SELECT PROCYANIDINS INDUCE γδ T CELL ACTIVATION AND PROLIFERATION

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TABLE OF CONTENTS

TABLE OF CONTENTS - CONTINUED

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Many pharmaceutical drugs in use today were originally identified in plants from traditional medicine. However, there remain many plants in traditional medicine that produce confusing immune responses and are therefore unlikely candidates for pharmaceutical drugs. The effects of some of the traditional medicines that induce these confusing immune responses may now be explained by recent advances in the characterization of our immune system, namely in our understanding of the unique functions of the γδ T cell. These γδ T cell functions include tissue repair and homeostasis, cancer infiltration and clearance, pathogen detection and cytokine response, and antigen presentation. Although there are currently therapies being studied to increase the effector function of γδ T cells, these techniques are only active on a limited population of γδ T cells, the human Vδ2 subset. Although these cells are potent effectors against pathogens and some cancers, Vδ2 T cells demonstrate a restricted tissue distribution and limited effector function in other γδ T cell host defense responses. As such, we screened compound libraries and traditional medicines for agonists with activity encompassing alternative γδ T cell subsets. Tannins derived from select plant species are able to fulfill this role as demonstrated by the activation and expansion of γδ T cell subsets not responsive to current γδ T cell expansion therapies. The ability of tannins to expand these γδ T cell populations will potentially increase the therapeutic range of γδ T cells and may be used as treatments for wound healing as well as in the clearance of solid tumor cancers.

INTRODUCTION

Mankind has studied and used plants as medicinal compounds for millennia. There is no recorded date for when this practice began; however, there is evidence for use of herbal remedies as healing agents throughout history. Although understanding of our immune system and drug interactions has clearly progressed over time, natural products remain a dominant source of drugs in all fields of medicine, indicating natural products have a high incidence of pharmaceutically relevant compounds. One component of plant extracts, which show effectiveness in medicine are the polyphenols, particularly tannins, which have a high protein binding affinity. The immunologic effect of these species of plant metabolites is often confusing and even contradictory, which has stunted research into the properties of these metabolites. Increasing evidence demonstrates select binding affinities of individual tannin species, which explains the discrepancies in immunologic function. Herein we develop a method for identifying natural products which contain relevant tannin species and describe the immune response of these species derived from a limited number of plant extracts, which selectively activate γδ T cells. This immune cell compartment is linked to innate immunity and effectively prevents or clears bacterial, viral, and cancer diseases. Although there are alternative γδ T cell activating therapies available, all function in a similar manner and contain a number of disadvantageous characteristics which inhibit their therapeutic application. The use of tannin-based therapies can potentially overcome some of the pitfalls associated with these current

therapies by using tannin-based drugs as either stand-alone treatments or in conjunction with currently available therapies.

Medicinal Uses of Plant-Derived Products

Historic Medicinal Use of Plants

Herbal medicine is the oldest form of medicine used by mankind, and many of today's drugs are directly derived from these same herbal plants. There is extensive archeological evidence for humanity's use of plants as medicines dating to prehistoric times. The earliest evidence of selective use of therapeutic herbs is found in a Neanderthal grave from 60,000 B.C. These remains were discovered with eight species of medicinal plants including *Achillea* (yarrow, anti-coagulant/pain reliever[1]) and *Ephedra altissima* (Ephedra, Table 1)[2]. Additionally, dried woody fruit from *Piptoporus betulinus* was discovered on the remains of a Swiss man from 3300 B.C. and were likely used to treat the parasites discovered in his intestines[3]. *Piptoporus betulinus* contains agaric acid, which is a powerful purgative and induces short bouts of diarrhea, which would have reduced the parasite concentration.

The extensive use of medicinal herbs in early human history is further demonstrated by the appearance of herbalism texts in many early civilizations. The earliest written texts describing the medicinal uses of plants originate from Mesopotamia (2700 B.C.) and Egypt (1550-1600 B.C.). These texts describe various medicinal effects of herbs including the following: senna, thyme, juniper, frankincense, cumin, pomegranate root, henbane, flax, oakgall, aloe, capers, caraway, coriander, elderberry,

fennel, garlic, peppermint, cedar, poppy, and lotus[1;4;5]. Many of these are still in use today as herbal remedies and provide sources for today's pharmaceutical drugs. Morphine, for example, is found in the poppy bud.

Other civilizations also use plants as part of their traditional medicine. China has developed a complex history of herbalism, which remains a popular form of medicine used not only in China, but also worldwide. The earliest treatise found on Chinese herbal medicine dates to approximately 200A.D.; however in this treatise, Emperor Shen Nong (2700 B.C.) is credited as the father of traditional Chinese medicine[6]. Shen Nong is recognized as testing hundreds of plants for medicinal uses. One of the more popular discoveries attributed to Shen Nong includes *Ma Huang* (ephedra, Figure 1)[7], which is in use today as a decongestant in its synthetic form, pseudoephedrine.

A neighbor to China, India also shares an ancient herbal tradition that utilizes many of the same plants. India's practice of medicine, Ayurveda, was developed from four sacred texts of ancient wisdom, the *Vedas*. The oldest book, the *Rig-Veda*, dating to 1500 B.C., contains herbal formulas derived from more than sixty different plants[8]. One of the herbs described in the *Rig-Veda* as an antipsychotic, *Rauwolfia serpentina*, contains reserpine, which is effective in the inhibition of norepinephrine receptor signalling[9].

The earliest recorded Greek use of herbal remedies comes from Hippocrates, who advocated using a few simple plants, such as garlic[10] and scammony, as medicines[4]. The use of plants in Greek medicine was later elaborated upon by Theophrastus (300 B.C.) who described both medicinal uses and growth conditions for a number of plants in two

large treatises, *Enquiry into Plants* (nine books) and *On the Causes of Plants* (six books). The most influential Greek contribution to traditional plant medicine comes from Dioscorides (60 A.D.), a surgeon in Nero's army. Dioscorides tested and compiled herbals that remained known as the *De materia medica* throughout the Middle Ages in which it acted as the definitive medical text of its time[11]. Some of the herbs documented in these texts include both an anti-cancer agent and an anesthetic (*Colchicum autumnale* [Colchicines], *Mandragora officinarum* [scopalmine], respectively, Table 1)

Other cultures, which do not have a well recorded history such as the native peoples of Africa[12] South America[13], North America[14] and the aboriginal tribes of Australia[15], all also traditionally include the use of plants for medicinal purposes. Although few of these traditional medicines are scientifically tested for bioactivity, some that have show potential for treatment of maladies including pain[12], high blood pressure[12], asthma [16], and malaria[17]. Most of the plants used for traditional medicines by these cultures, however are untested in the laboratory setting and, as with other untested traditional plant medicines, may provide additional sources of pharmaceutical drugs.

Pharmaceutical Drugs Developed from Traditional Herbal Medicines

The use of many of these traditional, plant-derived medicines continues today, not only in the form of herbal remedies, but also as pharmaceutical drugs. Present-day advances in chemistry and biochemistry allow for the isolation of the active components from the original herbal medicines. In fact, 74% of today's plant-derived drugs were discovered as a result of studies to isolate the active components of traditional medicines[18]. The biologic activities of plants encompass a large number of therapeutic

uses ranging from cough suppressants to antipsychotics. The most important role of plant-derived drugs in current medicine includes anti-cancer drugs and analgesics, wherein these plant-derived drugs are the primary sources for the majority of available therapies (Table 1). Many of the molecules isolated from these traditional medicines are additionally used as a model chemical for the production of new generations of pharmaceutical drugs. Therefore, plant-derived drugs often serve not only as a drug in their own right, but provide a template for the production of new generations of drugs with minor molecular variations resulting in different or improved effects.

Plant-derived chemicals also formed the basis for one of the earliest cancer chemotherapy options, colchicine[19] which although effective, has since been discontinued due to toxic side-effects. Other families of chemotherapeutic drugs derived from plant products include both the topoisomerase inhibitors I (camptothecin) and II (podophyllotoxin), the taxanes and the vinca alkaloids. The topoisomerase inhibitors prevent the unwinding of DNA for replication while the taxanes and the vinca alkaloids both prevent chromosome division during anaphase by either preventing cell restructuring (taxanes, Taxol®) or inhibiting the formation of microtubules (vinca alkaloids, Oncovin®). In fact, the most widely used chemotherapy agent, Taxotere, is a synthetic analogue of Taxol, which is isolated from the Pacific Yew tree (Table 1).

Another well-known pharmaceutical drug, morphine, an opiate, was first used as a pain reliever in 1600 B.C. in the form of poppy juice[5] and remains the most commonly used prescription analgesic today. Other opiates, heroin and codeine, have similar effects and are also commonly used. Unfortunately, the opiates are very addictive, which limits

their widespread use. Other analgesics, such as aspirin, are used in day-to-day applications. Aspirin, or acetylsalicylic acid, is the active component of willow bark and was chemically synthesized as a pain reliever in 1897[20].

It should also be noted that a large number of modern drugs are obtained from other, non-plant species such as fungi and bacteria[21-23], which are not covered here. These sources have also been used in traditional medicine. For more information on traditional usages of both plant and non-plant medicines, the reader is referred to *Herbal and Traditional Medicine*[24]. As a group, natural products comprise the majority of drugs in use today as either an original natural product-derived entity or as a chemical derived from one of these natural product sources[25], and sales from these natural products account for 30% of the worldwide drug market[23]. However, there remain a large number of traditional medicines that have yet to be tested, indicating a number of these natural-product medicines still hold valuable ingredients for today's pharmaceutical market.

Table 1. Common Pharmaceutical Drugs Derived from Botanical Sources

Botanical name	Common name	Indigenous use	Origin	Medical use	Active compounds
Adhatoda vasica	N/A	Antispasmodic, antiseptic,	India, Sri Lanka	cough suppressant	Bromhexine (Bisolvon®)*
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Chemotherapy	Vincristine (Oncovin®)*
Condrodendron tomentosum	N/A	Arrow poison	Brazil, Peru	Muscular relaxant [26]	D-Tubocurarine
Pausinystalia yohimbe	Yohimbe	aphrodisiac	Africa	Erectile dysfunction[27]	Yohimbine
Convolvulus scammonia	Scammony	purgative	Mediterranean	purgative	scammonin
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Chemotherapy	Podophyllotoxin* (Eposin®)
Cinchona	Cinchona tree	malaria	South America	Anti-malarial antipyretic	quinine†
Camptotheca acuminata	Chinese Camptotheca	N/A	China, Tibet	Chemotherapy	Camptothecin* (Hycamtin®)
Colchicum autumnale	Autumn crocus	Rheumatism, arthritis,gout	Europe	Gout Chemotherapy	Colchicines† (ColBenemid®)
Taxus brevifolia	Pacific Yew	N/A	North America	Chemotherapy	Paclitaxel* (Taxol®)
Cannabis sativa	Marijuana	Pain, fever, nausea	Eastern Europe, India	Antiemetic Appetite stimulant	Tetrahydrocannabinol* (Marinol®)
Papaver somniferum	Poppy	Pain, euphoric	Eurasia, Africa, North America	Analgesic	Morphine† Codeine†
Salix alba	White willow	Pain, fever	Europe	Analgesic antipyretic	Acetylsalicylic acid Aspirin®†
Rauvolfia serpentina	Serpent root	insanity	India, Southeast Asia	Antipsychotic hypertension	Reserpine* (Serpasil®)
Digitalis purpurea	Foxglove	Pulse regulation	Europe	antiarrhythmic	digitoxin†
Ephedra sinica	*Ma Huang*, Ephedra	Stimulant, asthma	Eurasia, Africa, North America	Stimulant, Bronchodilator, Decongestant	ephedrine†
Atremisia annua	Chinese wormwood	Skin diseases, malaria	China	antimalarial	artemisinin
Mandragora officinarum	Mandrake	Anesthesia Sleep aid	Europe	Nausea anasthesia	scopalamine

* FDA approved.

† Marketed prior to the revision of the Food and Drugs Act (1938). These chemical entities were at that time marketed and therefore grandfathered. Therefore, they are not technically FDA approved.

Identification of Novel Drugs from Plant Species:
The High Throughput Library Screen

With the advent of new tools and robotics, the pharmaceutical industry developed methods for screening thousands of compounds for potential drug candidates. One such method, the high-throughput library screen (HTS) utilizes natural products as their source and has identified a number of biologically active metabolites, which were ultimately developed into drugs. For information on the progression leading from source to drug, the reader is directed to reviews offered by two leading pharmaceutical companies, Merck[28] and GlaxoSmithKline (formerly Glaxo)[29]. More recently, changes were made to HTS library screens by replacing natural compounds with synthetic compounds, with variable results. Most early screens of these synthetic compound libraries proved ineffective at identifying novel agonists and were hence modified to incorporate natural product structures. These more "natural" synthetic libraries attempt to mimic the diversity observed in natural product libraries, underscoring the importance of natural-like diversity in drug candidates.

Early drug development focused on extracting the active components of known bioactive extracts. These drugs include morphine from poppies, aspirin from willow bark, ephedrine from ephedra, penicillin from *Penicillium notatum*, and many others. Advances in both chemical separation methods as well as screening assays over the past 30 years has enabled scientists to screen large amounts of natural products with previously unknown bioactivity and to isolate the active component[28].

Using natural products as a testing source does not come without associated complications. Identification of the active component in these natural product extracts is

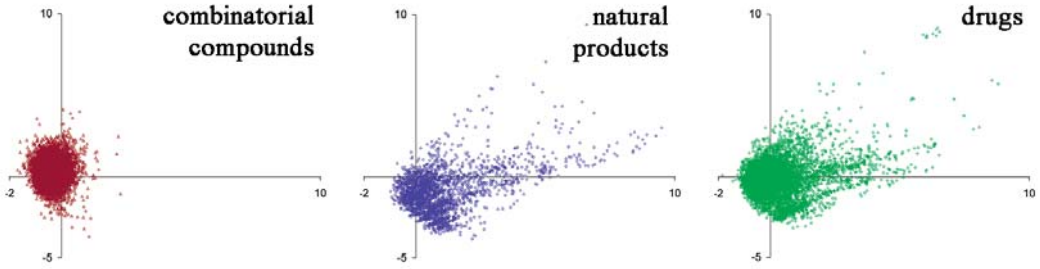
time consuming and costly. There are also often a number of patent rights issues accompanied with many plants, and the identified natural metabolite is often costly to produce. Therefore, many drug companies have begun producing synthetic compounds via combinatorial chemical synthesis. This process relies on solid-phase chemistry to modify basic structures, pharmacores, with additional chemical structures to generate new synthetic chemicals.

With the advent of combinatorial chemical synthesis, pharmaceutical companies are able to bypass many of the problems associated with natural product libraries. Namely, any chemicals found to have the desired effects were already known to be both readily synthesized and clear of patent issues. Due to these advantages, pharmaceutical companies largely adopted the use of combinatorial chemistry for the production of synthetic libraries in the 1990's. Unfortunately, these libraries provided few drug candidates. In fact, only one completely *de novo* synthetic compound has been identified, the antitumor compound Sorafenib®[25]. Further evidence of the negative impact these libraries has had upon the pharmaceutical industry is evidenced by the decreased number of drugs released; specifically, a 24-year low for the production of new drugs was set in 2004[25].

The reason for the ineffectiveness of these synthetic libraries is often attributed to the lack of diversity amongst the library compounds. Many compounds in the original synthetic libraries were simply minor modifications to a basic core structure. Therefore, these huge libraries contain very similar structures, most of which are not biologically relevant. Figure 1 describes the diversity of natural and combinatorial entities as

compared to pharmaceutical drugs. These analyses, performed by Feher and Schmidt[30], utilize principal component analysis to reduce the multidimensional data set, comprising the structural relationships of the three chemical entity types, to two dimensions. These two dimensions (x and y axis) explain 54% of the diversity among the three groups. As can be seen in Figure 1, combinatorial compounds demonstrate little diversity (clustered around the origin), whereas natural products and drugs demonstrate a large and similar diversity.

Figure 1. Analysis of Diversity in Natural and Combinatorial Chemical Entities as Compared to Pharmaceutical Drugs



Principal Component Analysis (PCA) of a random selection of combinatorial compounds (n = 13,506), natural compounds (n = 3287), and pharmaceutical drugs (n=10,968). Adapted from Feher and Schmidt 2003[30].

Due to the ineffectiveness of the first generation of synthetic libraries in generating pharmaceutical drugs, most synthetic libraries for drug screening now focus on producing more natural-like compounds[31]. This process relies on using pharmacores derived from natural or natural-like structures. A number of rules and standards were additionally developed to improve solubility, membrane permeability[32], and framework structures[33], which increase the potential for compound effectiveness. Using these standards and others as a guideline, computer models are produced to analyze each

chemical's drug potential before synthesis[34]. Utilizing these rules and standards, most libraries designed today are smaller, more diverse, and contain more bioactive molecules[35]. However, in respect to natural sources, these libraries still remain less diverse than natural product libraries since many structures produced naturally are difficult to produce via combinatorial chemistry. For example, one of the major differences between natural and combinatorial chemicals is ring structure diversity. Naturally-derived chemicals tend to be more rigid (non-aromatic) and contain more fused ring-ring bonds[30]. These structures are limited in synthetic libraries because the production of these moieties via combinatorial chemistry is difficult and costly and thus are rarely incorporated into synthetic libraries[30].

Whether the switch to natural-like chemical synthesis will improve the drug discovery rates of pharmaceutical companies remains to be seen. However, plant extracts contain many chemical moieties that still cannot be synthesized. Therefore, it is likely that key drug candidates will be overlooked in purely synthetic screens. For this reason, natural product libraries remain a valuable source for pharmaceutical drug screening.

Structure and Diversity of Plant Polyphenols

A number of plant metabolites demonstrate biologic activity on human cells, but one class of compounds of particular interest is the polyphenol. These secondary metabolites are unique to plants and therefore contain unique structures not found in either bacterial or mammalian systems. Of particular importance is a group of protein-binding polyphenols named tannins, which, originally, were named for their use in the tanning of leather or hides. There are a great number of processes for which the plant

employs tannins, ranging from herbivore protection to hormone regulation. Tannins are also found in high concentrations in many traditional plant medicines and the therapeutic effects of these traditional medicines trace to tannin interactions with the mammalian immune system. Tannins are classified as either condensed or hydrolysable depending on the structure of their core polyphenol and additionally have very different functions both in the plant and on mammalian cells. Recent evidence shows that tannins have preferential binding affinities for different protein sequences, which may explain some of the contradictory results observed when mammalian systems are treated with these plant metabolites.

For example, early studies on tannins demonstrated carcinogenic effects[36;37]. However, these studies were performed with tannic acid, a heterogenic and undefined mixture of tannins. This broad categorization of all tannins undoubtedly hindered studies on these plant metabolites. Furthermore, recent studies refuted the reports of tannin carcinogenity[38], and some authors further report cancer preventative properties of tannins[39]. Recent studies additionally describe incredibly diverse biochemical functions of individual tannins in both the plant and on mammalian systems. For this reason, current research now focuses of the activities of individual tannins. As a result, new focus was recently applied to the biologic activities of this polyphenol group in an effort to identify novel health-stimulating chemicals with success.

Polyphenol Classification

The polyphenol class of plant metabolites is characterized by the presence of multiple phenol rings. Polyphenols are further subdivided into lignin, hydrolysable tannins, and flavonoids (Figure 1.2). Polyphenols are derived using the shikimate pathway, a microbial and plant synthesis pathway for aromatic amino acids and other aromatic metabolites[40]. Each class of polyphenol is associated with specific functions in the plant ranging from scaffolding to host defense.

Lignin plays an important role in the cell wall of the plant and is the most abundant organic polymer after cellulose. This polyphenol forms a heterogeneous structure (Fig. 2) and acts in both the maintenance of the structural integrity of the cell wall as well as a scaffold for polysaccharides. The polysaccharides increase the hydrophobicity of the lignin structure, which facilitates water the transport throughout the tissues by preventing water absorption. This hydrophobic structure is crucial for capillary action, which allows water to pass through the lignin-lined capillaries without absorbing into plant cells[41;42].

The second class of plant polyphenols, hydrolysable tannins, is created using a sugar core surrounded by phenolic groups such as gallic acid residues. These residues can be subsequently modified by further addition of phenolic groups, oxidation reactions, or other polyphenols[43;44], thereby generating increasingly complex polyphenols. Hydrolysable tannins are so named because treatment with weak acids readily hydrolyses the gallic acid residues and produces phenolic acids with a carbohydrate core[45]. It is currently unclear what role hydrolysable tannins play in plant biology, however, they

likely play a role in numerous plant functions as indicated by highly diverse hydrolysable tannin structures as well as regulated production in plants. This indicates specialized functions by individual tannin species[46]. One indicated use of hydrolysable tannins in plant defense is as toxins to prevent herbivore predation, although characterizing these responses requires further study[46]. Most studies regarding the function of hydrolysable tannins focus on their effects in mammalian biology. Hydrolysable tannins show potential for therapies such as anti-cancer or anti-hypertensive agents[47]. These and other therapeutic applications for hydrolysable tannins will be covered in more detail in the following section.

Flavonoids are the final and most diverse family of plant polyphenols. These polyphenols comprise a functionally diverse family of over 9,000 described chemical entities with assorted activities throughout the plant world. Species from all orders of the plant kingdom, from the most simple to the most advanced, invest a significant amount of resources to the production of flavonoids[48], indicating the importance of these molecules in plant function. Flavonoids are further divided into chemical families including flavones, flavonols, anthocyanidins, procyanidins, and isoflavonoids. The basic flavonoids all demonstrate very similar three-ring structures (Fig. 2), which can be modified by the plant to produce a wide variety of phenol-rich rings with an incredibly diverse repertoire of functions. The largest and most varied flavonoids are composed of procyanidin monomers, which readily form large oligomers (also called condensed tannins). The incredible diversity available via modification of these ring structures

affords the plant with many unique metabolites used in day-to-day function, ranging from plant defense to gene regulation.

