories of innate lymphocytes, ILCs and innate T cells.
Innate Lymphoid Cells (ILCs)

Innate lymphoid cells are innate immune cells of lymphoid lineage that are distinguished from B and T cells due to their lack of somatic rearrangement of immunoglobulin and T cell receptors (TCRs). They are widely distributed throughout the body and are found in the blood, lymphatics, and tissues. One member of the ILC family, NK cells, has been extensively studied over a number of years and is known to play an important role in a wide variety of infections and other diseases. Other ILC populations have only more recently been studied and there is still much that is currently unknown about their development and activity, especially in humans. However, they appear to be important at barrier surfaces of the body and are involved in lymphoid organogenesis, tissue remodeling, antimicrobial immunity, and inflammation\textsuperscript{36-40}.

ILCs have been categorized into several different subsets in recent years. These subsets are based largely on their cytokine profile, which correlate with T helper (Th) cell subsets. ILC1s are characterized by the expression of Th1 cytokines, such as IFN$\gamma$. ILC2s express Th2 cytokines, such as IL-5 and IL-13, and are believed to play a role in allergic inflammation and host defense against helminths. ILC3s express Th17 cytokines and appear to be important for mucosal immunity by promoting the production of antimicrobial peptides. The different subsets of ILCs and their roles in immunity have been reviewed in detail elsewhere\textsuperscript{36-40}. For this dissertation, we will focus on the best studied member of the ILC family, the NK cell.
Natural Killer Cells

Human Natural Killer (NK) cells make up approximately 15% of peripheral blood lymphocytes and are present in abundance in bone marrow, liver, lung, spleen, and, to a lesser extent, secondary lymphoid tissues. NK cells are identified in humans by the expression of CD56 and lack of expression of CD3. In mice, they are identified by the expression of markers NK1.1 or DX5. As the name suggests, NK cells were first identified for their ability to kill (lyse) abnormal cells in an antigen-independent manner. NK cells lyse tumor cells, cells infected with viruses or bacteria, as well as allogeneic cells in transplant patients, playing an important role in all of these conditions. NK cells are one of the body's primary defenders against cancer and low NK-like cytotoxicity is associated with increased cancer risk, while high levels of tumor infiltrating NK cells are associated with favorable outcomes in cancer patients.

The cytotoxic activity of NK cells is controlled by a balancing act of activating and inhibitory signals mediated by a diverse and complex repertoire of receptors. Inhibitory receptors on NK cells, which include many killer-cell immunoglobulin-like receptor (KIR) family members in humans and Ly49 family members in mice, typically recognize MHC class I (MHCI) expression on normal, healthy cells from their host. MHCI expression is often downregulated on infected, malignant, and other stressed cells ("loss of self"), making them less capable of stimulating inhibitory receptors and more susceptible to NK cell-mediated killing. In addition to loss of MHCI expression, NK
cells often do not recognize MHCI from allogeneic cells, an important obstacle in the success of tissue and bone marrow transplants\textsuperscript{52}.

In contrast to the loss of ligands which stimulate inhibitory receptors, infected and malignant cells often express increased levels of ligands for activating NK receptors. Some important NK cell activating receptors include the natural cytotoxicity receptor family (NCRs), some Ly49 and KIR family members, LILR, CD2 family members, NKG2 family members (except NKG2A), CD16, TRAIL, and Fasl\textsuperscript{50,51,53}. The activation receptor NKG2D appears to play an especially important role in optimal recognition of tumor and infected cells by NK cells and their subsequent activation\textsuperscript{54}. NKG2D recognizes the ligands MICA, MICB, and UL16 binding proteins (ULBPs) in humans, as well as the ligands Rae1 and Mult1 in mice\textsuperscript{55}.

When the balance of activating and inhibitory signals from a target cell favors inhibition, the NK cell will leave the cell alone and move on to the next potential target. When the balance of signals favors activation, the NK cell will proceed to kill the target cell. Once a NK cell targets a cell for killing, the NK cell will induce apoptosis in the target cell by several mechanisms, including perforin/granzyme-induced apoptosis, Fas-FasL-induced apoptosis, and TRAIL-induced apoptosis\textsuperscript{56}. In addition to the killing of tumor cells, NK cell cytotoxicity is important for clearance of intracellular pathogens, by killing infected host cells and by direct antimicrobial activities\textsuperscript{57-59}. 
While the lytic activity of NK cells is very important for host defense, NK cells have additional roles during immune responses. One of these roles is the rapid expression of several cytokines upon activation, including IFNγ, which promotes the microbicidal activity of macrophages and supports the recruitment and function of adaptive T cells, such as cytotoxic T lymphocytes (CTLs). IFNγ secretion by NK cells is important for optimal clearance of a variety of pathogens, including fungal (Cryptococcus neoformans, Aspergillus fumigatus), bacterial (Francisella tularenia, Legionella pneumophila, Staphylococcus aureus, etc.), viral (HSV, RSV, etc.), and protozoan pathogens57, 60. In addition to its benefit during infectious disease, IFNγ expressed by NK cells restricts tumor angiogenesis, the production of new blood vessels which feed the tumor, and promotes adaptive antitumor immunity61, 62. The expression of IFNγ is critical for the optimal benefit of NK cells in the defense against many tumors and infections, suggesting that enhancing the production of this cytokine would benefit host defense in these types of diseases60, 62.

Effect of Aging on NK Cells

Aging influences NK cell numbers, activity, receptor expression, and maturation. First, while the NK cell percentage of lymphocytes does not change dramatically during aging, the number of NK cells/mm³ of human blood is reduced dramatically from cord blood to adult blood63. In general, NK cell function is skewed more towards cytokine production than cytotoxicity in neonates compared to adults. Human cord blood NK cells
are less cytotoxic than adult NK cells, but can be induced to have similar cytotoxic activity to adult NK cells by stimulation with IL-2. However, cord blood NK cells are responsive and express more CD69 and IFNγ than adult NK cells in response to IL-12 and IL-18, more IFNγ in response to a cocktail of ssRNA, IL-2 and IFNα, and similar IFNγ in response to a mitogen. In addition to these in vitro studies, a study looking at BCG vaccinated infants showed a significant contribution of NK cells to the IFNγ response, being the most responsive cell type to ex vivo restimulation (based on IFNγ production). However, during this same study, the authors found that CD8 T cells were the primary IFNγ-producers in vaccinated adults, suggesting the impact of NK cells, especially as it relates to IFNγ production, during BCG vaccination may decrease with age.

In addition to changes between neonates and adults, NK cell activity also changes from young adults to the elderly. When comparing young human adults to older adults, older NK cells have reduced activity, including reduced proliferation, IFNγ production, and possibly cytotoxicity. In a study in rats looking at the anti-metastatic activity of NK cells, NK cells from prepubescent male and female rats had greater activity than cells from mature rats, suggesting that age impacts the antitumor activity of NK cells. Altogether these data from cord blood, young adults, and older adults suggest that age is an important variable when studying NK cell activity.
These age-related changes in human NK cell activity could partially be due to changes in functional receptor expression important to these cells. The percentage of NK cells expressing the activating receptor NKG2D increases from cord blood to adult blood, while the percentage of cells expressing the inhibitory receptor NKG2A/CD94 decreases, with the balance between NKG2A and activating NKG2 molecules skewed more towards inhibition in neonates compared to adults. Other differences in surface receptor expression between newborns and adults include the reduced expression of KIRs, LIR-1, CD62L, CD54, CD8αα, granzyme B, and CD57 in newborns and reduced expression of NKp46, NKp30, and CD161 in adults. The relatively high expression of the inhibitory receptors NKG2A/CD94 and the lower expression of granzyme B in cord blood NK cells could account for the reduced cytolytic activity of cord blood NK cells. Differential expression of FasL or TRAIL likely does not account for the reduced cytolytic activity, as these molecules are expressed at similar levels on adult and cord blood NK cells. Furthermore, CD16 expression is similar between newborns and adults, suggesting similar IgG-mediated antibody dependent cytotoxicity. Altogether these data suggest that age significantly changes receptor expression of NK cells, which could lead to significant changes in functional activity.

Studies from mice suggest that the physiological environment may play an important role in the reduced activity of NK cells in older individuals. NK cells from aged mice do not expand and upregulate the expression of KLRG1 when stimulated in
vivo with PAMPs. However, when NK cells from old mice were adoptively transferred into young mice, they regained responsiveness to PAMPs similar to NK cells from younger mice. Conversely, when NK cells from young mice were transferred into older mice, they had reduced activity compared to NK cells transferred back to young mice. Immature CD27-expressing NK cells were more prevalent in the blood, spleen, and lungs of aged mice compared to young mice. However, when bone marrow NK cells from aged mice were transferred into young mice, they matured like young NK cells. Conversely, bone marrow stem cells from young individuals did not have enhanced maturation when transferred into aged mice. Treatment with IL-15/IL-15Ra brought the number of Granzyme B and KLRG1-expressing NK cells in aged mice to similar levels as young mice and brought target cell killing of aged NK cells back to similar levels as young mice. These data suggests that the reduced activity of NK cells in the elderly could partially be due to reduced IL-15 expression in these individuals. A better understanding of how the physiological environment in which NK cells reside changes with age could provide insight into why NK cells from different age groups behave differently.

**Bovine NK Cells**

In addition to humans and mice, the role of NK cells in host defense has been studied in other animal models, including cattle. It is believed that cattle can offer an important complementary model for immunology and this could also be true for the study of NK cells. As with humans, NK cells play an important role in bovine immunology.
Bovine NK cells are identified by the absence of CD3 and the expression of CD335 (NKp46)\textsuperscript{74}. While some T cells can express CD335, this marker is largely specific for NK cells in cattle\textsuperscript{75}. Bovine NK cells have been detected in the spleen, lung, liver, bone marrow, a variety of lymph nodes, and blood, with NK cells usually ranging from 2-10\% of peripheral blood mononuclear cells (PBMCs)\textsuperscript{73}. Cattle have similar numbers of lymph node NK cells as humans, and more than what is found in mice\textsuperscript{76}. This increase in lymph node NK cells is believed to be the result of cattle and humans having greater microbial exposure than mice, which are typically housed in cleaner environments, as microbial exposure is believed to attract naive NK cells to the lymph nodes, where they acquire the ability to respond to infections\textsuperscript{73}. Bovine NK cells in the lymph nodes contain perforin and can kill target cells, unlike human lymph node NK cells, which only acquire these features after activation\textsuperscript{76, 77}.

As with human and murine NK cells, bovine NK cells are cytotoxic and appear to be an important innate source of IFN\textgreek{g} in cattle in response to several important bovine pathogens. For example, bovine NK cells are stimulated by the mycobacterial proteins ESAT-6 and MPP14, BCG-infected DCs, and \textit{M. bovis}-infected macrophages to produce IFN\textgreek{g} and kill infected cells in a cell contact-dependent manner\textsuperscript{78-80}. In addition to mycobacterium, bovine NK cells directly recognize the protozoan \textit{Neospora caninum} and purified, IL-2 stimulated NK cells produced IFN\textgreek{g} and killed infected fibroblasts\textsuperscript{81}. NK cells are also the major early IFN\textgreek{g}-producing cell in response to another important
protozoal infection, *Babesia bovis*. The production of IFNγ by bovine NK cells can be stimulated by several mechanisms, including contact with infected cells, cytokines (such as IL-12), and by direct contact with the pathogen. Although IFNγ production and cytotoxicity appear to be important for NK cell-mediated host defense to many bovine pathogens, these cells can also mediate host defense through other, yet not well understood, mechanisms. For example, bovine NK cells reduce *M. bovis* replication in macrophages in a cell contact-dependent manner which does not appear to be mediated by IFNγ or the killing of infected macrophages. Further studies are needed to elucidate the full repertoire of effector functions available to bovine NK cells to protect hosts from infection.

While receptor expression on bovine NK cells has not been studied as thoroughly as in mice or humans, several studies have examined NK receptor expression in cattle. KIR genes have been identified in cattle and these KIRs appear to include both activating and inhibitory receptors. These bovine KIRs comprise three Ig-domains and one ITIM sequence, making them different from human KIRs, which typically have two Ig-domains and two ITIM sequences. It has yet to be determined whether or not bovine KIRs bind to MHCI. In cattle, there are several NKG2A genes and one NKG2C gene, but their functions are still unknown. In addition, receptors for the MIC protein are found on bovine NK cells and recombinant MIC activated these cells, suggesting that, as in humans, bovine NKG2D is an activating receptor which recognizes stress ligands. A
single Ly49 gene has also been found in cattle, but its ligand and possible function is unknown. While these studies suggest that bovine NK cell activity may be controlled in a similar manner to those in mice and humans, additional studies are needed to examine the control of NK cell activity in cattle. This knowledge could then be used to better understand NK cell activity during infection in cattle.

**Effect of Aging on Bovine NK Cells**

Bovine NK cells have been studied in newborn calves through to adults, and their numbers and activity changes during aging. One study found that NK cells are present at a lower frequency in neonate PBMCs compared to older calves and adults, but that the percentage of NK cells in PBMCs increases to older calf/adult levels after a couple of weeks. In another study, the numbers of CD335+ NK cells in the blood varied significantly during the first few months of life, with NK cell numbers increasing over the first 10-12 weeks then decreasing over the following 10 weeks. The numbers then underwent another rise and fall over the next 10 weeks, suggesting that calf NK cell numbers can be highly unstable in the blood. In the intestinal track of cattle, NK cell numbers increased with age in the jejunum over the first six months. In general, it appears that NK cell numbers in cattle increase with age from birth to young adulthood.

Although NK cell numbers are lower in very young calves, several studies suggest that NK cells from neonate calves are functional and likely play a critical role in host protection for calves during their first few weeks. In one study, NK cell activity was...
compared between neonate (less than 8 days old) and older calves, and while neonate calves had approximately one-third of the circulating CD335+ cells that the older calves had, the NK cells from neonates proliferated more robustly in response to IL-2 or IL-15 and produced more IFN\(\gamma\) than NK cells from older calves after culturing in IL-15 and stimulation with IL-12 and an antibody to NKp46\(^{89}\). Furthermore, BCG vaccination works best when given to neonates compared to adults and it is believed that this is the result of enhanced responses of neonatal NK cells, as well as other innate immune cells\(^{90}\). This enhanced response by neonatal NK cells to BCG may be mediated through direct interaction with BCG, DC-derived cytokines, or by cell-to-cell interactions with infected DCs, as described earlier.

As with humans, aging may also affect the cytotoxic potential of bovine NK cells. A greater percentage of bovine NK cells express perforin in young calves (1-6 weeks) compared to adults. In calves less than or equal to 6 weeks old, the majority of perforin+ cells were CD335+ NK cells. However, in adults, the majority of perforin+ cells were CD335- cells, likely T cells\(^{86}\). In addition, IL-15 expanded NK cells from neonates (less than 8 days old) were more effective at killing a bovine epithelial tumor cell line than those from older calves\(^{89}\). These data suggest that NK cell cytotoxicity may decrease with age in young calves. This contradicts the previously described data seen in humans, where cytotoxicity increased in adults compared to newborns. However, those human studies were performed using cord blood NK cells, which may not fully reflect the
activity of peripheral NK cells in very young individuals. Altogether, these data suggest that NK cells from very young calves are more responsive to certain stimuli than those from older animals and that NK cell activity can change rapidly in cattle during aging, even from neonate to older calves.

**Innate T Cells**

T cells are a major lymphoid cell population and represent probably the most studied cell in the immune system. While they are typically associated with antigen-specific adaptive immunity, some subsets of T cells respond rapidly in an antigen-independent fashion during innate immune responses. Among these T cell subsets include NKT cells, \( \gamma \delta \) T cells, and subsets of memory \( \alpha \beta \) T cells. Briefly, NKT cells are innate T cells found in blood and various tissues which recognize lipids presented by CD1d, a member of the CD1 family of MHC-like molecules, and are known for producing large quantities of cytokines, including Th1, Th2, and Th17 cytokines. They play a prominent role in the immune responses to several bacteria and also appear to have a role in viral infections, tumor immunology, and perhaps some allergic and autoimmune disorders. NKT cells and their role in disease have been reviewed in detail elsewhere\(^9\). For this dissertation, we will focus on memory \( \alpha \beta \) T cells and \( \gamma \delta \) T cells.
Generation and Characterization of Memory αβ T Cells

αβ T cells make up the largest proportion of T cells and express a TCR consisting of the α and β chain. After development in the thymus, αβ T cells enter the periphery as naive, mature T cells which traffic back and forth from the blood to lymph nodes, mucosa-associated lymphoid tissues, and the spleen. These naïve T cells proliferate upon activation, but do not have fully developed effector functions, such as cytotoxicity and cytokine production. During a primary immunological insult, DCs ingest, process, and transport antigens to regional lymph nodes, where they present those antigens on MHC molecules to T cells in the T cell cortex. CD4+ T cells recognize antigenic peptides presented on MHCII, while CD8+ T cells recognize peptides presented on MHCI. Naive T cells, which recognize a presented antigen, will proliferate and differentiate into effector T cells. In general, CD4+ T cells differentiate into cytokine producing helper T cells (Th), which support or suppress other effector T cell functions and antibody production, while CD8+ T cells differentiate into cytotoxic T lymphocytes (CTLs), which produce IFNγ and kill target cells expressing their cognate antigen on MHCI. Depending upon the immunological environment, CD4+ T cells can differentiate into one of four major Th subsets. The four major Th subsets are: Th1, which produce IFNγ and promote macrophage and CTL activity, Th2, which produce IL-4, IL-5, and IL-13 and promote allergic responses and helminth clearance, Th17, which produce IL-17 and
promote neutrophil recruitment and inflammation, and Treg, which produce IL-10 and suppress various immune functions\textsuperscript{93}.

After differentiation, some effector T cells traffic to the periphery and tissues, where they help mediate pathogen clearance, while others remain in the blood and lymphatic system. Upon clearance of the pathogen, the vast majority of the responsive effector T cells undergo apoptosis. However, a subpopulation of these cells differentiates into long-lived memory T cells. These memory T cells are able to rapidly respond to a secondary exposure by their cognate antigen, providing enhanced protection to secondary infections. Memory T cells can be divided into two major subsets, central memory T cells (Tcm) and effector memory T cells (Tem). Tcm cells express the lymph node homing receptors CD62L and CCR7, and are primarily found within the blood, bone marrow, and secondary lymphoid organs. They produce lower amounts of effector cytokines (IFN\textgreek{g}, IL-4, etc.) compared to Tem cells, but have longer telomeres and express more IL-2, suggesting that they are important in the long-term maintenance of memory T cell pools. Tem cells, on the other hand, lack the expression of CD62L and CCR7, and can be found in nonlymphoid tissues, as well as the blood. They also have greater cytotoxicity and effector cytokine production than Tcm cells\textsuperscript{94-96}. While many Tem cells recirculate, some take up permanent residence in tissues. These tissue resident memory T cells (Trm) are characterized by the expression of CD103 and their tissue residence is promoted by the downregulation of S1P\textsubscript{1}, the receptor for sphingosine-1-phosphate, which prevents them
from recirculating. This downregulation of S1P1 is promoted by TGFβ, IL-33, and TNF\textsuperscript{94, 97}. Many Trm cells localize to portals of entry into the body, such as the lung and intestinal tract, which places them in position to respond to the environment, allowing them to respond rapidly to secondary exposure with their cognate antigen upon its entry into the body through the mucosa\textsuperscript{94}. However, in addition to these antigen-specific responses, they can also participate during innate immune responses in an antigen-independent, bystander manner.

**Innate Activity of Memory T Cells**

Antigen-independent innate immune responses by memory T cells have been demonstrated in both mouse models and clinical studies. In humans, CD8+ memory T cells specific for influenza, CMV, and EBV are activated during an early HIV infection\textsuperscript{98}. The activation of memory T cells during these bystander responses is antigen-independent, but is dependent upon inflammation. Among the important bystander effects is the production of IFN\textgamma in response to innate IL-12, IL-18, and other cytokines in the absence of cognate antigen\textsuperscript{99, 100}.

Memory CD8+ T cells also express many receptors typically associated with NK cells. One of these NK receptors, NKG2D seems to be of particular importance during bystander memory CD8+ T cell responses. Exposure to IL-12, IL-15, and IL-18 could promote the development of cytotoxic CD8+ T cells from the memory T cell pool in the absence of TCR signaling and these cells could kill infected cells in an NKG2D-
dependent manner. This process was shown to be important for early pathogen clearance during *Listeria monocytogenes* infection in mice\textsuperscript{101}. Furthermore, during influenza infection in mice, non-specific memory T cells rapidly expanded and upregulated the expression of NKG2D. Neutralizing NKG2D enhanced infection, suggesting that memory T cells could also inhibit influenza infections in an NKG2D-dependent manner\textsuperscript{102}.

