이학석사 학위논문

BTG2 과발현에 의한 단핵구의 대식세포로의 분화 억제와 MCP-1 매개성 transmigration 억제 유도효과

Human B cell Translocation Gene 2 suppresses the differentiation of human monocytes into macrophages and MCP-1-induced transmigration of human monocytes

울산대학교대학원

의 과 학 과

이 명 화

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지도교수 한 기 훈

이 논문을 이학석사 학위 논문으로 제출함

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울산대학교 대학원
의 과 학 과
이 명 화

이명화의 이학석사학위 논문을 인준함

심사위원 한 기 훈 인

심사위원 류 재 원 인

심사위원 위 유 미 인

울 산 대 학 교 대 학 원

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Abstract

Background - Atherosclerosis is a chronic inflammatory disease that is caused by an excessive inflammatory response in the arterial wall. Inflammatory cells, most notably monocytes, play an important role in the development of atherosclerosis. During the development of atherosclerosis, circulating monocytes are recruited to and infiltrate the affected blood vessels through transmigration. Monocytes infiltrated to the arterial wall differentiate into macrophages that phagocytize oxidized LDL particles and secrete pro-inflammatory cytokines, such as TNF-α. Macrophages that uptake an excess amount of oxidized LDL are transformed into foam cells, which are responsible for the build-up of atherosclerotic plaques on arterial walls.

B cell translocation gene 2 (BTG2) is an anti-proliferative (APRO) gene that is expressed in various organs and tissues such as spleen, thymus, lungs, and stomach. BTG2 not only inhibits the biological activities of cancer cells, i.e. cell growth and proliferation, cell migration and invasion, including gastric cancer and breast cancer, but also promotes apoptosis of cancer cells. In the present study, we investigated the functional role of BTG2 in human monocytes.

Methods and Results - BTG2 was successfully overexpressed in monocytes RT-PCR and immunoblotting clearly showed that STAT6, a transcription factor for cellular, differentiation, was found to be upregulated as a time-dependent manner. RT-PCR and flow cytometry demonstrated that BTG2-overexpressing monocytes (tBTG2 monocytes) showed lower expression levels of surface markers, such as CD14 and CD36, suggesting the BTG2 may suppress monocytes differentiation. In addition, RT-PCR showed that the expression of integrins, mostly CD49, was significantly lower in tBTG2 monocytes. A transmigration assay confirmed that MCP-1-induced transmigration was inhibited when BTG2 was overexpressed in monocytes for 72 hours. Moreover, RT-PCR also showed that overexpression of BTG2 in monocytes directly affected the secretion of pro- and anti-

inflammatory cytokines. Anti-inflammatory cytokines, such as IL-10 and IL-1ra, were increased, while pro-inflammatory cytokines, such as TNF-α, MCP-1, and IL-12, were decreased. Our RT-PCR and real-time PCR confirmed that cultured human endothelial cells induce expressions of E-selectin, VCAM-1, and ICAM-1, and pro-inflammatory cytokines, such as endothelin-1, TNF-α, and IL-8 in response to hydrogen peroxide, which was significantly attenuated by pre-incubation with supernatants from overexpressed BTG2 monocytes. Therefore, anti-inflammatory cytokines secreted from tBTG2 monocytes may possibly modulate expressions of cell surface proteins and inflammatory cytokines in endothelial cells under hydrogen peroxide-triggered oxidative stress.

Conclusion - Overexpression of BTG2 in monocytes can inhibit their differentiation into macrophages as well as MCP-1-induced transmigration. In addition, BTG2 overexpression not only suppresses the pro-atherogenic activities of the monocytes themselves, i.e., decreases in the secretion of pro-inflammatory cytokines, such as TNF-α, MCP-1, and IL-12, but also actively increases the secretion of anti-inflammatory cytokines, such as IL-10 and IL-1ra. Such changes in the cytokine secretion profile of BTG2-overexpressing monocytes seem to protect neighboring cells, such as endothelial cells, under oxidative stress. Therefore, BTG2 expression in monocytes may retard the progression of atherosclerosis.

Keywords - B cell Translocation Gene 2 (BTG2), Monocytes/Macrophages, Human endothelial cell, Atherosclerosis, Inflammatory disease, Inflammatory cytokines, Monocyte chemoattractant protein-1 (MCP-1), C-C chemokine receptor type 2 (CCR2), Integrin

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Introduction

1. Progression of atherosclerosis

Atherosclerosis is one of the chronic inflammatory diseases of the arterial wall. Several risk factors, including hyperlipidemia, diabetes, hypertension, aging, and smoking, increase oxidative stress levels in vascular cells and activate the redox-sensitive signaling pathway and related transcription factors, leading to inflammation through the vascular cells and immune cells. It causes atherosclerosis and vascular dysfunction. The major cause of atherosclerosis is oxidation of liver-induced LDL by various factors such as reactive oxygen species (ROS), lipases, and myeloperoxidase. [1,2] When endothelium undergoes inflammation by oxidized LDL, proteins such as E-selectin expressed in the region of inflammation, recruit circulating monocytes in the blood which bind to adhesion molecules and transmigrate into the blood vessels. [3] Then, these monocytes entering the blood vessels differentiate into macrophages and begin to phagocytize the oxidized LDL. CD36, the oxidized LDL receptor, on the surface of macrophages binds to the oxidized LDL, which is then taken up by the macrophages. [4-7] Then, these macrophages secrete pro-inflammatory cytokines, such as TNF-α. Subsequently, several cytokines are secreted to induce the expression of adhesion molecules, such as E-selectin, to promote the recruitment of more monocytes to the blood vessels. Thus, a number of the macrophages that received the signal to uptake oxidized LDL differentiate into foam cells. In addition, cytokines and growth factors induced by the macrophages and foam cells cause the migration and proliferation of vascular smooth muscle cells to the intima and promote the apoptosis of both vascular smooth muscle and foam cells. The sticky lipids from the foam cells, vascular smooth muscle cells, and collagen accumulate to form an atherosclerotic plaque on the walls of the blood vessels, which weakens the blood vessels. (Figure 1).

Previously, many studies have been performed to induce apoptosis or anti-inflammation targeting monocytes, a major cause of atherosclerosis. [8-11] In the present study confirms

whether BTG2-overexpressing monocytes induce anti-inflammation. Thus, we confirm that BTG2-overexpressing monocytes have a positive effect on atherosclerosis.

