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High molecular weight polysaccharides are key immunomodulators in North American ginseng extracts: Characterization of the ginseng genetic signature in primary human immune cells

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ABSTRACT

Ethnopharmacological relevance: Ginseng (GS) has played a pivotal role in traditional Chinese medicine for thousands of years. Its use has become increasingly popular in North America, in part due to the many claims of its immune-enhancing properties. The immunopharmacology of the North American variety of GS and its extracts is needed to substantiate these claims.
Materials and methods: Human peripheral blood mononuclear cells were exposed to different North American GS extracts and microarray analysis was performed. The profile of cytokine response to GS extracts was established by ELISA, and Ingenuity Pathway Analysis was used to identify potential signaling pathways responsible for the transcriptional profile induced by GS. Fractionation of the aqueous and polysaccharide extracts was done to determine the molecular weight of the active immune modulatory ingredient(s).
Results: We found that GS induced a transcriptional profile of immunomodulation characterized by a net T_h1 immune response, with up-regulation of multiple pro-inflammatory cytokines (e.g., IFN-γ, IL-23A and IL-6) and down-regulation of TGF-β, IL-13 and the LPS co-receptor CD14. Ingenuity Pathway Analysis (IPA) revealed that the MAPK (ERK-1/2), PI3K, p38 and NF-κB cascades were key signaling pathways through which GS may trigger its immunomodulatory action. Furthermore, induction of such an immunomodulatory signature was recapitulated with the high molecular weight polysaccharides found in aqueous and polysaccharide GS extracts.
Conclusions: Based on our results, we conclude that high molecular weight polysaccharides in North American GS aqueous and polysaccharide extracts likely trigger the MAPK (ERK-1/2), PI3K, p38 and NF-κB signaling pathways in PBMC resulting in the induction of a T_h1 transcriptional profile. Our results may assist in optimizing GS-mediated immunomodulation and focus the search for compounds in GS extracts with specific immunomodulatory activities.

1. Introduction

Ginseng (GS) is the name given to several species of perennial plants that produce berry-like flowers and fleshy roots. The two most commonly recognized species of GS are the Asian GS *Panax ginseng* and the North American GS *Panax quinquefolius* (Ang-Lee et al., 2001; Attele et al., 1999). Asian GS is regularly consumed as an ingredient in tea, alcoholic beverages or candy (Choi, 2008). GS has also been an important part of traditional Chinese herbal remedies for thousands of years (Ang-Lee et al., 2001; Attele et al., 1999) due to claimed health benefits such as enhancement of immunity, stress relief and prevention of aging (Block and Mead, 2003; Wang and Yuan, 2008).

The current use of GS extends beyond Asia. In 2002, 4–5% of Americans aged 45–64 years of age, accounting for approximately 13 million individuals, used GS as a herbal supplement (Kaufman et al., 2002). In North America, GS has become the second-highest selling herbal supplement; in 2000, GS had gross retail sales of nearly $62 million (Jia and Zhao, 2009). In Canada, 17–32% of patients with cardiovascular disease reported use of alternative supplements and 6% of those using herbs reported GS use (Pharand et al., 2003). Additionally, GS is the 10th most used herbal supplement in HIV patients, accounting for 34% of individuals studied (Standish et al., 2001). With growing interest in the Western market for herbal medicine, an increasing amount of attention has focused on North American GS (Qi et al., 2011).

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Despite the wide use of North American GS, the mechanism of action and the effects of specific extracts from this root remain mostly unknown. The biological effects of GS have generally been attributed to ginsenosides, low molecular weight saponins found in GS (Attele et al., 1999). For example, ginsenosides have been shown to have anti-tumor and oxidative-stress prevention properties (Hwang and Jeong, 2010; King and Murphy, 2007; Luo et al., 2008; Wang and Yuan, 2008; Yoo et al., 2010). However, it is unclear if the immune effects of GS are due to ginsenosides or to other components in GS. In particular, polysaccharides isolated from GS, such as poly-furanosyl-pyranosyl-saccharides and monosaccharide have been claimed to have immune-enhancing effects (Biondo et al., 2008; Wang et al., 2004), justifying the use of preparations such as COLD-FX®, which contains 80% of poly-furanosyl-pyranosyl-saccharides from North American GS (Wang et al., 2004), and is marketed to prevent upper respiratory tract infections (McElhaney et al., 2006).

To explore the immunomodulatory capacity of distinct North American GS extracts, we examined the profile of gene expression induced on human peripheral blood mononuclear cells (PBMC) by water-soluble GS (*Panax quinquefolius*) fractions (i.e. aqueous extracts of GS equivalent to tea-like preparations of GS). We found that aqueous GS extracts induce a transcriptional profile characterized by an enhanced innate and adaptive T_h1 pro-inflammatory immune response—a profile corroborated at the protein level. These changes were induced mostly by high molecular weight fractions of polysaccharide extracts of North American GS.

2. Materials and methods

2.1. Cells

Informed consent from all individuals was obtained in compliance with the Office of Research Ethics at the University of Western Ontario. Ficoll-Hystopaque density gradient centrifugation was used to isolate human PBMC from healthy volunteers. Cells were cultured in RPMI-1640, supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 1% MEM non-essential amino acids, 1% 1 M Hepes and 1% sodium pyruvate.

2.2. Preparation of North-American GS extracts

Roots of *Panax quinquefolius* were collected from 7 different farms in Southwestern Ontario, Canada. A reference plant was deposited in The University of Western Ontario Department of Biology Herbarium, entry number 14723. Root samples were ground and soaked three times for five hours in water or an ethanol/water (75/25, v/v) solution at 40 °C. The solutions were then filtered and excess solvent was removed in a rotavaporizer under vacuum at 45 °C. Powdered aqueous extracts (10 g) were dissolved in 10 mL of distilled water. Four volumes of 95% ethanol were added to the solution in order to precipitate crude polysaccharides. Polysaccharide precipitate was collected by centrifugation (3000 rpm for 10 min) and freeze dried at −50 °C under reduced pressure. The crude polysaccharide (1 g) was re-dissolved in distilled water (10 mL) and treated with Sevag reagent (1:4 n-butanol:chloroform, v/v, 40 mL) to remove proteins. After re-precipitation by ethanol and freeze-drying, a final polysaccharide fraction was obtained. The content of the aqueous, ethanol and polysaccharide extracts have been previously described (Azike et al., 2011). These different types of North American GS extracts and their fractions were used throughout this work and are referred to as GS extracts or corresponding fractions. Lipopolysaccaride (LPS) contamination of GS extracts was ruled out by the Limulus test and further ensured by treatment with Polymyxin B.

2.3. Preparation of GS extracts fractionated according to molecular weight

Twenty mg of powdered GS extracts were dissolved in 1 mL of colorless media. Five hundred μL of GS extract at 20 mg/mL was placed in a 100 kDa Millipore Centrifugal Filter Unit (Billerica, MA). The filtrate was then put into centrifugal filter units with sequentially lower molecular weight cut offs (50 kDa, 30 kDa and 10 kDa). 50 μL of colorless media was used to wash each filter and remove any precipitate.

2.4. Reagents

COLD-FX® was purchased from commercial sources (Afexa Life Sciences Inc. Mississauga, ON). According to manufacturer's description, it contains CVT-E002, a proprietary ChemBioPrint® (CBP®) product of greater than 80% poly-furanosyl-pyranosyl-saccharides extracted by an aqueous method from *Panax quinquefolius*. *Staphylococcal aureus* enterotoxin E (SEE) was purchased from Toxin Technologies (Sarasota, FA). *Escherichia coli* lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO).

2.5. Functional assays

Human PBMC (2×10^5 cells) in 200 μL of media per well in 96-well U-bottom plates (Nunc, Rochester, NY) were stimulated for 20 h with increasing concentrations of GS extracts or COLD-FX®, by themselves or in the presence of LPS or SEE. Quantification of IL-1β, IL-6, IL-10, TNF-α, IFN-γ, IL-12p40, IL-12p70, IL-23 and IL-2 was performed using human OptEIA™ ELISA kits (BD Biosciences, San Diego, CA).

2.6. Monocyte isolation

$CD14^+$ monocyte isolation was performed using MACS human monocyte positive isolation kit (Miltenyi Biotec, Auburn, CA). $CD14^+CD16^+$ monocyte isolation was performed using EasySep negative selection kit (Stemcell Technologies, Vancouver, BC). Monocytes were then counted and plated at 50,000 cells in 200 μL of media per well in 96-well U-bottom plates and were stimulated with increasing concentrations of GS extracts or COLD-FX® for 20 h.

