

FOOD & FUNCTION

Blueberries reduce pro-inflammatory cytokine TNF- α and IL-6 production in mouse macrophages by inhibiting NF- κ B activation and the MAPK pathway

Chenghui Xie¹, Jie Kang¹, Matthew E. Ferguson², Shanmugam Nagarajan³, Thomas M. Badger¹ and Xianli Wu¹

¹ USDA Arkansas Children's Nutrition Center, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR, USA

² USDA Arkansas Children's Nutrition Center, Little Rock, AR, USA

³ USDA Arkansas Children's Nutrition Center, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Blueberries (BB) have been reported to attenuate atherosclerosis in apoE-deficient (ApoE^{-/-}) mice. The aim of this study was to evaluate the effects of BB in reducing pro-inflammatory cytokine production in mouse macrophages. ApoE^{-/-} mice were fed AIN-93G diet (CD) or CD formulated to contain 1% freeze-dried BB for 5 wk. TNF- α and IL-6 were lower in serum of BB-fed mice and TNF- α expression in aorta was down-regulated with BB feeding. Protein level and mRNA expression of TNF- α and IL-6 were significantly lower in the peritoneal macrophages from mice fed BB without or with LPS or oxLDL stimulation. RAW264.7 macrophages were treated with polyphenol-enriched extracts made from the sera of rats fed CD (SEC) or CD containing 10% BB (SEB). SEB significantly inhibited LPS-induced mRNA expression and protein levels of TNF- α and IL-6. Furthermore, SEB inhibited the phosphorylation of I κ B, NF- κ B p65, MAPK p38 and JNK. All of these are important signaling pathways involved in the production of TNF- α and IL-6.

Received: May 23, 2011

Revised: June 13, 2011

Accepted: June 28, 2011



Keywords:

Blueberry / Cytokine / Inflammation / MAPK / NF- κ B

Blueberries (BBs) have been implicated in preventing cardiovascular diseases [1], yet the *in vivo* experimental evidence that consumption of BBs reduces the risk of cardiovascular disease and the underlying mechanisms remained to be determined. In a recent publication by our group,

apolipoprotein E deficient- (apoE^{-/-}) mice fed 1% freeze-dried BBs developed significantly fewer atherosclerotic lesions in aorta and certain antioxidant enzymes were up-regulated [2]. Nevertheless, these findings did not rule out the possibilities that other mechanisms may also be involved.

It has been widely accepted that inflammation plays a critical role in the pathogenesis of atherosclerosis. The recruitment and activation of macrophages is considered as the most important early event in the development of atherosclerotic lesions. Activated macrophages release various pro-inflammatory cytokines that amplify the local inflammatory response in the lesion [3]. Tumor necrosis factor- α (TNF- α) and IL-6 are among the most potent inflammatory cytokines underlying atherosclerosis. Increases in TNF- α and IL-6 were proposed as predictive biomarkers of cardiovascular mortality [4, 5]. In apoE^{-/-} mice, IL-6 was found to exacerbate early atherosclerosis, and it was suggested that pro-inflammatory cytokines may participate

Correspondence: Dr. Xianli Wu, USDA Arkansas Children's Nutrition Center, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, 15 Children's Way, Little Rock, AR 72202, USA
E-mail: wuxianli@uams.edu
Fax: +1-501-364-3161

Abbreviations: ApoE^{-/-}, apolipoprotein E-deficient; BB, blueberries; CD, AIN-93G diet; I κ B, inhibitor of NF- κ B; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; oxLDL, oxidized LDL; SEB, sera of rats fed with CD containing 10% BB; SEC, sera of rats fed with CD; TNF- α , tumor necrosis factor- α

in early lesion development [6]. TNF- α was demonstrated to be actively involved in the progression of atherosclerosis in apoE^{-/-} mice, and thus may be a possible target for prevention of atherosclerosis [7].

BBs or BB extracts have been found to inhibit pro-inflammatory cytokine production in human subjects [8, 9]. Treatment of BV2 microglial cells with BB extracts has also been shown to reduce LPS-induced pro-inflammatory mediators such as TNF- α and IL-1 β [10]. The down-regulation effects by BB extracts were suggested to be partly through mediating nuclear factor κ B (NF- κ B) signaling pathways [11]. We now examined the effects of BBs on inhibiting pro-inflammatory cytokine production and the possible underlying mechanisms.

