Research Article

Sorghum [*Sorghum bicolor* (L.) Moench] Genotypes with Contrasting Polyphenol Compositions Differentially Modulate Inflammatory Cytokines in Mouse Macrophages

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This study sought to characterize and compare anti-inflammatory effects of twenty sorghum accessions with contrasting grain polyphenol concentrations but similar genetic backgrounds (based on a genomewide estimate of relatedness). Cell viability, tumor necrosis factor- (TNF-) α , and interleukin- (IL-) 6 were measured in RAW 264.7 macrophages treated with increasing doses (0, 15, 30, and 60 $\mu\text{g/mL}$) of sorghum ethanol extracts and stimulated with lipopolysaccharide (LPS). Extract dose had a significant effect on TNF- α and IL-6, with a trend of cytokines decreasing between 0 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$ of sorghum extract. Genotype also had a significant effect on the cytokines, with extracts from four accessions significantly decreasing TNF- α and/or IL-6. Cells treated with 3-deoxyanthocyanidin-containing accessions had less cytokine production than non-3-deoxyanthocyanidin accessions, whereas cells treated with proanthocyanidin-containing accessions had more cytokine production than cells treated with nonproanthocyanidin accessions. Additionally, there was a significant effect of the *Tannin1* allele on TNF- α and IL-6. Our results demonstrate that sorghum genotypes differentially modulate induction of inflammatory cytokine production in RAW 264.7 macrophages and that specialty grain has the potential to be tailored by controlling traits at the nucleotide level. This study adds to our knowledge of sorghum health effects and contributes to efforts aimed at developing health-promoting sorghum grain.

1. Introduction

Cereal crops provide the majority of nutrients to the world's population and thus have a significant impact on human health. However, most cereal breeding programs primarily focus on increasing grain yield and stress resistance because of the challenges involved in integrating human nutrition research [1, 2]. To breed cereal crops for human health, it is essential to understand the impact of different crop species, genotypes, and their associated composition on human nutrition by characterizing the health effects of their nutrient differences. Consumption of whole grain has been correlated with protective health effects related to several chronic inflammatory diseases, including obesity, type 2 diabetes, cancer, and cardiovascular disease [3–8]. Inflammation is known to be the underlying cause of many chronic diseases [9, 10],

and consuming foods with anti-inflammatory properties may help to prevent or attenuate the damage caused by inflammation. A large body of research demonstrates the anti-inflammatory effects of a variety of fruits [11–13], but these foods are a small contribution to daily food intake compared to grain products. Consumption of cereal grain with anti-inflammatory constituents has the potential to improve health outcomes in chronic disease.

A key feature of inflammation is the activation of inflammatory cells, especially monocytes and macrophages, which produce proinflammatory cytokines, including TNF- α and IL-6. These cytokines play crucial roles in inflammation, and excessive activation of the pathway leading to their production can result in chronic inflammation [14, 15]. Much of the research on sorghum polyphenol health benefits has been on its high antioxidant activity compared to commonly

consumed fruits [16, 17], but some studies suggest that sorghum grain has anti-inflammatory activity as well [18–22].

Many of the beneficial health effects of whole grain may be due to polyphenols in the bran [23–25]. Polyphenols are found in abundance in fruits, vegetables, tea, chocolate, red wine, and coffee, but also in certain grain crops, including varieties of wheat, rice, maize, and sorghum [24, 26–28]. Sorghum is one of the world's major cereal crops, feeding millions of people in Asia and Sub-Saharan Africa [29]. In the United States, it is used primarily as livestock feed, but it is also used in many specialty grain products and gluten-free food products [30–33]. It is a genetically and phenotypically diverse cereal crop, with some varieties containing no measurable amounts of polyphenols and others containing high levels of multiple polyphenols [34, 35], particularly proanthocyanidins and 3-deoxyanthocyanidins [36].

Most studies have only explored the health benefits of a small number of sorghum accessions, but over 45,000 sorghum accessions are available from the US National Plant Germplasm System's Germplasm Resources Information Network (GRIN) [37]. Using a large genetically diverse sorghum panel to explore the effects of natural variation of sorghum polyphenols on induction of inflammatory cytokine production will aid in discovering particularly beneficial accessions. Although several studies have been conducted comparing health effects between high and low polyphenol sorghum, none of them controlled for genetic background of the sorghum [19, 22]. Without adequate control of other genetic factors it may not be possible to attribute health effects to polyphenols *per se*. The goals of this study were to characterize and compare the induction of inflammatory cytokine production of twenty sorghum accessions with contrasting grain polyphenol concentrations but similar genetic backgrounds and to gain a broader understanding of the diversity of anti-inflammatory effects available in sorghum germplasm.

