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Aging influences the response of T cells to stimulation by the ellagitannin, oenothein B

Andrew G. Ramstead [a], Igor A. Schepetkin [a], Kimberly Todd [b], James Loeffelholz [b], James G. Berardinelli [c], Mark T. Quinn [a], Mark A. Jutila [a,*]

[a] Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717, United States
[b] Bozeman Deaconess Hospital, Bozeman, MT 59717, United States
[c] Department of Animal and Range Sciences, Montana State University, Bozeman, MT 59717, United States

Abstract

Several plant extracts, including certain polyphenols, prime innate lymphocytes and enhance responses to sec-ondary stimuli. Oenothein B, a polyphenol isolated from Epilobium angustifolium and other plant sources, en-hances IFNγ 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 exam-ined 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 cytokine production by T cells were seen. In T cells from human cord blood and bovine calves, oenothein B was unable to induce IFNγ production. However, oenothein B induced IFNγ production by T cells from adult humans and cattle. 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. 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.

Keywords:
Bovine
Human
Memory T cells
GM-CSF
Interferon-γ
Polyphenol

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 γδ 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 (IFNγ) 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 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 [8]. In the previous study [8], the majority of IFNγ-producing cells in bovine samples were CD335 (NKp46)-expressing NK cells. Additionally, we found that oenothein B enhanced IFNγ production by NK cells in human samples. However, in contrast to the bovine data, a non-NK cell population, specifically T cells (both γδ T cells and αβ T cells), produced IFNγ in response to stimulation by oenothein B in human samples. Furthermore, this oenothein B-responsive T cell population made up the majority of IFNγ-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 oenothein B in both humans and cattle. We show that oenothein 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 oenothein B is enhanced only in adults. Furthermore, we find that CD45RO + T cells isolated from cattle and humans produce more IFNγ, as well as granulocyte macrophage colony-stimulating factor (GM-CSF), in response to oenothein B than CD45RO − cells. These data indicate that the role of T cells in the modulation of immunity by oenothein 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 subject 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 oenothein B

Oenothein 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-μm 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 × 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* amebocyte lysate assay kit (Cambrex, East Rutherford, NJ, USA) was used to evaluate possible endotoxin contamination in purified oenothein B. Purified, endotoxin-free oenothein 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 × 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 FACSAria 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% CO_2 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 γδ 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% CO_2 before being used in the experiments described below.

2.5. T cell activation assays

To measure IL-2Rα 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% CO_2 in the presence of oenothein B (0, 15, 20, 25, or 40 μg/ml) or medium only for approximately 24 or 42 h. IL-2Rα and CD69 expression were then analyzed by flow cytometry. Brefeldin A (eBioscience) was added to some of the cultures to quantify IFNγ and GM-CSF expression by flow cytometry.

To measure the secretion of IFNγ 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% CO_2 in the presence of oenothein B (0, 20, or 40 μg/ml) or medium only for approximately 24 or 48 h. 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% CO_2 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.

2.6. 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 Inc., Raleigh, NC, USA), and γδ TCR (GD3.8). Human cells were stained with mAbs against CD69 (FN50, Biolegend), CD3 (UCHT1, Biolegend), CD45RO (UCHL1, eBioscience), and γδ TCR (11 F2, 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 μl/well samples at a cellular concentration of 1×10^6/ml or 5×10^5/ml were collected by the HTS sampler for analysis.

2.7. IFNγ and GM-CSF assays

Enzyme-linked immunosorbent assays (ELISA) were used to measure IFNγ and GM-CSF in cell supernatant fluids. A bovine IFNγ kit (MABTECH, Cincinnati, OH, USA), a human IFNγ 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 IFNγ 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 IFNγ was detected using a PE-conjugated mouse IgG1 mAb against bovine IFNγ (MCA1783E, AbD Serotec Inc.). Human IFNγ

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). IFNγ 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. Oenothein B enhances IL-2Rα expression by both adult and calf bovine T cells

To examine potential differences between oenothein 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 oenothein B. First, we compared oenothein B-induced activation using IL-2Rα as an activation marker, as described previously [3,8]. Consistent with our previous studies [8],

