Natural Whole Grain Components Effectively Control TNF alpha

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Dedication

This dissertation is dedicated to those that don't believe in themselves or non-believers in somebody trying to change. I started this journey uncertain of my immediate path, but knowing I needed more from life than what I had been getting from the previous 5 years. I knew what I liked, what I was capable of and where I wanted to be in 10 years. I pushed myself to start this journey by instinct, even though my good conscious and those around me thought otherwise. I needed change, and I needed a challenge. I didn't know it at the time, but I now I realize that I had stopped growing. Persistence pays dividends, but pushing your-self to achieve new goals such as this path, was truly rewarding. Dream and never give up trying to achieve your goals in life. Have faith in who you are and what you can do.
Change is always good in the end.

Abstract

The immune system plays a key role in recognizing self and non-self compounds to keep our bodies healthy. This leads to a balancing act to provide sufficient immunological response to appropriate antigenic (foreign) agents, and not over-responding which creates detrimental effects on the host. Beta-glucans from bacteria, fungi, and cereal grains are immune system stimulators that work through specific cell surface receptors to enhance the host response against antigenic organisms and tumors. One of the most significant signals the peripheral immune system utilizes to respond to an antigenic agent is the secretion of TNFα cytokine. This pleiotropic cytokine leads innate immune system defense, plays a key role in appetite, inflammation, and cancer, and regulates communication to other immune cells to respond in an organized configuration. First, this study demonstrated that beta-glucan from barley and oat as well as enzymatic treatment of beta-glucans from oats enhances immune stimulation through increased TNFα. I have shown through a model cell culture system (RAW 264.7 macrophages), a highly pure barley beta-glucan (>91%; 10 μg/ml) and oat beta-glucan (>97%; 300 μg/ml) stimulate macrophages to produce 0.57 +/- 0.19 and 0.49 +/- 0.17 fg/cell TNFα, respectively quantified by ELISA). However, treatment of barley (10 μg/ml) and oat (300 μg/ml) beta-glucans with lichenase (10 u/μg; 1 hr, 40 C) significantly ($p<0.05$) increased TNFα production only in the oat beta-glucan (300 μg/ml) samples (1.48 +/- 0.55 fg/cell). Uncontrolled production of TNFα has been well documented in patients with chronic diseases such as CVD, obesity, and diabetes. Drugs that suppress TNFα production have relieved many deleterious symptoms and disease progression associated with these chronic diseases. Second, this study demonstrated that phenolic acids associated with cereal bran reduce TNFα production. Bran extracts from wheat and barley containing phenolic acids can almost completely diminish (~87%) the TNFα production from macrophages stimulated by cereal and bacterial beta-glucans. Purified commercially available cinnamic and protocatechuic acid significantly ($p<0.05$) reduce TNFα production from bacterial and cereal beta-glucan stimulated macrophages. The combined effects of caffeic and ferulic acid significantly reduced TNFα (59-88%) from cereal and bacterial beta-glucan stimulated macrophages. Through a model cell culture system, I demonstrated that beta-glucans, cereal bran extracts, and multiple phenolic acids from cereal bran have the potential to regulate an important cytokine of the immune system.

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Literature Review

Introduction

Archeological records estimate plants contributed to over 50% of the calories in human diets prior to the Paleolithic-period (1). In historical human development, dietary intake was more heavily weighted by geographical location, climate, and ecology, prior to weapon inventions, so plants contributed significantly more to the human diets prior to the Paleolithic period occurring around 50,000 B.C. (2). Cereals, described as domesticated wild plants for human consumption, were first known to be cultivated between 15,000 and 12,000 B.C (3). However, plants were also gathered for rope (~40,000 B.C.), clothing (~25,000 B.C.), and medicine (>15,000 B.C.) much earlier (4). Charred remains of wild barley and wheat have been found between 16,300 –15,000 B.C. with large grinding stones dating to 13,000 – 10,000 B.C. (5). Centuries later, wheat, barley, and oats were introduced into Minnesota around 1849, just after the Louisiana Purchase agreement with France (6).

A diversity of plants, including grasses covered the earth, prior to and since the pre-Paleolithic period. Angiosperms include all flowering plants and some of the simplest are classified as monocotyledons (7). Monocotyledons have one embryonic leaf emerge upon germination of the seed, are usually characterized by parallel veins in the leaf, and have flowering parts found in multiples of 3, 6, or 9. Examples include grasses, sages, palms, and ginger plants (8). Cereals belong to the grass family (Gramineae or Poaceae), which are mostly herbaceous plants, including sedge, bamboo, reeds, and sugar cane (9, 10). In the late twentieth century, twenty percent of the earth was covered by grass, including lawn and forage, bamboo and the cereals. Grasses are characterized by a hollow stem leaves emerging at nodes, small flowers sheathed inside two glumes, and grouped into spikelets at the head of the plant (11). The fruiting body, caryopsis, is the reproductive seed of the plant or grain that is collected for food.

Structurally, most cereal grains have a similar overall morphology including three main structural components: endosperm, bran, and germ (12). Differences between each cereal genus represent distinct genetic phenotypes and biological characteristics within the spatial morphology of the grain. In general, the central portion of a grain (*endosperm*) consists of cells filled with starch, wrapped in proteins and a carbohydrate polymer matrix (13, 14). The outer portion of a seed (*bran*) consists of several different layers depending on the type of grain, but all contain an outer pericarp, seed coat (testa), and aleurone layer. Only a small portion of the seed (*germ*) is set aside for the potential development of a new shoot and root. The following paragraphs characterize the three main structural components generally found in whole grain cereals grown around the world.

The *endosperm*, the largest structure in the seed, mainly consists of starch and serves as an energy store for the growing shoots and roots that develop into a new plant (12). It's non-viable on its

own, but has gained much attention due to genetic manipulations of the plant, to create more raw materials for refined foods and animal feed. Most commercial breakfast cereals primary ingredients come from the starchy endosperm. The endosperm provides the primary source of gluten proteins for breads, starch for syrups, and soluble fibers from the cell walls. While the endosperm is primarily a storage tissue, the cell walls are composed of various mixed linkage β-glucans, arabinoxylans (pentosans), and minor amounts of lipids, nucleic acids and minerals (14). The quality of the endosperm proteins is considered nutritionally poor, but their biochemistry provides for unique developments in baking and health.

Storage proteins found in the endosperm encompass the prolamins and glutelins, normally accumulating with nitrogen availability in the soil, but over-accumulating during drought, disease, and early frost (15). These storage proteins give rise to the gluten complex, providing the functional properties to wheat dough (16), and consist of low molecular weight gliadins (~ 40 Kd) and high molecular weight (~100 Kd) glutenins (17). A complex interaction between appropriate amino acids, disulfide bonding, ratio of gliadin/glutenins, and an interaction with water allows these gluten proteins to yield a dough mixture with cohesive and elastic properties for making bread with a satisfactory loaf volume (18).

While the quality of protein in the bran is a higher grade, the reduced quality of storage proteins generally reflects a reduced level of lysine, methionine, cystine, and trytophan and a higher proportion of glutamic acid and proline, two important amino acids in gluten formation.

The *germ* is simply the embryonic plant destined for germination in favorable growth conditions, but because of these characteristics, contains a small quantity of enzymes (phytase), proteins (albumins, globulins), lipid (~10%), and nucleic acid (~4%; 20). Protein content in cereal germ ranges from ~20-25%. These proteins tend to have higher leucine, lysine and threonine amino acids and low tryptophan and methionine. Lipids present in the germ include linolenic, linoleic, oleic, and palmitic acids (21). The germ, like the bran, is more complex than the endosperm, comprise of a large number of tissues and storage compounds, but many of which are also significantly different from those encountered in the bran. Although the bran and germ appear to be similar, both contain proteins, lipid and phenolics, such as ferulic acid, but the extraordinary niacin reserves of the bran, for example, are totally absent from the germ. A large number of antioxidant and antimicrobial compounds have been identified in these tissues, but many remain unidentified (22; 23, and 24).

The *bran***,** comprised of the most complex tissues in the grain, also contains the highest concentration of biologically active compounds. The bran is a diverse array of compounds, containing multiple tissues, each with different compositions and biological activities. The dry weight of the bran, including all of its botanically distinct tissues, approaches 20% of the mature wheat kernel, but may be more or less in other cereal grains. Some components are irritating and indigestible in the upper gastrointestinal tract without further processing, but the bran contains elevated levels of antioxidants providing many health benefits to the plant and human consumers. Bran also contains a significant

amount of both insoluble and soluble fiber in the form of pentosans, cellulose and mixed-linkage β-glucans (25, 26, 27, and 28). Sterols and phytoestrogens are also stored in these tissues (29; 30, 31).

One of the bran tissues, the *aleurone layer*, constitutes approximately 50-70% of the bran weight and is, for example, the richest natural known source of niacin with many additional B-vitamins, minerals, arginine- and lysine-enriched proteins, and unique unsaturated lipids (32, 33, 34, 35, and 36). It is a source of enzymes during germination and it contains groups of bioactive compounds, including several cinnamic acids, flavonoids, tocopherols, tocotrienols, lignin, and pigments such as anthocyanins and proanthocyanidins. The nutritional properties of isolated aleurone layers are usually higher than those exerted by the intact, commercial bran fractions containing them. The aleurone layer of all cereals is the primary source of bioactive compounds in the bran and in general, all whole grain products.

Differences Among Common Cereal Grains

Some of the most economically important cereal grains grown around the world include corn (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*), rye (*Secale cereale*), oats (*Avena sativa*), and millet (*Pennisetum glaucum and other species) (37). Cereals found in other regions of the world include* eikorn, kamut, spelt, triticale, amaranth, tef, and quinoa. Compositional differences between the most common cereal grains in the U.S. can be found in Table 1. Some of the most distinct differences between these common grains are discussed in the following paragraphs.

Table 1. Gross compositional fractions of different cereal grains. (Values are based on % dry weight).

Cereal Grain	**Protein**	**Lipid**	**Starch**	**Fiber**	**Ash**
Wheat	12.2	1.9	71.9	1.9	1.7
Corn	10.2	4.6	79.5	2.3	1.3
Rice	8.1	1.2	75.8	0.5	1.4
Barley	10.9	2.3	73.5	4.3	2.4
Rye	11.6	1.7	71.9	1.9	2.0
Oats	11.3	5.8	55.5	10.9	3.2
Sorghum	11.0	3.5	65.0	4.9	2.6

Data from Lasztity, R. 1996 (19)

Wheat, the most common food grain in the U.S., ranks third behind corn and soybean crop acreage (38). Genetic diversity of wheat provides common varieties of winter/spring, red/white, hard/soft, and durham-wheat crops. The red varietals cover up to 75% of production in some years in the U.S. However, strong genetic improvements to row crops of corn and soybeans have slowly lowered wheat production since the early 1980s. Similar to barley and rye, wheat starch granules are of two types, large "A" granules (~20-30 µm dia.) and small "B" granules (~2-10 µm dia.). The diversity in granule size effects hydration, gelatinization and other processing parameters unique to these grains (39, 40). Starch composition and high contents of insoluble dietary fibers in the cell walls create differences in

digestibility, a feature of cereal starches well documented in animal nutrition, brewing and other industries (41, 42, 43, and 44). Wheat generally contains a higher composition of insoluble dietary fiber sources in the cell walls compared to other grains. "Resistant starch," common to cereal starches is receiving increased attention due to its undigestible nature in the small intestine, stimulation of satiety hormones, and fermentation products in the colon (45, 46, and 47). Gluten, the main storage protein in wheat, is the most abundant in wheat varieties and most common grains, provides the strength and structure to create bread with satisfactory loaf volume (48).

Barley, an under-utilized crop for food in the late twentieth century, has been mainly utilized for animal feed, production of malt, and fermentation for beer and ethanol (49). However, in recent years genetic manipulations have provided increased non-starchy polymers to barley varieties, including elevated β-glucans and pentosans (50, 51), a very large and softer, kernel exhibiting hulless and waxy starch characteristics, with exceptionally high antioxidant levels (52, 53, 54, 55, 56, and 57). The non-starchy carbohydrate polymers provide functionally distinct biological activities that show reductions in risk factors for some diseases (58). The cell walls in barley bran contain many small phenolic and poly-phenolic compounds that provide antimicrobial and antifungal activity as well as insect antifeedant activity (59, 60, 61, 62, and 63).

Oats contain approximately twice the quantity of lipids found in most barley varieties. Along with corn, these two cereal grains contain the most lipids with approximately one-third being polar phospholipids and glycolipid complexes in oat varieties (64, 65). Dependent on variety, oats contain some of the highest contents of globulin proteins (15-55%) and some of the lowest glutelins (23-50%). When processing, oats offer an unusual example of difficulties encountered when fractionating cereals (66). Although products such as "oat bran" are common items of commercial production, receiving almost excessive attention for presumed ability to modify serum chemistry, the products are neither "bran" nor endosperm, but rather a diluted version of the latter. The fractionation of oat bran from the endosperm cannot occur cleanly without including a considerable amount of starchy endosperm with the bran fractions, due to the soft texture of the oat kernel. Thus, oat bran is diluted with a considerable amount of easily digestible starch (67).

Rice is a major staple in many American diets, as it is for over 50% of the world's population, due to its low price, ready availability, lack of flavor, and ease of processing and preparation (68). According to recent figures, American rice consumption has increased 174% in the past 30 years, rivaling increases exhibited by corn (178%), and wheat (48%) in the same period (69). Oat consumption has declined by 9% in this same period in the U.S. diet. Rice is consumed primarily in milled form (69) in which bran and germ have been removed (12, 13). A distinctive feature of rice relates to the fact that the starch is similar to that of oats in having compound granules that are of similar composition and structure. Oat and rice starch are more digestible than that of other cereals, although the reasons remain tenuous (70). Rice bran is typically rich in many vitamins and lipid-soluble antioxidants such as tocopherols / tocotrienols (71, 72, and 73), but like many cereals it also contains a number of anti-nutrition factors in

the bran (embryo and germ), including phytin (phytate), trypsin inhibitors, oryzacystatin and haemagglutinin-lectin (74).

Rye, related to wheat through several characteristics, is quite different in composition from other cereals. The starch in rye is similar to that of wheat, but the endosperm includes substantially elevated levels of small phenolic acids and alk(en)ylresorcinols, including sterols, tocols, and lignans that are mainly responsible for the strong flavor and dark color (55, 56, and 57). These biologically active compounds are partitioned during fractionation and the bioactive concentrations are substantially different dependent upon mill-stream. Rye bran and flour are very different compositionally from wheat, containing high levels of soluble polymers, including both arabinoxylans and β-glucans, similar to some barley varieties (25, 26). Studies over several years continue to confirm the rather large variation in total and specific phenolic acid contents from different rye varieties grown in different locations (53, 52, 23, 75, 76, 77, and 78). The spatial relationship of these bioactive compounds is well organized, as in other grains. Cereal bran, for example may contain as much as 800-1000 µg / g of the most common phenolic acid, 3-methoxy-4-hydroxycinnamic acid, while the adjacent starchy endosperm may contain less than 100 µg / g, which is derived from fractionation procedures rather than essential biological compartmentalization (79). Moreover, several forms of hydroxycinnamic acids are present in these grains (80).

Corn in the U.S. is grown in white or yellow dent, flint, popcorn, and several ornamental varieties, including bloody butcher and blue-hopi (10, 12). Corn shares some environmental tolerances with sorghum, when grown under similar climates, and constituents of the bran and germ are distributed in similar spatial arrangements. While more corn acreage is planted in the U.S. compared to wheat, this increase in production is mainly due to the commercial refining of the endosperm (81). This trend is similar to the increase in rice consumption in the U.S, exceeding that of wheat by a large margin. The corn grain is much larger than other cereals, with polygonal starch granules, a lipid rich germ, and a thinner layer of bran compared to the other cereals (12). Protein contents are relatively close to oats and wheat, but low albumin and globulins are accompanied by high prolamins in the endosperm (19). But most commercial production of corn is for flour, grits, starch, modified starch, and sweeteners, like high fructose corn syrup and dextrose (69).

Sorghum, a very popular grain in other countries such as Africa, is not a common dietary food in the U.S (82). Sorghums ability to tolerate low precipitation and other harsh environmental conditions during the growing season, provide regions such as Alabama an opportunity to grow this grain, but it's mainly utilized for animal feed (83). However, ornamental broom-corn grown in many areas of the U.S. is actually *Sorghum vulgare*. The use of sorghum in the human diet in the Indian sub-continent is traditional in both baked goods and in alcoholic beverages. It appears in both red and white forms and flavors are very strong. Small amounts of mixed-linkage β-glucans are in both bran and germ (84) and the phenolic content of the bran is very high (85). The grain is highly variable genetically, and may contain extensive pigmentation due to carotenoids, tannins (a unique trait among the major cereals),

phenolic acids, flavonoids, and anthocyanins, among others. It is well known for its antimicrobial activities in the bran fractions (86). Phenolic compounds can occur in free or bound form and many have been identified by HPLC (87). Tannins are of particular interest because they have been implicated as antinutritional compounds in a number of animal systems (88).

An endemic grain to the upper Midwest region, harvest documents show wild rice being utilized by humans and migratory birds since early 1300 A.D. (89). Wild rice is different from white rice in having an abundance of phenolic constituents in the outer layers of the grain (90). Wild rice has received less effort devoted to breeding and genetic manipulations as compared to other grains, and its content of hydroxycinnamic acid derivatives, notably ferulic acid (3-methoxy-4-hydroxycinnamic acid), is much higher than other common grains. Where wheat and barley grains contain an average of 400-700 µg / g of this compound (91) in the entire grain, and perhaps 1000 µg / g in bran, wild rice has released almost 4000 µg / g on analysis (90). In the latter case, assuming typical compartmentalization within the grain, we might expect 2-3 times that amount in wild rice bran concentrates, and relatively the same in germ. It should be emphasized that wild rice also contains substantial qualities of other phenolics, many of which have not been completely identified, but include p-coumaric acid, sinapic acids, as well as associated glycosides (90). The phenolic acid profiles are similar to barley aleurone (92), wheat bran (93), and corn bran (94, 95, and 96), with several studies indicating large numbers of unidentified compounds.

Western Dietary Whole Grain Consumption

In the twentieth century, cereal grains provided the majority of the calories (primarily as starch) in the American diet, similar to other cultures (97). On a global scale, humans obtain ~56 percent of their calories and ~50 percent of protein from 8 cereal crops (98). Through different cultures and methods of preparation cereal grains have been utilized as food and beverage in a variety of ways. Barley, wheat, and rye have been utilized for bread and bread-like products, although only wheat and rye have the appropriate protein complex for yeast-leavened bread. Rye, barley, sorghum, and rice have been used in fermentative processes to produce whiskey, beer, sake, and miso (99). Cereals grains are closely related structurally and biochemically, but they are quite distinct from the other major dietary grains such as soy, peas, lentils, and canola which are botanically distinct, storing their seed reserves primarily as proteins, lipids, and fiber, with comparatively smaller amounts of starch. In western diets, most of the grains consumed in the diet, are refined grains that have been dominating the market place through several decades (100). Unfortunately, most of the nutrients and bioactive compounds are located in the outer portion of the grain. Refined grains consist mostly of starchy endosperm, containing little of the bioactive ingredients found within the germ and bran. Typical cereal grains are energy dense containing 50-80% starch, depending on the species, origin, and environmental growing conditions (101, 102). The increased use of refined products has provided high starch levels in commercial bread production, there

continues to be a shift towards high starch levels in breakfast cereals, and increasing consumption of sweeteners in many foods (103). Recent commercial processing developments include production of modified starch forms, including sweeteners, high amylase, waxy (high amylopectin), and resistant starches for various food applications (104). In 2000 Americans consumed, on average ~200 pounds of refined flour and other cereal products (unadjusted data) compared to ~135 pounds in the early 1970s (105, 106).

While Americans are consuming more refined cereals and sweeteners, recent market trends also show that they are choosing to supplement their food choices (107). The United States holds the largest portion of the world's functional food sales with grain and beverage products comprising the top two segments of the market (108). Functional foods and functional food ingredients are a high priority in the food industry due to consumer demands and the market potential for specific food products that could potentially improve human health (109, 108). Since the 1980's, the availability of information to consumers about healthcare, nutrition, exercise, and food has driven a dietary supplement market and a growing trend of "hands-on healthcare"(110). Currently, consumers choose to use fortified foods, over previous years of choosing dietary supplements, with fortified food sales up almost 60% since 1998, representing "health conscious" consumer selection and purchasing decisions at the grocery store (111). An increasing number of consumers want more foods containing vitamins, minerals, antioxidants, and fiber. These bioactive components are all found in whole grains.

Generally speaking, whole grains are the intact seed including the three major components in their original proportions: endosperm, bran, and germ. However, in an effort to increase the consumption of whole grains in western diets, the composition of whole grain products has been discussed and defined by different organizations, but the quantity of whole grains in processed products has not been as well defined. The American Association of Cereal Chemists define the term "Whole Grain": consisting of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components: the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the natural intact caryopsis." Sales of whole grain products are up with Americans increasing their use of fiber, and whole grain consumption has increased by almost 40% since the early 1990's (112, 113). Consumers associate the consumption of fiber with increased energy, prevention of disease, cancer and weight gain. However, heart disease (CVD), diabetes, high blood pressure, obesity, and cholesterol are still on the rise in many developed countries, including America (114).

Biologically Active Components of Whole Grain

The benefits of consuming whole grains have been well documented. These benefits include reduced risk factors for stroke, heart disease, type II diabetes, and reduced weight gain (115, 116, 117, and 118). Some counter-arguments point to the small magnitude of these positive benefits and the inability to tease –out the whole grain intake data from the fruit and vegetable data (119). However, increasing consumption of whole grains deserves recommendation for public health since they are nutrient dense, would most likely replace refined grains lacking natural nutrients, potentially reduce the intake of other calorie dense foods, and the lack of studies suggesting that whole grain consumption is detrimental to health. Whole grains are beneficial due to the concentrated sources of biologically active materials in the outer layers of the seed and the cell wall materials found throughout the grain. The following paragraphs will discuss the beneficial biologically active compounds in whole grains.

In the outer layers of the cereal grain, the seed produces many primary and secondary-compounds that lend color, protection, and strength to the bran (80). The aleurone layer of all cereals is the primary source of bioactive compounds in the bran and in general, all whole grain products. The aleurone layer constitutes approximately 50-70% of the bran weight, a rich source of niacin and other B-vitamins, minerals, arginine- and lysine-enriched proteins, and unique unsaturated lipids (32, 33, 34, and 35). The nutritional properties of isolated aleurone layers are usually higher than those exerted by the intact, commercial bran fractions containing them. The bran holds a substantial source of hydrolytic enzymes, which aids the germinating seed and it contains major groups of antioxidants, including several cinnamic acids, flavonoids, tocopherols, tocotrienols, lignin, and pigments such as anthocyanins and proanthocyanidins to protect the seed from herbivorous insects and photo damage (120).

Proteins

The nutritional characteristics of wheat proteins have been of somewhat limited interest historically, except for the recent interest in food allergens (121). While the storage proteins of the endosperm are composed for the most part of glutamic acid-rich, insoluble, lysine-deficient polymers that are prized primarily for their gluten-forming and related rheological properties, the proteins of the bran are metabolically active. Metabolically active proteins entail all enzymes found in the active bran layers during respiration, germination, and post-harvest reactions (122, 123). While not a complete list, these include proteases, α/β-amylases, glucanases, glucosidases, acid phosphatases, and others (124). Like other grain components, however, there is a biologically ordered distribution of protein types within the grain, and both the germ and bran are compositionally quite different from the endosperm that constitutes the majority of white flour (19).

The extraction of proteins from cereal grains results in the presence of four types and their ratios vary according to each cereal: albumins, globulins, glutelins, and prolamins (Table 2). Endosperm components in oats have been reviewed (125).

Table 2. Compositional distribution of proteins found in major cereal grains. (Values based upon Osborne (1907) solubility fractions;126).

Cereal Grain	Albumins	Prolamins	Globulins	Glutelins
Wheat	9-15	33-45	6-7	40-46
Corn	4-8	47-55	3-4	38-45
Rice	5-11	2-7	~10	77-78
Barley	~12	25-52	8-12	52-55
Oats	10-20	12-14	12-55	23-54
Rye	10-44	21-42	10-19	25-40
Sorghum	~4	~48	~9	~37

Data from (19, 127)

Wheat germ is a relatively small part of the grain, so the bran, and particularly the aleurone layer, is the primary contributor of non-endosperm proteins. Bran is well known as a negative modifier of gluten strength – the more bran present in flour, the less acceptable are the rheological properties (128, 129). Some of the rheological deficiencies of bran-rich (or whole grain) flours lie in the dilution effect expressed by the non-gluten bran components (e.g. fiber). There are suggestions however, that bran proteins may actively interfere with dough formation, but the evidence is spotty (130).

Nutritionally, however, bran is another matter – it is typically higher in total protein (15-20%) than the adjacent endosperm (typically 10-15%), and it usually contains elevated levels of those amino acids commonly considered to be nutritionally limiting in wheat products, especially lysine and other basic amino acids such as arginine and histidine (17, 61). Germ has elevated lysine levels (as much as 30-50% in some cereals (131) and both fractions (bran and germ) should be viewed as preferred sources of these basic amino acids. The aleurone layer contains the majority of the bran proteins, and recent assays of isolated aleurone layers indicate 16.9 – 20.8% (132).

Unfortunately for some consumers, some proteins pose problems for their digestive tracts. Glutamic acid and proline-rich specific sequences of storage proteins cause unwanted immunological activity in an increasing number of consumers (121). Cereal-related allergy has become a prevalent problem in our society due to cereals playing a central-role in our diet. An estimated 1 in 100 (~ 4%) Americans are required to avoid them due to celiac sprue, and many other people can be affected through ulcerative colitis or irritable bowel disease (133). Celiac disease is a multifactorial disease, which restricts an individual from eating food products that contain gluten, including wheat, spelt, barley, and rye. This is significant since gluten is the second most prevalent food ingredient next to sugar in the American diet (134). Wheat proteins, specifically the prolamins, gliadin and glutenins, appear to cause the majority of inflammatory problems for celiac patients, but the globular proteins, albumin and

globulin, have also demonstrated reactions *in vitro* (135). Wheat proteins belong to a family of plant proteins with similar characteristics that cause allergy and intolerance in effected individuals (136). Common characteristics include thermal stability and resistance to enzymatic proteolysis, with improved stabilization through binding of metals, lipids, and some hydrophobic signaling molecules. These interactions can happen in the gastrointestinal tract, but also happen during processing of salad dressings, sauces, and mayonnaises with casein, whey or soy proteins present. Many proteins unfold during interaction with lipid bilayers to reveal hydrophobic areas (121). These types of protein interactions can pose problems for the immune system in identifying self and non-self food epitopes in the gastrointestinal tract during absorption. Despite these interactions, most investigators agree that the one mechanism causing most problems comes from transglutaminase in the gastrointestinal tract, transforming glutamine to glutamic acid on proline-rich gluten peptides (121). This change (deamination) of an amino acid affects protein structure, stimulating the host immune system. However, help for the ~ 3 million celiac patients may not be so far off with the counter use of enzymatic therapy. Bacterial prolyl endopeptidases specifically break-up the proline-rich gliadin peptides and have shown significantly reduced reactions in animal models (137). This may pose a challenge for food scientists to build-a-better bread with significant loaf volume, without the all important proline/glutamine sequences in gliadins. Celiac sprue disease has recently been reviewed (138, 136).

Enzymes

Cells of the aleurone are the primary producer of most hydrolytic enzymes that are required for mobilizing seed reserves, including starch and other carbohydrates, proteins, lipids, and nucleic acids (139). Grain hormones stimulate these hydrolytic enzymes, which are capable of positive and negative effects on aleurone-enriched products, including nutrient release and enzymatic rancidity (140). However, this is not an issue for commercial producers, since significant inactivation of aleurone enzymes occurs when cereal grains are processed (141).

Sterols and Lignans

Sterols: Although traditionally associated with soy and corn oils, recent evidence suggests that wheat and other cereals may also be sources of significant amounts of plant sterols (142, 143). Sterols, stanols and their esters, including campesterol, campestanol, sitosterol and sitostanol, among others, have been associated with reduced serum cholesterol (144) and lower risks of colon cancer (132, 145, and 146). Pearling fines of hulless barley and rye contain > 2 mg/g of sterol compounds (147). More importantly, the level of total sterols in commercial concentrates of wheat aleurone layers is significantly

higher than that of either bran (by 25%) or whole wheat (~250%). Both sterol and stanol esters are deemed effective at 1.3 and 3.4 g/day in lowering cholesterol and at these levels may meet the allowable FDA health claims for cholesterol reduction and heart disease reduction (148). Most cereal grains contain significant levels of sterols and their esters (~50-100 mg/100g; 149) and isolated layers of wheat bran appear to contain over 200 mg/100g total plant sterols (132).

Lignans (Phytoestrogens): Lignans and neolignans are a large group of natural products characterized by propylbenzene units, derived from phenylalanine via dimerization (150). They are closely related to ferulic, coumaric, syringic and sinapic acids that are commonly found in cereal bran, being simple dimers of these and other cinnamic acids (150, 151). They are presumed precursors of lignin biosynthesis and are found in a wide range of plant species, including cereals and other commercial plant products. Lignans are a significant dietary component, primarily as physiological effectors of estrogen-like activity (152, 153). Although they are most commonly associated with soy and other legumes, they are abundant in many cereals, particularly rye, but including wheat (154, 155). Lignans are capable of binding to estrogenic receptors in mammalian cells, thereby modifying hormonal balance. High concentrations of lariciresinol, isolariciresinol, pinoresinol, and syringaresinol have recently been discovered in rye bran. All of these compounds, except isolariciresinol, are converted to enterolactone and enterodiol by intestinal bacterial enzymes (156, 157). As phenolic compounds they are also effective anti-oxidants (158, 159). Several laboratories have developed evidence that dietary intake of lignans assists in lowering breast and prostate cancer risks, and may also reduce risks of coronary heart disease (160). Using secoisolariciresinol (SECO) and matiaresinol (MAT) as primary markers of lignan content, Mazur and Adlerkreutz (1998) report that in some bran concentrates, these compounds exceed the total SECO+MAT content of whole wheat by more than ten times (381 vs 36 μg/100g) and endosperm flour by 40-fold (381 vs. 8 μg/100g; 161).

Vitamins

Cereal brans represent one of the richest natural sources of B vitamins, especially niacin in our food supply (162, 163, 164, and 36). Moreover, niacin is concentrated within the aleurone layer of bran, accompanied by the other water-soluble B vitamins such as thiamine, riboflavin, folic acid, pathothenic acid, as well as fat-soluble tocopherols (165). Isolated aleurone cell contents, for example, may contain as much as 1500 μ/g of niacin (32) and while there is considerable influence of both genetics and environment on its synthesis, aleurone cells remain a spectacular dietary source of all members of the B-vitamin group. In an effort to maximize white flour color, as well as flavor and bake volumes, the traditional milling process removes the bran, but this loss results directly in severe reductions of these vitamins.

Historically, widespread removal of bran from many cereals, including rice, corn and wheat, has led directly to several well-known dietary deficiency syndromes, including beriberi and pellagra (166). However digestion of some components of the grain such as the bran can be challenging for some gastrointestinal tracts. It has been known for some time (167, 168) that at least some of the wheat aleurone niacin is sequestered as an ester, which is both poorly characterized and difficult to digest (166). However, niacin in wheat aleurone (36) is easily digested after relatively mild pre-treatments that are designed to cleave the ester linkages. Interestingly, the niacin in wheat is associated with one or more antioxidants, and the complex also exerts some antibiotic effects (167, 168). Niacin deposits do not exist in wheat germ, although other members of the B-vitamin group show some partitioning into both bran and germ, the majority of the B-vitamins are stored primarily in the bran.

Minerals

The majority of the mineral complement in any cereal grain is sequestered primarily in the form of crystalline phytate complexes (169, 170). Typically the total mineral content is measured as "ash" after incineration at >550°C, but this technique belies a wealth of variation in composition, as well as distribution. While most of the minerals in wheat are concentrated as phytate salts in both the germ and bran, the overwhelming dominance of the latter as a proportion of the grain (~20% vs ~5%) dictates that grain minerals are, like most other biologically active constituents, a characteristic feature of bran (thus its decades-long role as a diagnostic tool for measuring bran content in flour) (171). Typical phytin crystals consist of a phosphate-rich myo-inositol skeleton to which are chelated an array of cations, including K, Mg, Ca, Fe, Zn, and Na. The ratios of these cations may vary, the total amount of phytin may vary, and the availability of the minerals to the digestive process has been debated for decades (172). Typical "ash" values for wheat bran are in the range of ~10g/100g (DM) and individual cations range from 10 - >900 mg/kg (DM) bran, including some of the most abundant, such as calcium and iron. The nutritional availability of these minerals are obviously dependent upon the chelating capacity of the inositol and of the processing conditions.

Lipids

Wheat does not contain the most significant source of lipids when comparing cereal grains, but wheat lipids are unique in food products, due to their ability to modify structure, texture, and flavors within dough while altering mechanical and sensory properties (173, 35). Spatial distribution of lipid constituents in the grain are found concentrated in the bran, with a substantial portion in the aleurone of certain grains and the remaining being distributed mainly to the germ or endosperm (174).