2. Materials and Methods

2.1. Plant Materials. We selected 20 sorghum accessions from a panel of 381 accessions that we previously evaluated for polyphenol concentrations [34]. The 20 accessions were selected based on contrasting polyphenol content and genetic relatedness. The panel primarily consisted of the sorghum association panel (SAP) [38], which includes accessions from all major cultivated races and geographic centers of diversity in Sub-Saharan Africa and Asia, as well as important breeding lines from the United States. Also included were 73 accessions selected based on the presence of a pigmented testa (which indicates presence of proanthocyanidins) using the GRIN data query. Seeds for all the sorghum accessions were obtained from GRIN and are readily available through GRIN.

The grain samples used for this experiment have previously been described [34]. Briefly, the panel was planted in late April 2012 at Clemson University Pee Dee Research and Education Center in Florence, SC, in a twofold replicated (hereafter referred to as biological replicates) complete randomized block design. Panicles were collected at physiological maturity between September and October

and mechanically threshed. Samples were phenotyped using near-infrared spectroscopy (NIRS) as previously described [34]. Total phenol, proanthocyanidin, and 3-deoxyanthocyanidin data were calculated based on dry seed and are expressed as mg gallic acid equivalent (GAE)/g, mg catechin equivalents (CE)/g, and absorbance (abs/mL/g), respectively. For the purposes of this study, we use a cut-off greater than 10 mg CE/g to define proanthocyanidin-containing varieties and greater than 50 abs/mL/g to define 3-deoxyanthocyanidin-containing varieties. Seed protein, fat, and starch are expressed as a percentage of dry seed weight. Seed weight is expressed as the total grain weight of 100 grains per accession.

2.2. Genomic Analysis. To select accessions with comparable genetic backgrounds, we used the genotypes of each accession to assess relatedness. Genotypes were available for the 381 accessions [34, 39]. Based on 404,628 SNP markers, the degree of genetic relatedness between accessions was calculated in a kinship matrix in a unified mixed linear model [40] using the statistical genetics package Genome Association and Prediction Integrated Tool (GAPIT) [41]. To validate proanthocyanidin effects on TNF- α and IL-6, we used a SNP (S4_61667908) on chromosome 4 that is tightly linked (~200 bp) to the causal loss-of-function mutation in the *Tannin1* gene. The wildtype allele *Tannin1*, along with two nonfunctional alleles *tan1-a* and *tan1-b*, is responsible for sorghum grain proanthocyanidin production [42].

2.3. Preparation of Sorghum Bran Extracts. Extraction was done according to the methods of Burdette et al. [19], with minor modifications. A tangential abrasive dehulling device (TADD; Venable Machine Works, Saskatoon, Canada) equipped with an 80-grit abrasive disk was used to remove the bran from the grain. Bran was mixed with 50% ethanol [10% (w/v)] and placed on a shaker at room temperature for three hours. Samples were then centrifuged at 5,000 rpm for 15 minutes and supernatant was poured through a 0.2-micromolar filter into a sterile container. Samples were refrigerated and protected from light until being ready to use.

2.4. Cell Cultures. The mouse macrophage cell line RAW 264.7 (TIB-71 from American Type Culture Collection (ATCC)) was cultured on 100 mm culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM, ATCC), supplemented with 10% fetal bovine serum (ATCC) and 100 I.U./mL penicillin and 100 μ g/mL streptomycin (ATCC) at 37°C in a humidified incubator with 5% CO₂.