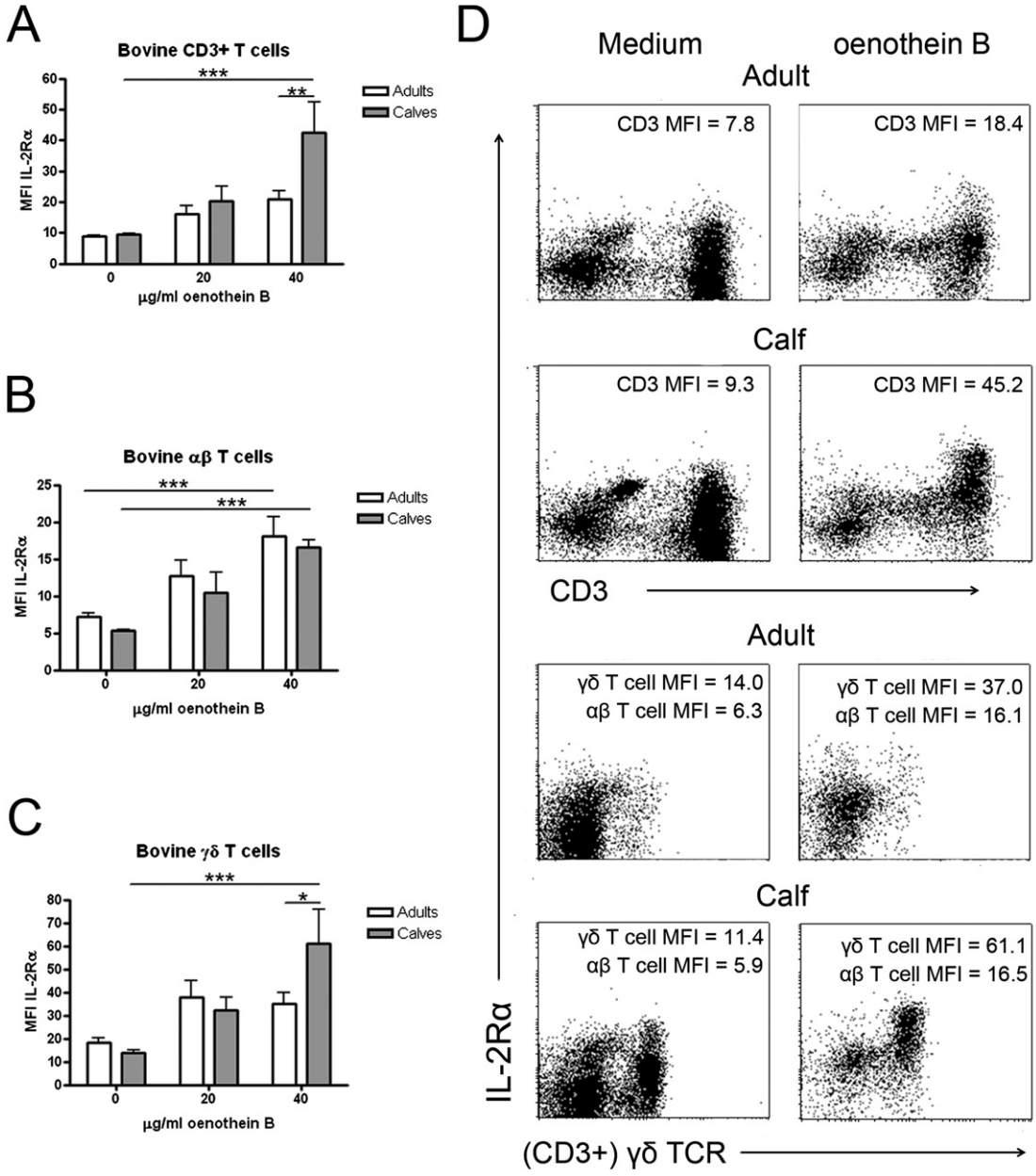


Fig. 1. Oenothein B induced IL-2Rα expression in bovine T cells from both adults and calves. PBMCs (10^5 cells/well) from calves (<12 weeks-old, N = 7) and adult cows (>2 years-old, N = 8) were treated with the indicated concentrations of oenothein B or X-VIVO medium only for 24 h and IL-2Rα expression 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, γδ T cells (CD3 + γδ TCR+), and αβ T cells (CD3 + γδ TCR−) treated with 40 µg/ml oenothein B or X-VIVO medium alone. *p < 0.05, **p < 0.01, ***p < 0.001.

we found that oenothein B enhanced IL-2Rα expression on calf lymphocytes. Likewise, treatment with oenothein B also enhanced IL-2Rα expression on T cells from adult cows (Fig. 1A and D). However, oenothein B induced less IL-2Rα expression on adult CD3 + T cells compared to calf T cells.

Within the CD3 + T cell population, γδ T cells and αβ T cells were then identified by flow cytometry and examined for IL-2Rα expression separately. The percentage of CD3 + T cells that were γδ 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, oenothein B induced less IL-2Rα expression on adult γδ T cells compared to cells from calves, but there was no significant difference in IL-2Rα expression on adult and calf αβ T cells (Fig. 1B, C, and D). These data suggest that oenothein B may be less effective at priming adult bovine γδ T cells to respond to IL-2, as compared to calf T cells, but equally effective at priming adult and calf αβ T cells.

3.2. Oenothein B induces IFNγ production by adult bovine T cells

Potential differences between oenothein B-induced IFNγ production by adult and calf T cells were also examined. Previous studies showed that bovine calf T cells (either αβ or γδ T cells) produced little, if any, IFNγ in response to oenothein B [8]. Results of the present study supported the results of the previous study, in that, there was a lack of significant IFNγ production by T cells from calves, with non-T cells producing the majority of oenothein B-induced IFNγ in these animals (Fig. 2A and B). In contrast, T cells from adult animals produced significantly more IFNγ than calf T cells (Fig. 2A). Furthermore, two color flow cytometric analysis showed that T cells made up the majority of IFNγ-producing cells in adult bovine samples (Fig. 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 oenothein B to enhance IFNγ production by bovine T cells increases with age.