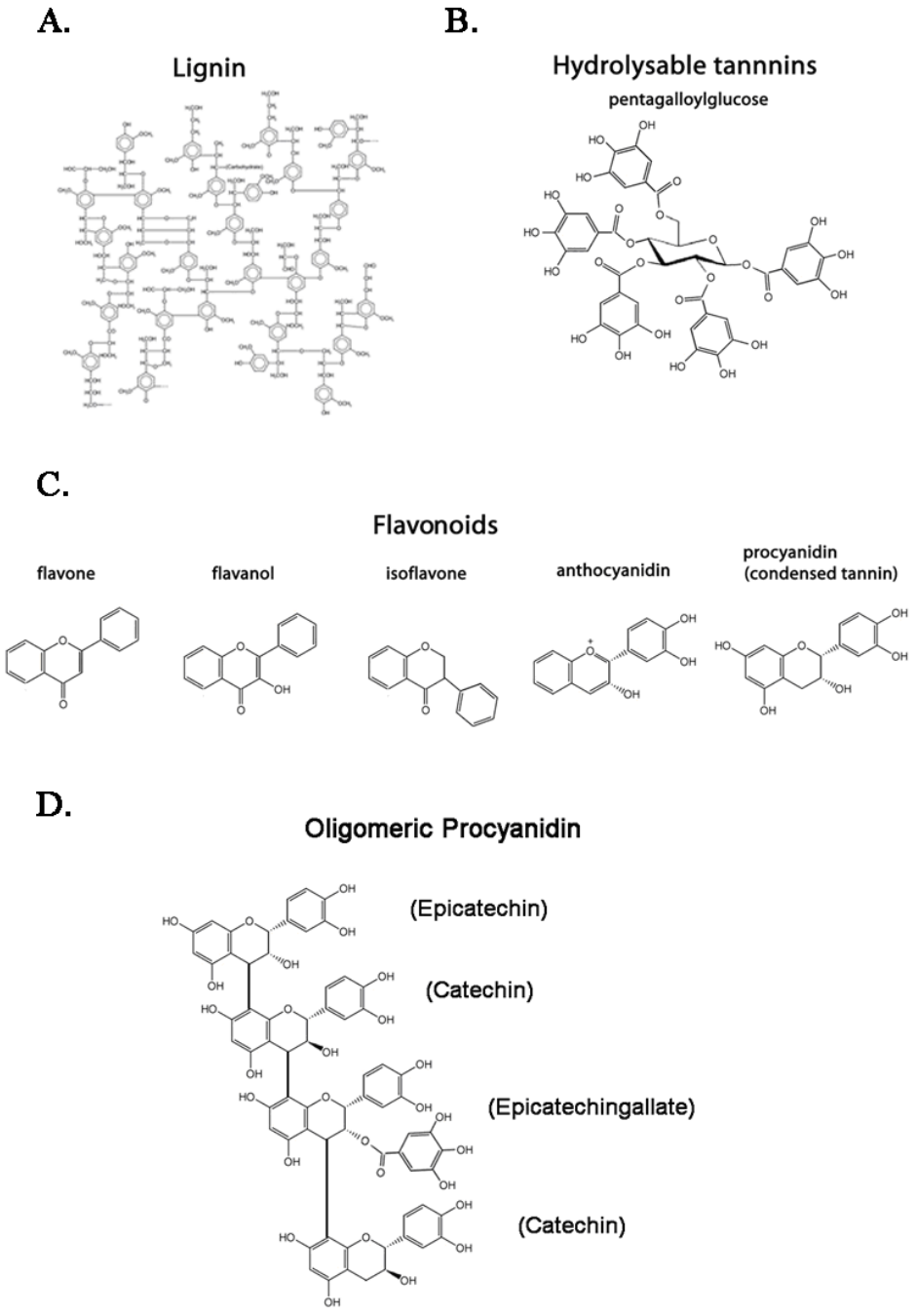
The flavonoids can be classified into two functional groups, colored and colorless. The colored tannins, which are predominantly anthocyanidins, are typically presented at the surfaces of flowers and leaves. These flavonoids add appropriate colors to aid in attracting pollinators[49] and also prevent insect predation since red is outside the visible spectrum for most insects[50]. Additionally, these colored flavonoids also prevent UV-induced cell damage[51]. The function of members of the colorless family of flavonoids are so named because they are not dependent upon their light emission and therefore are not necessarily colorless; in fact, they are often yellow[48]. In addition to the colored flavonoids, the colorless flavonoids also play a role in UV protection[52] but have a number of additional functions including the following: mitigation of temperature stress[53;54], heavy metal tolerance[55], reduction of oxidative stress[56], root nodule formation (nitrogen fixation)[57], mycorrhizal development (nutrient uptake)[58], fungal and bacterial[59] pathogen resistance, seed integrity[60], regulation of seed dormancy [61], phytohormone transport[62], regulation of gene expression[63] and toxic prevention of predation[64]. As seen from the number of functions attributed to flavonoids in the plant kingdom, these polyphenol complexes demonstrate incredibly diverse biologic activities, which are derived from the assorted flavonoid structures.

Of the colorless flavonoids, procyanidins represent a major fraction in both function[48] and content[65;66] of many plant tissues. The procyanidins, or condensed tannins, have the potential to produce highly diverse structures due to their formation of

oligomers, up to 28-mers have been identified[67], from combinations of monomer subunits, epicatechin or catechin. Furthermore, these oligomeric procyanidins are further modified by the addition of gallic acid residues. In grapes, for example, about 20% of the residues are galloylated[67]. By utilizing different procyanidin subunits and galloylation (see Fig. 2), oligomeric procyanidins are capable of generating a potential structural diversity reaching into the millions. This incredible capacity for diversity indicates the potential for functional diversity as well. This is, in fact, observed by both the wide range of activities observed by procyanidins in both the plant and mammalian systems.

Procyanidins, as well as hydrolysable tannins, are able to bind proteins with such high affinity due to their high incidence of polyphenolic nuclei. In particular these polyphenol subunits interact with the hydrophobic residues of proline[68]. These tannins aid in the defense of the plant by precipitating saliva proteins and preventing consumption. Tannins cause the dry, bitter taste associated with high-tannin foods such as non-ripe apples, grape skins, and strong teas. In addition to binding and precipitating salivary proteins, tannins will often also demonstrate a high affinity for other proteins in the mammalian system[69-71]. These interactions are currently being investigated in laboratory settings and evidence suggests that tannin species may account for the historic use of many plant extracts for medicinal benefit by their ability to bind specific proteins and trigger direct immune responses.

Figure 2. Structures of Common Polyphenol Subunits



A) Proposed structure for lignin, a complex, heterogeneous scaffold for carbohydrates. B) Pentagalloylglocose, the simplest hydrolysable tannin. The sugar core is oriented in the perpendicular plane. The five galloyl residues can be replaced, removed, or modified to increase diversity. C) Flavonoid families. D) Example of a tetrameric procyanidin, galloylated at the second procyanidin residue. Names of the monomeric form of each subunit are presented in parenthesis.

Health Benefits Attributed to Plant Tannins: In addition to studying tannin functions in the plant, a great deal of work is concentrated on the effect of both condensed and hydrolysable tannins on mammalian systems as well. In fact, a current literature search yields far more studies citing the effects of these polyphenols on other, non-plant species than on the plant itself (Pubmed search for the keyword "tannin"). Similar to the diverse activities in the plant, tannins also demonstrate a wide range of health benefits to mammals including antioxidant, anti-pathogen and anti-cancer activities as well as their immuno-stimulatory effects. Due to advances in tannin-chemistry and a greater understanding of these polyphenol complexes, a large number of these tannins have successfully been isolated from their original plant sources and are being developed into potential drugs for the treatment of a number of illnesses ranging from cancer to hypertension.

To achieve the diverse role tannins play within the plant system, they assemble into a large collection of three-dimensional structures. These unique three-dimensional structures result in highly active and specific molecular species that interact with plant molecules to generate the required diverse plant functions such as those previously discussed. Furthermore, many of the tannins which are, as a necessity, biologically active in the in the plant are most likely biologically active in the mammalian system, since there are many shared biochemical pathways and structures. This is evidenced by the many tannins already identified able to induce biologic effects on the mammalian system

(Table 2). These activities range from preventing urinary tract infection to anti-viral activities. Interestingly, all but one of these discoveries occurred within the last 10 years[72-81]. This indicates research into the direct effects of tannins, in particular individual, select tannin species, has only recently begun.

Table 2. Tannins with Characterized Health Benefits

Botanical name	Common name	Use in traditional medicine	Mode of action	Active component
Vaccinium oxycoccus	Cranberry juice	Urinary tract infection	Prevent bacterial colonization[82]	Procyanidins[72].
Magnolia lilflora	Magnolia tree	Antihypertensive, Allergies**	Angiotensin converting enzyme	Condensed and hydrolysable tannins[73]
Kola acuminata	Kola nut	Treatment of parasitic disease	Toxic to *Trypanosoma brucei brucei*	Oligomeric procyanidin[74]
Terminalia arjuna	Terminalia	Wound healing Blood pressure*, **	Epithelial regrowth	Tannin extract [75]
Woodfordia fruticosa	Fire Flame Bush	Numerous*	Topoisomerase II inhibitor	Woodforin C[76]
Vitis vinifera	Red wine	Solvent for many herbs*,**	Vasodilatation	Procyanidin[77]
Phyllanthus urinaria	Chamberbitter	Numerous*	Inhibit Herpes simplex 1 and 2 infection	geraniin[78]
Arbutus unedo	Strawberry tree	Hypertension	Decreases thrombin-induced platelet aggregation	Tannin extract [79]
Vitis vinifera	Grape seed	Not directly observed	Assists in tumor killing	Procyanidin[80]
Paeonia lactiflora	Peony	Numerous**	Inhibit nitric oxide synthase and cyclooxygenase activity	1,2,3,4,6-penta-O-galloyl-beta-D-glucose[81]

* Major source of Ayurvedic medicines[83]
** Major source of Traditional Chinese medicines[84]

Most plant-derived medicines in use by the pharmaceutical industry today are a result of the isolation of the active product from its traditional medicine source[18]. To make characterization of the multitude of chemical moieties in the plant a reliable pursuit, an isolated response must be seen from the traditional medicine. Therefore, plant medicines that illicit confusing effects, or those that seem to affect dissimilar diseases,

such as tannins, are typically discounted or viewed as a placebo effect. However, if the confusing effects of some tannins can be explained, characterization of the active compounds becomes a reliable avenue for the generation of novel drug candidates. One pharmacologically relevant function commonly found in plant tannins is their ability to regulate the inflammatory response. Understanding this mechanism would be of benefit to medicine, however the classical model of the immune system does not well explain the mode of action of these tannins. The recent characterization of unique mechanisms of immune function may shed light upon the inflammation modulatory activity of some tannins from traditional medicines.

Regulation of the Inflammatory Response by Select Tannin Preparations: The inflammatory response is a tightly regulated arm of the immune system which functions to recruit necessary cells to sites of infection for removal of the infectious agent and maintenance of tissue homeostasis. This response must be tightly regulated so as to specifically eliminate the infectious agent, but not destroy host tissue. Many diseases are associated with a disregulated inflammatory response including: allergies, asthma, autoimmune diseases (such as multiple sclerosis and lupus) and inflammatory diseases (including dermatitis, Crohn's disease and rheumatoid arthritis). Even in healthy individuals many infectious agents have developed methods of evading the immune response by producing metabolites to confuse or alter the proper inflammatory response and thereby enable a persistent infection[85-87]. Therefore, restoration of the inflammatory response has the potential to alleviate numerous diseases.

Many tannin preparations repair these disregulations in the inflammatory response and enable the proper host clearance of pathogen. However, these responses to tannin preparations are described by often opposing pro-inflammatory or anti-inflammatory host responses which are confusing with our current understanding of the immune system. For example, pine tree tannin alleviates inflammation in chemically induced inflammatory models[88] and also promotes inflammation in response to infection[89;90]. Additionally, studies on tannins from apple peel, grape seed, and cocoa demonstrate a similar ability to augment both pro-inflammatory[71;80;91] and anti-inflammatory[92-94] responses. This does not correlate with the system of grouping immune effectors as either pro- or anti-inflammatory. Therefore, using recent advances in our understanding of the tannin-induce inflammatory response, we propose tannins impart an improved responsiveness to inflammatory disfunction by increasing the function of the arm of the immune system responsible for correctly maintaining the inflammatory environment.

Attempts to describe the pro-inflammatory and anti-inflammatory functions of tannins have shed some light on the immune responses to these plant products, yet do not fully explain their activities. Some authors have identified individual tannin fractions that induce either anti- or pro-inflammatory responses[95-98]. These mixtures of pro-inflammatory and anti-inflammatory tannins in the same preparation may account for some of the conflicting results; however it is unlikely that in vivo regulation of inflammation is regulated solely by these mechanisms.

While this model of a mixture of pro- and anti-inflammatory tannins fits the classic model of either pro-inflammatory or anti-inflammatory effectors, there are some

inconsistancies. First, this model would be very susceptible to small changes in the tannin composition of the preprations, since this would skew the inflammatory response to either pro- or anti-inflammatory. The tannin preparations with immunoregulatory activity discussed above come from many different sources, and therefore are surely comprised of very different tannin populations. Therefore, the balance of pro-inflammatory and anti-inflammatory tannin species that would be required to regulate the inflammatory response would probably not occur in all of these tannin preparations. Secondly, evidence of a unique immune response comes from Cheshier et al., who describe the regulation of the inflammatory response as a restoration of Thelper1 and Thelper2 imbalance[89]. This observation is more in line with an elaborate host response, enabled by the tannin preparation, to correct imbalance as opposed to direct cell stimulation with a cocktail of pro-inflammatory and anti-inflammatory effector tannins.

Over the past 25 years, the characterization of a cell type now known to induce assorted immune responses, including the regulation of inflammatory responses, has occurred. This cell type, the γδ T cell is found both in circulation and resident in the tissues throughout the body. Furthermore, we show that this cell is able to directly respond to a limited number of tannin preparations both in vitro and in vivo. These data indicate that the inflammation regulatory activity observed in many traditional medicines is a direct result of stimulation of the γδ T cell population by the tannin components of these plants.

The γδ T Cell

The γδ T cell is an enigma in the field of immunology. Originally identified while sequencing an αβ T-cell receptor (TCR)[99], the γδ TCR is very similar in structure. Therefore, researchers originally believed it performed a role similar to the αβ T cell. However, the function of the γδ TCR is very different than that of the αβ TCR in that it does not recognize foreign antigen in the context of MHC[100] and therefore does not contribute to the classic adaptive immune response. The γδ TCR instead recognizes conserved epitopes of both self or non-self antigens and acts accordingly. This is indicative of a more innate function, allowing rapid response to pathogen invasion or cellular stress. Evidence of the γδ T cell innate response is demonstrated by the oligoclonality of the γδ TCR in the host. While αβ T cells are incredibly polyclonal, a minimum of 2.5×10^7 different αβ TCRs are expressed at any one time in the human, γδ T cells demonstrate restricted TCR diversity, best defined as oligoclonal[101]. This suggests that γδ T cells recognize a limited number of epitopes. Although the precise functions of γδ T cells in innate immunology are still vague, there is a great deal of evidence supporting γδ T cells as critical mediators in pathogen clearance, tumor sentry, and epithelium maintenance. The following will review the structure and unique functions of the γδ T cell as well as emerging therapeutic strategies for increasing the host's innate immune function by means of activating and/or expanding the γδ T cell population.

Structure of the γδ TCR

The γδ TCR is composed of two glycoprotein chains, the gamma (γ) chain, and its heterodimer, the delta (δ) chain. The framework structures of these chains are closely related to their αβ TCR cousins, with the γ chain demonstrating sequences structurally similar to the beta (β) chain and the δ chain being more similar to the alpha (α) chain. These conserved structures, namely organized immunoglobulin folds, allow association of the different chains into their respective heterodimers[102]. Like the αβ TCR, the γδ TCR also associates with the CD3 complex (CD3γ,δ,ε, and ζ), which is required and utilized by the γδ TCR for signaling responses[103]. Whereas the αβ CDR3 is limited in diversity since it must form a structure complementary to MHC class I or II and therefore must conform to a restricted conformation, the γδ T cell is not so restricted. Interestingly, for all of its similarity to the αβ TCR, the primary antigen binding domain of the γδ TCR demonstrates more similarity to the Ig heavy chain. This epitope binding domain, or complimentary-determining region 3 (CDR3) is larger and can bind a larger variety of epitopes, much like the immunoglobulin CDR3. Therefore, the γδ TCR can likely bind soluble antigen[100;104]. The generation of γδ T cells by the assembly of γ and δ chains is achieved by the same mechanism used to assemble and induce variation in B and αβ T cells, VDJ recombination[105].

VDJ Recombination and γδ T Cell Diversity

The γδ T cell utilizes VDJ recombination to generate the γδ TCR. Other than its use in forming the γδ TCR, VDJ recombination is also used by B cells to produce

immunoglobulin and by αβ T cells to generate αβ TCRs. VDJ recombination occurs by assembling DNA cassettes to form a unique sequence from which proteins of different conformation are synthesized. These cassettes, variable (V), diversity (D), and junctional (J), along with the constant region, form the basic structure of the cell receptor or immunoglobulin. Every species has a different number of each cassette type available for production of γδ and αβ TCRs or B cell immunoglobulin (Table 3).

Modification and insertion of different V, D, and J regions for the production of immunoglobulin or TCR generates a diverse repertoire of B and T cells able to recognize an almost unlimited number of potential epitopes. The immunoglobulin light chains, TCRα and TCRγ utilize only V and J regions, whereas the heavy chain of immunoglobulin, TCRβ, and TCRδ can additionally utilize one or more D regions. For B and αβ T cells, this creates a diverse repertoire of immunoglobulin or receptors to recognize foreign antigen. Binding of antigen leads to the expansion of the B or T cells containing the appropriate cell receptor. The expanded cells are then able to recognize and aid in the clearance of invading pathogen. These pathogen-reactive cells are then retained in the host to protect against subsequent infection. This forms the basis of the adaptive immune response. γδ T cells, however do not function in the classical adaptive immune response.

Table 3. Estimated VDJ Cassettes in Select Species

Mouse [106;107]

	αβ TCR		γδ TCR	
Segment	α chain	β chain	γ chain	δ chain
V	75	23	7	10
D	0	2	0	2
J	5	12	3	2

Human [107;108]

	αβ TCR		γδ TCR	
Segment	α chain	β chain	γ chain	δ chain
V	50	57	14	5
D	0	2	0	3
J	70	13	5	3

Chicken [106;109;110]

	αβ TCR		γδ TCR	
Segment	α chain	β chain	γ chain	δ chain
V	20	3	26	20-30
D	0	1	0	2
J	?	4	3	2

Bovine [111-113]

	αβ TCR		γδ TCR	
Segment	α chain	β chain	γ chain	δ chain
V	20	22	17	50
D	0	?	0	5
J	?	?	8	3

Estimated number of each V, D, or J cassettes available to assemble mouse, human, bovine, and chicken TCRs. Due to the interdispersed nature of the different segments within the chromosome, the values contained are estimates only, particularly the chicken and bovine species, for which the genome is incomplete.

Although γδ T cells can generate a higher receptor diversity than either αβ T cells or B cells[106], they actually demonstrate little diversity in vivo. Unlike the αβ T cell population, which contains $2.5x10^7$ different αβ TCRs[114], the γδ T cell population is composed of a relatively few clonal cell populations. These differences highlight the immune functions of these cell types. Whereas the αβ T cell must be able to respond to a diverse number of potential antigens to create an adaptive immune response, the γδ T cell population recognizes a limited number of conserved epitopes for a more rapid, innate response.

In all species studied to date, γδ T cells are the first T cell population to develop[115-117], and this will likely hold true for γδ T cell development in other species as well. However, the intricacies of this development process differ to some degree between species. Studies of murine γδ T cells demonstrate that the first T cells to appear during development are a clonal population of γδ T cells. These cells migrate to the epidermis where they reside throughout the life of the mouse[118]. Additional waves of clonal γδ T cells emerge from the thymus to populate the gut and then the reproductive tissues[118]. This controlled release of γδ T cell populations suggests a genetically programmed selection for VDJ recombination. Evidence for this is obtained by swapping the V chain groups in the mouse TCR loci. This alters the histone accessibility of different V chains, and instead of normal development, the order of γδ T cell waves is changed to produce the corresponding γδ T cell subsets at different times during development[119].

Unlike the clonal γδ T cell populations produced during murine embryogenesis, there is extensive VDJ rearrangement throughout embryogenesis of the bovine and chicken species[117;120]. This produces a highly diverse population of γδ T cells, which migrate throughout the body to designated tissues. In the mouse, the selective distribution of γδ T cells to different sites within the body suggests these γδ T cell populations are highly specific for recognition of epitopes specific for the designated tissue. Since similar tissue specificity is observed in both the bovine and the chicken coupled with increased TCR diversity, this suggests the bovine and chicken γδ T cell population is more promiscuous in terms of recognition of a variety of epitopes. This is increased complexity of the bovine and chicken γδ TCR loci compared to the mouse is presented in Table 3. Selection for a more or less diverse repertoire between species elicits phenotypic differences in the γδ T cell population and provides a method of categorizing species based on this TCR diversity.

Interspecies Prevalence of γδ T Cell Populations

γδ T cells are observed in all jawed vertebrates[106]. However, the complexity and abundance of γδ T cell populations greatly varies between species. In healthy humans and mice, γδ T cells typically contribute a minor fraction of the peripheral blood (0.5-5%)[106] whereas the peripheral blood of cattle, sheep[121], pigs[122] and chickens[117] contain as many as 30-60% γδ T cells. For this reason animals are classified as either γδ-high or γδ-low. This difference in γδ T cell concentration correlates with TCR loci complexity (Table 3). Complex γδ TCR loci are observed in γδ-high species[117;121] while simple γδ T cell loci

are found in γδ-low species[123;124]. The limited γδ TCR repertoire in γδ-low species correlates with a more diverse αβ repertoire. Six et al.[117] propose this is a result of selective evolutionary pressures producing preferential αβ and γδ TCR expression depending on the natural environment of the organism. Although the selective pressures that would cause this selection remain undefined, one possible explanation is that both ruminants and chickens are repeatedly challenged with pathogens. Specifically, due to the grazing nature of these γδ-high animals their gut mucosal immune system comes into contact with a very different environment. These animals more regularily come into contact with plant-derived foodstuff and soil microbes, and the immediate response provided by γδ T cells may be of greater benefit than would the αβ T cell.

The gross variation in the γδ T cell contribution between different species only affects the peripheral blood. Mucosal surfaces of all animals, even those with low concentrations of γδ T cells in the periphery, typically have high concentrations of γδ T cells in the lymphocyte population, up to 50%. This indicates a conserved and required function for γδ T cells in these tissues. These mucosal γδ T cells are part of the mucosal population of lymphocytes termed intraepithelial lymphocytes (IELs) and play a large role in the maintenance of the epithelial lining. The IEL population, including the γδ T cell portion, is a unique T cell population with functionality very different from both circulating αβ and γδ T cells. For this reason, γδ T cells can be functionally divided by their function and location in the body into two subsets, the tissue-specific and the circulatory γδ T cells.

Functional Characteristics of γδ T Cell Subsets and their Tissue Localization

γδ T cell populations are phenotypically categorized into functional classes by either their variable chain usage, as in the case of human and murine species, or the expression of WC1, a γδ T cell-associated scavenger receptor in ruminants. Broadly speaking, these functional classes are described as either pro-inflammatory or anti-inflammatory with the characteristic pro-inflammatory γδ T cell constituting the majority of the circulating γδ T cell population. Among the different species, this pro-inflammatory γδ T cell is Vγ1 and Vγ4 in mice, $WC1^{+}$ in bovine, and Vδ2 in humans (Table 4). These cells maintain the same function in all species; they express chemokine receptors for the invasion into inflamed tissues and produce large amounts of pro-inflammatory cytokines, such as IFNγ, in response to pathogens. Functionally, pro-inflammatory γδ T cells are implicated in the clearance of cancer cells, virus, bacteria, and protozoan parasites. Alternatively, tissue-localized γδ T cells are found in healthy tissues, where their primary role is in tissue maintenance. These γδ T cell subsets include Vγ5, Vγ6, and Vγ7 in the mouse, $WC1^{-}$ in the bovine, and Vδ1, Vδ3, Vδ5 in the human (Table 4). Tissue-specific γδ T cells play a role in maintaining tissue integrity by secreting growth factors to stressed epithelium during tissue damage and, depending on the environment, can be induced to recruit inflammatory responses for the elimination of pathogen.