Polyunsaturated fatty acids represent perhaps two-thirds of the total crude fat and approximately 15-20% of the total lipid is monounsaturated saturated fatty acids (132).

Aleurone lipids also differ substantially from other grain lipids (35). Hargin's work showed that 60->75% of the aleurone lipid is triglyceride, while the remaining aleurone lipids included non-polar lipids (7-12%, including sterylester, diglyceride, free fatty acids, monoglyceride and 6-O-acylsterylglycoside), glycolipids 2.2 to 9.8%, and phospholipids 13.8 – 17.9%. In contrast, endosperm non-starch lipids contained only 16.7 -26.2% triglyceride, with proportionally higher levels of non-polar lipids (13.3 – 19.5%), glycolipids (20.4 – 38.3%), and phospholipids (23.6 – 35.3%; 175). Once again, the aleurone layer is quite unique, with elevated levels of triglycerides, and its uniqueness offers potential opportunities, both nutritionally and in processing.

Soluble and Insoluble Fiber

Endosperm cell walls consist of dietary fiber sources, non-starchy carbohydrates that act as scaffolding during cell development. They also protect and aid movement of water and materials during germination (176). Non-starchy carbohydrates (i.e. β-glucans, arabinoxylans) demonstrate unique biological activity in the gastrointestinal tract in the purified and food matrix forms. Dietary fiber in general confers many health benefits to mammals, reducing risk factors for heart and gastrointestinal disease, and diabetes (115-118). While Americans are reminded to consume 20-35 grams of total fiber a day, the average consumer takes-in less than half this amount (177). Soluble fiber from oat and barley has received the attention of many investigators due to its ability to lower total and LDL cholesterol, reduce postprandial glucose and insulin, and slow gastric emptying (178, 179, 180, 181, and 182).

Wheat flour contains measurable amounts of both mixed linkage β-glucans, and arabinoxylans (183), although the former are not as abundant in wheat as in oats or barley (184). In bran, however, there are substantial amounts of both polymer groups, contributing as much as 9% or more of the total soluble fiber that can be extracted from wheat bran after preliminary abrasion processing (185). How much of these materials reside in clean aleurone cells, and how much is a product of other, adhering bran layers is difficult to determine. It is apparent, however, that approximately 50% of wheat aleurone layers are comprised of "total dietary fiber" (AOAC 991.43), mostly as insoluble fiber, with a relatively small amount (~4%) contributing to the soluble fiber values. While these numbers may appear high overall in comparison to the complete seed, 75% of wheat bran is aleurone tissue. Different varieties of wheat grain, their glucan, arabinoxylan, and related components have been assembled (186).

Oat bran has received attention from the public, media, and scientific community as a means to reduce risk associated with heart disease (CVD) (187). Oats are a good source of mixed linkage β-glucans, the purported cholesterol-lowering soluble fiber that is concentrated in oat and barley (188, 189, 190, and 191). Although the mechanism(s) by which serum lipids are modified and reduced by these

polymers is poorly understood, bran-rich oat products qualify for heart health claims, and its now realized by the FDA that barley beta-glucan is effective under the same qualifying health claim (192).

Barley β-glucans are also a concentrated source of cell wall β-glucans, especially in new waxy varieties (193). Waxy barley varieties were created through improved genetic breeding with new crops that yielded increased amylopectin and β-glucan contents. Much of the health related effects from cereal soluble fibers are attributed to β-glucans from barley and oat. Some of these health benefits include: cholesterol reduction, reduced post-pradial glucose and insulin, satiety, and immune stimulation (194, 195, 196, and 197).

While not many studies are available, β-glucans in whole grain oat and barley may provide functional benefits towards reducing weight gain when utilized in high fiber diets. Liu et al (2003) analyzed data from the Harvard Nurses Study and found an inverse relationship with weight gain and the consumption of whole grains (198). While this relationship was significant, more importantly was the direct relationship between increased consumption of refined grains and increased weight gain. Jenkins et al (1987, 1988) and others have suggested that reduction in weight gain with high fiber diets increases satiety through reduction of postprandial insulin and glucose (199, 200). A reduction in insulin and glucose from fiber appears to reduce hunger between meals and overall caloric intake of secondary meals. This may be due to effects of increasing viscosity that some soluble fibers have in the gastrointestinal tract or it may be due to the ability of some dietary fiber sources to modulate the endocrine system through gastroentestinal hormones (201). Soluble fibers stimulate specific cells through appropriate receptors in the gastrointestinal tract releasing gut hormones, such as cholecystokinin (CCK), gastrin, or GLP-1 (202). CCK has been suggested to be a long-term regulator of appetite and stimulated by leptin, fatty acids, tyrosine, and phenylalanine (203). CCK an endocrine hormone released upon certain foods entering the stomach and released into the duodenum, stimulates afferent neurons and organs in the gastrointestinal tract. While many functional food components have been studied, Holt et al (1995) found that meals containing high fiber cereals stimulated CCK to a greater extent than low fiber meals (204). Bourden et al (1999) and others have demonstrated similar elevated and sustained CCK plasma levels can occur after consuming low or high fat meals containing significant amounts of fiber (205, 206). While animal and human feeding studies demonstrate the connection between certain un-pure fibers from cereal and CCK secretion, no evidence exists demonstrating cereal fiber sources directly stimulate CCK secreting cells *in vitro*.

While not as well known, some soluble fibers from oat and barley stimulate the immune system (207). Recent evidence demonstrates soluble dietary fiber fermentation in the colon affects the highly integrated immune system along the gastrointestinal tract (208), increasing CD4+ T-cells in lymph (209) while others (210) show consuming high fermentable fiber diets produce higher CD8+ T-cells. This mechanism may work through production of short-chained fatty acids that traverse the intestinal wall to stimulate several components of the immune system (211, 212). But the most studied effects of

stimulating the immune system through components of whole grains would be through the use of β-glucans.

The first description of β-glucans interacting with the immune system was demonstrated in early 1900 under baron von Dungern utilizing *Saccharomyces cerevisiae* to show interaction with complement (213). However, not until 1941 was it realized that crude extracts of cell walls from *Saccharomyces cerevisiae* could stimulate the immune system without viability of yeast (214). Then in 1957 the scientific community understood that the carbohydrate and protein portion of cell wall extracts from bacteria and yeast stimulated the immune system (215). Benacerraf went on to obtain the Nobel Prize for his understanding of the immune system and contribution to medicine in 1980. A brief description of the immune system will follow to guide the reader in understanding the significance of the proposed experimental research.

Immune System

The immune system consists of several complex operating systems working in unison to keep foreign and infectious materials from gaining entry, infecting, and reproducing within the host body (216). Antigens elicit an immune response when they gain access beyond the bodies natural defense systems. Infectious organisms including bacterial, viral, fungal, small multicellular organisms, and non-living foreign materials are all considered antigens.

The immune systems first line of defense consists of barriers, such as the skin, mucus membranes, and cilia that line the mucus membranes (216). The skin cannot be penetrated unless there is access through cuts or abrasions. Mucus membranes trap antigens for removal by other components of the immune system, such as immunoglobulins (IgA, IgE, etc.). Cilia are hairs used to expel foreign materials along mucus membranes, sometimes with the aide of coughing. Stomach acidity aids in killing or damaging antigens before they can enter the intestinal tract. These barriers are supported by other operating systems.

The foundation of the immune system rests upon the activities of mature stem cells derived from hematopoiesis. Hematopoiesis gives rise to red blood cells, platelets, and leukocytes. Leukocytes are the white blood cells of the immune system. These are further subdivided into agranulocytes and granulocytes. Granulocytes mainly consist of neutrophils, with a minor portion consisting of eosinophils and basophils. Agranulocytes give rise to T and B cells as well as monocytes and macrophages.

The Innate and Acquired Systems

The immune system generally operates under two distinct systems, the innate and acquired (217a). The innate immune system is the first line of defense against antigenic materials. This system includes the barriers mentioned above, and cells called monocytes, macrophages, dendritic, and langerhans (217b). These cells have the responsibility of attracting, binding, engulfing, neutralizing, and signaling other cells of the immune system about the antigenic material. Monocytes circulate in the body and mature into macrophages, which can take residence in particular tissues of the host. Macrophages engulf antigenic material through a process of phagocytosis, destroying the antigen inside the cell. Other cells of this system, such as dendritic, langerhans, and macrophages, are termed antigen presenting cells (APC), due to specific signaling molecules presented on their surfaces, alarming other cells of the immune system to the type of antigenic material encountered.

Cells of the innate system utilize pattern recognition receptors to bind to antigenic material (218). These receptors are obtained from host genes, allowing immunity from this system to be passed on to the offspring. There are several hundred types of these receptors recognizing bacterial, viral, and fungal species through recognition of non-self carbohydrate, protein, and lipid materials, such as lipopolysaccharide (LPS), peptidoglycan, and RNA (219). A biological tagging system, where by all cells of the host are marked by the major histocompatibility complex (MHC) allow the immune system to recognize self or cells and materials from the host, from non-self or foreign materials from outside the host.

The second portion of the immune system, termed the acquired system, is generally considered to operate under an adaptive response (216). The cells of this system are T and B-lymphocytes making up 20-40 % of the total leukocyte population. T and B cells are derived from the bone marrow through hematopoiesis, but B cells mature in the bone marrow while T cells mature in the thymus. The adaptive response of this system is conferred through cell-mediated and humoral immunity. Cell-mediated immunity occurs through an orchestrated process of T cells and antigen presenting cells from the innate system. Anitgen presenting cells display antigenic markers on their surface to communicate and sensitize T cells to the foreign substance. T cells have a highly variably receptor system, termed clusters of differentiation (CD), allowing an estimated 10^{18} potentially different receptor forms. CD4+ T cells orchestrate the immune response, stimulating the cell differentiation and proliferation of cytotoxic CD8+ T and B cells, if needed, and can enhance the chemotactic and phagocytic activities of macrophages and neutrophils (220). CD8+ T cells are natural killer cells that secrete lymphotoxins upon binding to antigenic material. These cells are especially helpful in removing dead or cancerous cells. Memory T cells help post a defense for future encounters of similar antigenic organisms and suppressor T cells limit the proliferation of CD8+ T cells as a protective negative feedback mechanism.

Humoral immunity involves the activation of B cells and their antibody products that circulate through the blood and lymph fluids (221). Immature B cells become activated and mature with the

binding of antigen on their surface and a specific message from another cell, such as a cytokine from CD4+ T cells or macrophages. Once mature B cells are activated, they clone themselves to a specific population and produce similar antibodies against the encountered antigen. These antibodies are immunoglobulins (IgG), highly specific to the antigenic material. Antibodies inactivate or destroy the antigen by fixation, neutralization, agglutination, or precipitation by protein interaction. Some of the cloned population of B cells will remain with the host as memory cells for future encounters.

Cytokines

Cytokines are one of the principle components of cellular communication in the body between many cell types, including hematopoietic and non-hematopoietic cells (217). Cytokines consist of small cell-secreted polypeptide messengers, which are similar to hormones, acting over short distances or the whole organism. They regulate cell replication, differentiation, and activation (222). Cytokines mediate and regulate three systems in mammals, hematopoiesis, immunity, and inflammation. In the immune system, cytokines are produced by cells in response to an immune stimulus, such as an antigen or another cytokine (223). The same immune cell may influence the actions of many different cells based upon the production of one cytokine. Cytokines can influence the same cells in the same tissue (autocrine) producing them or they can influence different cells in the same tissue or area (paracrine; 217). The action of one cytokine can start a cascade of many cells producing other cytokines or the same one. The result of these different cytokines may act synergistically or antagonistically in the affected target cells. They are a critical part to the immune response during inflammation and infectious disease, to cell repair, and the cell cycle. Cytokines act by binding to specific cell membrane receptors, which activate an internal cell-signaling pathway (second messengers). This cascading signal stimulates internal proteins, which eventually control cell function by modulating transcripton factors and gene expression in the nucleus.

Cytokines are predominantly produced by macrophages and T cells, but are also produced in other types of cells (222). Interleukins (IL) one of the largest groups of cytokines stimulating immune cell proliferation and differentiation, are especially related to the acquired immune system. For example IL-1 is secreted by macrophages and T cells, stimulates T cells, and IL-2 stimulates T and B cells (224). However, B cells and some stromal cells also secrete interleukins. Interferon (IFN) inhibits viral replication and subsequent viral release in infected cells with assistance through stimulating macrophages and other antigen presenting cells. A subset of T cells, called T helper cells, secrete specific combinations of cytokines. Th1 cells produce specific interleukins (IL-2), interferons (IFN-γ), and tumor necrosis factors (TNFβ) that activate cytotoxic T cells and marcrophages. This process plays an important balance during cellular immunity and inflammation. Th2 cells secrete (IL-4, 5, 6 and 10) stimulating B cells to produce antibodies and regulation of each T helper system can be controlled by specific interleukins inhibiting a T helper system (225).

The cytokine tumor necrosis factor (TNF) was discovered in 1975 when it was noted that specific “factors” were produced in cancer patients in response to infection, thereby shrinking sarcoma tumors (226). The killing of tumor cells (apoptosis) was therefore was the first discovered function of TNF cytokines. TNF has two major secreted ligands in its family, alpha and beta. These ligands mainly consist of 233 amino acid type II transmembrane proteins, forming homotrimers cleaved from the cell surface by TNF alpha converting enzyme (TACE). This cleavage results in the production of soluble trimers of ~155 amino acids, each of which can bind to the TNF receptor (227). Both ligands operate through the same cell surface receptors (TNFR 1 and 2; 228). TNF beta (TNFβ) is involved with the regulation of cell proliferation, differentiation, apoptosis, and lipid metabolism, coagulation, and neurotransmission (229). It is secreted by activated T and B cells and aides TNF alpha during the immune response. TNF alpha (TNFα) is similar to TNFβ, but its secreted from the surface of CD4+ cells, such as macrophages, neutrophils, monocytes, and T cells (230). TNFα, a pleitropic cytokine that’s self-regulating accomplishes many tasks, including immune stimulation, proliferation, inhibition, and apoptosis. For example, TNFα increases the expression of autocrine acting growth factors, the responsiveness of target cells to these growth factors, and stimulates several internal signal pathways controlling proliferation (229). While TNFα is mainly produced by immune cells it affects target cells in most organ systems of the body. TNFα has the main function in the innate response, to orchestrate the mobilization of lymphocytes to rid the body of foreign antigens during an immune response. TNFα activates gene transcription in target cells through several tyrosine and serine/threonine receptor activated intracellular pathways (231, 232)

TNFα and Inflammation

Inflammation is a response of the immune system to tissue injury or infection (216). During acute inflammation, immune cells respond by increaseing blood flow to the damaged area, causing an increase in temperature, redness, pain, and swelling (233). Mast cells in the damaged tissues tend to be the first responders to injury or infection, initiating the immune stimulus. Mast cells, similar to cells of the innate immune system, have pattern recognition receptors (PRR) and other cell-surface receptors that recognize microorganisms and non-living foreign antigens. Upon stimulation of their cell-surface receptors, exocytosis is triggered and mast cells release granules containing histamine and cytokines, including TNFα. These chemical messengers communicate with cells of the innate immune system, recruiting monocytes, neutrophils, and macrophages to the injured area (234). These recruited cells migrate by chemotaxis passing through blood vessel walls and through tissue. This passage is made possible by histamine as well as TNFα by increasing fluid movement and retention in the damaged tissue area (235).

Multiple cell types are quickly involved when mast cells release TNFα. TNFα protects the host from infection and further damage by stimulating other immune cells to produce cytokines, including more TNFα (236). This cytokine increases vascular permeability allowing mobilized effector cells to migrate to the area, and stimulates clotting to isolate the injured area (237). These effects from TNFα initiate the healing process. Without TNFα animal models demonstrate that infection from several bacterial species produce septic shock and death (238). Therefore, the host needs this important cytokine, requiring a small quantity always be present (1.6 mg/ml human serum) for defense, but also to aide in circadian rhythms and assist with tissue and bone turn-over, stimulating fibroblasts and osteoblasts (229).

TNFα activation occurs through cell-surface receptors, internal signaling, and transcription of TNF and down-stream transcriptional gene products (239). Figure 1 demonstrates several signals through TNF receptor 1 (TNFR1) utilized by TNFα and β. Although there are two receptors, TNFR1 initiates the majority of the functional activities in the membrane and associated signaling pathways. Not all signaling pathways have been elucidated, but most of these in figure 2 have been agreed upon. Stimulation of TNFR1, will always lead to the activation of transcription factors NF-kappaB or c-Jun (JNK) in the cytoplasm (see figure 2). These transcription factors are responsible for the induction and expression of genes controlling growth, development, apoptosis, stress, and immune (inflammatory) responses (240). Once TNFα has stimulated TNFR1 the activated protein complex in the membrane is recognized by an adaptor protein TNF receptor activated death domain (TRADD; 241). TRADD recruits other adaptor proteins such as receptor interacting protein (RIP), TNFR associated factor (TRAF), and Fas associated death domain (FADD; 242). These adaptor proteins are responsible for gathering other protein/enzymes to the membrane, thereby initiating a cellular signal event. For example, FADD gathers caspase8 to the membrane, initiating a kinase cascade and subsequent apoptotic signal. TRAF, a centralized protein, controls several signaling pathways. TRAF can attract inhibitory proteins to the membrane thereby regulating apoptosis and gene transcription. TRAF can also activate the mitogen activated protein kinase (MAPK) through apoptosis stimulated kinase (ASK1) and c-JUN (243). ASK1, an apoptosis signal-regulating kinase induced by TNFα, stimulates JNK (244). When mammalian cells are stimulated by TNFα, there is rapid association between the three proteins TRAF2, ASK1, and c-Jun/JNK. C-Jun activation allows the concomitant activation of activator protein1 (AP-1) motifs

Lastly, the adaptor protein RIP is activated through TRADD, resulting in the well researched activation of NF-kappaB, which then translocates to the nucleus to bind DNA controlling many genes responsible for immune responses and pro-inflammatory mediators (247). NF-kappaB can only become activated by the release from IKKα/β subunits (248). When IκB kinase activates the IKKα/β complex through phosphorylation, an IkK protein is degraded and NF-kappaB is released (249).

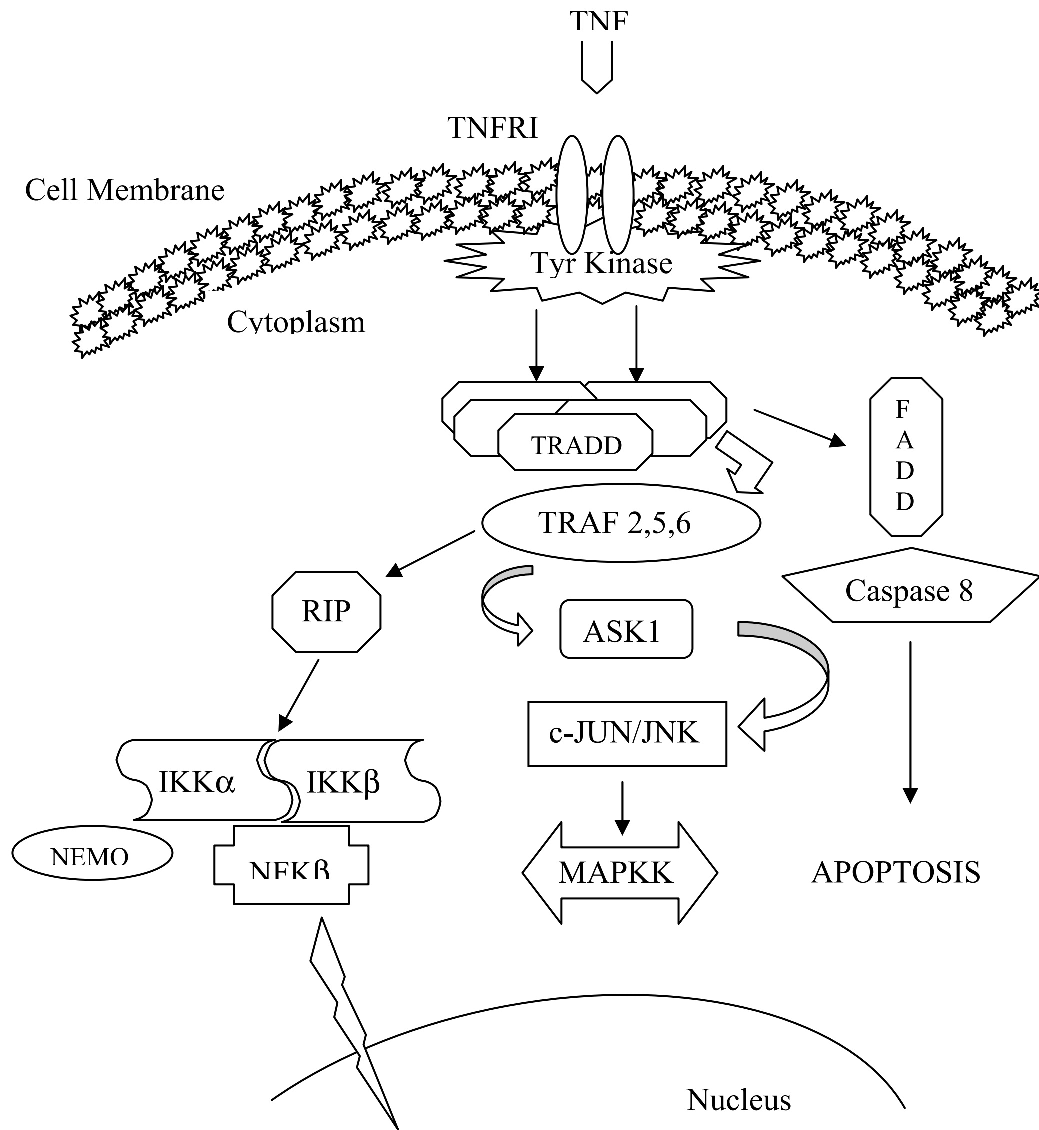

Figure 1. Common intracellular signaling pathways inside a macrophage cell for TNF R1 receptor.

While IKKβ was thought to be responsible for the activation of the complex between these two subunits, it's now known that IKKα also translocates to the nucleus, similar to NF-kappaB, binding DNA and directing transcription (250). At the level of gene transcription, the actions of TNFα are directed into gene products with minimal translational control (251).

While the requirement for TNFα participation in the immune system has been well established, there's a fine balance between TNFα immune production and unregulated production. It is the latter, too much TNFα, that can cause deleterious effects to the host, including chronic inflammation, which leads to continued release of inflammatory adhesion proteins, cytokines, and finally aberrant cell formation. Prolonged secretion of TNFα has now been associated with the pathogenesis of several diseases including sepsis, osteoporosis, multiple sclerosis, arthritis, cancer, hypertension as well as obesity, diabetes, inflammatory bowel, and cardiovascular disease (252, 240). The main TNFα secreting cell during inflammation, macrophages are a pivotal control and regulating cell in the immune system (222). Regulation of TNFα occurs through feedback systems such as suppressor cells, autocrine receptors, intracellular pathways, and secreted inhibitors (253). TNFα self-regulates by having a short-half life and autocrine receptors that stimulate intracellular inhibitory signaling pathways. One such inhibitory pathway includes the anti-apoptotic protein Bcl-XL, which inhibits TNFα gene transcription, by simultaneously down-regulating p38 MAP kinase and NF-kappaB signaling (254, 255). Several suppressor cells monitoring TNFα concentrations secrete inhibitory cytokines, including interleukin 10 (IL-10) and transforming growth factor beta (TGFβ), which work well to inhibit cells secreting TNFα (256). Alpha melanocyte stimulating hormone (αMSH), an effective anti-inflammatory peptide, inhibits the production of TNFα through effective NF-kappaB inhibition (257). While still unexplained, there appears to be a post-transcriptional inhibitory mechanism for TNFα that does not include NF-kappaB or p38 MAP kinase (258). The final inhibitors come from various synthetic chemical compounds, such as dexamethasone, carbocyclic nucleoside, Vioxx, Celebrex and other non-steroidal anti-inflammatories, and TNFα antibodies (259). However, pharmacological drugs that inhibit inflammation from TNFα stimulated eicosanoid production, such as Vioxx and Celebrex selectively inhibiting cyclooxgenase 2, have demonstrated deleterious effects to the host (260).

NSAIDS are best known for blocking cycloxygenase 1 and 2, two enzymes that mediate the metabolism of arachidonic acid (261). Arachidonic acid in this pathway produces prostaglandins, biologically active lipids from a family of eicosanoids (262). Prostaglandin synthesis from cell membranes is required in some tissues, such as the brain, gut, and kidneys, but their synthesis is increased in most tissues when inflammation or the production inflammatory mediators are present (263). COX-1 is required to maintain gastrointestinal integrity and COX-2 is mainly involved with inflammatory mediation. Pharmaceutical manufacturing facilities around the world continue efforts to design drugs that block TNFα, through inhibiting inflammatory mediators, or selective eicosanoids (264). The best

preventive action, may be to reduce production of inflammatory mediators, stop synthesizing and looking past the source of natural anti-inflammatory structures found in plants, and consume plant components and extracts with anti-inflammatory characteristics within our food supply (265.).

Source and Structure of β-glucans

Beta-D-glucans (β-glucans) comprise a group of non-digestible non-starchy polysaccharides widely found in natural sources such as oats, barley, rye, yeast, bacteria, algae, seaweeds and mushrooms (266). For flowering plants, like grasses and cereals, β-glucans are structurally important, helping to support intercellular compartments during development, and protect and aide movement of water and materials during germination (267).

β-glucans can be found in the cell walls of many biological species, including every biological Kingdom classification except Animalia (Campbell book). In cereal grains they are found mainly in the endosperm, but can also be in the bran and germ. β-glucan content in the various whole oat products are 5.5% in oat bran, rolled oats ~ 4%, and whole oat flour ~ 4%. In barley, β-glucans can reach 8 % in non-waxy and >15 % in waxy varieties (268). Cereal β-glucans consist of D-glucopyranosyl units of the sugar D-glucose joined by beta-linkages at positions 1 and 3 or 1 and 4 along the glucose molecule (269). This means that the bonds between the glucose units are beta-1, 3 or beta-1, 4 linkages (270). This type of beta-glucan, referred to as mixed-linkage (1→3), (1→4)-beta-D-glucan, comes from cereal grains. Most of the oat and barley β-glucan molecules consist of cellotriose and cellotetraose blocks separated by single (1→3)-linkages (271). There is, however, a smaller amount of sequences of (1→4)-linkages longer than the tetraose type. The (1→3)-linkages break-up the uniform structure of the β-D-glucan molecule, putting a twist to the developing polymer and making it soluble and flexible. In comparison, the non-digestible polysaccharide cellulose, also a β-glucan is insoluble. Cellulose consists only of (1→4)- β -D-linkages, which consist of linear strands that associate strongly with one another (272). Oat and barley β-glucans have generally been considered similar in structure both are linear, neutral, and unbranched. However, oat β-glucan consists of a greater number of β(1→3) linked cellotetraosyl oligimers (~ 33%) and less β(1→3) linked cellotriosyl oligimers than barley cultivars consisting of only ~ 25% β(1→3) linked cellotetraosyl oligimers (273).

Cereal β-glucans differ from other fungal and bacterial glucans, such as curdlan, a D-glucose sugar polymer containing all β(1→3) linkages to its backbone without any branching (274). Zymosan, a non-linear yeast β-glucan, contains a backbone of glucopyranosyl units comprised of β(1→3) linkages, similar to curdlan, but differing with considerable β(1→6) branching (275). Laminarin, an algal polymer, has structural similarity to Zymosan overall with much less β(1→6) branching.

β-glucan Immune Stimulation

β-glucans are important immune system modulators providing host protection from bacterial, viral, and fungal challenges, as well as necrosis of tumor cells (276, 277, 278, and 279). People with compromised immune systems, such as surgical and transplant patients are highly susceptible to infection from fungal, bacterial, parasitic, and viral organisms (280). Infectious agents, especially nosocomial, have become a problem of increasing concern over the last decade steming from a lack of effective anti-viral, bacterial, and fungal drugs (281). The need for pharmacological or functional food components that stimulate the immune system has also been prioritized by the lack of security over our food and water supplies in the U.S (282).

β-glucans assist the host by stimulating leukocytes in the immune system, including macrophages, monocytes, neutraphils, dendritic, langerhans cells (283, 284, and 285), and some T cells (286). The anti-infective and tumorcidal activities of β-glucans are based upon the cellular activation of phagocytosis and cytokine production in these cells (286, 287, and 288). β-glucans have increased the survival or abolished infections in animals challenged with *E.coli, Staphlococcus aureus, Candida albicans, Eimeria vermiformis, Streptococcus pneumoniae*, *Mycobacterium* tuberculosis, and Anthrax (*Bacillus anthracis*; 289, 290, 291, and 292). B-glucans have also been shown to stimulate host immune systems of amphibians and fish (293, 294). Several human models exist describing increased peripheral blood monocytes, polymorphonuclear monocytes, and macrophages from oral and IV glucan administration (295, 296, and 297).

Phagocytosis is the engulfment or eating of bacterial, viral, or fungal organisms, necrotic cells, debris, or clotting factors that cells, such as polymorphonuclear (PMN) or macrophages, encounter in the host (298). These phagocytic cells are generally attracted to the foreign material through chemotaxis. Chemotaxis, a sensory characteristic that cells use to detect chemical trails released by mast cells or complement proteins presented by other immune cells (299). This process is effective and efficient for most foreign invaders, except for encapsulated bacterial and some viral organisms, which require assistance in the form of antibody mediated immunity. Upon binding to foreign material, the macrophage may release cytokines, such as TNFα, if the appropriate cell surface receptor has been stimulated (see immune system section above).

In mammalian organisms, recognition and binding of foreign material or organisms occurs through cell surface receptors. Pathogen associated molecular patterns (PAMP) and specific conserved microbial structures allow pattern recognition receptors (PRR) to evaluate self from non-self on macrophages, monocytes, neutraphils, dendritic, and langerhans cells (300, 301). Pattern recognition receptors on macrophages, monocytes, neutrophils, and dendritic cells have become extremely important

detecting bacterial, fungal, viral, or cell wall polysaccharides then transmitting the appropriate immune response signal (302). One of the first attempts at identifying a β-glucan recognition receptor was in 1978 (303). However, this receptor (CR3) was further investigated and controversy followed the claim that this receptor was responsible for all glucan effects on the innate immune system (304, 305). In 2001, Brown and Gordon reported on finding the first non-opsonized independent receptor acting alone with out receptor CR3, called Dectin-1 (306).

The innate immune system of mammalian organisms utilizes three recognition patterns. These recognition patterns have been thought to recognize more broad based signals on their cell surface so that many foreign pathogens could be recognized (218). This broad-spectrum binding, rather than recognition of many ligands, such as antibodies, has labeled these receptors as pattern recognition. These patterns can be described in terms of recognizing the "microbial nonself," "missing self," and the "altered self."(307). The first receptor pattern is microbial nonself recognition, based upon the host recognizing conserved molecules of microorganisms (like β-glucan) or microbial metabolism that are unique to microorganisms, but not similar to the host. Most of these recognized microbial structures are called pathogen-associated molecular patterns (PAMP). Recognition is known to exist for lipopolysaccharide (LPS) from the gram-negative bacteria, peptidoglycan, lipotechoic acids from the gram-positive bacteria, mannose from microbial glycolipids and glycoproteins, bacterial DNA, bacterial N-formylmethionine, double-stranded RNA from viruses, and glucans from fungal cell walls. This allows the host to discriminate between "infectious nonself" and "noninfectious self."

The second receptor pattern, recognizing "missing self," relies on the ability of the host to evaluate "markers of normal self" and assess the addition of any non-self markers. This recognition is coupled to various inhibitory pathways that block immune responses toward the host. Markers of normal self are dictated through gene products and metabolic pathways products that are unique to the host and absent from microorganisms.

The third receptor pattern, recognizing the "induced self" detects markers of abnormal self that are induced upon infection or unknown allergic changes, especially viral infection and cellular transformation. Affected cells are marked "abnormal self" and eliminated by the immune system. These receptor patterns of recognition are not only expressed from the surface proteins on myeloid cells (macrophages and dendritic cells), but recognition is expanded by the rearrangement of receptors on T and B cells (308). Gordon (1999) reviewed specific macrophage receptors with associated stimulating ligands (309). Macrophage receptors not only recognize bacterial, viral, and fungal antigens, but glycoproteins, lipoproteins, heat shock proteins, senescent and apoptotic cells. The discovery of new pattern recognition ligands in the last ten years have demonstrated that macrophages and dendritic cells have more responsibility than just defense from exogenous ligands (310). They must control an internal environment of the mammalian system by interpreting, processing and expediting self from non-self, proinflammatory and anti-inflammatory compounds, potentiating and suppressive chemical signals.

There is two distinct classes of cell surface PRR's: phagocytic pattern recognition receptors and signaling pattern recognition receptors. Phagocytic PRR's include the mannose receptors and scavenger receptors. Signaling PRR"s include, toll-like receptors (TLR's), NOD protein receptors (NOD 1/NOD 2), and secreted PRR"s, such as CR3 (218**)**. Together these receptors detect the presence of conserved microbial molecules helping to keep the host free from infection.