Altogether, these data suggest that conventional memory T cells contribute to innate responses and should be considered members of the innate lymphocyte pool, participating in innate responses by cytokine production and cell contact-dependent killing of infected cells. It is currently not well understood just how important these bystander effects are to innate immunity. However, they may play a critical role in response to certain pathogens and further studies are needed to better understand the importance of antigen-independent innate responses by memory T cells. These responses could be very important in human immunology, as memory T cells make up a large percentage of the human T cell pool, especially in adults.

**Effect of Aging on Memory αβ T Cells**

As with NK cells, aging has a profound effect on the memory T cell pool. As individuals age, they become more exposed to various pathogens and other antigens, which leads to an increased accumulation of memory T cells in older individuals\textsuperscript{103}. In young individuals, the majority of αβ T cells express naive cell markers, while in older
individuals, more T cells express the effector and memory T cell marker CD45RO\textsuperscript{104}. This expansion of memory T cells could potentially lead to an increase in antigen-independent responses by T cells in older individuals compared to younger ones during infection. In support of this, a study comparing IFN\(\gamma\) production in cord blood and adult human PBMCs to the parasite \textit{Trypanosoma cruzi} and IL-15 found that IFN\(\gamma\) production was exclusive to the NK cell population in neonates, but in adults, CD56\(^+\) and CD56\(^-\) T cells also produced early IFN\(\gamma\)\textsuperscript{105}. Additional studies are warranted which examine how innate immune responses to different pathogens change with age due to an increase in memory T cell populations.

In addition to increased antigen exposure, aging also appears to promote the development of memory T cells by additional mechanisms. Reconstituted T cells from bone marrow transplants have a memory-dominated profile and produce IL-4, IL-5, and IFN\(\gamma\) in aged recipient mice, but not younger recipient mice\textsuperscript{106}. Furthermore, virtual memory T cells, which are antigen-inexperienced T cells expressing memory T cell markers, also expand with age\textsuperscript{107}. Homeostatic cytokine expression may contribute to these age-related microenvironmental effects on memory T cells. For example, IL-15 promotes virtual memory T cell development, as well as conventional memory T cell proliferation and survival, but can also lead to the accumulation of highly activated, hyporesponsive CD28- memory T cells in older individuals\textsuperscript{108,109}. Further studies are necessary to identify additional homeostatic factors influencing the development and
activity of memory T cells in older individuals, as well as how these factors may influence innate responses by memory T cells.

γδ T Cells

γδ T cells are an innate T cell subset, which express γ and δ TCR chains, and are thought to play a role in the bridging of innate and adaptive immunity\(^\text{110}\). Their contribution to T cell numbers in the blood is species-specific, as they are found in low numbers in the blood of rodents and humans, but are found in higher numbers in the blood of cows, sheep, and other hoofed animals\(^\text{111}\). The reason for this species variability in γδ T cell levels in the blood is not entirely known. In addition to being found in the blood, γδ T cells are found in high numbers in mucosal tissues at portals of entry into the body, including the intestines, lungs, and genitourinary tract, and make up a large percentage of intraepithelial lymphocytes (IELs) in the intestines of multiple species\(^\text{112}\). Therefore, they are positioned to be among the first responders to invading pathogens.

Subsets of γδ T cells are primarily identified by their TCR diversity. In humans, the primary subsets are the Vδ2 and Vδ1 subsets. Vδ2 γδ T cells make up the primary subset in the blood, while Vδ1 γδ T cells make up the primary subset in the mucosal tissues\(^\text{113, 114}\). In addition to TCR expression, subsets of γδ T cells in humans can be further distinguished by the expression of CD45RA and CD27, which divide γδ T cells, both Vδ2 and Vδ1, into four distinct subsets\(^\text{115}\). CD27+ CD45RA+ cells are described as
naive cells that have limited effector activity in terms of cytokine production and cytotoxicity, but have potential for proliferation and differentiation. Upon stimulation with IL-2 and the γδ T cell agonist isopentenyl pyrophosphate (IPP), these cells differentiate into CD27+ CD45RA- cells. These cells are described as central memory cells and have the greatest proliferation potential, as well as some cytokine producing and cytotoxic activity. At this point, central memory cells can further differentiate into one of two subsets. Upon additional stimulation with IL-2 and IPP, these cells differentiate into CD27- CD45RA- cells, which are described as effector memory cells. They have less proliferative potential than central memory γδ T cells, but have the most robust IFNγ production of the four subsets. These cells appear to play an important role in immune responses to *M. tuberculosis*, as TB-infected individuals have a lower percentage of this subset compared to healthy controls and individuals with active TB infections have lower percentages of this subset than those with inactive disease\textsuperscript{116}. Individuals infected with HIV also have reduced percentages of this subset. If central memory γδ T cells are stimulated with IL-15 instead of IL-2, they differentiate into CD27- CD45RA+ cells. These cells are terminally differentiated and have no proliferative capacity, but can produce some IFNγ and have the greatest cytotoxic activity of the four subsets\textsuperscript{115}.

Like NK cells, γδ T cells identify stressed or infected cells through a variety of receptors\textsuperscript{111}. These receptors include the γδ TCR itself, which recognizes proteins and small molecules on the surface of target cells. While ligands for γδ TCRs are still being
identified, the Vδ2 subset responds to non-peptide phosphoantigens, which can originate from endogenous or exogenous sources, in a γδ TCR-dependent manner. Numerous pathogens express phosphoantigens through the non-mevalonate biosynthesis pathway and infection by pathogens that produce high levels of these phosphoantigens, such as *M. tuberculosis*, results in a large expansion of Vδ2+ γδ T cells. In addition to exogenous phosphoantigens, γδ T cells can be stimulated by the endogenous phosphoantigen, isopentenyl pyrophosphate (IPP), which is a substrate in the mevalonate pathway of biosynthesis. When a cell is stressed, such as during malignancy or infection, IPP accumulates and is then presented on the surface of the cell. The recognition of infected or malignant cells by γδ T cells relies heavily on the recognition of IPP presented on the surface of these target cells. Recognition of these phosphoantigens by the γδ TCR requires antigen presentation and cell-to-cell contact; however, professional antigen presenting cells are not necessary and many cells, including γδ T cells themselves, can present phosphoantigens to the γδ TCR. This antigen presentation does not involve well described antigen presentation molecules, such as members of the MHC or CD1 family, but instead appears to involve the Ig superfamily protein, Butyrophilin 3A1. The understanding of phosphoantigen presentation to γδ T cells by Butyrophilin 3A1 is still in its infancy and is in the process of being better understood.
In addition to infection and cellular stress, the accumulation of IPP can be manipulated pharmacologically, allowing for the manipulation of human Vδ2 γδ T cell activity (Figure 2.1). Bisphosphonates, which are used to prevent the loss of bone mass during osteoporosis, inhibit the enzyme Farnesyl Pyrophosphate Synthase, which promotes the conversion of IPP to downstream products in the mevalonate pathway. The inhibition of this enzyme leads to the accumulation of IPP, which can then activate γδ T cells\textsuperscript{124}. In contrast, statins can prevent the accumulation of IPP by inhibiting the activity of the enzyme HMG-CoA reductase, which is found upstream of IPP synthesis in the mevalonate pathway. Statins have been shown to inhibit γδ T cell activation by bisphosphonates, as well as by influenza-infected cells\textsuperscript{120,125}. Considering how many patients take statins, the influence of this treatment on γδ T cell activity during infections, cancer, and other inflammatory diseases should be further examined.

In addition to the γδ TCR, γδ T cells also express various other receptors which contribute to their activation, such as NK receptors and various PRRs\textsuperscript{126-130}. The NK receptors NKG2D and the NCRs contribute to the recognition of tumor cells by γδ T cells and the recognition of these NK receptors to their ligands can activate γδ T cells, as well as act as co-receptors, enhancing γδ TCR-mediated responses\textsuperscript{127-130}. Stimulation of NKG2D on γδ T cells promotes degranulation and NKG2D is essential for the optimal cytotoxic activity of human γδ T cells\textsuperscript{129,130}. 
Figure 2.1 Pharmacological manipulation of mevalonate pathway and isopentenyl pyrophosphate accumulation. Statins inhibit HMG-CoA reductase, preventing the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid and inhibiting the production of isopentenyl pyrophosphate (blue). Bisphosphonates inhibit farnesyl diphosphate synthase, resulting in an accumulation of isopentenyl pyrophosphate.
γδ T cells are believed to contribute to pathogen clearance during many infections, including viral (influenza, CMV, HIV, etc.), bacterial (Staphylococcus aureus, E. coli, Mycobacterium tuberculosi, etc.), and protozoan (Leishmania, Toxoplasma godii) infections. Like NK cells, γδ T cells can directly lyse infected cells in a cell contact-dependent manner which can be mediated by the Fas-FasL, TRAIL, and perforin/granzyme B pathways. All three of these pathways were shown to be involved in the killing of influenza-infected human lung alveolar epithelial cells by Pamidronate-activated γδ T cells. The antibody-dependent cell-mediated cytotoxicity (ADCC) pathway, which is induced by the recognition of pathogen-specific antibodies by CD16, can also enhance γδ T cell activity.

In addition to their cytotoxic activity, γδ T cells also produce a variety of cytokines and chemokines in response to infections, which is important for their antimicrobial activity. IFNγ production by γδ T cells inhibits HCMV and influenza virus propagation and is also believed to be critical in host resistance to Plasmodium falciparum. In addition to IFNγ, IL-17 and TNFα produced by γδ T cells promotes the elimination of various bacteria and Plasmodium falciparum. Finally, IL-17, as well as IL-22, produced by γδ T cells induces the production of antimicrobial peptides and recruits neutrophils to the site of infection, as seen in several infection models. Therefore, γδ T cells may be critical in promoting the optimal production of antimicrobial peptides and recruitment of neutrophils to infected tissues.
In addition to enhancing innate immunity, γδ T cells also enhance the development of adaptive immunity in several ways. They promote the maturation of DCs, facilitate the development of αβ T cells, kill Tregs, and even migrate into secondary lymphoid tissues to act as B helper T cells\textsuperscript{144-147}. γδ T cells also have demonstrated professional antigen-presenting cell activities and are capable of taking up, processing, and presenting antigen from free viral particles and infected cells to both CD4 and CD8 αβ T cells\textsuperscript{148, 149}. Certain studies even suggest that γδ T cells may be more efficient than other APCs at cross-presentation of antigen to CD8+ cells\textsuperscript{148}. These data support the idea that γδ T cells serve as an important bridge between the innate and adaptive immune response.

After pathogen clearance, γδ T cells may also be beneficial in the resolution of inflammation and tissue recovery. In a murine model of influenza, γδ T cells were prevalent at the site of inflammation late during infection, after clearance of virus, suggesting that these γδ T cells were involved in the resolution of inflammation in this model\textsuperscript{150}. Consistent with this, studies from the early 2000's demonstrated that skin γδ T cells in mice, known as dendritic epidermal T cells (DETCs), were necessary for optimal wound healing, which was promoted by the production of Keratinocyte Growth Factor 7 (KGF-7) by these cells\textsuperscript{151}. In addition, γδ T cells in the intestine of mice protect against dextran sodium sulfate-induced inflammatory bowel disease and promote epithelial turnover\textsuperscript{152}. Human γδ T cells are also capable of producing several growth factors and
immunoregulatory proteins$^{153,154}$. These data suggest that $\gamma\delta$ T cells, in addition to their proinflammatory roles, may also promote tissue recovery and resolution of inflammation in humans and animals.

In addition to infectious disease and tissue healing, $\gamma\delta$ T cells also play an important, but complex, role in cancer. $\gamma\delta$ T cells lyse tumor cells from a variety of cellular origins$^{129}$. However, secreted factors produced by $\gamma\delta$ T cells can either be beneficial or detrimental to tumor responses and outcomes. The complex role $\gamma\delta$ T cells can play in response to cancer will be discussed in further detail in Chapter 5.

**Effect of Aging on $\gamma\delta$ T Cells**

$\gamma\delta$ T cells appear to mature throughout the lifetime of an individual until fully differentiated effector cells dominate the $\gamma\delta$ T cell pool in older individuals. In support of this, a study in 2004 identified a naive subset of $\gamma\delta$ T cells, which was described as CD45RO-, CD27 bright, and CD11a dull. This subset made up the majority of both the V$\delta$2 and V$\delta$1 $\gamma\delta$ T cell subsets in cord blood and infants, but the presence of this subset decreased with age and by approximately one year of age, it was only a small percentage of V$\delta$2 cells, although it was still a large percentage of V$\delta$1+ $\gamma\delta$ T cells. By adulthood, it was only a small subset of both V$\delta$2+ and V$\delta$1+ $\gamma\delta$ T cells$^{155}$. Another study found that the percentage of $\gamma\delta$ T cells expressing CD27, identifying naïve and central memory $\gamma\delta$ T cells, decreased with age from young adult to older adults, while the percentage of CD27- $\gamma\delta$ T cells increased in these older individuals, further supporting the concept of
increasing maturation of $\gamma\delta$ T cells over a lifetime\textsuperscript{156}. It is not entirely known what drives this maturation, but it could potentially be mediated by homeostatic cytokines, as well as by increasing exposure to exogenous and endogenous $\gamma\delta$ TCR ligands.

In addition to different maturation subsets, the percentage of $\gamma\delta$ T cells in the blood expressing the V\delta1 $\gamma\delta$ TCR changes with age. V\delta1 $\gamma\delta$ T cells make up a large percentage of the peripheral $\gamma\delta$ T cell population at the time of birth. However, over time, the V\delta2 subset expands and becomes the predominant peripheral subset, while the number of total V\delta1+ $\gamma\delta$ T cells in blood decreases from cord blood to child to adult\textsuperscript{155}. These data suggest that V\delta2+ cells expand more robustly than V\delta1+ cells over the lifetime of an individual and this is believed to be due to exposure of phosphoantigen-producing pathogens.

As can be expected from the described subset changes in the $\gamma\delta$ T cell pool with aging, $\gamma\delta$ T cell functions, including cytotoxicity, cytokine production, and proliferation is also affected by aging. $\gamma\delta$ T cells from elderly individuals express greater levels of the activation marker CD69 than $\gamma\delta$ T cells from young or middle age individuals, suggesting a more activated phenotype, possibly a result of chronic inflammation found in the elderly, a phenomenon known as inflammaging\textsuperscript{156}. $\gamma\delta$ T cells from infants express much lower levels of perforin than adult $\gamma\delta$ T cells, suggesting a reduced capability to lyse target cells. IFN$\gamma$ production by $\gamma\delta$ T cells increases with age, as $\gamma\delta$ T cells from children had enhanced production compared to cord blood $\gamma\delta$ T cells, but reduced compared to
adult γδ T cells. Finally, the expansion of γδ T cells in response to phosphoantigens and IL-2 decreases with age, except for the CCR7- subset of γδ T cells. In summary, maturation of γδ T cells during aging leads to differential effector functions, with IFNγ production and cytotoxicity increasing with age, but proliferation decreasing with age.

**Bovine γδ T Cells**

Unlike in humans and mice, γδ T cells make up a large percentage of PBMCs in cattle, especially in young calves. This is similar to other hoofed animals, such as sheep, bison, and goats. Bovine γδ T cells are broadly divided into subsets based on the expression of WC1, which can be further characterized by three isoforms of WC1, WC1.1, WC1.2, and WC1.3, with WC1.1 and WC1.2 expressed on largely non-overlapping populations. WC1+ bovine γδ T cells are found at high numbers in the blood and accumulate at inflammatory sites, while WC1- γδ T cells accumulate in mucosal tissues and splenic red pulp. Overall, WC1- γδ T cells are believed to be more immunoregulatory, while WC1+ cells are believed to be more inflammatory.

Unfortunately, the ligands recognized by the bovine γδ TCR have not been well characterized, as bovine γδ T cells have minimal responses to the best known human γδ T cell ligands, the phosphoantigens. However, many studies have examined the responses of these cells to other stimulants. Bovine γδ T cells express a number of different PRRs, including TLRs, scavenger receptors, C-type lectin receptors, and NOD receptors, which allow bovine γδ T cells to respond to numerous microbial products. For example,
stimulation with muramyl dipeptide, the NOD2 agonist, promoted proliferation and GM-CSF expression of bovine γδ T cells\textsuperscript{161}. Bovine γδ T cells may also be activated by NK cell receptors. Transcripts specific for NKG2D, as well as additional NK receptors, including KIR2DL1 and 2B4 (CD244), were measured in a bovine γδ T cell clone\textsuperscript{162}. Furthermore, a subset of bovine γδ T cells from the mesenteric lymph nodes and ileum of cattle bind bovine MIC proteins and produce IFNγ in response to stimulation by MIC, likely through NKG2D\textsuperscript{85}. Finally, the NCR CD335, typically used as an NK cell marker, can be upregulated on bovine γδ T cells \textit{in vivo} by viral infection and CD335+ γδ T cells produced robust amounts of IFNγ in response to IL-12 and IL-18\textsuperscript{75,163}.

Like NK cells, bovine γδ T cells appear to be important in the host response to several important bacterial and viral pathogens to cattle. They are early producers of IFNγ upon exposure to \textit{M. bovis}, \textit{Brucella abortus}, foot-and-mouth-disease (FMD), and possibly \textit{salmonella enterocolitis}, which activates infected macrophages\textsuperscript{111,163}. In addition to the production of IFNγ upon exposure to FMD, γδ T cells also upregulate CD335, perforin, and MHCII, lyse FMD-infected cells, and present antigen\textsuperscript{163}. Despite these data showing beneficial responses by bovine γδ T cells to counter infectious disease, they may not always be beneficial during infection. For example, despite being shown to produce IFNγ in response to \textit{M. bovis}, bovine γδ T cells appear to be regulatory during established \textit{M. bovis} infections, as depletion of γδ T cells results in increased antigen-specific proliferation of αβ T cells\textsuperscript{164}. Therefore, further analysis of the role of γδ
T cells during infection in cattle is necessary to determine what drives an inflammatory or regulatory γδ T cell phenotype during infection.

Effect of Aging on Bovine γδ T Cells

In cattle, γδ T cell numbers and activity has been compared between adults and young calves. In young calves, γδ T cells can be close to 60% of peripheral mononuclear cells\textsuperscript{165}. During the first 5 months of life, the percentage of γδ T cells decreases slightly, but absolute numbers stay relatively constant\textsuperscript{87}. By 150 days of age, these percentages decrease to adult levels (10-20%), but are still much higher than percentages in mice or humans\textsuperscript{166}. Despite decreases in percentages, absolute γδ T cell numbers in the blood remain fairly constant from calf to adult\textsuperscript{167}. In the small intestine, however, γδ T cells numbers increase significantly with age\textsuperscript{88}.

In addition to overall percentages, subsets of bovine γδ T cells in the peripheral blood also change with aging. The percentage of CD8+ γδ T cells increases with age\textsuperscript{168}. Furthermore, in young calves, the WC1.1 population is the dominant population of WC1+ γδ T cells. However, the percentage of WC1+ γδ T cells expressing the WC1.2 isoform increases with age until the percentage of cells expressing WC1.1 or WC1.2 is about even by 500 days of age\textsuperscript{169}. This change in WC1 subset distribution also affects γδ T cell activity. In a 2007 study, IFNγ production was induced in WC1+ γδ T cells from both adults (more than 2 years of age) and calves (less than 6 weeks old) by the use of IL-12 and IL-18. The authors found that the percentage of WC1+ cells producing IFNγ was
increased in the calves compared to the adults by almost 10 fold\textsuperscript{170}. This is consistent with a decreasing percentage of WC1.1 $\gamma \delta$ T cells in adults, as these cells produce more IFN$\gamma$ than WC1.2 $\gamma \delta$ T cells\textsuperscript{169}. Consistent with cytokine production, $\gamma \delta$ T cells from 1 week-old calves were found to have enhanced proliferation compared to 5-6 month-old steers upon stimulation with pokeweed mitogen. This was in contrast to CD4$^+$ and CD8$^+$ T cells, which had enhanced proliferation in the steer population. By 8 weeks of age, $\gamma \delta$ T cells from the calves had similar proliferation compared to the steers\textsuperscript{171}. In summary, $\gamma \delta$ T cells from cattle appear to have enhanced proliferation and cytokine production in younger individuals. However, these studies are few in number and may not reflect the behavior of bovine $\gamma \delta$ T cells to all stimulants. In addition, the effects of age on other effector functions of $\gamma \delta$ T cells, such as cytotoxicity and non-IFN$\gamma$ cytokine production, have not yet been examined and will likely be the focus of future studies.