2.7. Microarray analysis

Total RNA from cells of two independent donors was isolated using a Qiagen kit (Qiagen, Valencia, CA) and used to generate cDNA. cDNA samples were hybridized to the Affymetrix GeneChip Human Gene 1.0 sense target Array (Affymetrix, Santa Clara, CA). Expression levels were converted to fold changes by comparing expression levels between treatment and control sample arrays (Partek Incorporated, St. Louis, MO). Genes with a fold change less than 2 were filtered and eliminated from analysis. Profiled clusters of genes were identified and correlated to functional effects using Partek software (Partek Incorporated).

2.8. Ingenuity Pathway Analysis (IPA)

Affymetrix microarray data was analyzed using the IPA program that identifies relationships, mechanisms, functions, and pathways of biological relevance that are most significantly perturbed in a given dataset (Ingenuity Systems, Redwood City, CA).

2.9. Flow cytometry

FITC-labeled anti-CD14 antibodies were used to assess CD14 expression on the cell surface by flow cytometry. FITC-labeled anti-mouse IgG1κ antibodies were used as isotype controls.

2.10. Statistical analysis

Graphpad Prism (Graphpad Instat Software Inc., San Diego, CA), was used to perform ANOVA with post-hoc Bonferroni tests to determine the statistical significance of differences

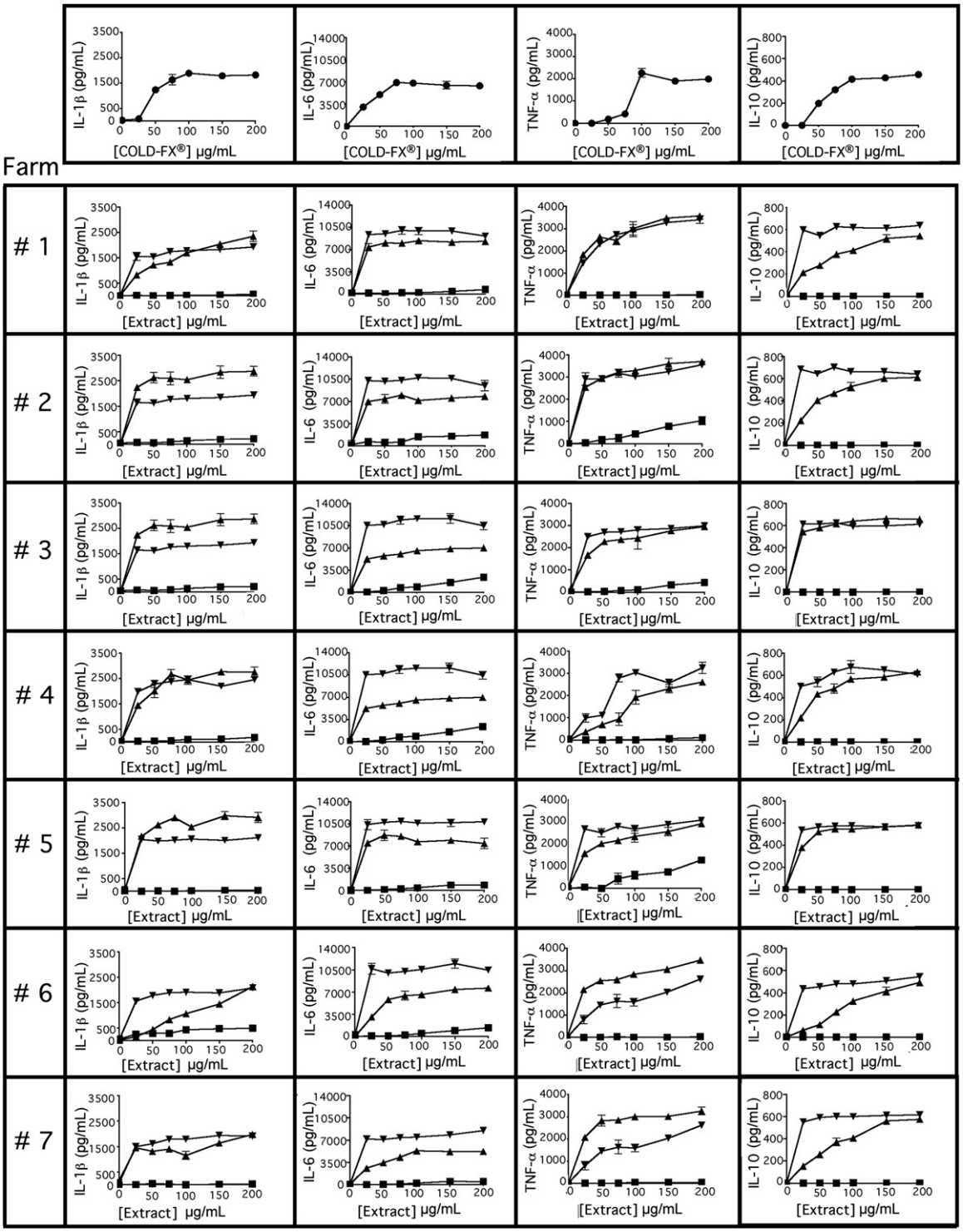

Fig. 1. *Cytokine response of human PBMC to aqueous, polysaccharide and ethanol GS extracts from 7 different North American GS farms*. Human PBMC were stimulated with increasing concentrations of COLD-FX® (top row graphs) or of aqueous (▲), polysaccharide (▼) and ethanol (■) extracts derived from the roots of North American GS plants obtained from 7 different farms for 20 h. IL-1β, IL-6, TNF-α and IL-10 in the supernatants were measured by ELISA. Data represent mean and standard deviation of triplicates, and are representative of 3 independent experiments. Differences between responses to aqueous or polysaccharide extracts vs. ethanol extracts were significant as determined by two-way ANOVA ($P < 0.001$).

among groups. Probability values of $P < 0.05$ were considered significant.

3. Results

3.1. Aqueous and polysaccharide extracts of North American GS induce a broad profile of cytokine production

To start identifying the immunomodulatory effects of GS we first measured the effect of the different North American GS extract formulations on immune cell activation, using induction of cytokine production as a read-out. We found that GS extracts induced the production of IL-6, IL-1β, TNF-α and IL-10 by human PBMC in a dose-dependent manner (Fig. 1). However, there was a marked difference in the magnitude of this effect between different types of GS extracts. Aqueous and polysaccharide extracts induced significantly more cytokine production than the ethanol extract ($P < 0.001$) (Fig. 1). COLD-FX®, used as a control, induced a similar profile of cytokine production as the aqueous and polysaccharide extracts. The cytokine response induced by GS extracts was corroborated using extracts prepared from seven different GS farms (Fig. 1). Furthermore, this response was not due to LPS contamination because no endotoxin was detected with the Limulus test and the cytokine response was resistant to polymyxin B (data not shown).

To determine the contribution of monocytes to the pro-inflammatory IL-6 response of PBMC to GS, two monocyte subsets were isolated. $CD14^+$ monocytes make up the majority of monocytes, approximately 90% in peripheral blood, the other 10% being the highly pro-inflammatory $CD14^+CD16^+$ monocytes, which have a higher potency of antigen presentation than preparations of monocytes based on $CD14^+$ expression only (Ziegler-Heitbrock, 2007). Interestingly, we found no significant difference between the amounts of IL-6 produced by either monocyte subset in response to GS extracts (Figs. 2A and B) but both monocyte subsets produced significantly less ($P < 0.05$) IL-6 in response to the aqueous or polysaccharide extract than PBMC with the same treatments. Interestingly, such a difference was lost when using COLD-FX® (Fig. 2C), suggesting that the IL-6 response to the GS extracts involves a different set of mechanisms.

Next, we tested whether the different GS extracts could inhibit the response to LPS. We observed that neither the GS extracts (polysaccharide, aqueous or ethanol) from 7 different GS farms nor COLD-FX® caused any change in the immune response to LPS as measured by IL-6 production (Fig. 3). This lack of effect was observed across a wide range of LPS and extract concentrations, suggesting that it was not due to saturation of the LPS response.

