Published in final edited form as:

Int Immunopharmacol. 2015 June ; 26(2): 367–377. doi:10.1016/j.intimp.2015.04.008.

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

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Abstract

Several plant extracts, including certain polyphenols, prime innate lymphocytes and enhance responses to secondary stimuli. Oenothlein B, a polyphenol isolated from *Epilobium angustifolium* and other plant sources, enhances IFN γ production by both bovine and human NK cells and T cells, alone and in response to secondary stimulation by cytokines or tumor cells. 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 oenothlein B responsiveness in T cells from cord blood, young, and adult donors. We found that oenothlein B stimulates bovine and human T cells from individuals over a broad range of ages, as measured by increased IL-2R α and CD69 expression. However, clear differences in induction of cytokine production by T cells were seen. In T cells from human cord blood and bovine calves, oenothlein B was unable to induce IFN γ production. However, oenothlein B induced IFN γ production by T cells from adult humans and cattle. In addition, oenothlein 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 oenothlein B than CD45RO[–] T cells. In summary, our data suggest that the immunostimulation of T cells by oenothlein B is influenced by age, particularly with respect to immune cytokine production.

Keywords

bovine; human; memory T cells; GM-CSF; interferon- γ ; polyphenol

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1. 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 [1–4].

Lymphocytes, such as NK cells and T cells, which play an important role in host defense against tumors and various pathogens, are stimulated by select polyphenols. For example, polyphenols from apples, cocoa, and other sources activate and enhance the proliferation and cytokine production of $\gamma\delta$ T cells and NK cells [3, 5–8]. 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 [3, 7, 8].

The production of interferon- γ (IFN γ) by lymphocytes is important during immune defense against tumors and a large number of viral, bacterial, and fungal infections [9–12].

Previously, we found that oenothien B, a dimeric, macrocyclic ellagitannin isolated from *Epilobium angustifolium*, enhanced IFN γ production by both NK cells and T cells from bovine calves and humans, either alone or in combination with additional stimuli [8]. In the previous study [8], the majority of IFN γ -producing cells in bovine samples were CD335 (NKp46)-expressing NK cells. Additionally, we found that oenothien B enhanced IFN γ production by NK cells in human samples. However, in contrast to the bovine data, a non-NK cell population, specifically T cells (both $\gamma\delta$ T cells and $\alpha\beta$ T cells), produced IFN γ in response to stimulation by oenothien B in human samples. Furthermore, this oenothien B-responsive T cell population made up the majority of IFN γ -producing cells in these human samples [8]. In the previous study, we did not investigate the bases for the difference between these two systems but hypothesized that age was likely a contributing factor. Specifically, the human donors in this study were all mature adults (at least 18 years of age), whereas the bovine donors were young animals (less than 6 months of age). While numerous studies find age-related differences in the response of immune cells to various agonists, the influence of age on the immunostimulatory activity of plant-derived polyphenols has not been well studied [13–17]. This could have important health implications, as individuals with a broad age range use polyphenol-containing supplements to support health and immunity, and currently, it is not clear whether these compounds are more or less effective for certain age groups.

Therefore, in the present work, we examined the effect of age on the immunostimulation of T cells by oenothien B in both humans and cattle. We show that oenothien B stimulates human and bovine T cells from neonate, young, and adult donors, but that the production of certain cytokines by T cells in response to oenothien B is enhanced only in adults.

Furthermore, we find that CD45RO⁺ T cells isolated from cattle and humans produce more IFN γ , as well as granulocyte macrophage colony-stimulating factor (GM-CSF), in response to oenothien B than CD45RO[–] cells. These data indicate that the role of T cells in the modulation of immunity by oenothien B may be influenced by age.

2. Materials and methods

2.1. 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 the parents of cord blood donors.

2.2. Isolation of oenothien B

Oenothien B was isolated and identified as described previously [4]. Briefly, fully blossomed *E. angustifolium* was 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- μ m filter. The filtrate was lyophilized to obtain the crude extract. The crude extract was re-dissolved and fractionated on a Sephadex LH-20 column (2.8 $\times$ 33 cm) using 80% methanol as an eluent. Fractions were collected, pooled based on elution profile (absorbance at 270 nm), evaporated to dryness, and re-chromatographed twice. The appropriate fractions for collection and pooling were identified as previously described [4]. The identity of the purified compound was confirmed by NMR and mass spectrometry, as described [4]. Purity was determined to be >95% by HPLC and mass spectrometry. A *Limulus* amoebocyte lysate assay kit (Cambrex, East Rutherford, NJ, USA) was used to evaluate possible endotoxin contamination in purified oenothien B. Purified, endotoxin-free oenothien B was resuspended in Dulbecco's PBS and stored at -80° C until use in the functional assays described below.