2.5. Cell Viability Assay. Cell viability was measured using the MTT Cell Proliferation Assay (R&D Systems), an indirect method of measuring metabolically active cells. RAW 264.7 cells were seeded in a 96-well plate at 1×10^5 cells/well and incubated overnight to allow cells to recover and adhere to the cell culture plate. Cells were pretreated for one hour with sorghum extracts (two biological replicates per accession) at concentrations of 60 μ g/mL, 30 μ g/mL, 15 μ g/mL, and 0 μ g/mL (negative control: 50% EtOH diluted with

cell culture medium to the same concentrations) and then stimulated with LPS at $1\text{ }\mu\text{g/mL}$ (diluted with cell culture medium) for an additional 18 hours. The MTT reagent was added to each well and cells were incubated for an additional 2 hours until purple dye was visible under the microscope. Detergent reagent was added and the plates were left in the dark at room temperature for 4 hours. Absorbance was measured at 570 nm in a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). Results are expressed as the percentage of absorbance of extract-treated cells versus negative controls. Viability less than 70% compared to the control was considered potentially cytotoxic.

2.6. Cytokine Assays. Cells were seeded in 96-well plates at 2×10^5 cells/well and incubated overnight to allow time to recover and adhere to the substrate. Cells were pretreated for one hour with sorghum bran extracts (two biological replicates per accession) at concentrations of $60\text{ }\mu\text{g/mL}$, $30\text{ }\mu\text{g/mL}$, $15\text{ }\mu\text{g/mL}$, and $0\text{ }\mu\text{g/mL}$ (negative control: 50% EtOH diluted with cell culture medium to the same concentrations) and then stimulated with LPS at $1\text{ }\mu\text{g/mL}$ (diluted with cell culture medium) for an additional 18 hours. Cell culture medium was collected and tested using TNF- α and IL-6 ELISA Ready-Set-Go! kits (eBioscience). Assays were carried out according to manufacturer's instructions. Absorbance was measured at 450 nm with wavelength subtraction at 570 nm in a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). Results are expressed as the percentage of cytokine levels in extract-treated cells versus negative controls (negative control: 50% EtOH diluted with cell culture medium to the same concentrations) and normalized with respect to cell viability for each extract.

2.7. Statistical Analysis. Statistical differences were assessed using analysis of covariance (ANCOVA) followed by Tukey's test to detect significant differences between treatments. P values of <0.05 were considered statistically significant. Data are expressed as the means $\pm$ standard deviation (SD) of results from three technical replicates and two biological replicates. All calculations were performed using R, a statistical computing language and environment [43].

3. Results

3.1. Targeted Set of Germplasm for Cell Activation Studies. To identify candidates with anti-inflammatory potential, we screened 381 sorghum accessions for high concentrations of proanthocyanidins and/or 3-deoxyanthocyanidins and used a kinship matrix to identify a subset of accessions with similar genetic background (high kinship value) but with contrasting polyphenol content (Figure 1). The resulting set included eleven proanthocyanidin-containing accessions (based on NIRS values greater than 10 mg CE/g or presence of a pigmented testa), eight 3-deoxyanthocyanidin-containing accessions (based on NIRS values greater than 50 abs/mL/g), and five low polyphenol accessions that did not contain either of the polyphenols (Figure 2). Three of the accessions contained both polyphenols. Total polyphenol concentrations

FIGURE 1: Heatmap and dendrogram of hierarchical clustering showing the estimated kinship among 20 sorghum accessions. Genetic relatedness between accessions was calculated in a kinship matrix using a unified mixed linear model in GAPIT. The darker the blue, the more related the accessions. The presence or absence of proanthocyanidins and 3-deoxyanthocyanidins is indicated in the top two rows.

in the panel ranged from 0 to 24 GAE/g , proanthocyanidin concentrations ranged from 0 to 42 mg CE/g , and 3-deoxyanthocyanidin concentrations ranged from 0 to 110 abs/mL/g .

3.2. Sorghum Extracts Do Not Reduce Cell Viability. To assess the effects of sorghum bran extracts on cell viability we used the MTT assay. RAW 264.7 macrophages were pretreated with sorghum extracts for one hour, followed by LPS for 18 hours. To characterize the effect of sorghum extracts on cell viability, we conducted an ANCOVA. Extract dose did not have a significant effect on cell viability ($P = 0.43$; Figure 3(a)), but genotype did ($P < 0.001$). A *post hoc* analysis was conducted to determine the effects of individual accessions on cell viability, and PI655978 was found to significantly increase cell viability ($P = 0.03$), whereas no accessions significantly decreased cell viability or dropped below 70% viability (Figure 3(b)).