As mentioned previously, human and mouse γδ T cell repertoires are more limited than the γδ-high species, but share similar immune function. Whereas the γδ-high

species' γδ T cell V regions demonstrate a large number of V regions with high sequence similarity[121;125], the V repertoires in the human and mouse do not[126]. This indicates that these TCR regions developed to recognize only a few critical epitopes. Therefore, expression of select V regions correlates to function and tissue distribution differences in the γδ T cells in these species. Functional differences between V gene usage in these γδ-low species are defined by a number of studies comparing gene expression[127] with cellular responses (Table 4), receptor profiles[128], antigen recognition[129;130], and trafficking[128;131;132]. Many of these different functions are attributed to the structural confirmation of the different Vγ or Vδ cassettes allowing for recognition of discrete epitopes by the TCR. Other functions of these subsets are defined by the expression of non-TCR receptors, indicating that the effectiveness of γδ T cells is not limited to TCR recognition, but that the γδ T cell has many receptors for sampling the environment.

Defining the bovine and chicken γδ T cell populations by TCR usage is likely an impossible task due to the highly developed γδ T cell repertoire of these γδ-high species. Whereas the γδ-low species produce only a few V genes that are selective for critical epitopes, the γδ-high species contain more V genes with similar sequence conformations. This commitment to generating a more diverse γδ T cell repertoire indicates that variable genes of similar conformation likely recognize similar epitopes and therefore probably share similar function and tissue localization. Support for this hypothesis is evidenced by the lack of a preferential distribution of Vγ chains in the tissues or the peripheral blood[125]. Furthermore, unlike the murine and human γδ T cell population, which contain only 1 or

2 D insertions, the bovine γδ T cell inserts up to 4 D cassettes, thereby increasing the potential diversity of the TCR without utilizing a different V region[125]. This ability to increase TCR diversity allows the γδ TCR to bind more epitopes while using the same V regions. In spite of the need to recognize a greater variety of epitopes via the γδ TCR due to the limited αβ TCR repertoire in these animals, the γδ T cell population in cattle and chickens remains oligoclonal. This indicates similar γδ T cell development creates oligoclonal γδ T cell populations in both the γδ-low and γδ-high species. Furthermore, since populations of γδ T cells in these different species share the same functional characteristics, similar epitopes are likely recognized by the γδ T cell populations of all species. Evidence of similar γδ T cell epitope recognition by different species will be provided in the section covering γδ T cell epitope recognition.

Regardless of the variety of TCRs utilized by ruminant γδ T cells, the expression of WC1, or lack thereof, correlates with distinct γδ T cell phenotypes[133]. Like the human and murine subsets, both inflammatory function and tissue localization can be categorized by the expression of WC1 on pro-inflammatory γδ T cells[134;135]. This may not come as a surprise since many other surface markers, indicative of function and/or tissue localization, are differentially expressed among the different pro-inflammatory and tissue-specific γδ T cell subsets of all species[131;132;135-137]. This underscores the divergent functions of these two γδ T cell subsets, and since both of these γδ T cell subsets are found in all species with similar function, there is a critical requirement for both pro-inflammatory and anti-inflammatory γδ T cells.

Pro-inflammatory, Effector γδ T Cell Functions: Circulating γδ T cells are generated during fetal development with high diversity. Selection of responsive γδ T cells is achieved by thymus-independent expansion of responsive γδ T cell populations[138]. These γδ T cell populations act as primary responders for invading pathogen including parasites, viruses and bacteria as well as against multiple cancers. These cells express chemokine receptors which upon infection, allow the pro-inflammatory γδ T cell to migrate to the site of infection and aid in pathogen removal[131]. The efficacy of these cells against pathogens and cancers is shown in vitro as well as in numerous animal models. The arsenal utilized by γδ T cells for the clearance of pathogen and cancer includes defensin secretion, Fas-Fas mediated apoptosis, perforin/granzyme production, cell recruitment, and production of pro-inflammatory cytokines. Due to their ability to recognize conserved antigen, these γδ T cells respond immediately and aid in the containment of pathogen until more effective adaptive immune responses are generated. In persistent illnesses, γδ T cells play a crucial role in controlling either pathogen or cancer.

The γδ T Cell Response to Bacterial Infections: The importance of pro-inflammatory γδ T cells in bacterial infections is seen during infection with *Francisella tularensis.* Tularemia induces a large expansion of pro-inflammatory γδ T cells, which can cause γδ T cell levels in the human to reach up to 50% of the total peripheral blood population[139]. γδ T cell expansion is caused by the recognition of bacterial products which additionally inducess the production of antibacterial products such as defensins[140]

and perforin[141] as well as inducing a pro-inflammatory response, which recruits other Th1 cells to clear the infection.

Cytokines released by γδ T cells during bacterial infection include IFNγ, TNFα, MIP1α, MIP1β, and RANTES. Using these cytokines, γδ T cells demonstrate a critical role in control of numerous bacterial pathogens. For example, during infection with *M. tuberculosis* γδ T cells are a key mediator of the Th1 response in the lungs during infection. They control the pro-inflammatory response and thereby the formation of granulomas by recruiting macrophages[142] and increasing the cytotoxic response from dendritic cells[143], both of which are key in controlling *M. tuberculosis* infection[144]. Furthermore, γδ T cells are able to directly kill both intracellular and extracellular *M. tuberculosis* via the release of granulysin/perforin[145]. The importance of pro-inflammatory γδ T cell subsets is also seen in many other bacterial infections where γδ T cell populations dramatically expand to fight the infection. These infectious agents include: *Salmonella*, *Legionella*, *Coxiella burnetii*, *Rickettsia, H. influenzae, N. meningitides, S. pneumoniae,* and *Listeria*[146].

The γδ T Cell Response to Viral Infections: γδ T cells play an important role in the control of both RNA and DNA virus infections with the exception of rotavirus[147]. This control comes mainly in the form of IFNγ production from pro-inflammatory γδ T cells. However, other cytokines including TNFα, IL-8, RANTES and MIP-1α are commonly observed[148]. γδ $TCR^{-/-}$ mice demonstrate an increased West Nile virus titer and viral dissemination into the CNS when compared to wild-type mice[149]. Mice depleted of γδ T

cells demonstrate severe HSV-1 induced epithelial lesions and mortality due to an inability to control viral replication[150]. Human Vδ2 are increased in Herpes simplex type 2 viral lesions, where they possess a pro-inflammatory profile as observed by up-regulation of IFNγ, TNFα, IL-8, RANTES and MIP1-α[151]. A similar pro-inflammatory response is seen during HIV infection. In fact, pro-inflammatory γδ T cells directly lyse HIV-infected cells[152]. Moreover, testing in the SIV model demonstrates that γδ T cells become activated and suppress viral replication in this disease as well [153]. However, the effects of Vδ2 T cells during prolonged HIV infection appear to be limited since the virus quickly depletes the Vδ2 population. In fact, the Vδ2 subset is the first T cell population to be depleted during HIV infection[154].

The γδ T Cell Response to Parasitic Infections: As with other pathogens, γδ T cells play an important role in maintaining a pro-inflammatory response against protozoan parasites. In fact, during the early stages of *Plasmodium falciparum,* the causative agent of malaria infection, the predominant source of IFNγ is the γδ T cell[155]. High IFNγ production at this early stage of infection correlates with decreased disease morbidity and mortality[156], demonstrating the importance of the immediate γδ T cell response to this pathogen.

Other protozoan parasites including *Trypanosoma cruzi*[157], *Toxoplasma gondii*[158] and *Cryptosporidium parvum*[159] are controlled, in part, by γδ T cells. γδ T cells were not shown to be critical for the control of one example of *Leishmania major* infection; however γδ T cells did produce IFNγ during infection as demonstrated by a decreased

amount of IFNγ in $\gamma\delta^{-/-}$ mice[160]. This indicates that the γδ T cells do respond to the pathogen and under different conditions, may help moderate this infection. Further investigation into the response of γδ T cells during *Leishmania* infection is therefore warranted.

The Anti-cancer Effects of γδ T Cells: A number of anti-cancer effects are known for γδ T cells. In fact, every subset of human γδ T cells studied shows some cytotoxic activity against at least one cancer cell line[108;161]. γδ T cells show cytotoxic responses against many types of cancer including: bladder cancers, breast cancers, colon carcinomas, lymphomas, myelomas, melanomas, nasopharyngeal carcinomas, neuroblastomas, pancreatic carcinomas, prostate cancers, renal cell carcinomas, and small-cell lung cancers[146]. Unfortunately, the direct mechanisms of cytotoxicity are still largely unclear. Recognition of some tumor cells may be due to increased expression of MHC-like ligands, MICA and MICB, although it is unlikely that these are the only recognition factors since both MICA and MICB are expressed on healthy cells[162]. Another possible recognition mechanism is endogenous phosphoantigen, which is up-regulated in tumor cells[163]. Phosphoantigens are a class of Vδ2-specific agonists that will be discussed in detail in a later section. However, the endogenous phosphoantigens are not particularly potent activators of Vδ2 T cells. For this reason, the concentrations of endogenous phosphoantigen required to activate γδ T cells in vivo may not be achieved by these tumor cells. Recently, a third method of tumor recognition by γδ T cells was identified. This surface molecule, F1 ATPase (F1), is up-regulated on tumor cells and, in

complex with apolipoprotein A-I, induces γδ T cell activation and tumor killing. These three mechanisms for tumor recognition by γδ T cells are probably not fully comprehensive regarding how tumor cells are recognized. As such, researchers are currently attempting to expand the known repertoire of γδ T cell tumor recognition.

Tissue-specific γδ T Cells: The second category of γδ T cell promotes quiescence and epithelial maintenance via the production of anti-inflammatory cytokines and growth hormones. These γδ T cell subsets are typically found in tissues such as the skin or mucosa. In the mouse, there are a number of different γδ T cell populations preferentially localizing to different tissues such as the gut, Vγ7, the skin, Vγ5, and the reproductive tract, Vγ6 (Table 3). In the human and bovine, this highly selective and preferential TCR distribution to tissues does not occur, but there remains a preferential, albeit less defined, distribution of γδ T cells in the tissues. In the human, there is an increased distribution of some Vδ-specific γδ T cells, Vδ1, Vδ3[164;165] and Vδ5[108] in the tissues, which are rare in the peripheral blood. Additionally, in the bovine, tissue distribution correlates with scavenger-receptor and CD8 expression, specifically the $WC1^-$ and $CD8^+$ subset[135]. These selective distributions of γδ T cell subsets indicate a similar mechanism for migration of select γδ T cell subsets to tissues in all species.

Due to the highly selective migration patterns of murine γδ T cell subsets and the availability of mice as an animal model, the functions of these γδ T cells are best described. Murine γδ T cells of the skin, Vγ5, and gut, Vγ7, secrete growth hormones in response to damaged or stressed epithelial cells, namely keratinocyte growth factor

(KGF)[166] and insulin-like growth factor 1(IGF-1)[167]. The importance of these tissue-specific γδ T cells in the mouse is observed by a reduced healing rate of damaged skin in the $\gamma\delta^{-/-}$ mouse compared to the wild-type. The same response is seen in gut healing, wherein $\gamma\delta^{-/-}$ mice develop spontaneous colitis[168] and are slower to recover from damage to the intestinal tract[169].

A similar γδ T cell response appears to occur in human tissues; however, due to the limited availability of human tissue samples, most of the γδ T cell responses must be implied from indirect observations. Patients with inflammatory bowel disease (IBD) are a common source for human γδ T cell studies and inflamed tissues from these patients possess an increased γδ T cell population compared to non-inflamed tissue[170]. Furthermore, IBD patients produce large amounts of KGF in the intestine and have an increased expression of KGF receptor in the intestinal tissues, supporting the role of KGF and γδ T cells in human mucosa maintenance[171]. This suggests that the γδ T cells in humans play a role similar in the gut as that role seen in mice. Finally, patients with atopic dermatitis have a decreased peripheral γδ T cell population[172], which additionally supports a role for human skin-specific γδ T cells similar to that in the murine model.

Tissue-specific γδ T cells appear to have different functions depending on the location of these cells. This is most evident by the distribution of murine γδ T cell subsets to different tissues. One of the most elaborate environments for tissue maintenance is the gut epithelium. The immune system must be able to respond to pathogen; however, classic methods of antigen recognition cannot apply since the

immune system must suppress activity against commensal bacteria. Immune responses in this environment are therefore distinct from elsewhere in the body, and specialized lymphocytes, intraepithelial lymphocytes (IELs) of which specialized γδ T cells are a large constituent, colonize the mucosal surface of the gut.

γδ T Cells, a Compartment of the IEL Population: The gut mucosa is protected by a large number of IELs, which can be found interspersed between epithelial cells directly facing the flora of the gut. Since the gut mucosa is the barrier between the outside environment and the organism, this tissue is the front line of defense against pathogen, and IELs play a key role in its defense. In fact, there are at least 15 IELs for every 100 epithelial cells in the mucosa[173]. IELs are comprised almost exclusively of T cells, which can be divided into one of 2 categories, Type A or Type B[174]. Type A T cells are most similar to classical T cells; they are αβ T cells expressing CD4 or the typical CD8αβ heterodimer. Alternatively, Type B T cells are αβ and γδ T cells expressing CD8αα, which demonstrate an unconventional phenotype.

CD8αα$^+$ αβ T cells and CD8αα$^+$ γδ T cells are both included in the same group, Type B IELs because, although the CD8αα$^+$ αβ T cells are MHC-dependant, whereas CD8αα$^+$ γδ T cells, like all γδ T cells, are MHC-independent, both CD8αα$^+$ αβ and CD8αα$^+$ γδ T cells demonstrate similar gene expression profiles[174] and oligoclonal TCR profiles[175;176] suggesting similar functions. Perhaps the most interesting aspect of Type B IELs is that some develop independent of the thymus. In nu/nu (athymic) mice, no Type A T cells are detected in the spleen, gut, or periphery of these mice. However, both

CD8αα$^+$ αβ and CD8αα$^+$ γδ T cells are found in the gut mucosa, albeit at levels lower than wild-type. This would indicate Type B IELs develop in a heretofore unidentified, thymus-independent mechanism[177].

Similar to other tissue-associated γδ T cells, the IEL population is responsible for maintenance of the epithelial layer. The gut Type B IELs play two opposing roles. First, they can be induced to a pro-inflammatory state to protect against infection but also prevent extended, over-exaggerated pro-inflammatory responses[174]. The role of γδ T cell IELs in particular is evidenced in γδ$^{-/-}$ mice. These mice experience a magnified inflammatory response in the gut during parasite infection, which can be relieved by γδ T cell adoptive transfer[169], indicating the importance of γδ T cell IELs in correcting inflammatory imbalance. Other studies support the theory that γδ T cell IELs suppress Th1 cytokine release from other T cells[178]. Furthermore, γδ T cell IELs, but not αβ IELs, produce KGF to aid in epithelial regrowth[179]. From these studies, it is apparent that γδ T cell IELs, like other tissue-specific γδ T cells, play a unique role in maintenance of epithelial integrity by both preventing excessive inflammatory responses and in healing the epithelium.

Pro-inflammatory Responses of the Tissue-specific γδ T Cell Population: Some of the pro-inflammatory functions attributed to the circulating set of γδ T cells can additionally be induced in the tissue-specific subsets[180]. In particular, there are examples of tissue-specific γδ T cells that are able to lyse tumor cells. In fact, they may have a higher propensity for certain cancers as, unlike circulating γδ T cells, these cells are able

to infiltrate solid tumors[181]. Additionally, tissue-specific γδ T cells express surface receptors indicative of proinflammatory function, and their engagement promotes pro-inflammatory responses. The functions of these receptors, including Toll-like receptors[182] and members of the NKG2 receptor family[183], will be discussed in a following section.

This dual function of the tissue-specific γδ T cell may at first appear contradictory to the classification of γδ T cells as either pro-inflammatory or anti-inflammatory. However, since tissue-specific γδ T cells are chiefly resident cells, unlike the circulating γδ T cells, their chemokine receptors are specific for migration to non-inflamed tissues[131;132] and thus their primary function is not as a pro-inflammatory effector. Instead, evidence indicates the tissue-associated γδ T cell functions to maintain the epithelial layer and as such encountered pathogen must be cleared to maintain epithelial integrity.

Therefore, a model of inflammation regulation is proposed for tissue-specific γδ T cell. Since tissue-γδ T cells act as sentinels of the epithelial layers, they are in a unique position to recognize pathogen invasion, which must be cleared to maintain mucosal integrity. The pro-inflammatory response from tissue-specific γδ T cells is likely induced upon pathogen recognition, which recruits pro-inflammatory cells to clear the pathogen. Then, subsequent damage from the pro-inflammatory response induces anti-inflammatory and tissue repair responses from the same tissue-specific γδ T cells. This model explains the inducible pro-inflammatory responses of tissue-specific γδ T cells and at the same time explains the anti-inflammatory, tissue repair responses commonly seen from these

cells during inflammation. Additional studies focusing on the inducible aspects of the tissue-specific γδ T cell population are required to further characterize the ability of these cells to regulate the inflammatory response.

Table 4. Immune Functions of Murine, Human, and Bovine γδ T Cell Subsets

Murine γδ T cell function Classification designation used by Heilig et al. (1986). Scheme by Garman et al. (1986) in parenthesis.	
Vγ1 **(Vγ1.1)**	Major peripheral recirculating γδ T cell Constitute 15% of γδ T cells in the lung Promote airway hyper-reactivity in the lung [184] Kill *Listeria*-infected macrophages via Fas-FasL
Vγ4 **(Vγ2)**	Pro-inflammatory functions Produces IFN-γ during coxsackie virus infection Kills coxsackie-infected monocytes via CD1d [185] Anti-inflammatory functions Suppress airway hyper-reactivity in the lung [186] Produce IL-10 when cultured with *Listeria*-treated macrophages[187] Constitute 45% of γδ T cells in the lung[188]
Vγ5Vδ1 **(Vγ3Vδ1)**	Primary γδ T cell population of the skin Wound repair and homeostasis[189] Constitutively produce insulin-like growth factor 1 (IGF-1)[190] During stress, produce keratinocyte growth factor-1 (KGF-1)
Vγ6Vδ1 **(Vγ4Vδ1)**	IELs of the tongue and reproductive tract Exhibit anti-inflammatory and tissue repair properties[191]
Vγ7 **(Vγ5)**	Majority of the IELs of the intestine Generated in the absence of a functional thymus Maintain the integrity of intestinal tight junctions[192]
Human γδ T cell function	
Vδ1	Major tissue-associated subset anti-inflammatory (IL-10, IL-11)[127] infiltrate and kill solid tumors[193]
Vδ2	Major circulating subset pro-inflammatory (IFNγ, TNFα, IL-17, IL-21, M-CSF)[127] Antitumor effectors[194]
Vδ3	Virtually non-existent in peripheral blood Vδ3 make up approximately half of the skin γδ T cell population[165] Antitumor effectors Increased population size in lupus patients[195]

Table 4. Continued

Bovine γδ T cell function	
WC1-, CD8$^+$	tissue-associated[134], anti-inflammatory[133]
WC1.1$^+$	peripheral blood[134], pro-inflammatory[133] produce IFNγ in response to *Leptospira* antigen[196] decreased concentration in blood during aging[196]
WC1.2$^+$	peripheral blood[134], pro-inflammatory[133] produce IFNγ in response to *A. marginale* surface protein[197]

Epitope Recognition of the γδ T Cell

The function of γδ T cells hinges on their ability to recognize conserved epitopes and immediately respond in the manners previously described (Table 4). Although a number of functional responses are attributed to the various γδ T cell subsets, identification of the precise mechanism of recognition is often difficult. As such, only a limited number of γδ T cell ligands are currently characterized. These identified ligands include both TCR ligands and non-TCR receptors uniquely expressed on γδ T cells. As would be expected of these innate immune cells, γδ TCRs recognize conserved patterns indicative of cellular stress or pathogen infection. The non-TCR receptors present on γδ T cells follow similar guidelines, and are in fact commonly expressed on other innate cells as well such as NK cells or monocytes.

γδ TCR Epitope Recognition: Due to the structure of the γδ TCR, it is not restricted to recognition of epitopes in the context of MHC. This is evidenced by its more immunoglobulin-like structure, which has the ability to bind antigen directly[100;104]. Therefore, the potential to recognize a large variety of soluble and membrane-bound

molecules has made identification of γδ TCR ligands difficult. Those few ligands identified resulted from thorough testing of known γδ T cell agonists such as mycobacterium extracts[198], stressed cells[199;200] or lymphomas[194]. Many γδ TCRs additionally require, or are believed to require, accessory proteins including apolipoproteins, a class of lipid-binding proteins found in the plasma. This requirement further increases the difficulty of γδ T cell epitope identification. Although only a limited number of γδ T cell epitopes are recognized, with those being limited to the mouse or human species, there appears to be a conserved trend in TCR subset recognition, indicative of similar immune function. Circulating, pro-inflammatory γδ T cells recognize bacterial products; these include phosphoantigens in human γδ T cells and cardiolipin in murine γδ T cells. Likewise resident, tissue-specific γδ T cells recognize cellular stress molecules including MICA by human γδ T cells and damaged epithelium by murine γδ T cells. Additionally, both γδ T cell subsets appear to recognize tumor cells, MICA and F-1/ApoA-I in human γδ T cells, underscoring the importance of γδ T cells in cancer immunology.

Although there are a few described ligands for γδ TCRs, there is only one available crystal structure of a γδ TCR with its ligand. These ligands, the non-classical MHCs, T10 and T22 (Table 1), are recognized by less than 1% of murine γδ T cells via a conserved CDR3 sequence. T22 is expressed on a variety of cell types, whereas T10 appears to be up-regulated on activated T and B cells as well as macrophages and dendritic cells. It is yet unclear what the function of T10/T22 recognition by the γδ T cell

plays in immunity. Analysis of the γδ T cell repertoire capable of binding to the T10/T22 ligand does not aid our understanding either, as it is expressed on clones of Vγ1, Vγ4, and Vγ7 T cells. Although this is the only TCR-ligand crystal structure available, a number of studies have indirectly demonstrated γδ T cell recognition of distinct epitopes by select TCR combinations with varying degrees of evidence.

Another set of defined γδ TCR epitopes are phosphoantigens, which activate human and non-human primate Vδ2 T cells. Since transfection of Jurkat T cells with Vδ2 TCR, but not Vδ1 TCR, activates these cells when cultured with phosphoantigen[198], it is clear that the TCR plays a role in the recognition of phosphoantigen. Additional testing of Vδ2 T cells identified the CDR3 region, in particular the J1.2 cassette, as crucial for Vδ2 recognition of phosphoantigen[201]. Direct association of the TCR and phosphoantigen has not been observed[202]. Since phosphoantigen by itself is too small to directly crosslink the TCR and thereby induce signaling, it is believed that the Vδ2 TCR does not directly recognize phosphoantigen, but requires presentation via a secondary molecule[202]. Phosphoantigens are currently in clinical trials for treatment of cancer and persistent infections; details of the effects of phosphoantigen therapy will be covered in a later section.