2.3. Human and Bovine Peripheral Blood Mononuclear Cell (PBMC) Preparations

Whole blood was collected from Holstein bull calves (< 12 weeks-old), adult (> 2 years-old) Holstein cows, and adult (4- to- 7 years-old) Angus and Angus X Hereford cows. All bovine blood was collected into sodium heparin tubes (BD Biosciences, San Jose, CA, USA). Whole blood from healthy human adult donors was collected in ACD solution A anticoagulant tubes (BD Biosciences). Human 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, USA) for bovine and human cells, as previously described [3] and per the manufacturer's instructions. Additionally, red blood cells were removed by hypotonic lysis after Histopaque separation.

2.4. Cell Sorting of PBMCs

Human CD45RO⁺ and CD45RO⁻ T cells were isolated by staining cell preparations with monoclonal antibodies (mAbs) against CD3 (UCHT1, Biolegend, San Diego, CA, USA) and CD45RO (UCHL1, eBioscience, San Diego, CA, USA) 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 (Sigma-Aldrich) medium 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, Pullman, WA, USA), CD4 (CC30), and $\gamma\delta$ TCR (GD3.8 [18]) and sorting using a FACSARIA cell sorter to achieve >98% purity. After sorting, bovine cells were incubated overnight in cRPMI medium with 10% FBS at 37° C and 10% CO₂ before being used in the experiments described below.

2.5. T Cell Activation Assays

To measure IL-2R α or CD69 expression, bovine and human blood mononuclear cells were isolated and incubated in cRPMI or X-VIVO 15 (Lonza, Walkersville, MD, USA) medium at 37° C and 10% CO₂ in the presence of oenothien B (0, 15, 20, 25, or 40 μ g/ml) or medium only for approximately 24 or 42 hrs. IL-2R α and CD69 expression were then analyzed by flow cytometry. Brefeldin A (eBioscience) was added to some of the cultures to quantify IFN γ and GM-CSF expression by flow cytometry.

To measure the secretion of IFN γ and GM-CSF, 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 oenothien B (0, 20, or 40 μ g/ml) or medium only for approximately 24 or 48 hrs. Supernatant fluids were then collected for analysis by ELISA.

To promote differentiation into CD45RO⁺ T cells, bovine PBMCs were cultured with 500 ng/ml Concanavalin A (ConA) (Sigma-Aldrich) and 1 ng/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ, USA) 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 oenothien B.

2.6. Flow Cytometric Analysis of Bovine and/or Human PBMCs

Bovine cells were stained with mAbs against surface molecules IL-2R α (LCTB2A, VMRD), CD3 (MM1A, Washington State University and VMRD), CD45RO (GC42A1, VMRD), CD335 (AKS1, AbDSerotec Inc., Raleigh, NC, USA), and $\gamma\delta$ TCR (GD3.8). Human cells were stained with mAbs against CD69 (FN50, Biolegend), CD3 (UCHT1, Biolegend), CD45RO (UCHL1, eBioscience), and $\gamma\delta$ 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, USA) 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). 75–80 μ l/well samples at a cellular concentration of 1 X 10⁶/ml or 5 X 10⁵/ml were collected by the HTS sampler for analysis.

2.7. IFN γ and GM-CSF Assays

Enzyme-linked immunosorbent assays (ELISA) were used to measure IFN γ and GM-CSF in cell supernatant fluids. A bovine IFN γ kit (MABTECH, Cincinnati, OH, USA), a human IFN γ kit (MABTECH), and a human GM-CSF kit (Biolegend ELISA Max) were used to perform ELISAs, according to the manufacturer's instructions. All samples were assayed in duplicate or triplicate.

To assay IFN γ 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-Aldrich) in PBS + 2% horse serum. Bovine IFN γ was detected using a PE-conjugated mouse IgG1 mAb against bovine IFN γ (MCA1783E, AbD Serotec Inc.). Human IFN γ 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 γ 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.

2.8. Statistical Analysis

Statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA, USA). The data were analyzed by Student's paired t-test or Two-way ANOVA, as indicated. *P* values under 0.05 were considered significant and *P* values under 0.05, 0.01, and 0.001 are indicated with asterisks.

3. Results

3.1. Oenothien B Enhances IL-2R α Expression by Both Adult and Calf Bovine T Cells

To examine potential differences between oenothien B-induced T cell responses in bovine adults and calves, we treated isolated PBMCs from Holstein bull calves (less than 12 weeks-old) and adult Holstein cows (at least 2 years-old) with oenothien B. First, we compared oenothien B-induced activation using IL-2R α as an activation marker, as described previously [3, 8]. Consistent with our previous studies [8], we found that oenothien B enhanced IL-2R α expression on calf lymphocytes. Likewise, treatment with oenothien B also enhanced IL-2R α expression on T cells from adult cows (Figures 1A and 1D). However, oenothien B induced less IL-2R α expression on adult CD3+ T cells compared to calf T cells.