3.3. Sorghum Accessions Differentially Modulate TNF- α and IL-6. To determine differences in cytokine response to increasing doses of sorghum extracts, RAW 264.7 macrophages were pretreated with sorghum bran extracts for one hour, followed by stimulation with LPS for 18 hours. We first analyzed the average effect across accessions for TNF- α and IL-6 production. Based on the mean value of all accessions, extract dose had a significant effect on both TNF- α ($P < 0.001$) and IL-6 ($P < 0.001$). A *post hoc* analysis showed that TNF- α decreased between $0\text{ }\mu\text{g/mL}$ and $15\text{ }\mu\text{g/mL}$ of

FIGURE 2: Polyphenol concentrations in the grain of 20 sorghum accessions. NIRS estimates of (a) total phenol concentrations (GAE/g), (b) proanthocyanidin concentrations (CE/g), and (c) 3-deoxyanthocyanidin concentrations (abs)/mL/g, ordered from lowest total phenols to highest ones. Error bars represent the means of triplicate experiments $\pm$ SD.

FIGURE 3: MTT cell viability assays of RAW 264.7 cells treated with sorghum bran extracts. Cells were seeded in 96-well plates, incubated overnight, and pretreated with sorghum bran extracts at concentrations of 60 μ g/mL, 30 μ g/mL, and 15 μ g/mL for 1 hour before LPS stimulation (1 μ g/mL) for 18 hours. Results are expressed as percentage of absorbance in extract-treated cells versus control cells (no extract added). (a) Mean viability value of all accessions and (b) value of each of the twenty accessions at extract concentrations of 15 μ g/mL (light gray), 30 μ g/mL (gray), and 60 μ g/mL (dark gray). Error bars represent the means of triplicate experiments $\pm$ SD. Accessions are ordered from lowest total phenols to highest ones. * $P < 0.05$.

extract, although it was not significant ($P = 0.78$), and increased between 15 μ g/mL and 30 μ g/mL of extract ($P = 0.06$), as well as 15 μ g/mL and 60 μ g/mL of extract ($P < 0.001$; Figure 4(a)). Similarly, IL-6 decreased between 0 μ g/mL and

15 μ g/mL of extract, although it was not significant ($P = 0.67$), and increased between 15 μ g/mL and 60 μ g/mL of extract ($P = 0.004$), as well as 30 μ g/mL and 60 μ g/mL of extract ($P < 0.001$; Figure 4(b)).

FIGURE 4: Sorghum bran extracts differentially modulate TNF- α and IL-6 production in RAW 264.7 cells. Cells were seeded in 96-well plates, incubated overnight, and pretreated with sorghum bran extracts at concentrations of 15 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, and 60 $\mu\text{g/mL}$ for 1 hour before LPS stimulation (1 $\mu\text{g/mL}$) for 18 hours. (a) Effect of extract dose on TNF- α , (b) effect of extract dose on IL-6, (c) effect of 3-deoxyanthocyanidin-containing accessions on TNF- α , (d) effect of 3-deoxyanthocyanidin-containing accessions on IL-6, (e) effect of proanthocyanidin-containing accessions on TNF- α , (f) effect of proanthocyanidin-containing accessions on IL-6, (g) effect of *Tannin1* on TNF- α , and (h) effect of *Tannin1* on IL-6. Results are expressed as percentage of extract-treated cells versus negative controls (no extract added). Error bars represent the means of triplicate experiments $\pm$ SD. For (a) and (b), concentrations share the same letter if they are not significantly different from each other.

TABLE 1: Nutritional traits of sorghum accessions that reduce cytokine production^{a,b}.

Trait	PI297139	PI329440	PI561072	PI655978	SAP ^c
Total phenols (mg GAE/g)	22.6 ± 2.0	ND	2.5 ± 2.2	5.0 ± 1.4	7.0 ± 5.9
Proanthocyanidins (mg CE/g)	41.7 ± 6.4	ND	0.5 ± 0.6	2.4 ± 1.2	7.7 ± 15.5
3-Deoxyanthocyanidins (abs/mL/g)	110.7 ± 29.9	19.4 ± 15.3	19.4 ± 3.0	95.2 ± 80.8	27.4 ± 24.1
Protein (%)	15.2 ± 0.4	11.2 ± 0.9	13.1 ± 1.1	12.2 ± 1.0	11.8 ± 2.4
Fat (%)	3.7 ± 0.4	2.7 ± 0.4	2.8 ± 0.1	3.7 ± 0.3	2.9 ± 0.8
Starch (%)	65.2 ± 0.1	68.5 ± 0.3	65.6 ± 0.9	66.1 ± 0.8	67.1 ± 1.8
Weight (g)	3.1 ± 0.1	2.2 ± 0.3	2.5 ± 0.3	2.8 ± 0.1	2.6 ± 0.5
Color	Red	Red	White	Yellow	Red, white, yellow, brown

^aData are the mean NIRS values ± SD of accessions grown in duplicate plots.