Female apoE^{-/-} mice were fed AIN-93G (CD) or AIN-93G containing 1% freeze-dried whole BB powder (15/group) (animal protocol, see Supporting Information) for 5 wk. The serum and thioglycollate-elicited peritoneal macrophages were collected. The serum IL-6 level of mice fed BB was 7.4 ± 0.9 ng/L, was lower than it in CD-fed mice at 31.3 ± 6.7 ng/L ($p < 0.01$, $n = 5$). The serum TNF- α concentration did not differ between the BB (0.6 ± 0.3 ng/L) and CD (0.9 ± 0.4 ng/L) groups. A quick screening using pooled aorta samples by RT-PCR array showed that mRNA expression of TNF- α was down-regulated in BB-fed mice (52% reduction, $p < 0.05$) (IL-6 was not detected) (for details, see Supporting Information).

Further measurements of gene expression and protein levels of IL-6 and TNF- α were performed in cultured

thioglycollate-elicited peritoneal macrophages from apoE^{-/-} mice (for detailed method, see Supporting Information). Gene expression of IL-6 and TNF- α were lower in peritoneal macrophages from mice fed BB compared with mice fed CD ($p < 0.05$) (Fig. 1A and B). When stimulated with LPS and the comparisons were made between CD and BB groups, TNF- α and IL-6 mRNA expression increased by ~ 120 -fold and ~ 550 -fold in macrophages from CD-fed mice, respectively, but only by ~ 16 -fold and ~ 6 -fold in macrophages from BB-fed mice, respectively ($p < 0.05$) (Fig. 1C and D). Similarly, the oxidized LDL (oxLDL) also induced marked increases in TNF- α and IL-6 mRNA expression by ~ 12 -fold and ~ 70 -fold in macrophages from CD mice; while in macrophages from BB mice, TNF- α expression increased ~ 3 -fold and IL-6 was unchanged ($p < 0.05$) (Fig. 1C and D). Without stimulation, the protein levels of IL-6 were lower in the macrophages from BB-fed mice ($p < 0.05$) (Fig. 1E), while TNF- α protein was barely detectable in macrophages and no difference was observed between the two diet groups, probably due to the detection limit and thus huge variations. Both IL-6 and TNF- α protein levels were significantly elevated in the macrophages from two diet groups with LPS or oxLDL stimulation. BB intervention attenuated the elevation of both IL-6 and TNF- α protein levels ($p < 0.05$) (Fig. 1E and F). It is clear that not only did BB reduce basal levels of these two pro-inflammatory cytokines; it also increased the resistance of secretion of these two cytokines by macrophages in response to inflammatory stimuli LPS and oxLDL. Since TNF- α and IL-6 are key players in the vascular inflammation underlying