Within the CD3+ T cell population, $\gamma\delta$ T cells and $\alpha\beta$ T cells were then identified by flow cytometry and examined for IL-2R α expression separately. The percentage of CD3+ T cells that were $\gamma\delta$ T cells had a range of 27.8% – 41.4% and a mean of 35.9% in calves. The percentage was much lower in the adults, with a range of 5.3% – 11.6% and a mean of 7.1%. Interestingly, oenothien B induced less IL-2R α expression on adult $\gamma\delta$ T cells compared to cells from calves, but there was no significant difference in IL-2R α expression on adult and

calf $\alpha\beta$ T cells (Figures 1B, 1C, and 1D). These data suggest that oenothien B may be less effective at priming adult bovine $\gamma\delta$ T cells to respond to IL-2, as compared to calf T cells, but equally effective at priming adult and calf $\alpha\beta$ T cells.

3.2. Oenothien B Induces IFN γ Production by Adult Bovine T Cells

Potential differences between oenothien B-induced IFN γ production by adult and calf T cells were also examined. Previous studies showed that bovine calf T cells (either $\alpha\beta$ or $\gamma\delta$ T cells) produced little, if any, IFN γ in response to oenothien B [8]. Results of the present study supported the results of the previous study, in that, there was a lack of significant IFN γ production by T cells from calves, with non-T cells producing the majority of oenothien B-induced IFN γ in these animals (Figures 2A and 2B). In contrast, T cells from adult animals produced significantly more IFN γ than calf T cells (Figure 2A). Furthermore, two color flow cytometric analysis showed that T cells made up the majority of IFN γ -producing cells in adult bovine samples (Figure 2B). This result was exactly the same as what we observed for adult human donors in our previous study [8]. These data suggest that the ability of oenothien B to enhance IFN γ production by bovine T cells increases with age.

3.3. Oenothien B Enhances IFN γ Production by Adult Bovine CD45RO $^{+}$ $\alpha\beta$ and $\gamma\delta$ T Cells

We then determined if memory T cells were an important source of oenothien B-induced IFN γ production in adult bovine T cells. Numerous studies have shown that memory T cells produce more IFN γ than naive T cells in response to both antigen-dependent and antigen-independent stimulation [17, 19, 20]. Furthermore, the percentage of memory T cells in PBMCs increases with age, correlating with pathogen exposure over the lifespan [17, 21]. Therefore, we evaluated whether T cells expressing the memory cell-marker CD45RO were an important source of oenothien B-induced IFN γ production in adult cows (4- to 7 years-old). Consistent with our previous results in bovine adults, cells expressing IFN γ in these animals were largely CD3 $^{+}$ T cells (Figures 3A and 3D). Furthermore, cells expressing CD45RO produced more IFN γ than cells that did not express CD45RO, suggesting that CD45RO $^{+}$ T cells were the major IFN γ -producing cells in response to stimulation with oenothien B (Figures 3B and 3D). These data indicate that oenothien B-induced IFN γ production by bovine T cells could be enhanced by increasing numbers of antigen-experienced CD45RO $^{+}$ T cells. However, not all CD45RO $^{+}$ cells produced IFN γ , and a small population of CD45RO $^{-}$ cells did express IFN γ in response to oenothien B, suggesting that the linkage of CD45RO expression to the capacity of oenothien B to stimulate IFN γ production in adult bovine T cells is not absolute. Finally, oenothien B was found to induce IFN γ production by both $\gamma\delta$ TCR $^{+}$ and $\gamma\delta$ TCR $^{-}$ cells (Figures 3C and 3D). These data indicate that oenothien B induces IFN γ production by both $\alpha\beta$ T cells and $\gamma\delta$ T cells from bovine adults, which is consistent with results reported for adult human T cells [8].

3.4. Oenothien B Enhances IFN γ Production by Purified Adult Bovine T Cells

To determine if differences in IFN γ production between adult and calf T cells were due to differences in the T cells themselves or differences in the composition of PBMCs, CD4 $^{+}$ T cells, $\gamma\delta$ T cells, and other T cells (CD4 $^{-}$ $\gamma\delta$ TCR $^{-}$) were sorted from bovine adults and calves by fluorescence-activated cell sorting (FACS). When these purified cells were

stimulated with oenothien B, CD4⁺ T cells from adults expressed more IFN γ in response to direct stimulation by oenothien B than those from calves (Figure 4). Neither $\gamma\delta$ T cells nor CD4⁺ $\gamma\delta$ -T cells from calves or adults expressed IFN γ in response to direct stimulation by oenothien B (Figure 4). These data suggest that oenothien B promotes greater IFN γ expression by adult bovine T cells, specifically CD4⁺ T cells, at least partially due to an apparent T cell-intrinsic difference between adult and calf T cells.