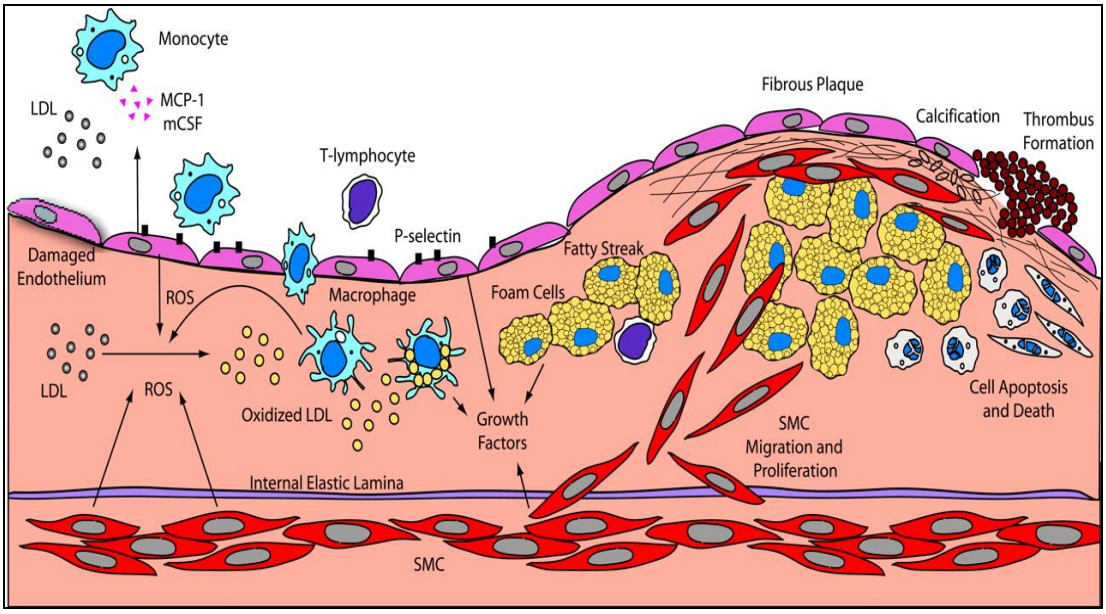

Figure 1. Progression of atherosclerosis [1]

2. Infiltration of circulating monocytes into the arterial wall

Monocyte chemoattractant protein 1 (MCP-1 or CCL2) is a chemotaxin expressed by a variety of cell types, including endothelial, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells as well as fibroblasts, that is induced by oxidative stress, cytokines, and growth factors..[12] MCP-1 has been found in both atherosclerotic plaques and endothelial cells. [13] Monocytes are a major cause of atherosclerosis and it is very important to inhibit the differentiation of monocytes. Infiltration of monocytes into the arterial wall, endothelium, is an initial step in atherosclerosis, and MCP-1 directs the infiltration of monocytes which differentiate into macrophages in arterial wall. [14] In addition and MCP-1 play other important roles in the initiation, and progression development of atherosclerosis. [15] MCP-1 induces the expression of many inflammatory genes that may be associated with atherosclerosis. MCP-1 also binds to the CCR2 receptor and induces the adhesion and chemotaxis of monocytes. [13, 15, 16] In addition, increases in both MCP-1 and its receptor CCR2 have been shown to play a central role in the development of atherosclerosis. (Figure 2)

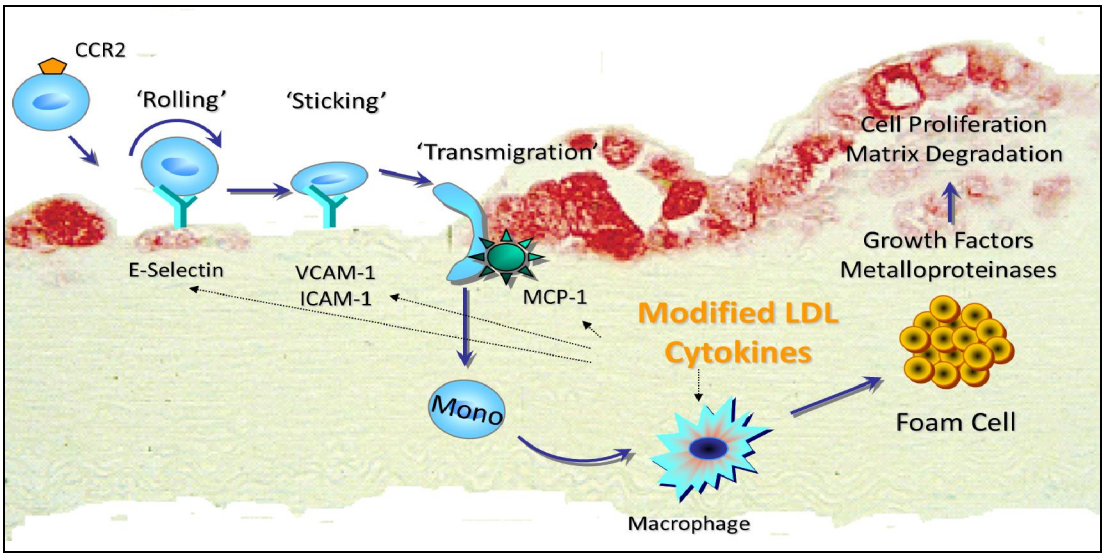

Figure 2. Monocyte infiltration into the arterial wall

3. Pro- and anti-inflammatory cytokines of atherosclerosis

Cytokines function in the defense against microbial infections, toxic agents, and trauma. In response, antibodies, immune complexes, and cytokines are produced by the cells of the innate immune system, such as monocytes, neutrophils, and NKT cells. Cytokines are categorized into several classes: interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), colony stimulating factors (CSFs), transforming growth factors (TGFs), and chemokines.[17] Cytokines are important for regulating both inflammatory and immune or pro-inflammatory cytokines. In addition, cytokines are often classified as pro- or anti-inflammatory cytokines. Atherosclerosis also has an already known pro- or anti-inflammatory or cytokines. [17, 18] Pro-inflammatory cytokines, such as IL-1, have been detected in the macrophages and endothelial cells around the atherosclerotic plaques and promote atherosclerosis. [18] To determine the effect of BTG2 overexpression on cytokines involved in atherosclerosis, we assessed the levels of various anti-inflammatory cytokines, including IL-10 and IL-1 receptor antagonist (IL-1RA), and pro-inflammatory cytokines, such as TNF-α, MCP-1, and IL-12 in BTG2-overexpressing monocytes.

	Pro- inflammatory cytokines	**Anti- inflammatory cytokines**
TNFR family	TNF-α, CD40L	
Interleukin family	IL-1,IL-2, IL-4, IL-6, IL-8, IL-12, IL-18	IL-1ra, IL-6, IL-9, IL-10
Chemokines/chemokine receptors	MCP-1/CCR2, Fractalkine/CX3CR1	
TGF-β family		TGF-β

Table 1. Pro- and anti-inflammatory cytokines involved in atherosclerosis [17]

4. The role of B cell Translocation Gene 2

B cell translocation gene 2 (BTG2) is a member of the BTG/Tob family and is a known anti-proliferation gene. Although it is celled BTG2 in human, this protein has different names in other species. BTG2 is called pheochromocytoma cell 3 (PC3) in rat and is called tetradecanoylphorbol acetate-inducible sequence 21 (Tis21) in mouse. BTG2 is expressed in various organs and tissues, including the spleen, thymus, lung, and stomach, and it plays important roles in cell differentiation, proliferation, DNA damage repair, and apoptosis. [19, 20] (Figure 3) Previous studies have shown that when BTG2 is overexpressed, it inhibits G1/S phase progression by downregulating cyclin D1 transcription independent of pRb [21, 22], and promotes G2/M phase arrest [23-25] and apoptosis.