Myeloid cells and some endothelial cells express phagocytic PRR's, such as scavenger receptors (SR). The first two mammalian scavenger receptors were found on macrophages and termed SR-AI and SR-A-II (311). Scavenger receptors have the important role in binding and clearing old tired cells, apoptotic, infected and senescent cells. Trans-membrane receptors are utilized for this type of removal and they can include C-type lectin domains and cysteine-rich sequences. Scavenger receptors also are involved in lipid metabolism and bind modified low-density lipoproteins (LDL). They do this through receptor-mediated endocytosis and are parcel to polyanionic ligands (312). Research in the area of atherogenesis is now concentrating in this area. They also contribute by clearing microorganisms and their antigens, such as lipoteichoic acid, foreign DNA and LPS. Scavenger receptors tend to be limiting in vertebrate animals, but are more prominent in invertebrates, such as Drosophila melanogaster. Recently these receptors have been described in their role of opsonin-mediated phagocytosis, a process that also involves toll-like receptors, CR3 and CD14 (313). Opsonin-mediated receptor phagocytosis and the cellular mechanisms involved, such as intracellular signaling, gene expression and activation of cytokines is currently unfolding. For several years it was thought that scavenger receptors worked through another receptor that bound the ligand of β-glucan. However, Rice et al demonstrated competitive exclusion binding with polyinosinic acid and polycytidylic acid confirmed the interaction of β-glucan with the scavenger receptor (314).

The mannose receptor (MR) is expressed on macrophages, dendritic cells, and certain endothelial cells in the vascular system (315). Mannose receptors bind to external mannose and fucose groups attached to microbial glycoproteins and glycolipids. Human host molecules are safe since human glycoproteins and glycolipids contain external N-acetylglucosamine and sialic acids. The mannose receptor is a type I transmemebrane receptor which consists of multiple C-type lectin-like domains (CTLD) all within a single polypeptide backbone (316). It has an N-terminal cysteine-rich domain, a fibronectin type II (FNII) domain, and 8 to 10 CTLD domains. It's within these C-type lectin domains, that b-glucan ligand binds (317). Two of the C-type lectin domains bind monosaccharides and the cysteine-rich domain binds sulfated sugars. It binds mannosyl, fucosyl, N-acetyl-glucosamine glycoconjugates through a calcium-dependent carbohydrate domain. Two ligands are required for binding and phagocytosis to occur. The mannose receptors role in innate immunity has been to clear old cells and self-created antigens. Self and non-self is recognized through the C-type lectin domains. Some of the first reports of this receptor described the clearance of *M. tuberculosis* and *C. albicans* (317). The

mannose receptor has also demonstrated adaptive immune recognition through dendritic cells phagocytizing antigens and presenting them to MHC class proteins (318).

Toll-like receptors (TLR's) a signaling pattern recognition receptor plays a fundamental role recognizing a wide range of ligands in the innate immune system (319). They originate from a discovery in Drosophila, where they also detected muliple ligands in the flies (320). TLR's bind microbes, peptidoglycan and LPS. Binding of PAMP's by TLR's cause intracellular membrane changes, which lead to transcription factor movement and cytokine modulation, such as TNF-alpha and the Interleukins. One of the first discovered, TLR4, was shown to bind lipopolysaccharide and stimulate NF-kappaB translocation to the nucleus (321). It has been suggested (322, 323), and disputed that stimulation of TLR2/4 with β-glucan causes inflammatory mediator production (324) as opposed to the stimulation of other β-glucan receptors. TLR2 appears to responds to β-glucans characterized by large MW and in particulate form, such as zymosan. TLR2 has been proposed as a requirement for zymosan stimulation (325) and it may work with TLR6 to produce cytokines (218). Therefore, the question remains unsolved whether the binding of the well-studied fungal β-glucan, Zymosan is to TLR2 or Dectin -1 that is often published producing the well-known production of TNFα. Most likely, the intracellular mechanisms work together to signal an immune response, since Brown et al (2003) showed that Dectin-1 and TLR2 were both required, to stimulate NF-kappaB and produce TNFα (325). Regardless of this dispute, the encompassing TLR family members identified stimulate many intracellular proteins including IkappaBα, NF-kappaB, c-JUN/JNK, p38 MAPK, TRAFF, TRIF, MyD88, and an interferon pathway (326).

Several TLR receptors work in pairs, such as TLR1/TLR2 providing specific recognition of bacterial lipoproteins and glycosylated molecules of certain parasites (307). TLR-4 recognizes LPS, and TLR-5 recognizes bacterial flagellin. As many as 11 TLR's have been identified on human macrophages, at least 3 in rats, and 13 in mice. They appear to provide detection outside of the immune system with presence in most tissues of the body (327). CD14 is one specific receptor that promotes TLR-4 and its ability to stimulate cytokine production (308, 309).

Among secreted protein receptors, complement receptor (CR3) is found on myeloid cells, cytotoxic and NK T cells (308, 309). It's responsible for the complement-derived phagocytosis of opsonized matter, without regard for host or origin of the matter (non-specific). When CR3 binds to the complement fragment iC3b on bacteria or yeast, phagocytosis occurs through recognition of iC3b fragment and another domain. The CR3 receptor has two subunits. The first has a lectin-like domain that recognizes microbial and yeast polysaccharides, such as β-glucan. This is located in the COOH-terminal end of the receptor. The other subunit domain is called CD11b and it's this domain that binds the iC3b complement fragment. This type of simultaneous recognition by CR3 will cause phagocytosis and degradation of the microbe or yeast once brought into the cell. However, iC3b is also present on some mammalian cells, such as erthrocytes and some tumor cells. They are not phagocytized and degraded because they do not have a polysaccharide component to stimulate the CD11b domain (329). This is the

first receptor thought to be "the β-glucan receptor" by several investigators over the last 25 years (303). It is therefore the non-specific β-glucan receptor. It's been shown that β-glucan can induce a primed state of CR3 for opsonized particles that would be phagocytized if the particle had iC3b fragment present (328). This primed state required tyrosine phosphorylation and a magnesium conformational shift in the CD11b domain. CR3 is also responsible for the specific interactions with Mycobacterium tuberculosis and yeast derived "zymosan." Zymosan, from purified yeast has β-glucan linkages of (1→3) and (1→6). CR3 is also responsible for recruitment of cells to inflammatory sites upon response to cytokines. CR3 expression is specific to body location. Alveoloar macrophages in the lung do not express it, but some specific sites, such as the microglia do. CR3 helps clear apoptotic cells, clotting components, senescent platelets, and denatured proteins. CR3 does not trigger the release of arachidonate or reactive oxygen species.

Dectin-1 is a C-type lectin-like receptor first discovered by Brown and Gordon (329). It has an undetermined amount of ligands and functions for the cells that contain them (330, 331). Dectin-1 was the first receptor to demonstrate that the inflammatory response from a foreign pathogen requires recognition by a specific receptor (325). Dectin-1 alone stimulates phagocytosis and oxidative bursts that help kill and control foreign antigens (332). Through subtractive cDNA cloning, five new genes were discovered in a specific motif that provided a polypeptide of 244 amino-acids long consisting of a carbohydrate binding domain at the COOH-terminal end (333). Dectin-1 is a small type-II transmembrane receptor with a single fold extracellular C-type lectin-like domain (312). The cytoplasmic domain contains an immunoreceptor tyrosine-based activation motif (ITAM). This requires that tyrosine kinases are involved and that tyrosine based signaling molecules will be phosphorylated. Dectin-1 recognizes several different types of β-glucans. These include laminarin, glucan phosphate, pustulan, lichenan, curdlan, galactan, barley β-glucan, cellulose, mannan, dextran and pullulan, but receptor recognition identifies the β(1→3) linkage (334). The human Dectin-1 homologue of the dendritic C-type lectin receptor has been found on murine cells (335). The human β-glucan receptor has been named hβGR and been shown to contain two alternatively spliced isoforms A and B, unlike its murine counterpart (336). Willment et al (2001) found six isoforms however, only two were confirmed to be active with zymosan. Dectin-1 receptor binding and activation occurs with a minimum size of 10-11 β-linked gluco-oligomers (331). Clusters of these small oligomers conjugated to the surface of liposomes, demonstrate competition with several sources of β-glucan for receptor affinity. PCR, cloning, and northern blotting was utilized to show that human dectin-1 was preferentially expressed on blood leukocytes and dendritic cells. However, Taylor et al utilized flow cytometry to demonstrate that many cell types of the immune system contain dectin-1 (337), but possibly highest concentrations are on lung alveloer macrophages (337, 338*)*. Cell systems utilized for *in vitro* investigations expressing dectin-1 so far include NIH3T3 and RAW 264.7, which are human and murine macrophages. The U937 cell line, which is CR3 specific, does not contain dectin-1 and may be the reason Dr. Czop and colleagues did not

discern this receptor (339). Dectin-1 also interacts with Toll-like receptors, complementing the bridge between the acquired and innate immune systems (TLR) (308, 340).

β-glucan Immune Stimulating Characteristics

The defining biological effects of β-glucans that specifically stimulate cells of the immune system are still being investigated (340). Suggested characteristics controlling immune stimulation and published in the literature are often inconsistent due to differences in molecular weight, source, purity, and stereochemistry of β-glucans. While receptor recognition is based upon identification of β (1→3) linkages, the details that have been uncovered by which other characteristics of β-glucans control immune stimulation to some degree, have been suggested to be related to (341, 342):

1) Molecular weight (MW; 343)
2) Polymer length (344)
3) Tertiary structure (345)
4) Stereochemistry of modified side groups off the main glucopyranosyl chain (346).
5) β (1→6) branching of the polymers (347).
6) Soluble, particulate, and aggregated polymers (348, 349, 350)

However, there is no agreement on a set of these requirements for biological activity. Molecular weight, β(1→3) linkages, β (1→6) branching, polymer length, and tertiary structures have received the most attention from multiple laboratories (343, 344, 347, and 351). For example, long, high molecular weight polymers, containing β(1→3) linkages, with significant β (1→6) branching have been regarded as the most stimulating, while smaller MW polymers (< 5000-10000) are generally inactive (352). But other labs have found that small aggregated insoluble particulates have also demonstrated highly stimulating responses *in vitro* and *in vivo* (349). Glucan-phosphate, considered an intermediate polymer (>10,000 MW), has had success stimulating NF-kappaB and specific receptors of immune cells, but not stimulating cytokine production (353). One characteristic often not discussed within the literature of β-glucan functionality, but has been brought up in the application of other nutraceuticals (354), regards how extraction methods often dictate functionality. Some β-glucan extraction methods have produced similar yields, but different molecular weights and vis-a-versa (355, 356). Douwes et al (1996) suggested that β-glucans form triple helices when extracted under hot neutral water conditions, but alkaline extractions did not, they produced random coils in the polymer. These different conformational changes could stimulate receptors to different degrees. Two different methods were tested by Wood et al (1994) demonstrating different viscosities, insulin, and glucose response patterns in animal models, from β-glucans extracted

with different methods (357). The tertiary conformation, size, MW, and solubility of β-glucan polymers may all be dependent upon the extraction method utilized**.**

Phenolic acids and Inflammation

One of the greatest causative factors in cardiovascular disease and some forms of cancer is inflammation (358, 359). Inflammation is an underlying problem not only in heart disease and cancer, but diabetes, high blood pressure, and obesity. In general, the longer chronic inflammatory conditions remain, the higher the risk to the individual. Recently, chronic diseases were estimated to claim more than 50% of the death in the world (114).

Epidemiological data has demonstrated that plant based diets rich in fruits, vegetables, and whole grains have positive benefits on human health and reduce risk for many chronic diseases (360, 361). It was initially thought that plant based dietary effects were derived from antioxidant vitamins, such as beta-carotene, but better methods and several disappointing studies lead many investigators to believe the benefit was from other sources, such as phenolics which provide flavor and color to many plant foods (362, 363). Simple phenolics and more complex polyphenolics produce several beneficial effects to human health including anti-viral (364), anti-microbial (365), anti-mutagenic (366), anti-thrombotic (367), antioxidant (368), and anti-inflammatory activities (369). Recently, investigations into plant phenolics have focused on specific sources, such as whole grains, legumes, nuts, olive oil, onions, garlic, apples, berries, tea, and red wine (370). All of these plant sources contain antioxidants in the form of phenolics and flavonoids (371).

Plant phenolics are secondary metabolites, biologically active compounds distributed widely in all plants. Primary plant metabolites are essential to the life of the plant, and secondary metabolites are utilized for defense, wound healing, and attractants for pollination (372). There have been >8000 phenolic structures identified, from simple phenolics to complex polyphenolic compounds (373).

Catagorized by structural characteristics, more than 10 catagories of phenolics and polyphenolics have been defined with flavonoids and simple phenols comprising the largest groups (Table 3; 374). Simple phenolics are organic compounds consisting of a benzene ring, containing alternating single and double bonds. Phenolic acids differ by containing one or more various functional groups often hydroxylated, methylated, or carboxylated (375). Phenolic acids are biologically synthesized from tyrosine and phenylalanine in the shikimic acid pathway (376), through thermal or enzymatic degradation of lignans (377). Phenolic acids are a diverse group of compounds extensively distributed in the plant kingdom. Cereal grains contain flavonoids, phenolic acids, and tannins, which include the hydroxycinnamic and hydroxbenzoic acids (373). Hydroxycinnamic acids, such as caffeic, p-coumaric, and ferulic acids found in honey, berries, tree bark, and whole grains, often occur as simple esters conjugated to alcohols, but hydroxybenzoic acids, such as gallic ellagic, and chlorogenic acid, often occur

as glucosides (378). In general, wheat and rye varieties mainly contain ferulic, sinapic, coumaric, and vanillic acids with smaller quantities of caffeic, protocatechuic, and syringic acids (379, 380). Corn generally has cinnamic acid, hydroxyphenylacetic acid, phenylacetic, and minor quantities of ferulic, coumaric, vanillic, and syringic (381). In barley, ferulic, sinapic, cinnamic, and coumaric acids with smaller amounts of vanillic, hydroxybenzoic acids are present (380). These particular phenolic acids are often linked to non-starchy polysaccharides in the cell walls of whole grains and referred to as 'bound phenolics', not free, althougt the degree of cross-linking in the cell walls to these polysaccharides varies between grains (382).

Table 3. Classification of phenolic and polyphenolic acids.

Phenolics >	Phenolic Acid >	Hydroxycinnamic >	Ferulic Acid
			Cinnamic Acid
			p-Coumaric Acid
		Hydroxybenzoic >	Gallic Acid
			Vanillic Acid
			Syringic Acid
			Protocatechuic Acid
	Flavonoids >	Flavonols	
		Flavones	
		Flavanols	
		Flavanones	
		Anthocyanidins	
		Isoflavanoids	
	Stilbenes		
	Coumarins		
	Tannins		

Plant extracts and the phenolic acids associated with them, have demonstrated effective antioxidant, anticarcinogenic, anti-thrombotic, and anti-inflammatory activities (361). Cancer models in cell culture and animals demonstrate phenolic acids inhibit carcinogenesis at the initiation, promotion, and progression stages, by reducing DNA-adduct formation, suppressing Phase I enzymes systems that activate compounds to carcinogens, and initiate Phase II detoxifying enzymes (383, 384, and 385). Phenolics are also known to scavenge free radicals, inhibit cell proliferation, and stimulate apoptosis or differentiation in abberant cells, thereby demonstrating that phenolic acids in general have the potential to inhibit all phases of carcinogenesis (386, 371).

Plant extract and phenolic acid activities that provide protective effects against initiation and progression of cancer have been associated with anti-inflammatory mechanisms. Studies utilizing several key plant extracts have demonstrated effective anti-inflammatory actions from phenolic acids. Pine bark extracts have been used in *ex vivo* and *in vivo* models decreasing NF-kappaB, metalloproteinase 9 (MMP-9) activities, and reduced cytokine release in treatment for asmtha. Grimm et al (2004) reported pine bark

extracts (*Pinus pinaster*) reduced NF-kappaB and MMP-9 in ex vivo human monocyte cultures, then demonstrated similar activities in human subjects given pine bark extracts for less than a week (387, 388). Pine bark extracts also reduced macrophage stimulation in the lungs of asmathics reducing cytokines TNFα and IL-1β (389). In clinical studies, Wiese et al (2004) utilized prickly pear cactus (*Opuntia ficus indica*) extracts to significantly reduce C-reactive protein in human serum after a two-week period of administration (390). Willow bark extracts have recently been used to reduce cycoloxygenase and TNFα *in vitro* and *ex vivo* (391). In these former studies, pycnogenol and phenolic acids were found to be active components demonstrating anti-inflammatory actions. Pycnogenol is a mixture of flavonoids, consisting of phenolic acids and procyanidins (392).

Plant extracts contain a wide variety of phenolic compounds with varying structures and molecular weights (393). Studies with isolated phenolic acids, proanthocyanins, and catechins from propolis, ginger, pine bark, and honey-suckle have reduced inflammatory mediators in different model systems (393a, 393b, and 28). Phenolic acids have demonstrated anti-inflammatory activity through the inhibition of NF-kappaB, cyclooxygenase-2 (COX-2), lipoxygenase, TNFα, IL-1β, and IL-6 (394). Protocatechuic, syringic, and ferulic acids significantly decreased edema in rats treated with tetradecanoylphorbol acetate (TPA; 395). Ferulic acid has decreased edema and associated cytokine release from mast cells in rat models (396), protected mice from beta-amyloid plaque formation in an alzheimers model (397), and inhibited NF-kappaB activation in RAW macrophage cell line (398).

Caffeic acid has inhibited COX-2 activity in human and rat models (399), reduced apoptosis through deactivation of NF-kappaB in a myocardial-ischemia injury to rabbit hearts (400), decreased inflammatory cytokines TNFα and IL-1β by inhibition of NF-kappaB in gastric epithelial cells stimulated by Helicobacter pylori (401), inhibits receptor mediated T cell activation with NF-kappaB and IL-2 inhibition (402), and significantly reduces the inflammatory cascade leading to pneumonitis in lung tissue after radiation treatment (403*).*

Benzoic and hydroxycinnamic acid derivatives, such as ferulic and caffeic acids, are structurally similar to salicylic acid derivatives (Table 1 and 2). Benzoic and hydroxycinnamic acid derivatives are also precursors to salicylic acid in biosynthetic pathways of plants (404, 405). Salicylic acid and its derivatives, constituents of willow bark, have a large collection of scientific literature demonstrating inhibition of pro-inflammatory mediators and free radicals, such as cyclooxygenase enzymes (COX 1 and 2), TNF-alpha, NF-kappa B, intercellular adhesion molecule –1 (ICAM-1), iNOS, and Erk kinase activity (406, 407, 408, and 409). Studies examining the role of salicylic acid derivatives inhibiting inflammation, focused on reducing prostaglandin production through COX 1 and COX 2 enzymes (410, 411). COX 1 and COX 2 enzymes have been well characterized and directly inhibited at the active site by aspirin (412). Salicylic acid cannot affect COX 1 and COX 2 enzyme activity, but acetylsalicylic acid (aspirin) does (413). Aspirin blocks inflammation and pain through acetylating the serine 530 in the active site of these enzymes (414). None of the present examined phenolic acids carry an acetyl group

and therefore, it is unlikely that these phenolic acids inhibit cytokine production through interfering with serine in the active site of COX 1 and 2 enzymes. However, curcumin, a di-ferulic phenolic acid has shown inhibition of these two enzymes, thereby reducing prostaglandin synthesis and subsequent cytokine transcription (415, 416). It's been noted that the receptor for COX 2 is much larger and more flexible than COX 1, leading to the binding of some related phenolic structures (417). While serine 530 would not be acetylated by curcumin or these present phenolic acids, the binding affinity and selectivity of the COX-2 active site for several different functional groups bound to phenolic-rings has been explored extensively to include exceptions to aspirins conformation (417).

More recent studies show salicylates reduce TNFα, through a NF-kappa B blockade, independent of COX 1 and COX 2 enzyme inhibition (418, 419). Yin et al (1998) was the first to discover that salicylic acid derivatives can also regulate inflammation by directly inhibiting cytokines, upstream of transcription by a signaling blockade (418). This mechanism occurs by blocking the IκB/NF-kappa B complex. If IκB is not phosphorylated, NF-kappa B cannot dissociate and translocate to the nucleus, stimulating gene transcription of cytokines.

Acetylsalicylic acid and curcumin have both demonstrated the inhibition of phospholipase A2 (PLA2) by directly inhibiting the enzymes active site, rather than through signaling blockades (420, 421).

Curcumin is a dimer of ferulic acid utilized in multiple clinical trials for multiple disease processes, including anti-inflammatory, anti-cancer, and anti-cataract disorders. Curcumins anti-inflammatory mechanisms work at the cellular level, similar to the hydroxycinnamic and hydroxybenzoic acids in cereal brans. The following paragraphs explain the absorption, metabolism, excretion, and anti-inflammatory mechanisms involved with curcumin in mammalian systems.

Curcumin

Curcumin (diferuloylmethane) is a commonly found compound in dietary and medicinal forms in Indian and Chinese cultures. Curcumin has been used medicinally for hundreds of years as a treatment for inflammation (422, 423). It's the parent compound derived from a mixture of similar structures commonly found in tumeric and ginger (424). Interestingly, crude curcumin extract is often a food adulterant added to inexpensive curry products. Tumeric and ginger plants grow in tropical regions were the rhizome is harvested, dried, and crushed. Consumption of curcumin has been shown to be safe and provide multiple beneficial effects to mammals, including anti-inflammatory, anti-angiogenic, antioxidant, anti-cancer, antimicrobial and wound healing properties (425, 426, 427, and 428).

Curcumin exists in several chemically distinct forms called curcuminoids. The most stable registered formulary name of curcumin is 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptodiene-3, 5-dione (Fig. 2). A dimer also exists, known as β-diketone with a molecular formula of $C_{21}H_{20}O_6$ and formula weight of 368.4. In the solid phase it exists as an orange crystal with a melting point of 181-182

ºC. Curcumin is not water soluble at neutral or slightly acidic pH, but is soluble in polar and non-polar organic solvents, such as ethanol and DMSO > 1 mg/ml (429, 430)

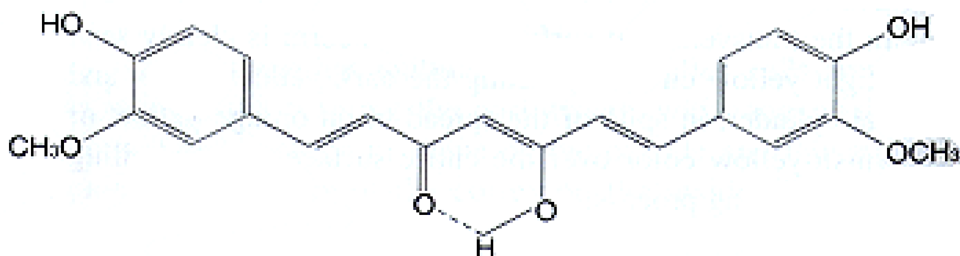

Figure 2. Curcumin structure (from citation 429).

Animal research demonstrates that only a small portion of orally administered curcumin is bioavailable (431). In rats, oral administration (2 g/kg) shows peak serum concentrations of 1 μg/ml in 45 minutes. Earlier studies (1978) of orally administered radioactive curcumin in rats, shows 60% absorption (432, 433). However, several reports agreed that only ~10% of oral curcumin is absorbed with 90% fecal recovery (434). Highest intraperitoneal absorption (100 mg/kg) in mice occurred with maximum plasma concentrations (2.25 μg/ml) within 15 min. In humans, oral administration (2g/kg) of curcumin showed peak serum concentrations 0.006 μg/ml at 1 hr (431). However, with the addition of piperine, which inhibits glucuronidation, absorption was increased (0.18 μg/ml). Some absorption problems are due to extract preparation prior to treatment, since certain curcumin extracts are unstable at neutral and basic pH, leading to ferulic acid and feruloylmethane (435). Transdermal absorption has also been shown to be relatively low (1.58 μg/cm^2 in 24 hr), since curcumin has relatively low lipophilicity (436).

Early studies of pharmacological distribution in gavaged rats found 67-85% unchanged curcumin excreted in the feces, negligible amounts in urine (0.005%), trace amounts in bile (0.0005%), and 0.015% of administered compound in the liver, kidney and fat (437, 438). Holder et al. demonstrated that 73% of radioactive curcumin was found in the feces and 11% in the bile after intraperitoneal injection (435). This suggested that most of the absorbed curcumin in the abdominal wall was partitioned to the bile. Ravindranath and Chandrasekhara (1981) showed that 90-100% of radiolabeled curcumin found in fecal and tissues samples was not parent compound (433). Curcumin undergoes rapid transformation in the gut intestinal wall during absorption (439). The transformation is a series of conjugations and reductions leading to O-glucuronide and sulfate conjugates (440). O-glucuronides are

most often excreted in the bile and could possibly be reabsorbed for enterohepatic circulation. Portal blood and the liver have been shown to contain only small traces of curcumin and its metabolites (441). The volume of distribution of curcumin in rats is approximately 1366 L/kg (442). Based on this, curcumin or its metabolites stay bound in tissues, plasma albumin or recirculated with slow excretion. However, intravenous administration showed only trace amounts of glucuronides and sulfate conjugates after 1 hr in the plasma (441). The volume of distribution is reduced to 783 L/kg with the addition of piperine (431, 442). Total clearance is also reduced (713 to 495 L/h) with the addition of piperine, suggesting that the addition of glucuronide to form conjugates delays the excretion of curcumin metabolites. No data was found for the distribution of curcumin or curcumin metabolites in fetal, placental, or mammary tissues.

The majority of curcumin is metabolized by reduction, glucuronidation, and sulfation (443). Current research shows that these processes mainly occur in the intestinal tract and liver. In the intestinal tract, most of the orally administered curcumin is quickly glucuronidated after absorption. This explains the low bioavailability and trace amounts found in plasma and tissues. Intraperotineal and intravascular administration of curcumin is biotransformed (<1 h) in the liver and to a minor extent in some tissues (444, 445).

Several different studies corroborate the finding that the majority of curcumin metabolites are in the glucuronidated or sulfated form (443, 444, and 445). It's been suggested that before glucuronidation, a P450 reductase acts on curcumin. An *in vivo* mouse model demonstrated that UDP-glucuronosyl transferase was responsible for one major glucuronide conjugate and three minor conjugate forms found in the plasma after Intraperotineal administration. These metabolites included curcumin-glucuronoside, dihydrocurcumin-glucuronoside, tetrahydrocurcumin-glucuronoside, hexahydrocurcumin-glucuronoside (444). In most cases biological activity of these curcumin metabolites are reduced, except for tetrahydrocurcumin, when compared to curcumin (446). Tetrahydrocurcumin has been shown to have a much stronger antioxidant activity compared to the parent compound. I*n vivo* rat models demonstrate tetrahydrocurcumin and hexahydrocurcumin are conjugated by the liver (445). Glucuronide conjugation was much greater in human intestinal fractions than rats. However, the conjugation in the rat liver was much more than the human activity. Also, the reducing ability of the intestinal and liver cytosol was 5-18 times greater in the human samples compared to the rat. This study also showed the involvement of P450 sulfotransferase 1A1 and 1A3 in both the human intestinal tract and liver.

It's been suggested that curcumin may exert its anticarcinogenic or mutagenic effects through blocking certain genes or endogenous enzymes that activate xenobiotic compounds. In rat liver, curcumins been shown to be a substrate and potent inhibitor of CYP1A1 and 1A2 enzymes, but a less potent inhibitor of 2B1 and 2B2 (447). However, *in vitro* human studies, curcumin produced 2-3 fold increases in CYP1A1 activities (448). Curcumin has also been shown to negate the inhibitory effects of bacterial endotoxin on CYP2C11 and CYP2E1, but not CYP3A2 in rat livers (446). Glutathione S-transferase, as measured in the cytosol of rat livers, was also significantly inhibited by curcumin in the

same study. This may help inhibit the activation of compounds such as dinitrobenzene. The concentration at which curcumin is administered has a strong effect on enzyme expression (449, 450). GSTP-1 is inhibited through the reduced binding of AP-1 on the gene promotor of glutahione S-transferase. However, this only occurs at high concentrations (> 2000 mg/kg/day).

Curcumins pharmacological effectiveness depends upon concentration, method of administration and composition. Human toxicity has not occurred from oral administration up to 8 g/day for 3 months (451). Reported side effects include orange discoloration of feces and some inhibitory cellular interactions with chemotherapeutic drugs (452). Cytotoxicity studies *in vitro* have demonstrated apoptosis in normal epithelial cells at 200 μM, but only 25 μM with squamous carcinoma cells (453). This discovery made curcumin a potential anti-cancer compound. Research has demonstrated that curcumin inhibits initiation, proliferation, and metastasis of cancer cells (454).

Historically, curcumin was initially utilized for its cholagogous affect on stimulating bile, pancreatic, and gastric juices (455**),** as well as its anti-inflammatory and anti-arthritic activity (456). Ayurvedic treatments included topical treatment for ulcers, wounds, eczema, and scabies (457), and traditional and western uses as a carminative for the treatment of digestive disorders including bloating, flatulence, and appetite loss (458). Later, curcumin was notably used to reduce the excretion of urinary mutagens from smokers (459) and reduce multi-nucleation in lymphocytes and fibroid development in submucosal tissue (460). Over the last four decades, curcumin has demonstrated the ability to help prevent inflammation, angiogenesis, free radical damage, and infection, revealing its potential to negate several disease mechanisms (425, 426, 427, and 428).

One of the most significant discoveries about curcumins mechanistic actions was NF-kappaB inhibition (Fig. 3; 461). Inhibition of NF-kappaB by curcumin occurs through the inhibition of IκκB kinase (462, 463). AP-1, another key transcription factor, is also down-regulated by curcumin, but by different mechanisms. Curcumin blocks AP-1 by preventing it from binding to specific DNA promoter regions (464). NF-kappaB and AP-1 are key transcription factors regulating gene products, such as cytokines, chemokines, and enzymes that contribute to the inflammation response and aberrant cellular proliferation (465). The inhibition of NF-kappaB suppresses multiple pro-inflammatory mediators, such as TNFα and IL-1β, ERK ½, arachidonic acid metabolism, and multiple enzymes, such as cyclooxygenase-2, and matrix metalloproteinase-9 (466, 467). The production of TNFα and IL-1β by lipopolysaccharide (LPS) has been significantly reduced by curcumin treatment in several investigations (468, 469). TNFα and IL-1β are well known stimulators of tyrosine kinase activity, phosphorylating IκκB and releasing NF-kappaB to act as a transcription factor. Inhibition of TNFα and IL-1β cytokines also blocks the down-stream induction of intercellular adhesion molecules (ICAM-1) and vascular adhesion molecules (VCAM-1; 470). These two intercellular proteins play pivotal roles in the development of vascular endothelial dysfunction, atherolsclerosis and high blood pressure (469**).**

Curcumin also controls other cytokines, such as reducing interferon gamma (IFN) and IL-12 from CD4+ T cells, while stimulating the production of IL-4 (471).

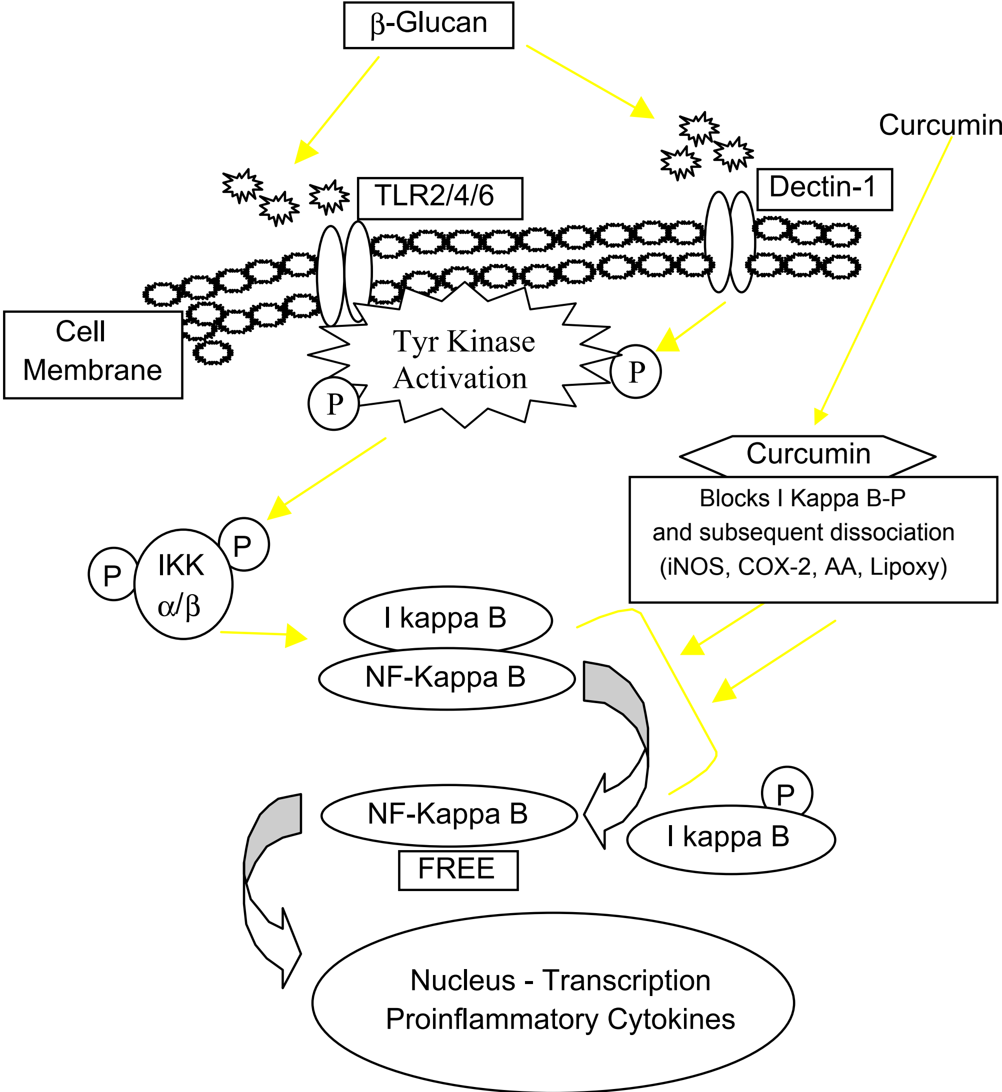

Figure 3. Inhibition of NF-kappa B by the di-ferulic acid curcumin.