^bNot detected (ND).

^cMean values of the entire sorghum association panel for comparison [34].

Next, we wanted to determine if there were genotypic differences affecting TNF- α and IL-6 production. Based on the mean value of all accessions, genotype had a significant effect on both IL-6 ($P < 0.001$) and TNF- α ($P < 0.001$). A *post hoc* analysis was conducted to determine the effect of individual accessions on cytokine modulation. TNF- α was significantly decreased in cells treated with extracts from PI329440 ($P < 0.001$), PI561072 ($P = 0.04$), and PI655978 ($P < 0.001$) compared to control cells. IL-6 was significantly decreased in cells treated with extracts from PI297139 ($P = 0.04$), PI329440 ($P < 0.001$), and PI655978 ($P < 0.001$) compared to control cells.

3.4. Sorghum Polyphenols Differentially Modulate TNF- α and IL-6. We hypothesized that sorghum accessions with high polyphenol content would decrease cytokine production in LPS-activated RAW 264.7 cells. To test this hypothesis, an ANCOVA was conducted. Surprisingly, there was only a marginally significant effect of 3-deoxyanthocyanidins on TNF- α ($P = 0.07$; Figure 4(c)) and no significant effect on IL-6 ($P = 0.13$; Figure 4(d)). However, there was a trend in which cells treated with 3-deoxyanthocyanidin-containing accessions had less cytokine production than cells treated with non-3-deoxyanthocyanidin accessions, so it may be that there was not enough statistical power to detect significant differences. There was a significant effect of proanthocyanidins on TNF- α ($P < 0.001$; Figure 4(e)) and IL-6 ($P = 0.04$; Figure 4(f)), with cells treated with proanthocyanidin-containing accessions producing more cytokines than cells treated with nonproanthocyanidin accessions.

To validate that differences in cytokines were due to proanthocyanidins, we took advantage of a previously identified variant in a major gene regulating grain proanthocyanidins in sorghum, *Tannin1* [42]. We used an ANCOVA to determine differences in cytokine production between cells treated with extracts from *Tannin1* accessions (proanthocyanidin-containing) and cells treated with extracts from *tan1-a* or *tan1-b* accessions (nonproanthocyanidin-containing). For TNF- α , there was a significant effect of the *Tannin1* allele ($P = 0.01$) and extract concentration ($P \leq 0.001$), as well as an interaction between them ($P = 0.04$; Figure 4(g)). For IL-6, there was

also a significant effect of the *Tannin1* allele ($P = 0.01$) and extract concentration ($P < 0.001$), but no significant interaction ($P = 0.44$) (Figure 4(h)). As expected, cells treated with extracts from accessions containing *Tannin1* had more cytokine production than cells treated with extracts from accessions containing *tan1-a* or *tan1-b*.

3.5. Accessions Differ in Nutritional Traits. In addition to ability to reduce induction of inflammatory cytokine production, other nutritional traits may influence the choice of accessions for use in the development of food-grade specialty varieties. Therefore grain protein, fat, starch, and weight were measured in the four accessions that inhibited both TNF- α and IL-6 production (Table 1). As a percentage of dry seed weight, protein ranged from 11.2 to 15.2%, fat ranged from 2.7 to 3.7%, and starch ranged from 65.2 to 68.5%. Seed weight ranged from 2.2 to 3.1g. Since grain color influences end-user acceptance, we characterized the appearance of the outer layer of the grain: two accessions were red, one was yellow, and one was white.