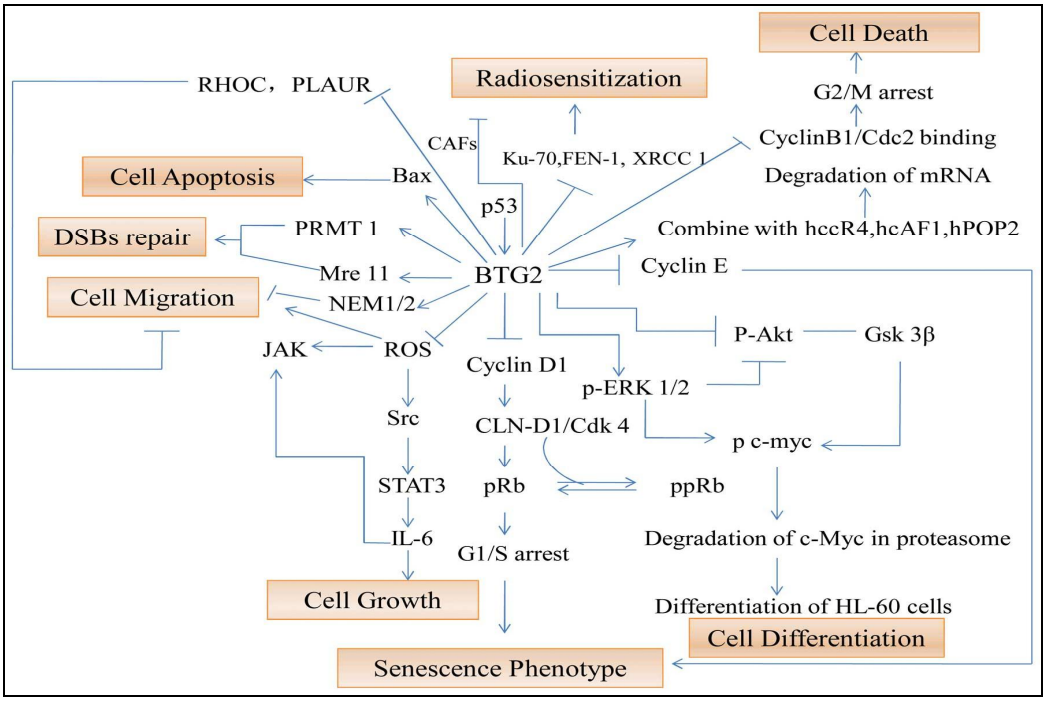

Figure 3. The signaling pathways involving B-cell translocation gene 2 [20]

5. Hypothesis

We hypothesized that expression of BTG2 will retard the progression of atherosclerosis by decreasing the number of monocytes entering the atherosclerotic plaque area. In the present study, we overexpressed human BTG2 in cultured human monocytes and investigated whether BTG2-overexpression change the cytokine secretion profile of the monocytes. We also tested whether BTG2 affected the differentiation of monocytes into macrophages and their transmigratory activities.

Materials and methods

1. Materials

The human monocyte THP-1 (American Type Culture Collection, Manassas, Virginia, USA), 293FT cell (Invitrogen by Thermo Fisher), Human umbilical artery endothelial cells (HUAECs, Lonza, Walkersville, INC), Endothelial cell Growth Medium (Lonza, Walkersville, INC), RPMI-1640, Dulbecco's Modified Eagle's Medium (GIBCO BRL, Grand Island, NY, USA) Fetal Bovine Serum (FBS) (GIBCO BRL, Grand Island, NY, USA), Penicillin streptomycin (GIBCO BRL, Grand Island, NY, USA), Phobol-12-Myristate-13-acetate (PMA, Thermo scientific, Waltham, MA, USA) Geneticin (GIBCO BRL, Grand Island, NY, USA), L-glutaMax (GIBCO BRL, Grand Island, NY, USA), Non- Essential Amino Acid (NEAA) (GIBCO BRL, Grand Island, NY, USA), HIT Competent Cells™-DH5α(Real Biotech Corporation RBC), EcoR1, Xbar1, BamH1 restriction enzyme (New England Biolabs), Lipofectamine®2000 Reagent, Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA), RNAiso plus (Takara, Kyoto, Japan), Roche reverse transcriptase system (Roche Diagnostics, Germany), LightCycler 1.5-SYBR GREEN 1 (Roche Diagnostics, Almere, Netherlands), Mini protease inhibitor cocktail tablets (Roche Diagnostics, Geramny), Bicinchoninic Acid (BCA) assay (Thermo scientific, Waltham, MA, USA), BTG2 antibody (Abcam, Cambridge, UK), STAT1, STAT3, STAT6, and conjugated secondary antibody (Cell Signaling, MA, USA), Mouse monoclonal anti-β-actin antibody (Santa Cruz Biotech, CA), Chemiluminescence (ECL) reagent, (Thermo scientific, Waltham, MA, USA),FITC Mouse Anti-Human CD14(BD Pharmingen™, USA),Human CD36/SR-B3 PE-conjugated Antibody (R&D systems Inc, USA), ChromPure Human IgG FC Fragment (Jackson Immuno ResearchInc, PA, USA), Luminex (R&D system Inc, USA), BTG2 siRNA (Santa Cruz Biotech, CA), Lenti-X™ concentrator (Clontech Laboratories, Inc.), Lentiviral coat protein vector pRSV, pMDLg, pMD2g (Addgene, Cambridge, MA), Trnaswell (Corning Incorporated, New York), MCP-1 (R&D system Inc, USA), Dil stain (Thermo scientific, Waltham, MA, USA).

2. Cell culture and transfection

Human THP-1 monocytes (American Type Culture Collection, Manassas, Virginia, USA) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 ng/mL streptomycin (PS) in a 5% CO_2 incubator at 37°C.

293 FT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 units/mL penicillin, 100 ng/mL streptomycin, 3.7 g/L sodium bicarbonate, 200 mM L-Glutamine, 10 mM non-essential amino acids (NEAA), and 500 μg/mL geneticin in a 5% CO_2 incubator at 37°C..

Human umbilical artery endothelial cells (HUAECs) were cultured in EGM in 100 mm culture dishes pre-coated with 0.1% gelatin in a 5% CO_2 incubator at 37°C.

For transfection, the THP-1 cells were maintained in 6-well plates at a density of 5×10^5 cells/well in RPMI 1640 medium containing 10% FBS and without PS. Next, equal amounts of 100 nM BTG2 siRNA and Lipofectamine™ RNAi max reagent were added to opti-MEM medium and gently mixed with diluted BTG2 siRNA, and then the mixture was incubated at room temperature for 25 minutes. Then, the lipofectamine reagent and BTG2 siRNA complexes were added to each well of the plate containing THP-1 cells. After 4 hours, the medium was changed to RPMI 1640 containing 10% FBS, and the plate was further incubated in a 5% CO_2 incubator at 37°C for up to 3 days.

THP-1 cells were maintained in T75 flasks containing RPMI 1640 medium with 10% FBS and 1% PS at 30–40% confluency. The cells were infected with Lentiviral BTG2 using 8 μg/mL polybrene in a CO_2 incubator at 37°C for 72 hours. HUAECs were incubated with THP-1 supernatant for 24 hours. Then, H_2O_2 was added, and the cells were incubated for 2 hours.