Upstream of NF-kappaB and AP-1, curcumin independently inhibits intracellular signaling that often modulates tyrosine kinase and serine/threonine activity (472). Curcumin inhibits pro-inflammatory JAK-STAT, JNK, MAPK, and nitric oxide (NO) synthase signaling pathways in rats and murine models (472, 473). These intracellular signaling pathways are involved in stimulation and transcription of immediate first response gene products leading to cellular proliferation, differentiation, or apoptosis of immune, cancer, and stem cells. Curcumins inhibition of JNK helps reduce apoptosis of cells in the eye lens and stimulates glutathione-S-transferase (GST), reducing cataract formation (474, 475). JAK-STAT pathway inhibition helps reduce the severity of T cell leukemias (476). Wound healing in normal and diabetic models can be enhanced through increased nitric oxide synthase and TGF-β1, stimulated and modulated by curcumin administration (477).

Pro-inflammatory enzymes, such as phospholipase A2 (PLA2), lipoxygenase, and cyclooxygenase (COX-1 and 2) contribute to the phenotypic expression of the inflammatory process. PLA2 helps form arachidonic acid from cellular membranes, and COX-2 enzymes metabolize arachidonic acid to produce pro-inflammatory prostaglandins (478, 479). Hong et al (2004) have demostrated that curcumin blocks arachidonic acid metabolism by COX-2 and PLA2 activities. Curcumin also acts to inhibit arachidonic acid incorporation into cell platelets thereby reducing the release of platelets into circulation, which exacerbates atherosclerosis (480, 481). Studies investigating the mechanistic activities of curcumin include inhibition of multiple cellular targets and gene products of inflammatory mediators, such as leukotrienes, thromboxane, prostaglandins, nitric oxide, collagenase, elastase, hyaluronidase, monocyte chemoattractant protein-1 (MCP-1), interferon-inducible protein, and interleukin-12 (469, 482).

Multiple studies have shown curcumin may have the best anti-inflammatory results during acute rather chronic conditions. Many studies support significant anti-inflammatory activity in rat paw-induced edema with single injections of carageenan (483, 484). Water-soluble sodium salts of curcumin appear to be one of the most effective anti-inflammatory components in these models (485). Combinations of curcumin with capsaicin, a compound similar to ferulic acid but with a longer carbon chain, demonstrates increased anti-inflammatory effectiveness in this combination, compared to curcumin alone (486). The disease-state physiology of the specific studied tissue has a direct affect on curcumin's pharmacology. Curcumin absorption is relatively low when first adminstered in crohn's disease and irritable bowel syndrome (IBS) patients, but over time absorption improves due to the reduction of TNF alpha cytokine (487). In these two gastrointesintal diseases, TNF alpha is responsible for the immediate inflammatory reactions in the gastrointestinal epithelium mediated by T-helper cells. Binding interactions between multiple compounds can be extremely beneficial, especially when they are neurotoxins. Curcumin significantly protects against lipid peroxidation induced by both lead and cadmium (451). In rat brains, the mechanism of chelating these two metals reduced both neurological tissue damage and lipid peroxidation.

While research into curcumin mechanisms has demonstrated a multitude of anti-inflammatory, antioxidant, and anticancer benefits, its utilization in new clinical trials has broadened the applications to include controlling carcinogenesis, radiation poisoning, oxidative stress, cataracts, and rheumatism (488, 489, and 490). Curcumin induces apoptosis through Bcl-2/death domains in cancer cells, but does not in normal control cells (491). Many cases of cystic fibrosis (CF) have mutations at specific alleles that don't allow normal genetic transcription to occur. Curcumin activates heterologous alleles that are assumed to have dysfunctional transmembrane conductance regulators (CFTR) that have remained inactive for the duration of the disease, thereby helping to treat CF patients (492). In human models, animal models, and in vitro curcumin increases superoxide dismutase and glutathione in blood and decreases glutathione peroxidase in smoking models. This proposed mechanism was through inhibition of phase I enzymes and induction of phase II enzymes thereby reducing the modification of cigarette components to carcinogenic compounds (493, 494).

As the mechanisms for curcumin effects are uncovered, it appears that the pharmacodynamics of curcumin is not only synergistic with drug therapy, but also inhibitory in some cases (452, 491, and 495). In the last five years, the combination of curcumin and chemotherapy drugs has documented both synergy and inhibitory effects. Curcumins deleterious effects on tumors are mediated through stimulating apoptotic pathways and within cancer cells. Co-administration of curcumin with cisplatin, sensitizes ovarian tumor cells to this chemotherapy drug and inhibits the tumors from becoming resistant to cisplatin (452). The mechanism was suggested to work through multiple cellular targets, one being the reduced production of IL-6 and NF kappa B, which appear to help tumor cells become drug resistant (495). Similar effects were revealed *in vitro* with prostate cancer cells (491). Curcumin enhanced the cytotoxicity of chemotherapeutic drug in prostate cancer cells by inducing genes p21 (WAF1/CIP1 proteins) and C/EBP beta. Multiple mechanistic targets in the cell were also estimated in this study, but the suppression of NF-kappaB activation was significant. Curcumin has demonstrated similar synergistic effects with immunosuppressive drugs, cyclosporine A, by enhancing the activity in rat cardiac allografts (496). Up to 70% inhibition of some chemotherapeutic drugs has occurred with camptothecin-, mechlorethamine-, and doxorubicin-induced apoptosis (497). These drugs work through generating reactive oxygen species (ROS) and activating JNK signaling pathways to induce tumor cell apoptosis. In some instances, curcumins antioxidant properties work in an opposing mechanism through tyrosine receptor v-Src protein kinase (498).

Curcumin has demonstrated multiple mechanisms to preventing disease and has become an effective nutraceutical applied to inflammation. Curcumin, the parent compound appears to be the most active constituent over its metabolites. But without a doubt, its application to the inhibition of multiple mechanisms in the inflammation process, make curcumin an excellent preventive compound. Positive benefits from current clinical trials and patents may provide an increased acceptance and utilization in western medicine. While several phenolic acids from some different plant sources have been investigated for their immunomodulatory activities, their remains questions about which metabolites in vivo are

active. Cereal grain bran extracts and related phenolic acids that cross-link and structurally support cell walls are an under-represented plant source for potential anti-inflammatory compounds and active metabolites.

Objectives and Rationale

This thesis is directed at investigating the functional characterization between non-starchy polysaccarides and bran components from cereal grains and the physiological response of a murine macrophage cell line.

A cell line was chosen for this study based upon the questions being asked, relating to cereal grain components directly modulating an immune response. Without isolating the cell line to monitor the biomarkers produced, other variables, such as half-life of cytokines, proteases, and naturally produced inhibitors, may ablate the true interaction of the materials with the immune system *in vivo*. The cell line for this system needed to be mammalian and the murine cell line chosen has been well characterized in the literature (499, 500, and 501), contains all relavent receptors known to date, that β-linked glucans use to stimulate TNFα and NF-kappaB (502), and secretes a multitude of immune cytokines (503).

The biomarker TNFα is well characterized as a keystone cytokine involved with host defense, inflammation, and becomes prolific if β-glucans stimulate certain cells. It was chosen since it is a pinnacle cellular protein, involved in the stimulation of multiple cells, tissues, cytokines, chemokines, and a regulating mechanism for inflammation, a problem underlying many chronic diseases. Controlling TNFα has been shown to assist patients with difficient immune systems, control insulin and leptin sensitivity, increase triglyceride utilization and decrease macrophage presense in obesity, and inflammation that is related to several chronic diseases.

Solvents, phenolic acids, β-glucans, and controls for all experiments were chosen based upon the questions and experimental procedures that required them as deemed necessary to investigate the following objectives.

With the former information in this literature review, I formulated the following hypothesis:

1) Increasing the availability and/or changing the conformation of β-(1-3) linkages in cereal β-glucans by enzymatic cleavage, to the available receptors on the RAW macrophage cell line would be more stimulating than untreated β-glucan in its extracted form and therefore produce more TNFα. Differences in linkage structure create changes in polymer structure, which may lead to different interactions with cell receptors and signaling pathways, hence creating a difference in macrophage response.

2) The extraction of phenolic acids, similar to those found in other plant species, from cereal brans could reduce TNFα production from β-glucan stimulated RAW macrophages. Since extraction methods often dictate functionality of compounds, if anti-inflammatory components are present in cereal brans, and interfering compounds reducing the inhibitory capacity of these anti-inflammatories are not extracted with them, then single phenolic acids as well as cereal brans should reduce the production of TNFα.

3). If cereal bran extracts reduce the production of TNFα from β-glucan stimulated RAW macrophages, then single and combinations of single phenolic acids similar in structure to curcumin and salicylic acid may also reduce TNFα from β-glucan stimulated RAW macrophages.

Objectives:

1. Evaluate the change in human serum TNFα concentrations with dietary introduction of barley β-glucan. Examine the relationship between feeding barley β-glucan containing 62 kdalton polymers and changes in serum TNFα concentrations over a 6-week feeding period.

2. Assess the effects of enzymatically treated cereal β-glucans on macrophages production of TNFa. Assess the inhibitory action of curcumin on the production of TNFα from macrophages stimulated by different β (1→3) glucans.

3. Evaluate extraction solvents for the release of compounds that could inhibit stimulated murine macrophages. Evaluate several extraction schemes dependent upon temperature and pH changes to provide cereal bran extracts the ability to inhibit β-glucan stimulated murine macrophages.

4. Evaluate the inhibition of TNFα secretion from β-glucan stimulated murine macrophages by several different phenolic acids found in cereal brans. Evaluate sensitivity of macrophages to different phenolic acids over temporal changes and concentration gradients, and compare the inhibitory capacity of phenolic acids acting on macrophages stimulated by oat and curdlan β-glucans.

Chapter 1

Evaluation of Human Serum TNFα Concentrations after Dietary Introduction of Barley β-glucan.

Introduction

The majority of the mammalian immune system is staged along the gastrointestinal tract protecting the internal environment and deciphering between safe compounds (self) and those that are not safe (non-self; 216). Many edible plant compounds fall into the non-self category and some are known to modulate the innate and acquired immune system (218a). Stimulation of the mammalian innate system can prime immune cells to produce cytokines that communicate, orchestrate and regulate other cells responsible for attacking microorganisms and tumors (318a). TNFα, one of the most important immune system cytokines utilized by mammals, stimulates multiple cells of the innate system to attack and remove microorganisms and foreign material, such as tumors (227, 229).

One plant compound known to stimulate TNFα in mammals is β-glucan (349). The interaction of β-glucans and the immune system has been investigated for over 40 years (343,344). Most of these investigations have utilized fungal sources of β-glucan (329). Advancements in understanding the mechanisms by which β -glucans modulate different parts of the immune system have occurred in recent years, but at present many cellular interactions explaining in vivo data remain unclear (329, 329a). Further advancements would benefit from collaborative experimental designs, which would remove problems such as investigators utilizing multiple sources and purity of β-glucan, different extraction processes, which dictate the functional-chemistry of these polymers, as well as the delivery systems utilized *in vivo*.

The most complete, peer-reviewed, reproducible data examining β-glucan interactions with mammalian immune system include: 1) the protective effects β-glucans instill within the host preventing pathogenic infections and 2) the anti-tumor activities by which β-glucan binds receptor CR3 and activates multiple leukocytes for tumor interaction (291, 328). Prevention of pathogenic infections occurs through activation of macrophages, monocytes, and dendritic cells (344, 309). It takes only one cell type to encounter β-glucan, releasing a cascade of cytokines, which in turn recruits other cells, thereby stimulating the immune system (337).

However, most β-glucan research has focused on the stimulation of the innate system over short 1-2 week periods with fungal β-glucans (287, 215a). My interest was to investigate the possibility of stimulating the innate immune system with cereal β-glucans over a period of 6 weeks. The aim of this study was to evaluate the change in human serum TNFα concentrations with dietary introduction of

barley β-glucan. The first objective was to examine the relationship between feeding barley β-glucan of 62 kdalton polymers and any changes in serum TNFα concentrations. The second objective was to examine serum TNFα concentrations over a 6-week feeding period.

Materials and Methods

Study Design

The purpose of this study was to determine if orally administrated barley β-glucan could increase serum TNFα levels in human subjects. This was a single blinded, parallel study conducted over a 6-week period at the General Clinical Research Center (GCRC) at the University of Minnesota. This study was part of a larger project evaluating the effect of barley β-glucan on cardiovascular disease biomarkers. Subjects interested in the study were in overall good health with one stipulation requiring elevated serum total cholesterol. Eligibility criteria included serum total cholesterol at 5.2mmol/L (200 mg/dL) or greater and meeting drug and health exclusion criteria. β-glucan treated subjects received ~ 8.5 grams barley product, which equated to 6 grams of concentrated barley β-glucan per day. The control group consumed ~ 6 grams of dextrose per day. Subjects had fasting blood draws at three different time points (baseline, midpoint, final) during the 6-week study to obtain plasma for TNFα analysis.

Subjects

Non-smoking hypercholesterolemic men and women between the ages of 22 and 65 were recruited around the University of Minnesota communities. Potential subjects were evaluated for cardiovascular disease risk factors, characterized by high serum total cholesterol. Exclusions were based on the presence of the following criteria: diabetes mellitus (fasting blood glucose > 126 mg/dL), BMI > 30 upon study entrance, recent bacterial infection (<2 weeks), chronic inflammatory disease, high fever or seizure in prior 2 months, cancer in previous 5 years, active weight loss > 5 kg in prior 3 months, concurrent or recent intervention study participation (within 30 days), renal, hepatic, or cardiovascular disease, history of drug or alcohol abuse in previous 6 months, lipid-lowering/anti-hypertensive/anti-inflammatory steroid medication use. To determine acceptance into the study, exclusion and inclusion criteria were analyzed following a telephone screening, a health history questionnaire and a serum total cholesterol screen.

Based on these criteria and serum total cholesterol, 84 men and women were qualified to have their blood drawn and analyzed for TNFα. 45 subjects were randomly assigned to the treated or control group. Subjects were asked to maintain normal activity and usual dietary habits through the course of the study. Diet was kept constant to reduce potential confounding effects on study biomarkers.

The University of Minnesota Institutional Review Board Human Subjects Committee approved this clinical trial. All subjects gave written consent after reviewing the study protocol and procedures.

Experimental Treatment

Subjects were randomly assigned to the low-MW or control group and consumed their normal dietary habits during the 6 weeks of the study. Participants in the low-MW group consumed six grams of isolated β-glucan, (as approximately 9 grams of total barley β-glucan product). At the beginning of the study (baseline) and 3 weeks, subjects were given a 21-day supply of assigned product, measured into 21 individually bagged portions for each day. Protocol indicated that the barley β-glucan product be consumed with the morning and evening meals, approximately half of the product each meal. Subjects were to blend the product into a beverage, excluding hot drinks or caffeinated beverages. Subjects were given small blenders to facilitate mixing their product into a beverage. They were to consume the product within 5-10 minutes of mixing. Any unused product was brought back upon final visit.

The barley β-glucan product contained a highly enriched non-starchy polysaccharide of (1→3)(1→4)-β-$_D$-glucan fiber with excellent gelling properties. Barley β-glucan content was assessed using an enzymatic assay procedure (Megazyme, International, Ireland). The barley product was classified based on molecular weight (MW). In this study, the barley product MW was 62,000 and through a patented process, the purity was ~75% barley β-glucan.

Study and Analytical Procedures

At baseline, 3 weeks and 6 weeks, subjects had fasting blood samples drawn. Plasma for TNFα analysis was obtained by centrifuging (1100xg; 4ºC) blood in vacutainer (EDTA; 7 ml) tubes. Separated plasma was transferred to 1.5 ml cryovials, placed on ice (<4 hr), and transferred to a freezer (-70ºC). TNFα was quantified by ELISA (monoclonal; BD OptEIA, detection limit: 5 pg/ml, cat. 550995, BD Biosciences, San Diego, CA.). Statistical analysis for each experiment was determined by ANOVA with statistical analysis between individual treatments determined by Tukey-Kramer post-test (Microsoft Excel™).

Results

Preliminary experiments with barley β-glucan samples, before the feeding trials, showed that the barley product hydrated quickly and mixed with hand blenders when combined with juice, yogurt and milk. A soft gel would form within 15 minutes upon standing if mixed with 8 ounces of beverage. It was not investigated how combinations of barley β-glucan samples combined with cooked oatmeal or cold-cereal/milk.

In this study, barley β-glucan consumed as a dietary supplement over a 6-week period, did not increase the production of quantifiable TNFα in human plasma samples to above baseline. There was no treatment effect within the barley β-glucan group between analysis points: baseline, mid-point, and final ($p>0.05$; Figure 1.1). There was also no significant difference between the baseline, mid-point, and final analysis points within the control groups ($p>0.05$).

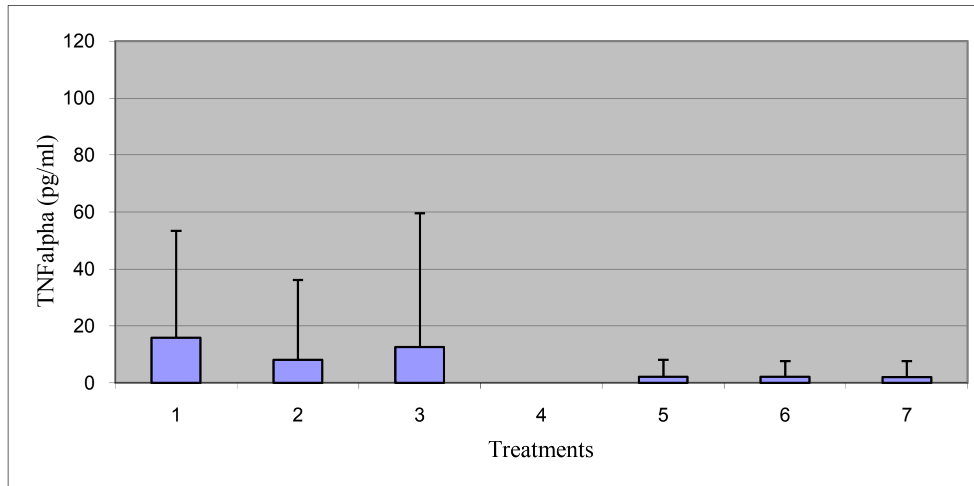

Figure 1.1 Quantified TNFα (pg/ml) from human serum samples +/- dietary barley β-glucan. 1. Baseline (0 weeks; treated group). 2. Mid-point (3 weeks; treated). 3. Final (6 weeks; treated). 4. Negative control. 5. Baseline (0 weeks; control untreated). 5. Mid-point (3 weeks; control untreated). 6. Final (6 weeks; control untreated). N = 42. Letters denote significant difference

β-glucan treatment had no statistically significant effect when comparing treated to control groups. While there was more quantifiable TNFα in each of the three β-glucan treated plasma points when compared to control values, there was not a significant difference between analysis points between treated groups and control groups ($p>0.05$; Figure 1.1). Separating both treated and control groups into two age sections, above 50 years of age and below 50 years of age, reduced the magnitude and variance around TNFα values in the below 50 years of age group (Figure 1.2 and 1.3). This separation of data created an increase in the magnitude and variance in the above 50 years of age section. However,

statistical significance patterns did not change from the original tested groups with the separation of data based upon age.

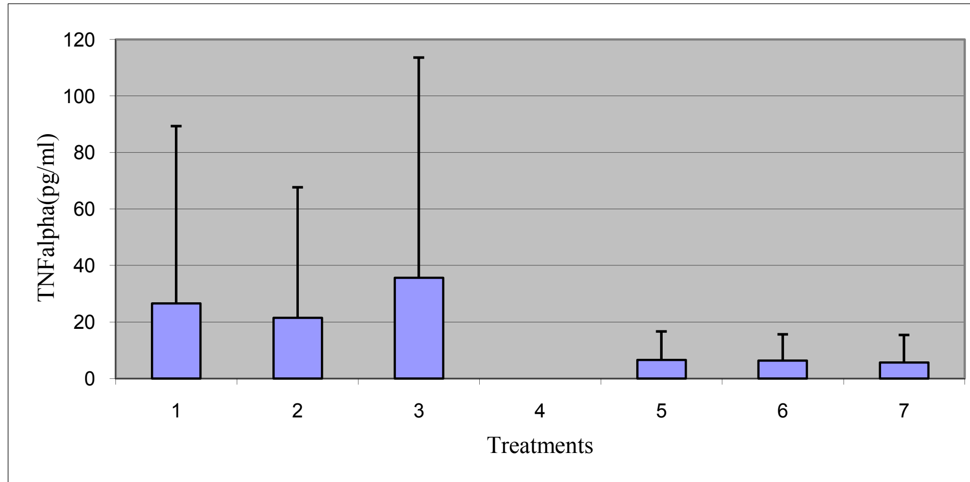

Figure 1.2 Quantified TNFα (pg/ml) in human serum samples from patients over 50 years of age. 1. Baseline (0 weeks; treated group). 2. Mid-point (3 weeks; treated). 3. Final (6 weeks; treated). 4. Negative control. 5. Baseline (0 weeks; control untreated). 5. Mid-point (3 weeks; control untreated). 6. Final (6 weeks; control untreated). N = 12-14. Letters denote significant difference

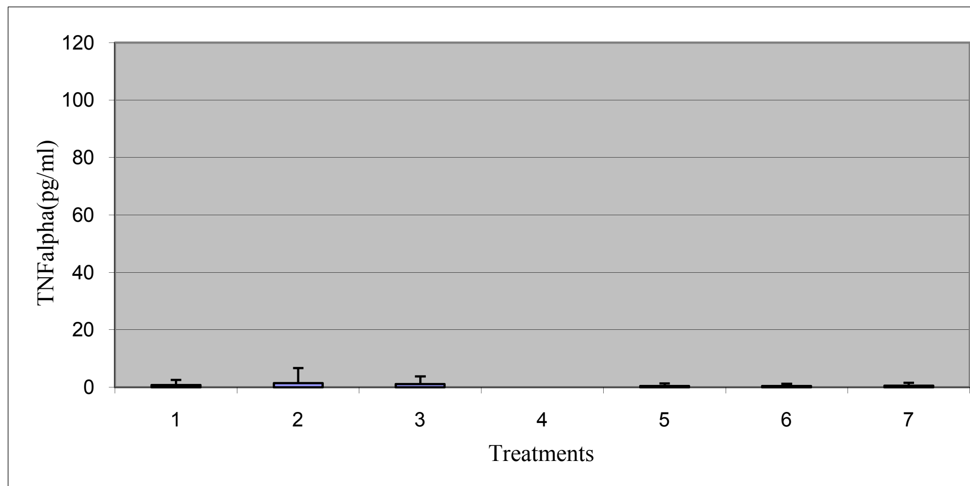

Figure 1.3 Quantified TNFα (pg/ml) in human serum samples from patients below 50 years of age. 1. Baseline (0 weeks; treated group). 2. Mid-point (3 weeks; treated). 3. Final (6 weeks; treated). 4. Negative control. 5. Baseline (0 weeks; control untreated). 5. Mid-point (3 weeks; control untreated). 6. Final (6 weeks; control untreated). N = 28-30.

Treated and control values were all above normal human clinical values (1.6 +/- 0.1 pg/ml). TNFα values in figures 1 and 2 are all above normal human plasma values including the control data.

Most of the magnitude and variance of TNFα values in figure 1 and 2 were due to 3 patients over 50 years of age. These three patients in figure 1.1 made up 94, 79, and 94 % of the variance at baseline, midpoint, and final analysis, respectively. Control values were ~ 4 times higher than normal at all analysis points. These elevated values were the result of 3 patients over 50 years of age in the control group. The magnitude of TNFα values in the under 50 years of age group were approximately normal. While there is an increase in TNFα values at week 3, it was not significant ($p>0.11$)

Discussion

The present study examined the role of orally administered barley β-glucan in stimulating TNFα production in humans. These experiments analyzed human plasma from patients consuming barley β-glucan as a supplement over a 6-week period. This work is related to chapter 2, which demonstrates the ability of β(1-3)(1-4)-glucans to stimulate murine macrophages to produce TNFα *in vitro*.

Multiple animal studies have demonstrated that orally administered fungal β(1-3) glucans and oat β(1-3)(1-4) glucans prevent host infection through immune stimulation before introduction of pathogenic organisms (216a, 216b). Yun et al (2003) reported that immune reactivity time was shortened and antibody concentration was significantly higher when orally administered oat β-glucan was paired with *Staphylococcus aureus* and *Eimeria vermiformis* infections (291). Human clinical trials have shown several forms of orally administered β(1-3) glucans protect against bacterial challenges, cancer, and radiation exposure (292, 281, and 295). The proposed mechanism behind infectious agents and tumors appears to be through stimulation of macrophage and monocyte proliferation and phagocytosis (284). Macrophages respond to bacterial, fungal, and tumor growth through receptor interaction and phagocytosis, while inducing cytokine synthesis and secretion to stimulate the activation and proliferation of cells in the innate immune system (325). TNFα, one of the most important cytokines synthesized, regulates cytokine production and cellular proliferation of other cells in the innate immune system through autocrine and paracrine action (227).

In the current experiment, oral barley β-glucan supplementation over a 6-week period did not increase serum TNFα levels within the treated group (Figure 1.1). There was no treatment effect within the barley β-glucan group between analysis points: baseline, mid-point, and final ($p>0.05$). There was a greater difference in absolute values between treated plasma points when compared to the control groups, suggesting that TNFα may have been stimulated by the barely β-glucan treatment. But these were not significant differences ($p>0.05$). Several subjects had elevated TNFα values in both the treated and control groups (Figure 1.2, 1.3). Historically, problems quantifying plasma or serum TNFα made it

difficult to evaluate normal concentrations and how disease affected this cytokine. But improved immunoassay's utilizing monoclonal antibodies, with more sensitive reporters, and identification of interfering compounds in the blood have provided more consistent measurements (227a, 218a). The ELISA kit utilized in this study had a minimum sensitivity of detection (5 pg/ml). ELISA assays with better sensitivity were not available when this study was undertaken. Due to enhanced immune stimulation with orally administered barley β–glucan in studies of the past 5 years, it was anticipated that TNFα stimulation above the normal could occur (230a, 231a).

The stability and detection of TNFα in human plasma and serum has been a concern immediately after blood draws (227a, 231a). TNFα has been shown to decrease immediately after removal from veins, but increase within 6 hours at 4ºC, or increase to a similar level within 12 hours at –20ºC storage (233a). This was demonstrated to occur from the difference in secreted monomeric forms and active oligomeric forms of TNFα (234a). TNFα changes from an active oligomeric trimer form to an inactive monomeric form upon storage outside of the body (235a). Inactive forms of TNFα have been shown to be present in plasma and not quantified by certain ELISA antibodies specific for the monomeric form. This was not a concern in this study given the specificity of the monoclonal antibody utilized to detect TNFα.

Peripheral blood mononuclear cells (PBMC) and monocytes are two cell types contributing to TNFα in blood samples. PBMC's can contribute to an increase in TNFα, if primed by soluble factors present in the blood before removal from a vein (236a). However, this would be derived from infectious, inflammatory, or specific disease states in the patient, not mishandled samples. This may explain the high TNFα values obtained from several subjects in the treated and control groups in this study. Endotoxin contamination, present in phlebotomist's collection (vacutainer) tubes have also been shown to stimulate monocytes in plasma samples (365). Collection materials were purchased from a commercial source and not tested for endotoxins.

The most stable storage conditions for TNFα, in plasma, has been shown to be –20 to -70ºC (233a). Plasma samples in this study remained on ice (0ºC) in a refrigerator for no more than 4 hrs at the hospital then transferred to -70ºC. The residence time of the plasma samples in the -70ºC was from 4 – 12 months before analysis. No protease inhibitors were utilized in these samples from the hospital. Therefore, losses of TNFα protein from natural protease activity may have contributed to some results in this study. However, TNFα collected from in vitro experiments in chapter 2, 3, 4 was quite stable over 16-hour treatments. Upon analysis of human plasma samples, only one thawing cycle was required per tube to obtain multiple readings of each sample to prevent destruction of proteins by repeated freeze-thaw cycling. The utilization of EDTA in the collection tubes and 1.1 M NaCl has been shown to stabilize and prevent masking of TNFα values from plasma proteins (38). No protease inhibitors were utilized and upon analysis, samples were only thawed once to prevent destruction of TNFα proteins by repeated freeze-thaw cycling. Cross reactivity of antibodies with other human cytokine, lymphokine, and growth

factors was negligible in BD Biosciences ELISA kit, which evaluated over 24 human factors (BD Biosciences Pharmingen).

It's not known to what magnitude above normal human levels (>1.6 pg/ml serum), barley β-glucan would raise quantifiable TNFα values. Human subjects receiving an oral dose (400 mg/day) of a branched soluble yeast β(1-3)(1-6)-glucan did not show changes in TNFα, IL-1, or IL-6 above normal values (239a). This particular study utilized β-glucan of 20,000 MW, quantified TNFα from serum and saliva (normal values 3 pg/ml). These results were similar to a small treatment group (n = 7) receiving barley β-glucan of 139,000 MW and a control group (n=7) in this study. No changes from normal baseline occurred in the control or treated groups (data not shown). The self-regulating nature of TNFα with its relatively short half-life may make it difficult to quantify after several hours of β-glucan administration (342a). Other studies that relate disease status with changes in TNFα values, include patients undergoing hyperglycemic clamps to investigate impaired glucose metabolism, had elevated TNFα values above normal clinical values (241a). Young (~ 22 y.o.) patients had TNFα values 3.40 pg/ml under normal conditions and with elevated blood glucose (hyperglycemic clamp) those values didn't change significantly (3.17 pg/ml). In a similar pattern, older (67 y.o.) patients TNFα values (~3.8 pg/ml) did not change under elevated blood glucose treatment. In chronic disease states, patients with cardiovascular disease express TNFα levels ranging from 0.5 - > 25 pg/ml (342a). It was suggested that problems with atherosclerosis, damage to the vascular system accounted for these increased levels. Long-term diabetic patients undergoing dialysis have very high TNFα levels (70.5 +/-32.3 pg/ml; 243a). Kidney damage accounted for these elevated TNFα values and also contributed to significant appetite loss.

Mechanisms explaining the inability of orally administered barley β-glucan to stimulate TNFα production in humans include abnormally high baseline values concealing the stimulation of the immune system, poor absorption of β-glucan polymers, inhibition of TNFα production by other cytokines in the gastrointestinal tract, and aggregation of polymers inhibiting absorption or receptor activation.

If immune stimulation occurred due to oral β-glucan supplementation, it might not be apparent upon first evaluation of data. Abnormally high baseline values (>1.6 pg/ml serum) are present masking any increase in TNFα levels due to oral β-glucan supplementation (Figure 1.2). In vitro studies from chapter 2 demonstrated that fungal β-glucans are approximately eight times more stimulating to macrophages of the immune system, than mixed linkage cereal β-glucans. Therefore the in vivo stimulation using barley β-glucans may only provide a small increase in TNFα. However, by dividing the treated and control groups into two age profiles, above 50 years of age and below 50 years of age, decreased the magnitude and variance around TNFα values in the younger age group (Figure 1.3). This separation of data created an increase in the magnitude and variance in the older age section. But the variance and average TNFα values were restored to normal in the youngest age group (Figure 1.3).

Statistical analysis of these new data presented no significant differences ($p>0.05$). It's now regarded as common clinical knowledge for the aging mammal to increase expression of TNFα and TNFα induced apoptosis over time (244a). In these older individuals, it may not be possible to detect an increase in TNFα, but possibly a decrease.