Human Vδ2 TCRs also recognize tumor cells via apolipoprotein (ApoA-I), in complex with F-1 ATPase (ApoA-I/F1). This complex was identified as a γδ TCR ligand by raising mAbs against a lymphoma line recognized by Vδ2 T cells. These mAbs were then screened for their ability to prevent Vδ2 T cells from responding to the tumor line[194].

The mAb that best inhibited Vδ2 proliferation was determined to be specific for extracellular ApoA-I, which stabilizes Vδ2 TCR recognition of the Apo A-I receptor, F1. This stabilized interaction of F1 and the Vδ2 TCR induces subsequent Vδ2 activation[194].

A number of molecules both induced by inflammation and produced by bacteria activate murine Vγ1 T cells. The first molecule identified was heat-shock protein 60 (HSP-60), but many others including cardiolipin, a phospholipid, were also identified (Table 5). Similar to the phosphoantigens, the recognition of cardiolipin is believed to require a carrier protein since serum is required for cardiolipin to induce Vγ1 T cell activation. This carrier protein was identified as Apolipoprotein H (ApoH)[200]. Cardiolipin, in complex with ApoH, activates Vγ1 TCR transfected hybridomas but not the parental hybridoma cell line without a TCR or αβ T cell hybridomas[200]. Since cardiolipin is expressed in some bacteria[203], its recognition by murine Vγ1 T cells likely induces a response similar to phosphoantigen and initiates a pro-inflammatory response to the pathogen.

Evidence of the difficulty in describing γδ TCR ligands is demonstrated with the tumor and stress-associated MICA and MICB, MHC-like receptors. Early experiments demonstrated that MICA and MICB ligands are stimulatory for γδ T cell subsets, namely Vδ1 T cells[204]. This was later discovered to be due, in large part, to the expression of the NK receptor, NKG2D on a number of γδ T cells[162;205]. Recent confirmatory studies from two groups confirmed the MICA/Vδ1 interaction. Zhao et al.[129] show recombinant Vδ1 TCR but not Vδ2 or Vδ3 specifically binds MICA and Wrobel et al.[161] show MICA

recognition by both NKG2D and Vδ1 are functionally relevant and synergistic via a series of MICA/Vδ1 TCR antibody blockade and cytolysis studies.

As mentioned earlier, γδ T cells function in the murine epidermis to maintain epithelial integrity by recognition of tissue damage via the production of growth factors, KGF and IGF-1, to initiate tissue repair. Although the actual epitope recognized by these cells has not been elucidated, the recognition is most likely via the TCR, since mice devoid of this cell cannot repair tissue damage, and reconstitution of this γδ T cell subset aids in epithelium repair[136;206].

Table 5. Ligands Recognized by the γδ TCR

γδ T cell subset	Putative ligand
(S)EGYEL sequence in CDR3 junction of murine Dδ2	Clones of Vγ1, Vγ4, and Vγ7 are specific for T22 and T10 non-classical MHC [207]
Murine Vγ1	HSP-60[208] cardiolipin[200]
Murine Vγ5	Damaged epithelium[167]
Human Vδ1 T cells	CD1c (MHC class 1-like, presents lipids)[209] MICA, MHC-like molecules (disputed, see text)
Human Vγ9(Jγ1.2)Vδ2	Phosphoantigens, metabolites of the isoprenoid biosynthesis pathway[130] F1-ATPase/Apo-1 complex[194]

The recognition of these assorted bacterial products, stressed or damaged cells, and cancers allows the γδ T cell to act quickly to resolve these potential problems for the immune system. However, responses to conserved antigens by the γδ T cell population are not limited to TCR expression. In fact, the γδ T cell expresses a wide variety of alternative cell-surface receptors for recognition of conserved pathogen and cancer

epitopes. These receptors provide additional means for sensing abnormalities in the environment and providing a rapid and innate cellular response.

Non-TCR Receptors Expressed on γδ T Cells: In addition to the γδ TCR ligands described in Table 1.2, γδ T cells express a number of cell receptors unique to innate immune cells including those for pathogen-associated molecular patterns (PAMPs) and Killer Inhibitory Receptors (KIRs), which further differentiates them from adaptive immune cells. These cellular receptors are used by innate cells to recognize conserved molecules such as stress-related proteins and pathogen sequences, the presence of which confirms the role of γδ T cells in the rapid, innate immune response to conserved epitopes.

NKG2D is expressed on most human γδ T cells, both Vδ1[204] and Vδ2[210] subsets, as well as αβ NKT and NK cells, and recognizes MICA, an MHC-like molecule, among other NKG2D ligands. As mentioned previously, conflicting reports exist as to whether human Vδ1 TCR additionally recognizes MICA/B. This is not in question with regard to NKG2D and MICA/B, for which there is a crystal structure[211]. NKG2D is a C-type lectin receptor responsible for activating NK-mediated cell lysis[212]. This activation is overridden by KIR receptors that sense the presence of MHC on target cells. MICA/B-NKG2D engagement therefore becomes an activating signal in the absence of MHC and leads to NK-mediated clearance of infected cells and/or tumors[161;213;214]. Stimulation of Vδ2 T cells, of which almost all express NKG2D, with NKG2D mAb or ligand (MICA) increases expression of both CD25 and CD69 activation markers and produces TNF-α,

but not IFNγ[210]. Although MICA/B is clearly up-regulated on DCs and epithelial cells during infection[214;215], these NKG2D ligands are constitutively expressed throughout the body[162], indicating a more elaborate function for this receptor/ligand pair in immunity than previously predicted. Other functional NK-specific receptors are found on subsets of γδ T cells including KIRG2A[155] and NKp44[216].

The γδ T cell-specific cell receptor, WC1, found in bovine and other ruminant species[217] is a 220kD cell surface receptor with structure sequence indicative of a scavenger receptor-like molecule. Although there are gene homologs identified in other species[218], the ruminant form is the only WC1 marker observed as protein to date. There are three identified isoforms of WC1: WC1.1, WC1.2, and WC1.3. WC1.1 and WC1.2 make up the majority of $WC1^+$ γδ T cells and are not co-expressed on the same γδ T cell. WC1.1 is the predominant WC1 molecule at birth and decreases to concentrations similar to WC1.2 after approximately 500 days[196]. WC1.3 is a minor γδ T cell population being additionally expressed on a limited number of $WC1.1^{+}$[196]. Implications for their function are seen in $WC1.1^+$ and $WC1.2^+$ γδ T cell selective responses to *Leptospira* antigen and the major surface protein 2 (MSP2) of *Anaplasma marginale*, respectively (see Table 4). Although formal testing of the recognition ligands in these cell populations is untested, the WC1 scavenger receptors may be responding to these pathogen components.

There is accumulating evidence for the role of PAMP recognition in γδ T cell immunology. A number of studies demonstrate γδ T cell reactivity to PAMPs including LPS[219], peptidoglycan[220], miramyl dipeptide (Hedges, in press), poly I:C[221], Pam3Cys[222], and PolyG3[182]. The direct role of γδ T cells in the recognition of these PAMPS is

evidenced by the expression of many PAMP receptors on γδ T cells including CD36, toll-like receptors (TLR2[222], TLR3[220;221], TLR8[182], TLR9[220]) and the TLR-signaling protein MyD88[220], as well as intracellular PAMP receptors (protein kinase regulated by RNA (PKR), NOD1, NOD2, NALP1[220]). In response to PAMPs, γδ T cells up-regulate pro-inflammatory cytokine production, RANTES, MIP-1α, MIP-1β IFNγ, TNFα, GM-CSF and IL-8[220], to both recruit and activate pro-inflammatory cells.

Antigen Presentation by γδ T Cells

Recent evidence also demonstrates γδ T cells from both cattle[223] and humans[224] have the potential to present foreign antigen in the context of MHCII. Furthermore, these γδ T cells express the necessary co-stimulatory factors B7 and CD40, to elicit αβ T cell activation and clonal expansion[224]. Previously, this is only demonstrated on professional antigen-presenting cells (APCs) such as B cells, monocytes, and dendritic cells. The potential for γδ T cells to not only recognize pathogen by use of their TCR or other surface molecules such as PAMPs, but also to process and display these antigens to $CD4^+$ αβ T cells suggests γδ T cell may play a larger role in the adaptive immune response than previously thought.

Although γδ T cells are likely less efficient at antigen presentation than classical APCs due to their smaller size, resulting in limited phagocytizing ability, their ability to present antigen suggests initiation of the adaptive immune response or memory recall response may not require the trafficking of cells to classic sites of antigen presentation such as the lymph nodes, but may begin earlier in the tissues. Although research into the

antigen presentation properties of γδ T cells has only recently begun, this suggests yet another important role of these versatile cells in the immune system and promises further use for γδ T cell-based therapies.

Development of Drugs for the Expansion of γδ T Cells

When considering the functions provided by γδ T cell populations in the immune system, it is clear how useful increasing the activity of these cells could be. Therefore, a great deal of effort is being invested in the development of therapies intended to increase γδ T cell effector function[152;225;226]. To date, there is only one reliable candidate for clinical expansion of γδ T cells, the phosphoantigen. Treatment of human γδ T cells, both in vivo and in vitro, with phosphoantigen dramatically expands the pro-inflammatory Vδ2 T cell population. These expanded Vδ2 T cells demonstrate effectiveness in treating cancers and persistent infections[225]. Unfortunately, there are many caveats to phosphoantigen-based therapies that may prevent its widespread use.

Phosphoantigens, also called prenyl phosphates, are members of the mevalonate and non-mevalonate pathway of cholesterol synthesis. The non-mevalonate pathway is utilized by plants, eubacteria, and gram-negative rods whereas the mevalonate pathway is utilized by mammals and archea[227]. Some bacteria, including *Listeria* and *Streptomyces*, use both pathways. Although metabolites from both pathways demonstrate Vδ2 T cell agonist activity (IPP and HDMAPP, see Figure 3)[146], phosphoantigens from the non-mevalonate pathway (HDMAPP) are one thousand times more potent[228]. For a detailed

description of the biosynthetic pathways of the mevalonate and non-mevalonate pathways, see the review by Eisenreich et al.[227].

It remains unclear how phosphoantigens are recognized by the Vδ2 TCR. Attempts to co-crystallize Vδ2 TCR with phosphoantigen were unsuccessful[229]. Studies modifying the Vδ2 TCR demonstrate that the CDR3 region is critical for phosphoantigen-induced Vδ2 activation[230]. However, activity is not regulated by a specific protein sequence in the CDR3 region[146]. Phosphoantigens are likely too small to induce crosslinking of the TCR alone, so it is possible that they are presented to the TCR via a secondary, heretofore unidentified carrier molecule[146].

There are many problems associated with the clinical use of phosphoantigens. First, they are only effective in expanding human and non-human primate Vδ2 T cells[231], and therefore, in vivo testing is difficult. Second, in vivo phosphoantigen therapy requires IL-2 as a secondary stimulation to achieve significant proliferation[232]. This requirement increases both cost of therapy and induces the activation of non-γδ T cell lymphocytes. Third, phosphoantigen treated Vδ2 T cells become anergic after repeated treatment. This is observed both in vivo and in vitro. In vivo results demonstrate a decreased phosphoantigen response in the peripheral blood after subsequent treatments. In fact, after three rounds of phosphoantigen/IL-2 therapy, responses are almost negligible[232]. Additionally, phosphoantigen anergy is observed in vitro by a limited number of rounds of phosphoantigen induced Vδ2 replication (unpublished observations). Finally, γδ T cell therapy is most likely to be effective against persistent infections such as *M. tuberculosis*, HIV and cancer. Unfortunately, Vδ2 T cells in these individuals are predisposed to an

anergic response to phosphoantigen stimulation prior to treatment since the expansion of the Vδ2 population in these individuals is greatly reduced compared to healthy persons[233-235]. Therefore, phosphoantigen expansion may not achieve sufficient Vδ2 T cell expansion to be effective against these diseases.

Bisphosphonate and Alkylamines: Bisphosphonates, specifically nitrogen-containing bisphosphonates, and alkylamines were originally identified as γδ T cell agonists with functional responses similar to the prenyl phosphates. Subsequent analyses of these responses show that the agonist activity is due to the blockade of metabolites downstream of IPP. This blockade thereby increases the endogenous concentration of IPP to a point where the Vδ2 T cells become activated. For this reason, alkylamines and bisphosphonates are categorized with the true phosphoantigens, prenyl phosphates, since the γδ T cell response is identical.

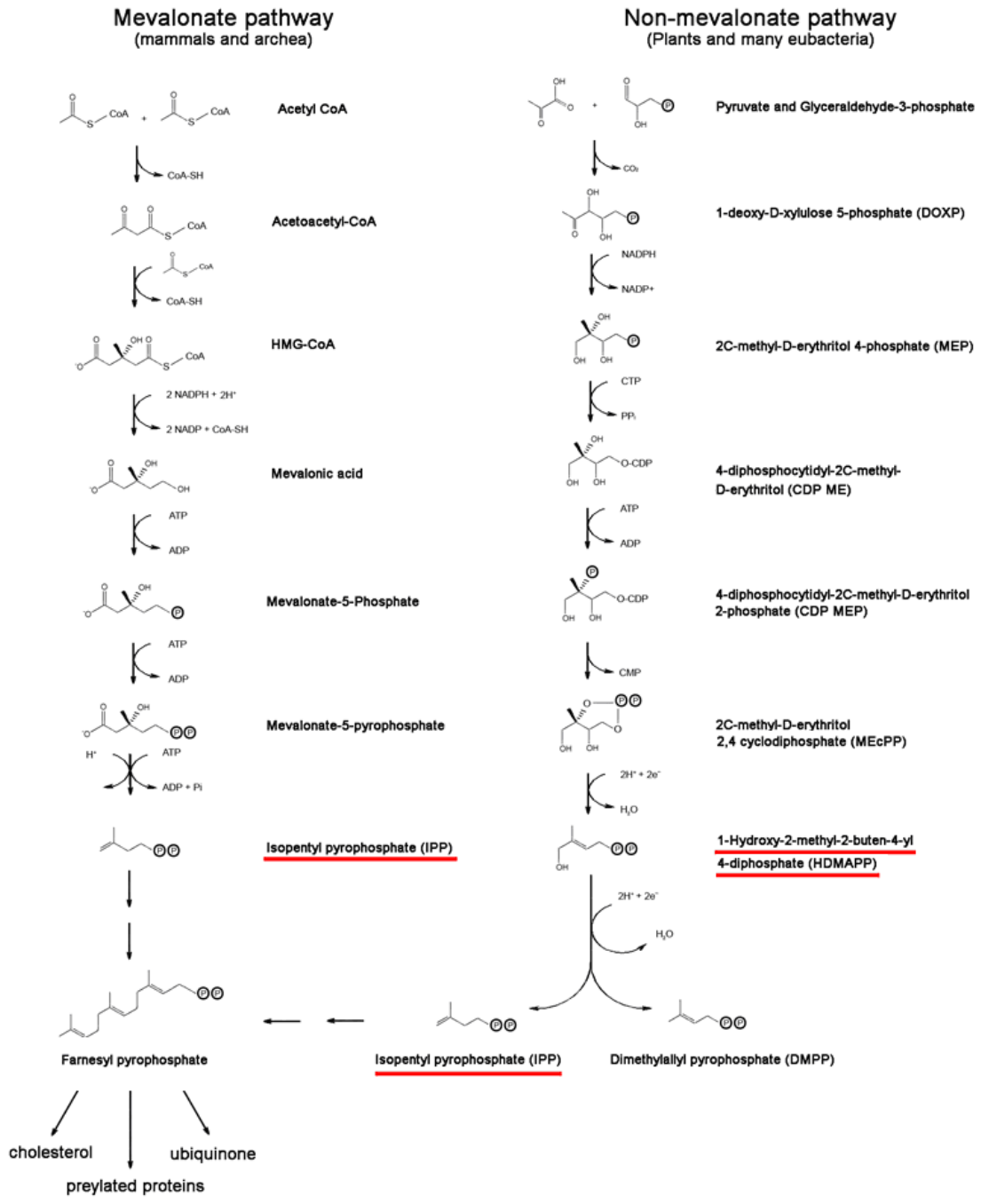
Bisphosphonates are clinically proven drugs for use in treating osteoporosis and Paget's disease. In fact, they were originally identified as γδ T cell agonists after acute proliferation of Vδ2 T cells was observed during bisphosphonate treatment for osteoporosis[236]. Bisphosphonates act by inhibiting bone resorption via blockade of osteoclast action, namely competitive inhibition of farnesyl pyrophosphate synthase (the enzyme responsible for converting IPP, see Fig. 3). Inhibition of the mevalonate pathway at this point of synthesis prevents protein prenylation. Unmodified Ras and Rho in the osteoclast is ineffective at maintaining contact with bone, and therefore cannot effectively

resorb it, thereby increasing bone density. Bisphosphonates are particularly effective at targeting osteoclasts since they have a high binding affinity for bone.

Alkylamines are structurally very similar to bisphosphonates except they have a positively charged amine group instead of the negatively charged phosphate group. These compounds are secreted by some bacteria and are found in tea and mushrooms as well[237]. Alkylamines act in a manner similar to bisphosphonates. Specifically, they increase endogenous IPP via the inhibition of the mevalonate pathway and in fact inhibit the same enzyme as bisphosphonates, farnesyl diphosphate synthase[238].

These results confirm that there is truly only one identified class of γδ T cell agonist, the prenyl phosphate. Alkylamines and bisphosphonates act by increasing the endogenous levels of prenyl phosphate. As with direct phosphoantigen stimulation, repeat treatment with bisphosphonates demonstrate a decreased effectiveness in γδ T cell expansion[236], suggesting this phenomenon is a response to all phosphoantigen-based treatments. In spite of the caveats of phosphoantigen therapy, these agonists are currently in clinical trials for the treatment of persistent illnesses including cancer and hepatitis infection.

Figure 3. Mevalonate and Non-mevalonate Pathways of Isoprenoid Biosynthesis



The mevalonate and non-mevalonate pathways for the production of isopentyl pyrophosphate (IPP), which is used as a metabolite for the biosynthesis of cholesterol, prenylated proteins, and ubiquinone, among others[227]. Intermediates of these pathways known to stimulate human Vδ2 T cells are underlined in red (IPP and HDMAPP).

Phosphoantigen clinical trials: The pharmaceutical company, Innate Pharma, is currently testing phosphoantigens in clinical trials for the treatment of a number of persistent infections. Currently, clinical trials use Phosphostim, a synthetic analogue of prenyl phosphates. This chemical, now named IPH 1101, is shown to be effective in expanding primate γδ T cells in vivo. However, as previously mentioned, repeat therapy is less effective as anergy is quickly observed[232].

Table 6. Phosphoantigen Clinical Trials

Trial number	Treatment
IPH 1101 201	Metastatic renal cell carcinoma
IPH 1101 202	Follicular lymphoma
IPH 1101 203	Hepatitis C virus
IPH 1101 204	Chronic myeloid leukemia
IPH 1101 205*	Solid tumors

* Currently being prepared for use with a cytolytic agent and is scheduled to be filed in late 2007[239].

The effectiveness of these phosphoantigen-based therapies remains to be seen considering the anergic response developed in both persistently infected individuals and those receiving repeated treatment. However, as of now, phosphoantigen is the only available therapy for the selective expansion of any γδ T cell population. The development of novel therapies to either alleviate phosphoantigen anergy and/or stimulate alternative γδ T cell subsets (i.e. Vδ1 T cells) would be of benefit for the many diseases controlled and influenced by γδ T cells in the human body.

Screening of Natural Products for γδ T cell Agonist Activity

In an effort to increase the therapeutic potential of γδ T cells, we screened natural product libraries for γδ T cell agonists. For this study, we used bovine γδ T cells under the premise that γδ T cells in both bovine and human systems function in a similar manner and therefore, likely respond to many similar agonists. In this study, we identified a number of plant extracts with γδ T cell agonist activity in both humans and cattle. These agonists, which induced activation and proliferation of both proinflammatory and tissue-specific subsets of γδ T cells, were contained in the procyanidin fraction. Further analyses of these tannins demonstrate agonist activity in oligomeric procyanidins. Since smaller procyanidins do not demonstrate γδ T cell agonist activity, the observed activity likely cannot be attributed to a general characteristic of the tannin, such as antioxidant activity. These results demonstrate that certain species of oligomeric tannins activate γδ T cells via a novel mechanism that, unlike the current γδ T cell therapies, is conserved in animal models and can activate a broader subset of human γδ T cells. The tannin-reponsive γδ T cells included not only the pro-inflammatory subset, but also the tissue-specific γδ T cell subset, implicating a potentially novel application in the regulation of inflammatory diseases. Furthermore, these studies corroborate the traditional use of many tannin-rich plants for the regulation of inflammatory diseases such as dermatitis and asthma.

IDENTIFICATION OF PLANT TANNINS AS γδ T CELL AGONISTS

Introduction

The γδ T cell is one of the primary lymphocytes considered to participate in innate immune responses. They are found in virtually all portals of entry into the body where they act as sentinels of the extracellular environment by responding rapidly to tissue damage[166;190] and pathogens through recognition of conserved pathogen-associated molecular patterns (PAMPs) and specific antigens[220;222;240]. Human γδ T cells can be categorized into two major, functionally distinct populations, Vδ1 and Vδ2 T cells, which are characterized by their respective TCR delta chain usage. Expression of surface molecules, such as chemokine receptors[241], varies between the γδ T cell subsets, as does their physical locale within the body. Vδ2 T cells are primarily found in the blood and lymphatics, while Vδ1 T cells are typically found in tissues, such as the gut mucosa and skin[242;243]. Vδ2 T cells are potent anti-microbial and anti-tumor effector cells[244-246]. Vδ1 cells have important immuno-regulatory functions and if properly stimulated can become potent effector cells[131].

Observations that the majority of Vδ2 T cells proliferate in response to intermediates of the cholesterol synthesis pathway (phosphoantigens) led to clinical trials testing these compounds as potential drugs for the treatment of some cancers and infections[247]. Other known γδ T cell agonists, such as bisphosphonates[248] and alkylamines[238], induce a similar response by increasing the endogenous concentration of phosphoantigens. Notably, phosphoantigen-based proliferation requires a co-stimulatory

factor for optimal Vδ2 T cell expansion, typically IL-2 or IL-15[249-251]. Though expansion of Vδ2 T cells *in vivo* is clearly demonstrated using these new drugs, the benefit of these expanded cells on host defenses is not fully understood due to a minimal or non-existent response in non-primate animal models[231]. Additional caveats for the clinical use of phosphoantigen-based drugs include the requirement for large amounts of agonist[232], induction of rapid cell anergy to treatment[232], and activation of only the Vδ2 subset, which must then traffic to the tumor or infection to be effective[131;232;236;243].