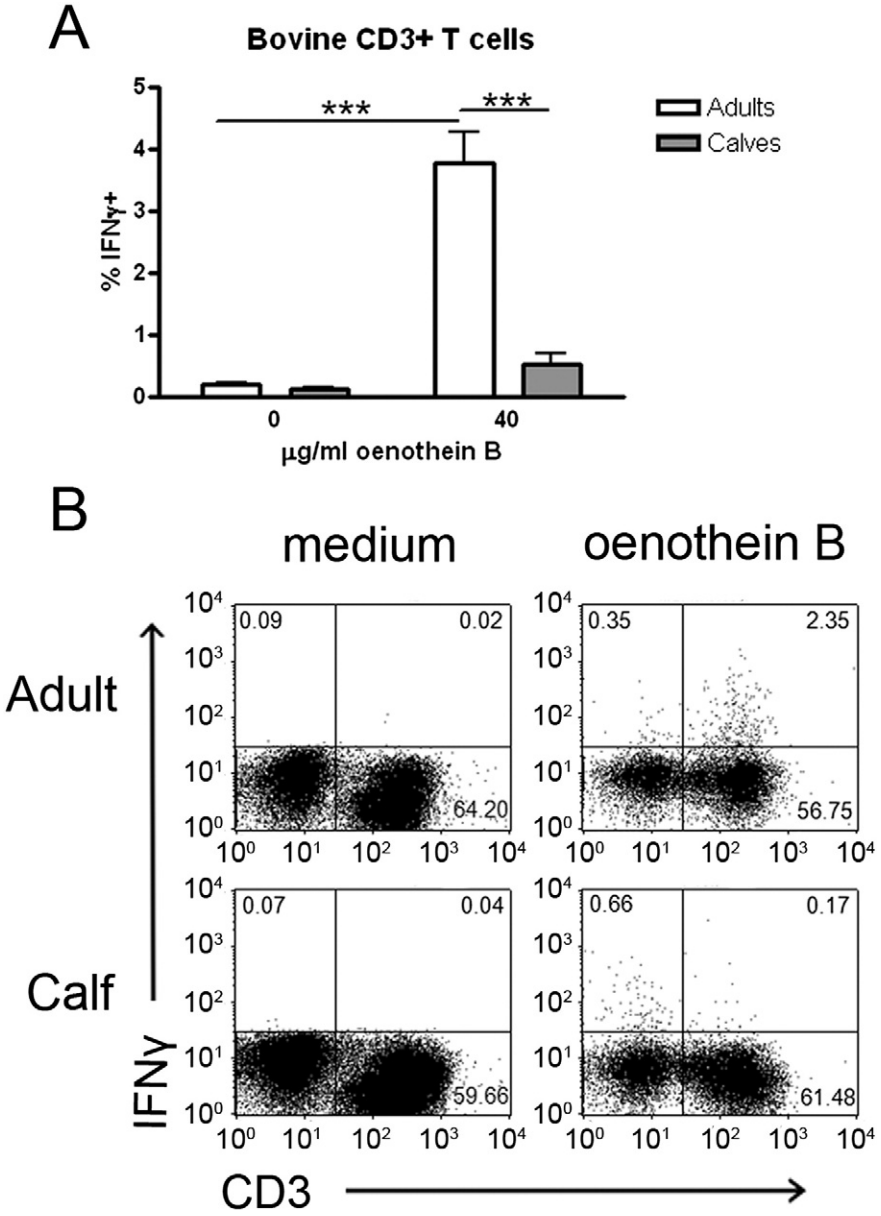


Fig. 2. Oenothein B induces more IFNγ production by adult bovine T cells than calf T cells. (A) PBMCs (10^5 cells/well) from calves (<12 weeks-old, N = 4) and adult cows (>2 years-old, N = 4) were treated with the indicated concentrations of oenothein B in X-VIVO medium for 42 h with brefeldin A added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

3.3. Oenothein B enhances IFNγ production by adult bovine CD45RO + αβ and γδ T cells

We then determined if memory T cells were an important source of oenothein B-induced IFNγ production in adult bovine T cells. Numerous studies have shown that memory T cells produce more IFNγ 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 oenothein B-induced IFNγ production in adult cows (4- to-7 years-old). Consistent with our previous results in bovine adults, cells expressing IFNγ in these animals were largely CD3 + T cells (Fig. 3A and D). Furthermore, cells expressing CD45RO produced more IFNγ than cells that did not express CD45RO, suggesting that CD45RO + T cells were the major IFNγ-producing cells in response to stimulation with oenothein B (Fig. 3B and D). These data indicate that oenothein B-induced IFNγ production by bovine T cells could be enhanced by increasing numbers of antigen-experienced CD45RO + T cells. However, not all CD45RO + cells produced IFNγ, and a small population of CD45RO − cells did express IFNγ in response to oenothein B, suggesting that the linkage of CD45RO expression to the capacity of oenothein B to stimulate IFNγ production in adult bovine T cells is not absolute. Finally, oenothein B was found to induce IFNγ production by both γδ TCR + and γδ TCR − cells (Fig. 3C and D). These data indicate that oenothein B induces IFNγ production by both αβ T cells and γδ T cells from bovine adults, which is consistent with results reported for adult human T cells [8].

3.4. Oenothein B enhances IFNγ production by purified adult bovine T cells

To determine if differences in IFNγ 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, γδ T cells, and other T cells (CD4 − γδ TCR −) were sorted from bovine adults and calves by fluorescence-activated cell sorting (FACS). When these purified cells were stimulated with oenothein B, CD4 + T cells from adults