To further characterize the immune properties of GS extracts we examined the effects of these extracts on the T cell response to superantigens. Superantigens such as SEE activate up to 20% of peripheral blood T cells as illustrated by an increase in IL-2 production (Chau et al., 2009) (Fig. 4). The addition of aqueous or polysaccharide GS extracts during SEE-induced T cell activation resulted in significant ($P < 0.05$) down regulation of the IL-2 response (Fig. 4). This effect was not significant for the ethanol extracts. Furthermore, such an activity was consistently seen across extracts from the seven GS farms tested.

3.2. Transcriptional profile induced by aqueous extracts of North American GS.

To broadly characterize the response of PBMC to an aqueous GS extract we performed DNA microarray analysis of this response. After exclusion of genes with a less than a 2 fold change over baseline, we found that 104 genes were up-regulated and 53 genes

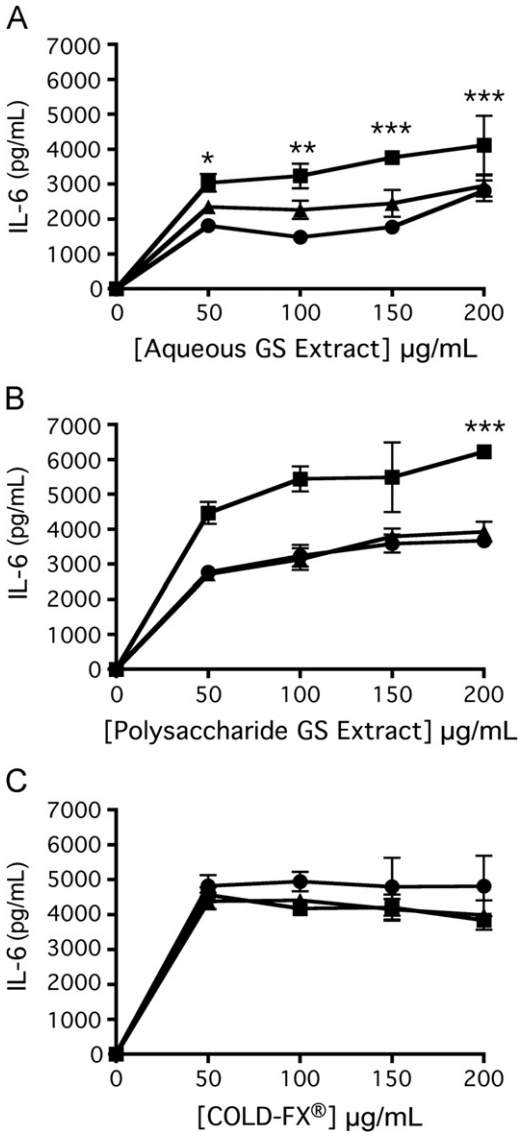

Fig. 2. *IL-6 responses of PBMC, $CD14^+$ monocytes and $CD14^+CD16^+$ monocytes to GS extracts and COLD-FX®.* Human PBMC (■), $CD14^+$ monocytes (▲) and $CD14^+CD16^+$ monocytes (●) from the same donor were stimulated with increasing concentrations of (A) aqueous GS extract, (B) polysaccharide GS extract and (C) COLD-FX® for 20 h. IL-6 accumulation in culture supernatants were measured by ELISA. Data represent mean and standard deviation of triplicates. Differences between the responses of $CD14^+$ and $CD14^+CD16^+$ monocytes were significantly different from those of PBMC as determined by two-way ANOVA, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

were down-regulated in response to the aqueous GS extract. Bioinformatics analysis of these genes revealed a transcriptional profile of immunomodulation (Tables 1 and 2). IFN-γ gene, coding for the primary T_h1 cytokine, was found to be the most up-regulated gene in response to GS. Formyl peptide receptor 3 (FPR3) was the most down-regulated gene. An overwhelming number of the regulated genes coded for cytokines, chemokines, or were genes required for proper immune function. These results were replicated in genetically unrelated donors and broad similarities in the heat map profile were detected in those donors (data not shown).

To control for the quality of the DNA microarray results, we examined the changes at the protein level for some of the most significantly regulated genes. As predicted by the microarray data, we found that IFN-γ, IL-12p40 and IL-23 were up-regulated by COLD-FX®, and the aqueous and polysaccharide GS extracts

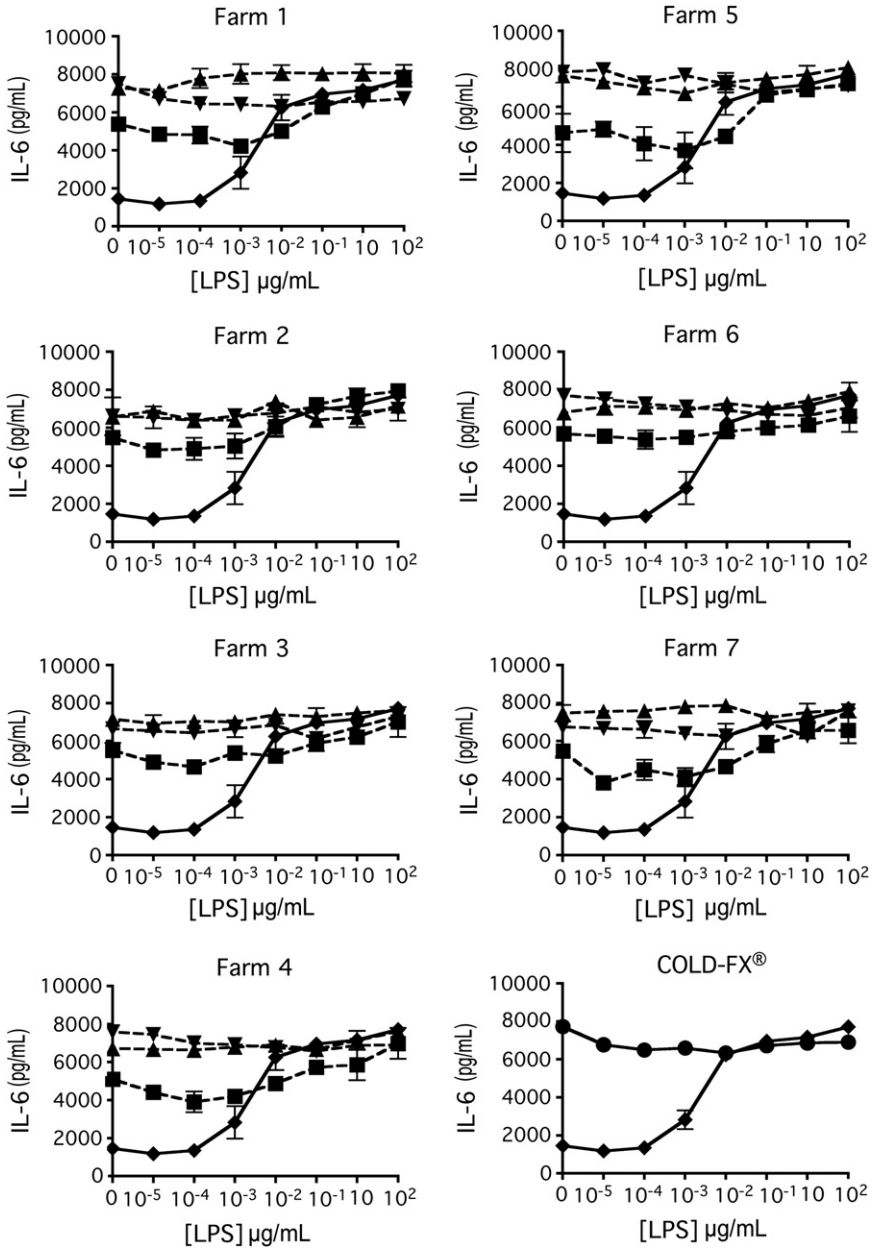

Fig. 3. *Aqueous, polysaccharide and ethanol GS extracts do not inhibit the innate immune response to LPS.* Human PBMC were stimulated with increasing concentrations of LPS alone (♦) or in the presence of 150 µg/mL aqueous (▲), polysaccharide (▼) and ethanol (■) GS extracts from 7 different farms or COLD-FX® (●) for 20 h. IL-6 accumulation in culture supernatants was measured by ELISA. Data represent mean and standard deviation of triplicates.

compared to ethanol GS extract (Fig. 5). CD-14 was identified in the microarray to be down-regulated and this down-regulation was confirmed at the protein level by flow cytometry. After 30 min (data not shown) and 4 h of incubation with the aqueous GS extract or COLD-FX®, there was a significant decrease ($P < 0.05$) in expression levels of cell surface-CD14, measured by mean fluorescents intensity (MFI) (Fig. 6).