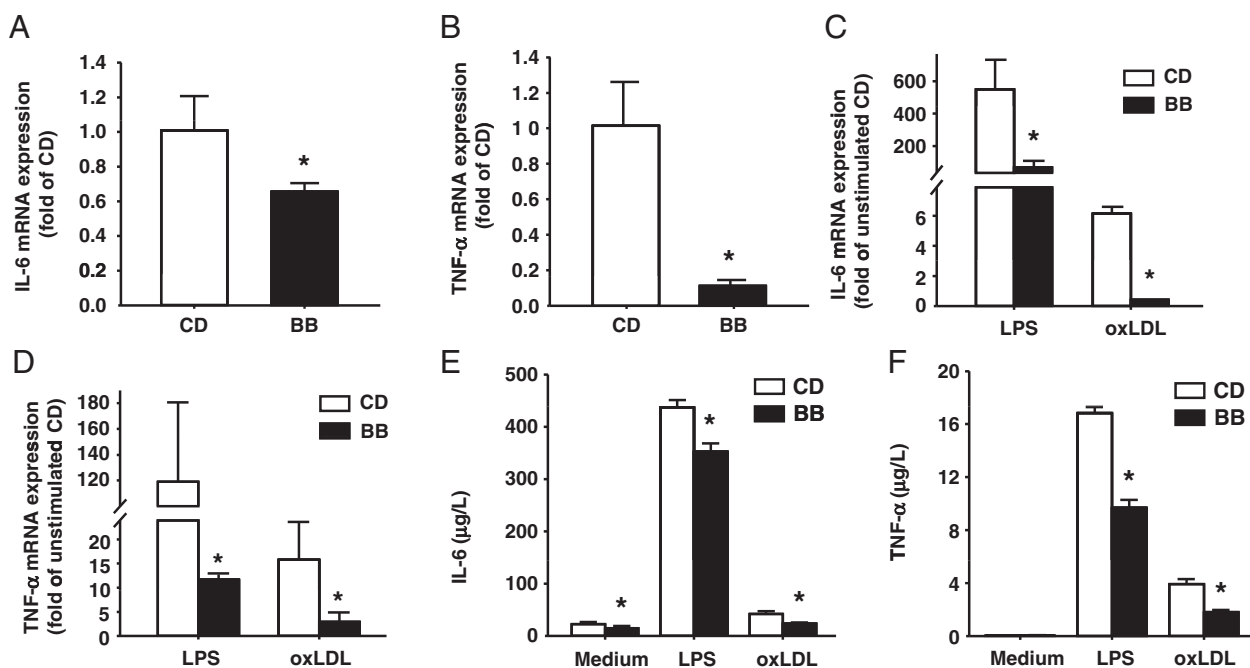


Figure 1. BB intervention suppressed TNF- α and IL-6 mRNA expression in thioglycollate-elicited peritoneal macrophages from apoE^{-/-} mice (A, B). Stimulated by LPS or oxLDL, TNF- α and IL-6 mRNA expressions were significantly increased, which were attenuated by BB (C, D). The protein levels without or with LPS or oxLDL stimulation in macrophages from BB fed mice were lower comparing to mice fed CD (E, F). Comparisons were made between CD and BB groups. Mean \pm SEM, * $p < 0.05$, $n = 5$.

atherosclerosis, reducing pro-inflammatory cytokine TNF- α and IL-6 could be an important mechanism underlying the protective effects of BB.

Owing to limited sources of peritoneal macrophages, the mechanistic study was conducted in murine macrophage cell line RAW 264.7. We came to realize that the results from RAW264.7 macrophages may not be directly applied to the animal models. However, they could provide us some good indications for the possible mechanisms. Polyphenol-enriched extracts from Sprague–Dawley rats fed CD (SEC) and CD containing 10% BB (SEB) were used as testing materials (animal protocol and preparation of extracts, see Supporting Information). While 1% BB was studied for in vivo studies, a higher level of 10% was used to obtain serum with sufficient bioactive material to test in vitro. RAW264.7 cells treated with SEB followed by LPS stimulation led to the reduction of both gene expression and protein levels of IL-6 and TNF- α (Fig. 2A–D), suggesting that SEB contains promising bioactive compounds to inhibit pro-inflammatory cytokine production. SEC treatment only reduced TNF- α protein level (Fig. 2D). It is worthwhile mentioning that until recently, treating cells with fruit or berry extracts is still a common approach in evaluating the bioactivities/mechanisms of a given fruit or berry including BB [11]. Polyphenols such as anthocyanin and proanthocyanidin are widely considered as major bioactive compounds in BB responsible for their protective effects against vascular diseases [12, 13]. However, it has been well known that absorption of polyphenols is very low [14]. So, the obvious drawback of this approach is that the compounds being

tested may not even exist in the body. In this study, we used polyphenol-enriched extracts prepared from rats fed BB. This approach was adopted for two major reasons. First, serum contained metabolites that expose to target tissue, hence are biological relevant. It would be ideal to use serum from apoE^{−/−} mice in this study; however, mice were not able to provide enough blood for the test. Rats were chosen because their metabolism was similar to mice and have been used as animal models in demonstrating the cardio-protective effects of BB recently [15, 16]. Second, since only a small portion of testing materials can be added in cell culture media, plus the protein in the serum may interfere with culture media, a concentrated extract, rather than original serum, was used to treat cells. Solid-phase extraction was used to concentrate polyphenols and to remove proteins.

Inhibiting NF- κ B and mitogen-activated protein kinase (MAPK) pathways have been suggested as the two major mechanisms underlying the attenuation of LPS-induced inflammatory cytokine production [17, 18]. Previous studies have shown that exposure of the murine macrophage cell line RAW 264.7 to LPS increases phosphorylation of the MAPK family members ERK1/2, p38 and JNK1/2 in a time-dependent manner [19]. NF- κ B plays a crucial role as transcription factor in regulating many of the pro-inflammatory cytokine genes [17]. LPS stimulation elicits a cascade leading to the activation of NF- κ B [20]. The down-regulation effects towards pro-inflammatory cytokines by BB extracts were suggested partly through mediating NF- κ B signaling pathways [11].