**Plant-derived Agonist Activity on Innate Lymphocytes**

**Immunotherapy Targeting Innate Lymphocytes**

Because of their lytic activity, NK cells and $\gamma \delta$ T cells have been targeted for immunotherapy in cancer patients. During these therapies, cytokines and other agonists are used to expand activated NK cell and $\gamma \delta$ T cell numbers. Unfortunately, while these types of therapies have had some success, these treatments have had limited efficacy in cancer patients overall\textsuperscript{172, 173}. 
There are several potential explanations for the problems current immunotherapy strategies to stimulate and expand human NK cells and γδ T cells have had in the clinic. Use of cytokines has been problematic because of toxicity with high doses. Phophoantigen-based therapies for γδ T cell immunotherapy only target the Vδ2 subset of γδ T cells, ignoring the potential benefit of the Vδ1 subset, and there is an exhaustion phenomenon that occurs with using phoshpoantigens repeatedly172, 173. Therefore, additional agonists for innate lymphocytes are needed which can enhance the activity of these current therapies and reduce the amount of these stimulants needed. A number of studies suggest that many plant extracts, some sold as nutritional supplements, could be a good source of novel NK cell and T cell agonists.

NK Cells

Several studies suggest that polyphenol extracts can enhance NK cell activation, proliferation, cytotoxicity, and cytokine production. The expression of CD69 on human NK cells, as well as NK cell proliferation in response to IL-15, was enhanced in vitro by polyphenols from unripe apple peels (APP)31. Increased cytotoxicity by murine and human NK cells was observed upon in vitro treatment with the popular polyphenol supplement resveratrol, as well as by the gall aqueous extract from Limoniastrum guyonianum Boiss174, 175. In addition to its in vitro activity, an increase in NK cell cytotoxicity was observed in resveratrol-fed mice176. A likely mechanism for the increased cytotoxicity by resveratrol-treated NK cells was the enhanced expression of
perforin and NKG2D, which was induced on a human NK cell line\textsuperscript{177}. To support these observations made \textit{in vitro} and in mice, clinical studies have found that the consumption of polyphenol-rich fruit juices or foods may enhance NK cell activity. A recent example showed that the consumption of a polyphenol-rich cranberry beverage may have enhanced mitogen-induced proliferation by NK cells\textsuperscript{27}. Another clinical study found increased NK cell cytotoxicity in individuals given a polyphenol-rich purple sweet potato leaves diet, similar to the results seen in resveratrol-fed mice\textsuperscript{178}. In all, these data suggests that treatment with immunomodulatory polyphenols have the potential to enhance NK cell activity in the human population, which could protect individuals from a variety of diseases.

\textbf{αβ T Cells}

Many of the studies which have examined the effect of polyphenols on αβ T cell activity have demonstrated a suppressive effect. In fact, suppression of αβ T cells may be critical for the observed benefit of polyphenols in some inflammatory disease models. In support of this idea, work by Skyberg et al. demonstrated that the ability for oral APP treatments to suppress inflammation in a dextran sodium sulfate model of colitis was lost in αβ T cell-deficient mice, suggesting that the suppression of αβ T cell activity was the primary benefit of APP treatment in this model\textsuperscript{19}.

Still, in addition to these studies demonstrating suppression of αβ T cells by polyphenols, other studies suggest that certain polyphenols, at least under certain
circumstances, activate αβ T cells. For example, in contrast to the previously described colitis study, APP, as it did with NK cells, enhanced resting human αβ T cell proliferation in response to IL-15. In addition to APP, lignin-like substances, which are high molecular weight polymerized polyphenols, induced T cell proliferation and the production of IFNγ, GM-CSF, and TNFα by murine CD4+ T cells in murine splenocytes, with these T cells being the major source of IFNγ and GM-CSF in polyphenol-treated splenocytes. When the mechanism for this activation of T cells by polymerized polyphenols was examined, it was found that antigen presentating cells were required for optimal cytokine production by T cells. Furthermore, blocking adhesion molecules involved in T cell-APC interactions inhibited the cytokine production by T cells, suggesting that cell-to-cell contact with APCs was required for the ability of polymerized lignin-like polyphenols to induce cytokine production by CD4+ T cells. Other polyphenols which can enhance αβ T cell activity are green tea polyphenols, resveratrol, and Cassia auriculata (CA)-derived polyphenols. Similar to its effect on NK cells, in vitro treatment of murine T cells with resveratrol promoted the production of several cytokines, including IFNγ, by CD8+ T cells, as well as increased CTL cytotoxicity. Green tea polyphenols also enhanced CD8+ T cell activity, strengthening their antitumor functions. Finally, CA-derived polyphenols fed daily to aged rats (24-26 months old) for 28 days increased T cell numbers and enhanced splenocyte proliferation to LPS, suggesting that treatment with polyphenols may be very useful in
reducing the T cell hyporesponsiveness observed in elderly individuals\textsuperscript{182}. Since $\alpha\beta$ T cells play such an important role in immunity, the influence of dietary polyphenols on their activity should be better understood.

**$\gamma\delta$ T Cells**

The localization of $\gamma\delta$ T cells to mucosal tissues, especially the gut, put these cells in ideal position to respond not only to pathogens, but to compounds taken orally or through other mucosal routes. Therefore, it is no surprise that numerous studies have examined the ability of numerous food products and nutritional supplements to influence the activity of these cells. In one study, APP suppressed the development of oral sensitization in mice and this suppression correlated with the expansion of $\gamma\delta$ T cells in the IELs, suggesting that polyphenol-induced expansion of $\gamma\delta$ T cells may prevent the development of food allergies\textsuperscript{16}. In contrast to the immunoregulatory effects of APP-treated $\gamma\delta$ T cells proposed in this study, the colitis study by Skyberg et al. suggested that oral treatment of APP may induce a proinflammatory response by $\gamma\delta$ T cells. In that study, APP-treated $\alpha\beta$ T cell-deficient mice, where there was no observed immunosuppression induced by APP, actually had increased cytokine production, including IFN$\gamma$, by mesenteric lymph node lymphocytes compared to untreated $\alpha\beta$ T cell-deficient mice. It is likely that $\gamma\delta$ T cells were a source of these cytokines, as their proportion amongst MLN lymphocytes in $\alpha\beta$ T cell-deficient mice increased fourfold\textsuperscript{19}. \textit{In vitro} studies in human PBMC cultures have also found that APP upregulates CD69 and CD11b on human $\gamma\delta$ T cells, promotes their
production of cytokines, and primes these cells to proliferate in response to suboptimal concentrations of phosphoantigens. Work by Daughenbaugh et al. showed that the ability of APP to enhance cytokine production by γδ T cells came from its ability to stabilize mRNA transcripts, allowing for rapid increased translation and generation of protein. It is possible that the stabilization of mRNA transcripts may be an important, conserved feature of polyphenols, allowing them to enhance the production of a wide variety of cytokines. In addition to APP, other polyphenol extracts activate γδ T cells, including polyphenols from cocoa, tea, Cat’s claw bark, among others.

Clinical studies in humans support some of the observations made in mice and human cell cultures. In one study, a juice concentrate made up of several fruits and vegetables increased γδ T cell numbers in human blood. Consumption of a polyphenol-rich tea beverage in another study primed γδ T cells to proliferate more robustly and produce more IFNγ to ex vivo ethylamine challenge. Finally, daily consumption of cranberry polyphenols for 10 weeks enhanced γδ T cell proliferation, and possibly IFNγ production, in response to ex vivo stimulation by PHA. In all, these data suggests that immunomodulatory food products containing polyphenols have the potential to enhance γδ T cell activity in the human population.

Oenothein B

Oenothein B is a polyphenol extracted from the Epilobium angustifolium (fireweed) plant. It is a cyclic ellagitannin with antitumor and antioxidant activity and
several studies over the past few decades have shown that it can also impact innate immune cell activity. One of the more attractive features of this polyphenol is that oenothein B is a pure compound with a known structure, unlike most polyphenol extracts, which could allow for further therapeutic development. In the 1990's, this compound was identified to inhibit tumor growth \textit{in vivo}^{28,29}. This inhibition of tumor growth was not believed to be caused by direct cytotoxicity to the tumor cells. Instead, oenothein B was found to stimulate macrophages and the production of IL-1 by oenothein B-stimulated macrophages was proposed to contribute to its observed antitumor effects in murine models\textsuperscript{28,29}. However, this was not examined in detail. In 2009, work by Schepetkin et al. supported these early studies and found that oenothein B activated both mouse and human neutrophils and monocytes/macrophages. Among the activities enhanced by oenothein B were intracellular calcium flux, the production of ROS, the activation of NF-kB, and the production of pro-inflammatory cytokines, including TNF\textalpha and IL-6\textsuperscript{33}. However, in contrast, subsequent studies have demonstrated an anti-inflammatory effect of oenothein B. A 2011 study by Kiss et al. found that myeloperoxidase release and production of ROS by activated neutrophils was inhibited by oenothein B\textsuperscript{186}. A 2012 study demonstrated that oenothein B inhibited NO production, NF-\textkappaB activity, and the production of IL-1\beta, IL-6, and TNF\textgreekalpha by a murine macrophage cell line pretreated with TLR2 and 4 agonists\textsuperscript{187}. A third study found that oenothein B inhibited IL-1\beta and IL-6 production by activated DCs and a 2013 study showed that oenothein B inhibited
neuroinflammation in response to systemic LPS treatment\textsuperscript{25,188}. Altogether, these data show that oenothein B has a complex influence on innate immune cells of the myeloid and granulocyte lineages. One possible explanation for the discrepancies in these results is the activation state of the cells at the time of treatment with oenothein B. For much of the work by Schepetkin et al., unstimulated, resting cells were used\textsuperscript{33}. However, in the studies demonstrating suppression by oenothein B, the authors used activated cells\textsuperscript{186-188}. Therefore, oenothein B may have differential effects on activated and resting immune cells. Surprisingly, despite all of these studies on the effect of oenothein B on neutrophils and macrophages, the effect of this compound on lymphocytes had not been previously studied. Therefore, we began to analyze the effect of oenothein B on lymphocytes.

Several plant-derived products which have been shown to inhibit tumor growth are also known to stimulate innate lymphocytes, such as NK cells and $\gamma\delta$ T cells\textsuperscript{176, 181, 185}. Therefore, we hypothesized that stimulation of innate lymphocytes could play an important role in the antitumor properties of these types of compounds. When we tested this hypothesis using oenothein B in a B16 melanoma tumor model, we saw a reduction in the mean tumor weight in wild-type mice treated with oenothein B. However, in mice that were $\gamma\delta$ T cell-deficient, oenothein B appeared to enhance tumor growth (Figure 2.2). These data suggested that $\gamma\delta$ T cells may have an important role in the antitumor activity of oenothein B and that this compound can induce both anti- and protumor effects.
In an alternative *in vivo* model, we tested the effect of oenothein B pre-treatment in a model of influenza A infection. In a preliminary experiment, we found that pretreatment with oenothein B intranasally reduced morbidity during influenza infection, measured by weight loss, in a dose-dependent manner (Figure 2.3). In both of these models, we hypothesized that oenothein B enhanced innate lymphocyte activity, which accounted for the benefit of oenothein B treatment. Unfortunately, our animal systems have not demonstrated the level of consistency required to fully test this hypothesis. This prompted us to focus on *in vitro* analysis to identify factors which may lead to inconsistencies in the immune-enhancing properties of oenothein B.

![B16 Melanoma](image_url)

**Figure 2.2** Inhibition of B16 tumor growth by oenothein B in a γδ T cell-dependent manner. 10-14 week old C57BL/6 mice and γδ T cell-deficient mice were injected subcutaneously with 5X10⁵ B16 melanoma cells on day 0. 50μg oenothein B or saline only was injected i.p. on days -1, 3, 7, and 11. On day 16, mice were humanely euthanized and tumor weights were recorded. N = 4 for all groups.
Factors Influencing Polyphenol Stimulation of Lymphocytes

Recent studies have already begun to examine what factors may influence the ability of certain polyphenols to stimulate immune cells. One factor that appears to be important is dose. In a 2001 study, which examined the effect of resveratrol on NK cell and T cell effector responses, low doses (less than 2.5 ug/ml) of resveratrol enhanced NK cell and T cell cytotoxicity, as well as the production of IFN\(\gamma\), IL-2, and IL-4 by T cells. However, higher doses (greater than 5 ug/ml) suppressed these responses\(^{175}\). This is consistent with the study by Skyberg et al., as higher doses of APP was more effective at suppressing colitis than lower doses\(^{19}\). Therefore, low amounts of polyphenols may favor immunostimulation, while large amounts, especially over a period of time, favor immunosuppression.

Another important factor in the immunomodulatory properties of polyphenols is molecular size, as T cells from murine splenocytes produced IFN\(\gamma\) and GM-CSF when stimulated by polymerized polyphenols with large molecular weights, but not their corresponding monomers\(^{179}\). Furthermore, procyanidin oligomers, but not monomers, stimulate \(\gamma\delta\) T cells\(^{15}\). In addition, the complete oenothein B molecule was necessary to stimulate monocytes, while molecular subunits of this molecule failed to do so\(^{33}\). Therefore, molecular stability and degradation \textit{in vivo} may impact the immunostimulatory properties of certain polyphenols. Importantly, while increasing
molecular size appears to have a significant effect on immunostimulation by polyphenols, small molecular weight polyphenols still have potent antioxidant activity\textsuperscript{189}.

Figure 2.3 Oenothein B reduced morbidity during influenza infection. Saline only or indicated concentrations of oenothein B were administered intranasally. 24hrs later, mice were infected intranasally with 1600 pfu of influenza strain PR8. Mice were weighed daily until day 14. (A) Experimental schematic. (B) Daily mouse weights as a % of starting body weight. N=5 for all groups.

In addition to molecular size, more subtle changes in polyphenol structure can have profound impacts on their activity. One study found that methylated, but not unmethylated, quercetin could enhance IL-1β production by monocytes in response to a
TLR2 agonist. In addition, not all methylated quercetin molecules had this activity, only certain ones\textsuperscript{190}. Since polyphenols can be methylated \textit{in vivo}, this could be an explanation as to why quercetin showed NK cell-enhancing activity \textit{in vivo}, but not \textit{in vitro}\textsuperscript{191}. These data suggest that small changes in polyphenol chemistry can have profound effects on their immunostimulatory activity.

While several polyphenol-specific factors have been identified which influence their ability to stimulate immune cells, very little research has been done to examine immunological factors which could impact their activity. However, previously described studies with oenothein B suggest that immune cells’ level of activation may be an important variable, as oenothein B appeared to activate resting cells while suppressing highly activated cells\textsuperscript{33, 186-188}. Consistent with this, APP enhanced cytokine production in αβ T cell-deficient mice with colitis. In these mice, colitis severity and proinflammatory cytokine production in untreated mice was low compared to untreated wild-type mice, suggesting that lymphocytes in these mice were not as activated, perhaps making them more susceptible to activation by APP. However, in wild-type mice, where colitis was more severe and lymphocytes produced more inflammatory cytokines, suggesting a higher state of activation, treatment with APP was suppressive and inhibited cytokine production\textsuperscript{19}. Therefore, a common behavior of immunomodulatory polyphenols may be the stimulation of resting immune cells, but the suppression of activated cells.
In addition to the activation state of immune cells, it was hypothesized that aging could also influence immune responses to polyphenols, as it influences responses to many other immune stimulants. For the rest of this dissertation, it is first determined that oenothein B stimulates lymphocytes, activates NK cells, αβ T cells, and γδ T cells, and promotes the production of IFNγ by these cells. Next, it is examined if age is a human and animal variable which influences the response of these innate lymphocytes to oenothein B, indeed showing that T cell responses to oenothein B are affected by age. Finally, a literature review is provided of the role of γδ T cells in cancer, which can either be beneficial or detrimental, as this may influence the effects of γδ T cell-stimulating polyphenols in tumor models. These studies provide additional insight into the immunomodulatory properties of oenothein B, as well as suggest additional variables to be taken into account when examining the immunomodulatory properties of oenothein B and, perhaps, other polyphenols.
CHAPTER THREE

OENO THEIN B, A CYCLIC DIMERIC ELLAGITANNIN ISOLATED FROM 
EPIL OBIUM ANGUSTIFOLIUM, ENHANCES IFNγ PRODUCTION BY 
LYMPHOCYTES

Contribution of Authors and Co-Authors

Manuscript in Chapter Three

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Abstract

Oenothein B is a polyphenol isolated from *Epilobium angustifolium* and other plant sources, which has been reported to exhibit immunomodulatory properties. Oenothein B is known to activate myeloid cells and induce the production of IL-1 and other cytokines. However, its effects on lymphocytes are unknown. In this report, we show that oenothein B stimulated innate lymphocytes, including bovine and human γδ T cells and NK cells, resulting in either increased CD25 and/or CD69 expression. We also demonstrate that oenothein B enhanced the production of interferon-γ (IFNγ) by bovine and human NK cells alone and in combination with interleukin-18 (IL-18), a response not observed with other commonly studied polyphenols. Furthermore, we demonstrate that oenothein B enhanced the production of IFNγ by human T cells. Since IFNγ contributes to antitumor, antibacterial, and antiviral cell responses, these data suggest an additional mechanism that could account, at least in part, for the immune enhancing properties of oenothein B.

Introduction

Nutritional supplements have been studied over many years for their ability to treat and prevent disease, including cancer and infections. Polyphenols represent a group of plant compounds found in many supplements that have been studied extensively for their role in promoting human health. Numerous studies have focused on the antioxidant
properties of polyphenols; however, the antioxidant effects of nutritional polyphenols in vivo are controversial\textsuperscript{192}. In addition, there are numerous studies that demonstrate biological activity of polyphenols beyond antioxidant activity, including modulating enzyme activity\textsuperscript{193}, receptor signaling\textsuperscript{194}, and immunity\textsuperscript{31, 32, 33, 183}.

Innate lymphocytes, such as NK cells and γδ T cells, play an important role in host defense against cancer and various pathogens, and enhancing the activity of these cells is an attractive option for immunotherapy\textsuperscript{195-197}. Results by our group and others have shown that some nutritional supplements are useful sources of novel agonists for innate lymphocytes and that the use of these supplements may represent a novel strategy to enhance the activity of these cells\textsuperscript{6, 16, 31, 33, 183}. For example, alkylamines from tea, apples, and wine, polysaccharides from Acai fruit and \textit{Funtumia elastica} bark, and other plant components have been shown to activate and enhance the proliferation of γδ T cells\textsuperscript{198-201}. In addition, we have recently found that certain polyphenols, such as oligomeric proanthocyanidins (OPCs) from apple peel, also stimulate innate lymphocytes, from different animals, including humans\textsuperscript{31}. However, not all polyphenols are capable of stimulating innate lymphocytes, and the size and structure of these compounds are important for their immunomodulating properties\textsuperscript{15, 179}.

NK cells and γδ T cells provide an early source of several cytokines, including interferon-γ (IFNγ) and IL-17\textsuperscript{202-204}. The production of IFNγ by lymphocytes is important in immune defense against various tumors and infections and could provide a possible
mechanism for the antibacterial, antiviral, and antitumor properties proposed for certain polyphenols\textsuperscript{205-207}. However, the induction of IFN\(\gamma\) by polyphenols is poorly understood or defined. In our earlier study of OPCs, we found no evidence for the induction of IFN\(\gamma\) in innate lymphocytes. Conversely, we have detected some IFN\(\gamma\) production from human PBMCs treated with oenothein B, a unique polyphenol with different structural and immunological properties than OPCs\textsuperscript{33}. Therefore, we investigated whether oenothein B might induce IFN\(\gamma\) production in innate lymphocytes or, based on our earlier studies that showed OPCs can enhance responses to secondary signals, possibly prime innate lymphocytes to respond more robustly to known inducers of IFN\(\gamma\), such as IL-18\textsuperscript{208}.

Briefly, oenothein B is a dimeric, macrocyclic ellagitannin isolated from \textit{Epilobium angustifolium}, as well as other plant sources. It has been studied for antitumor, antiviral, antibacterial, antioxidant, proinflammatory, and anti-inflammatory properties\textsuperscript{28, 33, 186, 187, 211}. Oenothein B has been reported to inhibit inflammatory responses by phagocytes induced by TLR agonists and other stimulants\textsuperscript{186, 187}. However, in the absence of additional stimulation, oenothein B promotes inflammatory responses by phagocytes.

In studies conducted in the early 1990’s, oenothein B was shown to reduce the growth of several tumors \textit{in vivo} and activate macrophages, promoting the production of IL-1\textsuperscript{28}. Induced IL-1 production was proposed to be important in the antitumor properties of oenothein B, although this has not been directly tested. We recently showed that oenothein B induces the production of IL-1, as well as other proinflammatory cytokines,
including IL-6 and tumor necrosis factor α (TNFα), by monocytes\textsuperscript{33}, responses not seen with OPCs. In addition, we showed that substructures of oenothein B did not stimulate phagocytes to the same extent as oenothein B\textsuperscript{33}, suggesting an important role for the complete structure in its immunological activity. To date, there are no reports on the effects of oenothein B on lymphocytes. We now show that oenothein B stimulates innate lymphocytes (γδ T cells and NK cells) and promotes their production of IFNγ. We also describe a novel priming effect of oenothein B on NK cells, leading to enhanced IFNγ production following IL-18 treatment. Finally, we describe a similar priming effect of oenothein B in response to a tumor cell line.