Animal studies have provided quality research demonstrating the protective effects of β-glucan against infectious agents and anti-tumor activities (291, 328). But specific factors that determine the potency of the polymers have not been completely elucidated. Molecular weight of glucan polymers has been suggested to be one factor controlling immune stimulation *in vitro*, but the best molecular weight for optimal absorption *in vivo* is not well-defined (329). Normal enterocytes responsible for absorption have been shown to facilitate the transportation of neutral β-glucan polymers to the basolateral membrane (346a). Hunter et al (2002) reported that a patented extraction and spray-drying process creating 1-2 μm particles were most stimulating to *in vivo* macrophages compared to other particles (348). Radiolabelling studies of different size fragments of β-glucan polymers have been found in the serum of animals (348a). An optimal polymer size of 18 kdaltons has been suggested, but macrophages have been shown to process larger polymers and display 25 kdalton fragments on their surfaces for immune recognition (341). While neutral polymers, such as cereal β-glucans are favored for absorption, the mechanism explaining absorption of β-glucan polymers is yet unresolved. Rice et al (2005) demonstrated that three different sources of β(1-3)-glucan were well absorbed and still detectable in serum after 24 hr of oral administration in rats (281). However, in addition to absorption, it has been found that the gut barrier does not completely prevent antigenic material from entering the internal environment (350a, 351a). The gut-associated lymphoid tissue (GALT) is an extensive network of specialized chemical messengers, cells, and tissues that maintain a dynamic balance (tolerance) of responding to pathogens or commensal bacteria and remaining unresponsive to food (352a). Two different members of GALT, sample the contents of the G.I. tract, translocating material into lymph tissue for identification and potential immune stimulation (353a, 354a). Peyer patches sample through dense collections of M cells in follicle-associated epithelium and dendritic cells of the innate system, sample gut contents by stretching their cellular processes between epithelial cells of the gut mucosa (355a, 356a). This may explain how polymers or fragments of polymers gain access to the gastrointestinal immune system. What remains to be identified is the mechanism by which β–glucan entered into the body through these three potential routes, stimulates the immune system and cytokine production. The average molecular weight of β-glucan in this study was determined to be 62 kdaltons with a smaller portion of large and small sized polymers. It is therefore possible that insignificant amounts of barley β-glucan entered the G.I. epithelial tissue stimulating the immune system.

Food, stability, or physical conformation may prevent the absorption or activity of β-glucan polymers in the lumen of the G.I. tract. Mixed linkage β-glucans from cereals are stable at low pH and should not be affected by stomach acid or digestive enzymes of α-specificity (357a). It has become well

established that the gel-forming properties of hydrated β-glucans can entrap large compounds, such as starch and sugars, removing their chemical reactivity and access with the epithelium of the G.I. tract (350b). This specific property has also been reported to inhibit G.I. absorption as aggregates of β-glucan polymers form upon hydration. Hunter et al (2002) found that aggregated β-glucans stimulated mouse macrophages in vivo significantly less than 1-2 μm spray-dried particles that won't aggregate (348). Physical conformation also affects the potency of these polymers (349a). Young et al (2003) reported that NaOH treatment of purified β-glucans before introduction into rats, significantly stimulated macrophage responses when compared to untreated β-glucans. Sodium hydroxide treatment was shown to denature the helices of the polymers. The same study demonstrated dissolved β-glucan polymers that were neutralized and allowed to sit for 9 days to re-anneal, lost potency, showing similar stimulation to untreated polymers. Barley β-glucans utilized in this study were not treated with sodium hydroxide and it was not determined if the barley β-glucan aggregated upon hydration in the drink mixtures.

While the former discussion establishes the entry of β-glucan polymers inside the host, the complex nature of eukaryotic organisms and their highly regulated immune systems may also pose problems for immune stimulation. The immune system requires multiple feedback mechanisms to regulate and protect tissues from over-stimulation (347a). Immunology dictates that continuous stimulation of the innate immune system leads to chronic inflammation and apoptosis. TNFα, a self-regulating cytokine will reduce its own receptor levels on cell surfaces, before feedback loops reduce gene transcription (347b). Interluekin 10 (IL-10) and transforming growth factor beta (TGF-β) are two cytokines generally considered anti-inflammatory, down-regulating TNFα and other cytokine production. (346b).

The cytokine IL-10 can suppress TNFα within minutes, while TGF-β inhibits after several hours (353b). Both appear to work through intracellular protein phosphorylation inhibition, including interactions with STAT3 and IkappaB/NFkappaB (347a). High TNFα concentrations can also stimulate IL-10 to create functional decoy receptors to bind TNFα and therefore inhibit pre-transcriptional and post-translational TNFα activities (337a). Again, more information linking the oral administration of β-glucans with the highly regulated immune system is required before our understanding of these mechanisms becomes clear. Therefore, if barley β-glucan in this study stimulated the immune system, potential inhibition exists by one or more of these counter-regulatory measures to occur by the time patients had blood drawn.

Utilizing food and food components as medicine has been the premise of some medical professionals and many nutraceutical organizations. The evidence demonstrating that β-glucans stimulate the immune system of mammals has become more validated in recent years (292, 281, and 295). Granted, a clear mechanism that directly links the entry of β-glucans from the G.I. tract and the direct stimulation of the immune system producing cytokines has yet to be discovered. If food and nutritional

organizations are going to continue to recommend specific foods containing β-glucans, then the complex interrelationship between food components in the gastrointestinal tract and the constant sampling and screening process by the immune system needs to be more clearly defined and supported by these organizations. Advances in technology, implementation of methods from other scientific disciplines, and a collaborative effort from investigators to utilize similar sourced materials of high purity will more clearly define this research.

Chapter 2

Dietary Components from Oat, Barley, and Ginger Modulate TNFα Production in Murine Macrophage

Introduction

Western dietary habits have been linked to many chronic diseases, including cardiovascular disease, diabetes, obesity, and cancer (114). While the consumption of certain dietary components has been associated with increased risk factors for chronic disease, whole grain components help reduce some of these same risk factors (45). For example, dietary fiber reduces total cholesterol and post-prandial glucose and insulin levels (115, 200). One benefit of dietary fiber that may not be as well known includes the immune stimulating properties of β-glucans.

β-glucans are cell wall polymers found in every biological kingdom taxa, except animalia (176). Concentrated forms can be found in cereal grains, fungi and some bacteria (45a, 46a). They are biological response modifiers that have successfully demonstrated immune stimulation *in vitro* and *in vivo* (291, 276). β-glucans specifically stimulate cells of the innate immune system, such as macrophages and monocytes. The immune cells respond through phagocytic activity and production of immune cytokines, such as tumor necrosis factor alpha (TNFα) and one or more interleukins (IL-1, IL-6) (287, 344).

TNFα initiates and regulates gene transcription and cytokine production in different cells in response to infectious agents or to mediate inflammation (229, 240). Regulating the quantity of TNFα produced in the body helps a host defend against infectious agents and control inflammation.

Curcumin, a diferuloyl methane compound found in turmeric and ginger has potent anti-inflammatory properties (428, 47a). Curcumin inhibits TNFα, cyclooxygenase-2, and prostaglandin PGE_2 (418a, 416). Among several effects, curcumin inhibits NF-*kappa* B from translocating to the nucleus and initiating gene transcription of immune cytokines (484, 419a).

The specific immune stimulating mechanisms of β-glucans have been suggested to be related to the molecular weight (MW), β(1→3) linkages, and β(1→6) branching of the polymer that interact with one or more cell surface receptors (218a, 329). However, most investigations into immune stimulation have utilized the predominant β(1→3) linked fungal β-glucans, such as zymosan, not cereal β-glucans (330). Soluble mixed-linkage cereal β-glucans are composed primarily of β(1→4) with fewer numbers of

β(1→3) linkages. If larger polymers and β(1→3) linkages are responsible for a more potent immune stimulating glucan, then it may follow that β-glucan polymers of increasing quantities of β(1→3) linkages, might stimulate cells of the immune system to a greater degree. Although cereal β-glucans contain fewer β(1→3) linkages, we hypothesize that to increase cereal β-glucan immune stimulation, the availability of β(1→3) linkages for cellular interaction must be increased.

In this study, the goal was to modulate the production of TNFα from macrophages with dietary food components. The first objective was to assess the effects of cereal β-glucans on macrophages of the immune system. The second objective was to modify these effects by treating the β–glucan with a glucan hydrolase. Finally, the third objective was to assess the inhibitory action of a common anti-inflammatory compound from the spice tumeric, on the production of TNFα from macrophages stimulated by different β(1→3) glucans.

Materials and Methods

Polysaccharide preparation

A patented process (50a) using an alkaline solution produced the mixed linkage oat β-glucan (>97% purity; MW ~150 kD). The patented process (50b) to produce mixed linkage barley β-glucan (>91% pure; MW ~139 kD) utilized hot water extraction. Both cereal glucans were solubilized and treated with trypsin (3%; 1 hr at 37°C, porcine GIBCO BRL, cat#15090-046), purified through anionic exchange columns (Diaion exchange resin, Mitsubishi Chem.), precipitated in 50% ethanol overnight (4°C), centrifuged (1000x*g*) and dried in a sterile hood. Oat β-glucan samples were dissolved (2 mg/ml) in sterile DMEM (Sigma cat.# D5648, St. Louis, MO.) medium at 65 °C for ~15 min using vigorous vortexing. Purified barley β-glucan was dissolved in sterile DMEM medium (2 mg/ml) at 75 °C for ~15 min. Laminarin (*Laminaria digitata*; Fluka #61430) was dissolved in sterile DMEM media (10 mg/ml) at 40 °C for 10 min. Zymosan A (Fluka # 97340) was prepared by first boiling for 30 min in 10 M NaOH, then washing three times in sterile 1x PBS. Zymosan was suspended in sterile DMEM media (5mg/ml) before use. Curdlan (Sigma #C-7821) was dissolved in dimethyl sulfoxide (DMSO; 20 mg/ml). Enzyme-treated cereal β-glucan was produced by incubating dissolved cereal glucan samples at 40°C for 1 h with glucan hydrolase (Lichenase; 18 u/mg; #E-LICHN, Megazyme Int. Ireland Limited). This reaction was stopped in a boiling water-bath (10 min). Amylose, amylopectin, and maltose were dissolved in DMEM medium (2 mg/ml) and cellulose, methyl-cellulose, carboxy methyl cellulose and cellotriose were all dissolved in DMSO (2 mg/ml).

Cell preparation and treatment

Murine macrophage/monocyte cell line (RAW 264.7) was obtained from the American Type Culture Collection (ATCC # TIB-71). Cells were cultured and maintained in sterile DMEM + 10% (heat inactivated) FBS at 37°C with 5% humidified CO_2. Isolated cells were plated (0.6×10^6) in single tissue culture dishes (35 X 10 mm, Starstedt, Inc., Newton, N.C.) in maintenance medium overnight before treatment. Adherent cells were treated with new medium, polysaccharides, or control conditions overnight (15 h). In the inhibitory experiments, macrophages were pretreated with curcumin for 2h, prior to the addition of β(1→3) glucans in the same tissue culture dishes.

Quantifying TNFα

Conditioned media from all experimental culture dishes were harvested on ice and centrifuged at 1100 xg, supernatants were stored in new sterile (1.5 ml) tubes at –25 °C until analysis (< 7 days). Cell numbers in all culture dishes were quantified by trypan blue after each experiment. TNFα was quantified by ELISA (BD OptEIA, cat. 555268, BD Biosciences, San Diego, CA.). Statistical analysis between treatments was determined by t-tests (Sigma plot software, version 5.0, SPSS, Inc.).

Results

The different β(1→3) glucans utilized in this study are summarized in Table 1. Listed with the mixed-linkage cereal β-glucans, are several β(1→3) linked polymers containing different quantities of this specific linkage.

Table 2.1 Different β1-3 glucans, documented to stimulate immune cells and utilized in this study.

β1-3 Glucan	***β Linkage***	***Percent β1-3 Linkage***	***Source***
Oat	(1-4), (1-3)	~30	*Avena* cereal grain
Barley	(1-4), (1-3)	~30	*Hordeum* cereal grain
Zymosan	(1-3), (1-6)	~70	*Saccharomyces* yeast
Curdlan	(1-3)	100	*Alcaligenes* bacteria
Laminarin	(1-3), (1-6)	~90 - 98	*Pheophyta* brown algae

All cereal β-glucans stimulated the macrophages cells at the concentration specified (Fig.2.1). Cereal β-glucan concentrations were experimentally determined to produce approximately similar macrophage responses in order to compare treatment effects within absorbance boundaries after enzyme treatment. Barley β-glucan stimulated macrophages to produce more TNFα than equal concentrations of oat. The oat and barley β-glucan dose-response treatments of macrophages show a linear response with increasing concentrations. Oat β-glucan treatments between 100 ug/ml and 200 ug/ml ($p<0.002$) and barley β-glucan treatments between 1 ug/ml and 5 ug/ml ($p<0.005$) were the only two comparisons that were significantly different from one another.

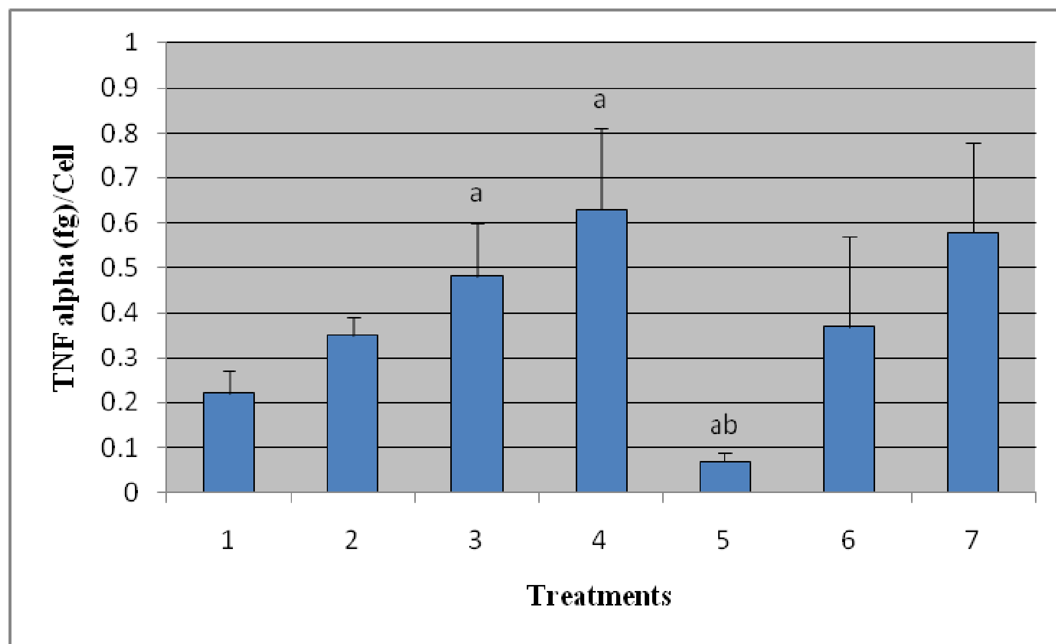

Figure 2.1 Dose response from macrophages treated with highly pure mixed linkage cereal β-glucans (n=6). Oat β-glucan: 1. 100 ug/ml. 2. 200 ug/ml. 3. 300 ug/ml. 4. 400 ug/ml. Barley β-glucan. 5. 1 ug/ml. 6. 5 ug/ml. 7. 10 ug/ml. Letters denote significant difference.

Both cereal β-glucans produced more TNFα than did laminarin (Fig 2.2) and enzyme-treated oat β-glucan produced significantly more TNFα when compared to non-enzyme-treated oat β-glucan and laminarin (Fig. 2.3; $p <0.0001$). Concentrations of enzyme-treated oat and barley β-glucans were chosen to allow for treatment effects that increase or inhibit production of TNFα while remaining in a quantifiable range. The enzyme-treated oat β-glucan stimulated the macrophage cells to produce ~330% more TNFα than non-enzyme-treated oat β-glucan and ~730% more than laminarin. The enzyme-treated barley β-glucan did not significantly affect the quantity of TNFα produced when compared to non-enzyme treated barley β-glucan ($p>0.30$). The enzyme-treatment of barley β-glucan decreased TNFα production by ~28%.

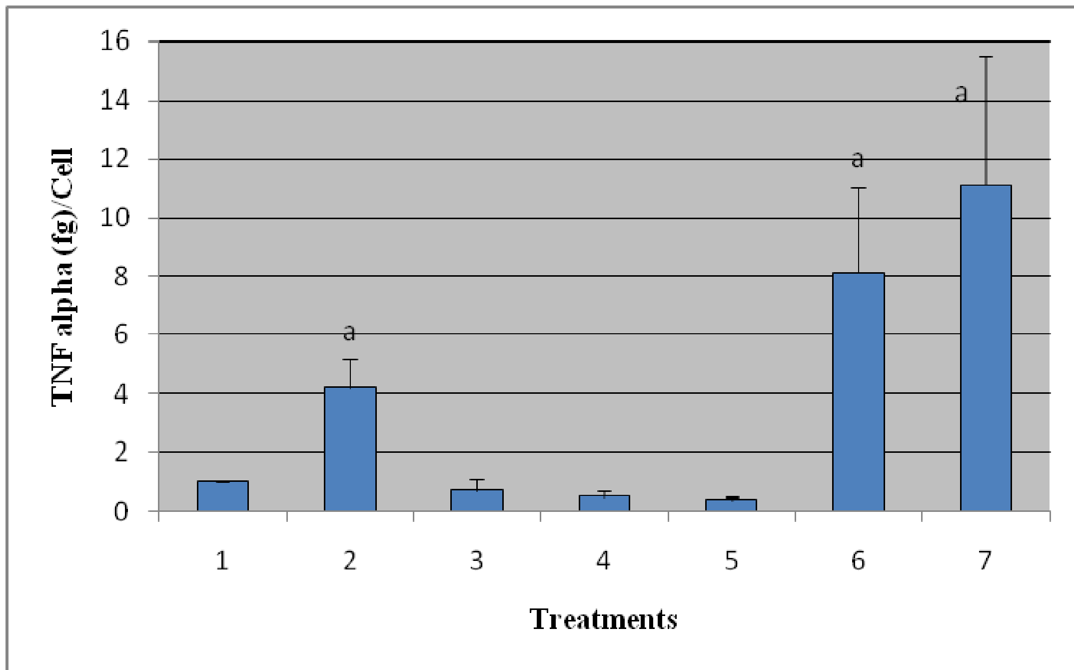

Figure 2.2 Macrophages treated with different β1→3 glucans (n=6). All treatments are standardized to oat β-glucan (300ug/ml). 1. Oat β-glucan (300ug/ml). 2. Enzyme-treated Oat β-glucan (300ug/ml). 3. Barley β-glucan (10ug/ml). 4. Enzyme-treated Barley β-glucan (10ug/ml). 5. Laminarin (200ug/ml). 6. Zymosan (50ug/ml). 7. Curdlan (50ug/ml). Letters denote significant difference.

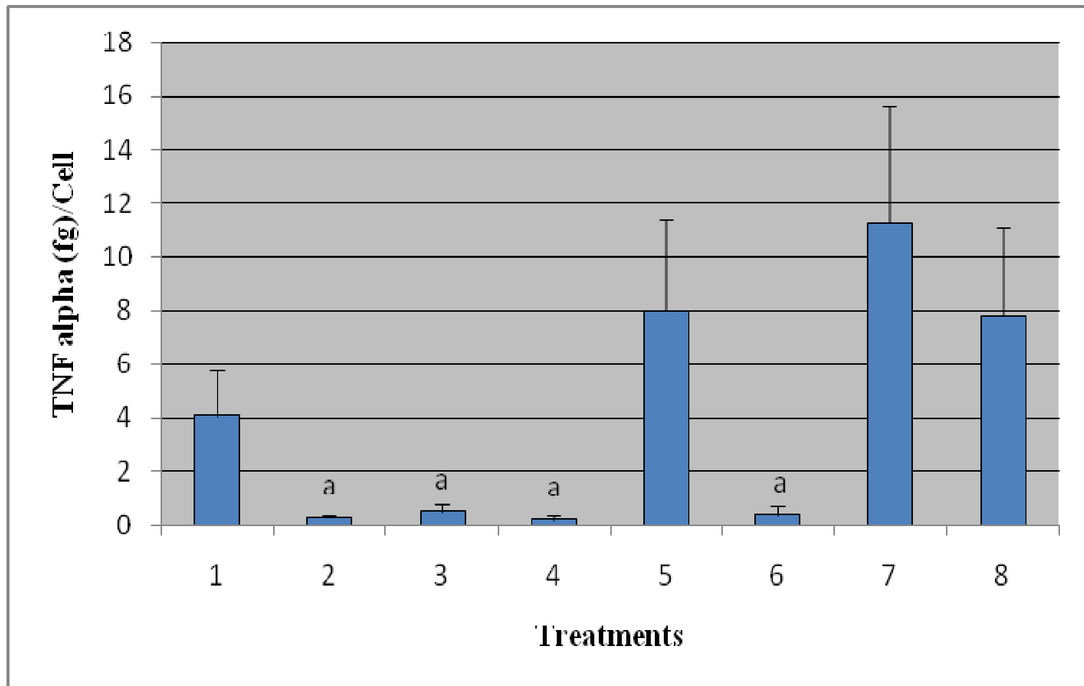

Figure 2.3 Macrophages treated with β (1→3) glucans +/- curcumin (n=6). Macrophages were pretreated (2h) with curcumin (9 ug/ml) before addition of β (1–3) glucans. All treatments were standardized to oat β-glucan (300ug/ml). 1. Enzyme-treated Oat β-glucan (300ug/ml). 2. Curcumin + enzyme-treated Oat β-glucan (300ug/ml). 3. Enzyme-treated barley β-glucan (10ug/ml). 4. Curcumin + enzyme-treated barley. β-glucan (10ug/ml). 5. Zymosan (50ug/ml). 6. Curcumin + zymosan (50ug/ml). 7. Curdlan (50ug/ml). 8. Curcumin + curdlan (50ug/ml). Letters denote significant difference.

Zymosan and curdlan stimulated macrophages to produce the most TNFα, including significantly more TNFα than either enzyme-treated oat or barley β-glucan ($p<0.05$). Curdlan produced significantly more (~109%; $p<0.0003$) and (~1500%; $p<0.0001$) TNFα than enzyme-treated oat β-glucan and barley β-glucan, respectively. Zymosan produced significantly more (~93%; $p<0.02$) and (~1400%; $p<0.0001$) TNFα than enzyme-treated oat β-glucan and barley β-glucan, respectively. Treatments between zymosan and curdlan were not statistically different ($p>0.65$).

Cellotriose, a small β-linked trimer fragment and other α and β linked polymers did not stimulate macrophages to produce significant quantities of TNFα above background levels of culture medium, except for amylopectin (Table 2.2). Maltose ($p>0.25$) and methyl cellulose ($p>0.89$) stimulated macrophages to produce more TNFα than culture medium alone, but it was not significant. The small stimulation from culture medium most likely occurs due to the proteins in the serum.

Table 2.2 Different α and β linked polymers evaluated for macrophage stimulation in this study.

Treatment	Polymer Characteristics	Concentration Range Evaluated	TNFα Production (fg/cell)
Amylose	α(1→4) R=OH	50 – 400 ug/ml	0.0
Amylopectin	α(1→4)(1→6) R=OH	50 – 400 ug/ml	<4.51 +/- 3.36*
Maltose	α(1→4) R=OH	50 – 400 ug/ml	<0.047 +/- 0.031
Cellulose	β(1→4) R=OH	50 – 400 ug/ml	0.0
Carboxy-Methyl-Cellulose	β(1→4) R=CH2-COOH	50 – 400 ug/ml	0.0
Methyl – Cellulose	β(1→4) R=CH3	50 – 400 ug/ml	<0.031 +/- 0.029
Cellotriose	β(1→4) R=OH	50 – 400 ug/ml	0.0
Culture Medium			0.029 +/- 0.030*

* significantly different

Curcumin significantly inhibited (81%; p<0.006) the production of TNFα by macrophages stimulated with enzyme-treated barley β-glucan and significantly reduced the production of TNFα by enzyme treated oat β-glucan by 93% (p<0.0001). Curcumin also significantly reduced the production of TNFα from zymosan by 94% (p<0.0002). Surprisingly, curcumin could not significantly reduce the production of TNFα by macrophages stimulated by curdlan (reduced ~15%; p>0.9). Control treatments of macrophages using only the solvents, such as 95% ethanol and DMSO, did not show any perturbation to the cell line by means of detectable TNFα secretion (data not shown).

Because excessive heating and shearing may modify glucan structure, heat sterilization was avoided when possible. While filter sterilization (0.2 um) was attempted, it removed dissolved glucans from treatment solutions and affected TNFα production. Bacterial contamination was monitored by microscopic inspection of all tissue culture dishes when counting macrophage cell numbers and by inspection of control tissue culture dishes left in incubator 24 hrs after harvesting each experiment. No bacterial contamination was noted in any dish (up to 39 h) and since bacterial generation times range from 15 to 30 min, we do not consider that the quantified TNFα collected in this study was adulterated by bacterial contamination.

Discussion

Several studies have shown that cells of the immune system bind, phagocytize, and induce immune cytokines from cereal β-glucan interactions (352, 51a). In this study, it was demonstrated for the first time, that the enzymatic treatment with a glucan hydrolase of highly pure (>97 %) mixed linkage cereal β-glucan, increases the production of TNFα from stimulated macrophages.

The separation of raw materials during purification helps to establish an important and more clearly defined cause and effect relationship between β-glucans and macrophages. Mixed-linkage β-glucans in this study were analyzed (β-glucan assay kit; Megazyme International, Inc. Ireland) to assess the purity of the oat (>97%) and barley (>91%) polymers (52a). An extra process of purification was also utilized to decrease protein contamination that could potentially stimulate macrophages. This included the use of trypsin and anionic exchange column purification, which reduced nitrogen-containing compounds to < 1.5% (Leco Corp., TruSpec™ N, data not shown).

Upon stimulating the macrophages with cereal β-glucans, approximately thirty-times more β-glucan from oat was required to produce the same quantity of TNFα from barley β-glucans (Fig. 2.2). This difference in macrophage response could be related to the individual structure, molecular weight, or the extraction method used to obtain the glucan polymers.

The structure of oat and barley β-glucans have been investigated thoroughly (53a, 54a). β-glucans are polysaccharide polymers that contain a backbone of repeating glucopyranosyl units. The main backbone shares similarity with cellulose, consisting of cellotriosyl and cellotetraosyl oligimers, interrupted by single β (1→3) linkages. Cereal β-glucans, referred to as mixed-linkage β-glucans, are polymers containing approximately 70% β(1→4) and 30% β(1→3) linkages (54a). Oat and barley β-glucans have generally been considered similar in structure. They are linear, neutral, and unbranched. However, oat β-glucan consists of a greater number of β(1→3) linked cellotetraosyl oligimers (~ 33%) and less β(1→3) linked cellotriosyl oligimers than barley cultivars consisting of only ~ 25% β(1→3) linked cellotetraosyl oligimers (355). Differences in linkage structure create changes in polymer structure, which may lead to different interactions with cell receptors and signaling pathways, hence creating a difference in macrophage response. Communication from cell surface receptors to nuclear gene transcription is accomplished through cell signaling pathways (55a). β-glucans use different receptors (Dectin-1, TLR 4) and signaling pathway proteins, such as nuclear factor *kappa* B (NF-*kappa* B), to promote TNFα production (309, 258).

Different extraction methods utilized in the literature to obtain β-glucans from cereals have reported mixed structural and functional results. In this study, two different extraction methods were utilized to obtain the oat and barley β-glucans. Rimsten et al (2003) utilized four different extraction

methods, and found that the average molecular weights and distribution of those weights were similar between all methods (355). Delaney et al (2003) have shown similar functional properties between oat and barley β-glucans extracted with the same method (191). Douwes et al estimated that glucans extracted under hot conditions form triple helices, while alkaline extractions do not, but they may form random coil fragments (356). These formations may change the functional properties of the polymers and therefore affect the physiological response elicited by the interaction of polymers with cell surface receptors. Wood et al (1994) demonstrated an inverse relationship between the viscosity of β-glucans and their physiological effects (reduced insulin and glucose; 357). While we did not measure triple helices or random coiled fragments in our β-glucan samples, functional changes due to extraction methods may have contributed to the different quantities of TNFα produced in this study.

The difference in molecular weight between oat (~150 kD) and barley (~139 kD) β-glucans, in this study, does not seem to justify the differences in TNFα production from macrophages. However, the presence of smaller oligomeric fragments generated through storage and handling, cannot be disregarded.

The modification of cereal β-glucan polymers with glucan hydrolase (lichenase) significantly increased the stimulating capacity of oat β-glucan compared to the untreated oat β-glucan (~330%; Fig.2.2). However, lichenase treatment had no significant effect on barley β-glucan to stimulate TNFα production. This may be explained by structural and functional differences between oat and barley β-glucans or by the greater quantity of oat β-glucan present in these samples. A greater quantity of oat β-glucan means more β(1→3) linkages for lichenase digestion compared to barley β-glucan. Lichenase or endo-β(1→4) glucanase, modifies polymers by hydrolyzing the β(1→4) bond after each β(1→3) bond it encounters (357a). The enzyme modification could theoretically reduce the size of the polymers and increase the exposure of the β(1→3) linkages. The importance of β(1→3) linkages in stimulating cells of the innate immune system, has been established (218a, 308). Roubroeks et al (2000) utilized the same enzyme, lichenase (Megazyme International, Inc., Ireland) with oat β-glucan under similar physical conditions and demonstrated a reduction in the weight-average molecular weight in the first hour of ~ 57% and after 24 h incubations a reduction of 98% (57a). Incubations of 1 h were utilized in this study with lichenase treatments and it increased the immune stimulating capacity of the oat β-glucan. Interestingly, Roubroeks et al (2000) did not find a significant accumulation of small oligomers. Therefore, it is not expected that we reduced the size of our cereal β-glucan polymers to small oligomeric fragments during 1 h lichenase treatments. However, small β(1→4) linked trimer fragments (cellotriose) were also evaluated for macrophage stimulation. Cellotriose did not stimulate macrophages to produce TNFα (Table 2.2).

The enzyme treatment may have "relaxed the structural conformation" of the polymer pieces to make the β(1→3) linkages more available for cell contact with the macrophages. Interestingly, this may have only applied to oat β-glucan under these parameters. Given that oat β-glucans contain more

cellotetraosyl segments compared to barley, then this also means that oat glucans contain less β(1→3) linkages when comparing similar size polymers between the two cereal glucans. Since an incomplete digestion most likely occurred in the span of 1 hr, oat β-glucans may have been digested to a greater degree than barley β-glucans, providing a difference in β(1→3) exposure. Low molecular weight glucans associating to form aggregates may also have occurred (275). If the oat β-glucan was digested to a greater degree, then a greater number of fragments of glucan may occur in the oat sample. Wood et al. (1994) demonstrated that a small number (~ 3%) of insoluble fragments of β-glucans were created after β-glucan digestion of oat and barley with lichenase (29). While oligomer fragments were not measured in this study, aggregated or branched glucans have been shown to stimulate immune cells more than unbranched or un-aggregated polymers (349).

For comparison, other β-linked polymers known to stimulate murine macrophages were included in the study. The two most potent stimulators of this murine macrophage cell line were curdlan and zymosan (Fig 2.2). Curdlan's an unbranched polymer (Table 1) consisting of only β(1→3) bonds and zymosan, a β(1→3) polymer with ~ 30% β(1→6) branching (274). Curdlan induced the macrophages to produce more TNFα (3.9 times) than the enzyme-treated oat β-glucan and at a much lower concentration (50 ug/ml). While zymosan didn't stimulate the macrophages as much (2.6 times), it also induced a greater amount of TNFα at a much lower concentration (50 ug/ml) than the enzyme-treated oat β-glucan. Less TNFα production from cereal β-glucan effects can be expected since several investigations have shown that the most active polymers contain highly branched structures with greater quantities of β(1→3) bonds (57a, 218a). Interestingly, laminarin, a polymer consisting of at least 90% β(1→3) bonds with less than 10% β(1→6) branching, stimulated macrophages to produce less TNFα than untreated oat or barley β-glucan. This may demonstrate the specific structural requirement for receptor interaction, requiring the appropriate combination of β(1→3) linkages and β(1→6) branching. But multiple mechanisms of action must occur considering the great diversity of polysaccarides that have shown immune stimulating potentials (275). While laminarin stimulated the macrophages to produce a small quantity of TNFα in this study, it has also demonstrated anti-inflammatory activities in plant cells (58a).

Several α-linked polymers were also evaluated for macrophage stimulation, but only one, amylopectin, significantly stimulated macrophages above background levels (culture medium) ($p<0.009$; Table 2.2). Amylopectin has been known to bind somatic cell receptors and be cleared from circulation *in vivo* by macrophages (59a). Drug-delivery, via liposomes to specific tissues in a host has been accomplished by conjugation with amylopectin (60a). Amylopectin does not contain β(1→3) linkages, but contains a unique stereo-chemistry of α(1→4) with (1→6) branched linkages that macrophages find important to apprehend. This specific stereo-chemistry may mimic carbohydrates from microorganisms or simply be recognized by the hosts immune system as non-self. Analysis of α and β linkages alone may not completely explain the stimulation of macrophages of the immune system, therefore, further

study into carbohydrate chemistry, receptor interaction, and cell signaling pathways should be encouraged. The stimulation of macrophages by β-glucans may be optimized through stereo-chemistry, specific ratios of β(1→3) and β(1→6) linkages, and molecular weights of the polysaccharides evaluated.