Next, we studied the molecular relationship of the top 20 up-regulated genes identified in the microarray analysis using Ingenuity Pathway Analysis (IPA). IPA generated a molecular network identifying which biological pathways were most greatly perturbed by those genes (Fig. 7). This resulted in the identification of several signaling molecules as being involved in the immune response to an aqueous GS extract including p38 MAPK, PI3K (complex), NF-κB (complex) and ERK 1/2.

3.3. The immunomodulatory effects of North American GS extracts are mediated by polysaccharides with a molecular weight higher than 100 kDa

Once we had determined the transcriptional profile induced by an aqueous North American GS extract, we proceeded to narrow down the identity of the compounds involved in this effect. To do this, whole aqueous and polysaccharide GS extracts were fractioned based on molecular weight. PBMC were then treated with different molecular weight fractions or fractioned extracts to determine the cytokine production induced by each fraction. We found that the profile of cytokine production, induced by whole aqueous or polysaccharide extracts was recapitulated when cells were stimulated with fractions containing compounds with a molecular weight greater than 100 kDa

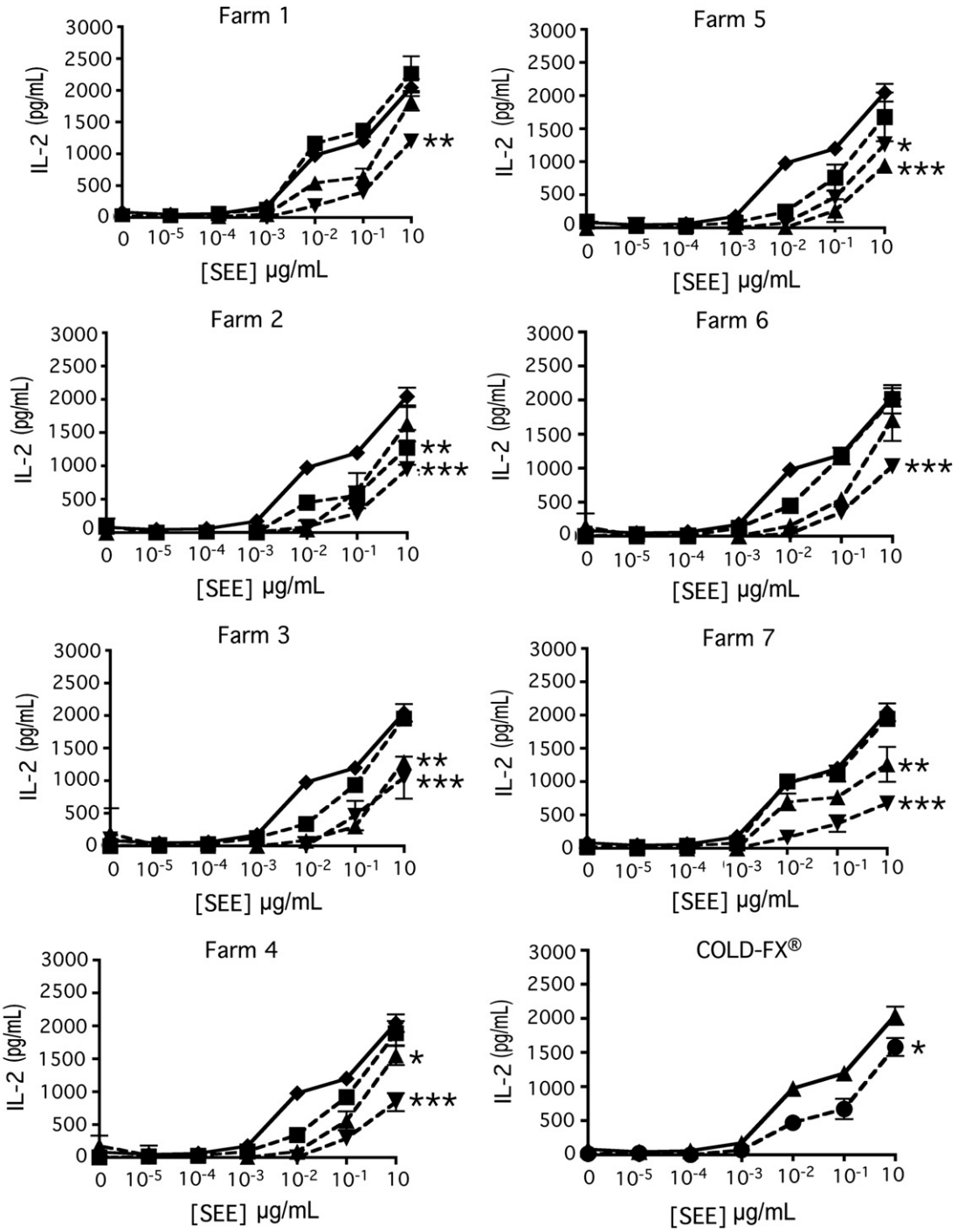

Fig. 4. *Aqueous and polysaccharide GS extracts down-regulate the IL-2 response to S. aureus superantigens (SEE).* Human PBMC were stimulated with increasing concentrations of SEE alone (♦) and with 150 µg/mL of aqueous (▲), polysaccharide (▼) and ethanol (■) GS extracts from 7 different farms or COLD-FX® (●) for 20 h. IL-2 accumulation in culture supernatants was measured by ELISA. Data represent mean and standard deviation of triplicates. Differences between GS extracts or COLD-FX® and the SEE alone group were significantly different as determined by one-way ANOVA. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

(Figs. 8A and B). The 0–10, 10–30, 30–50 and 50–100 kDa fractions produced significantly less ($P<0.01$) IL-6 than the whole aqueous or polysaccharide extracts and the greater than 100 kDa fraction.

Consistent with these results, the greater than 100 kDa fraction of the aqueous GS extracts (Fig. 9A) and of the polysaccharide GS extracts (Fig. 9B) had similar properties as the whole extract, failing to change the response to LPS (data not shown), but significantly ($P<0.001$) decreasing the IL-2 production in response to SEE by PBMC. Together, these data suggest that the active immunomodulatory component of GS extracts is a water-soluble polysaccharide of high molecular weight.

4. Discussion

In this paper, we report that high molecular weight polysaccharides within aqueous North American GS extracts have significant

Table 1
Up-regulated genes in human peripheral blood mononuclear cells in response to aqueous GS extracts.