3. Cloning of human BTG2 cDNA into a lentiviral vector

The human BTG2 cDNA inserted in pcDNA 3.1(-) was extracted by restriction enzymes such as Xba1 and BamH1, at 37°C overnight. The extracted BTG2 cDNA was seperated by 2% agarose gel using electrophoresis. Then, the separated BTG2 cDNA was purified from the agarose gel using a QIAGEN kit. Based on the weight of the agarose gel slice containing the BTG2 cDNA, three-volumes of Q1 buffer containing resin was added to the agarose gel slice and incubated at 50°C for 15 minutes. The supernatant was removed by centrifugation, and the resin-bound DNA was washed with PE buffer, and then dissolved in TE buffer. The purified BTG2 cDNA fragment was inserted into the pCDH-CMV-puro vector extracted with the same restriction enzymes, at a 2:1 insert to vector ratio, using T4 DNA ligase (incubated at 16°C for 8 hours). The ligated DNA mixture was added to HIT Competent Cells™-DH5α (Real Biotech Corporation) and the incubated on ice for 20 minutes. Then, the cells-DNA mixture was heat shocked at 42°C for 1 minute and incubated on ice for 20 minutes. After spreading on an LB plate containing 100 μg/mL ampicillin, the plate was incubated at 37°C overnight. The correct construction was verified by sequencing (Macrogen, Inc.).

293 FT cells were transfected by using Lipofectamine™ 2000 (Thermo Fisher). Briefly, 293 FT cells were maintained in 10 mm culture dishes at 70% confluence in medium containing 3.7 g/L sodium bicarbonate, 200 mM L-Glutamine, and 10 mM Non- Essential Amino Acid (NEAA), and antibiotics free. Then, lentiviral coat protein vector, pRSV, pMD2g, pPMLg, and pCDH-BTG2 were mixed, at a ratio of 2:2:2:4, with 40 μL of lipofectamine 2000 reagent. Then, the DNA-lipofectamine complex was added to the cells. After 4 hours, the medium was changed to complete medium, and the cells were incubated in a CO_2 incubator at 37°C for 36 hours. After incubation, the supernatant was filtered through a 0.45 nm syringe. Lenti X concentrator was added to the filtered supernatant, at a 1:3 ratio, and incubated at 4°C for 1 hour. After centrifugation at 1500 × *g* and 4°C for 1 hour, the supernatant was removed, and the pellet was dissolved in medium and stored at -70°C.

4. Immunoblotting assay

THP-1 cells were washed with Dulbecco's Phosphate Buffer Saline (DPBS) and lysed with lysis buffer containing 0.6% SDS, 100 μM PMSF, Mini protease inhibitor cocktail (1 tablet), and DPBS. Lysed THP-1 cells were incubated on ice and centrifuged at 13000 × *g* and 4°C for 30 minutes. Then, the proteins were extracted from the supernatant. The protein concentration was determined with the bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA, USA) as the absorbance at 562 nm. Equal protein (30 μg) was separated by 12% SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane (100 V on ice for 120 minutes). Then, the membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 hour. The blocked membrane was incubated with the following primary antibodies at 4°C overnight with gentle shaking: BTG2 (1:500; Abcam, Cambridge, UK), STAT1 (1:1000; Cell Signaling, Beverly, MA, USA), and STAT6 (1:1000; Cell Signaling) in TBST buffer containing 5% BSA, and STAT3 (1:1000; Cell Signaling) and β-Actin (1:2000; Santa Cruz Biotech, Santa Cruz, CA, USA) in TBST buffer containing 5% skim milk. After washing with TBS-T, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:2000; Cell Signaling) and HRP-conjugated anti-rabbit IgG (1:2000; Cell Signaling) in TBS-T buffer at room temperature for 2 hours with gentle shaking. The membrane was developed using Chemiluminescence (ECL) reagent (Thermo Scientific).

5. Analysis of the mRNA expression

Total RNA was isolated from THP-1 cells with RNAiso Plus (Takara, Kyoto, Japan). Then, 1 μg of the isolated RNA was reverse transcribed to synthesize the cDNA with the Roche Reverse Transcriptase system (Roche Diagnostics, Germany). The synthesized cDNA was amplified according to the following program: 32 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes. The specific primers used were as follows:

Human BTG2 :

5'-CAGAGCACTACAAACACCAC -3' (forward)

5'-AGACTGCCATCACGTAGTTC -3' (reverse)

Human STAT1 :

5’-AAGGAAGCACCAGAGCCAAT-3’ (forward)

5’-GCCCACTATCCGAGACACCT-3’ (reverse)

Human STAT3 :

5’-CCTTTGGAACGAAGGGTACA-3’ (forward)

5’-CGGACTGGATCTGGGTCTTA-3’ (reverse)

Human STAT6 :

5’-GTCTGACCGGCTGATCATTG-3’ (forward)

5’-GATGCCCCCAATCTCTGAGT-3’ (reverse)

Human CD14 :

5’-TCGGAAGACTTATCGACCAT-3’ (forward)

5’-GCATGGATCTCCACCTCTAC -3’ (reverse)

Human CD36 :

5’- GGCTGTGTTTGGAGGTATTC-3’ (forward)

5’- TTCATCATCACTTCCTGTGG -3’ (reverse)

Human IL-8 :

5’-TCTGCAGCTCTGTGTGAAGG-3’ (forward)

5’-AATTTCTGTGTTGGCGCAGT-3’ (reverse)

Human TNF-α :

5’-CCATCAGAGGGCCTGTACCT-3’ (forward)

5’-ATAGTCGGGCCGATTGATCT-3’ (reverse)

Human MCP-1 :

5’-GATCTCAGTGCAGAGGCTCG-3’ (forward)

5’-GTCCATGGAATCCTGAACCC-3’ (reverse)

Human Endithelin-1 :

5’-TGCCAAGCAGGAAAAGAACT-3’ (forward)

5’-TTTGACGCTGTTTCTCATGG-3’ (reverse)

Human IL-10 :

5’- CATCAAGGCGCATGTGAACT -3’ (forward)

5’- ACGGCCTTGCTCTTGTTTTC -3’ (reverse)

Human IL-1ra :

5’-CCGTCGGCCTCACCAATATG-3’ (forward)

5-CATGCAAGAATGGGAACAGG-3’ (reverse)

Human CD11a :

5’- AAGGATTCCTGCATCACTGT -3’ (forward)

5’- ATAGAGGCTTCCTGTGCTGT -3’ (reverse)

Human CD11b :

5’- CAACTTGGACACTGAAAACG -3’ (forward)

5’- CATGAGCCTGTGCTGTAGTC -3’ (reverse)

Human CD11c :

5’- CCTCCTGTTCACAGCCTTAG -3’ (forward)

5’- GGTTGGCAGCTGTTATCTTT -3’ (reverse)

Human CD49d :

5’- CCTACAACGTGGACACTGAG -3’ (forward)

5’- GATTGATCACTGAAGCGTTG -3’ (reverse)

Human CCR2 :

5’-CCAGTACATCCACAACATGC -3’ (forward)

5’- AAAGATGAACACCAGCGAGT -3’ (reverse)

Human E-selectin :

5’-GAGAGTGGAGCCTGGTCTTA-3’ (forward)

5’-CAGAGGTTTCTGGGTTCCTA-3’ (reverse)

Human VCAM-1 :