4. Discussion

4.1. Crop Improvement for Human Health. Improving the nutritional value of crops will benefit from an integrative approach [1]—a combination of biochemistry, genomics, high-throughput phenotyping, and statistics. Also important is human nutrition research that uses cell cultures, animal models, and human clinical trials to characterize the health effects of a food plant. By combining crop improvement and human nutrition tools, we were able to screen hundreds of sorghum accessions relatively quickly and cost-effectively, permitting a targeted study on cell activation properties of sorghum grain (Figure 5). Additionally, utilizing accessions that are readily available from crop genebanks allows for authentication of the accessions and reproducibility of the experiments.

The primary goal of surveying a large number of diverse varieties is to identify those with a trait of interest. We identified four out of the twenty sorghum accessions in our diverse panel that significantly decreased TNF- α and/or IL-6 in RAW 264.7 macrophages, making them good candidates

FIGURE 5: Workflow for screening and testing a large sorghum germplasm panel. 381 accessions were selected from a sorghum genebank, grown in South Carolina, and evaluated for polyphenol concentrations and relatedness. Twenty accessions were selected for *in vitro* testing. Four accessions were identified that reduce induction of inflammatory cytokines.

for use in developing specialty varieties. PI655978, which inhibited both TNF- α and IL-6 production, is a good source of high 3-deoxyanthocyanidin alleles for use in developing varieties that reduce induction of inflammatory cytokine production. Nutritional traits cannot be looked at in isolation but rather need to be considered in the context of other grain quality and agronomic traits, as well as the needs of growers and consumers. PI655978 is a yellow-grain sorghum with a slightly higher than average protein content (12.2%) and weight (2.8 g) compared to the entire SAP, a high concentration of 3-deoxyanthocyanidins (95.2 abs/mL/g), and no proanthocyanidins. These characteristics make this accession well suited for use in developing health-promoting specialty crop since light colored grain is generally more accepted by consumers than dark colored grain, proanthocyanidins can impart distasteful bitterness, and low grain weight is not desirable since grain weight is crucial to grain yield [44–46].

Interestingly, two low polyphenol accessions (PI329440 and PI561072) demonstrated ability to reduce induction of inflammatory cytokine production. Previous studies, conducted with a smaller number of sorghum varieties, did not find significant ability to reduce induction of inflammatory cytokine production in their low polyphenol controls [19, 22], which highlights the importance of exploring the cell activation effects of many genetically diverse sorghums in order to identify beneficial accessions. One strategy to

reduce confounding factors in future studies is to control for differences in genetic background using near isogenic lines, pairs of lines developed through backcrossing that only differ in the genomic region of interest [47].

Considerable effort has been aimed at identifying genetic markers in crops, with the goal of using them in marker-assisted breeding [48]. We found a significant effect of the *Tannin1* allele on TNF- α and IL-6, which demonstrates that marker-assisted breeding can be conducted down to the nucleotide level to develop health-promoting specialty crops.

4.2. 3-Deoxyanthocyanidins and Cell Activation. Sorghum 3-deoxyanthocyanidins are not widely found in nature, and sorghum is their only known dietary source [49–51]. They are a promising class of health-promoting polyphenols, with antioxidant and antiproliferative properties. Our results showed only a marginally significant effect of 3-deoxyanthocyanidins on TNF- α and no significant effect on IL-6 levels in LPS-activated cells. Our sample size of 3-deoxyanthocyanidin-containing accessions was small, though, so there was low statistical power to detect differences. We did, however, see a trend in which cells treated with 3-deoxyanthocyanidin-containing accessions had less cytokine production than cells treated with non-3-deoxyanthocyanidin accessions. Several studies have demonstrated anticancer properties of sorghum 3-deoxyanthocyanidins *in vitro* [52–55], so studying the effects of sorghum 3-deoxyanthocyanidins in *in vivo* cancer models will help to further our understanding of potential therapeutic properties of 3-deoxyanthocyanidins. Given that the effect of 3-deoxyanthocyanidin-containing accessions was only marginally significant, other constituents in the bran must also be contributing to the reduced induction of inflammatory cytokine production. For example, PI329440 is a low polyphenol sorghum, but addition of its extract significantly inhibited both TNF- α and IL-6 in cell cultures. In a recent study, phenolic acid derivatives isolated from sorghum grains decreased LPS-stimulated NO, iNOS, and COX-2 in RAW 264.7 cells [21]. Other phenolic compounds have been identified in sorghum bran, including flavones, flavanones, phlobaphenes, and anthocyanins [36], which may be contributing to the reduced induction of inflammatory cytokine production observed in this study. Follow-up studies that identify and test individual constituents in the bran will help to determine which constituents have health protective effects and thus could be targeted for specialty breeding.