3.5. Differentiation of Calf T Cells into CD45RO⁺ T Cells is not Sufficient to Enhance Oenothien B-induced IFN γ Production

CD45RO expression and IFN γ secretion is upregulated in T cells following mitogen (ConA/IL-2) stimulation *in vitro* [22, 23]. As such, it was then determined whether treatment with ConA to prestimulate T cells increased the capacity of these cells to produce IFN γ following oenothien B stimulation. Bovine calf PBMCs were stimulated with ConA and IL-2 for 4 days *in vitro* to simulate TCR stimulation and promote T cell-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⁺ T cells compared to unstimulated cells (Figure 5A). However, when these cells were subsequently re-stimulated with oenothien B, there was no increase in the percentage of IFN γ -producing cells in the ConA/IL-2 cultured T cells compared to T cells cultured in the absence of ConA/IL-2 (Figure 5B). These data suggest that the increased frequency of previously activated, CD45RO⁺ T cells alone is not sufficient to explain the increase in IFN γ production by adult T cells in response to oenothien B.

3.6. Oenothien B Enhances IFN γ and GM-CSF 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 oenothien B to stimulate human adult and cord blood T cells was evaluated. Oenothien B activated cord blood T cells, as measured by increased expression of CD69, although the activation of cord blood T cells was not as robust as oenothien B-stimulated adult T cells (Figure 6). When human adult and cord blood mononuclear cells were stimulated with oenothien B, adult, but not cord blood, mononuclear cells produced IFN γ (Figure 7A). Furthermore, oenothien B induced IFN γ production by adult, but not cord blood, T cells (Figures 7A and 7C). These data were consistent with our data from bovine calves and adults.

In addition to IFN γ , oenothien B induced GM-CSF production by adult human mononuclear cells and T cells but not cord blood mononuclear cells and T cells (Figures 7B and 7C). Together, these data suggest that, while oenothien B can activate both cord blood and adult T cells, oenothien B induces more IFN γ and GM-CSF production by T cells from human adult mononuclear cell cultures compared to neonate mononuclear cell cultures, supporting our observations for adult and young cattle.

3.7. Oenothien B Induces More Cytokine Production by Human Memory T Cells Than Naive T Cells

In bovine adults, CD45RO⁺ memory T cells produced more IFN γ than CD45RO[−] cells. To see if this was also the case in humans, we compared the ability of oenothien B to stimulate human CD45RO⁺ and CD45RO[−] T cells. When human adult PBMCs were stimulated with oenothien B, CD69 expression was upregulated on both CD45RO⁺ and CD45RO[−] T cells, with greater expression on CD45RO[−] cells (Figure 8A). In contrast, IFN γ and GM-CSF expression by T cells was enhanced in CD45RO⁺ T cells compared to CD45RO[−] cells (Figure 8B).

To further support these results, CD45RO⁺ and CD45RO[−] T cells were FACS sorted from adult human donors. When these purified cells were stimulated with oenothien B, CD45RO⁺ T cells expressed more IFN γ and GM-CSF in response to direct stimulation by oenothien B than CD45RO[−] T cells (Figure 8C). These data suggest that, as with bovine T cells, oenothien B promotes greater cytokine expression by human CD45RO⁺ memory T cells, despite enhancing CD69 expression on both CD45RO⁺ and CD45RO[−] T cells. Data from sorted T cells also indicate that oenothien B directly induces more cytokine production by CD45RO⁺ T cells compared to CD45RO[−] T cells, even in the absence of macrophages and other non-T cell PBMCs. Finally, the activation of human T cells, like in bovine T cells, does not always correlate with cytokine expression by oenothien B-stimulated T cells.

4. Discussion

Plant polyphenols have been studied extensively for numerous health benefits, including immunostimulation [7, 8, 24]. However, there is much that is still unknown about their immunostimulatory properties, including what factors may influence their activity. In this study, we show that age may be an important factor, as while T cells from both young and adult individuals are stimulated by oenothien B, they differ in their responses. In cattle, $\gamma\delta$ T cells from calves express more IL-2R α in response to oenothien B than adult T cells, while $\alpha\beta$ T cells from calves and adults express similar levels of IL-2R α in response to oenothien B. Certain polyphenols can prime T cells to proliferate in response to IL-2, likely due to the upregulation of IL-2R α [3]. Our data suggest that oenothien B may prime $\alpha\beta$ T cells from both young calves and adult cattle equally to IL-2, but may better prime $\gamma\delta$ T cells from young calves. In humans, T cells from both cord blood and adults express the activation marker CD69 in response to oenothien B, with adult T cells expressing higher levels.

In the context of cytokine production, T cells from young individuals, whether from human cord blood or bovine calves, produce significantly less IFN γ and GM-CSF in response to oenothien B. Similar results have been observed for other T cell agonists [25, 26]. For example, stimulation of human infant T cells with phytohemagglutinin (PHA) leads to similar proliferation, but reduced IFN γ production, compared to adult T cells [25]. These observations with oenothien B could, at least in part, be due to the accumulation of memory T cells over time [21], as we also found CD45RO⁺ T cells produce more of these cytokines compared to naive-phenotype T cells in both mixed and sorted cultures. However, even though CD45RO⁺ T cells express several times more cytokines in response to oenothien B than CD45RO[−] cells, CD45RO[−] T cells from adults still appear to produce more IFN γ in

response to oenothien B than CD45RO⁻ T cells from younger individuals. Therefore, other factors in the adult microenvironment may play an important role. This would be consistent with results by Hanna-Wakim and coworkers, who found that CD45RO⁺ CD4⁺ T cells from adults produced more IFN γ in response to staphylococcal enterotoxin B than CD45RO⁺ CD4⁺ T cells from young children [26].