5’-GTCTCATTGACTTGCAGCAC-3’ (forward)

5’-TTGTCAGCGTAGATGTGGTC-3’ (reverse)

Human ICAM-1 :

5’-GTCCCCCTCAAAAGTCATC-3’ (forward)

5’-TAGGCAACGGGGTCTCTAT-3’ (reverse)

Human GAPDH :

5’-GACCCCCTTATTGACCTC-3’ (forward)

5’-GCTAAGCAGTTGGTGGTG-3’ (reverse)

Realtime PCR was performed using a Light Cycler 1.5 (Roche Diagnostics, Almere, Nethelands) with SYBR-Green. The amplification conditions were as follows: 95°C for 15 seconds, 60°C for 5 seconds, and 72°C for 25 seconds. After 45 cycles of the melting point determination, dissociation curves were obtained to verify the specificity of the reaction. The specific primers used were used as follows:

Human BTG2 :

5’-CAGAGCACTACAAACACCAC -3’ (forward)

5’-AGACTGCCATCACGTAGTTC -3’ (reverse)

Human IL-8 :

5’-TCTGCAGCTCTGTGTGAAGG-3’ (forward)

5’-AATTTCTGTGTTGGCGCAGT-3’ (reverse)

Human TNF-α :

5’-CCATCAGAGGGCCTGTACCT-3’ (forward)

5’-ATAGTCGGGCCGATTGATCT-3’ (reverse)

Human MCP-1 :

5’-GATCTCAGTGCAGAGGCTCG-3’ (forward)

5’-GTCCATGGAATCCTGAACCC-3’ (reverse)

Human Endithelin-1 :

5’-TGCCAAGCAGGAAAAGAACT-3’ (forward)

5’-TTTGACGCTGTTTCTCATGG-3’ (reverse)

Human E-selectin :

5’-GAGAGTGGAGCCTGGTCTTA-3’ (forward)

5’-CAGAGGTTTCTGGGTTCCTA-3’ (reverse)

Human VCAM-1 :

5’-GTCTCATTGACTTGCAGCAC-3’ (forward)

5’-TTGTCAGCGTAGATGTGGTC-3’ (reverse)

Human ICAM-1 :

5’-GTCCCCCTCAAAAGTCATC-3’ (forward)

5’-TAGGCAACGGGGTCTCTAT-3’ (reverse)

Human GAPDH :

5’-GACCCCCTTATTGACCTC-3’ (forward)

5’-GCTAAGCAGTTGGTGGTG-3’ (reverse)

The Ct value (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) for the target cDNA was corrected by the Ct value for GAPDH and was expressed as ΔCt. Data shown are the percent changes in the amount of mRNA, which was calculated with the following formula : (fold changes)=$2^{(\Delta Ct \text{ of untreated cells} - \Delta Ct \text{ of treated cells})}$

6. Flow cytometry

THP-1 cells were maintained in 6-well plates at a density of 5×10^5 cells/well in RPMI 1640 medium containing 10% FBS and 1% PS and infected with lantiviral BTG2 using 8 μg/mL polybrene in a CO_2 incubator at 37°C. Then, cells were blocked with 10 μg/mL human IgG FC fragment at room temperature for 30 minutes on a shaker. After blocking, the cells were stained with FITC mouse anti-human CD14 or human CD36/SR-B3 PE-conjugated antibody at room temperature for 2 hours on a shaker. The cells were washed twice with cold DPBS and resuspended in DPBS. Cells were suspended in 300 μL of cold DPBS and analyzed by flow cytometry.

7. Transmigration assay

THP-1 cells were maintained in T25 flasks containing RPMI 1640 medium with 10% FBS and 1% PS at a density of 5×10^5 cells and infected with lentiviral BTG2 using 8 μg/mL polybrene in a CO_2 incubator at 37°C, and the cells were stained with Dil for 10 minutes at 37°C. Then, the cells were transferred to an 8 μm Transwell (Corning Incorporated) for a transmigration assay. MCP-1 (R&D Systems, Inc., USA) was added at 10 ng/mL in serum free RPMI 1640 and incubated for 4 hours. Cells that migrated to the basal side were visualized by fluorescence microscopy (100×).

8. Statistical analysis

Data are expressed as mean ± SD. Differences between two groups were evaluated by the unpaired *t*-test, and the differences among more than two groups were evaluated by two-way

ANOVA using SPSS (version 10.0; SSPS Inc., Chicago, IL, USA) P values less than 0.05 were considered to be statistically significant.

Results

1. Verification of BTG2 overexpression using a Lentivirus in THP-1 monocytes, and BTG2-overexpressing (via Lentiviral BTG2) THP-1 monocytes show decreased cell growth and differentiation

To induce overexpression of BTG2, a BTG2-lentiviral construct was made. RT-PCR and realtime PCR (Figure 1A), and immunoblotting (Figure 1B) showed that BTG2 was successfully overexpressed in THP-1 monocytes following infection with a lentiviral BTG2, which varied according to the amount of infecting virus.

In addition, cell counting showed an approximately 2-fold time-dependent decrease in cell growth when BTG2 was overexpressed in monocytes with a lentiviral BTG2 (Figure 2A). Furthermore, these BTG2-overexpressing monocytes differentiated into macrophages after incubation with 5 nM PMA for 24 hours, and their growth was reduced by up to 1.5-fold (Figure 2B).

2. Overexpression of BTG2 in THP-1 monocytes increases STAT6 expression

Human THP-1 monocytes were infected with MOCK (control) or lentiviral BTG2 for 0, 24, 48, or 72 hours. RT-PCR and immunoblotting showed that STAT6 increased when BTG2 was overexpressed in THP-1 monocytes (Figure 3A). In addition, we suppressed BTG2-overexpression by transfecting THP-1 monocytes with 100 nM of BTG2 siRNA. Complete siRNA-mediated BTG2 inhibition suppressed the observed increase in STAT6 (Figure 3B). Compared with MOCK (control monocytes), STAT6 expression was 4-fold higher in BTG2-overexpressing monocytes.

3. Expression levels of CD14 and CD36 in THP-1 monocytes are reduced by BTG2 overexpression

RT-PCR showed that the expression of CD14 and CD36 in THP-1 monocytes decreased when BTG2 was overexpressed (Figure 4A). Compared with MOCK, CD14 expression was

reduced approximately two-fold and CD36 was reduced approximately three-fold in BTG2-overexpressing monocytes.

To demonstrate the protein levels of CD14 and CD36 on the surface of THP-1 monocytes, we used flow cytometry. Compared with control monocytes, CD14 was dramatically reduced by up to three-fold (Figure 4B), and CD36 was maximally reduced by five-fold in BTG2-overexpressing monocytes (Figure 4C). In addition, when BTG2-overexpressing monocytes were differentiated into macrophages by incubation with 5 nM PMA for 24 hours, CD36 expression was similarly reduced by ~four-fold (Figure 4C).

4. Overexpression of BTG2 in THP-1 monocytes downregulates the expression of integrins associated with cell adhesion and MCP-1-induced transmigration

To determine whether the integrins associated with cell adherence and transmigration are affected by BTG2 overexpression in monocytes, we performed RT-PCR. The results showed that BTG2 lentivirus-infected monocytes exhibited time-dependent decreases in the levels of cell surface proteins associated with adhesion, including integrin α subunits, CD11b, and CD11c. In addition, CD49d was similarly reduced in monocytes overexpressing BTG2. RT-PCR showed that the expression of CCR2 was decreased by 50% compared with the levels in control monocytes (Figure 5A).