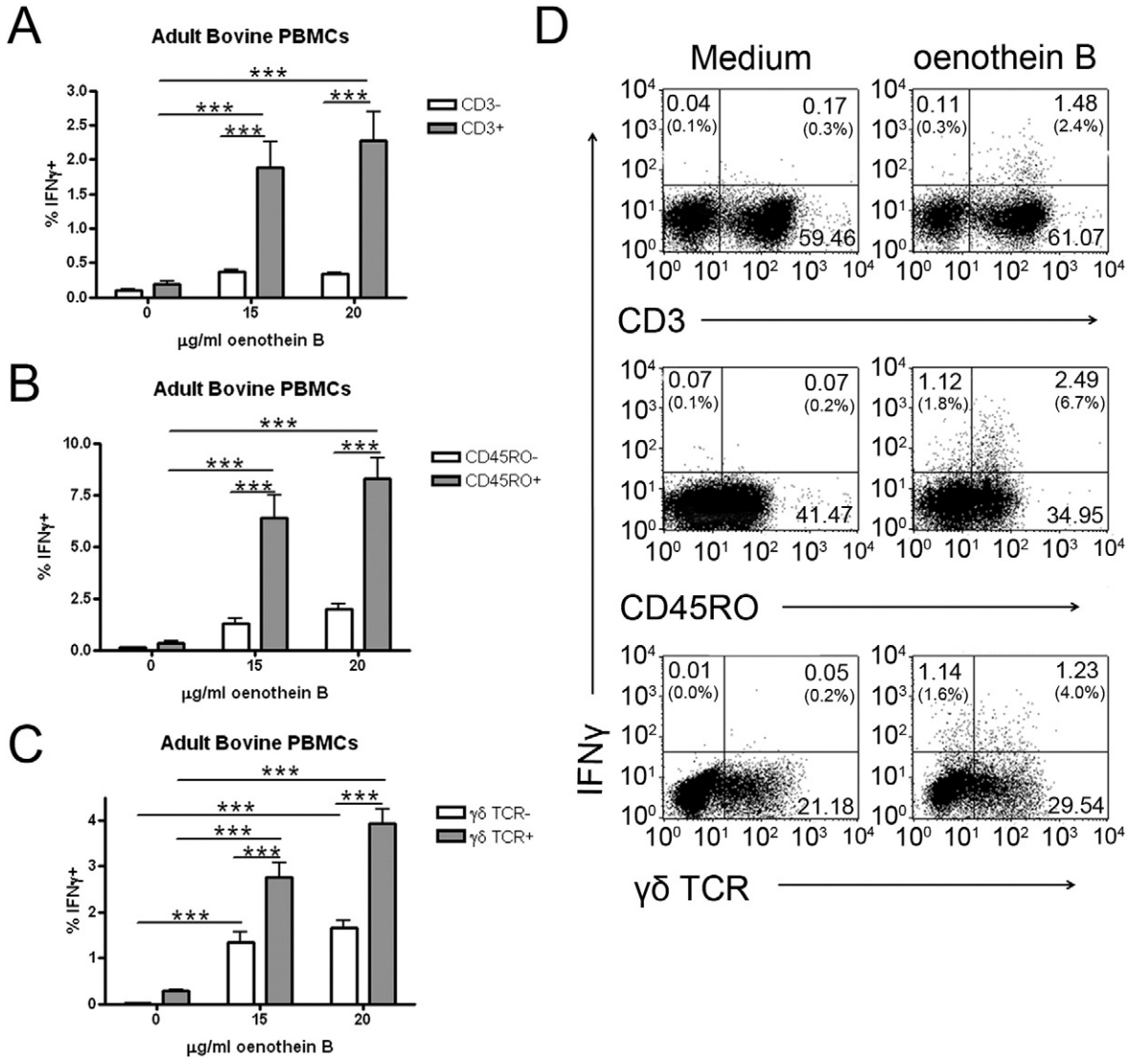


Fig. 3. Oenothein B induces more IFNγ production by bovine CD45RO + T cells than CD45RO − T cells. PBMCs (10^5 cells/well) isolated from adult cows (4 to 7 years-old, N = 2) were treated with the indicated concentrations of oenothein B or X-VIVO medium alone for 24 h, with brefeldin A added for the final 6 h. 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. ***$p < 0.001$.

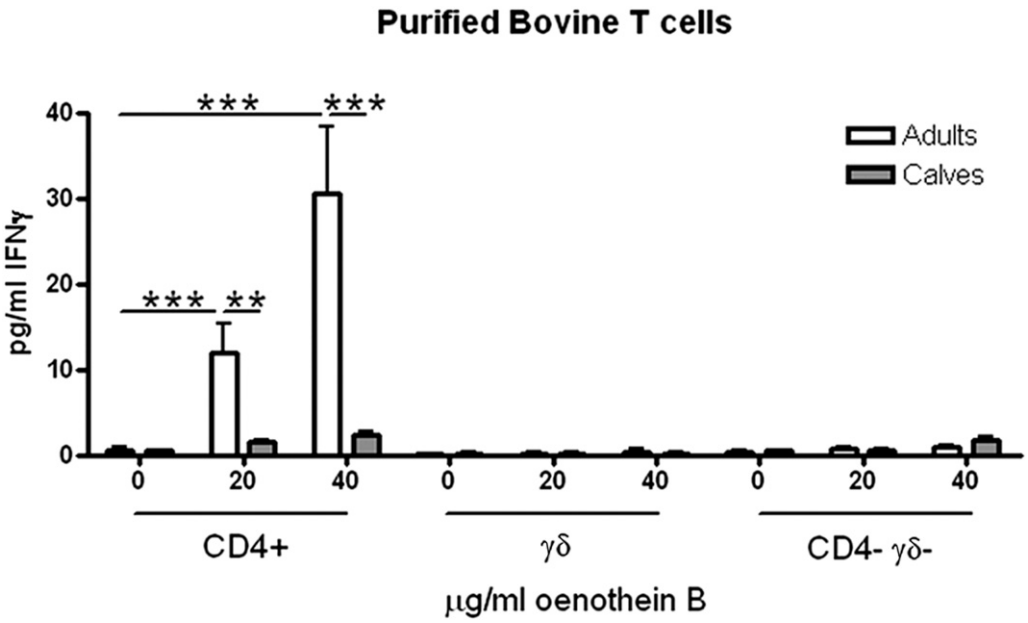


Fig. 4. Oenothein B enhances IFNγ production by purified bovine CD4+ T cells from adults, but not calves. CD4+ T cells, γδ T cells, and all other (CD4− γδ TCR−) CD3+ T cells were sorted from bovine adults (>2 years-old, N = 5) and calves (<12 weeks-old, N = 5), plated (10^4 cells/well), and treated with indicated concentrations of oenothein B or X-VIVO medium alone. After 24 h, 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 < 0.01, ***p < 0.001.