Rank	Gene symbol	NCBI reference sequence ID	Description	Fold change range of up-regulation
1	IFNG	NM_000619	Interferon, gamma	53.2–55.9
2	MMP1	NM_002421	Matrix metallopeptidase 1 (interstitial collagenase)	14.3–16.7
3	MMP10	NM_002425	Matrix metallopeptidase 10 (stromelysin 2)	12.5–13.0
4	CXCL9	NM_002416	Chemokine (C-X-C motif) ligand 9	7.5–23.1
5	CXCL10	NM_001565	Chemokine (C-X-C motif) ligand 10	5.6–11.0
6	CSF2	NM_000758	Colony stimulating factor 2 (granulocyte-macrophage	4.9–5.3
7	CSF3	NM_172220	Colony stimulating factor 3 (granulocyte)	5.2–8.6
8	IL1F9	NM_019618	Interleukin 1 family, member 9	5.2–23.5
9	IL24	NM_006850	Interleukin 24, transcript variant 1	4.7–8.5
10	IL23A	NM_016584	Interleukin 23, alpha subunit p19	4.2–6.7
11	IL20	NM_018724	Interleukin 20	2.8–3.8
12	F3	NM_001993	Coagulation factor III (thromboplastin, tissue factor)	3.7–18.2
13	IFIT1	NM_001548	Interferon-induced protein with tetratricopeptide repeats 1	3.7–15.6
14	MIR146A	NR_029701.1	Microrna 146a (MIR146A), microRNA	3.6–5.5
15	IL6	NM_000600	Interleukin 6 (interferon, beta 2)	3.5–5.1
16	INHBA	NM_002192	Inhibin, beta A	3.5–20.2
17	TNF*	NM_000594	Tumor necrosis factor	3.3–3.4
18	IFIT2	NM_001547	Interferon-induced protein with tetratricopeptide repeats 2	3.3–8.0
19	RNF152	NM_173557	Ring finger protein 152	2.0–3.2
20	TNFSF15	NM_005118	Tumor necrosis factor (ligand) superfamily, member 15	3.2–8.5
21	RHCG	NM_016321	Rh family, C glycoprotein	3.2–9.1
22	STOM	NM_004099	Stomatin, transcript variant 1	2.9–3.1
23	IFIT3	NM_001031683	Interferon-induced protein with tetratricopeptide repeats 3	3.1–10.2
24	HERC5	NM_016323	Hect domain and RLD 5	3.1–11.1
25	RTP4	NM_022147	Receptor (chemosensory) transporter protein 4	3.1–4.8
26	MIR221	NR_029635.1	MicroRNA 221, microRNA	3.0–3.9
27	GJB2	NM_004004	Gap junction protein, beta 2, 26 kDa	3.0–9.4
28	CXCL2	NM_002089	Chemokine (C-X-C motif) ligand 2	3.0–5.0
29	OAS3	NM_006187	2′-5′-Oligoadenylate synthetase 3, 100 kDa	3.0–9.6
30	RSAD2	NM_080657	Radical S-adenosyl methionine domain containing 2	3.0–17.2
31	MX1	NM_002462	Myxovirus (influenza virus) resistance 1, in interferon-inducible protein p78 (similar to mouse)	3.0–8.1
32	MIR155	NR_030784.1	MicroRNA 155, microRNA	3.0–6.7
33	IL1R1	NM_000877	Interleukin 1 receptor, type I	2.9–8.2
34	GEM	NM_005261	GTP binding protein overexpressed in skeletal muscle	2.9–4.0
35	IFI35	NM_005533	Interferon-induced protein 35	2.8–5.8
36	C13orf29	NR_027701	Chromosome 13 open reading frame 29	2.7–3.1
37	USP18	NM_017414.1	Ubiquitin specific peptidase 18	2.7–14.7
38	TFPI2	NM_006528	Tissue factor pathway inhibitor 2 (TFPI2	2.7–5.4
39	CD69	NM_001781	CD69 molecule, transcript variant 1	2.7–3.2
40	USP18	NM_017414	Ubiquitin specific peptidase 18	2.7–11.4
41	DHX58	NM_024119	DEXH (Asp-Glu-X-His) box polypeptide 58	2.6–5.5
42	SPINK1	NM_003122	Serine peptidase inhibitor, Kazal type 1	2.6–4.5
43	CXCL11	NM_005409	Chemokine (C-X-C motif) ligand 11	2.6–17.1
44	CFB	NM_001710	Complement factor B	2.6–13.5
45	BST2	NM_004335	Bone marrow stromal cell antigen 2	2.6–3.6
46	PNPT1	NM_033109	Polyribonucleotide nucleotidyltransferase 1	2.6–7.7
47	IFI44	NM_006417	Interferon-induced protein 44	2.6–8.6
48	GPRC5A	NM_003979	G protein-coupled receptor, family C, group 5, member A	2.5–4.9
49	CFB	NM_001710	Complement factor B	2.5–12.7
50	OAS1	NM_016816	2′,5′-Oligoadenylate synthetase 1, 40/46 kDa	2.5–6.2
51	PRIC285	NM_001037335	Peroxisomal proliferator-activated receptor A interacting complex 285	2.5–3.9
52	HSH2D	NM_032855	Hematopoietic SH2 domain containing	2.5–2.1
53	STAT2	NM_005419	Signal transducer and activator of transcription 2, 113 kDa	2.5–3.2
54	LY6E	NM_002346	Lymphocyte antigen 6 complex, locus E	2.5–5.1
55	DDX58	NM_014314	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2.5–5.4
56	PGAP1	NM_024989	Post-GPI attachment to proteins 1	2.5–3.9
57	ZBP1	NM_030776	Z-DNA binding protein 1 (ZBP1),	2.4–2.5
58	HERC6	NM_017912	Hect domain and RLD 6	2.4–8.5
59	PMAIP1	NM_021127	Phorbol-12-myristate-13-acetate-induced protein 1	2.4–4.0
60	GBP5	NM_052942	Guanylate binding protein 5	2.4–4.1
61	CXCL3	NM_002090	Chemokine (C-X-C motif) ligand 3	2.4–3.3
62	PARP9	NM_031458	Poly-(ADP-ribose) polymerase family, member 9	2.4–5.4
63	IFI44L	NM_006820	Interferon-induced protein 44-like	2.4–9.6
64	PRKD2	NM_016457	Protein kinase D2	2.3–2.1
65	IFIH1	NM_022168	Interferon induced with helicase C domain 1	2.3–5.8
66	ISG20	NM_002201	Interferon stimulated exonuclease gene 20 kDa	2.3–2.9
67	LIF	NM_002309	Leukemia inhibitory factor (cholinergic differentiation factor)	2.1–2.3
68	IRF7	NM_004031	Interferon regulatory factor 7	2.3–2.5
69	PRF1	NM_005041	Perforin 1 (pore forming protein)	2.1–2.3
70	SLC38A5	NM_033518	Solute carrier family 38, member 5	2.3–2.9
71	TM4SF1	NM_014220	Transmembrane 4 L six family member 1	2.3–4.7
72	CFB	NM_001710	Complement factor B	2.3–9.1
73	RASGRP3	NM_170672	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	2.3–4.0

Table 1 (*continued*)

Rank	Gene symbol	NCBI reference sequence ID	Description	Fold change range of up-regulation
74	SLC4A10	NM_001178015	Solute carrier family 4, sodium bicarbonate transporter, member 10	2.3–3.4
75	GNG2	NM_053064	Guanine nucleotide binding protein (G protein), gamma 2	2.3–2.5
76	AREG	NM_001657	Amphiregulin	2.2–3.6
77	PPM1K	NM_152542	Protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1 K	2.2–3.3
78	AREG	NM_001657	Amphiregulin	2.2–4.1
79	MX2	NM_002463	Myxovirus (influenza virus) resistance 2 (similar to mouse)	2.2–4.7
80	EPSTI1	NM_001002264	Epithelial stromal interaction 1 (breast)	2.2–7.0
81	APOL1	NM_145343	Apolipoprotein L, 1	2.2–3.4
82	TXN	NM_003329	Thioredoxin (TXN	2.2–6.2
83	CCNA1	NM_003914	Cyclin A1 (CCNA1), transcript variant 1,	2.2–3.5
84	EREG	NM_001432	Epiregulin	2.2–3.9
85	MOV10	NM_020963	Mov10, Moloney leukemia virus 10, homolog (similar to mouse)	2.2–2.6
86	PARP14	NM_017554	Poly-(ADP-ribose) polymerase family, member 14	2.2–3.7
87	DDX60	NM_017631	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	2.1–6.6
88	CMPK2	NM_207315	Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	2.1–7.8
89	RGS1	NM_002922	Regulator of G-protein signaling 1	2.1–2.9
90	TDRD7	NM_014290	Tudor domain containing 7	2.1–4.2
91	CCL3	NM_002983	Chemokine (C-C motif) ligand 3	2.1–4.3
92	OASL	NM_003733	2′-5′-Oligoadenylate synthetase-like	2.1–3.9
93	TMEM140	NM_018295	Transmembrane protein 140	2.1–2.4
94	OAS2	NM_002535	2′-5′-Oligoadenylate synthetase 2, 69/71 kDa	2.1–4.3
95	SLFN12L	NM_001145027	Sapiens schlafen family member 12-like	2.0–2.1
96	PML	NM_033240	Promyelocytic leukemia	2.1–3.3
97	FNIP2	NM_020840	Folliculin interacting protein 2	2.1–4.6
98	DUSP5	NM_004419	Dual specificity phosphatase 5	2.1–2.6
99	STAP1	NM_012108	Signal transducing adapter family member 1	2.1–4.4
100	CCL20	NM_004591	Chemokine (C-C motif) ligand 20	2.0–12.0
101	LAMP3	NM_014398	Lysosomal-associated membrane protein 3	2.0–4.2
102	CCRN4L	NM_012118	CCR4 carbon catabolite repression 4-like (*Saccharomyces cerevisiae*)	2.0–3.8
103	CHRNA6	NM_004198	Cholinergic receptor, nicotinic, alpha 6	2.0–3.3
104	TNFRSF9	NM_001561	Tumor necrosis factor receptor superfamily, member 9	2.0–3.2

Table 2
Down-regulated genes in human peripheral blood mononuclear cells in response to aqueous GS extracts.