Microscopy showed the transmigration of monocytes treated with 10 ng/mL MCP-1 (Figure 5B). In this experiment, BTG2 was overexpressed in THP-1 monocytes for 72 hours, and then the cells were stained with Dil solution for 10 minutes. Next, an equal volume of cells was transferred to the transwell (8 μm pore size), and serum free medium and 10 ng/ml MCP-1 were added to the bottom well. After four hours of incubation, the transmigrated cells were observed under a fluorescence microscope (100×) and counted. The number of transmigrated BTG2-overexpressing THP-1 cells was approximately 2.5-fold lower than the number of migrated control cells.

5. Secretion of pro- and anti-inflammatory cytokines in BTG2-overexpressing THP-1 monocytes

The previous results showed that overexpression of BTG2 inhibited the activity of the monocytes. Next, we examined the effect of BTG2 overexpression on the secretion of inflammatory cytokines (Figure 6). The monocytes were infected with Lentiviral BTG2 for 0, 24, 48, or 72 hours, and then the levels of various cytokines were evaluated. The levels of pro-inflammatory cytokines, such as TNF-α and MCP-1, were decreased, while the secretion of anti-inflammatory cytokines, such as IL-10 and IL-1ra, was increased.

6. H_2O_2-Induced endothelial cells change to the balance of surface proteins and cytokines by secreted cytokines in THP-1 monocytes

We hypothesized that the cytokines secreted by BTG2-overexpressing monocytes will affect endothelial cell surface protein expression and inflammatory cytokine secretion. In this experiment, BTG2 was overexpressed in THP-1 monocytes for 72 hours, and then endothelial cells were cultured with the medium from a for 24 hour BTG2-overexpressing monocyte culture. Then, the endothelial cells were treated with H_2O_2 (0, 10, 100, and 200 μM) for 2 hours. RT-PCR (Figure 7A) and real-time PCR (Figure 7B) demonstrated that the mRNA levels of surface proteins on the endothelial cells, such as E-selectin, VCAM-1, and ICAM-1, were decreased. Conversely, the mRNA levels of pro-inflammatory cytokines, such as endothelin-1, TNF-α, and IL-8, were also decreased compared to cells incubated with control culture medium. Furthermore, the mRNA levels of pro-inflammatory cytokines and chemoattractants, such as MCP-1, were decreased when compared with the levels in the controls.

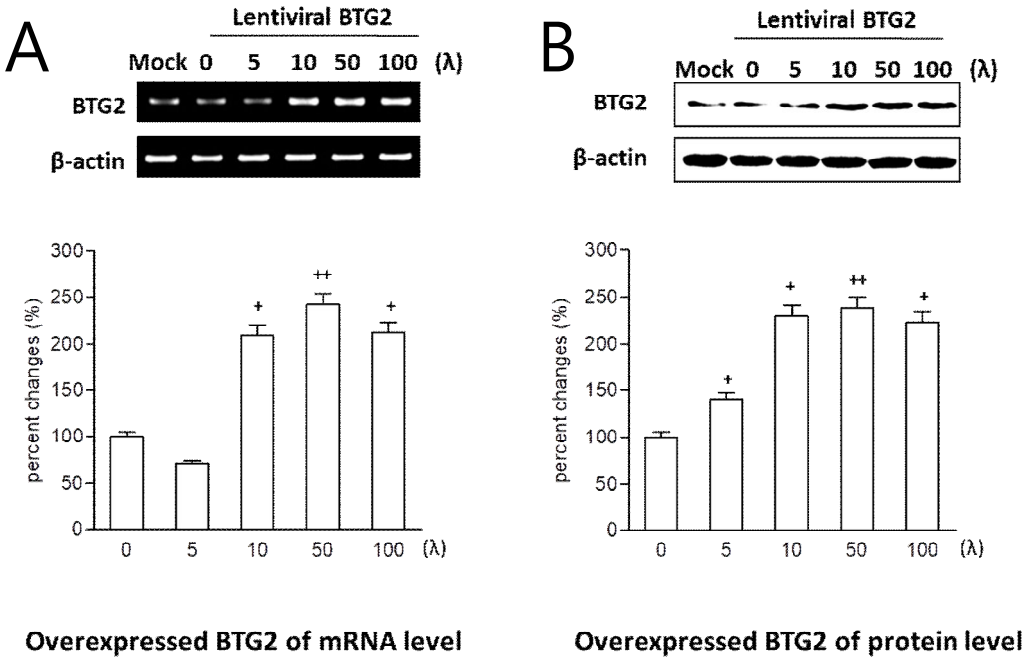

Figure 1. Overexpression of BTG2 in THP-1 monocytes

Human THP-1 monocytes were infected with 0 - 100λ of lentiviral BTG2 stock overnight, and then BTG2 mRNA and protein expression levels were evaluated. BTG2 mRNA levels were quantified by RT-PCR and real-time PCR (A), and BTG2 protein levels in cell lysates were detected by immunoblotting (B). Data are the mean ± SD values of triplicates and were analyzed with the unpaired *t*-test. (+; $p<0.05$, level of significance)