We initiated a drug discovery effort to identify novel γδ T cell agonists that overcome the shortcomings of phosphoantigen-based drugs. Traditional medicine utilizes extracts from plants and microbes to treat diseases, many of which were shown to contain pharmaceutically relevant components. In fact, 47% of the small molecule drugs developed over the last 25 years are either natural products or derived from natural products[25]. Phosphostim (Innate Pharma, Marseille, France), a γδ T cell agonist in clinical trials, is an example of a drug designed from the structure of naturally occurring phosphoantigens[130;252]. Due to the dominance of pharmaceutically relevant compounds in nature, in an ongoing effort we initiated a screen of >100,000 natural products, including common nutritional supplements, using a FACS-based assay, which measures bovine γδ T cell activation via up-regulation of the IL-2 receptor chain α (IL-2Rα). Bovine cells were used in the primary screens, since, among other reasons, they do not robustly respond to phosphoantigens[253], thus avoiding the detection of these common γδ T cell agonists. Here we report non-phosphoantigen agonist activity that was observed for both bovine and human γδ T cells in specific plant species including extracts of non-ripe

Malus domestica (apple) peel, *Uncaria tomentosa* (Cat's Claw), *Angelica sinensis* (Dong Quai), and *Funtumia elastica* (Yamoa). We traced activity in the apple and Cat's Claw extracts to the tannin fraction, a class of polyphenols characterized by their capacity to bind proteins. Further analysis identified the bio-active population of tannins as procyanidins. Detailed dose analyses suggested that superior activity existed in the apple extract. In studies on human cells, apple-derived tannins demonstrated agonist activity for human Vδ1 and Vδ2 T cells as well as NK cells. These studies suggest that unique tannin agonists for innate lymphocytes may account, at least in part, for the immuno-modulatory properties of some plant extracts. They also suggest that a tannin-based drug may complement the phosphoantigen-based drugs, thereby enhancing the therapeutic potential of γδ T cells.

Materials and Methods

Preparation of Bovine and Human PBMCs

Whole blood was collected from 1-3 month old bull Holstein calves into sodium heparin tubes (Becton Dickinson, Franklin Lakes, NJ) and healthy human adult donors with ACT tubes (Becton Dickinson). Leukocytes were separated from whole blood using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) for bovine cells as previously described[133] and human cells as per the manufacturer's instructions. Additionally, bovine red blood cells were removed by hypotonic lysis. All experiments were performed in accordance with National Institute of Health guidelines and approved by the Institutional

Animal Care and Use Committee and Institutional Review Board of Montana State University.

Preparation of Plant Extracts

Non-ripe *Malus domestica* (apple) peel (APP; Apple Poly, Littleton, CO), *Uncaria tomentosa* (Cat's Claw; Raintree Nutrition, Carson City, NV), *Angelica sinensis* (Dong Quai; Nature's Way Products, Springville, UT), and *Funtumia elastica* (Yamoa; NHC, Oxford, United Kingdom) were typically suspended in room temperature water (Cat's Claw 10 ml/g, Dong Quai 5.0 ml/g, APP 8.0 ml/g, and Yamoa 3.0 ml/g), although use of near boiling water had no detectable impact on the preparations, and were agitated for 5 minutes prior to centrifugation to remove insoluble particles. Extracts were sterile filtered (0.2μm) and stored at -80^{o}C until use. Extracts were lyophilized to determine their approximate dry weights: Cat's Claw 17.6 mg/ml, APP 84.8 mg/ml, Dong Quai 87.0 mg/ml, Yamoa 32.1 mg/ml.

FACS-based IL-2Rα and CFSE Analysis of Human and Bovine PBMCs

PBMC preparations were suspended in X-VIVO 15 medium (Cambrex, East Rutherford, NJ) at 1x10^{6} cells/ml for bovine cells or 2x10^{6} cells/ml for human cells. For the measurement of IL-2Rα expression, cells were cultured for 48h at 37^{o}C, 10% CO_2 in the presence or absence of known or test agonists, and the expression of IL-2Rα measured by multi-color FACS (see below). To measure cell proliferation, bovine or human PBMCs were labeled with 2.5 μM CFSE for five minutes in Hank's Balanced Salt Solution (HBSS; Mediatech, Herndon, VA), washed, and then cultured at 37^{o}C, 10% CO_2

in X-VIVO 15 medium in the presence or absence of known or test agonists for 5 days. Human CFSE cultures were typically treated with low-dose human rIL-15 (IL-15; Peprotech, Rocky Hill, NJ) at the beginning of the culture period. For these cultures, all samples, including controls, received 1.0 ng/ml IL-15. Cell division was quantified by determining the percentage of cells that had divided at least once, based on a 50% or greater reduction in CFSE intensity, using multi-color FACS. Positive control agonists were Concanavalin A (Con A; 0.5 ng/ml; Sigma-Aldrich) with human rIL-2 (1.0 ng/ml; Peprotech), or lipopolysaccharide (LPS; 10 μg/ml, *E. coli O111:B4*; Sigma-Aldrich), and negative controls included appropriate buffers diluted in the assay medium.

Cells were analyzed by FACS using a FACSCalibur equipped with an High-Throughput Screening (HTS) loader (Becton Dickinson) or a FACSCanto (Becton Dickinson). Bovine cells were stained with monoclonal antibodies (mAbs) against IL-2Rα (LCTB2A, VMRD) and/or γδTCR (GD3.8)[254]. Human cells were stained with IL-2Rα (MEM-181, Serotec, Raleigh, NC), γδTCR (11F2, Becton Dickinson), CD19 (HIB19, Becton Dickinson), CD94 (HP-3B1, Beckman Coulter, Fullerton, CA), CD56 (B159, Becton Dickinson), CD3 (UCHT1, Beckman Coulter), Vδ1 TCR (T58.2, Endogen), and/or Vδ2 TCR (IMMU389, Beckman Coulter) mAbs. These mAbs were directly labeled (FITC, PE, PE-Cy5, PE-Cy7, APC, or APC-Cy7) or indirectly labeled with goat-anti-mouse PE, FITC or APC (Jackson ImmunoResearch, West Grove, PA). Second stage reagents alone were used to determine the level of background staining in the FACS analyses.

HPLC Analysis of Cat's Claw

An aqueous extract of Cat's Claw (1.3 mg in 100 μl) was applied to a reverse phase analytical C18 column (4.6x250mm Grace Vydac, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL). The extract was eluted with a linear gradient of 0% CH_3CN/0.08% TFA to 100% CH_3CN/0.08% TFA at a flow rate of 1ml/min. UV absorbance was measured at a wavelength of 254 nm. Fractions were collected at one minute intervals, dried (Speed Vac, Savant Instruments, Holbrook, NY), and suspended in PBS.

CD69 Gene Expression

Bovine CD69 gene expression was determined using the Quantigene assay (Panomics, Freemont, CA) following the manufacturer's procedure. Briefly, bovine PBMCs ($2x10^6$cells/ml) were cultured with the HPLC separated Cat's Claw fractions (1:20 dilution) for 4h in complete RPMI medium (cRPMI; RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 10mM HEPES buffer (Mediatech), 50 IU/ml penicillin (Mediatech), 50 μg/ml streptomycin (Mediatech), 2 mM L-glutamine (Mediatech), and non-essential amino acids (Mediatech)) before lysis with supplied buffer. Lysates were added to prepared 96-well plates containing hybridization probes (provided by the manufacturer) for bovine CD69 mRNA. Presence of hybridized CD69 mRNA was detected with luminescent DNA probes using an LB960 Centro (Berthold Technologies, Bad Wildbad, Germany). CD69 gene expression was represented by the fold increase in CD69 signal compared to medium controls.

Separation of Tannins from Plant Extracts

Aqueous extract of Cat's Claw (10 ml) was treated with 5 ml of polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich) slurry in 0.0625 M HCl at room temperature overnight. The resulting slurry was filtered through a 0.2 μm membrane to remove PVPP and PVPP-bound tannins. The eluent was brought to a pH of 7.2 with NaOH. The tannin-bound PVPP was washed with water before treatment with 0.1 M NaOH for 60 minutes to remove bound tannins. The solution was again filtered to remove PVPP and brought to a pH of 7.2 with HCl. Removal of tannins from Yamoa, Dong Quai, and APP was performed by incubating 1.0 ml of extract with 100 mg PVPP for 15 minutes at 4°C. The unbound fractions were separated from the tannin-bound PVPP by centrifugation. The supernatant fluid was treated with an additional 100 mg PVPP. This process was repeated a total of five times, reserving samples from each round of depletion.

Tannin fractions from APP, Cat's Claw, and Dong Quai extracts were subjected to a modified Folin-Ciocalteu assay[255] to measure total phenolics. Briefly, 2 ml of each extract was transferred into a bed volume of 3 ml PVPP and incubated for 1 h. The non-tannin fraction was collected by washing with 10 ml of water. Tannins were eluted with 10 ml 1.0 M NaOH. Extracts and fractions thereof were serially diluted 1:1 to 1:4050 in water before mixing 500 μL of diluted sample with 250 μL 1 N Folin-Ciocalteu reagent (Sigma-Aldrich) and 1.25ml 20% sodium carbonate. The samples were mixed and incubated at room temperature for 40 minutes before absorbance was read at 725 nm

using a DU800 spectrophotometer (Beckman Coulter). Tannic acid (Sigma-Aldrich) dilutions (40 μg/ml to 200 μg/ml) were freshly prepared to generate a standard curve.

To separate procyanidins by size, 1.5 ml of APP (approximately 150 mg) was adsorbed to LH-20 resin and washed with 30 ml water. To elute, 30 ml of 25% MeOH, 50% MeOH, 75% MeOH, 95% MeOH and 70% acetone were used in succession and the fractions collected. 95% MeOH and 70% acetone fractions were collected in two 15 ml fractions. Activity was predominantly found in the later fractions, therefore these fractions were used for analyses. All fractions were lyophilized and resuspended in 750 μl water.

Toll-like Receptor Activation and Limulus Assays

Apple extract was tested on THP1-Blue-CD14 cells (InvivoGen, San Diego, CA) for Toll-Like Receptor (TLR) agonist activity according to the manufacturer's protocol. THP1-Blue-CD14 cells express human TLR1-10, over-express CD14, and are transfected with a reporter plasmid containing secreted embryonic alkaline phosphatase (SEAP) under the control of both an NF-κB and AP-1 inducible promoter. TLR activation was determined by quantifying SEAP activity. Briefly, THP1-Blue-CD14 cells at a concentration of $2x10^6$ cells/ml were cultured in cRPMI containing 10% FBS, glucose (4.5 mg/ml), zeocin (200 μg/ml), and blasticidin (10 μg/ml) (all from Invivogen, San Diego, CA) followed by PMA (50 ng/ml) treatment for 18 h. PMA was used to differentiate the THP1 cells to induce expression of TLRs. Cells were washed to remove residual PMA and the glucose, zeocin, and blasticidin treatment was discontinued. Cells

were stimulated with LPS (a TLR 4 agonist), as a positive control, and different concentrations of APP or Cat's Claw extract in cRPMI for 24 h at 37°C and 10% CO_2. Supernatant fluid was removed and added to QUANTI-Blue colorimetric assay reagent for 24 h at 37°C and 10% CO_2. After 24 h, samples were read at an OD of 655 nm by a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). All samples were run in quadruplicate, from which averages and standard deviations were determined.

A kinetic turbidimetric Limulus Amebocyte Lysate (LAL, Associates of Cape Cod, East Falmouth, MA) assay was performed on APP to determine the presence of endotoxin in the extract as per the manufacturer's instructions. Briefly, APP was diluted 1:6 in LAL Reagent water and analyzed for endotoxin using LAL Reagent resuspended in Glucashield buffer. LAL agglutination was measured using a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale, CA). Samples were analyzed in triplicate.

Protease Digestion of Tannin-associated Proteins

APP (42.4 mg) was treated with PVPP (50 mg) and either PBS, chymotrypsin (20 U; Sigma-Aldrich), or trypsin (50,000 U; Sigma-Aldrich) for 1 h at 37°C. PVPP was washed, and the tannins eluted with NaOH.

Alkaline Phosphatase Inactivation of Phosphoantigens

APP extract was passed over a column of polyvinyl spheres containing immobilized alkaline phosphatase (MoBiTec, Goettingen, Germany). The phosphoantigens 1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP; Echelon,

Salt Lake City, UT) or isopentenyl pyrophosphate (IPP; Sigma-Aldrich) (both at 500 μg/ml) or APP (42.4 mg/ml) were equilibrated with reaction buffer (final concentrations: 25 mM Tris/HCl, 0.05 mM $ZnCl_2$, 0.5 mM $MgCl_2$, pH 8.0) and passed over the column twice. Control samples for were equilibrated with reaction buffer but not passed over the column.

Cell Separation Using Magnetic Beads and Flow Cytometry

PBMCs from bovine and human donors were sorted to create purified cell populations via magnetic bead separation (Miltenyi Biotec, Bergisch Gladbach, Germany), as per the manufacturer's instructions. Monocytes from bovine and human PBMCs were depleted with magnetic beads conjugated to CD14 mAb using an LD column (Miltenyi Biotec). Purification of human γδ T cells was achieved using either positive or negative sorting techniques. Briefly, human PBMCs were treated with mAbs either to the γδTCR or to a cocktail of mAbs not expressed on γδ T cells (Miltenyi Biotec; positive and negative sorting, respectively). The labeled cells were then linked to magnetic beads and transferred through LS columns (Miltenyi Biotec). The γδ T cells were then collected as the column bound or un-bound fractions (positive and negatively sorted γδ T cells, respectively). Bovine γδ T cells were sorted from PBMC by labeling with γδ TCR mAb (GD3.8) and magnetic beads conjugated with anti-mouse antibodies (Miltenyi Biotec) as per the manufacturer's instructions or by using a FACS Vantage SE (Becton Dickinson) as previously described[133]. All sorted cells were cultured under the

conditions noted for PBMC cultures except sorted human γδ T cell cultures which were cultured between $1x10^5$ to $5x10^5$ cells/ml.

Purities of magnetically sorted cells were verified by FACS prior to culture. CD14 depletion was confirmed as less than 0.5% monocyte contamination using CD14 mAbs (human: UCH-M1, Santa Cruz Biotechnology, Santa Cruz, CA; bovine: CAM36A, VMRD, Pullman, WA). γδ T cell purity was determined using γδTCR mAbs (human: 11F2; bovine: GD3.8).

HPLC Separation of Tannin-enriched LH-20 Fraction and Commercial Tannins

Oligomeric tannin, MeOH fraction from LH-20 coulumn, from APP was prepared as described previously and loaded onto a normal phase HPLC column so separate the procyanidins by size[256].

Determination of Lymphocyte Survival by FACS Light Scatter

To determine the amount of toxicicty induced by APP or individual tannins after culture, cells were analyzed by FACS. Live lymphocytes were measured by FFC/SSC light scatter and the ratio of live to dead (cells outside the range of live cells) were calculated. The average ratio of live to dead cells in the medium control wells was considered maximum (100%). The percentage of live cells in the tannin treated wells was then calculated as the ratio of live cells in the treated culture divided by the ratio of live cells in the medium control wells.

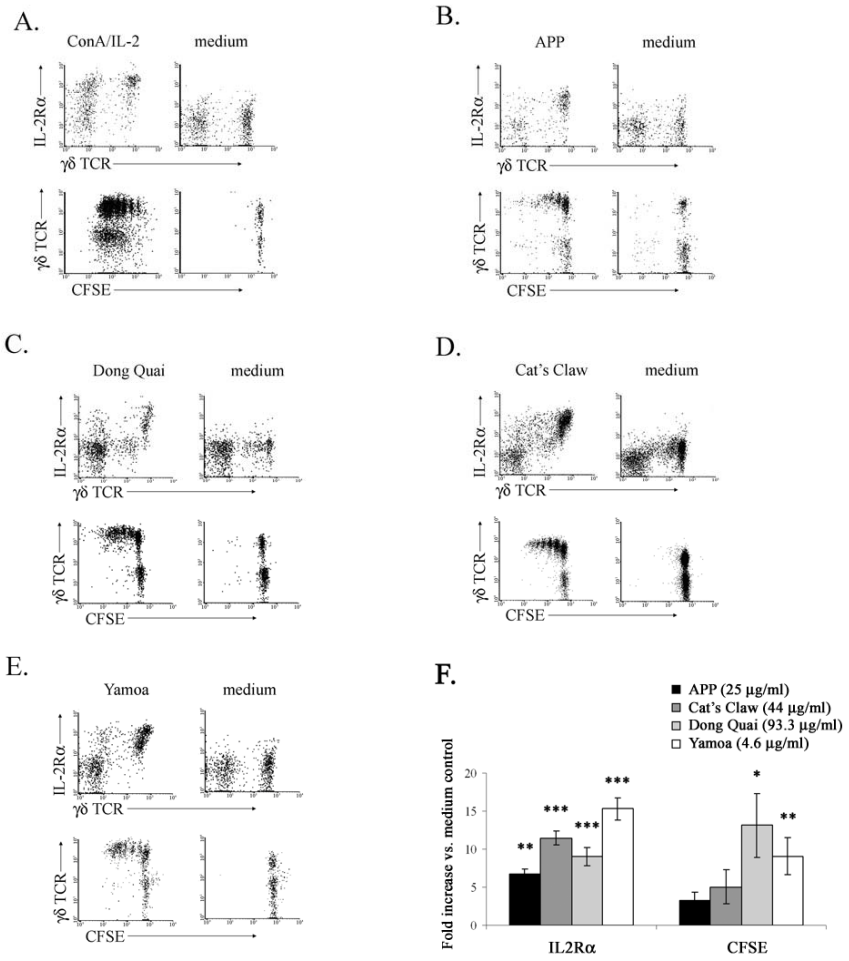
Results

Extracts from Common Dietary Plant Supplements Induce Activation and Cell Division from γδ T Cells in Bovine PBMC Preparations

Flow cytometry-based assays to determine cell activation (IL-2Rα up-regulation) and proliferation (cell division based on CFSE staining) were used to screen extracts of plants commonly considered having immunomodulatory activity. Bovine γδ T cells were used for these screens to minimize the potential of identifying phosphoantigens since these agonists do not induce robust responses in the bovine model[253]. Potent activity was identified in water-soluble extracts from four plant species: non-ripe *Malus domestica* fruit peel (apple polyphenols; APP), *Uncaria tomentosa* bark (Cat's Claw), *Angelica sinensis* root (Dong Quai), and *Funtumia elastica* bark (Yamoa) (Fig. 4). Each extract induced IL-2Rα expression on γδ T cells after 48h in culture and induced limited cell division, as detected in a 5-day CFSE assay (Fig. 4). Activity of each extract was specific to γδ T cells and furthermore, nearly all γδ T cells became activated, suggesting activity was not limited to a single γδ T cell subset. In cattle, there are two major γδ T cell subset classes, the tissue-associated γδ T cell subset, which has similarities to Vδ1 cells in humans, expresses CD8 and comprises up to 20% of the peripheral γδ T cell population, whereas the predominant blood subset lacks CD8 and expresses the workshop cluster 1 family of glycoproteins[133;134]. Therefore, to confirm that extract activities were not subset specific, additional 3-color analyses were performed on 48h cultures using CD8 (CC58)[257] to differentiate the primary bovine γδ T cell subsets. These analyses validated the observations in Figure 4 (data not shown), demonstrating the agonist activity in APP,

Cat's Claw, Dong Quai, and Yamoa extracts were not limited to one of the primary bovine γδ T cell subsets.

Figure 4. Plant Extracts Induce the Activation and Proliferation of Bovine γδ T Cells



Freshly collected bovine PBMCs were cultured with ConA/IL-2 *(A)* or plant extracts (APP [10.6 μg/ml; *B*], Cat's Claw [44.0 μg/ml; *C*], Dong Quai [435.0 μg/ml; *D*], Yamoa [160.5 μg/ml; *E*]) and analyzed by flow cytometry in IL-2Rα or CFSE two-color FACS assays and the fluorescence intensity on a $\log_{10}$ scale. *F*. The average fold increase in IL2Rα expression or cell division in bovine γδ T cells treated with plant extracts as compared to medium controls. Error bars represent the standard error from a minimum of 5 independent experiments for each compound and assay. *P*-values for differences in means are *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Identification of Plant Tannins as Agonists for γδ T Cells

As a first step in the identification of the specific agonists in the plant preparations, the Cat's Claw extract was fractionated on a C-18 HPLC column (Fig. 5A, top). Activity in the collected fractions was measured with bovine PBMCs by cellular expression of surface IL-2Rα or by CD69 gene expression (Fig. 5A, bottom). The broad range of γδ T cell agonist activity observed in the HPLC fractions correlated with the incomplete separation seen in the elution profile, demonstrating a widespread distribution of the active component. A similar result was seen with the APP extract, but initial HPLC separation of the activity in the Yamoa extract failed (data not shown). To gain additional insight into the chemical nature of the active component of Cat's Claw, we dialyzed the extract against a 50kD cut-off membrane. The active fraction was retained (data not shown), suggesting the active component formed large complexes in aqueous solution. Since polyphenols commonly form large, non-dialyzable polymeric complexes in aqueous solution[258;259], and the immunomodulatory activity of many plant extracts resides in the polyphenol fraction[47;259], we hypothesized that the broad activity profile from samples collected during the HPLC run may be from a group of polyphenolic complexes.