Materials and Methods

Ethics Statement

All animal experiments were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of Montana State University (protocol identification: 2009-3, 2011-61). Human subjects testing was performed in accordance with a protocol approved by the Institutional Review Board of Montana State University (approval identification: MJ032609), and written, informed consent was obtained from all individuals. No specific permits were required for the described field studies involving \textit{E. angustifolium}. According to the Gallatin National Forest Office (Montana), collection of limited amounts of plant...
Isolation of Oenothein B  Oenothein B was isolated and identified as described previously. Briefly, fully blossomed *E. angustifolium* were collected and the dried plant material (400 g) was extracted with 80% methanol at room temperature for 3 days. The combined extracts were concentrated, and any precipitates were removed by filtration through a 0.22-μm filter. The filtrate was lyophilized to obtain the crude extract or subjected to concentration and fractionation on a Sephadex LH-20 column (2.8 × 33 cm) using 80% methanol as an eluent. The relevant fractions were pooled and evaporated to dryness, re-chromatographed twice, and compound identification was performed by NMR and mass spectrometry, as described. Purity was determined to be >95% by HPLC and mass spectrometry, as described. A *Limulus* amebocyte lysate assay kit (Cambrex, East Rutherford, NJ) was used to evaluate possible endotoxin contamination in purified oenothein B. Purified oenothein B found to be free of endotoxin was stored at -80°C until used in the functional assays described below.

**Human and Bovine Peripheral Blood Mononuclear Cell Preparations**  Whole blood was collected from 1- to 3-month bull Holstein calves into sodium heparin tubes (BD Biosciences, San Jose, CA) and from healthy human adult donors with ACD solution B anticoagulant tubes (BD
Biosciences). Mononuclear cells were separated from whole blood using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) or Ficoll-Paque™Premium (GE Healthcare, Piscataway, NJ) for bovine and human cells, respectively, as previously described and per the manufacturer’s instructions\textsuperscript{31}. Additionally, bovine red blood cells were removed by hypotonic lysis after Histopaque separation.

Flow Cytometric Analysis and Cell Sorting of Bovine and/or Human PBMCs PBMCs were suspended in X-VIVO 15 serum-free medium or RPMI 1640 medium containing supplements and 10% FBS (cRPMI (31)). Cells were then cultured with or without oenothein B at 37°C and 10% CO\textsubscript{2}. Bovine cells were stained with antibodies against IL-2R\textalpha (LCTB2A, VMRD), CD335 (AKS1, AbDSerotec), \(\gamma\delta\) TCR (GD3.8\textsuperscript{212}), or a bovine monocyte antigen (BN180). Human cells were stained with antibodies against CD69 (FN50, Biolegend), CD25 (M-A251, BD Pharmingen), CD3 (UCHT1, Biolegend), CD56 (MEM-188, Biolegend and CM55B, eBioscience), CD8 (HIT8a, Biolegend), \(\gamma\delta\) TCR (11F2, BD Biosciences), V\(\delta\)2 (B6, BD Pharmingen), or IL-18R\textalpha (H44, Biolegend). All antibodies were directly labeled or indirectly labeled using goat anti-mouse FITC, PE, or APC (Jackson ImmunoResearch Laboratories, West Grove, PA). After staining, cells were analyzed using a BD Biosciences FACSCalibur with high throughput sampling (HTS).

Removal of bovine CD335\textsuperscript{+} cells, \(\gamma\delta\) T cells, and monocytes from PBMC preparations was performed using flow cytometric sorting. Briefly, bovine CD335\textsuperscript{+} cells,
γδ T cells, and monocytes were stained with monoclonal antibodies (mAb) against CD335 (AbDSerotec), γδTCR (GD3.8), and monocytes (BN180), respectively. Negative cells were then purified using a BD Biosciences FACSaria cell sorter to achieve >95% purity. As a control, unsorted bovine PBMCs were collected from the FACSaria for undepleted controls (controlling for the effects of the sorting procedure). Human NK cells were also sorted. Briefly, NK cells were isolated by staining cell preparations with CD3 (UCHT1, Biolegend) and CD56 (CM55B, eBioscience) and sorting CD3-/CD56+ cells using the FACSaria cell sorter to achieve >95% purity. After sorting, human cells were allowed to rest overnight in cRPMI with 10% FBS at 37°C and 10% CO2 before being used in the experiments described below.

**IL-18 Activation Assays** To test for priming effects by oenothein B, bovine and human PBMCs were isolated and incubated in X-VIVO 15 medium at 37°C and 10% CO2 in the presence of oenothein B (0-40 µg/ml) or medium only for approximately 24 hrs (bovine cells) or 48 hrs (human cells). Cells were then washed with Dulbecco’s PBS and resuspended in X-VIVO 15 medium in the presence or absence of recombinant human (rhu) IL-18 (R&D Systems, Minneapolis, MN). A fraction of the cells were then incubated approximately 18 hrs, and the supernatant fluids were collected for IFNγ quantification by ELISA (see below). Other cells were treated with brefeldin A (eBioscience), incubated for 6 hrs, stained for intracellular IFNγ using anti-IFNγ antibodies, and analyzed by flow cytometry (see below).
Sorted human NK cells were resuspended in X-VIVO 15 medium and plated in a 96-well plate at 5 X 10^4 cells/well. Cells were treated with oenothein B (20 µg/ml), rhu IL-18 (100 ng/ml), both, or medium only. Cells were incubated for 24 hrs and supernatant fluids were collected for IFNγ quantification by ELISA (see below).

**K562 Assay** K562 (chronic myelogenous leukemia) human cell line was from American Type Culture Collection (Manassas, Virginia). Human PBMCs were isolated and incubated in X-VIVO 15 medium at 37°C and 10% CO2 in the presence of oenothein B (20 µg/ml) or medium only for approximately 24 hrs. Cells were then washed with X-VIVO 15 and subsequently cultured in X-VIVO 15 in the presence or absence of K562 target cells. To measure soluble IFNγ, cells were co-cultured for 42 hours at 37°C and 10% CO2. Supernatant fluids were then collected for IFNγ quantification by ELISA (see below). To measure intracellular IFNγ, cells were co-cultured for 24 hours at 37°C and 10% CO2 with brefeldin A added for the final 6 hours. IFNγ quantification was then performed by flow cytometry (see below).

**Measurement of IFNγ** Enzyme-linked immunosorbent assays (ELISA) were used to measure IFNγ in cell supernatant fluids. A bovine IFNγ kit (MABTECH, Cincinnati, OH) and a human IFNγ kit (Biolegend ELISA Max) were used to perform ELISAs, according to the manufacturer’s instructions. All measurements were performed in duplicate or triplicate.
To measure IFNγ production by flow cytometry, leukocytes were isolated as described above. Cells were treated with brefeldin A and incubated for 6 hrs at 37°C and 10% CO₂. Bovine and human lymphocytes were stained as described above. Cells were then fixed with 2% paraformaldehyde for at least 10 min, washed once with PBS + 2% horse serum, and then washed once with 0.2% saponin (Sigma) in PBS + 2% horse serum. Bovine IFNγ was detected using a PE-conjugated mouse IgG1 mAb against bovine IFNγ (MCA1783E, ABD Serotec Inc., Raleigh, NC), whereas human IFNγ was detected using a PE-conjugated mouse IgG1 mAb (clone 4S.B3, Biolegend). For isotype controls, cells were stained with a PE-conjugated mouse IgG1 antibody (Biolegend). IFNγ antibodies and isotype controls were resuspended in 0.2% saponin. Cells were stained for 20 min at room temperature. After staining, cells were washed, then analyzed using a FACSCalibur with HTS.

**Statistical Analysis** Statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA). The data were analyzed by Student’s paired t-test, Student’s unpaired t-test, One-way ANOVA, or Two-way ANOVA as indicated.
Results and Discussion

Oenothein B Activates Human and Bovine Lymphocytes

Previously, we and others have found bovine PBMCs to be a useful model for the testing of novel innate lymphocyte agonists$^{31,213}$. The bovine model has also been used to study infections by *Mycobacterium* species and *Salmonella* species since it better reflects human diseases than rodent models$^{214-216}$. To determine if oenothein B stimulated lymphocytes, we first evaluated IL-2Rα expression as a marker for activation of bovine PBMCs. IL-2Rα was upregulated on both bovine γδ T cells and NK cells after stimulation with oenothein B (20-40 μg/ml) for 24 hours *in vitro* (Figure 3.1A and Figure 3.2). Doses and timepoints were based upon preliminary dose and kinetic analyses (data not shown). We then examined if similar responses were seen in human PBMCs, using CD69 expression as a marker for activation. In these studies, oenothein B stimulation for 2 days *in vitro* induced CD69 expression on human CD3+ T cells, γδ T cells, CD8+ T cells, and CD3- CD56+ NK cells (Figure 3.1B and Figure 3.2) at similar doses known to stimulate monocytes$^{33}$. Within the human γδ T cell population, both Vδ2+ (major circulatory subset) and Vδ2- (mainly Vδ1+ cells (217)) subsets were activated by oenothein B (Figure 3.1B), which is similar to responses induced by OPCs$^{31}$. In addition, we also examined CD25 expression on human PBMCs. Interestingly, oenothein B stimulation induced CD25 expression on T cells, but not NK cells (Figure 3.3).
Figure 3.1 Oenothein B induces IL-2Rα or CD69 on bovine and human lymphocyte subsets. (A) Bovine PBMCs (10^5 cells/well) were treated with the indicated concentrations of oenothein B in X-VIVO medium for 24 hrs, and IL-2Rα expression on γδ T cells and NK cells was measured by multi-color flow cytometry. NK cells were defined as non-γδ T cells that expressed CD335. The graphs represent pooled data from 3 individuals. Each treatment was analyzed in triplicate and error bars indicate SEM. Significance compared to untreated cells (0 µg/ml) was determined by One-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001 (B) Human PBMCs (10^5 cells/well) were treated with the indicated concentrations of oenothein B in cRPMI medium for 48 hrs. CD69 expression on lymphocytes, which included CD3+ T cells, CD8+ T cells, γδ T cells, and NK cells, was then measured by flow cytometry. The graphs represent pooled data from 5 individuals. Each treatment was analyzed in triplicate and error bars indicate SEM. Significance compared to untreated cells (0 µg/ml) was determined by One-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001
Figure 3.2 Oenothein B induces IL-2Rα or CD69 on bovine and human NK cells. (A) Bovine PBMCs (10^5 cells/well) were treated with 20 μg/ml oenothein B in X-VIVO medium for 24 hrs, and IL-2Rα expression on NK cells was measured by multi-color flow cytometry. Representative examples of two-color flow cytometry plots comparing IL-2Rα staining on oenothein B-treated and untreated bovine NK cells (gated on CD335+ cells) from each animal are shown. (B) Human PBMCs (10^5 cells/well) were treated with 40 μg/ml oenothein B in cRPMI medium for 48 hrs. CD69 expression on NK cells (gated on CD3- CD56+ cells) was then measured by flow cytometry. Representative examples of two-color flow cytometry plots comparing CD69 staining on oenothein B-treated and untreated human NK cells from each donor are shown.
Figure 3.3 Oenothein B induces CD25 on human T cells. Human PBMCs (10^5 cells/well) were treated with the indicated concentrations of oenothein B in X-VIVO medium for 42 hrs. CD25 expression on lymphocytes, which included γδ T cells (CD3+/γδ TCR+), NK cells (CD3-/CD56+), and αβ T cells (CD3+/γδ TCR-), was then measured by flow cytometry. The graph represents pooled data from 5 individuals. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance compared to untreated cells (0 µg/ml) was determined by One-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001

Oenothein B Primes Bovine PBMCs to Respond to IL-18

To examine the effects of oenothein B on IFNγ production in the bovine model, bovine PBMCs were treated with oenothein B for two days and secreted IFNγ was measured by ELISA. Similar to our studies on OPCs, we did not find significant amounts of IFNγ produced by oenothein B-treated bovine PBMCs (data not shown). However, in our original studies with OPCs, we found that OPC-treated γδ T cells had enhanced responses to secondary signals, such as IL-2 and TCR agonists. In addition, others have
found that feeding bovine calves polyphenols from pomegranate can enhance mitogen-induced IFNγ production by PBMCs. Therefore, we hypothesized that oenothein B might enhance or prime responses to an inducer of IFNγ.

As such, we tested if oenothein B treatment of bovine lymphocytes enhanced responses to the IFNγ-inducing cytokine, IL-18. We also tested several well-studied polyphenols, epigallocatechingallate (EGCG), resveratrol, curcumin, and theaflavin digallate (TFDG), all of which are potent antioxidants, to determine if such a response was a common property of polyphenols. When oenothein B–treated cells were subsequently treated with suboptimal doses of IL-18, IFNγ production was greatly enhanced compared to IL-18 or oenothein B alone (Figure 3.4). These data suggested that oenothein B could prime immune cells for enhanced IFNγ production in response to low doses of IL-18. Resveratrol and curcumin did not enhance IFNγ production in response to IL-18, but rather appeared to suppress the response, which would be consistent with previous studies describing their immunosuppressive properties. Both EGCG and TFDG enhanced IFNγ production in response to IL-18 in one of the calves tested, but their effect was not as consistent or as robust as oenothein B. The level of priming by oenothein B and the amount of IFNγ produced varied between animals. It is likely that these observed differences between the three calves were due to animal-specific responses to oenothein B, as our preliminary studies with IL-2Rα suggested that PBMCs
from individual calves can respond differently to oenothein B. Based on these results, we focused our subsequent studies on oenothein B and its effect on IFNγ production.

Presence of CD335+ Cells is Essential for Oenothein B Priming to IL-18

After observing enhanced IFNγ production by bovine cells pre-treated with oenothein B, we then determined which cells were important for this response. Since oenothein B has been shown to be a potent monocyte agonist, we first examined if these cells were essential for the priming responses. Monocytes were removed by flow cytometric sorting, and the priming response was again evaluated. Priming responses were still observed in monocyte-depleted PBMCs, although the level of priming was reduced in two out of three experiments (Figure 3.5A). These results suggested that monocytes likely contributed to the response in the mixed population, but were not required for the response.

We then examined the importance of NKp46+ cells, since they are a major source of IFNγ induced by IL-12 and IL-18 in bovine lymphocytes. NKp46, also known as CD335, is a NK cell marker, although it is expressed by other minor leukocyte-populations, including some γδ T cells. To test the importance of these cells, we depleted cells expressing CD335 from bovine PBMCs and found that nearly all of the oenothein B-induced IFNγ priming response was absent compared to undepleted PBMCs (Figure 3.6A).
Figure 3.4 Oenothein B primes bovine PBMCs to respond to IL-18. Bovine PBMCs (10^5 cells/well) were treated with oenothein B (40 µg/ml and 20 µg/ml), EGCG (40 µg/ml and 20 µg/ml), resveratrol (50 µg/ml and 25 µg/ml), curcumin (40 µg/ml and 20 µg/ml), theaflavin digallate (50 µg/ml), or X-VIVO medium alone for approximately 24 hrs. Cells were then washed and treated with 10 ng/ml rhu IL-18, 100 ng/ml rhu IL-18, or X-VIVO medium alone for approximately 24 hrs. After incubation, soluble IFNγ levels were measured by ELISA. The data are expressed as mean +/- SEM of three independent experiments. Statistical significance was measured by Two-way ANOVA with Bonferroni post-test. a^p<0.05 compared to 10ng/ml IL-18 only, b^p<0.05 compared to 100ng/ml IL-18 only.
Because CD335 is expressed on some γδ T cells, we examined whether γδ T cells contributed to the oenothein B-induced IFNγ response. Removal of γδ T cells reduced, but did not eliminate, the priming response (Figure 3.5B). This result suggested that, like monocytes, γδ T cells contributed to, but were not required for, the response and further suggested that γδ TCR-/CD335+ cells were the primary source of IFNγ in these assays. As a final approach to confirm these results, multi-color intracellular cytokine analyses were performed. As shown in Figure 3.6, oenothein B-primed, IL-18-treated CD335+ cells expressed IFNγ (Figure 3.6B and 3.6C). The percentage of CD335+ cells was also enhanced by oenothein B (Figure 3.6C). However, this was likely due to activated monocytes adhering to the sample plates and being removed from the CD335- population rather than an expansion of CD335+ cells. Collectively, these data indicate that CD335+ NK cells are the major source of IFNγ produced in response to oenothein B and IL-18 in the bovine system.
Figure 3.5 Effect of monocyte and γδ T cell depletion on oenothein B-priming of bovine PBMCs. Bovine PBMCs (10^5 cells/well) were depleted of (A) monocytes or (B) γδ T cells and treated with 20 µg/ml oenothein B or X-VIVO medium alone for 24 hrs. Cells were then washed and treated with 10 ng/ml rhu IL-18 or medium alone for 18 hrs. After incubation, IFNγ levels in the supernatant fluids were measured by ELISA. The data are expressed as mean +/- SEM of three independent experiments comparing depleted PBMCs to un-depleted controls tested concurrently. All samples were tested in duplicate or triplicate. Statistical significance was measured by Two-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001
Figure 3.6 Oenothein B primes bovine CD335+ cells to respond to IL-18. (A) Bovine PBMCs (10^5 cells/well) were depleted of CD335+ cells and treated with 20 µg/ml oenothein B or X-VIVO medium alone for 24 hrs. Cells were then washed and treated with 10 ng/ml rhu IL-18 or medium alone for 18 hrs. After incubation, IFNγ levels in the supernatant fluids were measured by ELISA. The data are expressed as mean +/- SEM of three independent experiments comparing depleted PBMCs to un-depleted controls tested concurrently. All samples were tested in duplicate or triplicate. Statistical significance was measured by Two-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001 (B) Bovine PBMCs (10^5 cells/well) from a new calf were treated with the indicated amounts of oenothein B or X-VIVO medium alone for 24 hrs. Cells were washed and treated with 10 ng/ml rhu IL-18 or X-VIVO medium alone for 6 hrs in the presence of brefeldin A. IFNγ production was measured by intracellular flow cytometry. The data are expressed as mean +/- SEM. All samples were tested in triplicate. Statistical significance was measured by Two-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001 (C) Representative examples of two-color flow cytometry plots comparing IFNγ staining on CD335+ cells.
Oenothein B Induces IFNγ Production by Human Innate Lymphocytes

In our previous studies with oenothein B, we showed that treatment of human PBMCs with oenothein B promoted some IFNγ production, in contrast to the response we observed with bovine cells. However, potential sources of this cytokine were not identified. We first confirmed our previous results in human PBMCs by cytokine ELISA (Figure 3.7A). We then examined if lymphocytes were a source of this induced IFNγ. Human T cells, including γδ T cells and CD8+ T cells, produced IFNγ in response to oenothein B (Figure 3.7B and 3.7C). The percentages of NK cells positive for IFNγ staining also increased in three out of five donors tested (Figure 3.7B), but minimal staining was observed compared to that seen in T cells (Figure 3.7C). Thus, unlike the bovine system, oenothein B-induced IFNγ production was not restricted to the human NK cell population.

In the bovine system, NK cells were primed to produce enhanced IFNγ in response to IL-18. We tested whether the same could be true for human NK cells. First, multi-color flow cytometry showed that IL-18 receptor was increased on oenothein B-treated human NK cells in two out of five donors (Figure 3.8A). Similarly, further analyses showed that in two, possibly three, of five human PBMC preparations, IFNγ production was increased in oenothein B-primed, IL-18-treated human NK cells compared to cells treated with oenothein B or IL-18 alone (Figure 3.8B and 3.8C).
We then tested if oenothein B could enhance IFNγ production in response to the NK cell target leukemic cell line, K562. Others have shown that stimulation by K562 cells induces IFNγ secretion by NK cells and that this response can be enhanced by the presence of a second stimuli\textsuperscript{220}. Consistent with these reports, pretreatment of human PBMCs with oenothein B enhanced IFNγ production in response to K562 cells compared to untreated PBMCs (Figure 3.9A) and NK cells were the major cell population primed by oenothein B for enhanced IFNγ production (Figure 3.9B).