expressed more IFNγ in response to direct stimulation by oenothein B than those from calves (Fig. 4). Neither γδ T cells nor CD4− γδ− T cells from calves or adults expressed IFNγ in response to direct stimulation by oenothein B (Fig. 4). These data suggest that oenothein B promotes greater IFNγ 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 oenothein B-induced IFNγ production

CD45RO expression and IFNγ 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 IFNγ following oenothein 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 (Fig. 5A). However, when these cells were subsequently re-stimulated with oenothein B, there was no increase in the percentage of IFNγ-producing cells in the ConA/IL-2 cultured T cells compared to T cells cultured in the absence of ConA/IL-2 (Fig. 5B). These data suggest that the increased frequency of previously activated, CD45RO+ T cells alone is not sufficient to explain the increase in IFNγ production by adult T cells in response to oenothein B.

3.6. Oenothein B enhances IFNγ 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 oenothein B to stimulate human adult and cord blood T cells was evaluated. Oenothein B activated cord blood T cells, as measured by increased expression of CD69, although the activation of cord blood T cells

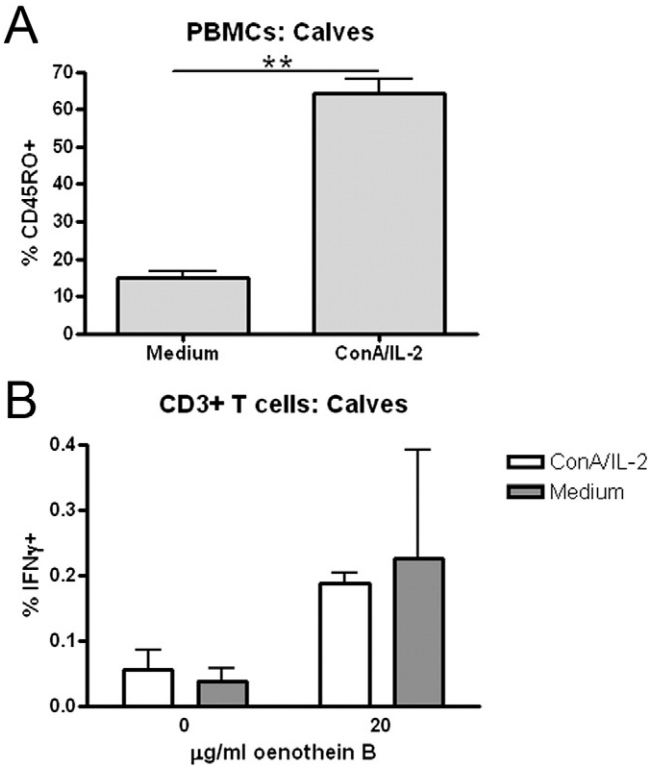


Fig. 5. Enhancing CD45RO expression *in vitro* does not enhance oenothein B-induced IFNγ production by calf T cells. PBMCs (10^5 cells/well) from calves (<12 weeks-old, N = 3) were treated for 4 days with ConA (500 ng/ml) and recombinant human IL-2 (1 ng/ml) or X-VIVO medium only. After 4 days, medium 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 h with brefeldin A added for the final 18 h. (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 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.