Interestingly, sorted bovine CD4⁺ T cells, but not other T cells, could directly produce IFN γ in response to oenothien B. Therefore, bovine adult $\gamma\delta$ T cells and non-CD4⁺ $\alpha\beta$ T cells may require indirect stimulation for optimal IFN γ production, as our data shows that these cells produce IFN γ in mixed PBMC cultures, but not in sorted cultures. While it is currently unknown why only sorted CD4⁺ T cells could produce IFN γ in response to oenothien B in cattle, Yamanaka and coworkers found that certain large polyphenols could bind to CD4 and that blocking CD4 inhibited cytokine production by murine splenocytes in response to those polyphenols [24]. It is possible that oenothien B has similar activity. Future studies should examine the importance of accessory cells, such as monocytes and NK cells, for the optimal production of IFN γ by adult T cells in response to oenothien B, as well as the role of CD4.

Currently, the mechanism for the disparity in cytokine production between adult and young T cells has yet to be determined. However, one possible explanation involves methylation at certain cytokine promoter sites, which can prevent gene transcription and is reduced in memory T cells compared to naive T cells, as well as adult cells compared to cord blood cells [27, 28]. Alternatively, mRNA stability and availability also changes with age. Cairo and coworkers found that GM-CSF mRNA is less stable in human cord blood mononuclear cells compared to adults upon treatment with PMA and PHA [29]. Another study found that mRNA for IFN γ and several other T cell cytokines was less stable in CD3/CD28 stimulated CD4⁺ T cells in young mice compared to older mice [30]. Furthermore, steady state IFN γ RNA content was shown to be lower for naive CD8⁺ T cells compared to memory T cells in mice [31]. Therefore, increased accessibility of promoters and stability of mRNA may allow for greater IFN γ and GM-CSF production by adult and memory T cells in response to a non-specific agonist like oenothien B, and possibly other polyphenols.

While our experiments focus on memory T cells in the blood, they can also migrate into a number of tissues, including the lung and gut [32]. This suggests that they may be prime targets for immunomodulatory food products. While typically associated with adaptive immunity, memory T cells can also play an important role in innate immunity through antigen-independent, bystander effects [19, 20, 33, 34]. Therefore, they may be another innate-like lymphocyte population, in addition to $\gamma\delta$ T cells and NK cells, whose numbers and activity could be influenced by polyphenols. This possibility should be examined in future studies.

Studies comparing very young and adult individuals *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. However, cattle are large enough to handle and feed at a very young age and are often used in age-related and diet-influenced immunity studies [14, 16, 35–39]. Furthermore, the current study and other studies suggest that bovine lymphocytes are predictive for the influence of polyphenols on

human lymphocytes [3, 8]. Therefore, our data suggest that cattle may be a useful animal model for studying the effects of polyphenols on immunity *in vivo*, particularly when comparing very young individuals to adults.

In conclusion, these data are the first to our knowledge that demonstrate age-related differences in immune cell responsiveness to polyphenols. Further work is required to determine if our observations with oenothien B also hold true for other immunomodulatory polyphenols, as well as *in vivo*. These data have implications for optimizing the use of dietary polyphenols to enhance immunity in both humans and animals and suggest that the ages of individuals should be taken into account when examining the effect of consumption of polyphenol-rich supplements on immunity.