Tannins, a major component of plant polyphenols, are characterized by their ability to bind and precipitate proteins. Therefore, utilizing this peptide-binding nature of tannins, we removed the tannin fraction of the Cat's Claw sample using the peptide mimetic, polyvinylpolypyrrolidone (PVPP)[260]. The γδ T cell agonist activity in the Cat's Claw extract was removed by the PVPP column, as determined by an absence of IL-2Rα up-regulation in bovine γδ T cells treated with the pre-cleared extract (Fig. 5B). APP,

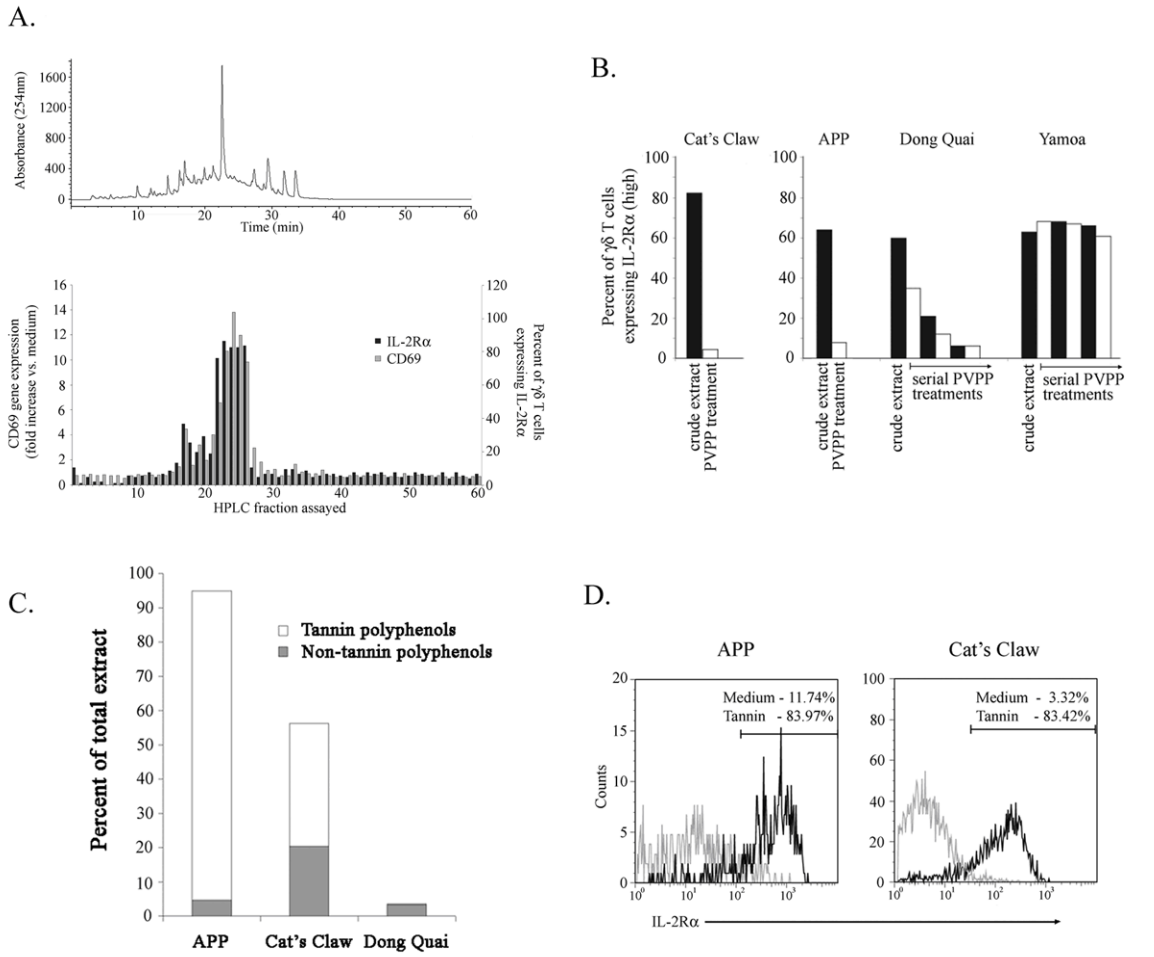
Dong Quai, and Yamoa extracts were additionally treated with PVPP to produce tannin-depleted extracts, which were similarly assayed for agonist activity. As shown in Fig. 5B, a single treatment with PVPP removed >90% of the bioactivity in APP. PVPP also removed activity in the Dong Quai extract, although four rounds of depletion were required to remove activity. In contrast, five sequential rounds of PVPP tannin-depletion of the Yamoa extract had minimal impact on its bio-activity (Fig. 5B) indicating the active component in Yamoa was not a tannin. The active component of the Yamoa extract is being characterized in other studies (Jutila, in preparation), and will not be further discussed.

To measure the amount of polyphenols comprising the putative tannin agonist-containing extracts (APP, Cat's Claw and Dong Quai), we performed a series of Folin-Ciocalteu assays[255]. These assays quantify the amount of polyphenols in a preparation. Therefore, Folin-Ciocalteu assays were used to measure the polyphenol contribution in the entire extracts as well as the tannin and non-tannin fractions. Using tannic acid as a standard for polyphenol weight, we determined the amount of polyphenols contributing to the total dry weight in each of the extracts. Polyphenols contributed to 95% of the total weight of the APP, 56% of the Cat's Claw, and 4% of the Dong Quai extracts (Fig. 5C). Next, we determined the amount of tannins contributing to the total polyphenols in the extracts by separating tannins and non-tannin polyphenols with PVPP prior to Folin-Ciocalteu assay. Tannins contributed to the majority of polyphenols detected in the APP and Cat's Claw extracts, 95.1% and 63.7% respectively. Conversely, the polyphenols in the Dong Quai were predominantly non-tannin (90.6%) in character. Interestingly, the

Dong Quai extract contained less than 1% tannins by weight, though PVPP treatment did remove activity (Fig. 5C).

To confirm that tannins in APP, Cat's Claw, and Dong Quai extracts were the active γδ T cell agonist, the tannin fractions were isolated and assayed for activity. Tannins were separated from each whole extract with PVPP, and then eluted from the PVPP with NaOH. The resulting tannin fractions were assayed for bioactivity via detection of IL-2Rα expression on bovine γδ T cells. The eluted tannin fraction from Dong Quai demonstrated little bioactivity (data not shown). In contrast, culture with tannin fractions isolated from Cat's Claw and APP up-regulated IL-2Rα expression on γδ T cells (Fig. 5D), thereby verifying that the γδ T cell agonist in these two extracts was a tannin. Analysis of the PVPP-precipitated tannin fraction via HPLC resulted in a broad elution profile similar to that shown in Fig. 5A (data not shown), confirming the heterogeneous nature of the tannin fraction in these extracts.

Figure 5. Identification of Tannins as the Agonist Component of Select Plant Extracts



A, Cat's Claw was analyzed by HPLC to characterize the active component(s). The elution profile from a C-18 HPLC column is represented by absorbance at 254nm over time in minutes *(top)*. Fractions from the HPLC column were collected at 1 minute intervals, and their activity on bovine γδ T cells was analyzed by IL-2Rα cell surface expression (FACS) and CD69 gene expression *(bottom)*. *B*, Plant extracts were repeatedly treated with PVPP to remove tannins. The resulting tannin-depleted fractions were then assayed for agonist activity by measuring IL-2Rα expression in γδ T cells after 48h culture with bovine PBMCs. *C*, Polyphenol content was determined for APP, Cat's Claw, and Dong Quai by Folin-Ciocalteu assay. Percentage values represent the fraction of polyphenols comprising the whole extract. The composition of the polyphenol fraction for each extract is further characterized as either tannin (white) or non-tannin (shaded). *D*, IL-2Rα expression was measured on γδ T cells after incubation with PVPP eluents of APP (36.4μg/ml) or Cat's Claw (113.8μg/ml). Data are representative of at least five experiments.

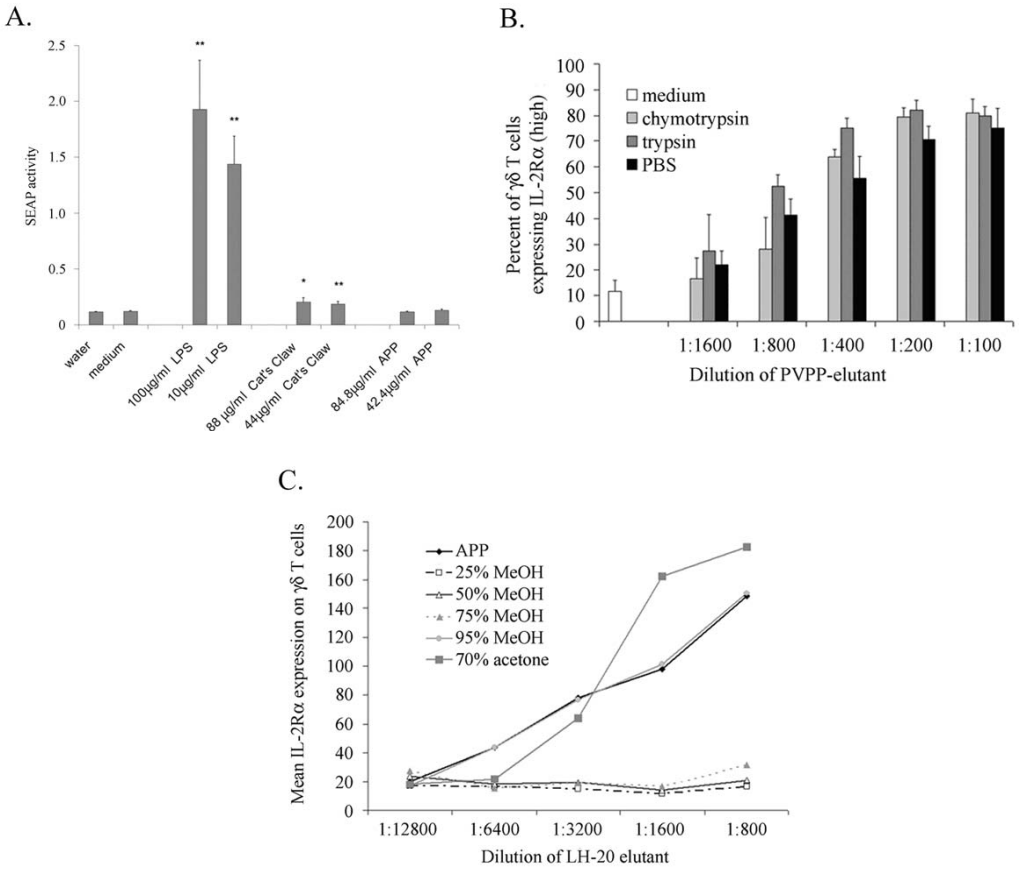
We next tested the Cat's Claw and APP extracts for TLR agonist contaminants, which would potentially confound interpretation of the extract's activity, using the human TLR reporter cell line THP1-CD14-Blue (InvivoGen, San Diego, CA). This cell line reports TLR1-10 activity (with the exception of TLRs 3 and 9) via NFκB/AP1 transcription of secreted alkaline phosphatase (SEAP). No apparent TLR agonist activity was detected in the APP extract, and only minimal TLR agonist activity was detected in the Cat's Claw extract (Fig. 6A). To confirm that the APP extract did not contain endotoxin at concentrations below detection levels in the THP1-CD14-Blue reporter, we performed a turbimetric limulus assay. APP was determined to contain less than 16 endotoxin units per μg (concentration in culture equivalent to less than 20 ng/ml *E. coli O55:B5* LPS). These data demonstrated that the principal activity observed in the tannin fraction of APP, and most likely Cat's Claw, was not due to TLR agonists.

Although PVPP is a fairly specific adsorbent for tannins, proteins, including HAP I and HAP 2 from the apple, associate with tannins and are co-adsorbed to PVPP[261]. Therefore, to exclude the possibility of protein eliciting the γδ T cell agonist activity, we treated APP with proteases. For these experiments, APP was treated with trypsin or chymotrypsin, adsorbed to PVPP, eluted, and bioassayed via CFSE (data not shown) and IL-2Rα assays (Fig. 6B). Protease treatment of APP had little effect on the agonist activity eluted from subsequent PVPP tannin, demonstrating that the agonist activity was most likely from tannins and not tannin-associated proteins.

Tannins can be classified as either hydrolyzable or condensed. Although structurally different, both types of tannins are adsorbed to PVPP. Therefore we sought to

determine to which of these tannin groups the active component of Cat's Claw and APP belonged. To this end, we treated the PVPP-eluant (total tannins) from Cat's Claw with HCL/mercaptoethanolamine at 90°C for 2h, a process that hydrolyzes procyanidins[262]. Hydrolysis of the procyanidins removed γδ T cell activity (data not shown), supporting the concept of procyanidins as the active component in Cat's Claw. As an additional test, we sought to confirm the γδ T cell agonist activity of procyanidins by purifying non-degraded, procyanidins. We chose to separate procyanidins from APP because it contained fewer non-tannin contaminants and showed no TLR agonist activity. Consequently, we adsorbed APP to a Sephadex LH-20 (LH-20; a 25-100μm bead size lipophilic crosslinked dextran-based resin) column, which specifically adsorbs condensed, but not hydrolyzable tannins. The adsorbed tannins were then eluted with MeOH and acetone[263]. In addition to separating whole procyanidins, use of the LH-20 column additionally allows for partial separation of procyanidins by size. Monomers and dimers preferentially elute in a weaker, 75% MeOH solution, whereas trimers and oligomeric procyanidins are concentrated in the 95% MeOH and 70% acetone fractions[256]. We therefore applied this selective elution method to APP adsorbed to LH-20 and tested the subsequent fractions by IL-2Rα (Fig. 6C) and CFSE (data not shown) assays. We observed γδ T cell activity in the 95% MeOH and acetone fractions, indicating that the predominant γδ T cell agonists in these tannin preparations were trimers or larger (Fig. 6E). We also confirmed that the 95% MeOH and 70% acetone fractions were predominantly larger procyanidins by TLC (data not shown).

Figure 6. Biochemical Analysis of the Cat's Claw and APP Plant Extracts



A, THP1-CD14-Blue cells were cultured with APP (42.4 or 84.8 µg/ml), Cat's Claw (44 or 88 µg/ml), LPS (10 or 100 µg/ml, [5,000 and 50,000 EU/ml, respectively]) or medium alone. TLR agonist activity was quantified by SEAP secretion. *P*-values for differences in means are *, $P < 0.05$; **, $P < 0.01$. *B*, APP was incubated with chymotrypsin, trypsin, or PBS in conjunction with PVPP for 1h. The resulting slurry was washed and the tannins eluted from the PVPP. The collected tannins were cultured for 48 h with bovine PBMCs, and then analyzed for IL-2Rα expression on the γδ T cell population. Data represent mean values from 3 different experiments. *C*, APP procyanidins adsorbed to LH-20 resin were eluted with increasing concentrations of MeOH and a final wash with 70% acetone. The fractions were dried, resuspended in water, and dilutions were cultured with PBMCs for 48h. IL-2Rα expression on γδ T cells from the cultures was analyzed to determine activity in the procyanidin fractions.

Collectively, these analyses indicated that the γδ T cell agonist activities in Cat's Claw and APP, and possibly Dong Quai, were due to procyanidins. In contrast, distinct non-tannin γδ T cell agonists existed in the Yamoa extracts as determined by the inability of PVPP to remove γδ T cell agonist activity in the Yamoa extract (Fig. 5B).

Effects of Plant Tannins on Human Cells

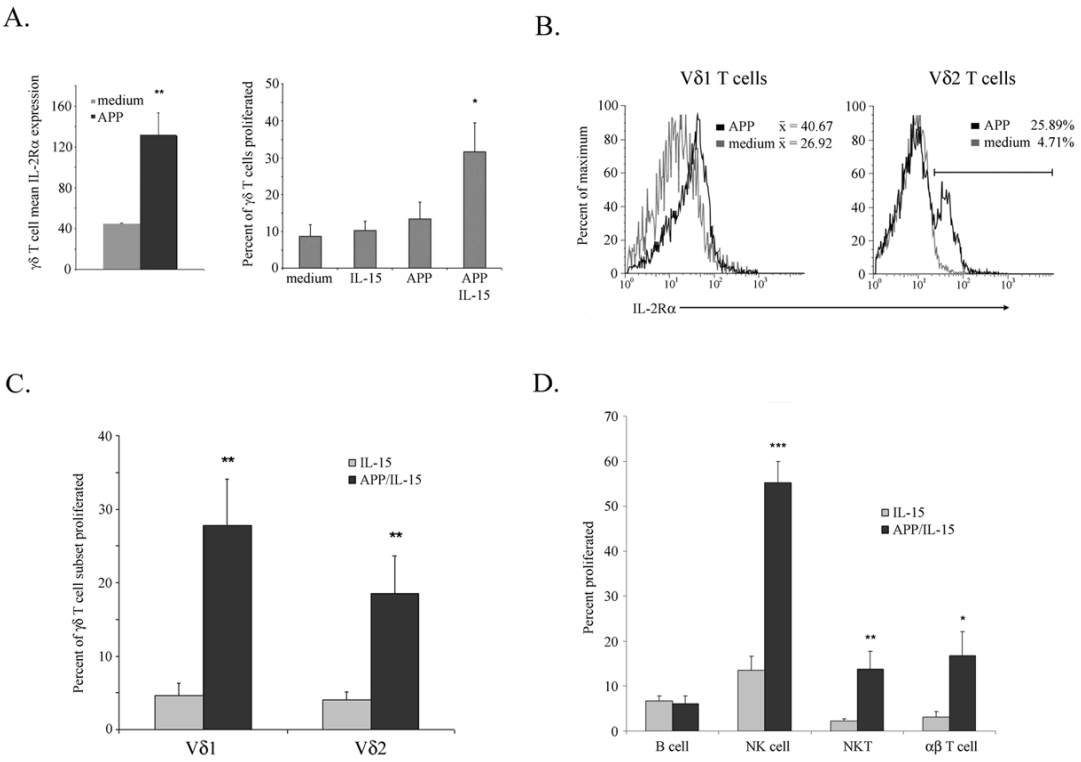
The γδ T cell agonist activity of plant tannins were then characterized on human cells. APP was selected for these analyses since it was more enriched for tannins and demonstrated no detectable TLR agonist activity by either THP1-CD14-Blue reporter cell or limulus assays. Additionally, APP is predominantly composed of tannins and therefore contains fewer impurities than Cat's Claw. Activity in the procyanidin fraction from APP was confirmed in human cells, and this activity was comparable to the total APP preparation (data not shown). As shown in Fig. 7A, APP induced IL-2Rα up-regulation on human γδ T cells, results comparable to those observed in bovine γδ T cells (Fig. 4B). Contrary to the response observed in bovine γδ T cells, wherein a portion of the γδ T cells proliferated in response to APP alone, human γδ T cells required the addition of low-dose IL-15 (1 ng/ml; Fig. 7A) or IL-2 (0.1 ng/ml; data not shown) to significantly proliferate. This co-stimulatory requirement for the proliferation of γδ T cells was similar to the well-defined phosphoantigen, 1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP)[249]. However, in contrast to HDMAPP and other phosphoantigen-based agonists, which activate only the Vδ2 subset of γδ T cells, APP induced IL-2Rα up-regulation and proliferation of both Vδ1 and Vδ2 T cells (Fig. 7B and 4B). Unlike the γδ T cell-specific

proliferative response to APP observed in bovine PBMCs, populations of human non-γδ T cells also responded during culture with the extract. The responding cells included the majority of NK cells and fractions of NKT cells and αβ T cells. There was no significant change in APP-induced B-cell proliferation in this assay (Fig. 7D) although B cells are shown to respond to other tannin preparations[91]. Of the responding αβ T cells, naïve cells (CD45RO negative cells) were predominant (data not shown). The effective dose range for human cells was approximately 1-20 μg/ml, with concentrations >40 μg/ml being toxic, which compared closely to the effective dose range for bovine cells of 1-40 μg/ml (data not shown).

Enhancement of the Phosphoantigen Agonist Response

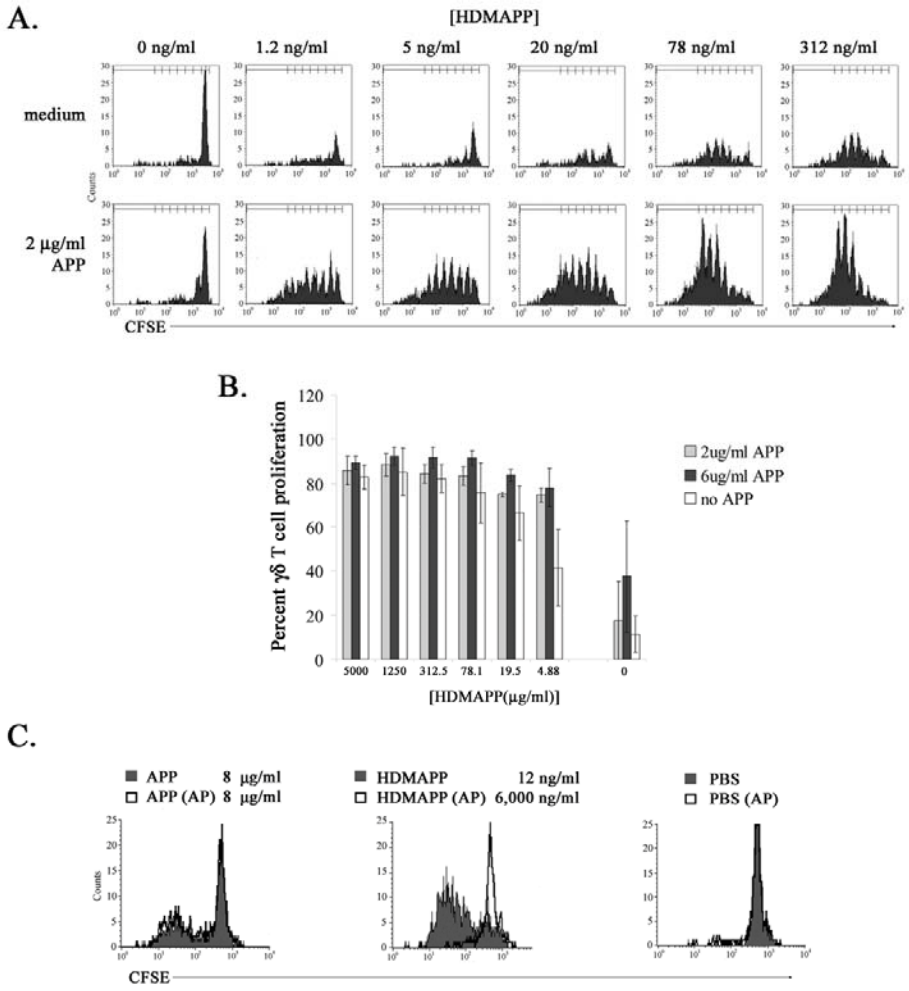
To determine if γδ T cell proliferation driven by apple tannins was synergistic with the phosphoantigen HDMAPP, human PBMC cultures containing low-dose IL-15 (1 ng/ml) and various concentrations of HDMAPP were additionally cultured with or without 2 μg/ml APP. In the presence of APP, γδ T cells responded nearly as robustly to 1.2 ng/ml HDMAPP as they did to 312 ng/ml HDMAPP alone (Fig. 8A), demonstrating a synergistic effect of APP with phosphoantigen-directed proliferation. Since plants generate phosphoantigens[227], these agonists were a potential contaminant in plant extracts. To address the potential of phosphoantigen activity in the APP, we inactivated potential contaminating phosphoantigens with alkaline phosphatase[130;264]. Alkaline phosphatase treatment of APP had no effect on its bioactivity, whereas the activity of HDMAPP was abolished (Fig. 8B).

Figure 7. Characterization of the Human Response to Plant Tannin



Human PBMCs were treated with PBS or APP (4 to 16 μg/ml) and activation (IL-2Rα expression) or proliferation (CFSE) was measured after 48 h or 5 days of culture, respectively. *A*, Flow cytometry was used to determine the effect of APP treatment on γδ T cell activation (IL-2Rα expression, left graph) and proliferation (CFSE assay) with or without IL-15 (1 ng/ml) (right graph). Data represent mean values from at least four individuals. *B*, Activation profiles (IL-2Rα expression) of human Vδ1 and Vδ2 T cells treated with APP are shown. Data are representative of three independent experiments. *C*, Proliferation (CFSE assay) of Vδ1 and Vδ2 T cell subsets was measured in response to APP and medium controls (all containing 1 ng/ml IL-15). Values represent the average percent of Vδ1 or Vδ2 T cells that proliferated in samples from four donors. *D*, Proliferation (CFSE assay) of NK cells ($CD3^-$, $CD94^+$), NKT cells ($CD3^+$, $CD94^+$), αβ T cells ($CD3^+$, γδ TCR^-) and B-cells ($CD3^-$, $CD94^-$) was measured in IL-15-containing (1 ng/ml) PBMC cultures with either APP or medium alone. *P*-values for differences in means are *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 8. Human γδ T Cell Responses to the Phosphoantigen HDMAPP are Augmented by Plant Tannins



A, Proliferation of CFSE-labeled human PBMCs was measured in response to varying concentrations of HDMAPP with or without 2 µg/ml APP. All cultures, including medium alone, contained 1ng/ml IL-15. Responses by γδ T cells were determined using γδ TCR mAbs in two color FACS analyses as described in the materials and methods. Data are representative of three independent experiments from different donors. Average γδ T cell proliferation in the three donors is presented in *B*, with error bars representing the standard deviation. *C*, Responses of human γδ T cells to APP and HDMAPP preparations that were passed over immobilized alkaline phosphatase (AP), as measured in the two-color FACS-based CFSE assay with 1ng/ml IL-15. Overlay histograms of gated γδ T cells are presented. Shaded histograms represent buffer-treated samples and solid black lines represent alkaline phosphatase-treated samples. Data are representative of two independent digestions using PBMCs from three different donors.