NK cell numbers and activity can vary significantly from donor to donor. To address whether the inconsistency seen in PBMC preparations in response to oenothein B and IL-18 might be due to variable numbers of NK cells between PBMC samples or variable influences by other cells within the mixed populations on the NK cells, human NK cells were sorted, and then equal cell numbers were treated with oenothein B alone, IL-18 alone, or a combination of both. IFNγ production was measured 24 hrs later by ELISA. As shown in Figure 3.10, oenothein B alone directly induced IFNγ production by NK cells and there was an increase in IFNγ production with the combined treatment in all donors tested, although the amount of IFNγ produced varied between donors. This variability in IFNγ production by NK cells has been observed in other studies and may have a genetic component\textsuperscript{220, 221}. 
Figure 3.7 IFNγ production by human lymphocytes in response to oenothein B. (A) Human PBMCs (10^5 cells/well) were treated with the indicated concentrations of oenothein B or X-VIVO medium alone for 48 hrs, and soluble IFNγ levels in supernatant fluids were measured by ELISA. The graph represents data from ten individuals, with each sample plated in triplicate. Statistical significance was measured by One-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001 (B) Human PBMCs (10^5 cells/well) were treated with oenothein B or X-VIVO medium alone for 6 hrs in the presence of brefeldin A. The percent of total CD3+ T cells, γδ T cells, CD8+ T cells, and NK cells positive for IFNγ staining was then determined by flow cytometry. The graphs represent data for five individuals, with each treatment analyzed in triplicate. Statistical significance was determined by paired Student's t-test. *p < 0.05, **p<0.01, ***p<0.001 (C) Representative examples of two-color flow cytometry plots comparing IFNγ staining on oenothein B-treated and untreated human lymphocytes.
Figure 3.8 Priming of human NK cells to IL-18 by oenothein B. (A) Human PBMCs (10^5 cells/well) were treated with the indicated concentrations of oenothein B or X-VIVO medium alone for 48 hrs, and expression of IL-18R on human NK cells was determined by flow cytometry. Significance was determined by One-way ANOVA with Bonferroni post-test. *p < 0.05, **p<0.01 (B) Human PBMCs (10^5 cells/well) were treated with 20 µg/ml oenothein B or X-VIVO medium alone for 48 hrs. Cells were washed with PBS and treated with 50 ng/ml rhu IL-18 for 6 hrs in the presence of brefeldin A. IFNγ staining [mean fluorescent intensity (MFI)] on human NK cells was determined by flow cytometry. The graphs represent data from five individuals, with each treatment analyzed in triplicate. (C) Representative examples of two-color flow cytometry plots comparing IFNγ staining on gated human NK cells treated with 20 µg/ml oenothein B, 50 ng/ml rhuIL-18, both, or X-VIVO medium alone. *p<0.05, **p<0.01, ***p<0.001
Figure 3.9 Priming of human NK cells to K562 cells by oenothein B. (A) Human PBMCs (10^5 cells/well) were treated with 20 µg/ml oenothein B or X-VIVO medium alone for approximately 24 hrs. Cells were then washed and co-cultured with or without K562 cells at effector:target (E:T) ratios of 10:1 and 1:1 for approximately 42 hrs. After incubation, soluble IFNγ levels were measured by ELISA. The data represent pooled results from three donors and are expressed as mean +/- SEM. Samples were analyzed in duplicate. Statistical significance was measured by Two-way ANOVA with Bonferroni post-test. (B) Human PBMCs (10^5 cells/well) were treated with 20 µg/ml oenothein B or X-VIVO medium alone for approximately 24 hrs. Cells were then washed and co-cultured with or without K562 cells at an effector:target (E:T) ratio of 1:1 for approximately 18 hrs. After incubation, brefeldin A was added to the culture for 6 hrs. IFNγ expression by NK cells (CD3-/CD56+), T cells (CD3+), and others (CD3-/CD56-) was then measured by intracellular flow cytometry. The data represent pooled results from two donors and are expressed as mean +/- SEM. Samples were analyzed in duplicate. Statistical significance was measured by One-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001
Figure 3.10 Oenothein B directly primes purified human NK cells to produce IFNγ. Human NK cells (5 X10⁴ cells/well) were sorted and treated with 20 μg/ml oenothein B, 100 ng/ml rhu IL-18, both, or medium alone. After 24 hrs, soluble IFNγ was measured by ELISA. The graph represents soluble IFNγ levels in culture supernatant fluids from three separate experiments with three different donors. Error bars indicate SEM. Each sample was analyzed in triplicate. Significance was determined by One-way ANOVA with Bonferroni post-test. *p < 0.05, **p<0.01, ***p<0.001

These data further suggest that, as with bovine cells, oenothein B treatment has the potential to augment IFNγ production by human NK cells alone and in response to IL-18. In addition, these data suggest that oenothein B can directly prime these cells to respond to IL-18.

Collectively, our results show that, in addition to monocytes, oenothein B stimulates subsets of bovine and human lymphocytes, including NK cells, CD8+ T cells, and both Vδ2+ and Vδ2- γδ T cells, by upregulating IL-2Rα and/or CD69 on these cells. We also show that oenothein B promotes the production of IFNγ by human lymphocytes, specifically γδ T cells and CD8+ T cells. Furthermore, we demonstrate that IFNγ
production by NK cells can also be induced by oenothein B, although this response was not as robust or consistent as that seen in T cells. Interestingly, differences in the capacity of oenothein B to induce IFN\(\gamma\) production by T cells was observed between human and bovine cells, as oenothein B alone did not directly induce significant IFN\(\gamma\) secretion by bovine T cells as it did with human T cells. These data suggest that certain polyphenols may exert species-specific effects and that immunomodulatory effects of polyphenols demonstrated in one species may not always be conserved in other species. Thus, analysis of the immunomodulating properties of polyphenols cannot rely solely on animal testing, and a combination of animal and human cell testing is required to identify relevant, conserved responses.

A possible explanation for some of the differences observed between human and bovine T cells in these studies could be due to differences in ages, as young calves were used for our bovine studies while adults were used for our human studies. It has been shown that IFN\(\gamma\) secretion by T cells can increase with age, correlating with an increase in CD45RO\(^+\) T cells\(^{222}\). Therefore, future studies could examine the effects of aging on these responses. It is possible that lymphocyte responses to certain polyphenols in young bovine calves are more reflective of those that might occur in children, suggesting a potential new use for this animal model in the study of the effects of dietary polyphenols on neonatal and adult lymphocytes.
A potentially important and conserved response to oenothein B is enhanced IFN\(\gamma\) secretion following exposure to suboptimal IL-18 concentrations, which was observed in both bovine and human NK cells. The synergistic effect of oenothein B and IL-18 for enhancing IFN\(\gamma\) production by NK cells was observed in mixed PBMC cultures, NK cell-depleted PBMCs, as well as sorted NK cells. Our earlier studies demonstrated that oenothein B could induce IL-12 production by monocytes\(^{33}\), which others have found synergizes with IL-18 to produce IFN\(\gamma\)\(^{223}\). Thus, this could provide an explanation for oenothein B’s ability to enhance IL-18-induced IFN\(\gamma\) production in some of our experiments; however, the enhanced production of IFN\(\gamma\) observed in sorted NK cell cultures suggests a direct effect on NK cells by oenothein B. Additionally, oenothein B enhanced IFN\(\gamma\) secretion in response to an NK cell target cell line, suggesting that the ability of oenothein B to enhance IFN\(\gamma\) secretion is not restricted to IL-18, but also occurs upon co-culture with tumor cell targets.

In conclusion, our results expand upon previous studies suggesting that oenothein B stimulates innate and antitumor immunity, and further characterizes this activity, suggesting that lymphocyte activation and IFN\(\gamma\) production may contribute to these responses. The production of IFN\(\gamma\) by lymphocytes and other cells enhances antitumor immunity by a number of mechanisms, and it will be important to examine whether lymphocytes and/or IFN\(\gamma\) play an important role in the antitumor properties of oenothein B \textit{in vivo}. In addition, IFN\(\gamma\) production is a vital step in the host defense against
numerous pathogens, including viruses and intracellular bacteria. Therefore, our data also suggest a potential mechanism whereby oenothein B could enhance antiviral and antibacterial immunity \textit{in vivo}. Thus, it will also be important to examine if oenothein B enhances host defense against various pathogens whose clearance relies on lymphocyte activity and IFN\(\gamma\) production. Further work is also necessary to identify the receptors and signaling pathways involved in these immune stimulatory effects of oenothein B. Finally, these studies suggest that oenothein B may be a promising candidate for therapeutic development to supplement immunotherapies, especially those involving IL-18.

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AGING INFLUENCES THE RESPONSE OF T CELLS TO STIMULATION BY THE ELLAGITANNIN, OENOTHEIN B.

Contribution of Authors and Co-Authors

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Several plant extracts, including certain polyphenols, prime innate lymphocytes and enhance responses to secondary stimuli. Oenothein B, a polyphenol isolated from *Epilobium angustifolium* and other plant sources, is one of these polyphenols. Oenothein B enhances IFNγ production by both bovine and human NK cells and T cells, alone and in response to secondary stimulation by cytokines and a tumor cell line. Innate immune cell responsiveness is known to be affected by aging, but whether polyphenol responses by these cells are also impacted by aging is not known. Therefore, we examined oenothein B responsiveness in T cells from cord blood, young, and adult donors. We found that oenothein B stimulates bovine and human T cells from individuals over a broad range of ages, as measured by increased IL-2Rα and CD69 expression. However, clear differences in induction of cytokines by T cells were seen. In human cord blood and bovine calves, oenothein B was unable to induce IFNγ production by T cells. However, in human and bovine adults, oenothein B induced IFNγ production by T cells and T cells were the most prevalent IFNγ-expressing cell type. In addition, oenothein B induced GM-CSF production by human adult T cells, but not cord blood T cells. Within the responsive T cell population, we found that CD45RO+ memory T cells expressed more cytokines in response to oenothein B than CD45RO- T cells, supporting the enhanced production of cytokines by T cells in older individuals. In summary, our data suggest that the
immunostimulation of T cells by oenothein B is influenced by age, particularly with respect to immune cytokine production.

**Introduction**

Plant polyphenols are known to modulate innate immunity. While many of these compounds suppress inflammation, some can act as agonists for innate immune cells. Lymphocytes, such as NK cells and T cells, which play an important role in host defense against cancer and various pathogens, are stimulated by select polyphenols. For example, polyphenols from tea, apples, cocoa, and others activate and enhance the proliferation and cytokine production of γδ T cells and NK cells. Furthermore, these compounds are capable of antigen-independent priming of lymphocytes, enhancing their responses to secondary stimuli, even in the absence of a significant response to the compounds themselves.

The production of IFNγ by lymphocytes is important during immune defense against tumors and a large number of viral, bacterial, and fungal infections. In a previous study, we found that oenothein B, a dimeric, macrocyclic ellagitannin isolated from *Epilobium angustifolium*, enhanced IFNγ production by both NK cells and T cells from bovine calves and humans, either alone or in combination with additional stimuli. During this study, the majority of IFNγ-producing cells in our bovine samples were CD335 (NKp46)-expressing NK cells. We also found that oenothein B enhanced IFNγ production by oenothein B is influenced by age, particularly with respect to immune cytokine production.
production by NK cells in our human samples. However, in contrast to our bovine data, a non-NK cell population, specifically T cells (both $\gamma\delta$ T cells and $\alpha\beta$ T cells), produced IFN$\gamma$ in response to stimulation by oenothein B in human samples. Furthermore, this oenothein B-responsive T cell population made up the majority of IFN$\gamma$-producing cells in these human samples$^{224}$. At this time, we did not investigate the basis for the difference in the two systems, but felt age was likely a contributing factor. Specifically, the human donors were all mature adults (at least 18 years old), while the bovine donors were young animals (less than 6 months old). While numerous studies find age-related differences in the response of immune cells to various agonists, the influence of age on the immunostimulation activity of plant-derived polyphenols has not been well studied$^{63, 89, 155, 170, 222}$. This could have important health implications, as individuals with a broad age range use polyphenol-containing supplements to support health and immunity and it is currently unknown if these compounds are more effective for certain age groups.

Therefore, we examined what effect age had on the immunostimulation of T cells by oenothein B in both humans and cattle. We show that oenothein B stimulated human and bovine T cells from neonate, young, and adult donors, but that cytokine production by T cells in response to oenothein B was enhanced in adults. Consistent with this, we found that CD45RO+ T cells isolated from both cows and humans produced more IFN$\gamma$, as well as GM-CSF, in response to oenothein B than CD45RO- cells. These data suggested that
the role of T cells in the modulation of immunity by oenothein B may be influenced by age.

**Materials and Methods**

**Ethics Statement**

All animal experiments were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of Montana State University. Human subjects testing was performed in accordance with a protocol approved by the Institutional Review Board of Montana State University, and written, informed consent was obtained from all adult donors and cord blood donors.

**Isolation of Oenothein B** Oenothein B was isolated and identified as described previously\(^3^3\). Briefly, fully blossomed *E. angustifolium* were collected and the dried plant material (400g) was extracted with 80% methanol at room temperature for 3 days. The combined extracts were concentrated, and any precipitates were removed by filtration through a 0.22-μm filter. The filtrate was lyophilized to obtain the crude extract or subjected to concentration and fractionation on a Sephadex LH-20 column (2.8 \times 33 cm) using 80% methanol as an eluent. The relevant fractions were pooled and evaporated to dryness, re-chromatographed twice, and compound identification was performed by NMR and mass spectrometry, as described\(^3^3\). Purity was determined to be >95% by HPLC and mass spectrometry, as described\(^3^3\). A *Limulus* amebocyte lysate assay kit
(Cambrex, East Rutherford, NJ) was used to evaluate possible endotoxin contamination in purified oenothein B. Purified oenothein B found to be free of endotoxin was stored at -80°C until used in the functional assays described below.

Human and Bovine Peripheral Blood Mononuclear Cell Preparations Whole blood was collected from 6-week old bull Holstein calves, adult (>2 years old) Holstein cows, and adult (4-7 years old) Angus and Angus X Hereford cows. All bovine blood was collected into sodium heparin tubes (BD Biosciences, San Jose, CA). Whole blood from healthy human adult donors was collected in ACD solution A anticoagulant tubes (BD Biosciences). Cord blood was collected in sodium heparin anticoagulant tubes (BD Biosciences). Mononuclear cells were separated from whole blood using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) for bovine and human cells, as previously described and per the manufacturer’s instructions. Additionally, red blood cells were removed by hypotonic lysis after Histopaque separation.

Cell Sorting of PBMCs Human CD45RO+ and CD45RO- T cells were isolated by staining cell preparations with monoclonal antibodies (mAbs) against CD3 (UCHT1, Biolegend) and CD45RO (UCHL1, eBioscience) and sorting using a FACS Aria cell sorter to achieve >98% purity. Staining with CD3 was used to distinguish CD45RO+ and CD45RO- T cells from CD45RO+ and CD45RO- non-T cells. After sorting, human cells
were incubated overnight in cRPMI with 10% FBS at 37°C and 10% CO₂ before being used in the experiments described below.

Bovine T cells were isolated by staining cell preparations with mAbs against CD3 (MM1A, Washington State University and VMRD), CD4 (CC30), and γδ TCR (GD3.8212) and sorting using a FACSaria cell sorter to achieve >98% purity. After sorting, bovine cells were incubated overnight in cRPMI with 10% FBS at 37°C and 10% CO₂ before being used in the experiments described below.

**T Cell Activation Assays** To measure IL-2Rα or CD69 expression, bovine and human PBMCs were isolated and incubated in cRPMI or X-VIVO 15 medium at 37°C and 10% CO₂ in the presence of oenothein B (0-40 µg/ml) or medium only for approximately 24 hrs. IL-2Rα and CD69 expression was then analyzed by flow cytometry (see below). Some samples were incubated for an additional 18 hrs in the presence of brefeldin A (eBioscience) to quantify IFNγ and GM-CSF expression by flow cytometry (see below). To measure IFNγ and GM-CSF secretion, bovine and human PBMCs, as well as sorted human and bovine cells, were incubated in cRPMI or X-VIVO 15 medium at 37°C and 10% CO₂ in the presence of oenothein B (0-40 µg/ml) or medium only for approximately 24 hrs. Supernatant fluids were then collected for quantification by ELISA (see below).

To promote differentiation into CD45RO+ T cells, bovine PBMCs were cultured with 500 ng/ml Concanavalin A and 1 ng/ml recombinant human IL-2 in X-VIVO 15
medium at 37°C and 10% CO₂ for 4 days. Medium was replaced after 4 days and PBMCs were cultured 2 additional days in X-VIVO 15 medium only before stimulating with oenothein B as described above.

Flow Cytometric Analysis of Bovine and/or Human PBMCs Bovine cells were stained with mAbs against surface molecules IL-2Rα (LCTB2A, VMRD), CD3 (MM1A, Washington State University and VMRD), CD45RO (GC42A1, VMRD), CD335 (AKS1, AbDSerotec), and γδ TCR (GD3.8212). Human cells were stained with mAbs against CD69 (FN50, Biolegend), CD3 (UCHT1, Biolegend), CD45RO (UCHL1, eBioscience), CD56 (MEM-188, Biolegend and CM55B, eBioscience), and γδ TCR (11F2, BD Biosciences). All mAbs were directly labeled or indirectly labeled using goat anti-mouse FITC, PE, or APC secondary mAbs (Jackson ImmunoResearch Laboratories, West Grove, PA) that were not cross-reactive to human or bovine antibodies. To control for non-specific staining by secondary mAbs, cells labeled with both primary and secondary mAbs were compared with cells labeled with secondary mAbs only. After staining, cells were analyzed using a BD Biosciences FACSCalibur with high throughput sampling (HTS).

Measurement of IFNγ and GM-CSF Enzyme-linked immunosorbent assays (ELISA) were used to measure IFNγ and GM-CSF in cell supernatant fluids. A bovine IFNγ kit (MABTECH, Cincinnati, OH), a human IFNγ kit (MABTECH, Cincinnati, OH),
and a human GM-CSF kit (Biolegend ELISA Max) were used to perform ELISAs, according to the manufacturer’s instructions. All measurements were performed in duplicate or triplicate.

To measure IFN$\gamma$ and GM-CSF production by flow cytometry, leukocytes were isolated, treated, and stained for cell surface molecules as described above. Cells were then fixed with 2% paraformaldehyde for at least 10 min, washed once with PBS + 2% horse serum, and then washed once with 0.2% saponin (Sigma) in PBS + 2% horse serum. Bovine IFN$\gamma$ was detected using a PE-conjugated mouse IgG1 mAb against bovine IFN$\gamma$ (MCA1783E, ABD Serotec Inc., Raleigh, NC). Human IFN$\gamma$ was detected using a PE-conjugated mouse IgG1 mAb (clone 4S.B3, Biolegend). Human GM-CSF was detected using a PE-conjugated rat IgG2a mAb (clone BVD2-21C11, Biolegend). For isotype controls, cells were stained with a non-specific, PE-conjugated mouse IgG1 antibody (Biolegend) or rat IgG2a antibody (Biolegend). IFN$\gamma$ antibodies and isotype controls were resuspended in 0.2% saponin. Cells were stained for 25 min at room temperature. After staining, cells were washed and then analyzed using a FACSCalibur with HTS.

**Statistical Analysis** Statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA). The data were analyzed by Student’s paired t-test, Student’s unpaired t-test, One-way ANOVA, or Two-way ANOVA as indicated.
Results

Oenothein B Stimulates and Induces IFNγ Production by Bovine Adult T Cells

To examine potential differences between adults and calves in relation to the stimulation of T cells by oenothein B, we stimulated isolated PBMCs from Holstein bull calves approximately 6-weeks old and adult Holstein cows at least 2 years old with oenothein B. First, we compared oenothein B-induced activation of adult and calf T cells, using IL-2Rα as an activation marker, as we have done in previous studies. Treatment with certain polyphenols enhance T cell proliferation in response to IL-2, likely due to the upregulation of IL-2Rα, which promotes the formation of the high affinity IL-2R complex. Previous studies showed that oenothein B enhanced IL-2Rα expression on calf lymphocytes. Similar to these results in calves, treatment with oenothein B enhanced IL-2Rα expression on T cells from adult cows. However, oenothein B induced less IL-2Rα expression on adult CD3+ T cells compared to calf T cells. CD3+ T cells were then separated into γδ T cells and αβ T cells and examined for IL-2Rα expression. Interestingly, oenothein B induced less IL-2Rα expression on adult γδ T cells compared to calves, but there was no significant difference in IL-2Rα expression on adult and calf αβ T cells. These data suggest that oenothein B may be less effective at priming adult bovine γδ T cells to
respond to IL-2, compared to calf T cells, but equally as effective at priming adult and calf αβ T cells.

Next, potential differences between adults and calves in relation to IFNγ production by T cells in response to oenothein B were examined. Previous results found that considerable IFNγ was produced by bovine calf PBMCs following oenothein B treatment, plus IL-18. However, T cells (either αβ or γδ T cells) produced little, if any, of the induced IFNγ. NK cells were consistently the major source of IFNγ in the calf preparations. The lack of significant IFNγ production by T cells from calves, with non-T cells producing the majority of oenothein B-induced IFNγ, was repeated here (Figure 4.2A and 4.2B). However, in contrast, T cells from adult animals produced significantly more IFNγ than calf T cells (Figure 4.2A). Indeed, upon two color FACS analysis, T cells made up the majority of IFNγ-producing cells in these adult samples (Figure 4.2B). This result was exactly as we observed for adult human donors in our previous study. These data suggested that the ability of oenothein B to enhance IFNγ production by bovine T cells increases with age.