Acknowledgments

We would like to thank Dr. Robyn Klein (Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT) for plant identification. We thank Kerri Jones and the Johnson Family Livestock Facility for providing bovine calf blood. We also thank Larissa Jackiw for her assistance in FACS sorting, as well as Dr. Jodi Hedges for helpful scientific discussion. This work was supported by grants from the National Institutes of Health [NCCAM AT0004986-01]; National Institutes of Health COBRE [P30 GM110732]; the M.J. Murdock Charitable Trust; and the Montana Agricultural Experiment Station. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Skyberg JA, Robison A, Golden S, Rollins MF, Callis G, Huarte E, et al. Apple polyphenols require T cells to ameliorate dextran sulfate sodium-induced colitis and dampen proinflammatory cytokine expression. *J Leukoc Biol.* 2011; 90:1043–54. [PubMed: 21693591]
2. Okuyama S, Makihata N, Yoshimura M, Amakura Y, Yoshida T, Nakajima M, et al. Oenothien B suppresses lipopolysaccharide (LPS)-induced inflammation in the mouse brain. *Int J Mol Sci.* 2013; 14:9767–78. [PubMed: 23652834]
3. Holderness J, Jackiw L, Kimmel E, Kerns H, Radke M, Hedges JF, et al. Select plant tannins induce IL-2R α up-regulation and augment cell division in $\gamma\delta$ T cells. *J Immunol.* 2007; 179:6468–78. [PubMed: 17982035]
4. Schepetkin IA, Kirpotina LN, Jakiw L, Khlebnikov AI, Blaskovich CL, Jutila MA, et al. Immunomodulatory activity of oenothien B isolated from *Epilobium angustifolium*. *J Immunol.* 2009; 183:6754–66. [PubMed: 19846877]
5. Holderness J, Hedges JF, Daughenbaugh K, Kimmel E, Graff J, Freedman B, et al. Response of $\gamma\delta$ T cells to plant-derived tannins. *Crit Rev Immunol.* 2008; 28:377–402. [PubMed: 19166386]
6. Ramiro-Puig E, Pérez-Cano FJ, Ramos-Romero S, Pérez-Berezo T, Castellote C, Permanyer J, et al. Intestinal immune system of young rats influenced by cocoa-enriched diet. *J Nutr Biochem.* 2008; 19:555–65. [PubMed: 18061430]
7. Daughenbaugh KF, Holderness J, Graff JC, Hedges JF, Freedman B, Graff JW, et al. Contribution of transcript stability to a conserved procyanidin-induced cytokine response in $\gamma\delta$ T cells. *Genes Immun.* 2011; 12:378–89. [PubMed: 21307878]
8. Ramstead AG, Schepetkin IA, Quinn MT, Jutila MA. Oenothien B, a cyclic dimeric ellagitannin isolated from *Epilobium angustifolium*, enhances IFN γ production by lymphocytes. *PLoS One.* 2012; 7:e50546. [PubMed: 23226309]
9. Scharton-Kersten TM, Sher A. Role of natural killer cells in innate resistance to protozoan infections. *Curr Opin Immunol.* 1997; 9:44–51. [PubMed: 9039777]
10. Culley FJ. Natural killer cells in infection and inflammation of the lung. *Immunology.* 2009; 128:151–63. [PubMed: 19740372]

11. Wallin RP, Sundquist VS, Bråkenhielm E, Cao Y, Ljunggren HG, Grandien A. Angiostatic Effects of NK Cell-Derived IFN- γ Counteracted by Tumour Cell Bcl-xL Expression. *Scand J Immunol.* 2014; 79:90–7. [PubMed: 24313893]
12. Ninomiya T, Takimoto H, Matsuzaki G, Hamano S, Yoshida H, Yoshikai Y, et al. V γ 1+ $\gamma\delta$ T cells play protective roles at an early phase of murine cytomegalovirus infection through production of interferon- γ . *Immunology.* 2000; 99:187–94. [PubMed: 10692035]
13. Le Garff-Tavernier M, Béziat V, Decocq J, Siguret V, Gandjbakhch F, Pautas E, et al. Human NK cells display major phenotypic and functional changes over the life span. *Aging Cell.* 2010; 9:527–35. [PubMed: 20477761]
14. Elhmouzi-Younes J, Storset AK, Boysen P, Laurent F, Drouet F. Bovine neonate natural killer cells are fully functional and highly responsive to interleukin-15 and to NKp46 receptor stimulation. *Vet Res.* 2009; 40:54. [PubMed: 19549488]
15. De Rosa SC, Andrus JP, Perfetto SP, Mantovani JJ, Herzenberg LA, Herzenberg LA, et al. Ontogeny of $\gamma\delta$ T cells in humans. *J Immunol.* 2004; 172:1637–45. [PubMed: 14734745]
16. Price SJ, Sopp P, Howard CJ, Hope JC. Workshop cluster 1+ $\gamma\delta$ T-cell receptor T cells from calves express high levels of interferon- γ in response to stimulation with interleukin-12 and -18. *Immunology.* 2007; 120:57–65. [PubMed: 17034425]
17. Chipeta J, Komada Y, Zhang XL, Deguchi T, Sugiyama K, Azuma E, et al. CD4+ and CD8+ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. *Cell Immunol.* 1998; 183:149–56. [PubMed: 9606999]
18. Wilson E, Walcheck B, Davis WC, Jutila MA. Preferential tissue localization of bovine $\gamma\delta$ T cell subsets defined by anti-T cell receptor for antigen antibodies. *Immunol Lett.* 1998; 64:39–44. [PubMed: 9865600]
19. Berg RE, Crossley E, Murray S, Forman J. Memory CD8+ T cells provide innate immune protection against *Listeria monocytogenes* in the absence of cognate antigen. *J Exp Med.* 2003; 198:1583–93. [PubMed: 14623912]
20. Soudja SM, Ruiz AL, Marie JC, Lauvau G. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity.* 2012; 37:549–62. [PubMed: 22940097]
21. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol.* 2014; 14:24–35. [PubMed: 24336101]
22. Rothstein DM, Sohen S, Daley JF, Schlossman SF, Morimoto C. CD4+CD45RA+ and CD4+CD45RA– T cell subsets in man maintain distinct function and CD45RA expression persists on a subpopulation of CD45RA+ cells after activation with Con A. *Cell Immunol.* 1990; 129:449–67. [PubMed: 1696528]
23. Reem GH, Cook LA, Henriksen DM, Vilcek J. Gamma interferon induction in human thymocytes activated by lectins and B cell lines. *Infect Immun.* 1982; 37:216–21. [PubMed: 6179877]
24. Yamanaka D, Tamiya Y, Motoi M, Ishibashi K, Miura NN, Adachi Y, et al. The effect of enzymatically polymerised polyphenols on CD4 binding and cytokine production in murine splenocytes. *PLoS One.* 2012; 7:e36025. [PubMed: 22540016]
25. Frenkel L, Bryson YJ. Ontogeny of phytohemagglutinin-induced gamma interferon by leukocytes of healthy infants and children: evidence for decreased production in infants younger than 2 months of age. *J Pediatr.* 1987; 111:97–100. [PubMed: 3110393]
26. Hanna-Wakim R, Yasukawa LL, Sung P, Fang M, Sullivan B, Rinki M, et al. Age- related increase in the frequency of CD4(+) T cells that produce interferon-gamma in response to staphylococcal enterotoxin B during childhood. *J Infect Dis.* 2009; 200:1921–7. [PubMed: 19909079]
27. Kersh EN, Fitzpatrick DR, Murali-Krishna K, Shires J, Speck SH, Boss JM, et al. Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *J Immunol.* 2006; 176:4083–93. [PubMed: 16547244]
28. White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO– T cells. *J Immunol.* 2002; 168:2820–7. [PubMed: 11884451]