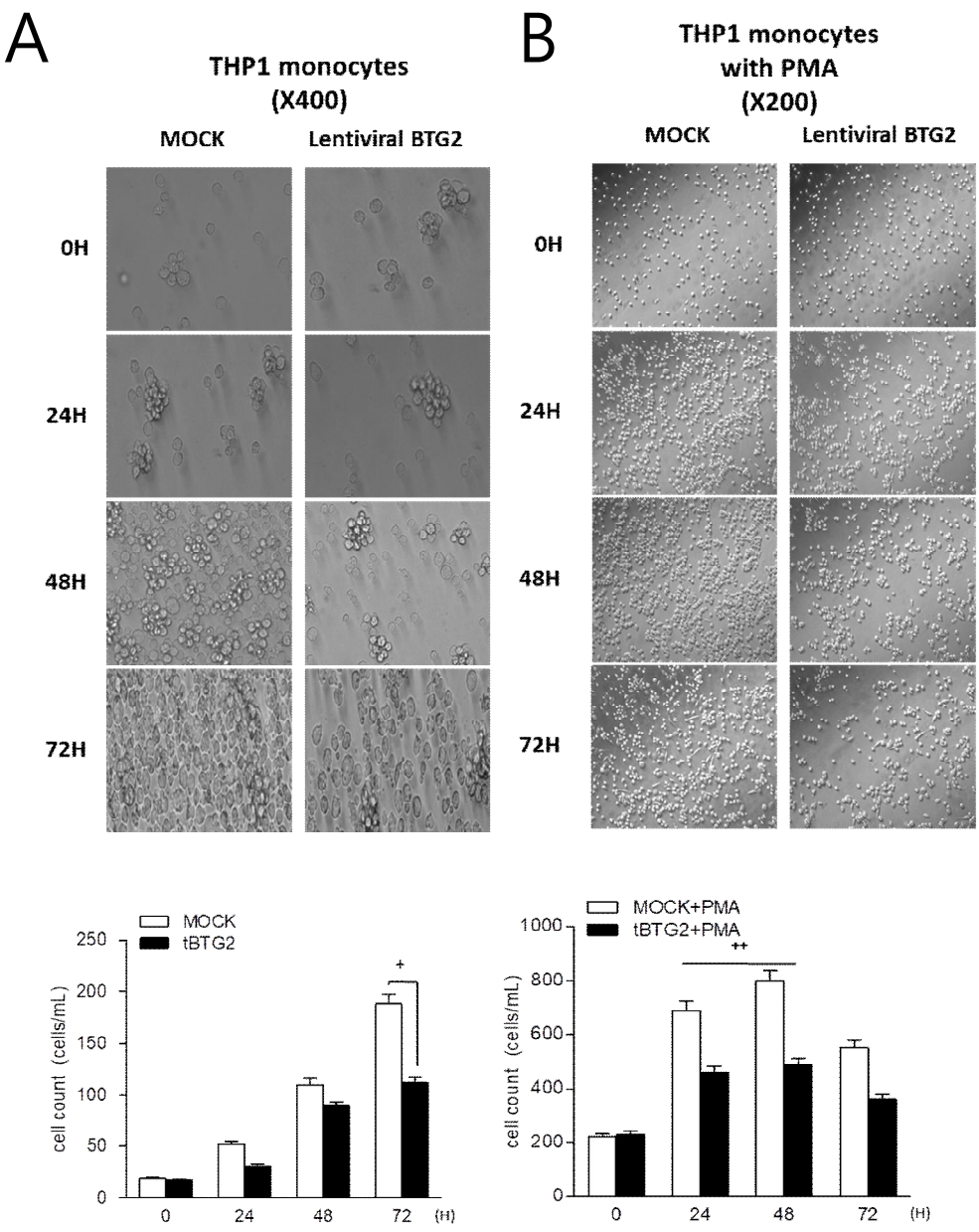

Figure 2. Overexpression of BTG2 reduces monocytes growth and differentiation into macrophages

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock for 0, 24, 48, and 72 hours (A) and then treated with 5 nM PMA for 24 hours to induce macrophage differentiation (B). Images visualized using a microscope (400X and 200X). Data are the mean ± SD of triplicates and were analyzed with the unpaired *t*-test. ($^{+}p < 0.05$. vs. control)

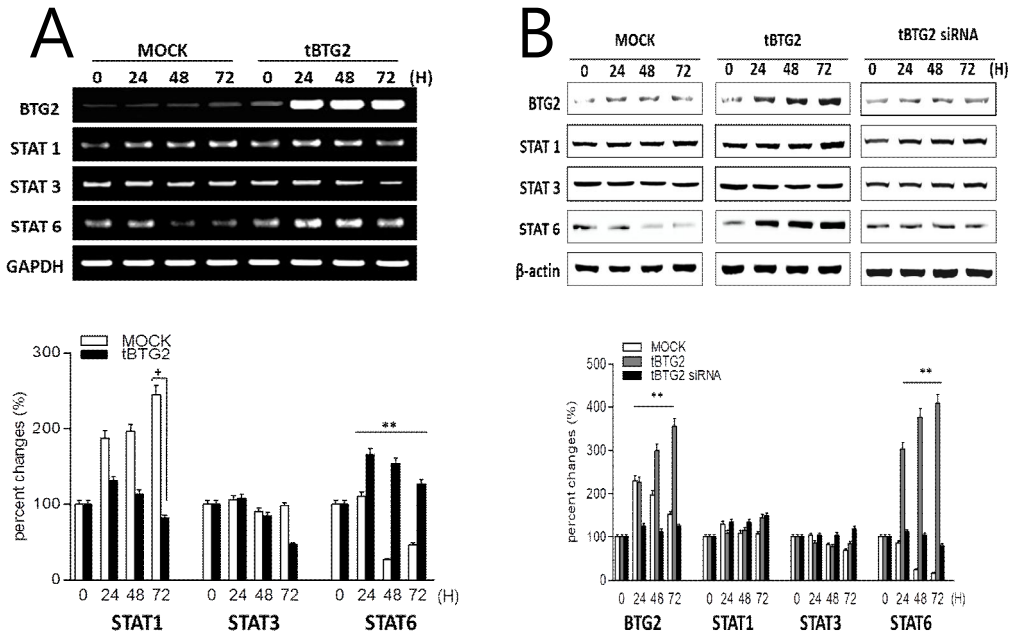

Figure 3. mRNA and protein expression of transcription factors in BTG2-overexpressing THP-1 monocytes

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock for 0, 24, 48, and 72 hours. Then, the cells were harvested, and mRNA levels were analyzed by RT-PCR (A). BTG2-overexpressing THP-1 monocytes were lysed with a 0.6% SDS lysis buffer, and proteins were detected by immunoblotting (B). Data are the mean ± SD values of triplicates and were analyzed with the unpaired *t*-test. ($^{+}p < 0.05$, $*p < 0.05$. vs. control)