Oenothein B Enhances IFNγ Production by Adult Bovine CD45RO+ αβ and γδ T Cells

Next, it was determined if memory T cells were an important source of this oenothein B-induced IFNγ in adult bovine T cells. Numerous studies have found that memory T cells produce more IFNγ than naive T cells in response to both antigen-dependent and antigen-independent stimulation. Furthermore, the percentage of
memory T cells in PBMCs increases with age, correlating with pathogen exposure over the lifespan\textsuperscript{103, 222}. Therefore, it was examined if T cells expressing the memory cell-marker CD45RO were an important source of oenothein-B induced IFN\textgamma in adult cows (4-7 year old cows). In these experiments, cells expressing IFN\textgamma in these animals were largely CD3+ T cells, consistent with our earlier results (Figure 4.3A and 4.3D). Furthermore, cells expressing CD45RO produced more IFN\textgamma than cells that did not express CD45RO, suggesting that CD45RO+ T cells were the major IFN\textgamma-producing cells in response to stimulation with oenothein B in these animals (Figure 4.3B and 4.3D). These data suggested that IFN\textgamma production by bovine T cells in response to oenothein B could be enhanced by increasing numbers of antigen-experienced CD45RO+ T cells. However, not all CD45RO+ cells produced IFN\textgamma and a small population of CD45RO- cells did express IFN\textgamma in response to oenothein B, suggesting that the linkage of CD45RO expression to the capacity of oenothein B to stimulate IFN\textgamma production in T cells in adult cattle is not absolute. Finally, oenothein B was found to induce IFN\textgamma production by both $\gamma\delta$ TCR+ and $\gamma\delta$ TCR- cells (Figure 4.3C and 4.3D). These data suggest that, consistent with studies with adult human PBMCs, oenothein B induces IFN\textgamma production by both $\alpha\beta$ T cells and $\gamma\delta$ T cells from adults\textsuperscript{224}. 
Oenothein B Enhances IFNγ Production by Purified Adult Bovine T Cells

To determine if differences in IFNγ production between adult and calf T cells was due to differences in the T cells themselves, or differences in the composition of PBMCs, CD4+ T cells, γδ T cells, and other T cells (CD4- γδ TCR-) were sorted from

Figure 4.1 Oenothein B induces IL-2Rα expression on bovine T cells from both adults and calves. Bovine PBMCs (10^5 cells/well) from calves (<12 weeks old) and adult cows (>2 years old) were treated with the indicated concentrations of oenothein B or X-VIVO medium only for 24 hrs and IL-2Rα expression on CD3+ T cells (A), αβ T cells (B), and γδ T cells (C) was measured by multi-color flow cytometry. αβ T cells were characterized as CD3+ cells that did not express γδ TCR. The graphs represent pooled data. Each treatment was analyzed in triplicate and error bars indicate SEM. Significance compared to untreated cells (0 µg/ml) and between calves and adults was determined by Two-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001
Figure 4.2 Oenothein B induces more IFNγ production by adult bovine T cells than calf T cells. (A) Bovine PBMCs (10⁵ cells/well) from calves (<12 weeks old, N=4) and adult cows (>2 years old, N=4) were treated with the indicated concentrations of oenothein B in X-VIVO medium for 42 hrs with brefeldin A added for the final 18 hrs. The percent of CD3+ T cells which were IFNγ+ was measured by multi-color flow cytometry. The graph represents pooled data. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance was determined by Two-way ANOVA with Bonferroni post-test. (B) Representative examples of two-color flow cytometry plots of adult and calf bovine PBMCs treated with 20 µg/ml oenothein B or X-VIVO medium alone.
bovine adults and calves. When these purified cells were stimulated with oenothein B, CD4+ T cells from adults expressed more IFNγ in response to direct stimulation by oenothein B than those from calves (Figure 4.4A). Neither γδ T cells nor other T cells from calves or adults expressed IFNγ in response to direct stimulation by oenothein B (Figure 4.4B and 4.4C). These data suggest that oenothein B promotes greater IFNγ expression by adult bovine T cells, at least CD4+ T cells, due to T cell-intrinsic differences between adult and calf T cells.

Differentiation of Calf T Cells into CD45RO+ T Cells is not Sufficient to Enhance Oenothein B-induced IFNγ Production

CD45RO expression and IFNγ secretion is upregulated in T cells following mitogen (ConA/IL-2) stimulation \textit{in vitro}\textsuperscript{225,226}. As such, it was then determined whether treatment with ConA to increase CD45RO expression, also increased the capacity of the same cells to produce IFNγ following oenothein B stimulation. Bovine calf PBMCs were stimulated with ConA and IL-2 for 4 days \textit{in vitro} to simulate TCR stimulation and promote differentiation into CD45RO+ cells. Control PBMCs were cultured without ConA or IL-2. After 4 days, cells were allowed to rest in the absence of ConA/IL-2 for 2 additional days. As expected, treatment with ConA/IL-2 resulted in an increased percentage of CD45RO+ cells compared to unstimulated cells (Figure 4.5A). However, when these cells were subsequently re-stimulated with oenothein B, there was no increase
Figure 4.3 Oenothein B induces more IFNγ production by bovine CD45RO+ T cells than CD45RO- T cells. Bovine PBMCs (10^5 cells/well) isolated from adult cows (n=2) were treated with the indicated concentrations of oenothein B or X-VIVO medium alone for 24 hrs, with brefeldin A added for the final 6 hrs. The percentage of CD3+ (A), CD45RO+ (B), or γδ TCR+ (C) cells producing IFNγ was measured by multi-color flow cytometry. The graphs represent pooled data. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance was determined by Two-way ANOVA with Bonferroni post-test. (D) Representative examples of two-color flow cytometry plots of PBMCs treated with 20 µg/ml oenothein B or medium alone. *p<0.05, **p<0.01, ***p<0.001
Figure 4.4 Oenothein B enhances IFNγ production by purified bovine CD4+ T cells from adults, but not calves. CD4+ T cells (A), γδ T cells (B), and all other (CD4- γδ TCR-) CD3+ T cells (C) were sorted from bovine adults (N=3) and calves (N=3), plated (10^4 cells/well), and treated with indicated concentrations of oenothein B or X-VIVO medium alone. After 24 hrs, soluble IFNγ were measured by ELISA. The graphs represent pooled data. Error bars indicate SEM. Each sample was analyzed in duplicate. Significance was determined by Two-way ANOVA with Bonferroni post-test. ***p<0.001
Figure 4.5 Enhancing CD45RO expression *in vitro* does not enhance oenothein B-induced IFNγ production by calf T cells. Bovine PBMCs (10^5 cells/well) from calves (<12 weeks old, N=3) were treated for 4 days with ConA (500 ng/ml) and IL-2 (1 ng/ml) or X-VIVO medium only. After 4 days, medium was replaced with X-VIVO only for an additional 2 days. PBMCs were then washed with X-VIVO and treated with the indicated concentrations of oenothein B in X-VIVO medium for 42 hrs with brefeldin A added for the final 18 hrs. (A) The percent PBMCs which were CD45RO+ was calculated in cells not treated with oenothein B. Significance was determined by Paired T test. (B) The percent of CD3+ T cells which are IFNγ + was measured by multi-color flow cytometry. The graph represents pooled data. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance was determined by Two-way ANOVA with Bonferroni post-test. **p<0.01
in the percentage of IFN\(\gamma\)-producing cells in the ConA/IL-2 cultured T cells compared to T cells cultured in the absence of ConA/IL-2 (Figure 4.5B). These data suggest that the increased frequency of CD45RO\(^+\) T cells alone is not sufficient to explain the increase in IFN\(\gamma\) production by adult T cells in response to oenothein B.

Oenothein B Enhances IFN\(\gamma\) Production by Human Adult T Cells, but not Cord Blood T Cells

To determine if the observations made with bovine lymphocytes also held true in human lymphocytes, the ability of oenothein B to stimulate human adult and cord blood T cells was tested. Oenothein B could activate cord blood T cells, as measured by increased expression of CD69, although the activation of cord blood T cells was not as robust as oenothein B-stimulated adult T cells (Figure 4.6). When human adult and cord blood PBMCs were stimulated with oenothein B, adult PBMCs, but not cord blood PBMCs, produced IFN\(\gamma\) (Figure 4.7A). Furthermore, oenothein B induced IFN\(\gamma\) production by adult, but not cord blood, T cells (Figure 4.7B and 4.7C). These data were consistent with our data comparing bovine calves and adults. In addition to IFN\(\gamma\), oenothein B induced GM-CSF production by adult human PBMCs and T cells, but not cord blood PBMCs and T cells (Figure 4.8). Altogether, these data suggested that, while oenothein B can activate both cord blood and adult T cells, oenothein B induces more IFN\(\gamma\) and GM-CSF production by T cells from human adult mononuclear cell cultures.
compared to neonate mononuclear cell cultures, supporting our observations in bovine lymphocytes.

Figure 4.6 Oenothein B induces CD69 expression on human cord blood T cells. Human blood mononuclear cells (5X10⁴ cells/well) isolated from cord blood (n=3) and sex-matched adult donors (n=3) were treated with the indicated concentrations of oenothein B or cRPMI medium only for 42 hrs and CD69 expression on CD3+ T cells was measured by multi-color flow cytometry. The graphs represent pooled data. Each treatment was analyzed in triplicate and error bars indicate SEM. Significance compared to untreated cells (medium) and between cord blood and adult samples was determined by Two-way ANOVA with Bonferroni post-test. **p<0.01, ***p<0.001
Figure 4.7 Oenothein B induces IFNγ expression on human adult, but not cord blood, T cells. (A) Human blood mononuclear cells (5X10^4 cells/well) from cord blood (n=3) and adult donors (n=3) were treated with the indicated concentrations of oenothein B or cRPMI medium alone for 24 hrs, and soluble IFNγ levels in supernatant fluids were measured by ELISA. The graph represents data from each sample, with each sample plated in triplicate. Statistical significance between adults and cord blood was measured by Two-way ANOVA with Bonferroni post-test. (B) Human blood mononuclear cells (5X10^4 cells/well) isolated from a cord blood and adult donor were treated with the indicated concentrations of oenothein B or X-VIVO medium alone for 24 hrs, with brefeldin A added for the final 6 hrs. The percent of CD3+ T cells expressing IFNγ was measured by multi-color flow cytometry. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance between cord blood and adult samples was determined by Two-way ANOVA with Bonferroni post-test. (C) Representative examples of two-color flow cytometry plots of human adult and cord blood PBMCs treated with 20 μg/ml oenothein B or medium alone. *p<0.05, **p<0.01, ***p<0.001
Oenothein B Induces More Cytokine Production by Human Memory T Cells Than Naive T Cells.

In the bovine experiments described earlier, CD45RO+ memory T cells produced more IFNγ than CD45RO- cells. To see if this was also true in humans, the ability of oenothein B to stimulate human CD45RO+ and CD45RO- T cells was compared. When human adult PBMCs were stimulated with oenothein B, CD69 expression was upregulated on both CD45RO+ and CD45RO- T cells, with greater expression on CD45RO- cells (Figure 4.9A).

In contrast to CD69, IFNγ and GM-CSF expression by T cells was enhanced in CD45RO+ T cells compared to CD45RO- cells (Figure 4.9B). To further confirm these human results, CD45RO+ and CD45RO- T cells were sorted from adult human donors. When these purified cells were stimulated with oenothein B, CD45RO+ T cells expressed more IFNγ and GM-CSF in response to direct stimulation by oenothein B than CD45RO- T cells (Figure 4.9C). These data suggested that, as in the cow, oenothein B promotes greater cytokine expression by CD45RO+ memory T cells, despite enhancing CD69 expression on both CD45RO+ and CD45RO- T cells. The sorted T cell data also suggest that oenothein B enhances greater cytokine production by CD45RO+ T cells compared to CD45RO- T cells even in the absence of macrophages and other non-T cell PBMCs.
Figure 4.8 Oenothein B induces GM-CSF expression on human adult, but not cord blood, T cells. (A) Human blood mononuclear cells (5X10⁴ cells/well) from cord blood (n=3) and adult donors (n=3) were treated with the indicated concentrations of oenothein B or cRPMI medium alone for 24 hrs, and soluble GM-CSF levels in supernatant fluids were measured by ELISA. The graph represents data from each sample, with each sample plated in triplicate. Statistical significance between adults and cord blood was measured by Two-way ANOVA with Bonferroni post-test. (B) Human blood mononuclear cells (5X10⁴ cells/well) isolated from cord blood (N=2) and adult (N=2) donors were treated with the indicated concentrations of oenothein B or cRPMI medium alone for 24 hrs, with brefeldin A added for the final 6 hrs. The percent of CD3+ T cells expressing GM-CSF was measured by multi-color flow cytometry. The graph represents pooled data. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance between cord blood and adult samples was determined by Two-way ANOVA with Bonferroni post-test. (C) Representative examples of two-color flow cytometry plots of human adult and cord blood PBMCs treated with 20 µg/ml oenothein B or medium alone. *p<0.05, **p<0.01, ***p<0.001
Altogether, these data are consistent with our bovine data, where activation of T cells, as measured by increased expression of an activation marker, does not always correlate with cytokine expression by oenothein B-stimulated T cells.

**Discussion**

Plant polyphenols have been studied extensively for numerous health benefits, including enhanced immune activity. Polyphenols isolated from several sources activate lymphocytes, including NK cells and T cells, promoting the production of various proteins, including IFN$_\gamma$.$^{32,179,224}$ The production of IFN$_\gamma$ by T cells is critical for immune defense against many viral, bacterial, and other infections.$^{60,205,206}$ It is also critical for defense against tumors.$^{207}$ Therefore, enhanced production of IFN$_\gamma$ by T cells may play an important role in the proposed ability for certain polyphenols and other food products to protect against infection and tumors. However, there is much that is still unknown about the immunostimulatory properties of polyphenols, including what factors may influence their activity.
Figure 4.9 Oenothein B enhances more cytokine production by human CD45RO+ memory T cells than naïve T cells. Human PBMCs (10^5 cells/well) isolated from adult donors (n=3) were treated with the indicated concentrations of oenothein B or cRPMI medium alone for 24 hrs, with brefeldin A added for the final 6 hrs. CD69 (A), as well as IFNγ and GM-CSF (B) expression on CD3+ T cells was measured by multi-color flow cytometry. The graphs represent pooled data from three individuals. Each treatment was analyzed in duplicate and error bars indicate SEM. Significance between CD45RO+ and CD45RO- CD3+ T cells was determined by Two-way ANOVA with Bonferroni post-test. (C) Human CD45RO+ and CD45RO- CD3+ T cells (10^5 cells/well) were sorted and treated with indicated concentrations of oenothein B or cRPMI medium alone. After 24 hrs, soluble IFNγ and GM-CSF were measured by ELISA. The graphs represent pooled data of soluble IFNγ levels in culture supernatant fluids from three separate experiments with different donors and soluble GM-CSF levels from two separate experiments with different donors. Error bars indicate SEM. Each sample was analyzed in triplicate. Significance between CD45RO+ and CD45RO- CD3+ T cells was determined by Two-way ANOVA with Bonferroni post-test. *p < 0.05, **p<0.01, ***p<0.001
One factor that appears to be important for stimulation of T cell cytokine production by polyphenols is the size of the polyphenol molecule. Work by Yamanaka and coworkers found that stimulation of murine splenocytes by polymerized polyphenols with large molecular weights, but not their corresponding monomers, enhances cytokine production, including IFN\(\gamma\) and GM-CSF, by T cells\(^{179}\). Furthermore, we have found that procyanidin oligomers, but not monomers, stimulate innate lymphocytes\(^{15}\). The importance of molecular size is consistent with the activity of oenothein B, as it is a large polyphenol and molecular subunits of this compound do not have the same immunostimulatory capabilities\(^{33}\). An additional factor may be the chemical modification of polyphenol structures, such as methylation. A recent study found that some methylated quercetin molecules, but not unmethylated quercetin, could enhance TLR2-induced IL-1 production by a monocyte cell line. Furthermore, not all methylated quercetin molecules enhanced IL-1 production, suggesting an exquisite level of chemical specificity in modulation of innate immune cell activity by polyphenols\(^{190}\). It will be interesting to determine if such chemical specificity also exists for immunostimulation of T cells and other lymphocytes by polyphenols.

In this study, we propose that age may be an important human and animal factor influencing the immunostimulatory properties of polyphenols. The immune system of both humans and animals changes significantly as individual's age from birth to adulthood to old age. Infants and young individuals have immature immune systems and
are more susceptible to a number of different diseases\textsuperscript{227, 228}. T cells and other lymphocytes change their behavior with aging\textsuperscript{70, 87, 170, 222}. Subset distribution, receptor expression, and other features of T cells differ between young individuals and adults, and their responsiveness to various agonists also changes\textsuperscript{105, 155, 169, 170, 222}. Our data suggest that T cell responsiveness to large-molecular weight polyphenols can also change with age.

Here, we show that T cells from both young and adult individuals are stimulated by oenothein B, but differ in their responses. $\gamma\delta$ T cells from calves express more IL-2R\textalpha in response to oenothein B than adult T cells, while $\alpha\beta$ T cells from calves and adults express similar levels of IL-2R\textalpha in response to oenothein B. This suggests that oenothein B may prime $\alpha\beta$ T cells from both young calves and adults equally to IL-2-induced proliferation, but may better prime $\gamma\delta$ T cells from young calves. We also show that T cells from both human and bovine adults secrete IFN\textgamma and GM-CSF in PBMCs treated with oenothein B. However, T cells from young individuals, either bovine calves or human cord blood, produce less of these cytokines in response to oenothein B. This is true for both $\alpha\beta$ and $\gamma\delta$ T cells. Altogether, these data suggest that the benefit of oenothein B, and perhaps other polyphenols, on T cell activity may change with age, possibly promoting enhanced T cell proliferation and expansion in young individuals while promoting enhanced cytokine production in older individuals.
In addition, we show that CD45RO+ T cells, in mixed cultures as well as sorted, produce more IFN\(\gamma\) and GM-CSF in response to oenothein B than CD45RO- T cells in both cows and humans. This could account, at least in part, for the enhanced cytokine secretion by adult T cells, as CD45RO+ memory T cells accumulate with age as a result of increased antigen exposure over time\(^{103}\). However, when we used ConA and IL-2 to increase the percentage of CD45RO+ cells in calf PBMCs, IFN\(\gamma\) production by these T cells in response to oenothein B was not significantly enhanced. Therefore, in addition to an increased percentage of antigen-experienced T cells in adults, other factors in the adult microenvironment may play an important role in enhancing cytokine production by adult T cells in response to oenothein B. This would be consistent with our data that shows that, even though CD45RO+ T cells express several times more cytokines in response to oenothein B than CD45RO- cells, CD45RO- T cells from adults appear to produce more IFN\(\gamma\) and GM-CSF in response to oenothein B than CD45RO- T cells from younger individuals. However, an alternative explanation for the enhanced cytokine production by adult CD45RO- T cells could be the loss of CD45RO expression on some activated T cells, as CD45RO expression is known to sometimes drift\(^{229}\).

The mechanism for the enhanced antigen-independent cytokine production of adult T cells compared to young T cells has yet to be determined. One possible explanation, as oenothein B is known to stimulate myeloid cells, would be enhanced IFN\(\gamma\) production by memory and adult T cells in response to myeloid cell-derived
cytokines, such as IL-12 and IL-18. However, our data with sorted T cells would suggest that oenothein B can directly enhance cytokine production by adult and memory T cells. Furthermore, our IL-2Rα and CD69 data suggest that naive T cells and T cells from young individuals are just as responsive to stimulation by oenothein B as adult and memory T cells, if not more so, so enhanced stimulation by oenothein B is not a likely explanation. Another possible explanation involves methylation at cytokine promoter sites in adult and memory T cells. Methylation at certain CpG sites within DNA are known to prevent gene transcription. Studies have found that reduced methylation at sites within the \textit{ifn}\textgamma\ promoter in memory T cells compared to naive T cells results in enhanced \textit{ifn}\textgamma\ transcription and cytokine production in memory T cells. Furthermore, inhibiting DNA methylation enhances IFN\textgamma\ production by naive T cells\textsuperscript{230}. In addition, another study found that CD45RO- CD4+ T cells from adult T cells had reduced methylation at the \textit{ifn}\textgamma\ promoter compared to cord blood T cells\textsuperscript{231}. It is possible that reduced methylation at cytokine promoter sites in adult T cells allow for enhanced IFN\textgamma\ and GM-CSF production by adult and memory T cells in response to oenothein B. However, this would need to be tested experimentally.

In addition to their presence in blood, CD45RO+ memory T cells migrate into a number of different tissues, including portals of entry into the body such as the lung and gut\textsuperscript{94}. This suggests that they may be prime targets for immunomodulatory food products. While mostly studied for their antigen-specific responses, memory T cells can also play
an important role in innate immunity through antigen-independent, bystander effects. These bystander effects include the early production of IFNγ, as well as the killing of infected cells by NKG2D-dependent mechanisms\textsuperscript{99-102}. Therefore, although they are typically associated with adaptive immunity, they can also be characterized as innate lymphocytes. Therefore, in addition to γδ T cells and NK cells, CD45RO+ memory T cells may be another innate-like lymphocyte population whose numbers and activity could be influenced by polyphenols and this should be taken into account when examining enhancing effects of polyphenols on innate immunity. Further studies are needed to determine what effect polyphenols have on CD45RO+ T cells and their activity in vivo, as well as the possible importance of CD45RO+ T cells for certain polyphenols to optimally enhance immunity to various pathogens and cancers. This could be an important aspect that has been largely overlooked in mouse models, as mice are typically housed in pathogen-free conditions and have low CD45RO+ memory T cell numbers compared to humans and cows of a similar maturity level.