Curcumin over the last ten years has demonstrated its potential as an anti-oxidant, anti-cancer, and anti-mutagenic compound through *in vitro* and *in vivo* experimentation (418a, 416). Here we utilized the anti-inflammatory mechanism of curcumin to investigate the potential to inhibit cytokine production from macrophages stimulated by different β-glucans. Curcumin has been shown to block the signaling pathway ERK 1/2 and downstream activation of NF kappa B (485, 62a). It's now established that certain β-glucan receptors utilize a tyrosine activation motif to initiate the phosphorylation of signaling proteins inside immune cells, which then activate NF kappa B to translocate to the nucleus and promote gene transcription (328, 63a). In this study, curcumin significantly inhibited TNFα production by enzyme-treated barley β-glucan and significantly reduced the production of TNFα by enzyme-treated oat β-glucan and zymosan (Fig.2.3). However, curcumin could only inhibit a small portion of the production of TNFα by curdlan. The possibility exists that curdlan avoids significant inhibitory action from curcumin by utilizing different cell receptors and/or activate another signaling pathway inside the cell that promotes inflammatory cytokine production. It has been suggested that β-glucans have unusual intracellular signaling and this has yet to be more thoroughly investigated (329, 341). Evidence supplied over the last 20 years, suggest that there are multiple receptors that β-glucans can interact with to stimulate an immune response (303, 337).

In this study, it was demonstrated *in vitro* how dietary food components can control an important cellular mediator of the immune system. For the first time, the modification with a glucan hydrolase of a highly pure (>97%) mixed linkage oat β-glucan, was shown to significantly increase the production of TNFα from stimulated macrophages. Macrophage stimulation from fungal sources may still be greater, but the new potent stimulating effects of modified cereal β-glucans on macrophages in this study, demonstrate the need for new *in vivo* investigations. Just as important, the dietary constituent, curcumin, significantly decreased the production of TNFα from macrophages stimulated by several β-glucan sources, except curdlan.

Chapter 3

Cereal Bran Extracts Inhibit Beta-Glucan-Stimulated Macrophages

Introduction

Plants and plant-derived compounds have been utilized globally by most cultures for over 10,000 years (360a). These include structural, reproductive, and storage tissues of the plants, and their extracts, utilized for food, technology, and medicine (361a). The medicinal attributes of plant-derived compounds still provide pharmaceutical companies with over 20% of their products (362a). Plants have also provided humans with nutrients, including various macromolecules (e.g. proteins, starches, fiber, etc...), vitamins, minerals, and a broad range of phytochemicals (363a). The seeds of cultivated grasses, such as whole cereal grains, have provided the human diet with a large portion of its protein and carbohydrates (364a). Breeding and refining over the last 50 years has reduced the nutritional and functional value of these harvested grains, but many bioactive compounds are still present.

Epidemiological studies suggest that plant compounds, such as flavonoids may play a protective role in cancer and cardiovascular disease (365a). Flavonoids are polyphenolic compounds, found in several aerial parts of the plant, but concentrated sources exist in reproductive tissues of the seeds (393).

Whole grains contain many phenolic compounds, including those of the shikimic acid pathway (Table 3.1). Like most cereal grains, wheat and rye contain significant amounts of ferulic, sinapic, coumaric, and vanillic acids with smaller quantities of caffeic, protocatechuic, and syringic acids (379, 380). Corn generally has cinnamic acid, hydroxyphenylacetic acid, phenylacetic acid, and minor quantities of ferulic, coumaric, vanillic, and syringic (381). In barley, ferulic, sinapic, cinnamic, and coumaric acids predominate, with smaller amounts of vanillic, hydroxybenzoic acids (381). These particular phenolic acids are often linked to non-starchy polysaccharides in the cell walls of whole grains and are referred to as 'bound phenolics', not free, although the degree of cross-linking in the cell walls to these polysaccharides varies among types and varieties of grains (382).

Plant extracts, and the phenolic acids associated with them, have demonstrated effective antioxidant, anticarcinogenic, anti-thrombotic, and anti-inflammatory activities (361). The antioxidant affects on mammalian systems delivers a buffer from free radical development and ingested contaminants, providing some degree of protection from chronic disease (367a). Some of these same phenolics reduce inflammatory mediators, providing additional support to the prevention of chronic diseases (368a). Studies with isolated phenolic acids, proanthocyanins, and catechins from propolis, ginger, pine bark, and honey-suckle have reduced inflammatory mediators in different model systems (393a, 411).

Table 3.1. Phenolic acid compounds from select edible grain species and legumes (393, 379, 380 and 381).

Compound Class	Carbon Skeleton	Barley	Wheat	Oat	Rye	Legumes
Phenolic Acids	C_6-C_1	X	X	X	X	X
Hydroxy-cinnamic Acids	C_6-C_3	X	X	X	X	X
Avenanthramide	C_6-N-C_3-C_6			X		
Flavonoid Anthocyanins	C_6-C_3-C_6	X	X	X	X	X
Lignan	$(C_6\text{-}C_3)_2$	X	X	X	X	X
Lignin	$(C_6\text{-}C_3)_n$ $(C_6)_n$	X	X	X	X	X
Al(ken)nylresorcinols	C_6- C_4-$C_{3\text{-}9}$-C_5	X	X		X	

Phenolic acids have demonstrated anti-inflammatory activity through the inhibition of NF-kappaB, cyclooxygenase-2 (COX-2), lipoxygenase, TNFα, IL-1β, and IL-6 (23, 24). However, most anti-inflammatory studies with phenolic acids have not included the central cytokine, TNFα.

As discussed in Chapter 1, inflammatory cytokines such as TNFα, IL-1 β, and IL-6 are cellular mediators of inflammatory pathways (369a). TNFα plays a central role in orchestrating immunological reactions to secrete cytokines, which communicate and stimulate pathways leading to the induction of inflammation (370a). Controlling these cellular mediators of inflammation, specifically TNFα, provides a means of reducing health-risks and symptoms associated with several chronic diseases (370b). Cardiovascular disease has been improved by reducing COX-1 and 2 with salicylic acid derivatives (371a). Insulin sensitivity and glucose utilization has been improved in obese individuals by blocking TNFα receptors (371b).

The aim of this study was to evaluate multiple cereal bran extracts that inhibit the production of TNFα from stimulated murine macrophages. Small phenolic, phenolic acid and flavonoid compounds contained in the bran of cereal grains can be removed with appropriate solvent systems. The first objective was to evaluate several extraction procedures, dependent upon solvent, temperature, and pH for their toxicity and stimulating capacity to macrophages. To examine the capacity of candidate cereal bran

extracts to inhibit inflammatory cytokines, β -glucan was utilized as a macrophage stimulant to produce TNFα. The second objective was to evaluate cereal bran extracts for their ability to inhibit TNFα secretion from β-glucan stimulated murine macrophages. Inhibition of TNFα by cereal bran extracts was then compared to curcumins inhibitory effects.

Materials and Methods

Polysaccharide preparation:

A modified extraction process, based on alkaline conditions, produced the mixed linkage oat β-glucan (>97% purity; MW ~150 kD, 24). β-glucan samples were dissolved (2 mg/ml) in sterile DMEM (Sigma cat. D5648, St. Louis, MO.) medium at 65 °C for ~15 min using vigorous vortexing. Curdlan, a linear bacterial β (1-3)-glucan polymer (Sigma C-7821) was dissolved in dimethyl sulfoxide (DMSO; 20 mg/ml). Enzyme-treated oat β -glucan was produced by incubating dissolved cereal glucan samples at 40°C for 1 h with glucan hydrolase (Lichenase; 18 u/mg; E-LICHN, Megazyme Int. Ireland Limited). This reaction was stopped in a boiling water-bath (10 min).

Cereal bran extract preparation:

Barley bran (3g: CDC Alamo - waxy hulless) and wheat bran samples (3g: Oxen - hard red spring) were combined with 50, 80, or 95% ethanol (30 ml) in erylenmeyer flasks**,** the pH was adjusted to 5.2, 7.0, or 8.8 with glacial acetic acid or sodium hydroxide, and the samples were shaken overnight (~15h) at 35 ºC, except for the mixtures that were combined and pH adjusted in the same manner, but with an addition of a heating step up to 90 ºC, before shaking overnight. To harvest bran extracts, mixtures were allowed to settle (1h) at 25 ºC, the aqueous layer was transferred into a clean glass tube (50 ml), and stored at 4 ºC. Bran extracts were utilized within 4 days for optimal cytokine inhibition.

Cell preparation and treatment:

Murine macrophage/monocyte cell line (RAW 264.7) was obtained from the American Type Culture Collection (ATCC # TIB-71). Cells were cultured and maintained in sterile DMEM + 10% (heat inactivated) fetal bovine serum (FBS) at 37°C with 5% humidified CO_2. An additional heating step for 30 min. at 65 ºC was added for FBS to reduce stimulation of macrophages from medium alone. Isolated cells were plated (0.65×10^6) in single tissue culture dishes (35 X 10 mm, Starstedt, Inc., Newton, N.C.)

in maintenance medium overnight before treatment. Control treatments of adherent cells contained one or more of the following: new medium, cereal bran extracts, curcumin, polysaccharides, or treatment solvents. In the inhibitory experiments, adherent macrophages were pretreated with cereal bran extracts or curcumin for 2h, prior to the addition of β (1→3) glucans in the same tissue culture dishes then incubated overnight (15h). Curcumin (diferuloylmethane), a potent anti-inflammatory compound that inhibits multiple intracellular immune mediators, is commonly found in dietary and medicinal forms. Its chemical structure consists of two ferulic acids, the dominant phenolic acid found in cereal brans.

Quantifying TNFα

Conditioned media from all experimental culture dishes were harvested on ice and centrifuged at 1100 x g. Supernatants were stored in new sterile (1.5 ml) tubes at –25 °C until analysis (< 7 days). Cell numbers in all culture dishes were determined after each experiment. TNFα was quantified by ELISA (monoclonal; BD OptEIA, cat. 555268, BD Biosciences, San Diego, CA.). Statistical analysis for each experiment was determined by ANOVA with statistical analysis between individual treatments determined by Tukey-Kramer post-test (Microsoft Excel™).

Results

Several different solvent combinations were created to extract anti-inflammatory compounds from cereal bran through manipulation of pH, temperature, and ratio of water/ethanol/methanol (Table 3.2). Methanol was not used since concentrations required for extractions became toxic to the macrophage cell line. Cereal bran from barley, wheat, rye, buckwheat, and oats were investigated. Buckwheat and oat bran extracts had no affect on TNFα production from macrophages treated alone or with β-glucan treatment. After multiple solvent systems and experimental trials, only two solvent extracts were found to be effective with two cereal brans: solvent 5C for barley and 4B for wheat (Table 3.2). These two systems were chosen, based upon TNFα production from macrophages treated with cereal bran extracts alone (Figure 3.1). All other solvent extracts, including rye (4A, 5A, 5C, 6B), stimulated the macrophages to an inconsistent, but greater degree (data not shown).

Table 3.2 Solvent scheme utilized in this study to obtain optimal extracts from barley and wheat bran. NT = "not tested."

Extract	**% Ethanol**	Solvent #1	Solvent #2	Solvent #3	Solvent #4	Solvent #5	Solvent #6
		pH 5.2	pH 7.0	pH 8.8	pH 5.2	pH 7.0	pH 8.8
A	50% Ethanol	NT	90 °C	NT	overnight 35 °C	overnight 35 °C	NT
B	80% Ethanol	90 °C	90 °C	90 °C	overnight 35 °C	overnight 35 °C	overnight 35 °C
C	95% Ethanol	NT	90 °C	NT	NT	overnight 35 °C	NT

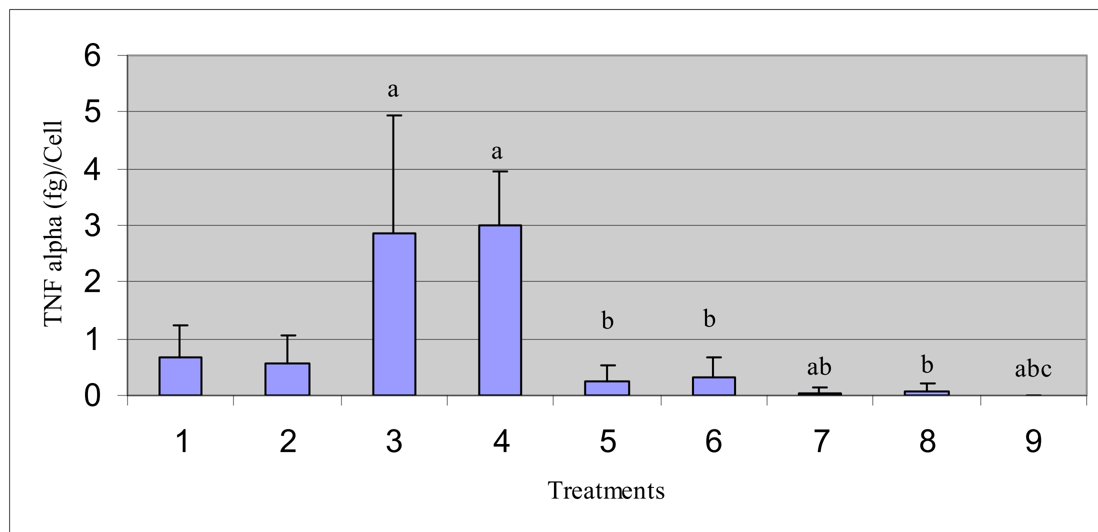

Figure 3.1 Macrophages treated with cereal bran extracts or curcumin only (no glucan stimulation). 1. Barley Bran (30 ug/ml) 5C. 2. Barley Bran (60 ug/ml) 5C. 3. Barley Bran (30 ug/ml) 4B. 4. Barley Bran (60 ug/ml) 4B. 5. Wheat Bran (40 ug/ml) 5C. 6. Wheat Bran (80 ug/ml) 5C. 7. Wheat Bran (40 ug/ml) 4B. 8. Wheat Bran (80 ug/ml) 4B. 9. Curcumin (9 ug/ml). Letters denote significantly different

Curcumin, a natural pro-inflammatory cytokine inhibitor, did not stimulate macrophage cells to produce TNFα when used alone (without β -glucan) in experiments (Figure 3.1). Curcumin reproduced similar results from chapter 2, when utilized in experiments with oat and curdlan β -glucan as TNFα stimulators to macrophages. Curcumin significantly ($p<0.01$) inhibited TNFα production from

macrophages stimulated by oat glucan (93.5%), but only reduced TNFα production from curdlan-stimulated macrophages by 17% (Figure 3.2 and 3.3).

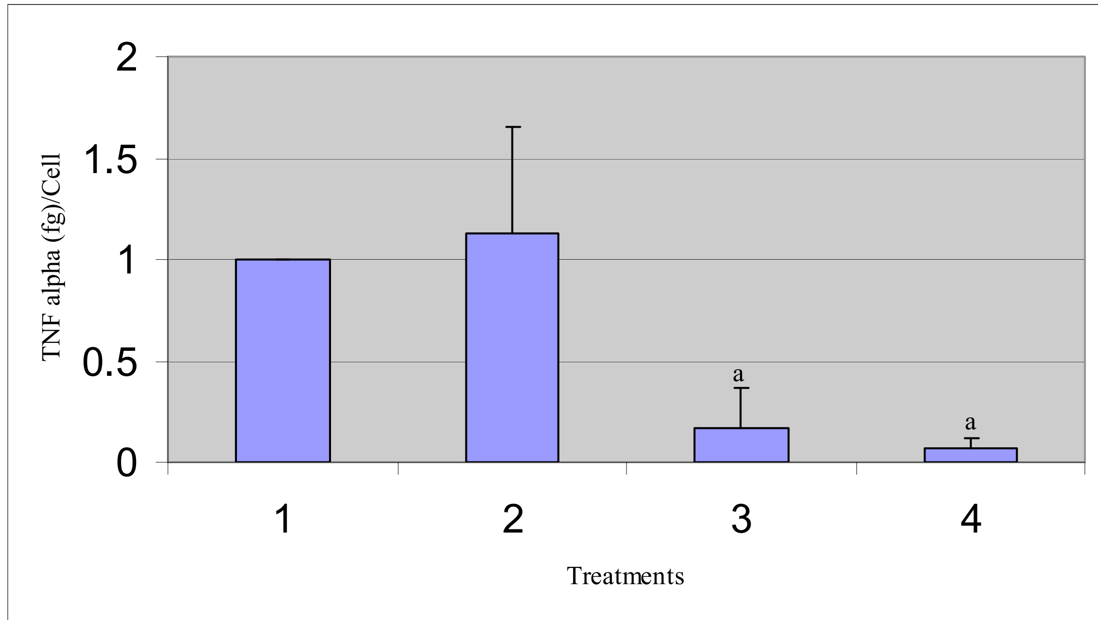

Figure 3.2 Macrophages pretreated with barley bran extracts or curcumin (2h), then stimulated with oat β -glucan (300 ug/ml). All data normalized to oat β -glucan. 1. Oat β -glucan (300 ug/ml). 2. Barley bran (30 ug/ml) + Oat β -glucan (300 ug/ml). 3. Barley bran (60 ug/ml) + Oat β -glucan (300 ug/ml). 4. Curcumin (9 ug/ml) + Oat β -glucan (300ug/ml). Letters denote significantly different

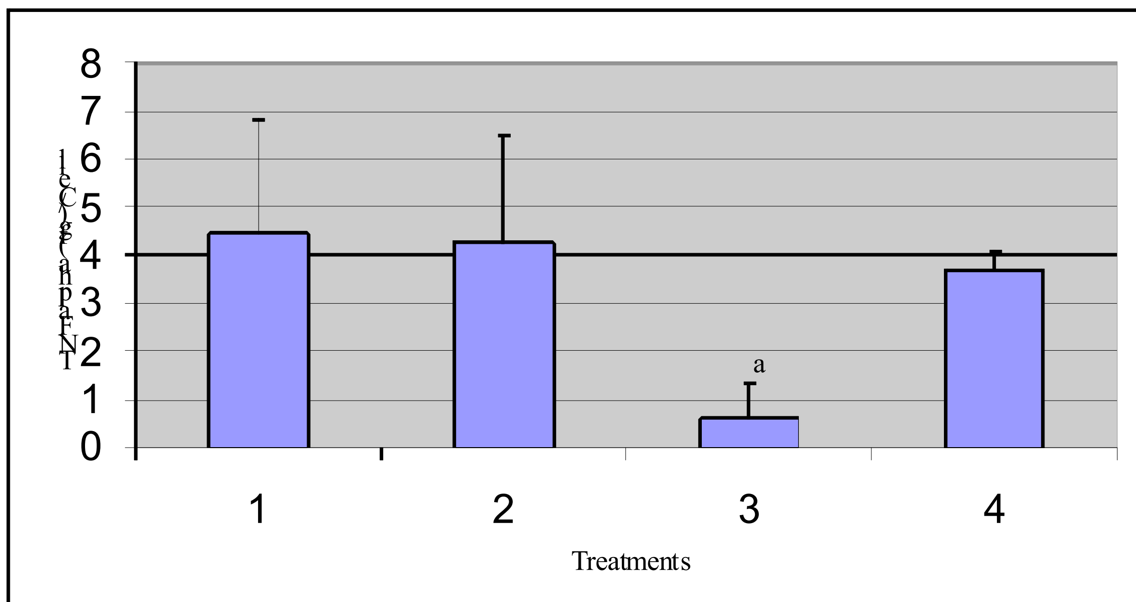

Figure 3.3 Macrophages pretreated with barley bran extracts or curcumin (2h), then stimulated with curdlan (50 ug/ml). All data normalized to oat β -glucan. 1. Curdlan (50 ug/ml). 2. Barley bran (30 ug/ml) + Curdlan (50 ug/ml). 3. Barley bran (60 ug/ml) + Curdlan (50 ug/ml). 4. Curcumin (9 ug/ml) + Curdlan (50 ug/ml). Letters denote significantly different.

Barley bran extracts (BB) in both 5C and 4B alone stimulated macrophages to produce TNFα (Figure 3.1). BB extract (30 ug/ml) in 5C-stimulated macrophages to produce slightly more (12%) TNFα than 60 ug/ml of extract 5C, but the opposite occurred with extract 4B. However, BB extracts in 4B-stimulated macrophages considerably more than extract 5C (Figure 3.1). Therefore, BB extracts with solvent 5C were utilized in inhibitory anti-inflammatory experiments were β-glucans stimulated macrophages.

Wheat bran extracts (WB) in 5C and 4B induced macrophages to produce much less ~42.5% in 5C and 98.6% in 4B, respectively) TNFα than barley bran extracts (Figure 3.1). WB extracts in 4B constituted a satisfactory treatment, demonstrating only slight TNFα production by macrophages of ~4-6 % more than curcumin alone. The 4B solvent system for WB extracts was therefore utilized in inhibitory anti-inflammatory experiments with β-glucan-stimulated macrophages.

BB extracts (60 ug/ml) reduced TNFα production from oat β -glucan-stimulated macrophages 10% less than that of oat β -glucans-stimulated macrophages pretreated with curcumin (Figure 3.2). Total TNFα production from oat β -glucan-stimulated macrophages was inhibited by BB extracts (60 ug/ml) ~83%, but stimulated 13% at 30 ug/ml when compared to oat β-glucan alone. A significant finding in this study was BB extracts (60ug/ml) inhibited TNFα production from curdlan-stimulated macrophages to a greater degree (~70% more) than curcumin treatments (Figure 3.3). Total TNFα production from curdlan-stimulated macrophages inhibited by BB extracts (60 ug/ml) was reduced by ~ 87%, when compared to curdlan-stimulated macrophages alone.

WB extracts (80 ug/ml) were not as effective at reducing TNFα production as BB extracts and curcumin. WB extracts reduced TNFα production by ~26% from oat β -glucan-stimulated macrophages, much less than BB extracts at ~83% or curcumin at 93.5% inhibition (Figure 3.4). However, TNFα production was reduced by 18% (40 ug/ml) and 43% (80 ug/ml) when curdlan-stimulated macrophages were pretreated with WB extracts (Figure 3.5). While the difference was not significant with WB extracts, both cereal bran extracts appear to have the potential to inhibit TNFα production to a greater degree than curdlan-stimulated macrophages pretreated by curcumin (~17%).

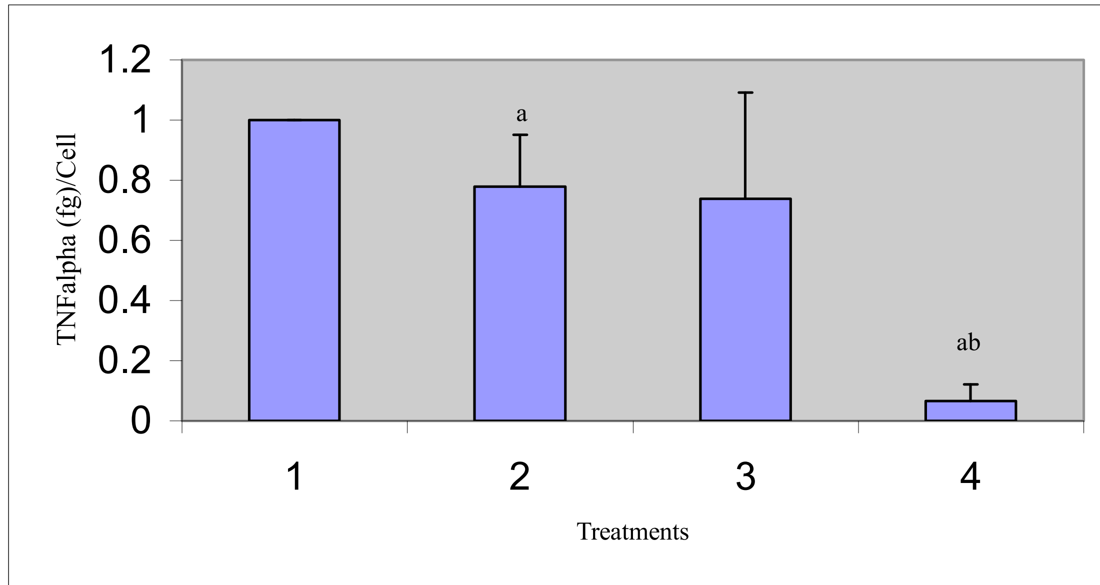

Figure 3.4 Macrophages pretreated with wheat bran extracts 4B or curcumin (2h), then stimulated with oat β -glucan (300 ug/ml). All data normalized to Oat β -glucan. 1. Oat β -glucan (300 ug/ml). 2. Wheat bran (40 ug/ml) + Oat β -glucan (300 ug/ml). 3. Wheat bran (80 ug/ml) + Oat β -glucan (300 ug/ml). 4. Curcumin (9 ug/ml) + Oat β -glucan (300 ug/ml). Letters denote significantly different.

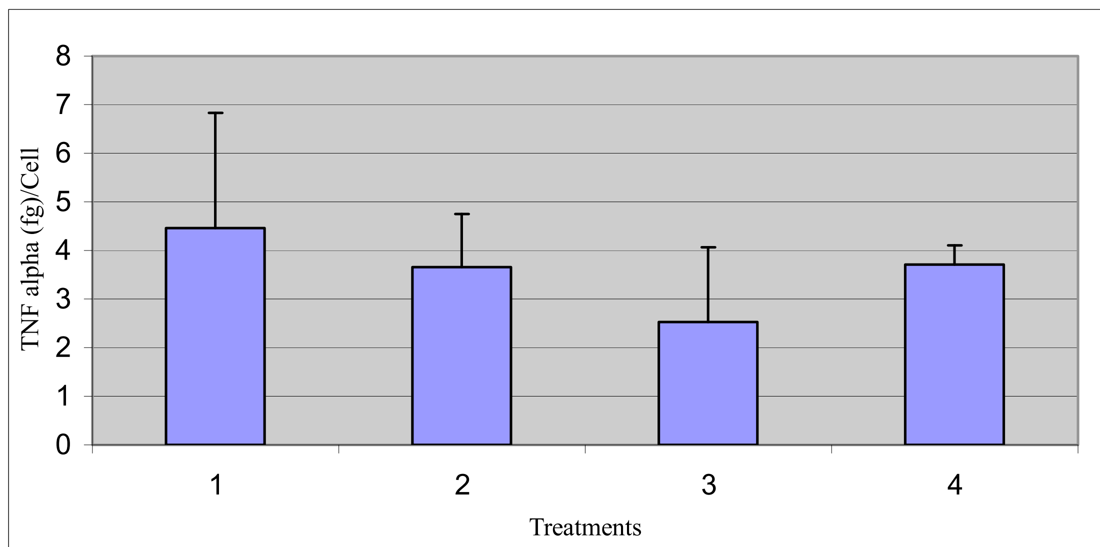

Figure 3.5 Macrophages pretreated with wheat bran extracts 4B or curcumin (2h), then stimulated with curdlan (50 ug/ml). All data normalized to oat β -glucan. 1. Curdlan (50 ug/ml). 2. Wheat bran (40 ug/ml) + Curdlan (50 ug/ml). 3. Wheat bran (80 ug/ml) + Curdlan (50 ug/ml). 4. Curcumin (9 ug/ml) + Curdlan (50 ug/ml).

After completion of the previously described experiments in this study, two un-tested cereal bran extracts (Barley Bran 4B and Wheat Bran 5C) that were not used in anti-inflammatory experiments due to direct stimulation of macrophages when utilized alone, were then evaluated with oat and curdlan β -

glucans (Figure 3.6). When macrophages were pretreated with BB 4B extracts then treated with oat β -glucan, less TNFα production occurred when compared to macrophages treated with BB extract 4B alone (Figure 3.1 and 3.6). But TNFα production was almost doubled (98% more) when BB 4B extracts combined with oat β -glucan was compared to oat β -glucan-stimulated macrophages alone (Figure 3.6). The only significant ($p<0.001$) reduction in TNFα occurred once again using BB extract 5C with oat β -glucan-stimulated macrophages, just as before (~88%; Figure 3.6). The opposite effect occurred with experiments utilizing WB extracts in 5C and 4B with curdlan-stimulated macrophages. When macrophages were pretreated with WB extracts 5C and 4B before introduction of curdlan, both solvent extracts reduced TNFα production relatively equally (~55 and 52%, respectively) below TNFα production from curdlan-stimulated macrophage alone. However, the combination of curdlan and either of the WB (5C or 4B) extracts was still more than macrophages treated with WB extracts alone.

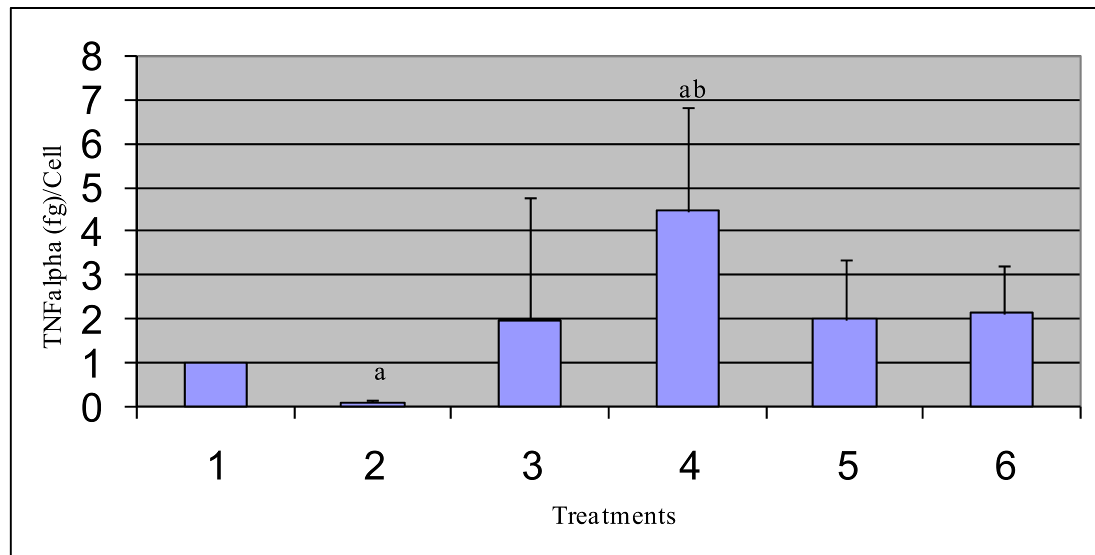

Figure 3.6. Macrophages pretreated with cereal bran extracts (2h), then stimulated with oat β -glucan (300 ug/ml) or curdlan (50 ug/ml). All data normalized to oat β -glucan. 1. Oat β -glucan (300 ug/ml). 2. Barley bran (60 ug/ml) 5C + Oat β -glucan (300 ug/ml). 3. Barley bran (60 ug/ml) 4B + oat β -glucan (300 ug/ml). 4. Curdlan (50 ug/ml). 5. Wheat bran (80 ug/ml) 5C + Curdlan (50 ug/ml). 6. Wheat bran (80 ug/ml) 4B + Curdlan (50 ug/ml). Letters denote significantly different.

Bacterial contamination was monitored 24-48 hours beyond cell harvesting, and was not considered to contribute to TNFα production in these experiments. The only contaminating issues presented in these experiments, came before cell culture treatments from buckwheat and rye extracts in 50% ethanol (pH 5.2 and 7). All other cell treatments were free of microorganisms. See further discussion in previous chapter for monitoring procedures (Chapter 2).

Discussion

The present study examines the role of cereal bran extracts and curcumin to inhibit TNFα production by β -glucan-treated murine macrophages. These experiments first evaluated the effectiveness of two solvent systems for extracting anti-inflammatory compounds from cereal bran's. Second, they demonstrated their subsequent effectiveness in inhibiting TNFα production from glucan-stimulated macrophages in comparison to the inhibitory activities of curcumin (Figure 3.2 – 3.5). As shown in the previous chapter curcumin (an extract of ginger) significantly inhibits TNFα production from β-glucan-stimulated murine macrophages.

Plants contain genetically expressed compilations of functionally active metabolites (372a). Primary metabolites are essential to the life of the plant, and secondary metabolites are utilized for defense, wound healing, and attractants for pollination (373a). Many of these secondary metabolites have been shown to positively affect the physiology of mammalian health (374a). Epidemiological studies show that consumers eating plant-based diets, including whole grain compounds, have lower incidences of chronic diseases (115, 119). Furthermore, studies show an inverse relationship between the consumption of concentrated sources of secondary metabolites, such as flavonoids and phenolic acids, and the risk factors for cardiovascular disease and colon cancer (377a, 377b). The antioxidant capacities of secondary metabolites may provide a mechanism for this protective effect against chronic diseases (378a). However, over the last ten years, evidence has accumulated that indicates that chronic diseases, such as cancer, cardiovascular disease, obesity and diabetes are all linked by inflammation (379a).

Individuals with obesity and type II diabetes have abnormal cytokine production and increased inflammatory signaling pathway stimulation (370b). Cardiovascular disease characterized by increased vascular adhesion proteins, leads to lymphocyte accumulation and cytokine production within the arterioles (381a). Inflammatory cytokines, such as TNFα, IL-1 β, and IL-6 are cellular mediators of inflammatory pathways (382a, 382b). Controlling these cellular mediators of inflammation, specifically TNFα, provides a means of reducing health-risks and symptoms associated with several chronic diseases (370a, 230)

Plant extracts contain a wide variety of flavonoids and phenolic acids with varying molecular weights (393). These bioactive compounds are found in the bark, roots, stems, and flowers of fruits, vegetables, and cereals. Plant extracts containing phenolic acids, proanthocyanins, and catechins from propolis, ginger, pine bark, and honey-suckle show reduced inflammatory mediators in different model systems (393a, 411). But the extracts from cereal bran's have yet to be examined for the ability to inhibit TNFα, the central cytokine involved in inflammatory pathways of the mammalian immune system.

Curcumin is a plant extract from ginger, consisting of several curcuminoid compounds. A large collection of literature has described its anti-inflammatory activities through intracellular inactivation of

cytokines (Figure 3.7), adhesion molecules, and enzymes in the arachidonic acid pathway, which lead to prostaglandin formation and subsequent inflammation (383a).