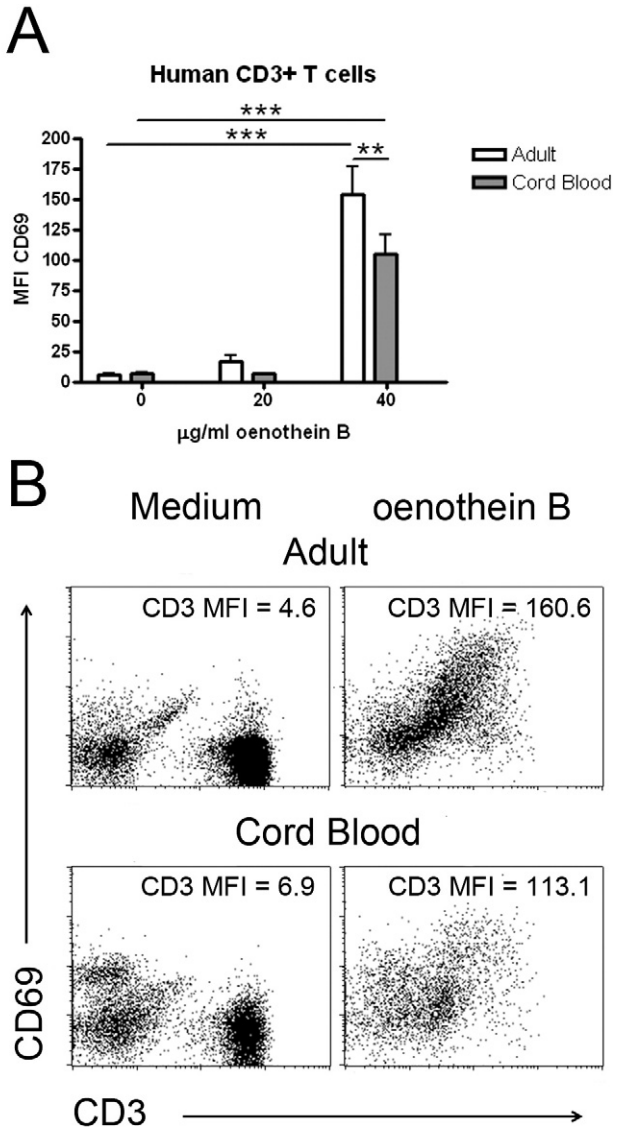


Fig. 6. Oenothein B induces CD69 expression on human cord blood T cells. (A) Human blood mononuclear cells (5×10^4 cells/well) isolated from cord blood (N = 3) and sex-matched adult donors (N = 3) were treated with the indicated concentrations of oenothein B or cRPMI medium only for 42 h and CD69 expression 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 oenothein B or cRPMI medium alone. $^{**}p < 0.01$, $^{***}p < 0.001$.

was not as robust as oenothein B-stimulated adult T cells (Fig. 6). When human adult and cord blood mononuclear cells were stimulated with oenothein B, adult, but not cord blood, mononuclear cells produced IFNγ (Fig. 7A). Furthermore, oenothein B induced IFNγ production by adult, but not cord blood, T cells (Fig. 7A and C). These data were consistent with our data from bovine calves and adults.

In addition to IFNγ, oenothein B induced GM-CSF production by adult human mononuclear cells and T cells but not cord blood mononuclear cells and T cells (Fig. 7B and C). Together, these data suggest that, while oenothein B can activate both cord blood and adult T cells, oenothein B induces more IFNγ 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. Oenothein B induces more cytokine production by human memory T cells than naive T cells

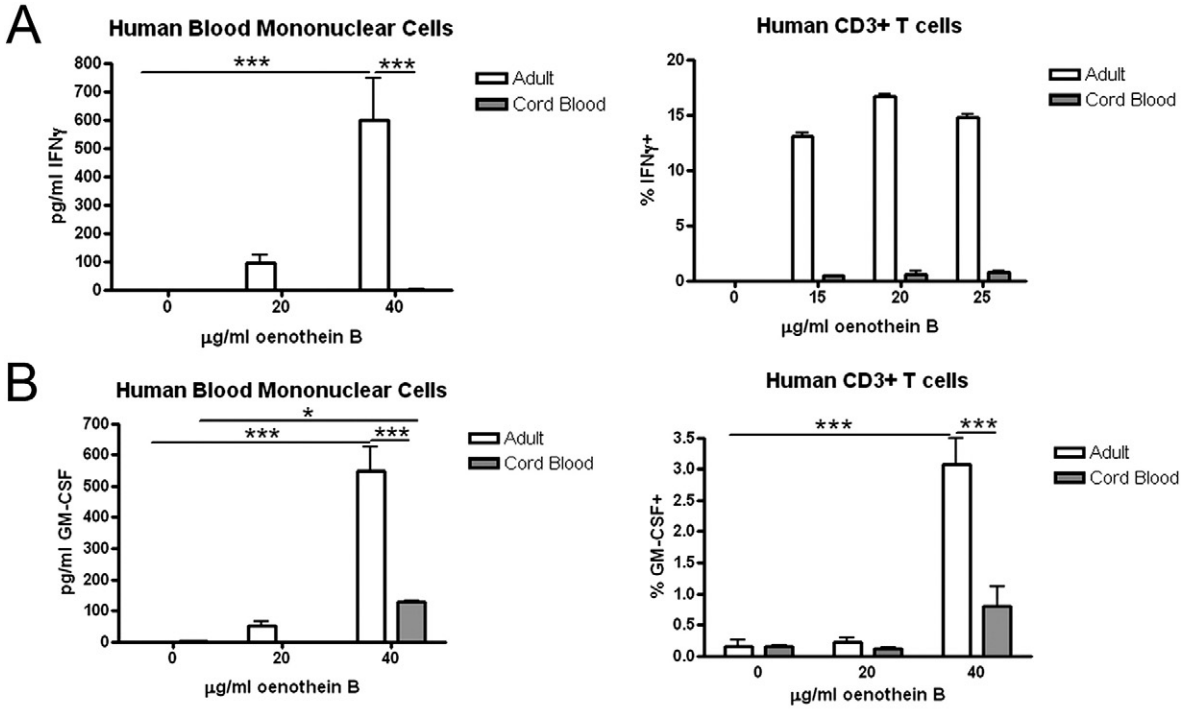
In bovine adults, CD45RO+ memory T cells produced more IFNγ than CD45RO− cells. To see if this was also the case in humans, we compared the ability of oenothein B to stimulate human CD45RO+ and CD45RO− T cells. When human adult PBMCs were stimulated with oenothein B, CD69 expression was upregulated on both CD45RO+ and CD45RO− T cells, with greater expression on CD45RO− cells (Fig. 8A). In contrast, IFNγ and GM-CSF expression by T cells was enhanced in CD45RO+ T cells compared to CD45RO− cells (Fig. 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 oenothein B, CD45RO+ T cells expressed more IFNγ and GM-CSF in response to direct stimulation by oenothein B than CD45RO− T cells (Fig. 8C). These data suggest that, as with bovine T cells, oenothein 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 oenothein 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 oenothein 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 oenothein B, they differ in their responses. In cattle, γδ T cells from calves express more IL-2Rα in response to oenothein B than adult T cells, while αβ T cells from calves and adults express similar levels of IL-2Rα in response to oenothein B. Certain polyphenols can prime T cells to proliferate in response to IL-2, likely due to the upregulation of IL-2Rα [3]. Our data suggest that oenothein B may prime αβ T cells from both young calves and adult cattle equally to IL-2, but may better prime γδ T cells from young calves. In humans, T cells from both cord blood and adults express the activation marker CD69 in response to oenothein 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 IFNγ and GM-CSF in response to oenothein 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 IFNγ production, compared to adult T cells [25]. These observations with oenothein 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 oenothein B than CD45RO− cells, CD45RO− T cells from adults still appear to produce more IFNγ in response to oenothein 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 IFNγ in response to staphylococcal enterotoxin B than CD45RO+ CD4+ T cells from young children [26].