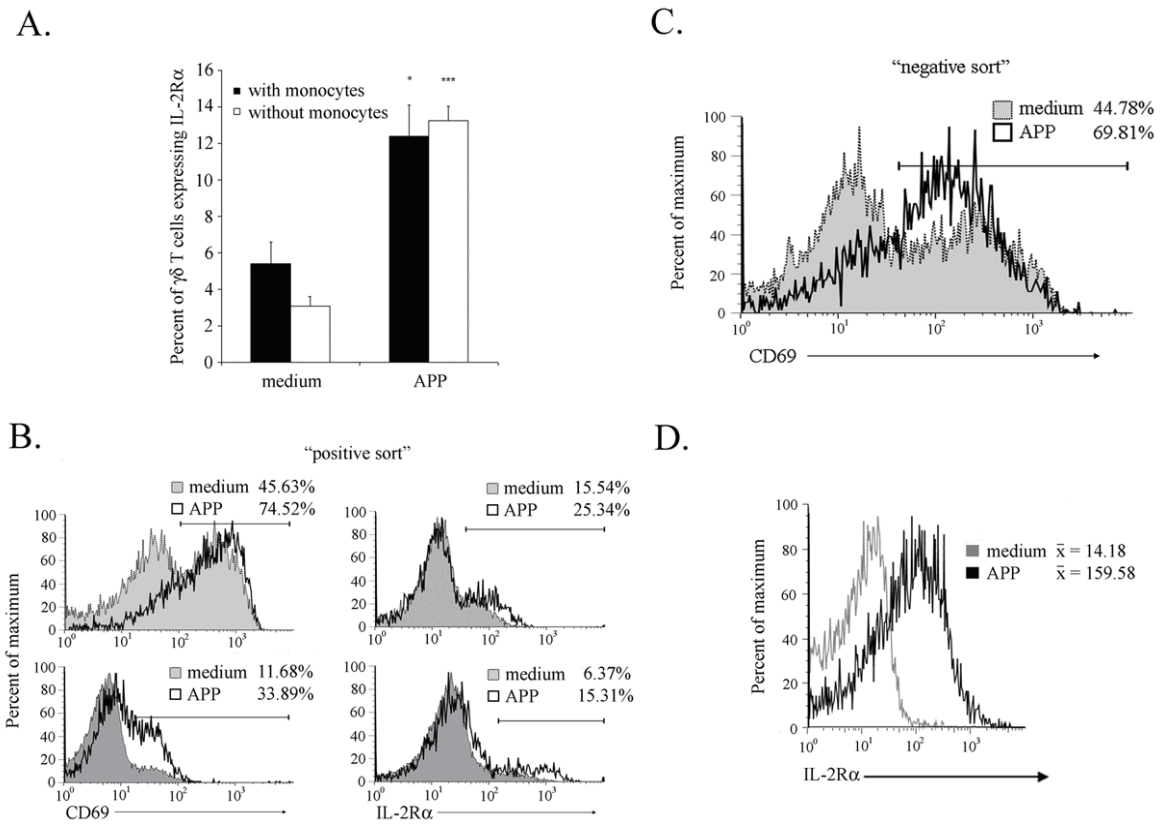
Plant Tannins Prime γδ T Cells to
Respond More Robustly to Secondary Stimulation

In a concurrent study, we observed an increase in gene expression (Mip1α, GM-CSF) in bovine γδ T cells after only 4 hours in culture with APP[71]. Due to this immediate response, these observations indicated γδ T cells respond directly to procyanidins. Therefore, to establish if γδ T cells responded directly to APP or required signaling through an intermediate cell type, bovine and human PBMC preparations were subjected to various cell separation procedures prior to culture with APP. Since some tannin preparations impact monocyte cytokine secretion[265], we first compared activation profiles between monocyte-depleted and non-depleted PBMCs from the same bovine or human donor. APP induced equivalent IL-2Rα up-regulation on γδ T cells in both monocyte-containing and -depleted bovine (data not shown) and human (Fig. 9A) cultures. To verify that γδ T cells responded directly to APP, human PBMCs were sorted via positive (Fig. 9B) and negative (Fig. 9C) procedures using magnetic beads prior to culture with APP or medium alone. Cell activation was measured by CD69 and IL-2Rα expression in the positively sorted cells (Fig. 9B). Negatively sorted γδ T cells (Fig 9C) were assayed for CD69 expression only. Notably, a large fraction of the γδ T cells from the medium controls in both the positively and negatively sorted human γδ T cell cultures were activated (Fig. 9B and 9C, medium controls). PBMC cultures from the same donors assayed in parallel did not demonstrate increased baseline activation marker (CD69 and IL-2Rα) expression (data not shown), suggesting the activation was a result of purified human γδ T cells cultured under the conditions used herein. Regardless of the high

baseline activation in the purified γδ T cell cultures, there was a considerable increase in activation marker expression (CD69 and IL-2Rα, though changes in CD69 were greater) in APP-treated cultures, demonstrating direct activity for human γδ T cells in the absence of accessory cells.

To obtain a purified population of γδ T cells that does not inherently become activated, we sorted bovine γδ T cells via FACS[71] (Fig 9D) and used these cells as another test to confirm the direct activity of APP on γδ T cells. Bovine peripheral γδ T cells were sorted by flow cytometry to 97.25% purity, treated with PBS or APP, and analyzed for IL-2Rα expression. As shown in Fig. 9D, sorted bovine γδ T cells expressed low IL-2Rα levels following culture in medium alone. The APP-treated, sorted γδ T cell cultures induced IL-2Rα up-regulation to a level comparable to that seen on γδ T cells in a non-sorted culture (see Fig. 4B). As further confirmation of the purified bovine γδ T cell response to APP, cells were positively sorted using magnetic beads (99.1% purity). These cells responded to APP culture in a manner similar to the FACS-sorted bovine γδ T cells; a negligible expression of IL-2Rα was detected in the control population, whereas the APP-treated (10 μg/ml) cells increased IL-2Rα expression greater than 9-fold (data not shown).

Figure 9. Plant Tannins Induce γδ T Cell Activation in the Absence of Accessory Cells

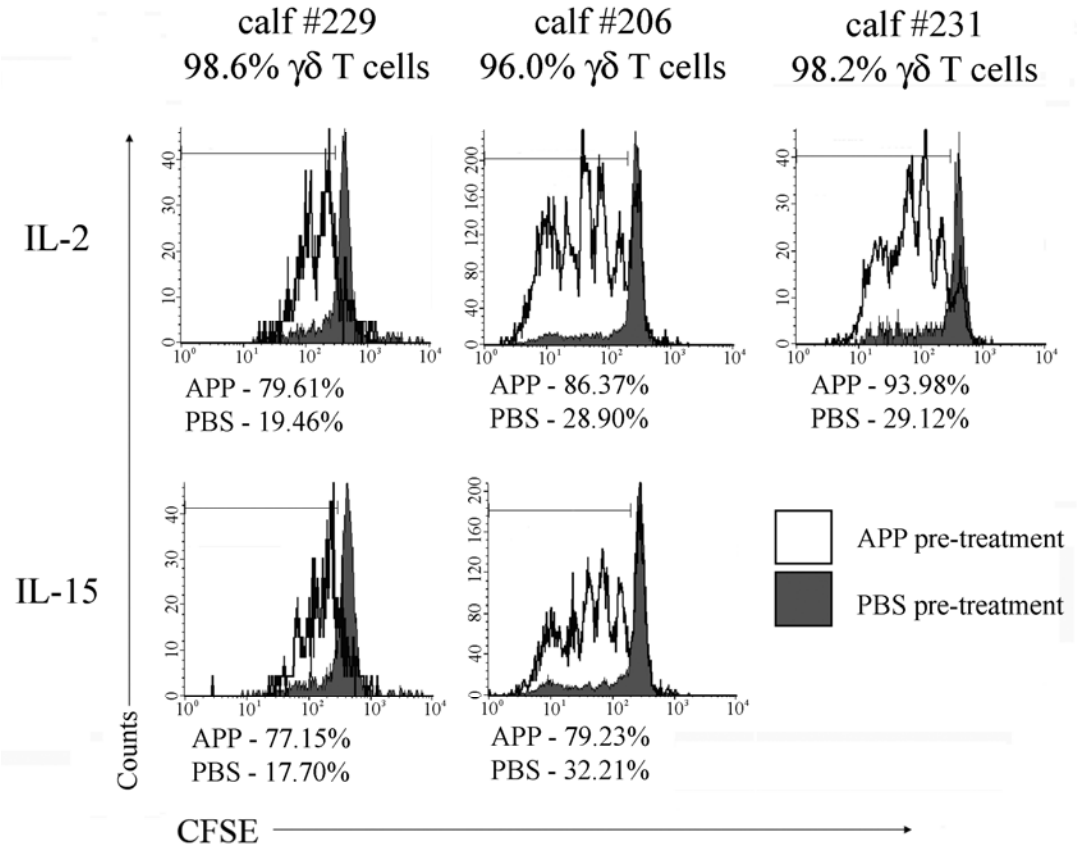


Human and bovine γδ T cells were sorted to varying degrees of cell purity and cultured with APP or medium alone for 48 h prior to FACS analysis of activation marker expression on γδ T cells. *A*, Monocyte-depleted and non-depleted human PBMCs from four donors were incubated with medium control or APP, and the effect on IL-2Rα expression determined by FACS. Data represent the concentration of APP inducing maximal activation (4-16 μg/ml). Differences in means were significant at *p*-values < 0.05* or < 0.001***. *B*, Positively-sorted γδ T cells (91.14%, top; 83.63%, bottom) obtained from different donors were plated with either APP (12 μg/ml) or medium alone and then were analyzed by FACS for CD69 and IL-2Rα expression. *C*, Human γδ T cells were collected by negative sort (94.18%) and then cultured with 14 μg/ml APP or medium alone prior to analysis for CD69 expression. *D*, IL-2Rα expression was measured on sorted bovine γδ T cells (97.25%) after culture with medium control or APP (31.3 μg/ml).

Since γδ T cells responded directly to APP with increased expression of activation markers, we next sought to determine if APP treatment additionally conferred an augmented proliferative status upon the sorted γδ T cell cultures. We first tested monocyte-depleted human and bovine PBMC cultures to determine if monocytes were required for γδ T cell proliferation in response to APP. Both human and bovine monocyte-depleted cultures demonstrated γδ T cell proliferation when treated with APP (data not shown), demonstrating APP-treated γδ T cells could proliferate in the absence of monocyte-derived growth factors. Next, we tested purified γδ T cell cultures (>96% purity) to determine if APP-treated γδ T cells could proliferate without the presence of any accessory cells. Sorted bovine γδ T cell cultures were selected for these experiments because high baseline activation was not observed in purified cultures, unlike the sorted human γδ T cell cultures. Initial findings demonstrated that sorted bovine γδ T cells did not proliferate well following APP treatment alone (data not shown), though as shown in Fig. 9D, APP induced IL-2Rα expression. We then tested whether addition of IL-2 or IL-15 would drive cell division in APP pre-treated cells (priming assay). To this end, we cultured FACS-sorted, CFSE-labeled bovine γδ T cells for 48 h in culture medium containing either PBS or APP (priming event), washed, and then replaced the media with either IL-2 (1 ng/ml) or IL-15 (10 ng/ml). After an additional three days of culture with the cytokines, the amount of cell division in APP-primed or PBS-treated cells was compared via FACS analysis. As shown in Fig. 10, APP-primed γδ T cells from three different animals responded robustly (77-94% responding cells) to either IL-2 or IL-15,

whereas the PBS-primed cells showed minimal response to either cytokine (18-32% responding cells). Thus, APP directly primed γδ T cells to proliferate in response to IL-2 or IL-15 in the absence of accessory cells.

Figure 10. APP-primed, Purified γδ T Cell Cultures Proliferate in Response to IL-2 and IL-15.



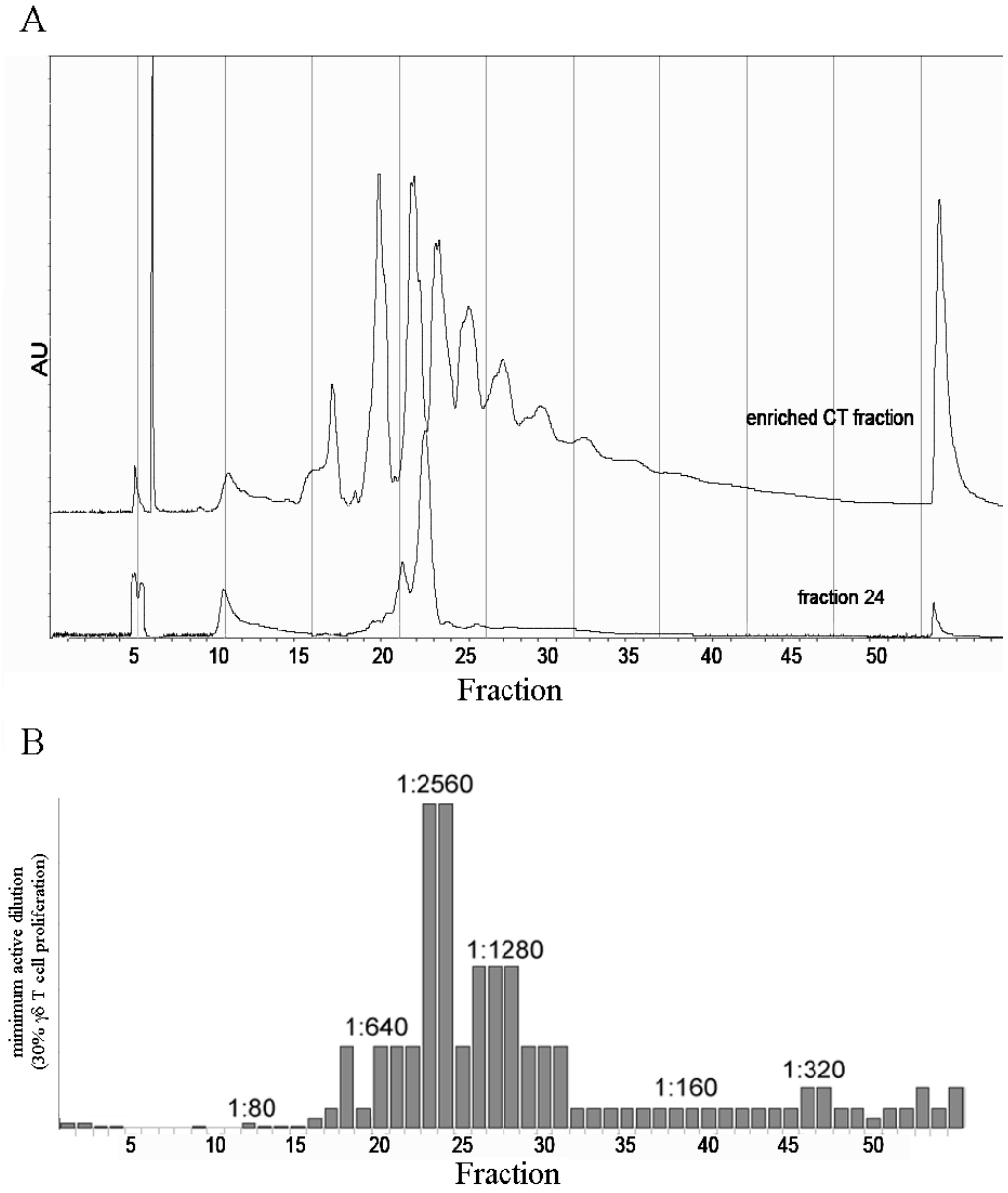
Sorted bovine γδ T cells were labeled with CFSE and cultured with APP (25 μg/ml) or PBS in culture medium. After 48 h, the medium was replaced with fresh medium containing IL-2 or IL-15, and then cells were cultured for an additional 72-96 h before FACS analysis. All cultures were plated in triplicate and *p*-values calculated from the ratio of proliferating to non-proliferating γδ T cells in both of the PBS- and APP-treated controls ($p<0.001$ for all experiments). Data displayed are pooled from the triplicates collected for each treatment and calf.

Characterization of the Agonist Activity Induced by Individual Procyanidin Species

To identify the active tannin species responsible for γδ T cell agonist activity, we separated the oligomeric-tannin enriched APP fraction from Figure 6C by normal-phase HPLC (Fig. 11A for the HPLC tracing). Using this method of fractionation, tannins can be separated by the number of subunits; monomers are eluted first, followed by dimers, and so forth. Fractions were collected at 1 minute intervals for the duration of the 60 minute fractionation.

To determine the active oligomeric tannin in the APP preparation, these fractions were tested for γδ T cell agonist activity, γδ T cell expression of IL-2Rα, during bovine PBMC culture (Fig. 11B). Peak agonist activity was observed in fraction #24, although other fractions demonstrated some activity on bovine γδ T cells. To confirm efficient separation of fraction #24, it was reapplied to the same HPLC separation method (Fig. 11A). The elution profile for fraction #24 appeared as we predicted with a predominant peak corresponding with a unique procyanidin oligomer. There was additionally a minor population of a smaller size procyanidin as observed in the smaller peak elution prior to the major peak, indicating that although HPLC fractionation did enrich the active component from APP, fraction #24 was not a purified chemical entity.

Figure 11. HPLC Fractionation of Procyanidins



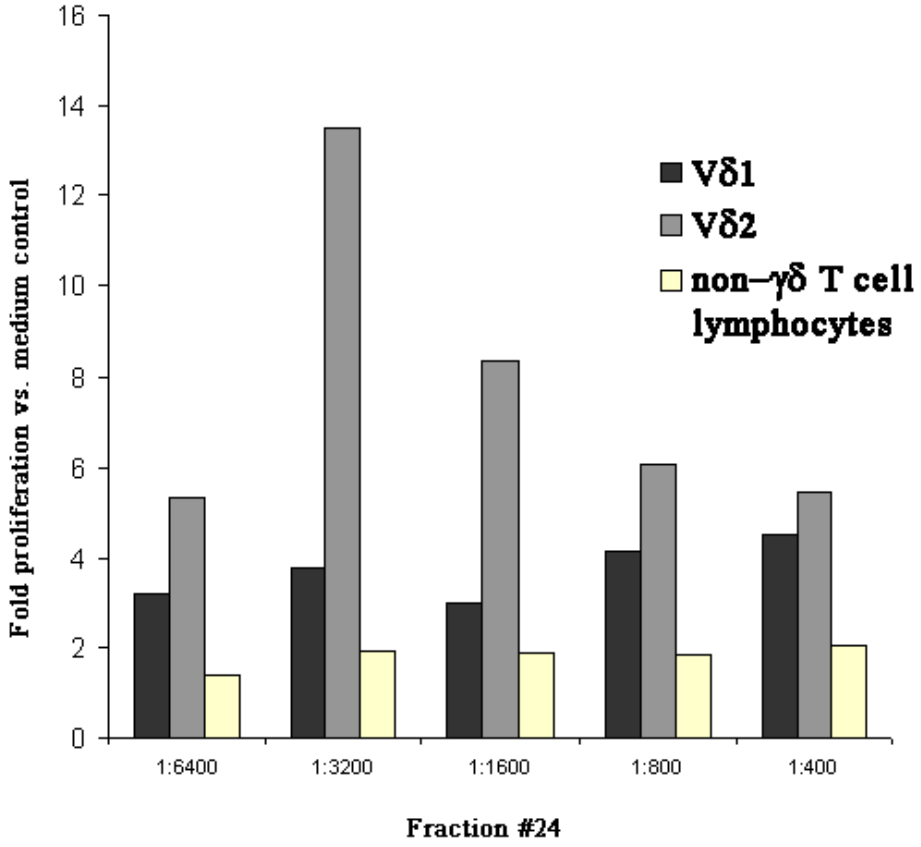
HPLC fractionation of procyanidins from APP confirms the oligomeric nature of the active tannin fraction. The enriched oligomeric procyanidin preparation was further fractionated with a C18 HPLC column. *A*, HPLC absorbtion profile @ 254nm for the initial, procyanidin fractionation and the biologically active fraction, #24. *B,* Expression of bovine IL2Rα was measured in the presence of fractions obtained from the HPLC run. Data represent the lowest dilution of each fraction in medium required to achieve 30% proliferation.

Three fractions from the HPLC run, #19, #24, and #27, were then tested for their ability to proliferate human γδ T cells. Similar to the γδ T cell agonist activity observed in bovine PBMCs. Fraction #24 was the most potent of these fractions, although other fractions demonstrated some activity (data not shown). These results indicated two properties of the active tannin component. First, the same tannin fractions induced similar γδ T cell agonist activity in both human and bovine species. This indicated γδ T cells in different species may respond to the same tannin(s). Second, these results indicated the active tannin was likely in the range of the trimeric or tetrameric species. This can be determined by the HPLC elution profile (Fig. 11A). Procyanidins sequentially elute using this method. The monomers elute first, followed by increasingly larger procyanidins as observed by peaks in the elution profile[266]. Based on this elution profile, we predicted the active procyanidin was in the trimer or tetramer range. To confirm this, we tested fraction #24 using mass spectrometry. Unfortuantely, there was not enough material remaining to confirm the procayinidin size.

Analysis of the Vδ1 and Vδ2 T cell subsets was additionally performed during culture with fraction #24. As with the total tannin preparation, APP, both of these subsets were induced to proliferate during culture with fraction #24. However, unlike the total tannin preparation, which induces non-γδ T cell proliferation (Fig. 7D), this activity was greatly reduced in fraction #24 (Fig. 12). This suggested the isolated tannin was a more specific γδ T cell agonist than the total tannin preparation. Additionally, unlike the γδ T cell subset response in the total tannin preparation, fraction #24 was more active on Vδ2 T cells than on Vδ1 (Fig 12). The total tannin preparation induces 27.5% of the Vδ1 T

cells to proliferate whereas only 18.5% of Vδ2 T cells proliterate (Fig 7C). The opposite response is observed in fraction #24 wherein a preperential proliferative response is observed in the Vδ2 T cell population. A similar response was observed in the second donor's cells treated with fraction #24.