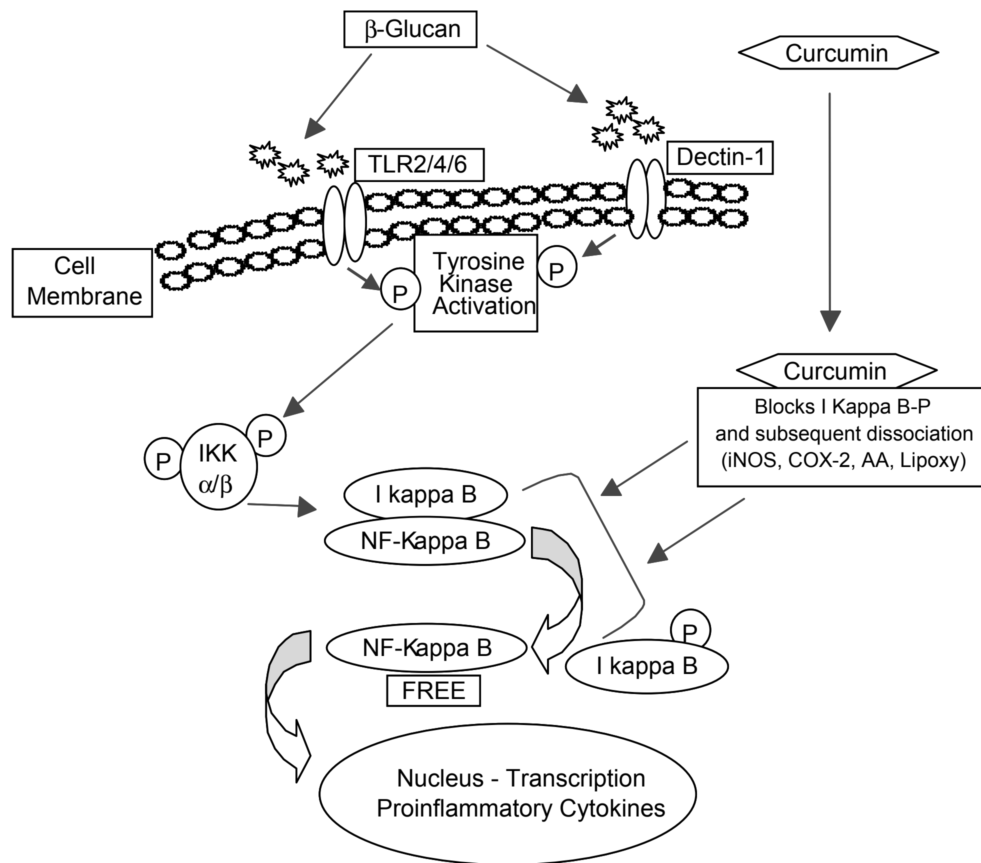

Figure 3.7. Inhibitory effect of curcumin on I-kappa B/NF-KappaB, preventing TNFα production from macrophages.

As reported in Chapter 2, curcumin demonstrated its anti-inflammatory activity by significantly ($p<0.001$) reducing TNFα production by 93.5% from oat β -glucan-stimulated macrophages (Figures 3.2 – 3.5). However, curcumin only reduces TNFα production by 17% from curdlan-stimulated macrophages (Fig 3.3 and 3.5). This difference in inhibition may be explained through the interconnections between glucan receptors and intracellular signaling systems. Curdlan, a (1>3), β -D-glucan, similar in potency to zymosan, stimulates macrophages to produce TNFα to a greater degree than

oat β -glucans. The increased stimulatory capacity of curdlan and zymosan comes from their specific stereochemical structures interacting with glucan receptors to stimulate more than one intracellular signaling pathway of RAW 264.7 macrophages (274). Curdlan β-glucans have been shown to bind other glucan receptors (Dectin-1 and TLR-2), but the intracellular signaling has not been completely investigated (274). Differences in the ability of curcumin to inhibit TNFα production from either oat or curdlan β -glucan also has yet to be completely explained. Due to the central role of NF-kappa B in regulating transcriptional activities of pro-inflammatory mediators, the best mechanism explaining curcumins action on oat β -glucan and curdlan-stimulated macrophages is curcumins ability to block the phosphorylation of IkB complex (Figure 3.1; 384a). Curcumin inhibits the phosphorylation of key proteins, such as STAT3, in the cell signaling pathway, and IkB complex (384a). If phosphorylation of IkB complex occurs, NF-kappa B dissociates and becomes activated. An activated NF-kappa B translocates to the nucleus to initiate gene expression of many pro-inflammatory cytokines, including TNFα (258).

The ideal solvent system utilized in this study, would extract the appropriate inhibitory compounds from cereal bran to significantly inhibit TNFα production, but would not initiate macrophages to produce TNFα. Since plant extracts contain diverse mixtures of compounds, the inhibitory effects may come from one or the synergistic effects of more than one compound (385a). Cereal bran's contain concentrations of bioactive flavanols, anthocyanins, and phenolic acids (385b). Phenolic acid compounds are concentrated and have the greatest diversity in the aleurone layer of cereal bran's (33). Phenolic acids found in barley and wheat, consist of derivatives of benzoic and hydroxycinnamic acids. It's been estimated that 75-85% of the phenolic compounds are bound to carbohydrates in the cell walls of these bran layers (33, 33a**).** Esterase activity from microbes release bound phenolic acids from carbohydrate polymers in the lower gastrointestinal tract (34a). But the utilization of solvent systems that alter pH and temperature, have helped remove bound-phenolic acids from several cereal bran's (33a). Collins and others have found that in oat extracts, acidified solvents were more concentrated in phenolic acids than neutral solvents (35a). In this study, acidified solvent systems (e.g. 4B: acidified 80% ethanol) used to extract wheat bran were required to obtain sufficient concentrations, appropriate stereochemistry, or quality of inhibitory compounds to reduce TNFα production (Figure 3.4 and 3.5). But in the case of barley bran, solvent extract 4B (acidified ethanol) produced an extract of unknown composition that when used alone, stimulated TNFα production from macrophages (Figure 3.1 and 3.6). Solvent system 5C (neutralized ethanol) used on barley bran did not require the reduced pH to extract sufficient inhibitory compounds, but benefited from an increase in the percentage of alcohol above 80% (Figure 3.2, 3.3 and 3.6). Experimental controls utilizing 50, 80, and 95% ethanol, as well as DMSO did not show stimulation to macrophages through quantifiable TNFα production (data not shown).

The greatest inhibition of TNFα production from a cereal bran extract was BB extract 5C (95% ethanol). This was a significant finding, given that BB extract 5C over-came the inability of curcumin to significantly ($p<0.0001$) inhibit curdlan-stimulated macrophages (Figure 3.3). These extracts reduced TNFα production from curdlan-stimulated macrophages to less than 14% (Figure 3.3). WB extracts did not inhibit TNFα production to the same magnitude as BB extracts, but did provide greater inhibition than curcumin with curdlan-stimulated macrophages (Figure 3.5). In this specific case, the inhibition was approximately half of the inhibition of BB extracts. However, curcumin's inhibition over TNFα production from oat β -glucan-stimulated macrophages was significantly ($p<0.01$) more when compared to WB extract effects. Given that BB extracts prevailed over the more potent curdlan-stimulated macrophage to significantly inhibit production of TNFα, surprisingly these extracts did not inhibit TNFα production more than curcumin when macrophages were stimulated by oat β -glucan (Figure 3.3).

Previous studies examining the anti-inflammatory mechanisms of phenolic acid derivatives, such as curcumins effective inhibition of inflammatory mediators, most likely explains the results of these cereal bran extracts (384a, 465).

Phenolic acids have demonstrated the capacity to inhibit the dissociation of intracellular Ikappa B/NF-kappa B complex, stimulate the gene transcription of inhibitory compounds, and inhibit the post-transcriptional activation of MAP kinase activating protein within macrophages. Plant extracts containing phenolic acid derivatives have also demonstrated anti-inflammatory activity through the inhibition of pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and lipoxygenase (411). These enzymes are involved with acting on polyunsaturated fatty acids, such as arachidonic acid, leading to the formation of pro-inflammatory prostaglandins and leukotrienes (34b) The gene transcription of these pro-inflammatory enzymes modulate the phosphorylation of I-kappa B, dissociating it from NF-kappa B (35a). However, not all signaling pathways have been clearly investigated therefore it would be incorrect to suggest that all proinflammatory enzymes and proteins are exclusively controlled by NF-kappa B. Recently, pine bark extracts (Pycnogenol) reduced prostaglandin PGE2 production, through inhibition of COX 1 and 2 (411, 36a). Prostaglandin E2 synthesis can lead to the production of inflammatory mediators TNFα and IL-1 β (37a). The active ingredient in these pine-bark extracts was ferulic acid, pineresinol, and matairesinol. Ferulic acid is often the most abundant phenolic acid in cereal bran's (385b). Barley and wheat bran's contain many small benzoic and hydroxycinnamic acid derivatives that are structurally similar to ferulic acid and curcumin (385b). The anti-inflammatory activity demonstrated by these barley and wheat extracts may be explained by one or more phenolic acid derivatives blocking the dissociation of I-Kappa B/NF-kappa B complex.

The inhibition of TNFα by barley and wheat bran extracts could have also occurred by flavonoids stimulating the transcription of I-kappa B. Glucocorticoids are powerful immunosuppressive drugs that have been shown to specifically interfere with NF-kappa B (37b). Dexamethasone mimics glucocorticoid action by stimulating the gene transcription of I-kappa B, therefore creating more I-kappa

B to bind any released NF-kappa B and preventing transcription of proinflammatory mediators. Ternatin, an anti-inflammatory flavonoid, mimics the effects of dexamethasone when *in vitro* cells are treated with ternatin and lipopolysaccharide (38a). Flavonoids present in these bran extracts may be stimulating the production of I-kappa B, thereby reducing the production of TNFα.

One regulatory mechanism that the mammalian immune system, (i.e. macrophages) utilizes to control inflammatory cytokine production is through secreting anti-inflammatory cytokines IL-10 and TGF-α, known inhibitors of TNFα (38b). While literature explaining the stimulation of these anti-inflammatory cytokines by small phenolics does not appear to exist, it is possible that other chemical compounds in the bran extracts may act as modifiers, since phenolic glycolipids have been shown to significantly stimulate production of inhibitory TGF β in monocytes (39a).

One of the most recent discoveries of the inhibitory activities of purified small phenolic acids showed that caffeic acid, also present in cereal bran's, inhibited p38 mitogen-activated protein kinase (MAPK; 374). This specific serine/threonine protein kinase reacts to external stimuli, usually from series or inflammatory signals, leading to gene transcription, differentiation, and apoptosis (39b). The inhibition of p38 MAP kinase activity has been known to significantly reduce TNFa production. The inhibition of AP-1, a transcriptional factor controlled by a MAP kinase with caffeic and ferulic acid has been shown to reduce TNFa post-transcriptionally (40a). This type of inhibition occurs after receptor binding, NF-kappa B translocation, and gene transcription. Therefore, phenolic acids present in barley and wheat bran may inhibit TNFa production through blocking intracellular phosphorylation before gene transcription or after gene transcription.

This study demonstrated that the inhibition of TNFα production from murine macrophages stimulated by oat and curdlan β-glucan, can be achieved through the extraction of inhibitory compounds from barley and wheat bran's. While it is uncertain which compound or mixture of compounds was responsible for the inhibition of TNFα, these extracts demonstrated significantly more inhibition than the potent anti-inflammatory curcumin, a compound currently used in several clinical trials. Future studies should focus on the identification of the compounds responsible for these strong effects, synergies exerted by combinations of multiple compounds found in cereal bran's, and the metabolic modification by phase I and II systems in mammalian organisms which may change the functional capacity to inhibit inflammation. Interactions of specific phenolic acid compounds with macrophages are further explored in Chapter 4.

Chapter 4

Cereal Bran Phenolic Acids Inhibit TNFα Production
from Murine Macrophages

Introduction

Over the last 3 years, studies have implicated pro-inflammatory mediators, which play a central role in tissue inflammation, in the etiology of several chronic diseases (120a). Cardiovascular disease, diabetes, obesity, irritable bowel, and cancer are all linked to inflammatory mediators. The biological and functional characteristics of each disease, such as increased C-reactive protein, reduced GLUT 4 receptor signaling, reduced insulin receptor signaling, and reduced mucosal proliferation can all be regulated through inflammatory mechanisms, such as adhesion molecules, leukotrienes, cytokines, and complement proteins (121a, 122a, 114). Collectively, these inflammatory responses have a substantial impact on human health, particularly in Western cultures.

Current approaches to preventing inflammation involve interference with the mechanistic actions of inflammatory mediators or by preventing their production. Some non-steroidal anti-inflammatory drugs (NSAIDS), such as aspirin (acetylsalicylic acid), have been used for over 100 years to interfere with the functional aspects of inflammatory mediators (414). NSIADS and salicylates interfere by competitively blocking the arachidonic acid receptor of cyclooxygenase (COX) 1 and 2 (412). Arachidonic acid receptor binding can lead to the production of prostaglandin E_2 and eventually to pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukins (IL). TNFα, an acute phase protein, stimulates cascades of other inflammatory cytokines and prolonged elevated levels are associated with increased mortality (123a). Unfortunately, not all NSAIDS reduce pro-inflammatory cytokines. Lin et al (2004) demonstrated that two common anti-inflammatory drugs, indomethacin and ibuprofen, do not inhibit TNFα production (124a).

Salicylates and some phenolic acids have been shown to exert anti-inflammatory characteristics by preventing the production of pro-inflammatory mediators (409, 419, 393a). Yin *et al*. (418) was the first to publish salicylic acid derivatives could also regulate inflammation by directly inhibiting cytokines, upstream of transcription by a signaling blockade. This mechanism involves blocking the IκB/NF-kappa B complex. If IκB is not phosphorylated, NF-kappa B cannot dissociate and translocate to the nucleus to stimulate gene transcription of cytokines.

Salicylates are biologically synthesized from, and are structurally similar to, hydroxycinnamic and benzoic acids found in cereal brans (125a). In the previous chapters it was shown that TNFα secreted from glucan-stimulated macrophages could be inhibited significantly by cereal bran extracts and curcumin. Curcumin, a diferuloyl phenolic acid has demonstrated robust anti-inflammatory activity (125b). Given these findings, and that cereal bran extracts contain significant quantities of phenolic acids, it is hypothesized that hydroxycinnamic and benzoic acids found in cereal bran may reduce TNFα from β-glucan-stimulated murine macrophages.

This study examined the inhibition of TNFα secretion from β-glucan-stimulated murine macrophages by several different phenolic acids, particularly those commonly found in cereal brans. The first objective was to evaluate sensitivity of macrophages to different phenolic acids over temporal changes and concentration gradients. The second objective was to compare the inhibitory capacity of phenolic acids acting on macrophages stimulated by oat and curdlan β-glucans.

Materials and Methods

Polysaccharide preparation:

See chapter 3.

Phenolic acid preparation:

Benzoic and cinnamic acid derivatives (0.5g; Table 1) were dissolved in 95% ethanol, precipitated (re-crystalized) with the addition of sterile dH_2O, centrifuged (1100x *g*), and dried under sterile conditions. Dried pellets were weighed and re-dissolved in 95% ethanol (2 mg/ml) and stored at 4 ºC. These phenolic acid preparations were utilized within 4 days for optimal cytokine inhibition.

Cell preparation and treatment:

Murine macrophage/monocyte cell line (RAW 264.7) was obtained from the American Type Culture Collection (ATCC # TIB-71). Cells were cultured and maintained in sterile DMEM + 10% (heat inactivated) fetal bovine serum (FBS) at 37°C with 5% humidified CO_2. An additional heating step for 30 min. at 65 ºC was added for FBS to reduce stimulation of macrophages from medium alone. Isolated cells were plated (0.5×10^6) in single tissue culture dishes (35 X 10 mm, Starstedt, Inc., Newton, N.C.) in maintenance medium overnight before treatment. Control treatments of adherent cells contained one or more of the following: new medium, phenolic acids, polysaccharides, or treatment solvents. In the inhibitory experiments, adherent macrophages were pretreated with phenolic extracts for 0 min, 30 min, 1 h, or 2 h, prior to the addition of oat or curdlan β-glucans in the same tissue culture dishes then incubated overnight (15h).

Quantifying TNFα:

Conditioned media from all experimental culture dishes were harvested on ice and centrifuged at 1100 x g, supernatants were stored in new sterile (1.5 ml) tubes at –25 °C until analysis (< 7 days). Viable cell numbers in all culture dishes were determined by trypan blue after each experiment. TNFα was quantified by ELISA (BD OptEIA, #555268, BD Biosciences, San Diego, CA.). Statistical analysis for each experiment was determined by ANOVA with statistical analysis between individual treatments determined by Tukey-Kramer post-test (Microsoft Excel™).

Results

To investigate the anti-inflammatory characteristics of nine phenolic acids (Table 4.1, 4.2) applied to murine macrophages, the effects of single compounds, combinations of multiple compounds, and various pretreatment times were examined. Preliminary experiments with macrophages treated with phenolic acids only, demonstrated that four benzoic acid derivatives (p-anisic, syringic, vanillic, and p-hydroxybenzoic acid) stimulated macrophages to produce TNFα to a high degree (data not shown). Therefore, these four phenolics were not utilized further in anti-inflammatory experiments with β-glucan treated macrophages. No significant ($p<0.01$) TNFα production occurred when macrophages were treated with the other five phenolic acids without β-glucan (Figure 4.1). No significant TNFα

production occurred when control solvents (95% ethanol, DMSO) were applied alone to murine macrophages and compared to untreated macrophages ($p<0.05$).

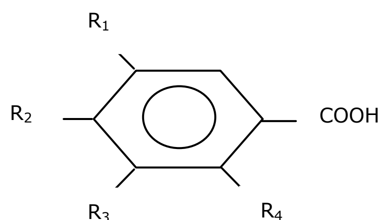

Table 4.1 Benzoic acid derivatives.

Compound	R1	R2	R3	R4
p-Hydroxybenzoic	H	OH	H	H
Protocatechuic	H	OH	OH	H
Salicylic	H	H	H	OH
AcetylSalicylic	H	OH	H	OCOH
Vanillic acid	H	OH	OCH_3	H
Syringic acid	OCH_3	OH	OCH_3	H
p-Anisic acid	H	OCH_3	H	H

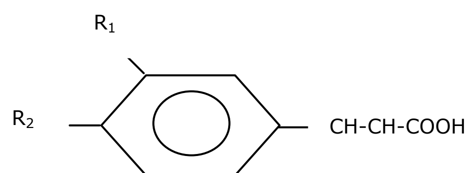

Table 4.2 Cinnamic acid derivatives.

Compound	R1	R2
Caffeic Acid	OH	OH
Ferulic Acid	OCH3	OH
Cinnamic	H	H
p-Coumaric	H	OH

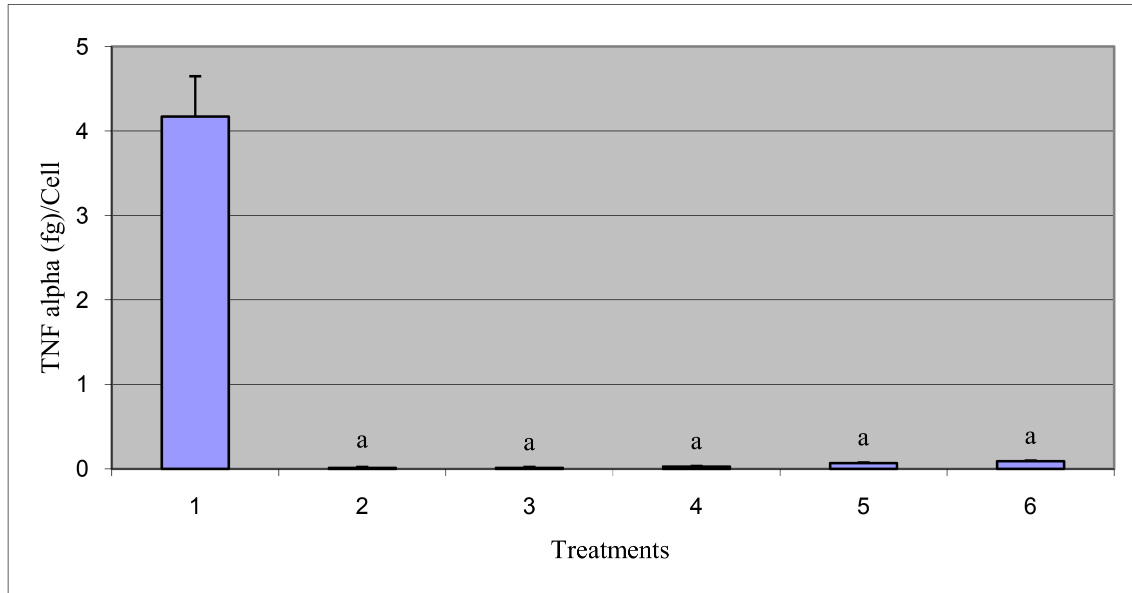

Figure 4.1 TNFα secreted by murine macrophages, after treatment with either curdlan or individual phenolic acids: 1. Curdlan (50 µg/ml). 2. Caffeic acid (25 µg/ml). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml). 6. Protocatechuic acid (25 µg/ml). (n=4) Letters denote significant difference ($p<0.05$)

All phenolic acids inhibited TNFα production from treated macrophages stimulated by oat β-glucan (Figure 4.2, 4.3, 4.4, and 4.5). Phenolic acid inhibition of TNFα production from these treated macrophages varied between 45 – 80%. The greatest inhibition of TNFα production (~80%) from oat β-glucan-stimulated macrophages, were cells treated with cinnamic acid (Figure 4.3). Protocatechuic and caffeic acid treatments also inhibited TNFα production to a large degree, ~77 and 75%, respectively. Depending on pretreatment times, the maximum inhibition of TNFα with ferulic and p-coumaric acids was ~63% for both and the minimum ~45% and ~47% respectively. p-Coumaric and protocatechuic acid stimulated cell numbers the greatest over all time points, when compared to cell numbers from other treatments and to the control (data not shown).

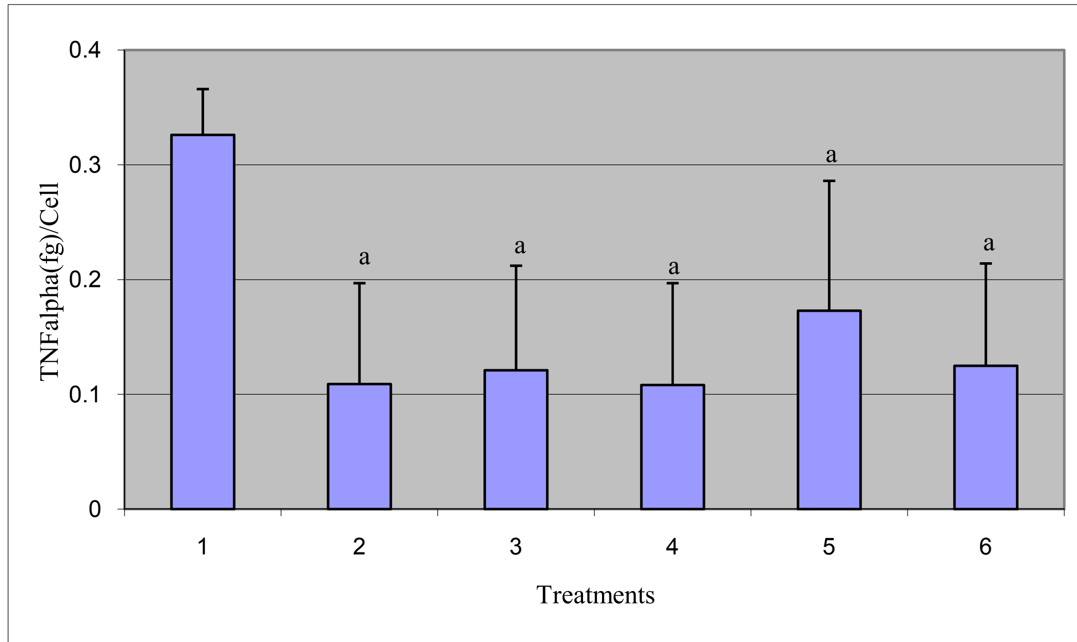

Figure 4.2 TNFα secreted by oat β-glucan-stimulated murine macrophages treated with phenolic acids without pretreatment. 1. Oat β-glucan (300 µg/ml). 2. Caffeic acid (25 µg/ml). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml).6. Protocatechuic acid (25 µg/ml). (n=4) Letters denote significant difference ($p<0.05$)

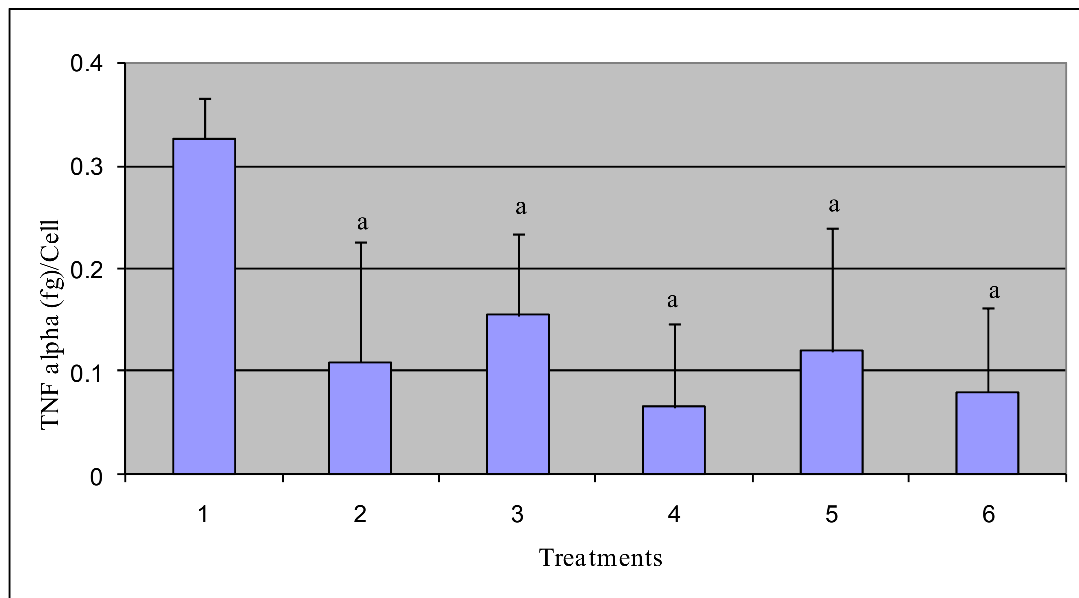

Figure 4.3. TNFα secreted by murine macrophages pretreated with phenolic acids 30 min., before addition of oat β-glucan (300 ug/ml) to all treatments (2-6). 1.Oat β-glucan (300 ug/ml). 2. Caffeic acid (25 ug/ml). 3. Ferulic acid (25 ug/ml). 4. Cinnamic acid (25 ug/ml). 5. p-Coumaric acid (25 ug/ml). 6. Protocatechuic acid (25 ug/ml). (n=4) Letters denote significant difference ($p<0.05$)

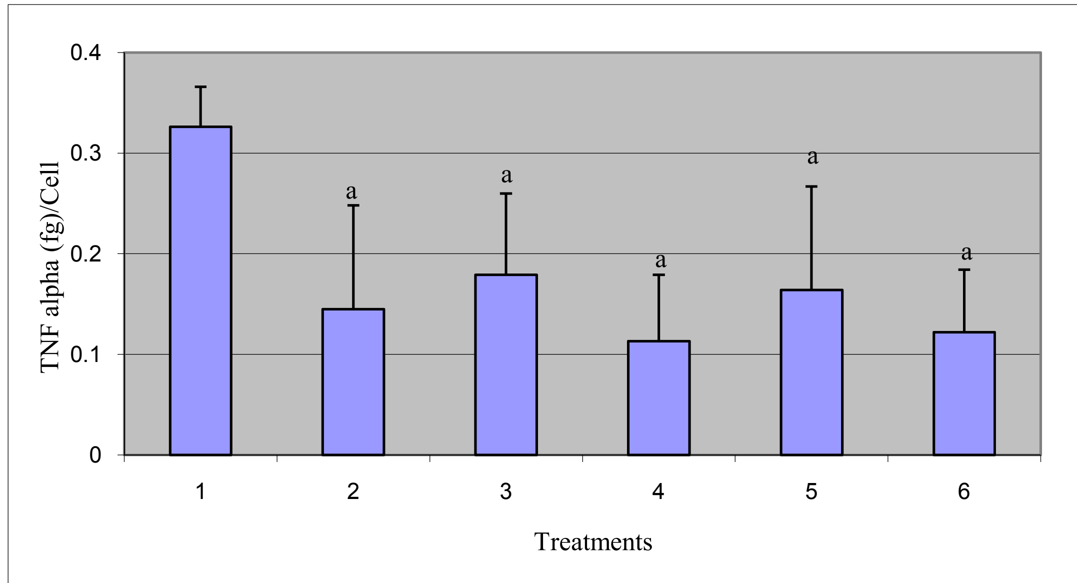

Figure 4.4 TNFα secreted by murine macrophages pretreated with phenolic acids 1 h, before addition of oat β-glucan (300 ug/ml) to all treatments (2-6). 1. Oat β-glucan (300 ug/ml) alone. 2. Caffeic acid (25 ug/ml). 3. Ferulic acid (25 ug/ml). 4. Cinnamic acid (25 ug/ml). 5. p-Coumaric acid (25 ug/ml). 6. Protocatechuic acid (25 ug/ml). (n=4) Letters denote significant difference ($p<0.05$)

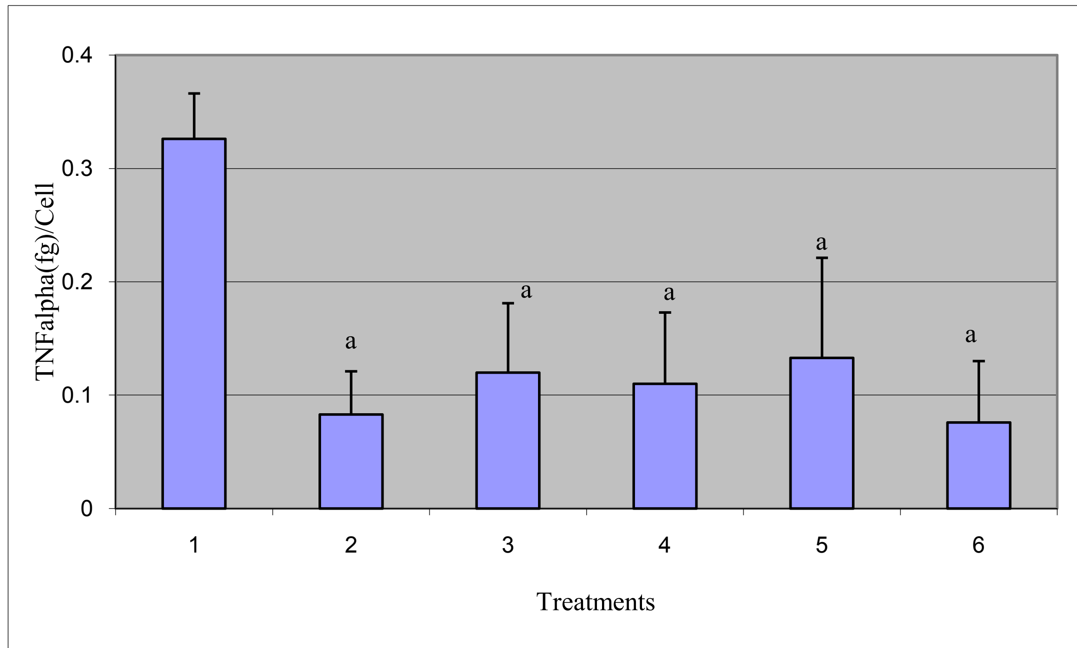

Figure 4.5 TNFα secreted by murine macrophages pretreated with phenolic acids 2 h, before addition of oat β-glucan (300 µg/ml) to all treatments (2-6). 1. Oat β-glucan (300 µg/ml) alone. 2. Caffeic acid (25 µg/ml). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml). 6. Protocatechuic acid (25 µg/ml). (n=4) Letters denote significant difference ($p<0.05$)

The greatest inhibition of TNFα production from oat β-glucan stimulated macrophages did not always come from the longest pretreatment (2 h) times (Figure 4.2, 4.3, 4.4, 4.5). Macrophages treated with phenolic acids without pretreatment time, inhibited TNFα production by more than 60%, except in the case of p-coumaric acid. Macrophages pretreated with caffeic, ferulic, and protocatechuic acid had maximum inhibition of TNFα production at 2 h. However, maximum inhibition of TNFα production with cinnamic and p-coumaric acid was 30 min pretreatment. The inhibition of secreted TNFα, between pretreatment times for each phenolic acid treatment, varied by 15 – 19 %.

The combined effects of two phenolic acids each at one half the concentration (w/v) of a single phenolic treatment, was examined with caffeic and ferulic acid at one time point (Figure 4.6). The pretreatment of oat β-glucan-stimulated macrophages for 1h resulted in ~88% inhibition of TNFα production.