A
Human Blood Mononuclear Cells
pg/ml IFNγ
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Adult
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Human CD3+ T cells
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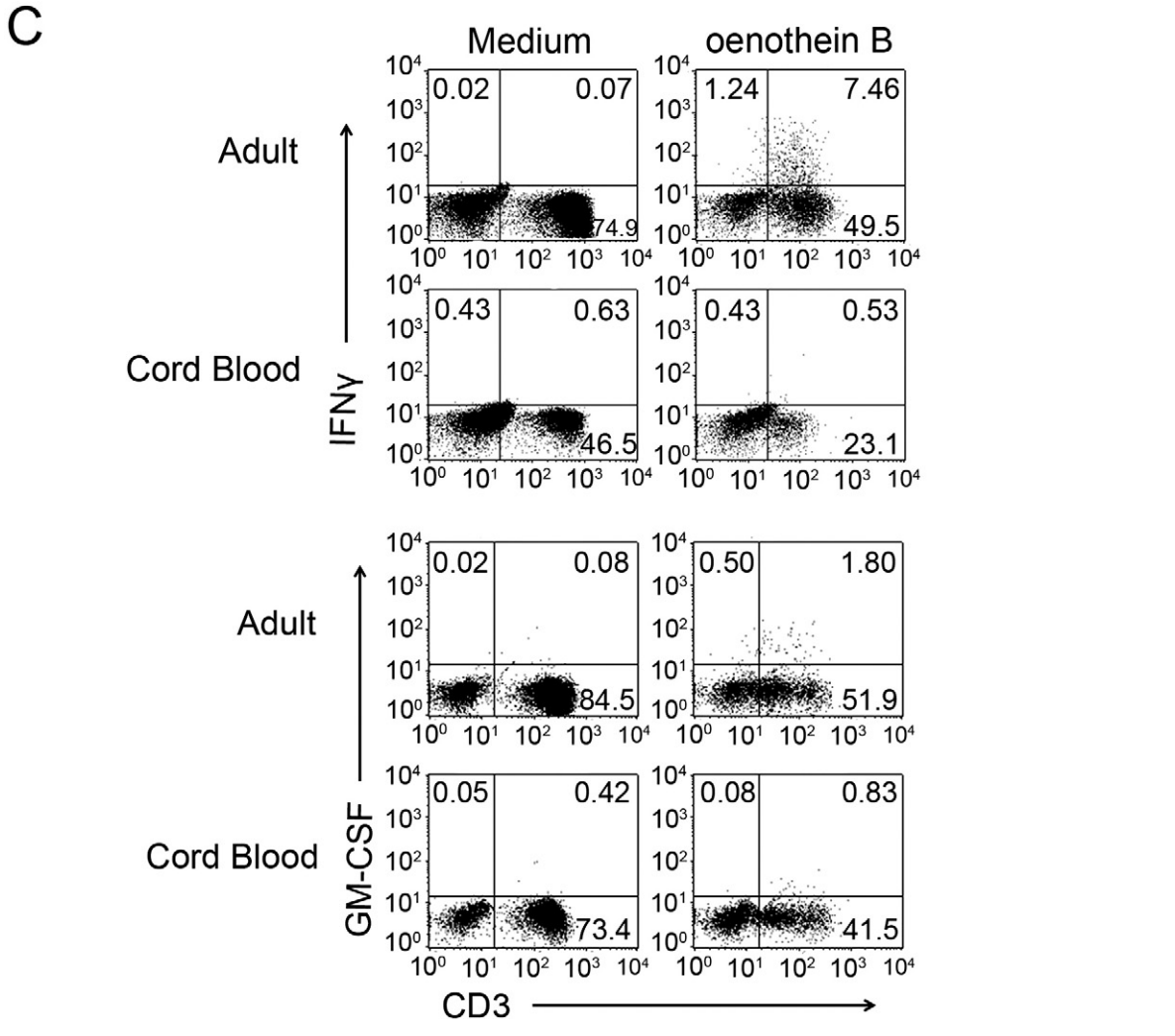
C
Medium
oenothein B
Adult
Cord Blood
IFNγ
0.02
0.07
74.9
1.24
7.46
49.5
0.43
0.63
46.5
0.43
0.53
23.1
Adult
Cord Blood
GM-CSF
0.02
0.08
84.5
0.50
1.80
51.9
0.05
0.42
73.4
0.08
0.83
41.5
CD3