LPS stimulates the activation of MAPK, including ERK, JNK, and p38 pathways. These pathways are used to directly or indirectly phosphorylate transcription factors such as Elk-1, c-Jun, ATF-1. Activation of NF- κ B mainly occurs via I κ B kinase-mediated phosphorylation of inhibitory molecules, including I κ B α . Optimal induction of NF- κ B target genes also requires phosphorylation of NF- κ B proteins, such as p65 [21]. For signaling pathway analysis, two commercial PathScan ELISA kits were employed to determine the effects of BB on NF- κ B and MAP kinase signaling pathways (for detailed method, see Supporting Information). SEB selectively reduced phosphorylation of I κ B, NF- κ B p65, MAPK p38 and JNK (Fig. 3), indicating the in vivo metabolites following BB consumption were able to inhibit NF- κ B and MAPK pathways. These could be important mechanisms contributing to the reduction of IL-6 and TNF- α associated with BB feeding. Notably, Erk1/2 was inhibited with SEC treatment as well, suggesting that certain endogenous compounds in rat serum unrelated to dietary intake could also influence MAPK pathway. This may partly explain the reduction of TNF- α production in the macrophages treated with SEC (Fig. 2D). Based on these results, the mechanisms by which BB inhibited TNF- α and IL-6 production were summarized (Supporting Information Fig. 1, see Supporting Information), in which BB were proposed to reduce phosphorylation of several key components in NF- κ B and MAPK pathways.

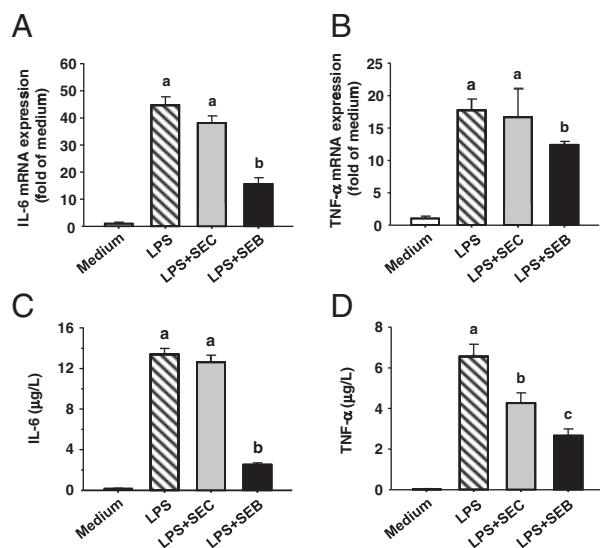


Figure 2. SEB treatment suppressed LPS-induced TNF- α and IL-6 mRNA expression (A, B) and protein levels (C, D) in RAW264.7 macrophages. SEC treatment did not show inhibitory effects against mRNA expression of IL-6 and TNF- α as well as IL-6 production, but reduced the TNF- α protein level (D). Mean \pm SEM, means with different letters are significantly different ($p < 0.05$, $n = 5$).

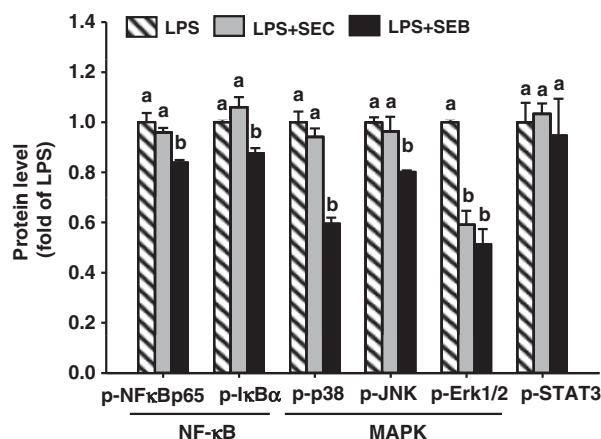


Figure 3. Effects of SEB in inhibiting LPS-induced phosphorylation of IκB, NF-κB p65, p38 and JNK. Mean ± SEM, means with different letters are significantly different ($p < 0.05$, $n = 5$).

Another question raised by our studies is the nature and identity of bioactive components in SEB that may be responsible for the observed anti-inflammatory effects. Our initial attempts to identify these compounds using HPLC with various conditions (column and mobile phases) failed to yield meaningful difference between SEB and SEC (data not shown), which may indicate at least two possibilities: the concentration of bioactive compounds was very low; and/or the bioactive compounds may not have UV absorption. Further characterization of bioactive compounds is currently ongoing at our lab.