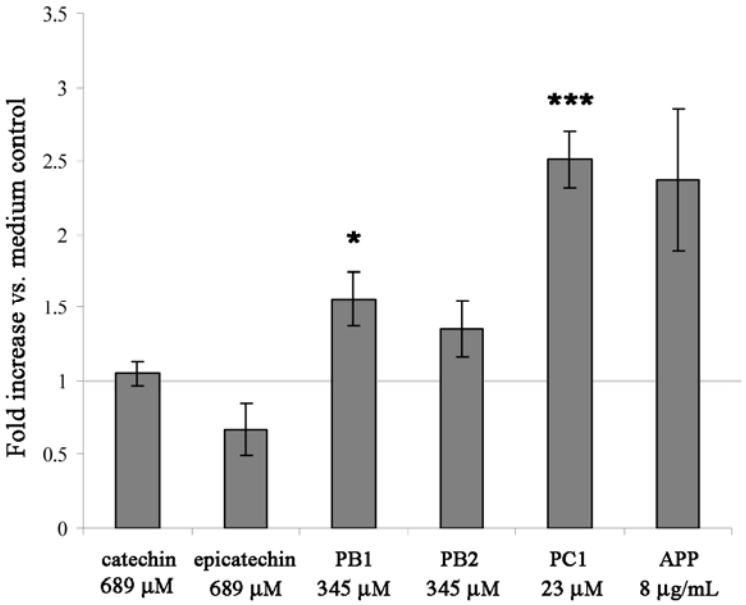
Figure 12. Procyanidin HPLC Fraction #24 from APP is a Selective γδ T Cell Agonist.



Human PBMCs were CFSE-labeled, treated with various concentrations of Fraction #24 or medium only, and were cultured for 5 days in the presence of low-dose IL-15 (1 ng/ml). Proliferation was measured in γδ T cell subsets (Vδ1 and Vδ2) and non-γδ T cell lymphocytes. Bars represent the fold increases for each of these populations vs. medium controls under the various dilutions of fraction #24 indicated. Lymphocyte survival was >75% for all dilutions tested. Results are representative from two donors.

Since separation of procyanidins by HPLC did not produce a purified procyanidin species (see Fig. 11A), we undertook a different approach to determine γδ T cell active procyanidin species. To this end, we tested commercially available trimeric procyanidin, Procyanidin C1 (PC1), for γδ T cell agonist activity. We also tested commercially available monomeric and dimeric procyanidins to confirm the lack of activity observed during HPLC fractionation. For these analyses, we CFSE-labeled human PBMCs and cultured them with monomeric, dimeric, or trimeric procyanidins as well as APP or medium only controls (Fig. 13). The tannins tested were the commercially available, purified procyanidin components of APP[267], which consisted of epicatechin, catechin, procyanidin B1 (PB1), procyanidin B2 (PB2), or PC1. The monomers, epicatechin and catechin, were tested at concentrations from 689 mM to 5.1 mM. No γδ T cell agonist activity was seen in these cultures. The dimeric procyanidins, PB1 and PB2, were tested at concentrations from 345 μM to 2.5 μM. Moderate γδ T cell agonist activity was observed in these cultures. Finally, the trimeric procyanidin C1 was cultured at concentraions ranging from 230 μM to 1.7 μM. As was predicted from the HPLC separation, these were the most active procyanidins tested, with peak activity at 23 μM. Interestingly, in all three donors tested, the peak activity in PC1 was greater than that of the total tannin preparation, APP.

Figure 13. Purified Procyanidin Dimers and Trimer Induce Human γδ T Cell Proliferation.

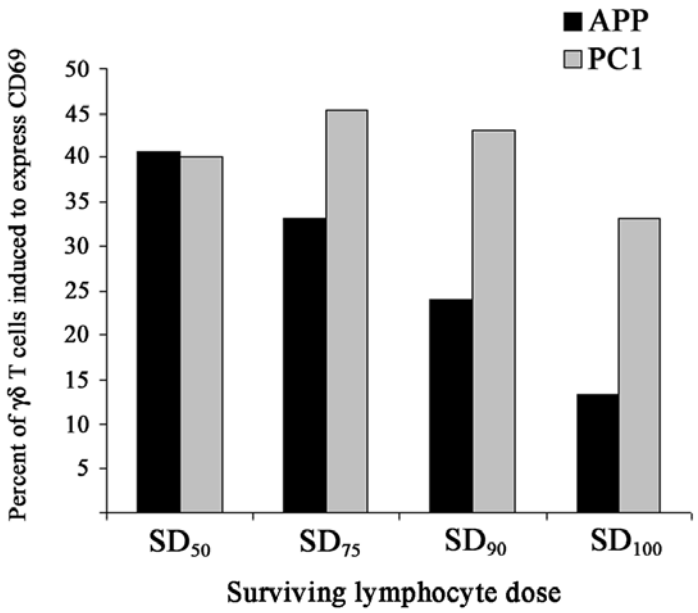


Human PBMCs from 3 donors were CFSE-labeled and cultured with IL-15 (1 ng/ml) and either APP, purified tannins, or medium only. Dilutions of tannins inducing peak responses were selected and compared for their γδ T cell proliferative ability. Data represent the average fold increase of γδ T cell proliferation in the procyanidin-treated samples versus the medium control (1 ng/ml IL-15) wells. Error bars represent the standard error of the mean. *P*-values for differences in means are *, $P < 0.05$; ***, $P < 0.001$.

Since one of the limiting factors observed during APP-induced γδ T cell proliferation was the amount of lymphocyte cell death in the range of agonist activity, we compared the total tannin preparation, APP, to PC1 to determine if isolating the active tannin improved cell survival. For these analyses, human PBMCs were cultured with concentrations of APP or PC1 ranging from 200 µg/ml to 1.5 µg/ml and 232 µM to 1.73 µM, respectively. After 2 days of culture, γδ T cell activation (CD69 expression) and lymphocyte survival (light scatter) were analysed under each culture condition. γδ T cell

activation was then calculated at tannin a dose observing 50%, 75%, 90%, and 100% cell survival ($SD_{50\text{-}100}$) as compared to medium controls. These results indicated the PC1 fraction was less toxic than the total tannin fraction, APP (Fig. 14).

Figure 14. Procyanidin C1 is less Toxic than Total APP Tannins

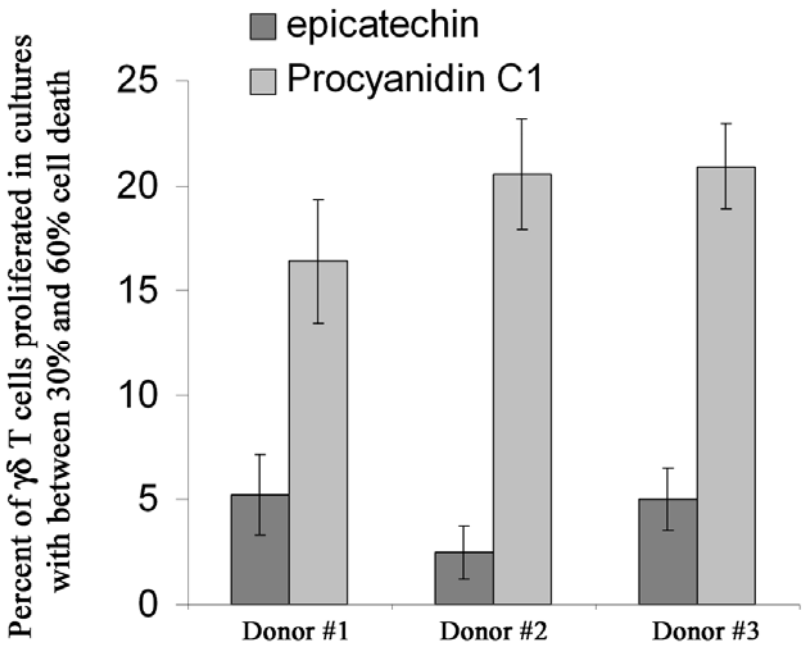


Human PBMCs were cultured for 48 h in the presence of medium alone, APP, or purified procyanidin C1 at various concentrations. After this culture period, the ratio of surviving cells to dead/dying cells was determined by light scatter (FACS) where medium control was used as maximum survival. γδ T cell activation was measured at the four concentrations inducing 50%, 25%, 10%, and 0% lymphocyte death (SD_{50}-SD_{100}, respectively). Data represent the percentage of $CD69^{bright}$ γδ T cells at each tannin concentration minus the percentage of $CD69^{bright}$ γδ T cells in the medium controls.

Since one of the primary functions of γδ T cells is recognition of cellular stress[242], it is possible the γδ T cell response during culture with tannins is due to recognition of cellular stress during non-specific cell death induced by tannins. Therefore, we measured the amount of γδ T cell activation during culture with semi-toxic (SD_{50}) concentrations of

both epicatechin and PC1 (Fig. 15). Concentrations were found to be 23.0 μM and 515-688 μM for PC1 and epicatechin, respectively. At these concentrations, epicatechin-induced γδ T cell activation was comparable to those observed at lower concentrations of catechin and in medium controls (data not shown), therefore indicating a unique property of PC1 to stimulate γδ T cells not provided by its subunit, epicatechin.

Figure 15. γδ T cell Activation Cannot be Explained by Procyanidin-induced Cell Death



Semi-toxic concentrations of Procyanidin C1, but not its monomeric subunit, induce γδ T cell proliferation. CFSE-labeled human PBMCs were cultured with IL-15 (1 ng/ml) and medium alone or various concentrations of purified epicatechin or purified Procyanidin C1 for 5 days. Cultures were analyzed for lymphocyte survival by light scatter (FACS) where the maximum survival was medium only. Cell cultures between 30-60% lymphocyte death were analyzed for γδ T cell proliferation. Bars represent the AVG γδ T cell proliferation and SD from each of the 3 cultures found to induce this range of cell death in culture.

Discussion

γδ T cells are one of four lymphocyte populations thought to be important in innate immunity, with the other three being NK cells, NKT cells, and B1 cells. Recently, clinical approaches were developed to expand the number and/or function of human γδ T cells as a potential therapeutic modality for some cancers[225] and to increase innate resistance to infection[242;245]. The current phosphoantigen "drugs" expand the major γδ T cell subset found in blood (Vδ2), which display potent anti-tumor cell activity and immune function[247;268;269]. However, these drugs have no effect on the tissue-predominant human γδ T cell subset, Vδ1[198]. Thus it is unclear whether phosphoantigen-based drugs will induce the desired effect within tissues, such as the gut mucosa, the skin, and other portals of pathogen entry into the body[131;243]. Here we show that procyanidins derived from the unripe peel of the apple fruit are agonists for both human Vδ1 and Vδ2 T cells, leading to increased expression of IL-2Rα and cell proliferation. The procyanidins were active on both human and bovine γδ T cells but showed broader leukocyte subset specificity with human cells. In particular, the human NK cell population was largely responsive to the procyanidins. Of the two major human γδ T cell subsets, a greater percentage of Vδ1 T cells responded to the procyanidins, suggesting that this tissue population might be more responsive to a tannin-based drug preparation *in vivo*. If so, this would indicate the potential use of this agonist in therapies for IBD, eczema, or other diseases mediated by γδ T cells. Though APP was not particularly effective in driving Vδ2 T cell proliferation unaided, the extract greatly augmented (nearly 300-fold) the

proliferative responses of these cells to phosphoantigens and therefore has the potential to be used in combination with phosphoantigens for clinical therapy.

Studies in animals show tissue γδ T cells not only serve as effector cells, but also participate in immunoregulation[242], tissue homeostasis[190], and epithelial wound repair [270]. Increasing these functions would directly and indirectly enhance host innate and downstream adaptive immune responses. Here, we found that the tissue-associated γδ T cells in both human (Vδ1 T cells) and bovine ($CD8^+$ γδ T cells) respond to APP. Likewise, Akiyama *et al*. show that mouse mucosal γδ T cells expand in response to apple procyanidins *in vivo*[271], which we have confirmed with *in vitro* assays (Jutila, M.A., unpublished results). Since these responses to tannins are conserved in various species, tannin recognition may represent an ancient mechanism used by these cells to sense and respond to their environment. One would expect plant tannin-based agonists to be variably represented in the diet of each of these animals, including humans. Ruminants are exposed to a very high load of plant derived tannins and this may account for, in part, the very large numbers of γδ T cells seen in these animals.

The best-defined agonists that drive γδ T cell expansion are phosphoantigens, but they are specific to human and non-human primate cells[231] making *in vivo* studies difficult. Limited studies in non-human primates demonstrate proof-of-principle that γδ T cells expand *in vivo*[232], but efficacy studies have not been reported in these animals. To circumvent the difficulties inherent in using non-human primates for *in vivo* studies, efficacy studies were performed in SCID mice reconstituted with phosphoantigen-expanded human Vδ2 T cells. These studies demonstrate phosphoantigen-expanded Vδ2

T cell effector function against tumors[268] and bacteria[244]. However, challenge studies in immuno-competent animals have not been performed. Until these studies are performed, understanding the effectiveness of phosphoantigen-expanded γδ T cells in a clinical setting will remain unresolved.

Since the γδ T cell response to procyanidins is conserved in a wide range of species, there are multiple models available to test the efficacy of these procyanidins in infectious disease, wound repair/healing, and cancers. Limited work already published on the extracts examined in this report support the likelihood for success in these experiments. For instance, Cat's Claw is a traditional South American medicine used to treat a variety of illnesses including allergies, cancer, colitis, dysentery, and urinary tract infection[272]. Interestingly, these illnesses are commonly associated with γδ T cell regulatory activities[242;273-276], suggesting the described immunostimulatory properties of the Cat's Claw plant could be partially due to the activation of γδ T cells. Preparations containing the apple procyanidins described herein are also recognized for similar immunostimulatory properties[70;94;271;277]. Additionally, apple tannins increase the rate of hair regrowth and show potential for use in baldness therapy[278]. These results suggest apple tannins directly stimulate skin γδ T cells, which are well known for their ability to promote the health of the epidermis[167]. The increased hair growth when treated with apple tannins would indicate tissue-associated γδ T cells may also be responsible for promoting the health of hair follicles. These observations on the immunological effects of Cat's Claw and apple tannins support the prospect for procyanidins to be used as a therapy to increase γδ T cell function in innate immunity and wound healing.

The results of this study may also provide another explanation for the effect of tea consumption on human γδ T cell activation. Kamath *et al.* demonstrate an *in vivo* γδ T cell priming event corresponding to tea intake[237]. After 14 days of tea consumption, the authors show that γδ T cells become more responsive to *in vitro* phosphoantigen-based stimulation. They attribute the effect of tea to L-theonine, which is converted to alkylamines by the liver. The alkylamines then purportedly drive the expansion/priming of the phosphoantigen-responsive γδ T cells. However, tea is a rich source of immunomodulatory tannins, and we have found that hot water extracts of black tea induce bovine γδ T cell responses (Jutila M.A., unpublished observations). The tannin fraction of tea may prime human γδ T cells, in a manner akin to the APP response described herein (Fig. 8), making them more responsive to *in vitro* phosphoantigen stimulation. Thus, tannins derived from tea may account, at least in part, for the priming effects on γδ T cells observed *in vivo* following tea consumption.

A major implication of these observations is that a tannin-based drug may enhance the effectiveness of current phosphoantigen-based drugs and other approaches to increase innate responses by lymphocytes. Enhancement of phosphoantigen responses may be of particular benefit to individuals afflicted with hyporesponsive Vδ2 populations due to either repeat phosphoantigen treatment[232] or persistent infection[233-235]. As shown in Fig. 8A, APP improves the proliferation of HDMAPP stimulated γδ T cells. Therefore, in these settings, it may be possible to increase the number of expanding Vδ2 cells by co-administering a tannin preparation. The effects of a tannin agonist would likely extend to other human lymphocytes as well.

As shown in Fig. 8, NK cells were actually more responsive to APP in the CFSE assay than γδ T cells. Furthermore, γδ T cell agonist activity can be separated from the NK agonist activity (Fig. 12). This would indicate NK-inducing tannins may be found elsewhere in the APP tannin preparation, although this requires further testing. NK cells are important in a variety of innate functions, including roles as potent anti-viral and anti-tumor cells as well as producers of IFN-γ. Currently, attempts to expand NK cells for clinical benefit focus on cytokine-based expansion and are therefore troubled with toxicity due to non-target cell activation[279;280]. The *in vitro* data provided herein suggest the potential for tannin-derived agonists to aid, in a manner akin to phosphoantigen/IL-2 stimulation, the cytokine-based expansion of this innate cell population. A fraction of αβ T cells also responded within our assays. Analysis of CD45RO expression suggested that the majority of the responding αβ T cells were, surprisingly, naïve T cells. This suggests that some primary antigen-driven αβ T cell responses might be enhanced by these tannins, but this requires further testing.

A tannin-based drug has the potential to not only be used therapeutically, but prophylactically as well. A concern with the prophylactic use of any immunomodulatory compound is the induction of chronic inflammation. Current phosphoantigen-based drugs lead to overt cytokine release in humans[281;282] including the pro-inflammatory cytokines TNF-α and IFN-γ, which would contribute to systemic inflammation in the patient. In a concurrent study, we found that the tannin preparation tested here up-regulated CD11b on γδ T cells, but, importantly, did not alter transcript levels for TNFα. However, GM-CSF and MIP1α gene transcription was induced[71]. Thus, activation occurs following tannin

treatment, but the response appears to be far more subtle than that observed with phosphoantigens. Our data suggests that under a prophylactic treatment regimen, tannin agonists prime but do not overtly activate cells. Upon introduction of an insult, such as exposure to a mucosal pathogen, we postulate that "primed" γδ T cells would respond faster and more robustly to secondary agonists such as PAMPs and cytokines secreted from other innate cells, such as macrophages.

These processes define the initial definitions of the active tannin(s) responsible for γδ T cell activation. Clearly, monomeric tannins are ineffective γδ T cell agonists as are the dimeric forms tested. Procyanidin C1 on the other hand demonstrated agonist activity similar to the crude APP extract (Fig. 13). Furthermore, it is clear that not all plant species produce procyanidins with γδ T cell agonist activity. In screens of common dietary supplements, potent agonist activity was detected in extracts of the plants described here, but the majority of plants tested contained minimal to no γδ T cell agonist activity. In our ongoing drug discovery efforts, we are characterizing eight plant extracts with potent non-tannin γδ T cell agonist activity (Palecanda, A. and Jutila, M. unpublished observations). Yamoa is an example of one such plant; although it produces complex tannins (data not shown), its activity for γδ T cells resides in its non-tannin fraction (Fig. 6A).

A portion of the γδ T cell agonist activity can be found in the trimeric procyanidin from APP (Fig. 13), although there may be other active procyanidins in the APP tannin preparation. These large procyanidins are prohibitive to synthesize, but may be amendable to bulk purification procedures. Techniques of bulk tannin purification

previously developed by the food industry may be adaptable to purify the tannin complex involved in these responses[283;284]. Utilizing these tannin isolation procedures for purification and characterization of the minimal tannin complex from APP is a primary focus in further evaluating the potential for employment of these compounds as therapeutic and/or prophylactic agents.

Questions remain regarding the specific mechanisms of tannin activity on leukocytes. Originally defined as low-affinity and non-specific protein binding complexes with anti-oxidant activity, tannins are increasingly portrayed as having high-affinity counter receptors[285;286]. An example of an immunologically relevant tannin-protein specific interaction is found in tannic acid. Tannic acid is able to block the chemokine CXCL12 from binding to its receptor, CXCR4. The action of tannic acid is specific to CXCL12/CXCR4 given that other chemokines/chemoattractants are not effected by this tannin preparation[69]. Furthermore, apple tannins block FcεR1/IgE binding[70] and prevent epidermal growth factor signaling, presumably through physically blocking the receptor[277]. Similar polyphenols from tea bind CD11b on monocytes, which we recently found for APP as well[71]. Characterizing a potential receptor mediated response triggered by APP in γδ T cells was well beyond the intent of this initial study. However, the data presented here are consistent with APP acting through one or, perhaps a restricted number of, receptors on the T cell and not through a non-specific mechanism, such as anti-oxidant activity. Assuming the active agonist represents a fraction of the total crude material, activity is likely within ng/ml levels. This level of activity is consistent with signaling through a specific receptor. Studies describing the specific binding of

tannic acid to CXCR4 show this interaction competitively inhibits CXCL12 binding at concentrations (IC_{50}, 360 ng/ml)[69] similar to those we predict for the active component of APP. The restrictive pattern of gene regulation induced by APP in γδ T cells, selective up-regulation of IL-2Rα, CD69, MIP1α, and GM-CSF shown here and by Graff, J. and Jutila, M.[71], is also consistent with a receptor-mediated event. We are currently investigating the receptors that may be involved in these responses.

The effective dose range of crude APP required to induce IL-2Rα on γδ T cells was quite limited (1-40 μg/ml for bovine cells and 1-20 μg/ml for human cells), but this was due to toxicity (induction of apoptosis) of the preparations at higher concentrations under the assay conditions tested. Separation of the active component, PC1, increased the specific γδ T cell agonist activity at less toxic concentrations (Fig. 14), although a considerable amount of toxicity remained (data not shown). This level of toxicity is consistent with other tannin preparations[69]. These in vivo data suggest a relatively narrow therapeutic and non-toxic range for tannins. However, *in vivo* studies in rats and mice demonstrate high-dose oral administration without toxic effects. Apple tannins provided to mice *ad libitium* in their drinking water at 1.0% w/v lead to expansion of γδ T cells in the gut mucosa but this dose does not induce obvious toxicity[271]. Furthermore, when APP is given orally to rats at concentrations up to 2,000 mg/kg, all animals survive, gain body weight, and overt inflammation of the gut mucosa or any other organ is not observed over a 14 day period[287]. The reason such high doses of APP administered orally are not toxic may be because the gut appears to regulate the uptake of orally administered tannins and prevents toxic levels from making it to the bloodstream. Specifically, plasma uptake of

tannins in rats plateaus at 10.2 μg/ml (1,000 mg/kg oral dose) and does not increase when rats are fed 2,000 mg/kg of a tannin preparation[288]. This suggests oral administration may naturally prevent overdose, and regulate tannin concentrations in the plasma at optimal mitogenic concentrations.

In summary, although phosphoantigens are potent stimulators of human γδ T cells, they possess limitations that may prevent their reliable expansion of γδ T cell populations for potential treatment of a variety of diseases. Due to the implications γδ T cells may play in medicine, namely regulation of the inflammatory response and cancer surveilance, the search for additional γδ T cell agonists capable of improving cell function is warranted. Many of today's drugs are resultant of isolating active compounds from traditional plant medicines, thereby emphasizing the high content of pharmaceutical compounds in plants. Herbal medicines are of particular intertest in the search for novel γδ T cell agonists. The cellular function of these unique lymphocytes is now being realized and many of these functions correlate with previously confusing effects observed in herbal medicines. As such, many of the heretofore uncharacterized traditional medicines, particularly those that induce responses indicative of γδ T cell stimulation, should now be revisited as potential γδ T cell agonists. The results presented here indicate select tannins, such as those derived from Cat's Claw bark and non-ripe apple peel, represent an example of γδ T cell agonists found in medicinal plants. Undoubtably other plant compounds have the potential to augment the γδ T cell response. Plant extracts from Yamoa and Dong Quai, as well as other plants, demonstrate unique agonist

activities which may be used to further develop the expanding field of γδ T cell therapeutics.

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