Rank	Gene symbol	NCBI reference sequence ID	Description	Fold change range of down-regulation
1	FPR3	NM_002030	Formyl peptide receptor 3	from −13.4 to −10.8
2	GAPT	NM_152687	GRB2-binding adapter protein, transmembrane	from −6.3 to −5.3
3	CCR2	NM_001123396	Chemokine (C-C motif) receptor 2	from −7.0 to −6.2
4	CSF1R	NM_005211	Colony stimulating factor 1 receptor	from −18.3 to −6.1
5	STEAP4	NM_024636	STEAP family member 4	from −6.0 to −3.8
6	VCAN	NM_004385	Versican (VCAN), transcript variant 1,	from −13.8 to −5.9
7	LILRA2	NM_001130917	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2	from −5.2 to −4.6
8	TGFBI	NM_000358	transforming growth factor, beta-induced, 68 kDa	from −4.9 to −4.8
9	LILRA1	NM_006863	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1	from −4.6 to −3.7
10	CCR2	NM_001123041	Chemokine (C-C motif) receptor 2	from −5.4 to −4.1
11	CCR1	NM_001295	Chemokine (C-C motif) receptor 1	from −3.8 to −2.7
12	CCL24	NM_002991	Chemokine (C-C motif) ligand 24	from −6.3 to −3.4
13	LRRC25	NM_145256	Leucine rich repeat containing 25	from −3.6 to −3.5
14	FN1	NM_212482	Fibronectin 1 (FN1), transcript variant 1	from −4.4 to −3.6
15	PLXDC2	NM_032812	Plexin domain containing 2	from −3.6 to −3.0
16	CD14	NM_000591	CD14 molecule (CD14), transcript variant 1	from −4.2 to −3.3
17	MPEG1	NM_001039396	Macrophage expressed 1	from −3.3 to −2.5
18	LILRB1	NM_006669	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	from −3.0 to −2.8
19	PECAM1	NM_000442	Platelet/endothelial cell adhesion molecule	from −4.5 to −3.0
20	SLC8A1	NM_021097	Solute carrier family 8 (sodium/calcium exchanger), member 1	from −3.2 to −2.9
21	PPARG	NM_138712	Peroxisome proliferator-activated receptor gamma	from −3.3 to −2.7
22	CTNND1	NM_001085458	Catenin (cadherin-associated protein), delta 1	from −2.7 to −2.3
23	HMOX1	NM_002133	Heme oxygenase (decycling) 1	from −3.4 to −2.7
24	MIR223	NR_029637.1	microrna 223, microRNA	from −2.7 to −2.3
25	TNS1	NM_022648	Tensin 1	from −7.1 to −2.7
26	CMKLR1	NM_001142343	Chemokine-like receptor 1	from −2.7 to −2.6
27	C17orf60	NM_001085423	Chromosome 17 open reading frame 60	from −2.6 to −2.5
28	CXCL16	NM_022059	Chemokine (C-X-C motif) ligand 16	from −2.6 to −2.5
29	ENG	NM_000118	Endoglin (ENG), transcript variant 2	from −4.2 to −2.6

Table 2 (*continued*)

Rank	Gene symbol	NCBI reference sequence ID	Description	Fold change range of down-regulation
30	GPNMB	NM_001005340	Glycoprotein (transmembrane) nmb	from −9.9 to −2.6
31	MAFB	NM_005461	v-Maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	from −2.7 to −2.5
32	IFI30	NM_006332	Interferon, gamma-inducible protein 30	from −2.5 to −2.4
33	SIGLEC10	NM_033130	Sialic acid binding Ig-like lectin 10	from −2.5 to −2.2
34	FAM198B	NM_001128424	Family with sequence similarity 198, member B	from −2.4 to −2.1
35	ARHGAP18	NM_033515	Rho GTPase activating protein 18	from −2.7 to −2.4
36	CFP	NM_002621	Complement factor properdin (CFP	from −2.9 to −2.4
37	CD300E	NM_181449	CD300e molecule	from −2.6 to −2.4
38	VNN2	NM_004665	Vanin 2, transcript variant 1	from −2.8 to −2.4
39	TM6SF1	NM_023003	Transmembrane 6 superfamily member 1	from −2.4 to −2.3
40	PLXNB2	NM_012401	Plexin B2	from −3.6 to −2.3
41	NLRP12	NM_144687	NLR family, pyrin domain containing 12	from −5.3 to −2.3
42	CX3CR1	NM_001337	Chemokine (C-X3-C motif) receptor 1	from −2.7 to −2.3
43	HIP1	NM_005338	Huntingtin interacting protein 1	from −3.1 to −2.3
44	SLC37A2	NM_198277	Solute carrier family 37 (glycerol-3- phosphate transporter), member 2	from −5.0 to −2.3
45	CYP27A1	NM_000784	Cytochrome P450, family 27, subfamily A, polypeptide 1	from −6.3 to −2.2
46	KIAA1598	NM_001127211	KIAA1598, transcript variant 1	from −2.4 to −2.2
47	KIAA1539	BC004406	KIAA1539 (cDNA clone MGC:10998 IMAGE:3)	from −2.1 to −2.0
48	EEPD1	NM_030636	Endonuclease/exonuclease/phosphatase family domain containing 1	from −4.4 to −2.1
49	CD300C	NM_006678	CD300c molecule	from −2.1 to −2.1
50	SERPINE1	NM_000602	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	from −3.0 to −2.1
51	QSOX1	NM_002826	Quiescin Q6 sulfhydryl oxidase 1	from −2.5 to −2.2
52	GLUL	NM_002065	Glutamate-ammonia ligase	from −2.2 to −2.0
53	CD9	NM_001769	CD9 molecule	from −2.4 to −2.0

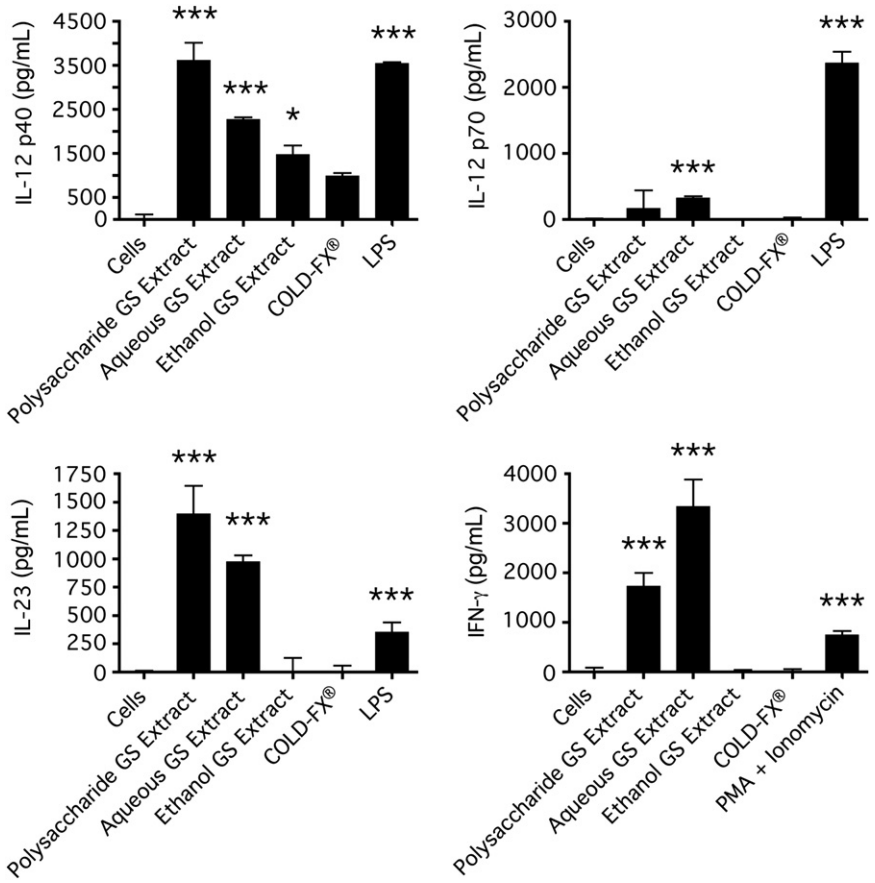

Fig. 5. *IL-12, IL-23 and IFN-γ responses of PBMC to different GS extracts and COLD-FX®.* Human PBMC were stimulated with 150 μg/mL of aqueous, polysaccharide and ethanol GS extracts or COLD-FX®, 100 ng/mL of LPS or 50 ng/mL of PMA and 1 mg/mL of ionomycin. IL-12p40, IL-12p70, IL-23 and IFN-γ accumulation in culture supernatants was measured by ELISA. Data represent mean and standard deviation of 3 independent experiments. Differences between cytokine production of control, COLD-FX® and GS extracts was determined by one-way ANOVA, **: $P < 0.01$, ***: $P < 0.001$.