Overall, the present study showed that BB effectively inhibited inflammatory cytokines TNF- α and IL-6 production in mouse macrophages. The down-regulatory effects by BB were partly mediated through inhibiting NF-κB activation by reducing phosphorylation of NF-κB p65 and IκB proteins, as well as inhibiting MAPK p38 and JNK phosphorylation. This study also indicated that sera from rats fed BB contained promising in vivo bioactive compounds, though their actual forms are yet to be determined.

This study was supported by USDA (6251-51000-005-02S).

The authors have declared no conflicts of interest.

References

- [1] Neto, C. C., Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. *Mol. Nutr. Food Res.* 2007, 51, 652–664.
- [2] Wu, X., Kang, J., Xie, C., Burris, R. et al., Dietary blueberries attenuate atherosclerosis in apolipoprotein E-deficient mice by upregulating antioxidant enzyme expression. *J. Nutr.* 2010, 140, 1628–1632.
- [3] Libby, P., Inflammation in atherosclerosis. *Nature* 2002, 420, 868–874.
- [4] Szekanecz, Z., Pro-inflammatory cytokines in atherosclerosis. *Isr. Med. Assoc. J.* 2008, 10, 529–530.
- [5] Gotsman, I., Stabholz, A., Planer, D., Pugatsch, T. et al., Serum cytokine tumor necrosis factor- α and interleukin-6 associated with the severity of coronary artery disease: indicators of an active inflammatory burden? *Isr. Med. Assoc. J.* 2008, 10, 494–498.
- [6] Huber, S. A., Sakkinen, P., Conze, D., Hardin, N. et al., Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler. Thromb. Vasc. Biol.* 1999, 19, 2364–2367.
- [7] Brannen, L., Hovgaard, L., Nitulescu, M., Bengtsson, E. et al., Inhibition of tumor necrosis factor- α reduces atherosclerosis in apolipoprotein E knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 2004, 24, 2137–2142.
- [8] Basu, A., Du, M., Leyva, M. J., Sanchez, K. et al., Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *J. Nutr.* 2010, 140, 1582–1587.
- [9] Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A. et al., Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* 2007, 51, 675–683.
- [10] Lau, F., Bielinski, D., Joseph, J., Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. *J. Neurosci. Res.* 2007, 85, 1010–1017.
- [11] Lau, F. C., Joseph, J. A., McDonald, J. E., Kalt, W., Attenuation of iNOS and COX2 by blueberry polyphenols is mediated through the suppression of NF-κB activation. *J. Funct. Food* 2009, 1, 274–283.
- [12] Chong, M. F., Macdonald, R., Lovegrove, J. A., Fruit polyphenols and CVD risk: a review of human intervention studies. *Br. J. Nutr.* 2010, 104, S28–S39.
- [13] Neto, C. C., Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. *Mol. Nutr. Food Res.* 2007, 51, 652–664.
- [14] Manach, C., Williamson, G., Morand, C., Scalbert, A. et al., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 2005, 81, 230S–242S.
- [15] Ahmet, I., Spangler, E., Shukitt-Hale, B., Joseph, J. A. et al., Survival and cardioprotective benefits of long-term blueberry enriched diet in dilated cardiomyopathy following myocardial infarction in rats. *PLoS One* 2009, 4, e7975.
- [16] Ahmet, I., Spangler, E., Shukitt-Hale, B., Juhaszova, M. et al., Blueberry-enriched diet protects rat heart from ischemic damage. *PLoS One* 2009, 4, e5954.
- [17] de Winther, M. P., Kanters, E., Kraal, G., Hofker, M. H., Nuclear factor kappaB signaling in atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 2005, 25, 904–914.

- [18] Muslin, A. J., MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. *Clin. Sci. (London)* 2008, 115, 203–218.
- [19] Xagorari, A., Roussos, C., Papapetropoulos, A., Inhibition of LPS-stimulated pathways in macrophages by the flavonoid luteolin. *Br. J. Pharmacol.* 2002, 136, 1058–1064.
- [20] Baeuerle, P. A., Henkel, T., Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.* 1994, 12, 141–179.
- [21] Viatour, P., Merville, M. P., Bours, V., Chariot, A., Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem. Sci.* 2005, 30, 43–52.