4.3. Proanthocyanidins and Cell Activation. Proanthocyanidins are not commonly found in high concentrations in cereal crops, but many sorghum varieties are rich sources of this polyphenol [56]. They are also a promising class of health-promoting polyphenols but their effects are more complex. Cells treated with proanthocyanidin-containing accessions showed a trend towards lower production of TNF- α and IL-6 at 15 μ g/mL of extract compared to 0 μ g/mL but higher cytokine production compared to nonproanthocyanidin accessions. A study in 2010 found that high concentrations of a proanthocyanidin-containing sorghum accession

slightly induced induction of inflammatory cytokine production in PBMC cells; however, the same study also found that this accession reduced induction of inflammatory cytokine production in a mouse model of ear inflammation, demonstrating the complexity of the effects on cytokine modulation and the need for disease specific and tissue specific research [19]. PI297139 is the only high proanthocyanidin accession in our study that significantly reduced induction of inflammatory cytokine production. This accession also contains a high concentration of 3-deoxyanthocyanidins, which may have been responsible for the reduced induction. It is also possible that there were differences in the proanthocyanidins in this accession compared to other accessions, which led to differing effects on induction of inflammatory cytokine production *in vitro*. Several structural variations of sorghum proanthocyanidins have been described, including differences in the monomer repeats, interflavan bonds, patterns of hydroxylation and glycosylation, and degree of polymerization [36, 57]. The degree of polymerization highly influences absorption of proanthocyanidins. Small proanthocyanidin compounds are absorbed in the small intestine, whereas large ones pass through the small intestine into the large intestine where they are catabolized by intestinal bacteria before they are absorbed [58]. For this reason, it has been suggested that health benefits derived from proanthocyanidins may be largely due to their effects on intestinal bacteria [59]. Several studies have found that proanthocyanidins from grape seeds attenuate inflammation in colitis animal models in part by improving the profile of beneficial gut bacteria [60, 61], and a recent study found that a high proanthocyanidin sorghum bran diet may protect colitis-induced rats by enriching colon bacteria [62]. Further study on proanthocyanidin structural variation in PI297139 and the effects of its grain extracts in colitis animal models could help clarify dietary effects of sorghum proanthocyanidins.

5. Conclusions

The RAW 264.7 cell activation model provides a good starting point for understanding the biological activity of a test compound and is a good high-throughput screening method to characterize the diversity of cell activation effects within a crop species. This can act as a guide in the selection of a subset of accessions for use in more complex disease models. Overall this study provides evidence that sorghum extracts significantly and differentially modulate induction of inflammatory cytokines TNF- α and IL-6, with 3-deoxyanthocyanidin and proanthocyanidin having a significant effect on their production. Additionally, in showing that the modulation of cytokines varies between accessions, this study demonstrates the importance of exploring diverse germplasm within a plant species to discover the full health-promoting potential of this crop. Finally, the effect of the *Tannin1* allele on cytokines shows the potential of tailoring grain for beneficial end-uses by controlling traits at the nucleotide level. The results reported here can inform future sorghum studies, which should focus on testing with *in vivo* disease models such as a colitis mouse model, while reducing confounding factors

through the use of near isogenic lines. This study contributes to the growing body of evidence that sorghum accessions differentially modulate induction of cytokine production and this difference is related to polyphenol profile and other active compounds.

Abbreviations

abs:	Absorbance
ANOVA:	Analysis of variance
CE:	Catechin equivalent
GAE:	Gallic acid equivalent
GAPIT:	Genome Association and Prediction Integrated Tool
GRIN:	Germplasm Resources Information Network
IL-6:	Interleukin-6
MLM:	Mixed linear model
NIRS:	Near-infrared spectroscopy
TNF- α :	Tumor necrosis factor alpha.

Disclosure

Davina H. Rhodes present address is as follows: USDA-ARS-Center for Grain and Animal Health Research, 1515 College Avenue, Manhattan, KS 66502, USA. The United Sorghum Checkoff Program only provided financial support and had no involvement in the study design; in the collection, analysis, and interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication.

Competing Interests

The authors declare that they have no competing interests.

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