29. Cairo MS, Suen Y, Knoppel E, van de Ven C, Nguyen A, Sender L. Decreased stimulated GM-CSF production and GM-CSF gene expression but normal numbers of GM-CSF receptors in human term newborns compared with adults. *Pediatr Res.* 1991; 30:362–7. [PubMed: 1720233]
30. Pioli C, Pucci S, Barile S, Frasca D, Doria G. Role of mRNA stability in the different patterns of cytokine production by CD4+ cells from young and old mice. *Immunology.* 1998; 94:380–7. [PubMed: 9767421]
31. Grayson JM, Murali-Krishna K, Altman JD, Ahmed R. Gene expression in antigen-specific CD8+ T cells during viral infection. *J Immunol.* 2001; 166:795–9. [PubMed: 11145652]
32. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol.* 2013; 31:137–61. [PubMed: 23215646]
33. Chu T, Tyznik AJ, Roepke S, Berkley AM, Woodward-Davis A, Pattacini L, et al. Bystander-activated memory CD8 T cells control early pathogen load in an innate-like, NKG2D-dependent manner. *Cell Rep.* 2013; 3:701–8. [PubMed: 23523350]
34. Sckisel GD, Tietze JK, Zamora AE, Hsiao HH, Priest SO, Wilkins DE, et al. Influenza infection results in local expansion of memory CD8(+) T cells with antigen non-specific phenotype and function. *Clin Exp Immunol.* 2014; 175:79–91. [PubMed: 23937663]
35. Kampen AH, Olsen I, Tollersrud T, Storset AK, Lund A. Lymphocyte subpopulations and neutrophil function in calves during the first 6 months of life. *Vet Immunol Immunopathol.* 2006; 113:53–63. [PubMed: 16772096]
36. Graham EM, Thom ML, Howard CJ, Boysen P, Storset AK, Sopp P, et al. Natural killer cell number and phenotype in bovine peripheral blood is influenced by age. *Vet Immunol Immunopathol.* 2009; 132:101–8. [PubMed: 19525013]
37. Oliveira RA, Narciso CD, Bisinotto RS, Perdomo MC, Ballou MA, Dreher M, et al. Effects of feeding polyphenols from pomegranate extract on health, growth, nutrient digestion, and immunocompetence of calves. *J Dairy Sci.* 2010; 93:4280–91. [PubMed: 20723701]
38. Doherty ML, Monaghan ML, Bassett HF, Quinn PJ, Davis WC. Effect of dietary restriction on cell-mediated immune responses in cattle infected with *Mycobacterium bovis*. *Vet Immunol Immunopathol.* 1996; 49:307–20. [PubMed: 8677633]
39. Caroprese M, Albenzio M, Marino R, Santillo A, Sevi A. Dietary glutamine enhances immune responses of dairy cows under high ambient temperature. *J Dairy Sci.* 2013; 96:3002–11. [PubMed: 23498025]

Highlights

- Oenothien B stimulates T cells from both young and adult humans and cattle.
- Oenothien B promotes IFN γ and GM-CSF production by T cells from adults only.
- Oenothien B-induced IFN γ is produced by both $\alpha\beta$ and $\gamma\delta$ T cells in adults.
- Oenothien B-treated CD45RO⁺ cells produce more IFN γ and GM-CSF than naive T cells.