Our data further emphasizes the utility of the bovine system to study dietary polyphenols effects on the immune system and, in particular, the influence of age on the impact of dietary polyphenols. Studies comparing very young and adult individuals \textit{in vivo} are difficult, as experiments on human children are rarely done for obvious ethical reasons and mice usually need to reach several weeks of age before they are large enough to handle and treat. In addition, pathogen-free housing and the inbred nature of laboratory
mice are significant variables when comparing results to outbred animals and humans. However, studies examining age-related changes of the bovine immune system are common\textsuperscript{86, 87, 89, 170}. Domestic cattle are also large enough to handle and feed at a very young age and are often used in diet-influenced immunity studies. In one study examining the effect of feeding polyphenol-rich pomegranate extracts to calves during the first 70 days of age, the authors found that feeding pomegranate extract enhanced PHA-induced IFN\textgreek{g} and IL-4 production by PBMCs\textsuperscript{30}. It would be of great interest to determine if T cells were the primary source of these cytokines and if using older animals would have affected these results. Furthermore, this study and others suggest that bovine lymphocytes are predictive for the influence of polyphenols on human lymphocytes\textsuperscript{31, 224}. Therefore, cattle may be a useful model for studying the role of age, particularly between very young individuals to adults, on the immunomodulatory properties of food products, such as polyphenols.

These data are the first to our knowledge to demonstrate differences in immune cell responsiveness to polyphenols with age. Further work is required to determine if our observations with oenothein B also holds true for other immunomodulating polyphenols, especially those which stimulate T cells. In addition, further work is required to determine if our observations also hold true \textit{in vivo}. These data has implications for optimizing the use of dietary polyphenols to enhance immunity in both humans and
animals and suggest that subject age should be taken into account when examining the
effect of consumption of polyphenol-rich supplements on immunity.

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CHAPTER FIVE

COMPLEX ROLE OF γδ T CELL-DERIVED CYTOKINES AND GROWTH FACTORS IN CANCER.

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: Andrew G. Ramstead

Contributions: Primary author of the manuscript.

Co-author: Mark A. Jutila

Contributions: Co-wrote and edited manuscript.
During our preliminary experiments with the B16 melanoma tumor model, we saw a reduction in the mean tumor weight in wild-type mice treated with oenothein B, but an increase in mean tumor weight in γδ T cell-deficient animals. These data suggested that γδ T cells may have an important role in the antitumor activity of oenothein B and that this compound had both anti- and protumor effects. Unfortunately, inconsistencies in our animal systems lead to inconsistencies in these results and an inability to fully test these hypotheses. A major inconsistency in our animal system was the observed activity of γδ T cells during B16 melanoma growth. In experiments conducted in 2010, saline-treated γδ-/- mice consistently had smaller tumors than saline-treated wild-type C57BL/6 mice (Figure 5.1A). This suggested that the γδ T cells in our model were pro-tumor and potentially inhibited tumor immunity. However, in experiments conducted in 2011, B16 tumors grew larger in untreated γδ-/- mice compared to wild-type mice (Figure 5.1B). Similar results were seen in 2012, when saline-treated γδ-/- mice consistently had larger tumors than saline-treated wild-type mice (Figure 5.1C). Therefore, in our 2011 and 2012 experiments, γδ T cells appeared to have antitumor activities, opposite of what was observed in 2010, but similar to what has been observed in the literature. Altogether, the γδ T cells in our mice appeared to shift from a protumor, regulatory phenotype to an antitumor, inflammatory phenotype. This variability in γδ T cell responses may have had a profound impact on the ability of oenothein B to influence tumor growth. This contradictory γδ T cell behavior in the context of tumor immunology has been observed
in other murine studies, as well as in the clinic. In this chapter, I present a published review of the literature examining the complex role of γδ T cells in cancer.

Figure 5.1 Changes in B16 melanoma growth in γδ T cell-deficient mice compared to wild-type mice over time. (A) In 2010, C57BL/6 (N=20) and γδ T cell-deficient (N=15) animals were injected with 5 X 10^5 B16 melanoma cells subcutaneously on day 0. Mice were injected i.p. on days -1, 4, 7, and 11 with saline. Mice were euthanized on day 16 and tumor weights were recorded. (B) In 2011, C57BL/6 (N=10) and γδ T cell-deficient (N=10) animals were injected with 1 X 10^6 B16 melanoma cells subcutaneously on day 0. Mice were euthanized on day 14 and tumor weights were recorded. (C) In 2012, C57BL/6 (N=4) and γδ T cell-deficient (N=3) animals were injected with 5 X 10^5 B16 melanoma cells subcutaneously on day 0. Mice were injected i.p. on days -1, 4, 7, and 11 with saline as described in part A. Mice were euthanized on day 16 and tumor weights were recorded. All mice were male and 8-14 weeks old. *** p < 0.001
Abstract

γδ T cells are innate lymphocytes, which recognize and kill a range of tumor cells and are currently being explored as a target for tumor immunotherapy. However, γδ T cells have a complex role in cancer and can promote, as well as inhibit, tumor growth. In addition to tumor cell killing, γδ T cells express a number of cytokines and other soluble factors in response to tumors. Soluble factors expressed by γδ T cells in these settings include IFNγ, TNFα, IL-4, IL-10, TGFβ, IL-17, and a number of growth factors. These factors have differing, sometimes opposing effects on anti-tumor immunity and tumor angiogenesis, and likely contribute to the complex role of these cells in cancer. Here, we review studies in both mice and humans examining differential cytokine secretion by γδ T cells in response to tumors and tumor immunotherapy, and discuss the influence of these γδ T cell-derived factors on tumor growth.

Introduction

Immunity has long been known to impact cancer development in a diverse manner. Effective immune surveillance of cancerous tumors can suppress tumor growth, but improper and/or prolonged immune activity can actually contribute to its initiation and progression\textsuperscript{232,233}. Immune responses at tumor sites are a balancing act of inflammatory and regulatory responses mediated by many different immune cells and cytokines, many of which display dual functionality by both promoting and inhibiting...
tumor growth. Among the immune cells, γδ T cells may be important during the establishment of the tumor microenvironment and development of tumor immunity.

Recently, the role of γδ T cells in tumor immunity has received considerable attention and research. γδ T cells from both humans and mice infiltrate tumor sites, lyse tumor cells, and prevent the growth of a variety of cancers. Tumor cell recognition by γδ T cells is largely mediated through the recognition of membrane-bound phosphoantigens, such as isopentenyl pyrophosphate (IPP), by the γδ T cell receptor (TCR) and/or the recognition of stress ligands on the tumor cell through the TCR and NKG2D. Due to their anti-tumor activity, therapeutic strategies aimed at harnessing and enhancing the anti-tumor properties of these cells have been developed and used in the clinic. These therapies often require IL-2 combined with synthetic phosphoantigens or bisphosphonates, such as zoledronate, which stimulate γδ T cells by enhancing cellular accumulation of IPP. While these therapies show potential, optimal results have not yet been achieved. Several recent reviews have examined the anti-tumor activity of γδ T cells and their potential for immunotherapy (Table 1).

Despite the evidence demonstrating anti-tumor responses by γδ T cells, the exact role these cells play in cancer is not entirely clear. In mice, the absence of γδ T cells sometimes leads to enhanced tumor growth, but in some cases leads to a reduction in tumor burden. In human patients, infiltration of γδ T cells into the tumor is
associated with better prognosis in some cancers\textsuperscript{250}, but not in others\textsuperscript{251}. These data suggest that, depending upon the tumor, \(\gamma\delta\) T cells can promote, inhibit, or possibly have no significant effect on tumor growth. These differential roles are likely mediated, at least in part, by the diverse repertoire of cytokines and other secreted factors that are induced in these cells, which can be categorized as either inflammatory or regulatory\textsuperscript{252}. A better understanding of the diverse roles of \(\gamma\delta\) T cells and their secreted factors in cancer should allow for better manipulation of these cells for immunotherapy. In this review, we will summarize the literature with regard to different cytokines and other secreted factors expressed by \(\gamma\delta\) T cells in response to tumors and examine how these factors could impact tumor immunity and immunotherapy.

\section*{\(\gamma\delta\) T Cell-associated Factors Which Enhance Anti-tumor Immunity}

\(\gamma\delta\) T cells are an important early source of the inflammatory cytokines IFN\(\gamma\) and TNF\(\alpha\) in many infections and other disease models\textsuperscript{253}. The expression of IFN\(\gamma\) and TNF\(\alpha\) by \(\gamma\delta\) T cells is promoted by numerous stimuli, including TCR agonists, ligands to NKG2D, and certain cytokines, such as IL-12 and IL-18\textsuperscript{130, 235, 254, 255}. IFN\(\gamma\) and TNF\(\alpha\) are also important cytokines in anti-tumor responses and inhibit tumor growth through several mechanisms, including the enhancement of anti-tumor immunity and inhibition of tumor angiogenesis\textsuperscript{256, 257, 258}. Human \(\gamma\delta\) T cells express IFN\(\gamma\) and TNF\(\alpha\) upon exposure
to tumor cell lines of numerous origins\textsuperscript{235,259,260}, suggesting these cytokines may play a role in $\gamma\delta$ T cell responses to tumors.

Table 5.1 Select recent reviews for $\gamma\delta$ T cells and cancer

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In mice, $\gamma\delta$ T cells appear to be an important early source of tumor-induced IFN$\gamma$ and the expression of IFN$\gamma$ is essential for optimal anti-tumor responses by these cells\textsuperscript{202,238}. The early production of IFN$\gamma$ by murine $\gamma\delta$ T cells can enhance MHCI expression on
tumors as well as enhance CD8+ T cell responses\textsuperscript{202, 261} suggesting γδ T cells could be important for augmenting downstream adaptive immune responses to tumors. These data suggest that early IFNγ secretion by γδ T cells is important for some anti-tumor responses in mice.

Results in humans suggest a far more complicated story in regard to the role of γδ T cell-derived IFNγ and TNFα in anti-tumor responses. In cancer patients, the expression of both IFNγ and TNFα by γδ T cells is modulated, but not always enhanced. Peripheral γδ T cells from breast cancer patients produce enhanced amounts of TNFα compared to healthy controls, which is thought to be beneficial\textsuperscript{262}. However, peripheral γδ T cells from patients with nasopharyngeal cancer and melanoma produce reduced amounts of IFNγ and TNFα, which could contribute to defective anti-tumor immune responses in these patients\textsuperscript{263, 264}. Upon the removal of melanoma, IFNγ and TNFα expression by γδ T cells is enhanced, suggesting that the reduced expression of these cytokines by γδ T cells is mediated by tumor-associated factors, which benefit the tumor\textsuperscript{265}. In support of this, mesenchymal stem cells, which are commonly found in tumor microenvironments, were shown to inhibit IFNγ and TNFα expression by peripheral γδ T cells through the production of prostaglandin E2, which was induced by γδ T cell-derived IFNγ and TNFα\textsuperscript{266}. In cancer patients undergoing immunotherapy with zoledronate and IL-2, serum levels of IFNγ increase after treatment\textsuperscript{267}. This increase in IFNγ expression by γδ T cells may be an important factor for successful γδ T cell immunotherapy, as clinical responses
to immunotherapy with zoledronate and IL-2 in one clinical trial correlated with increasing numbers of an effector memory γδ T cell phenotype, which could produce IFNγ\(^241\). However, in another clinical trial using infusions of zoledronate-activated γδ T cells in multiple myeloma patients, IFNγ was not believed to be important for the anti-tumor activity even though serum levels of IFNγ increased after treatment\(^268\). Collectively, these data suggest that the expression of IFNγ and TNFα is important in certain cancers for anti-tumor responses by γδ T cells, and that downregulation of γδ T cell-derived IFNγ and TNFα may help facilitate immune escape by tumors. However, further studies are needed to better determine their importance in human patients, particularly in response to immunotherapy.

**γδ T Cell-associated Factors Which Suppress Anti-tumor Immunity**

As mentioned above, γδ T cells may not always play a beneficial role in anti-tumor immunity. Instead, in some settings they likely have a regulatory role, suppressing anti-tumor responses and enhancing tumor growth. This response is not species-specific, in that immunosuppressive γδ T cells have been described in both mouse tumor models and human cancers\(^237, 248\). Furthermore, their activity appears to be at least partially mediated by certain cytokines.

In a study by Seo and coworkers\(^269\), murine γδ T cells infiltrating B16 melanoma tumors after 5 days were shown to inhibit NK and NKT cell activity and express large
amounts of IL-4 and IL-10, but not IFN\(\gamma\). The supernatant fluids from cultures of these cells did not reduce NK and NKT cell cytotoxicity, but did reduce their proliferation, suggesting that soluble IL-4 and IL-10 contributed to the inhibition of NK and NKT cell activity by \(\gamma\delta\) T cells in this model. Additional studies supported this observation and showed that \(\gamma\delta\) T cell-derived IL-4 and IL-10, as well as TGF\(\beta\), could inhibit anti-tumor immunity and promote tumor growth in mice. For example, using the B16 melanoma model, Hao and coworkers\textsuperscript{270} showed that the V\(\gamma\)1 subset of murine \(\gamma\delta\) T cells promoted tumor growth through the production of IL-4. These V\(\gamma\)1 \(\gamma\delta\) T cells reduced the expression of IFN\(\gamma\) and perforin within the tumor. Also, IL-4 inhibited the expression of NKG2D and perforin by V\(\gamma\)4 \(\gamma\delta\) T cells, which was important for the tumor-promoting activity of these V\(\gamma\)1 \(\gamma\delta\) T cells. Seo and coworkers\textsuperscript{248} showed that tumor-infiltrating \(\gamma\delta\) T cells from MM2 tumor lesions in mice expressed IL-10 and TGF\(\beta\), but not IFN\(\gamma\) or IL-4. \(\gamma\delta\) T cells isolated from the tumor lesions, as well as the spleens, of these MM2 tumor-bearing mice inhibited the cytotoxic activity of NK cells and CD8\(^+\) T cells. Neutralizing IL-10 and TGF\(\beta\) inhibited some of the suppressive effects of these \(\gamma\delta\) T cells, suggesting these cytokines participated in the suppressive activity of these cells. Depletion of these \(\gamma\delta\) T cells by the use of a specific antibody enhanced anti-tumor immunity and reduced tumor growth. Finally, a study by Ke and coworkers\textsuperscript{249} also described an immunosuppressive function for \(\gamma\delta\) T cells in tumor responses, as \(\gamma\delta\) T cells suppressed responses to an EL4 leukemia tumor cell line modified to express OVA, and IL-10
appeared to play a role in the suppression. Collectively, these data strongly suggest that at least certain subsets of murine γδ T cells can express IL-4, IL-10, and TGFβ in response to certain tumors, inhibiting anti-tumor immunity.

Immunosuppressive γδ T cells may also play an important role in human cancers. In a study by Peng and coworkers\textsuperscript{237}, the Vδ1 subset of tumor-infiltrating γδ T cells from human breast cancer could suppress DC maturation and T cell effector functions, which included proliferation, IL-2 secretion, and CD8+ T cell anti-tumor responses in a mouse xenograft model. This suppressive activity was mediated, at least in part, by a soluble factor or factors. The suppressive activity was present in isolated fractions with greater than 100 kDa molecular mass and could be inactivated by heat, but not DNAse or RNAse. However, the factors were not identified. When these cells were stimulated by tumor cells and anti-CD3 antibody, they expressed cytokines typically associated with pro-inflammatory responses, including IFNγ, GM-CSF, and IL-6, but not IL-1β, TNFα, IL-12, IL-2, IL-4, IL-10, or TGFβ. These Vδ1 γδ T cells made up a large percentage of tumor-infiltrating lymphocytes in breast and prostate cancer, suggesting that they may be important in promoting an immunosuppressive microenvironment in these cancers. However, Vδ1 γδ T cell-infiltration into necrotizing melanomas has correlated with increased survival\textsuperscript{250}, suggesting that the development of suppressive Vδ1 γδ T cells may be specific for certain cancers. Even though the suppressive effects of these cells were not mediated by IL-10 or TGFβ, these results resemble those found in mice by Seo and
coworkers\textsuperscript{248}, where infiltrating $\gamma\delta$ T cells suppressed the activity of CD8$^+$ T cells by secreted factors. Interestingly, stimulation of these suppressive breast cancer V$\delta$1 $\gamma\delta$ T cells by a TLR8 agonist could reverse the suppression of anti-tumor responses\textsuperscript{237}.

Even though human $\gamma\delta$ T cells may secrete different soluble factors than murine $\gamma\delta$ T cells, which suppress anti-tumor immunity, certain human peripheral $\gamma\delta$ T cells do express IL-4, IL-10, and TGFβ upon activation\textsuperscript{254, 271}. In one study, culture of human $\gamma\delta$ T cells with IPP or Daudi lymphoma cells \textit{in vitro} under Th2-polarizing conditions (rhIL-4, anti-IL-12) resulted in reduced IFN$\gamma$ and TNF$\alpha$ production and enhanced IL-4 production by these $\gamma\delta$ T cells\textsuperscript{254}. In the absence of these polarizing conditions, $\gamma\delta$ T cells primarily secreted IFN$\gamma$. Furthermore, a study by Gaafar and coworkers\textsuperscript{262} showed that while $\gamma\delta$ T cells from breast cancer patients produced very little IL-4, expansion of these cells by zoledronate and IL-2 led to increased production of IL-4 by these cells compared to expanded $\gamma\delta$ T cells from healthy controls. Therefore, IL-4, IL-10, and TGFβ production by human $\gamma\delta$ T cells may also play a role in suppressing anti-tumor responses, like they do in mice. However, additional studies are needed to confirm this possibility.

Collectively, the results summarized above support the idea that certain human $\gamma\delta$ T cells, at least in some cancers, can behave as regulatory cells within the tumor microenvironment and suppress anti-tumor responses and promote tumor growth, with secreted factors important for their activity.
In addition to their role in tumor responses, a renewed interest in \(\gamma\delta\) T cells has also emerged due to the discovery that \(\gamma\delta\) T cells are an important innate source of IL-17, particularly in the mouse. Secretion of IL-17 by murine and human \(\gamma\delta\) T cells is promoted by TCR and pattern recognition receptor (PRR) stimulation, along with the cytokines IL-1, IL-6, IL-23, and TGF\(\beta\) (272). Previous studies describing the role of IL-17 on tumor growth have had conflicting results, suggesting both pro-tumor and anti-tumor functions for this cytokine (273). Murine \(\gamma\delta\) T cells have been identified as a major source of IL-17 in several tumor models, which are summarized below.

In some studies, a detrimental role for \(\gamma\delta\) T cell-derived IL-17 in tumor responses has been suggested. Specifically, expression of IL-17 by tumor-infiltrating \(\gamma\delta\) T cells in a model of fibrosarcoma in Balb/c mice promoted tumor angiogenesis and, subsequently, enhanced tumor growth (274). Consistent with this, others have found that IL-17 enhanced the expression of vascular endothelial growth factor (VEGF), an important growth factor in angiogenesis (275). As such, the promotion of tumor angiogenesis may be an important and detrimental function of IL-17+ \(\gamma\delta\) T cells. Importantly, the local tumor microenvironment was important for the expression of IL-17 by these \(\gamma\delta\) T cells, as cells from the tumor tissue had enhanced IL-17 production compared to normal skin and cells from the spleen and draining lymph nodes of tumor-bearing mice did not increase IL-17 production. Furthermore, IL-6, TGF\(\beta\), and IL-23 were all involved in the promotion of
IL-17 by these γδ T cells. Another study examining lung metastasis showed that the expression of IL-17 enhanced metastasis and reduced survival in experiments involving the Lewis lung carcinoma model\textsuperscript{276}. In these experiments, IL-17 was primarily produced by γδ T cells and the secretion of IL-17 by γδ T cells was induced by IL-1. Enhanced tumor growth in the lung induced by IL-17 may have been mediated by the reduced potential of antigen presenting cells to promote Th1 immunity. However, based upon the study by Wakita and coworkers\textsuperscript{274}, angiogenesis may also have played a role. These data suggest that IL-17 production by γδ T cells clearly promotes tumor growth in some settings.