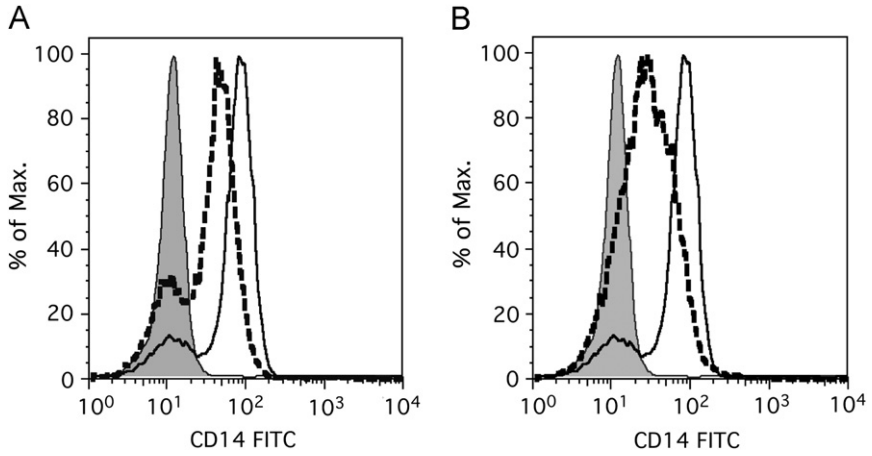

Fig. 6. *Aqueous GS extract decreases surface CD-14 expression*. Human PBMC were stimulated for 4 h with 200 μg/mL of aqueous extract (A) or COLD-FX® (B). CD-14 expression was measured by flow cytometry using an isotype control (shaded histogram), no treatment (continuous line histogram in A and B), or treated with aqueous extract (dashed line histogram in A) or COLD-FX® (dashed line histogram in B). Data represent mean and standard deviation of triplicates. Differences between responses to control and aqueous GS extract or COLD-FX® were significant as determined by one-way ANOVA ($P < 0.05$).

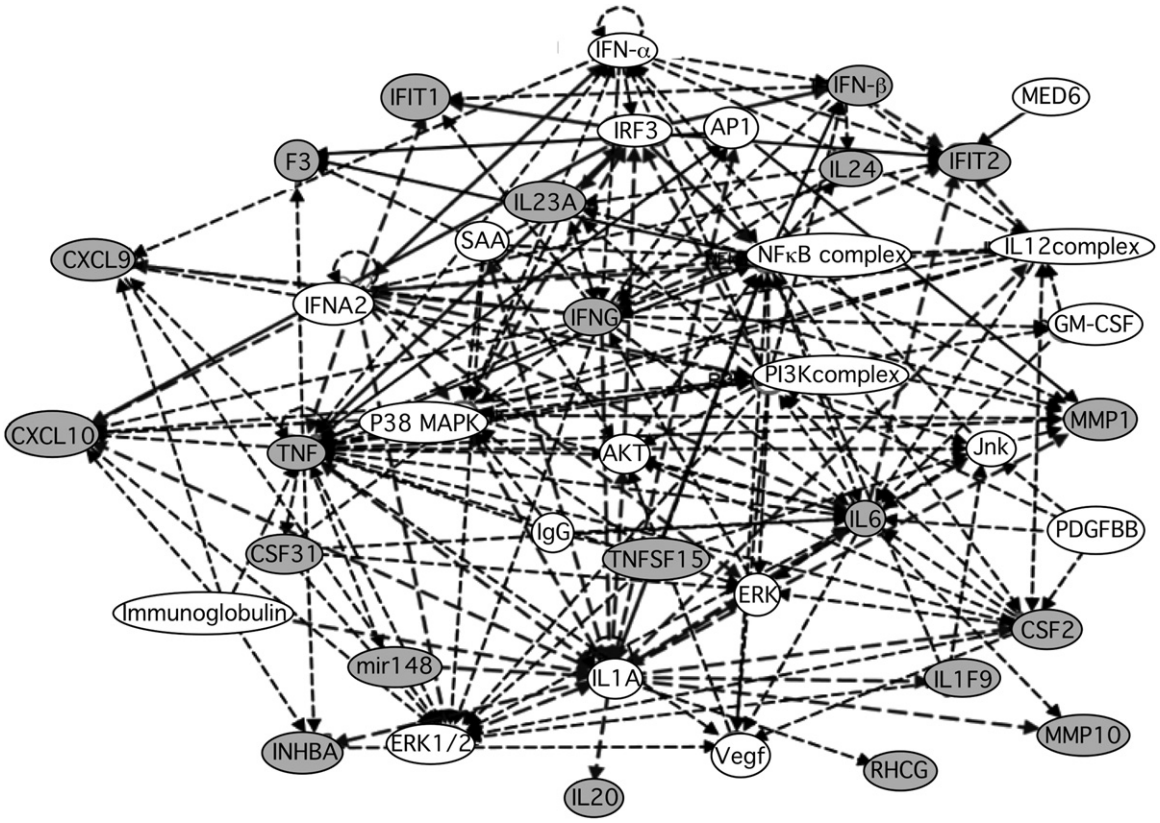

Fig. 7. *Ingenuity Pathway Analysis of the top 20 up-regulated genes after exposure to an aqueous GS extract*. Shaded molecules indicate the genes that were directly up-regulated in response to aqueous extract. Unshaded molecules indicate genes inferred to be up-regulated upon treatment with aqueous extract.

immunomodulatory properties characterized by the induction of pro-inflammatory cytokines along a T_h1 profile. Such a response results from broad transcriptional imprinting for immunomodulation. Based on bioinformatics analysis, North American GS immunomodulation is likely mediated by MAPK (ERK 1/2), PI3K, p38 and NF-κB signaling, that leads to broad cytokine production including IL-6, IL-2, IL-1β, TNF-α, IFN-γ, IL-12 and IL-23. To our knowledge this work is the first to report the transcriptional profile of primary human immune cells in response to an aqueous North American GS extract.

The molecules responsible for the immunomodulatory effects of GS have long been thought to be the low molecular weight steroidal saponins called ginsenosides (Attele et al., 1999; Choi, 2008; Lu et al., 2009; Qi et al., 2011). However, it has been recently shown that polysaccharides in GS may also play a role (Hwang and Jeong, 2010; King and Murphy, 2007; Luo et al., 2008; Wang and Yuan, 2008; Yoo et al., 2010). Based on previous studies, the aqueous extract of GS contains a complex mixture of water-soluble components including polyacetylenic alcohols, ginsenosides and polysaccharides (Assinewe et al., 2002; Azike et al., 2011) while the polysaccharide extract contains only the polysaccharides present in the aqueous extract. Fractionation of the aqueous or polysaccharide extracts by molecular weight allowed us to link immunomodulation with the high molecular

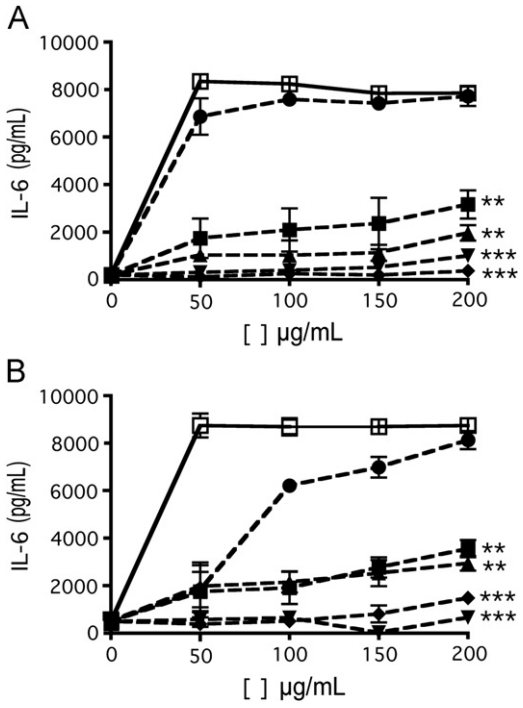

Fig. 8. *The immunomodulatory properties of aqueous and polysaccharide GS extracts are mediated by compounds with a molecular weight higher than 100 kDa.* Human PBMCs were stimulated with increasing concentrations of fractionated aqueous extract (A) or polysaccharide extract (B) of MWs < 10 kDa (■), 10–30 kDa (▲), 30–50 kDa (▼), 50–100 kDa (♦) and > 100 kDa (●) or whole extracts (□) for 20 h. IL-6 concentration in culture supernatants was measured by ELISA. Data represent mean and standard deviation of triplicates and are representative of 2 independent experiments. **: $P < 0.01$; ****: $P < 0.001$.

