Blueberries reduce pro-inflammatory cytokine TNF-α and IL-6 production in mouse macrophages by inhibiting NF-κB activation and the MAPK pathway.
Supplement information files (SIF): Blueberries reduce pro-inflammatory cytokine TNF-α and IL-6 production in mouse macrophages by inhibiting NF-κB activation and the MAPK pathway

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Materials and methods

Chemicals and Reagents

LPS and zeocin were purchased from Invivogen (San Diego, CA). oxLDL was purchased from Academy Biomedicals (Houston, TX). All solvents were obtained from Fisher Scientific (Pittsburg, PA).

Animals

The animal protocols were approved by the Animal Care and Use Committee of the Arkansas Children’s Hospital Research Institute (permission number; 3035). Female apoE⁻/⁻ mice and Sprague-Dawley rats were obtained from Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA), respectively. Animals were maintained in sterile microisolator cages and fed autoclaved-pelleted diet and water ad libitum.

AIN-93G (CD) or AIN-93G containing 1% freeze-dried whole blueberries (BB) powder (provided by VDF/FutureCeuticals) was made by Harlan Teklad (Madison, WI). Diet formulation was presented in an earlier paper [1]. ApoE⁻/⁻ mice were fed CD or BB for 5 weeks. Three days prior to sacrifice, 3% thioglycollate was injected ip into the mice for peritoneal macrophage collection. At the end of 5 weeks, mice were euthanized using CO₂. The sera from
all mice were collected and peritoneal macrophages from five mice per group were collected and 
cultured.

Sprague-Dawley rats were fed CD or CD containing 10% freeze-dried BB for 6 weeks. Diet 
formulation was described previously [2]. At the end of 6 weeks, rats were euthanized using 
CO₂ and the sera from all rats were collected. These sera were used to produce polyphenol-
enriched extracts described later in the method section.

Cell culture

Thioglycollate-elicited peritoneal macrophages were plated in RPMI-1640 supplemented with 
10% (v/v) FBS, 2 mM L-glutamine, penicillin, streptomycin, and sodium pyruvate. Non-
adherent cells were removed after 2 h and macrophages used after 48 h.

RAW264.7 macrophage cell line (Invivogen, San Diego, CA) were cultured in DMEM 
supplemented with 10% (v/v) FBS (Hyclone, Logan, UT) and zeocin (200 µg/mL).

Protein measurement in serum

TNF-α and IL-6 were measured in the serum by Mouse Proinflammtory 7-Plex Assay Ultra-
Sensitive Kit obtained from Meso Scale Discovery (MSD) (Gaithersburg, MD). The cytokine 
assays were performed with an ultra-low-noise charge-coupled device (CCD) Sector Imager 
2400 provided by MSD (Gaithersburg, MD) according to manufacturer’s protocol.

RT-PCR array analyses of aorta samples

Pooled aorta samples (4/group, 3 pools) were used for RT-PCR array. The sample preparation 
and the PCR-array using custom real-time PCR-array (SA Biosciences, Frederick, MD) were 
performed according to the method described previously by our group [1].
Preparing polyphenol-enriched extracts from sera

Sera from rats fed CD or CD containing 10% freeze-dried BB were used to prepare polyphenol-enriched extracts by following procedures: 5 mL of pooled sera from each treatment group were extracted with hexane (5 mL×2) to remove lipids. The left sera (1 mL of sera/cartridge) were loaded into pre-equilibrated solid phase extraction (SPE) cartridges, of which sorbent is styrene-divinylbenzene (Strata-X, 500 mg, 6 mL) from Phenomenex (Torrance, CA). The cartridge was eluted with water containing 0.2 % formic acid and total polyphenols were recovered by acetonitrile containing 0.2 % formic acid. The acetonitrile solution was dried under N₂ flow and reconstituted with 500 µL of methanol. After centrifugation, the supernate of MeOH solution was taken and dried under N₂ flow and then re-dissolved in 200 µL of DMSO for cell treatment. The final solution from sera of mice fed CD was referred as SEC and that from mice fed CD containing 10% BB was referred as SEB.

Real-time RT-PCR analysis

Procedures of real time RT-PCR were described previously [3]. Real-time PCR primers (from Integrated DNA Technologies, Coralville, IA) were as follows: β-actin sense (GGCTATGCTCTCCCTCACG), β-actin antisense (CGCTCGGTACGGATCTTCAT), TNF-α sense (ACAAGGCTGCCCCGACTAC), TNF-α antisense (TGGAAGACTCCTCCCAGGTATATG), IL-6 sense (TGGAGTCACAGAAGGAGTGGCTAAG), and IL-6 antisense (TCTGACCACAGTGAGGAATGTCCAC).

TNF-α and IL-6 ELISA
RAW-Blue cells or peritoneal macrophages (5x10⁶ cells/well) were pretreated with various concentrations of the indicated reagents for 1 h before LPS stimulation. After 18 h stimulation, supernatant was collected; TNF-α and IL-6 in the supernatant was determined by ELISA using Duoset ELISA kits (R&D, Minneapolis, MN) according to the previously described method [3].

**Signaling pathway analysis**

Raw-Blue cells treated with indicated reagents were lysed in RIPA buffer with protease and phosphatase inhibitors (Cell Signaling, Danvers, MA). Cell lysates were centrifuged at 10,000 rpm for 15 min to remove cell debris. Protein concentrations were determined by Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). The lysate was used for signaling analysis by two PathScan Sandwich Elisa Kits (Cell Signaling): PathScan Multi-Target Sandwich Elisa Kit, which was designed to measure phospho-NF-κB-p65, phospho-SAPK/JNK, phospho-p38 MAPK, phosphor-Stat3 and phospho-IκBα; as well as PathScan Total p44/42 MAPK (Erk1/2) Sandwich Elisa Kit for phospho-p44/42. The assays were conducted according to manufacturer’s protocol.

**Statistical analysis**

Data were expressed as mean value ± SEM unless otherwise mentioned. Student’s t test was used to analyze differences between groups. One-way ANOVA with the Student-Newman-Keuls Method was used to compare more than two groups. A value of \( p < 0.05 \) was considered as a significant difference. Statistic analyses were performed by SigmaStat statistical software (SigmaStat 3.5).
References in Materials and Method:


Figure 1.

Proposed mechanisms of blueberries in reducing TNF-α and IL-6 production in macrophages.