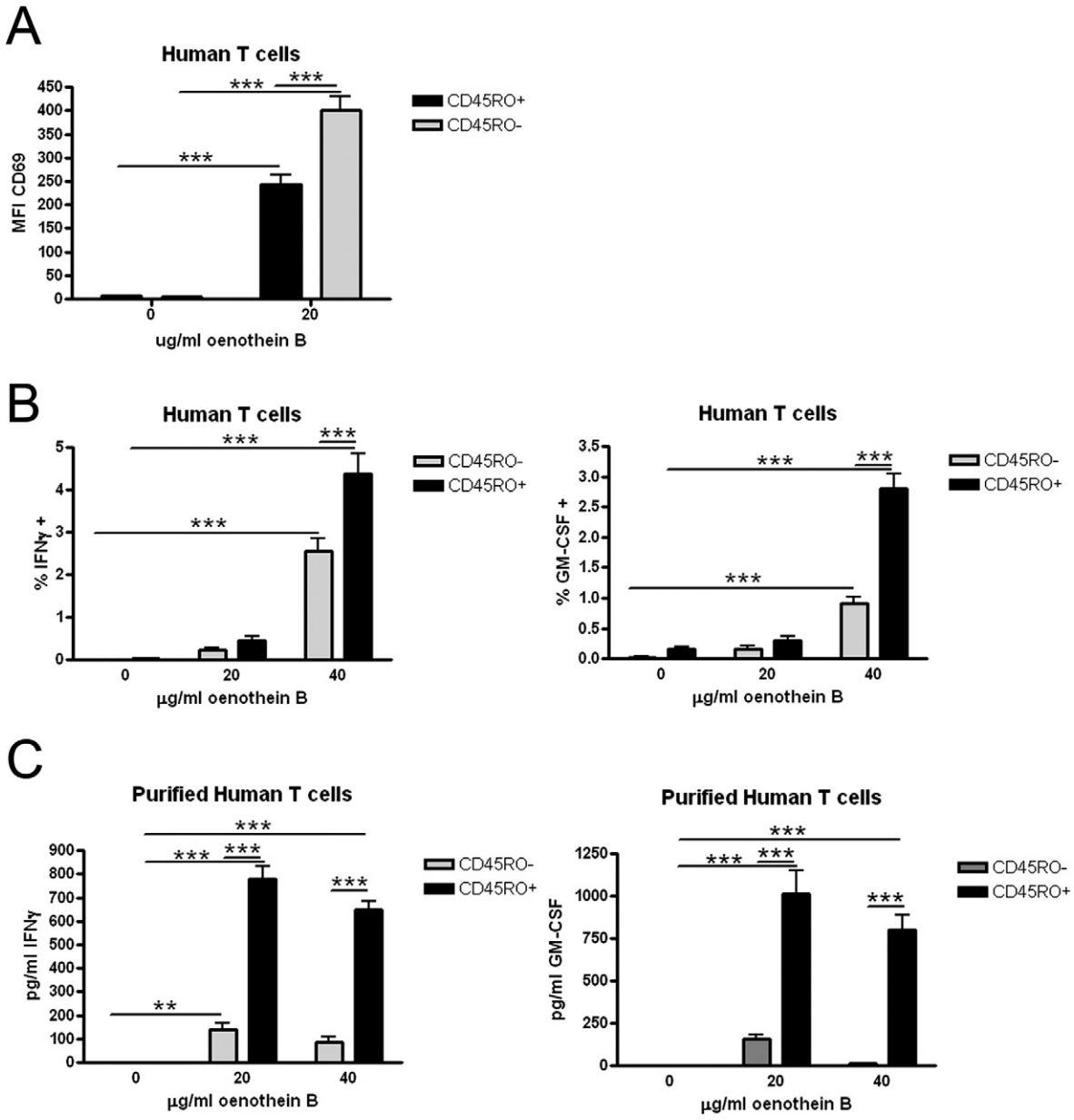

Fig. 8. Oenothein B induces more cytokine production by human CD45RO + memory T cells than naïve T cells. Human PBMCs (10^5 cells/well) isolated from adult donors (N = 3) were treated with the indicated concentrations of oenothein B or cRPMI medium alone for 24 h, with brefeldin A added for the final 6 h. 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. 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 48 h, 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. $^{**}p < 0.01$, $^{***}p < 0.001$.

Interestingly, sorted bovine CD4 + T cells, but not other T cells, could directly produce IFNγ in response to oenothein B. Therefore, bovine adult γδ T cells and non-CD4 + αβ T cells may require indirect stimulation for optimal IFNγ production, as our data shows that these cells produce IFNγ in mixed PBMC cultures, but not in sorted cultures. While it is currently unknown why only sorted CD4 + T cells could produce IFNγ in response to oenothein 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 oenothein B has similar activity.

Fig. 7. Oenothein B induces IFNγ 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 oenothein B or cRPMI medium alone for 24 h, and soluble IFNγ (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 IFNγ, N = 2 for GM-CSF) and adult (N = 1 for IFNγ, N = 2 for GM-CSF) donors were also treated with the indicated concentrations of oenothein B or medium alone for 24 h, with brefeldin A added for the final 6 h. The percent of CD3 + T cells expressing IFNγ (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 oenothein B or medium alone. $^{*}p < 0.05$, $^{***}p < 0.001$.

Future studies should examine the importance of accessory cells, such as monocytes and NK cells, for the optimal production of IFNγ by adult T cells in response to oenothein 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 IFNγ 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 IFNγ 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 IFNγ and GM-CSF production by adult and memory T cells in response to a non-specific agonist like oenothein 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 γδ 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 oenothein 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.

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