Fig. 1.

Oenothien B induced IL-2Rα expression in bovine T cells from both adults and calves. PBMCs (10^5 cells/well) from calves (< 12 weeks-old, N=7) and adult cows (> 2 years-old, N=8) were treated with the indicated concentrations of oenothien 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 as mean fluorescence intensity (MFI) 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. (D) Representative examples of two-color flow cytometry plots of bovine adult and calf CD3⁺ T cells, $\gamma\delta$ T cells (CD3⁺ $\gamma\delta$ TCR⁺), and $\alpha\beta$ T cells (CD3⁺ $\gamma\delta$ TCR⁻) treated with 40 μ g/ml oenothien B or X-VIVO medium alone. *p<0.05, **p<0.01, ***p<0.001

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Fig. 2.

Oenothien B induces more IFN γ production by adult bovine T cells than calf T cells. (A) PBMCs (10^5 cells/well) from calves (< 12 weeks-old, N=4) and adult cows (> 2 years-old, N=4) were treated with the indicated concentrations of oenothien B in X-VIVO medium for 42 hrs with brefeldin A added for the final 18 hrs. The percent of CD3+ T cells that were IFN γ + were 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 μ g/ml oenothien B or X-VIVO medium alone. ***p<0.001

Fig. 3.

Oenothein B induces more IFN γ production by bovine CD45RO+ T cells than CD45RO- T cells. PBMCs (10^5 cells/well) isolated from adult cows (4- to- 7 years-old, 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 $\gamma\delta$ TCR+ (C) cells producing IFN γ 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 μ g/ml oenothein B or medium alone. ***p<0.001

Fig. 4.

Oenothien B enhances IFN γ production by purified bovine CD4+ T cells from adults, but not calves. CD4+ T cells, $\gamma\delta$ T cells, and all other (CD4- $\gamma\delta$ TCR-) CD3+ T cells were sorted from bovine adults (> 2 years-old, N=5) and calves (< 12 weeks-old, N=5), plated (10^4 cells/well), and treated with indicated concentrations of oenothien B or X-VIVO medium alone. After 24 hrs, soluble IFN γ 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<0.01, ***p<0.001

Fig. 5.

Enhancing CD45RO expression *in vitro* does not enhance oenothein B-induced IFN γ production by calf T cells. PBMCs (10^5 cells/well) from calves (< 12 weeks-old, N=3) were treated for 4 days with ConA (500 ng/ml) and recombinant human 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 of PBMCs that 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 that were IFN γ + 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

Fig. 6.

Oenothien B induces CD69 expression on human cord blood T cells. (A) Human blood mononuclear cells (5×10^4 cells/well) isolated from cord blood (N=3) and sex-matched adult donors (N=3) were treated with the indicated concentrations of oenothien B or cRPMI medium only for 42 hrs and CD69 expression on CD3⁺ T cells was measured as mean fluorescence intensity (MFI) 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 (0 µg/ml) and between cord blood and adult samples was determined by Two-way ANOVA with Bonferroni post-test. (B) Representative examples of two-color flow cytometry plots of human adult and cord blood mononuclear cells treated with 40 µg/ml oenothien B or cRPMI medium alone. **p<0.01, ***p<0.001

Fig. 7. Oenothien B induces IFN γ and GM-CSF expression on human adult, but not cord blood, T cells. Human blood mononuclear cells (5×10^4 cells/well) from cord blood (N=3) and adult donors (N=3) were treated with the indicated concentrations of oenothien B or cRPMI medium alone for 24 hrs, and soluble IFN γ (A, left panel) and GM-CSF (B, left panel) levels in supernatant fluids were measured by ELISA. The graph represents pooled data, with each sample plated in triplicate. Human blood mononuclear cells (5×10^4 cells/well) isolated from cord blood (N=1 for IFN γ , N=2 for GM-CSF) and adult (N=1 for IFN γ , N=2 for GM-CSF) donors were also treated with the indicated concentrations of oenothien B or medium alone for 24 hrs, with brefeldin A added for the final 6 hrs. The percent of CD3⁺ T cells expressing IFN γ (A, right panel) and GM-CSF (B, right panel) 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 mononuclear cells treated with 20 µg/ml oenothien B or medium alone. * $p < 0.05$, *** $p < 0.001$

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Fig. 8.

Oenothien B induces 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 oenothien B or cRPMI medium alone for 24 hrs, with brefeldin A added for the final 6 hrs. CD69 (A), as well as IFN γ and GM-CSF (B), expression on CD3⁺ T cells was measured by multi-color flow cytometry. The graphs represent pooled data. 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 oenothien B or cRPMI medium alone. After 48 hrs, soluble IFN γ and GM-CSF were measured by ELISA. The graphs represent pooled data of soluble IFN γ 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. ** $p < 0.01$, *** $p < 0.001$

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