However, other studies in opposition to the results described above demonstrate a beneficial role for IL-17+ γδ T cells in the inhibition of tumor growth. In a mouse model of bladder cancer, treatment with \textit{Mycobacterium bovis} BCG enhanced IL-17 expression by γδ T cells, which was essential for optimal neutrophil recruitment into the tumor and a reduction in tumor growth\textsuperscript{277}. In another study with a number of different tumor models, the early infiltration of IL-17-producing γδ T cells into the tumor bed of chemotherapy-treated tumors was associated with the subsequent infiltration of IFNγ-producing CD8+ T cells and the suppression of tumor growth\textsuperscript{278}. In these experiments, both IL-17 and IFNγ were necessary for the inhibition of tumor growth. Based on these results, it has been proposed that immunotherapy aimed at polarizing γδ T cells to express IL-17 might be useful in enhancing the efficacy of chemotherapy\textsuperscript{240}. Interestingly, in both studies where
anti-tumor immunity was enhanced by γδ T cell-derived IL-17, other cells played an important role for the beneficial response. In the bladder cancer study, neutrophils were important, whereas in the chemotherapy study, IFNγ-secreting CD8+ T cells were important. Therefore, it is possible that in the absence of these other responses, IL-17 production by γδ T cells could lose its benefit and, therefore, enhance tumor growth as described above. Further studies are needed to better clarify the role of γδ T cell-derived IL-17 on tumor growth and determine if γδ T cell production of IL-17 has relevance to human cancers.

Potentially Underappreciated Role of γδ T Cell-derived Growth Factors in Tumor Immunity

Tumors have been described as wounds that do not heal, and numerous growth factors, including keratinocyte growth factor (KGF), play a role in their progression. In addition to pro- and anti-inflammatory cytokines, γδ T cells are a source of a number of growth factors. This has been well defined in the mouse, where skin-associated γδ T cells are a major source of KGF and are essential for optimal wound healing. In humans, γδ T cells produce transcripts and/or proteins for a number of growth factors, including KGF, IGF-1, EGF, FGF-9, ANG, PDGF, and VEGF. Furthermore, in human peripheral Vδ2 γδ T cells, the expression of FGF-9 is enhanced by IPP. As such, the expression of growth factors by tumor-infiltrating γδ T cells could potentially represent a significant response that promotes tumor growth in some settings.
Expression of Growth Factors in Human γδ T Cells In a study by Shilbach and coworkers\textsuperscript{154}, human Vδ2 and Vδ1 T cells were expanded and found to produce a number of growth factors, including IGF-1, EGF, PDGF, ANG, and VEGF. When these cells were cultured with a neuroblastoma cell line, the Vδ1 cells produced reduced amounts of these growth factors while Vδ2 cells produced slightly increased amounts. These data prompted the authors to suggest that Vδ1 γδ T cells may be better at promoting anti-tumor responses to this type of tumor, partially due to their reduced expression of growth factors. The expression of VEGF by γδ T cells, particularly in response to a tumor cell, is intriguing as VEGF is vital for tumor angiogenesis, growth, and metastasis\textsuperscript{280}. In addition to direct VEGF expression by γδ T cells, KGF and FGF-9 are capable of promoting VEGF expression in other cells in a paracrine manner\textsuperscript{281,282}. Therefore, γδ T cells may also stimulate VEGF expression indirectly by the expression of other growth factors. These data suggest that γδ T cells may participate in the production of growth factors within the tumor microenvironment, functions that have not yet been attributed to γδ T cells.

A recent clinical study examining the treatment of patients with zoledronate and IL-2 observed an increase in VEGF levels in these patients in addition to an expansion of γδ T cells and other immune cells\textsuperscript{267}, supporting the possible role for γδ T cell-derived growth factors in human cancer. Interestingly, the increase in VEGF was more pronounced in patients with solid tumors compared to those with leukemia. It is unknown
whether \( \gamma \delta \) T cells played a direct role in this increase of VEGF production. However, these data would be consistent with the previously discussed studies demonstrating that activated \( \gamma \delta \) T cells express VEGF, as well as factors which can indirectly promote the expression of VEGF. Importantly, elevated VEGF levels in these patients correlated with a lack of success of the therapy. Even if \( \gamma \delta \) T cells were not important for this enhanced VEGF expression, it appears to be an important obstacle to overcome in optimizing \( \gamma \delta \) T cell immunotherapy. Further studies are warranted to determine if \( \gamma \delta \) T cells are an important source of tumor-promoting growth factors in mice or humans.

Influences on Differential Cytokine Secretion by \( \gamma \delta \) T Cells in Tumor Studies

Differential cytokine production and behavior by \( \gamma \delta \) T cells clearly is an important variable in mouse studies examining the role of \( \gamma \delta \) T cells in cancer, but there are important caveats to consider in defining these roles. Differences in mouse strain, age, and other factors (source, housing, etc.) in these studies may influence \( \gamma \delta \) T cell cytokine secretion and subset distribution, which could influence the effect of \( \gamma \delta \) T cells on tumor growth in these experiments. For example, a study on West Nile Virus demonstrated that the numbers and behavior of \( V\gamma 1 \) and \( V\gamma 4 \) \( \gamma \delta \) T cells in mice could vary with age\(^{283} \). In addition, epidermal \( \gamma \delta \) T cells from Balb/c mice were shown to produce less IFN\( \gamma \) in response to IL-12 and IL-18 than those from C57BL/6 mice\(^{284} \). Therefore, in mouse studies examining the role of \( \gamma \delta \) T cells in cancer, it is likely important to further examine
γδ T cell responses and subsets within the specific mice used for the study in the absence of tumor cells, as variations in these factors would likely lead to variable tumor responses by the γδ T cells.

Conclusions

In response to tumor cells, γδ T cells produce a variety of cytokines that both inhibit and enhance anti-tumor immune responses, which likely accounts for some of the conflicting reports about the role of these cells in anti-tumor immunity (Figure 5.2). Amongst these cytokines, IFNγ, and possibly TNFα, contribute to the ability of γδ T cells to inhibit tumor growth. In contrast, the expression of IL-4, IL-10, TGFβ, other unknown factors, and possibly growth factors, by γδ T cells suppress anti-tumor immunity and enhance tumor growth. The expression of IL-17 by γδ T cells appears to have conflicting effects on tumor growth, which could be dependent upon the type of cancer or other factors, such as tumor-infiltration of other cell types or the use of chemotherapy. The expression of these cytokines by γδ T cells influences downstream adaptive immune responses to tumors, consistent with the described ability of γδ T cells to link innate and adaptive immunity. γδ T cell-derived IFNγ and IL-17 enhance CD8+ T cell responses, while IL-10, TGFβ, and other γδ T cell-derived soluble factors inhibit them. Therefore, in addition to their lytic activity, several studies suggest that the influence of γδ T cells on
adaptive immune responses to tumors is an important part of their role in anti-tumor immunity.

Differential cytokine production by $\gamma\delta$ T cells may also be important in $\gamma\delta$ T cell immunotherapy. Stimulation of $\gamma\delta$ T cells with synthetic phosphoantigens or bisphosphonates may only enhance $\gamma\delta$ T cell-responses already influenced by the tumor environment, beneficial or not, which could account for the variable effectiveness of these therapies. Therefore, the identification of therapeutic options which enhance and favor the production of beneficial anti-tumor cytokines and soluble factors by $\gamma\delta$ T cells, while minimizing or removing detrimental factors, may be key to unlocking the maximum potential of $\gamma\delta$ T cell immunotherapy. A good example of this concept can be found in the study by Peng and coworkers, where they were able to reverse the immunosuppressive phenotype of tumor-infiltrating $\gamma\delta$ T cells by stimulating them with a TLR8 agonist. Other options may include the use of additional cytokines to further enhance the anti-tumor activity of $\gamma\delta$ T cells. For example, the addition of IL-18 to zoledronate and IL-2 enhances IFN$\gamma$ and TNF$\alpha$ expression by $\gamma\delta$ T cells compared to zoledronate and IL-2 alone. The use of anti-VEGF and other anti-angiogenesis therapies may inhibit any pro-angiogenesis responses induced by $\gamma\delta$ T cells or $\gamma\delta$ T cell immunotherapy. Furthermore, chemotherapy might also have the potential to enhance the effectiveness of $\gamma\delta$ T cell immunotherapy, as discussed by Hannani and coworkers. In conclusion, in order to better understand the complex role of $\gamma\delta$ T cells in cancer and
improve the effectiveness of γδ T cell immunotherapy, additional studies are needed that examine the cytokine profiles of γδ T cells in response to tumors and immunotherapy, as well as identify ways to best manipulate this profile for the benefit of the patient.

Figure 5.2 Summary of the influence of γδ T cell-derived cytokines and growth factors on tumor growth.
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Author Disclosure Statement

No competing financial interests exist.
Supplements containing plant polyphenols are consumed by many people to improve health, with various benefits attributed to these compounds. It is important that the proposed benefits of these molecules be studied for proof of benefit, as well as for optimization of that benefit. Therefore, many studies have examined the effects of polyphenols in more detail, but there is still much that is currently unknown.

In this dissertation, the immunomodulatory polyphenol oenothein B was found to stimulate lymphocytes, including NK cells, γδ T cells, and αβ T cells, inducing the expression of IL-2Rα, CD69, and IFNγ (Chapter 3). Oenothein B has been described to have both pro- and anti-inflammatory effects on macrophages and neutrophils, but this is the first study to examine its influence on lymphocytes. Interestingly, oenothein B primed NK cells from both cattle and humans, in both mixed PBMC and purified cell cultures, to produce enhanced IFNγ in response to a cytokine, IL-18, or a tumor target cell. This priming effect on bovine and human NK cells by oenothein B does not appear to be a common property of polyphenols, as similar doses of several other commonly studied polyphenols did not affect IFNγ production by bovine NK cells in response to IL-18 (Chapter 3).
In addition to NK cells, T cells, both αβ and γδ, produced IFNγ in response to stimulation with oenothein B (Chapters 3 and 4). Aging impacted oenothein B-induced IFNγ production by T cells, as αβ and γδ T cells from bovine calves produced little IFNγ in response to oenothein B, but αβ and γδ T cells from bovine adults produced significantly more IFNγ than calf T cells upon treatment with oenothein B (Chapter 4, Table 6.1). Similar results were seen in human PBMCs, as adult αβ and γδ T cells, but not cord blood cells, produced IFNγ, as well as GM-CSF. In both cows and humans, the CD45RO+ T cell population produced more cytokines in response to oenothein B than CD45RO- T cells. This increase in cytokine production does not appear to be simply related to oenothein B’s ability to stimulate these cells, as oenothein B upregulated IL-2Rα and CD69 expression on T cells from both young and old individuals, as well as both CD45RO+ and CD45RO- T cells (Chapter 4). These results could have important implications for in vivo murine studies, as laboratory mice are often kept in specific-pathogen free conditions and used at a young age, resulting in a relatively low number of CD45RO+ memory T cells. If these cells are major cytokine-producing cells in response to oenothein B in adults, this could potentially lead to differential effects on adult immunity by oenothein B in mice compared to humans and cattle.

Like T cells, NK cell responses to oenothein B may also be impacted by aging, as IFNγ production by NK cells in response to oenothein B appeared to be reduced in bovine adults (Chapter 4, Table 6.1). IFNγ production in response to oenothein B was
Table 6.1 Summary of oenothein B-induced IFNγ production by lymphocytes

<table>
<thead>
<tr>
<th>Induction of IFNγ Production by Oenothein B</th>
<th>Cattle</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NK cells</td>
<td>αβ T cells</td>
</tr>
<tr>
<td>Adult Calves</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>↑IFNγ</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

dominated by NK cells in bovine calves, but was dominated by T cells in bovine and human adults. Interestingly, these results are similar to what has been observed for IFNγ responses in response to BCG vaccination in humans. Altogether, these data suggest that a polyphenol's ability to enhance innate immunity and protection from disease could be different for children and adults. For NK cell-mediated protection, oenothein B may be more effective in younger individuals, while for T cell-mediated protection, especially if cytokine production is important, oenothein B may be more effective in adults.

The production of IFNγ by NK cells and T cells is known to be important in these cells' ability to limit tumor growth, through the enhancement of cellular cytotoxicity, the reduction of tumor angiogenesis, and other mechanisms (Chapter 5). Therefore, these data suggest a new potential mechanism of action for the proposed antitumor effects of oenothein B. They also suggest a potential benefit for oenothein B to counter infectious
disease, as IFNγ production by NK cells and T cells are critical for the clearance of numerous pathogens (Chapter 2).

In addition to age and the presence of CD45RO+ T cells, the phenotypic state of γδ T cells may also influence the effects of oenothein B on innate immunity *in vivo*. Oenothein B may have been effective in our early B16 melanoma experiments because γδ T cells were of a more regulatory phenotype, as indicated by reduced tumor growth in saline-treated γδ-/- mice. However, when γδ T cells were of a more inflammatory phenotype in later studies, as indicated by enhanced tumor growth in γδ-/- mice, this benefit may have been lost (Chapter 5). It is possible that oenothein B has a similar activity as TLR8 agonists[^237], which can convert regulatory γδ T cells to more inflammatory cells. This hypothesis would be consistent with the loss of benefit in γδ-/- mice, as well as in later studies when γδ T cells did not need to be converted to an inflammatory phenotype.

To our knowledge, the influence of age on the immunomodulatory effects of polyphenols had not been previously studied, especially comparing children to adults. While these types of studies may be difficult to perform in humans (ethical reasons), as well as mice (difficult to use when very young), our data suggest that domestic cattle may be a very useful model in studying the influence of age on the enhancement of immunity by dietary polyphenols. These data has implications for optimizing the use of dietary polyphenols in both humans and animals and suggest that subject age should be taken
into account when examining the effect of consumption of polyphenol-rich supplements on immunity.
CHAPTER SEVEN

FUTURE STUDIES

While these data provide some insight into the effects of oenothein B on innate lymphocytes and the influence of age on these effects, there is still much that needs to be explored. For example, it is currently unknown why CD45RO+ T cells and T cells from adults produce more IFNγ and GM-CSF in response to oenothein B. As discussed in chapter 4, one possible explanation could involve methylation at cytokine promoter sites, as ifnγ promoter sites in adult and memory T cells have been shown to have reduced methylation compared to young and naïve T cells. As methylation at promoter sites is known to inhibit gene expression, T cells with reduced methylation at the promoter sites may be more capable of producing IFNγ and GM-CSF in response to oenothein B. This could be tested by the treatment of T cells from young individuals with 5-azacytidine, as 5-azacytidine is incorporated into the DNA of proliferating cells and prevents methylation. Therefore, co-treatment with 5-azacytidine could potentially enhance IFNγ and GM-CSF production by T cells which normally do not produce these cytokines in response to oenothein B. Preliminary experiments have currently not shown enhanced IFNγ production by bovine calf T cells treated with 5-azacytidine in response to oenothein B (Figure 7.1). Therefore, it is possible that reduced methylation is not sufficient to explain the enhanced IFNγ production by adult T cells.
Figure 7.1 5-Azacytidine does not enhance IFNγ production by oenothein B-stimulated bovine calves. αβ T cells (CD3+ γδ TCR-) (A) and γδ T cells (CD3+ γδ TCR+) (B) were FACS sorted from bovine calves (N=2), plated (4 X 10⁴ cells/well), and treated with indicated concentrations of oenothein B or X-VIVO medium alone, with or without the indicated concentrations of 5-Azacytidine. After 72 hrs, soluble IFNγ were measured by ELISA. The graphs represent pooled data. Error bars indicate SEM. Each sample was analyzed in duplicate.
However, additional experiments and experimental controls are needed to confirm these preliminary results.

In addition, studies in this dissertation primarily focus on αβ T cells as a single population, and do not examine potential differences between the CD4+ and CD8+ T cell subpopulations in detail. Furthermore, the relative contribution of these T cell subsets, as well as γδ T cells, to the overall production of IFNγ and GM-CSF by adult PBMCs should be examined. This could be done by depleting CD4+, CD8+, or γδ T cells from adult human and bovine PBMCs and comparing oenothein B-induced cytokine production by PBMCs lacking these populations to whole PBMCs by ELISA. These experiments could then determine which T cell subset was the most important for oenothein B-induced cytokine production in adult PBMCs. It is likely that different subsets of T cells will have different contributions in humans and cattle, as these species have different compositions of these subsets.

Data in chapter 3 suggested that the production of IFNγ by bovine calf NK cells could be enhanced by the presence of other mononuclear cells which did not produce significant IFNγ themselves, such as monocytes and, possibly, γδ T cells. Another study also demonstrated an important role for antigen presenting cells for cytokine production by T cells in response to polymerized polyphenols. Therefore, although purified NK cells and T cells were shown in these studies to produce IFNγ in response to oenothein B in the absence of other cells, the potential for other mononuclear cells to enhance
cytokine production by NK cells and T cells should be examined. This could be examined by adding different FACS sorted mononuclear cell subsets to sorted NK cells and T cells, looking for synergy in oenothein B-induced IFNγ production between different cells. Furthermore, the importance of cell-to-cell contact or the secretion of soluble mediators for this synergy could then be tested with transwell experiments.

In chapter 3, it was shown that oenothein B enhanced IFNγ production by human NK cells in response to a tumor cell line, the K562 cell line. It was also hypothesized that oenothein B could enhance tumor cell killing by NK cells. However, preliminary experiments with K562 cells using the CD107a mobilization assay were inconclusive, with some doses of oenothein B reducing CD107a mobilization on NK cells and another enhancing it (Figure 7.2). As a result, it was decided to focus on the IFNγ response for these studies. However, it is still possible that oenothein B enhances NK cell killing of tumor cells. It is possible that K562 was not the ideal cell type to perform these experiments due to its high susceptibility to NK cell killing, with more than 60% of NK cells expressing CD107a on their surface in response to K562 cells in the absence of oenothein B. Therefore, this experiment should be repeated using a broad range of tumor cell lines with varying susceptibility to NK cell killing to determine the effect of stimulation with oenothein B on NK cell-mediated killing of tumor cells.
Figure 7.2 Influence of oenothein B on NK cell degranulation in response to a tumor cell line. Human PBMCs (N=1) (10^5 cells/well) were treated with oenothein B or cRPMI medium alone for 48 hrs. Cells were then washed and co-cultured for 4 hrs with or without K562 cells at a 1:1 effector:target (E:T) ratio in cRPMI. CD107a expression on the surface of NK cells (CD3^- CD56+) was then determined by flow cytometry. Each sample was analyzed in triplicate. Statistical significance was determined by Two-way ANOVA with Bonferroni post-test. **p<0.01 ***p<0.001

Finally, there is currently no evidence to suggest that oenothein B enhances IFNγ production in vivo, as it does in vitro. However, preliminary experiments suggest that treatment with oenothein B can limit B16 melanoma growth, as well as reduce morbidity to influenza A infection (Chapter 2). Future experiments are needed to first confirm oenothein B’s ability to counter tumor growth and infectious disease in vivo. Experiments should then determine if NK cells and T cells, as well as IFNγ, are critical for oenothein B’s ability to counter tumor growth and infectious disease. One study found that
treatment with Acai berry polysaccharides enhanced protection to pulmonary infection by intracellular bacteria and that this enhanced protection was dependent upon the enhanced production of IFN$\gamma$ by NK cells and $\gamma\delta$ T cells$^6$. It is possible that oenothein B has similar activity. In addition, these studies should also determine if oenothein B’s ability to counter tumor growth and infectious disease in vivo is impacted by age or the presence of CD45RO+ T cells, as suggested by these in vitro studies.

However, it is also possible that the mouse may not be the ideal model to test the in vivo activity of oenothein B. Unlike human and bovine PBMCs, oenothein B induced little IFN$\gamma$ production by murine splenocytes in vitro, either alone or in combination with IL-18 (Figure 7.3). In contrast, it appeared to inhibit IL-18-induced IFN$\gamma$ production. Therefore, it is possible that oenothein B does not have the same immunomodulatory properties with mice as it does with humans and cattle. Therefore, as described previously, domestic cattle may be a better in vivo animal model. Cattle have already been shown to be an effective model for testing the in vivo immunomodulatory effects of polyphenols, as feeding bovine calves polyphenols from pomegranate was found to enhance mitogen-induced IFN$\gamma$ production by PBMCs$^{30}$. Similar experiments could be performed which administer oenothein B, or oenothein B-containing plants, to calves and/or adult cows. PBMC responses to IL-18, tumor cells, and other agonists could then be compared between oenothein B-treated and untreated animals.
Figure 7.3 Oenothein B does not prime murine splenocytes to produce IFNγ in response to IL-18. Spleens from 6 week-old (A) or 13 week-old (B) male human TL4 mice were homogenized and mononuclear cells were isolated by Lympholyte M gradient. Splenocytes (10^5 cells/well) were then treated with indicated concentrations of oenothein B or X-VIVO medium only. After 24 hrs, cells were washed and treated with indicated concentrations of recombinant mouse IL-18 or X-VIVO medium only. After 24 hrs, soluble IFNγ levels in culture supernatant fluids were determined by ELISA. The graph represents results from one experiment. Error bars indicate SEM. Each sample was analyzed in triplicate. Significance compared to 0 µg/ml oenothein B samples was determined by Two-way ANOVA with Bonferroni post-test. **p<0.01, ***p<0.001
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