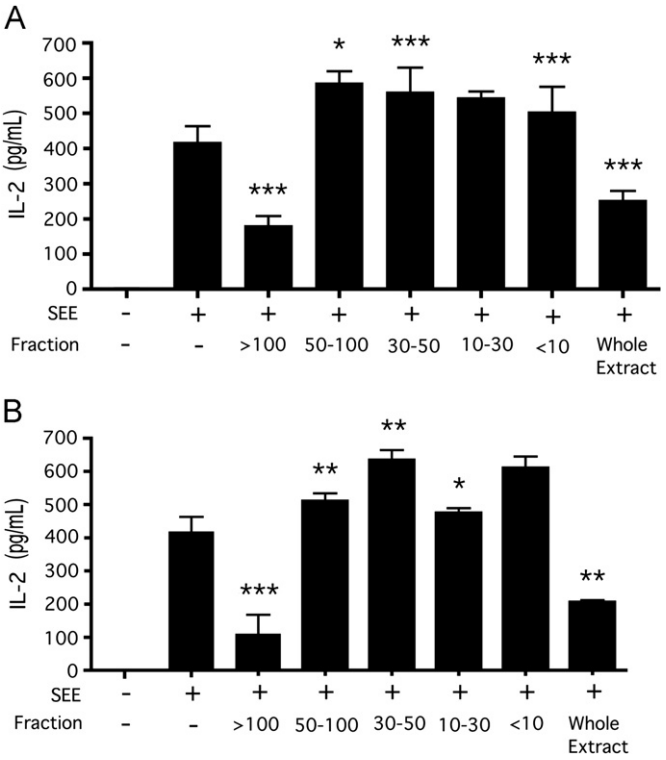

Fig. 9. *The "molecular weight greater than 100 kDa" fraction of aqueous and polysaccharide GS extracts recapitulate immunomodulation of T cells responses by aqueous and polysaccharide GS extracts.* Human PBMC were stimulated with 0.1 ng/mL of SEE in the absence or presence of 25 µg/mL of fractionated or whole aqueous extracts (A) or fractionated or whole polysaccharide extracts (B). IL-2 concentration in culture supernatants was measured by ELISA. Differences between responses to fractions or whole extract were significantly different from SEE alone as determined by one-way ANOVA *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

weight fraction of GS. This fraction contains a complex mixture of polysaccharides with molecular weights greater then 100 kDa (Wang et al., 2001). In contrast, fractions containing polysaccharides of molecular weight less than 100 kDa had little immunomodulatory effects. These low molecular weight fractions likely contained ginsenosides as they have molecular weights between 5 and 15 kDa (Chen et al., 2008). Together, this reinforces the idea that immunomodulation is not primarily induced by ginsenosides.

One interesting finding from this work is that the immunomodulatory properties of polysaccharides within North American GS extracts are not recapitulated by COLD-FX®, a preparation that contains 80% of poly-furanosyl-pyranosyl-saccharides from North American GS (Wang et al., 2004). COLD-FX® induced less cytokine production by PBMC and had less effect on the IL-2 response of T cells stimulated with SEE than either the aqueous or polysaccharide extracts. It is possible that it is the complex mix of polysaccharides found in the aqueous and polysaccharide GS extracts that have a broader profile of effects by acting on more cell types than COLD-FX®. This is suggested by the observation that the response to COLD-FX® was similar between both $CD14^+$ and $CD14^+CD16^+$ isolated monocytes and PBMC. However, the response of monocytes to GS extracts was only half of the response observed with PBMC. While poly-furanosyl-pyranosyl-saccharides in COLD-FX® show immunomodulatory properties, there are other polysaccharides from GS that have shown immune effects, including panaxans, ginsan (Shin et al., 2002), arabinogalactans (Schepetkin and Quinn, 2006), rhamnogalacturonan II (Shin et al., 1997), galactose, arabinose, rhamnose, glucose, fucose and mannose (Assinewe et al., 2002). While the evidence points to a complex mixture of polysaccharides isolated from North American GS to be the active immunomodulatory compounds, further work is necessary to better understand exactly which polysaccharides and in what proportions may be most beneficial for immune modulation.

The mechanism of action of GS polysaccharides is unknown. Many plant polysaccharides and phenols have been shown to bind to CD14 and TLR-4 (Delehanty et al., 2007; Yoon et al., 2003) but this may be secondary to the presence of LPS. Of interest, CD14 was one of the molecules identified by our microarray experiments as being down-regulated in response to GS treatment, a result confirmed by flow cytometry. CD14 is a GPI-anchored membrane protein that acts as the main receptor for LPS together with TLR-4 and MD2 (Kitchens, 2000). Down-regulation of surface CD14 may indicate modulation of this receptor upon interaction with GS or could be a secondary event following exposure to GS. Our data suggest mostly the latter, occurring at the transcriptional level. In any case, this effect is not sufficient in vitro to decrease the response to LPS.

Network analysis to determine the signaling pathways most perturbed by GS extracts suggested that the GS-induced immune modulation occurs through activation of the NF-κB, PI3K, p38 and MAPK (ERK1/2) signaling pathways. These pathways are involved in the production of pro-inflammatory cytokines (Buhrmann et al., 2011; Kovac et al., 2011; Sun et al., 2011) and have previously been shown to be induced by GS extract stimulation in PBMC (Toth et al., 2010). In addition, these pathways are also involved in other biological activities of GS such as decreasing ischemic reperfusion injury (Liu et al., 2010), killing of tumors (Ming et al., 2011; Wong et al., 2010) and reducing damage from oxidative stress (Liu et al., 2010). Similarly, PI3K signaling after GS treatment has been linked to the decrease of ischemic reperfusion injury (Tsutsumi et al., 2011; Wang and Yuan, 2008) as well as to glucose uptake (Lee et al., 2011; Shang et al., 2008). Activation of

p38 and MAPK after treatment with GS has been implicated in inhibiting angiogenesis in human endothelial cells (Jeong et al., 2010) as well as causing preferential apoptosis to cancer cells (Kim et al., 2011). Therefore, there seems to be a commonality of signaling events triggered by GS across different cell types.

Among the genes up-regulated by GS, IFN-γ is the top one, coding for the primordial T_h1 cytokine IFN-γ. Of interest, while the IL-2 response of peripheral blood T cells to SEE was reduced by GS, the IFN-γ response was not. This suggests that the IFN-γ response induced by GS extracts may come from sources other than activated T cells, likely NK cells. In this context, induction of a T_h1 profile of immunity supports the claim to use GS extracts to combat respiratory viral infections, as it would enhance cellular immunity.

It is important to note that the pro-inflammatory effect of GS extracts balanced with the induction of IL-10, an anti-inflammatory cytokine. Production of IL-10 may counteract the pro-inflammatory T_h1 profile, and explain the findings of other groups that have reported a humoral T_h2 response to GS exposure (Wang et al., 2001; Zhou and Kitts, 2002). The magnitude of the IL-10 response varies between extracts and this may explain the differences among reports.

In summary, we show here that a complex mixture of high molecular weight polysaccharides present in water-soluble extracts of GS mediate most of the immunomodulatory properties of North American GS. These properties include the induction of a pro-inflammatory T_h1 cytokine production profile and IL-10. This profile of response is determined by a strong immunomodulatory transcriptional signature secondary to signaling through the MAPK (ERK 1/2), PI3K, p38 and NF-κB pathways. Our work thus provides a comprehensive model for the immunomodulatory effects of North American GS extracts on primary human immune cells.

Acknowledgments

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