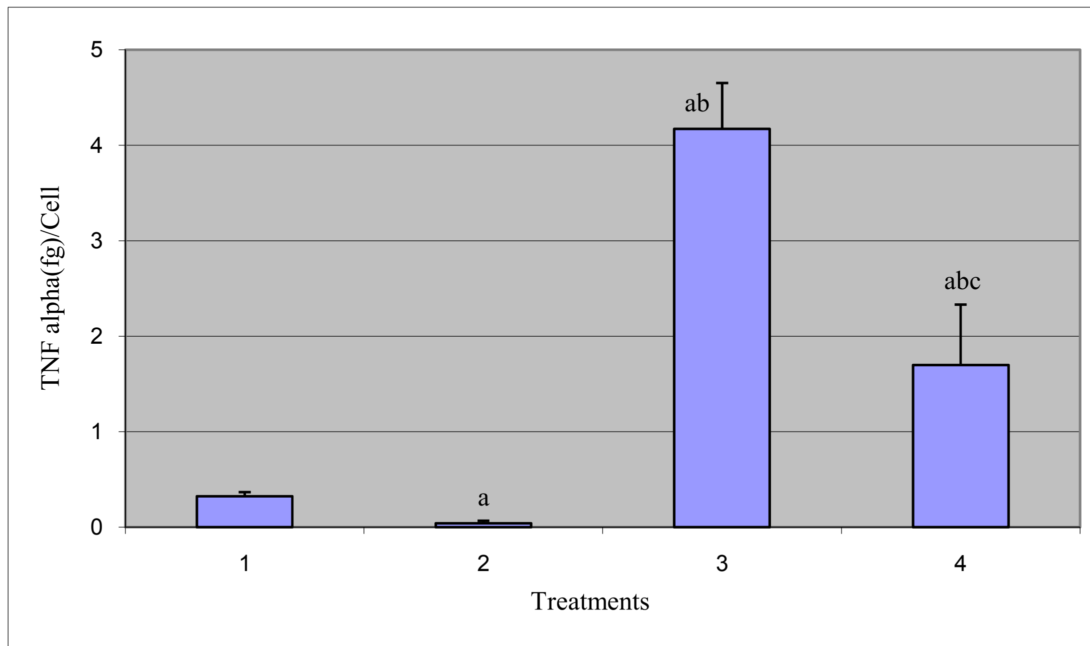

Figure 4.6 TNFα secreted by murine macrophages pretreated with a combination of caffeic/ferulic acids 1 h, before addition of oat β-glucan or curdlan. 1. Oat β-glucan (300 μg/ml) alone. 2. Caffeic/ferulic acid (12.5 μg/ml each) + Oat β-glucan (300 μg/ml). 3. Curdlan (50 μg/ml) alone. 4. Caffeic/ferulic acid (12.5 μg/ml) + Curdlan (50 μg/ml). (n=4) Letters denote significant difference ($p<0.05$)

All phenolic acids reduced TNFα production from macrophages stimulated by curdlan, although not as much compared to oat β-glucan stimulated macrophages (Figures 4.2 – 4.5 and 4.7 – 4.10). Phenolic acid inhibition of TNFα production from curdlan-stimulated cells ranged from ~22 - 47 %. As in the case of macrophages stimulated by oat β-glucan, the greatest inhibition of TNFα production from cells stimulated by curdlan was by cinnamic acid and protocatechuic acid, 47% and 40% respectively.

The 1 h and 2 h pretreatment times for, ferulic acid and p-coumaric acid respectively, demonstrated the least amount of TNFα inhibition (~22%), although ferulic acid response was still significant.

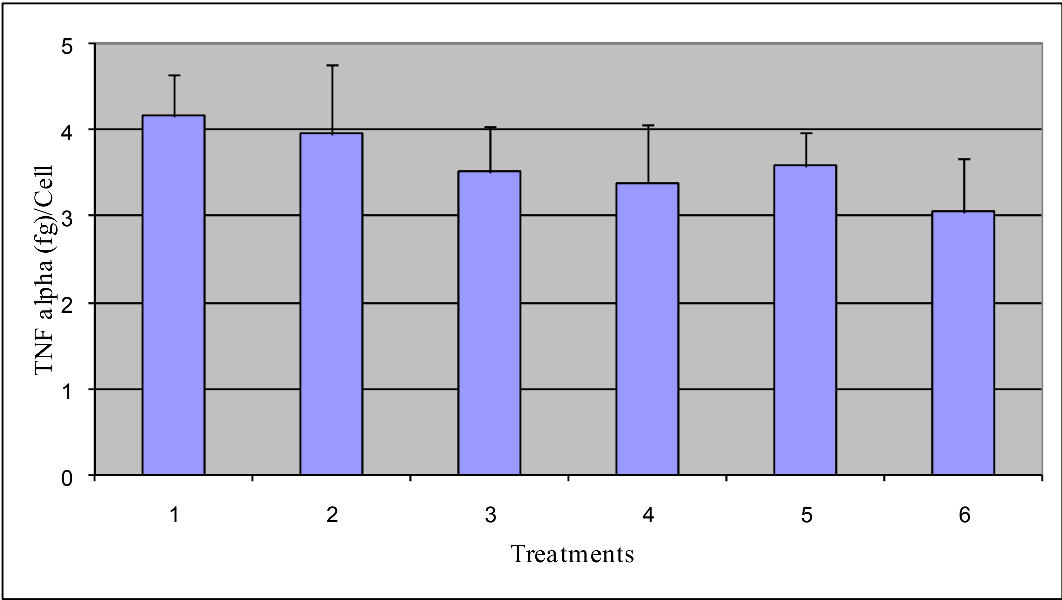

Figure 4.7 TNFα secreted by curdlan-stimulated murine macrophages treated with phenolic acids, with out pretreatment time. 1. Curdlan (50 µg/ml) alone. 2. Caffeic acid (25 µg/ml each). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml). 6. Protocatechuic acid (25 µg/ml).(n=4) Letters denote significant difference ($p<0.05$)

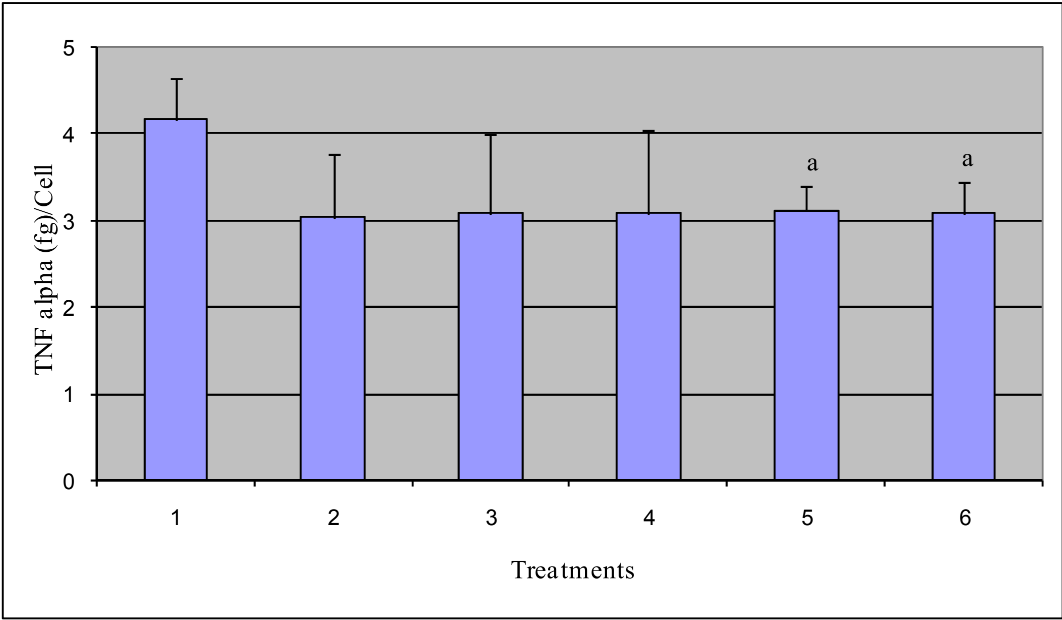

Figure 4.8 TNFα secreted by curdlan stimulated murine macrophages pretreated with phenolic acids 30 min., before addition of curdlan to all treatments (2-6). 1. Curdlan (50 µg/ml) alone. 2. Caffeic acid (25 µg/ml). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml). 6. Protocatechuic acid (25 µg/ml). (n=4) Letters denote significant difference ($p<0.05$)

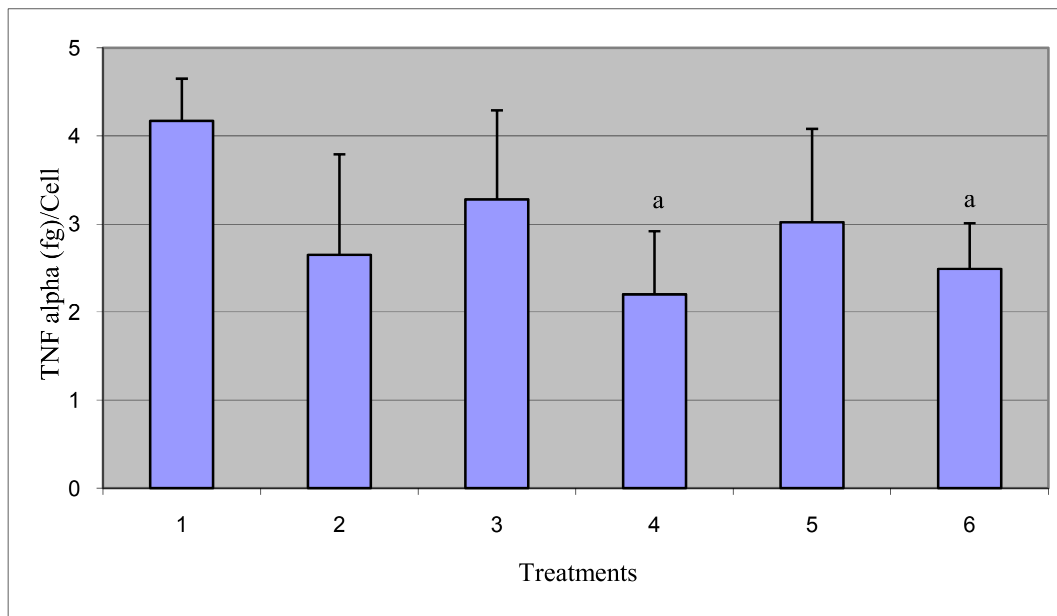

Figure 4.9 TNFα secreted by curdlan stimulated murine macrophages pretreated with phenolic acids 1 h, before addition of curdlan to all treatments (2-6). 1. Curdlan (50 µg/ml) alone. 2. Caffeic acid (25 µg/ml). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml). 6. Protocatechuic acid (25 µg/ml). (n=4) Letters denote significant difference ($p<0.05$)

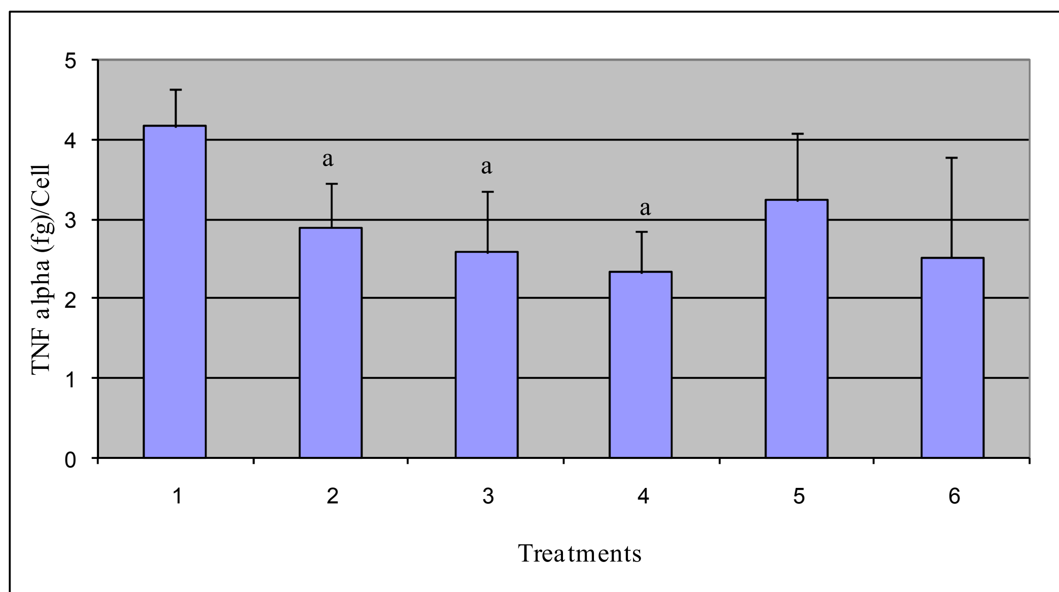

Figure 4.10 TNFα secreted by curdlan stimulated murine macrophages pretreated with phenolic acids 2 h, before addition of curdlan to all treatments (2-6). 1. Curdlan (50 µg/ml) alone. 2. Caffeic acid (25 µg/ml). 3. Ferulic acid (25 µg/ml). 4. Cinnamic acid (25 µg/ml). 5. p-Coumaric acid (25 µg/ml). 6. Protocatechuic acid (25 µg/ml). (n=4) Letters denote significant difference ($p<0.05$)

Curdlan-stimulated macrophages treated with phenolic acids without a pretreatment period demonstrated the lowest TNFα inhibition (Figure 4.7). Contrary to the results obtained with oat β-glucan, curdlan-stimulated cells pretreated for 30 min did not show maximum inhibition of TNFα (Figure 6). Maximum inhibition of curdlan-stimulated cells occurred at 1h pretreatment for caffeic, p-coumaric, and cinnamic acids. Macrophages pretreated for 2h with ferulic acid had the greatest TNFα inhibition (Figure 4.8). Interestingly, protocatechuic acid inhibited TNFα production to a similar degree at 1 and 2 h of pretreatment (Figure 4.7 and 4.8).

The combined effects of caffeic and ferulic acid, each at one half the concentration (w/v) of the single phenolic treatments, demonstrated the most inhibition of TNFα production (59%) from curdlan-stimulated macrophages (Figure 4.6).

Discussion

This study examined the role of benzoic and hydroxycinnamic acid derivatives in opposing β-glucan stimulated murine macrophages from secreting TNFα. These experiments defined the inhibition of TNFα secretion from 5 of the most active phenolic acids (Tables 4.1 and 4.2; Figures 4.1-4.10). This work directly follows the previous chapter on cereal bran extracts inhibiting TNFα from macrophages. More specifically, the previous chapter demonstrated that barley and wheat bran extracts, containing multiple derivatives of benzoic and hydroxycinnamic acids significantly reduce TNFα from β-glucan-stimulated murine macrophages.

While barley and wheat bran contain many flavanols, anthocyanin, and phenolic acids, benzoic and hydroxycinnamic acid derivatives are structurally similar to salicylic acid derivatives (Table 4.1 and 4.2). Benzoic and hydroxycinnamic acid derivatives are also precursors to salicylic acid in biological pathways, making them particularly interesting for further examination in this study (404). A large collection of scientific literature has demonstrated salicylic acid inhibition of pro-inflammatory mediators and free radicals, such as cyclooxygenase enzymes (COX 1 and 2), TNF-alpha, NF-kappa B, intercellular adhesion molecule –1 (ICAM-1), iNOS, and Erk kinase activity (126a, 409). Collectively, these biological mediators induce symptoms and enhance the progression of several chronic diseases.

Previous studies have examined the role of salicylic acid derivatives in inhibiting inflammation, and have been focused on reducing prostaglandin production through COX 1 and 2 enzymes (411). However, more recent studies show that salicylates reduce TNFα through a NF-kappa B blockade, independent of COX 1 and 2-enzyme inhibition (418, 419). Specific phenolic acids found in food, such as honey (chrysin) and olives (oleocanthal), also have exhibited anti-inflammatory mechanisms by

inhibiting NF-kappa B and decreasing IL-1β, and IL-6 (393a). Caffeic and ferulic acids have been utilized in a similar manner to reduce NF-kappa B activity within U937 cells, but were much weaker in murine epidermis models as topical agents (127a, 127b). However, the multiple phenolic acids that cross-link and structurally support cell walls from cereal brans have not been extensively examined for their potential anti-inflammatory characteristics by reducing TNFβ production. The present study supports accumulating evidence in the literature that demonstrate the ability of bioactive components from foods to modulate specific inflammatory mediators, such as TNF-alpha, IL-1β, IL-6, and IL-8 (128a).

Stability of phenolic and polyphenolic acid compounds under *in vitro* conditions of changing pH and temperature, have proven to be problematic in some studies (129a, b). Velljo et al (2004) demonstrated that hydroxycinnamic acid derivatives are relatively stable in acidic conditions at 37 °C with losses of only 6-25%. However, when exposed to gastrointestinal digestive enzymes with reduced pH, greater losses (>75%) occurred. Stability and effectiveness of the benzoic and hydroxycinnamic acid derivatives in this study, at a pH of 7.2-7.4, demonstrated that up to 2 h pretreatment and 15 h treatment periods, significant inhibition of TNFα production occurred (Figure 4.5 and 4.10). The same phenolic acids prepared on Day 1 were often utilized in consecutive experiments on Day 2 with reproducible inhibition of TNFα.

In these experiments, β-glucan-stimulated macrophages were sensitive to all 5 phenolic acids. Preliminary experiments with multiple benzoic and hydroxycinnamic acid derivatives (total of 8) demonstrated reduced viable cell numbers with phenolic acid concentrations ~ 40 μg/ml. This indicated cellular necrosis or apoptosis *in vitro*. Therefore, the experimental phenolic acid concentrations utilized in this study were set at a concentration ~25 μg/ml. The concentrations utilized in these experiments are below therapeutic serum levels used to treat inflammatory disorders; therapeutic levels of salicylates in human serum utilized to decrease inflammation in rheumatic disorders are 150 – 300 μg/ml (414).

The greatest inhibition of TNFα production from a single phenolic acid treatment was from oat β-glucan-stimulated macrophages pretreated for 30 min with cinnamic, caffeic, and protocatechuic acid (Figure 4.3). These 3 phenolic acids may exhibit sufficient stereo-chemical similarities to salicylates to obtain this magnitude of inhibition through similar inhibitory mechanisms (Table 4.1 and 4.2).

Significant ($p<0.05$) inhibition of TNFα production occurred with relatively short phenolic acid pretreatments (Figure 4.2 and 4.3). The simultaneous addition of phenolic acids and β-glucan stimulators to macrophages in this study generated a significant degree of TNFα inhibition (Figure 4.2). This effect may initially be due to physical interaction with β-glucans or blockade of glucan cell surface receptors by phenolic acids, preventing β-glucan/receptor interaction. Recently, salicylic acid has been shown to significantly inhibit TNFα secretion from LPS-stimulated macrophages within 15 min (409). Katerinaki et al (2006) noted that TNFα inhibition in 15 min was associated with a direct decrease in NF-kappa B

activity. Maximum inhibition with 30 min pretreatment, suggests inhibitory intracellular signaling to IKKβ/NF-kappa B complex achieving inhibition of TNFα transcription.

In the second set of experiments, curdlan-stimulated macrophages were sensitive to all phenolic acids and responded by down-regulating TNFα production by 47% compared to curdlan alone (Figures 4.7-4.10). While the magnitude of this inhibition did not compare to inhibition of oat β-glucan-stimulated cells in this study, different magnitudes in TNFα production by macrophages stimulated by oat or curdlan glucans are also present (see previous chapters). In comparison to curcumin, a potent anti-inflammatory compound which reduced TNFα production from curdlan-stimulated macrophages by ~10% in the previous chapter, this magnitude of inhibition was considerably greater. Differences in TNFα inhibition may be explained by higher affinity binding of glucan receptors for curdlan compared to oat glucans or to completely different receptors (see previous chapter discussion).

While caffeic and ferulic acid treatments alone were not the most effective inhibitors of TNFα production in this study, the combination of the two (12.5 ug/ml each) on both oat- and curdlan-stimulated macrophages presented the greatest inhibition in this study (Figure 4.6). This combined effect provided an additional reduction in TNFα (>22%) from oat β-glucan stimulated cells, and (>12%) from curdlan stimulated cells, when compared to single phenolic acid treatments (Figures 4.4 and 4.6). Although these experimental conditions are not precisely as reported for other published systems, 88% inhibition of TNFα production from oat-stimulated macrophages and 59% inhibition from curdlan-stimulated macrophages provides equal and arguably better cytokine inhibition than other studies utilizing phenolic acids from other plant sources (393a, 419, 128a, 368).

The mechanisms controlling the reduction of TNFα production from these glucan-stimulated macrophages have not been completely investigated. However, zymosan, a highly stimulating branched β-glucan polymer, and lipopolysaccharide (LPS), a very immunogenic glycolipid that makes up the cell wall of gram negative bacteria, appear to be the main structural antigenic compounds for inducing macrophages or monocytes to produce inflammatory mediators (130a). Although some receptors are shared by several β-glucans, glucan receptors have been shown to require unique binding with incomplete kinase signaling (340, 131a). Phenolic acids are quite diverse in the plant kingdom and are found in most human's diets, since they are present in fruits, vegetables, and cereals (132a). Four of the five different phenolic acid compounds investigated in this study have been described as inhibiting inflammatory mediators, but not all of these studies included TNFα in the design (128a, 133a). Salicylic acid derivatives are also phenolics, and given the relatively similar structural chemistry and unique biological relationship that salicylates share with hydroxycinnamic and benzoic acid derivatives, some relationships may be drawn from salicylate studies as well from curcumin research (125a). Therefore, the inhibition of TNFα production in this study may be explained through mechanisms that include inhibition of phosholipases, COX 1 and 2 enzymes, inhibition of IkB/NF-kappa complex, or concentration dependant mechanisms not investigated here.

Phospholipases catalyze lipid substrates in the biological formation of eicosanoids, such as prostaglandins and leukotrienes (133b). Prostaglandins and leukotrienes participate in the formation of inflammatory mediators. Phospholipase A_2 (PLA2) specifically plays an important role in controlling the production of arachidonic acid from phospholipids and triacylglycerol, leading to the production of inflammatory cytokines (134a). Acetylsalicylic acid and curcumin have both demonstrated the inhibition of PLA2 through directly inhibiting this enzymes active site (133b, 421). This could explain some of the inhibition of TNFα production from these phenolic acids found in cereal brans.

COX 1 and 2 enzymes have been well characterized and directly inhibited at the active site by acetylsalicylic acid (10). Salicylic acid cannot affect COX 1 and 2 enzyme activity, but aspirin does (418). Aspirin blocks inflammation and pain through acetylating the serine 530 in the active site of these enzymes (414). None of the phenolic acids examined in this study carry an acetyl group and it is therefore unlikely that these phenolic acids inhibit cytokine production through interfering with serine in the active site of COX 1 and 2 enzymes. However, curcumin, a diferulic phenolic acid has shown inhibition of these two enzymes, thereby reducing prostaglandin synthesis and subsequent cytokine transcription (135a). It's been noted that the receptor for COX 2 is much larger and more flexible than COX 1, leading to the binding of some related phenolic structures (136a). While serine 530 would not be acetylated by curcumin or the phenolic acids used in this study, the binding affinity and selectivity of the COX-2 active site for several different functional groups bound to phenolic-rings has been explored extensively to include exceptions to aspirin's conformation (136a).

The most commonly considered mechanism by which TNFα is inhibited by phenolic acids would be direct inhibition of NF kappa-B activation (418). NF kappa-B, a transcription factor bound to the inhibitory protein IκB in the cytoplasm, is only released upon IKK kinase activity to IκB. If IκB phosphorylation occurs, NF-kapppa B dissociates and translocates to the nucleus, stimulating transcription, and turning on pro-inflammatory cytokine genes (404, 419). Sodium salicylate and acetylsalicylate both are shuttled into cells, bind IKK proteins, and inhibit the inducible activation of NF kappa-B (18). Curcumin, chrysin, and caffeic acid have all demonstrated the ability to enter cells and inhibit NF-kapppa B mediated inflammatory signaling (393a, 368). This mechanism of inhibition may be the best explanation of TNFα inhibition demonstrated in this study.

Lastly, the possibility of concentration-dependant inhibition may play a role, since salicylates demonstrate evidence of two different mechanisms, based upon concentration (414). Xu et al (1999) reported the direct inhibition of prostaglandins and leukotrienes, through COX-2 inhibition, but only when salicylate and acetylsalicylate concentrations were < 5 mmol/L (137a). This inhibition occurred at the COX-2 mRNA level, accompanied by reduced COX-2 promoter activity. However, the induction of NF kappa-B was not monitored and cannot be ruled-out as a potential mechanism in this study. At higher salicylate concentrations (>5 mmol/L), transcriptional activators, such as NF kappa-B have been

significantly inhibited in more than one study (418, 411, 138a). These studies reported inhibition of both IKK alpha and beta kinase activity and IκB phosphorylation activity. Concentrations of salicylates > 5 mmol/L considered "supra"-pharmacological, show disruptive action to gastrointestinal tissue and toxicity to others (139a). In the present study, only one phenolic acid concentration was used. This concentration was chosen based upon preliminary experiments of TNFα inhibition, < 5 mmol/L, and below therapeutic doses used for human anti-inflammatory activity.

In conclusion, this study demonstrated that 5 different phenolic acids found in cereal brans inhibit TNFα production by murine macrophages stimulated by oat and curdlan β-glucans. Although this study only used a single phenolic acid concentration, it was non-toxic, below therapeutic levels of effective salicylate concentrations, and significantly inhibited TNFα production, especially when phenolics acids were utilized in combination. This study demonstrates the ability of phenolic acids from cereal brans to achieve anti-inflammatory activity by significantly inhibiting a central cytokine in chronic inflammation that contributes to multiple chronic diseases. Future studies investigating the activity of phase II biotransformed phenolic acids and new delivery systems to achieve tissue specific anti-inflammatory activity have the potential to abolish symptoms and stop the progression of several chronic diseases.

Chapter 5

Conclusions and Summation

The WHO (2003) has recently estimated that chronic diseases account for more than 50% of the mortality in the world. In the U.S., National Vital Statistics (2004) reports that > 61% of deaths are due to chronic diseases, such as cancer, heart disease, diabetes, and obesity. Close to a 30% increase in infectious agents have claimed other lives associated with lung and kidney disorders in the United States. The one central biological system that ties these statistics together is the mammalian immune system. A stronger more responsive immune system protects against infectious agents and weakened immune systems in the young, elderly patients in hospitals that are experiencing increased nosocomial agents, and the nation's food and water supply that are potential targets of terrorism. Chronic diseases such as heart disease and cancer are linked by various degrees of inflammation. Inflammation is also a problem for diabetes, hypertension, and obesity patients. In general, the longer chronic inflammatory conditions remain in an individual, the higher the risk of developing a chronic disease.

Consumers are becoming more educated about healthcare, food, and nutrition. Many are utilizing a "hands-on" approach to their own health and seeking a physiological harmony for their bodies. Market research shows consumer spending for organic products has continued to increase over the last 5 years with accompanied increase in natural wellness products. Consumers are seeking whole and fresh foods as ways to improve their health and wellness. Based upon spending, whole grains market has become more important to U.S. consumers than European. Whole grains, fiber, vitamins, and minerals continue to top the list of demands from health-conscious consumers. Whole grains contain many bioactive compounds that provide beneficial effects to humans.

This project evaluated the interaction of cereal grain components with an *in vitro* mammalian immune cell system and *in vivo* human immune system. Historically, non-starchy polysaccharides from cell walls of cereal grains have effectively reduced cholesterol and stimulated the immune system. In this study, one objective was to examine β-glucans from barley and oat for their ability to stimulate an immune biomarker from murine macrophages, before and after enzyme treatment. In a second objective, the same human immune biomarker was monitored after orally administered barley β-glucan was fed to a group of subjects. Cereal bran contains many flavonoid and phenolic acid compounds, some with structural similarity to aspirin. A third objective was to demonstrate that bran components from barley and wheat cereals could modulate this same biomarker, a common thread in immune stimulation and inflammation. Lastly, benzoic acid and hydroxycinnamic acid derivatives were utilized as single and combinations of these compounds could reduce the production of this biomarker *in vitro*.

TNFα, a central protein (cytokine) of the mammalian immune system, plays a key role in orchestrating immunological reactions. A pleitropic cytokine that's self-regulating, it accomplishes many tasks, including immune stimulation, proliferation, inhibition, and apoptosis. TNFα deficient knock-out animal models demonstrate significantly reduced resistance to disease and high rates of mortality. Knock-out animal models that over-express TNFα demonstrate insulin resistance, reduced glucose utilization, various degrees of inflammation, and chronic disease, including atherosclerotic plaques, diabetes, hypertension, and weight gain. A balance of TNFα production or more appropriately, the ability to regulate TNFα production would keep this pivotal cytokine in check and expressed when needed.

In my experiments, cell wall β-glucans extracted from oat and barley stimulated murine macrophages *in vitro* to produce greater quantities of TNFα compared to untreated control cells, similar to other published literature. Lichenase treatment of both oat and barley β-glucans only increased the stimulating capacity of oat β-glucans, quantified by increased TNFα production from macrophages. These differences could be explained by different extraction methods and solvents utilized for each of these cereal β-glucans. However, this increase in potency of oat β-glucans may have been due to the larger contribution of β(1-3) linkages in the oat samples compared to the barley β-glucan samples. After several reviews of the literature, there was not one study that duplicated this treatment of oat and barley β-glucan with lichenase to evaluate the capacity of these polysaccharides to stimulate macrophages *in vitro* or *in vivo*.

Orally administered barley β-glucan treatment consumed over a 6-week period, did not significantly stimulate plasma TNFα when compared to all time points. There was no treatment effect between baseline, mid-point, and final sampling plasma. This trend was similar within the control (dextrose) treated group. When values from treated subjects at all time points were compared to control values, there was also no significant difference between plasma TNFα. However, greater TNFα values were quantified under the treated group compared to control values, suggesting that there may have been a treatment effect. When treated and control data for TNFα were separated into two sections based upon the age of 50 years, the youngest section had TNFα values in both the treated and control groups return to near normal physiological values with minimal variance. However, in the oldest section, quantifiable TNFα was increased in both the treated and control groups when compared to values of all ages pooled together. As humans age, it appears normal that plasma levels of TNFα also increase, but these particular values in this study may have also been affected by undetectable disease states suggested by the large magnitude of several individual values. A few studies in the literature have demonstrated that orally administered fungal β-glucans stimulate the innate and acquired immune systems.

Controlling cellular mediators of inflammation, specifically TNFα, provides a means of reducing health-risks and symptoms associated with several chronic diseases. Experimental studies reducing TNFα with anti-sense oligomers, intracellular pathway blockades, or anti-inflammatory drugs,

demonstrate increased insulin and glucose sensitivity, reduced COX-1 enzymes, reduced tissue inflammation, and increased mucosal cell proliferation. Bran extracts from barley and wheat were effective at reducing the TNFα production from oat and curdlan β-glucan stimulated macrophages similar to the anti-inflammatory compound, curcumin. However, barley bran extracts significantly ($p<0.05$) inhibited TNFα production, compared to wheat bran extracts or curcumin when macrophages were stimulated with the potent β(1-3) linked curdlan. Interestingly, although several bran-extracts from rye, barley, and wheat stimulated macrophages to produce TNFα alone without β-glucan treatment, one of these extracts (wheat) still inhibited TNFα production when curdlan was also added to the same macrophages and compared to curdlan treated macrophages alone. Plants contain many different primary and secondary metabolites to aid in plant growth, maintenance, and defense. In this study, the extraction of one or multiple compounds from barley and wheat bran significantly reduced the production of TNFα better than curcumin, a compound currently utilized in FDA clinical trials.

Cinnamic acid treatment or protocatechuic acid treatments alone, demonstrated the greatest and statistically significant inhibition of TNFα from oat β-glucan stimulated macrophages. The same phenolic acid treatments utilizing curdlan-stimulated macrophages were less effective inhibitors when compared to oat β-glucan stimulated cells. The inhibition from 3 phenolic acids equaled barley bran extracts inhibitory capacity when oat β-glucan was stimulating macrophage cells. However, single phenolic acid treatments of curdlan-stimulated macrophages could not reproduce the inhibitory capacity of barley bran extract. But the combination of caffeic and ferulic acid treatments together on curdlan stimulated macrophages reduced TNFα production to similar levels as barley bran extracts. These data suggest that a combination of two or more phenolics acids can control TNFα production to a greater degree than single phenolic treatments of the same dose or curcumin alone.

Epidemiological data has demonstrated that plant based diets rich in fruits, vegetables, and whole grains have positive benefits on human health and reduce risk for many chronic diseases. In the last 10 years, journal articles have continued to enlighten the historically puzzling picture of β-glucan stimulation on mammalian immune systems and the unclear connection of antioxidant benefits to the prevention of chronic diseases. Whole grains clearly contain multiple bioactive compounds that can be used to provide immune stimulation or anti-inflammatory affects through TNFα regulation, thereby reducing the progression of chronic disease. I have demonstrated that the enzymatic treatment of oat β-glucan with a natural cereal enzyme can increase the production of TNFα from murine macrophages and that this increase can be almost completely inhibited by extracts of barley bran and a combination of two specific phenolic acids found in most bran tissues of cereal grains.

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