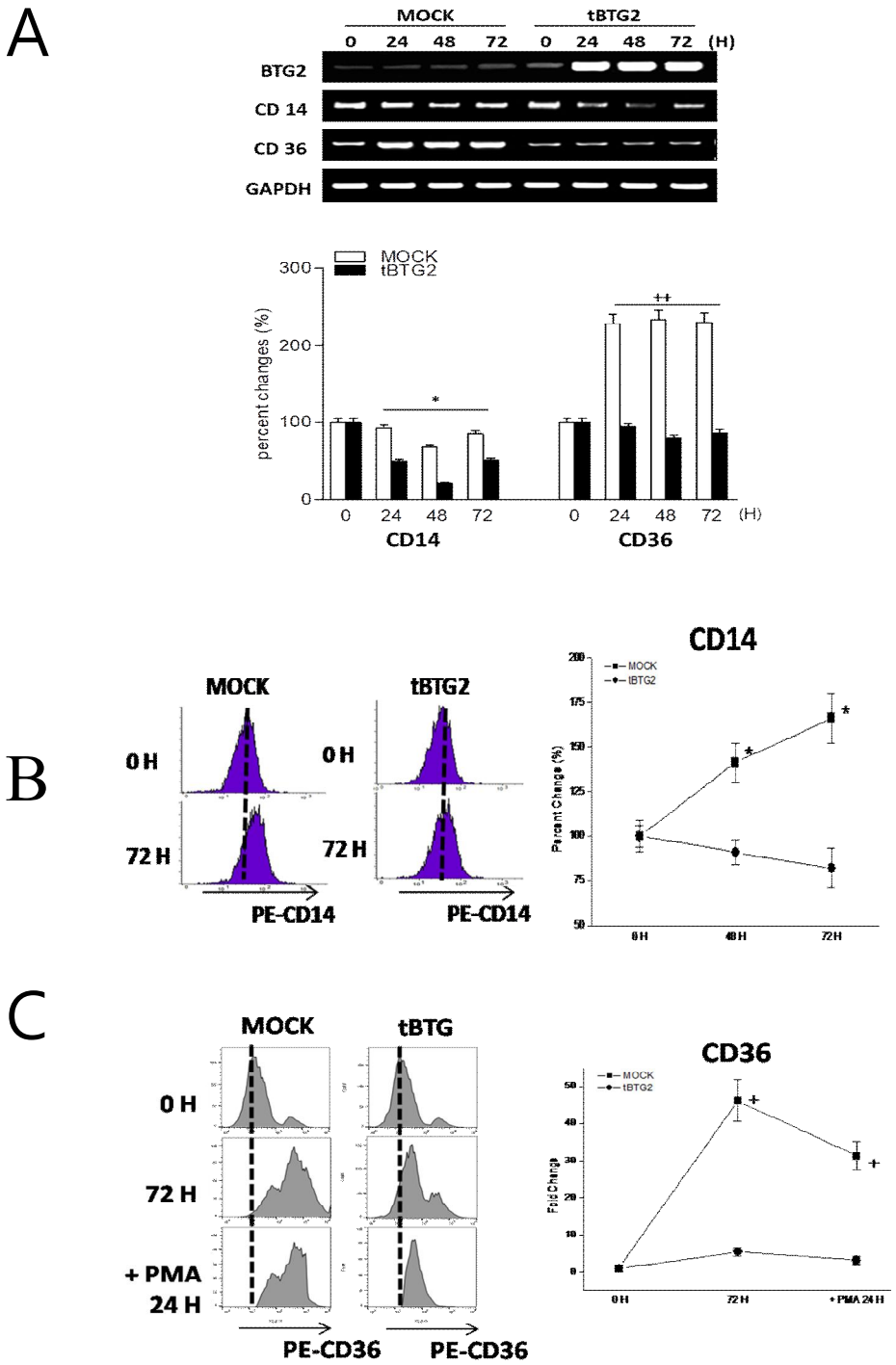

Figure 4. Overexpression of BTG2 in monocytes inhibits the expression of surface proteins such as CD14 and CD36

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock for 0, 24, 48, and 72 hours. Then, the cells were harvested, and mRNA levels were analyzed by RT-PCR

(A).

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock for 0, 24, 48, and 72 hours. Then, the cells were treated with PMA for 24 hours to induce macrophage differentiation. Next, BTG2 overexpressed cells were blocked with a human IgG FC fragment (10 μg/mL) at RT for 30 minutes. Then, the cells were incubated with a FITC-conjugated mouse anti-human CD14 antibody (B) or a human CD36/SR-B3 PE-conjugated antibody (C) at room temperature for 2 hours. The cells were washed and resuspended in DPBS, and fluorescence was analyzed by flow cytometry.

(n=3, *; $p<0.05$, $^{+}$; $p<0.05$ vs. the control)

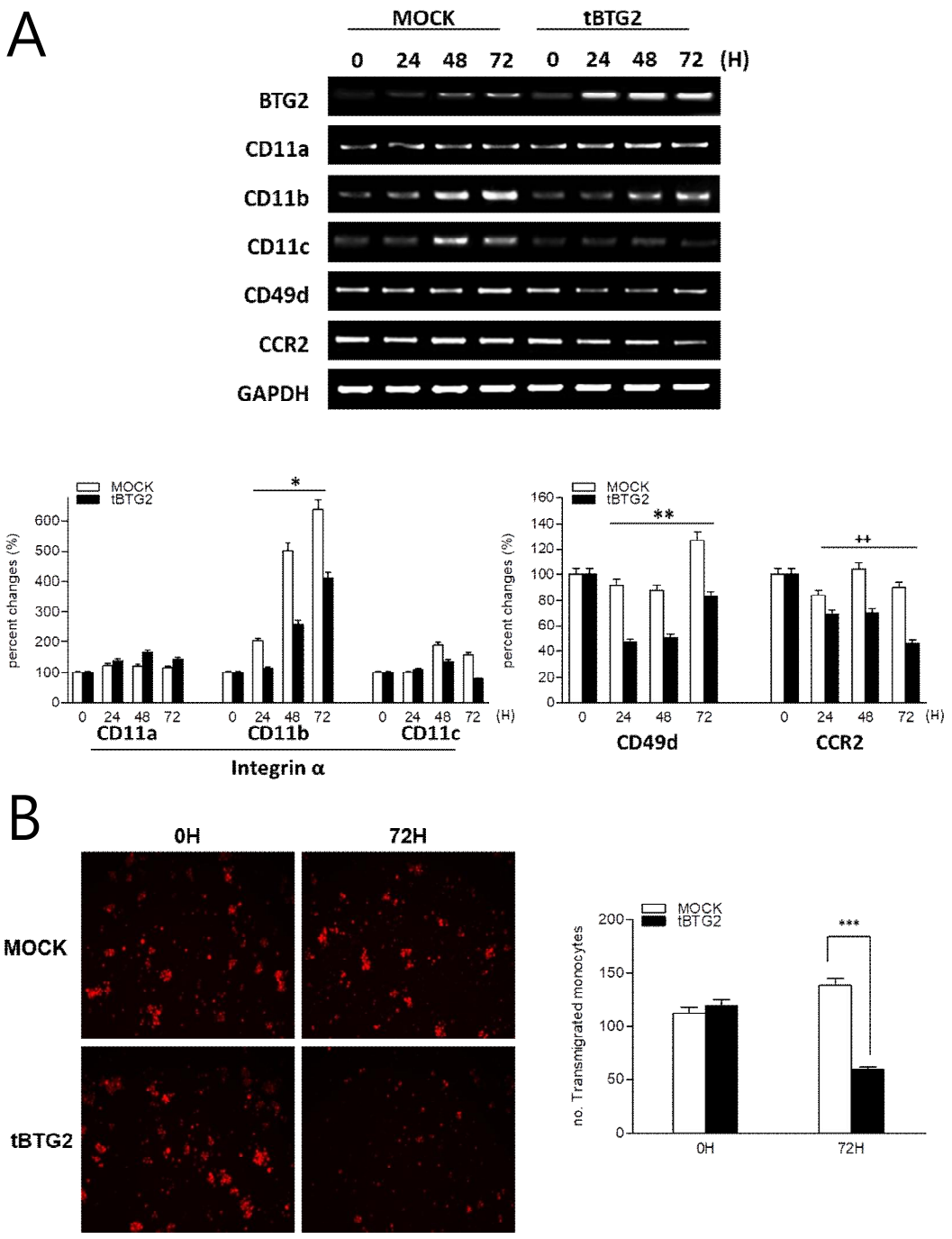

Figure 5. Overexpression of BTG2 in THP-1 monocytes downregulates the expression of integrins associated with cell adhesion and MCP-1-induced transmigration

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock or MOCK infected (control) for 0, 24, 48, or 72 hours. Then, the monocytes were harvested, and mRNA levels were analyzed by RT-PCR (A).

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock or MOCK infected (control) for 0 or 72 hours. Then, infected THP-1 monocytes were stained with Dil solution for 10 minutes, and stained cells were transferred to a Transwell. Serum-free medium was added to the bottom of the Transwell along with 10 ng/mL MCP-1 and incubated for 4 hours. Cells were visualized with a fluorescence microscope (100X) (B). Data are the mean ± SD values of triplicates and were analyzed with the unpaired *t*-test.
(+; $p<0.05$, level of significance)

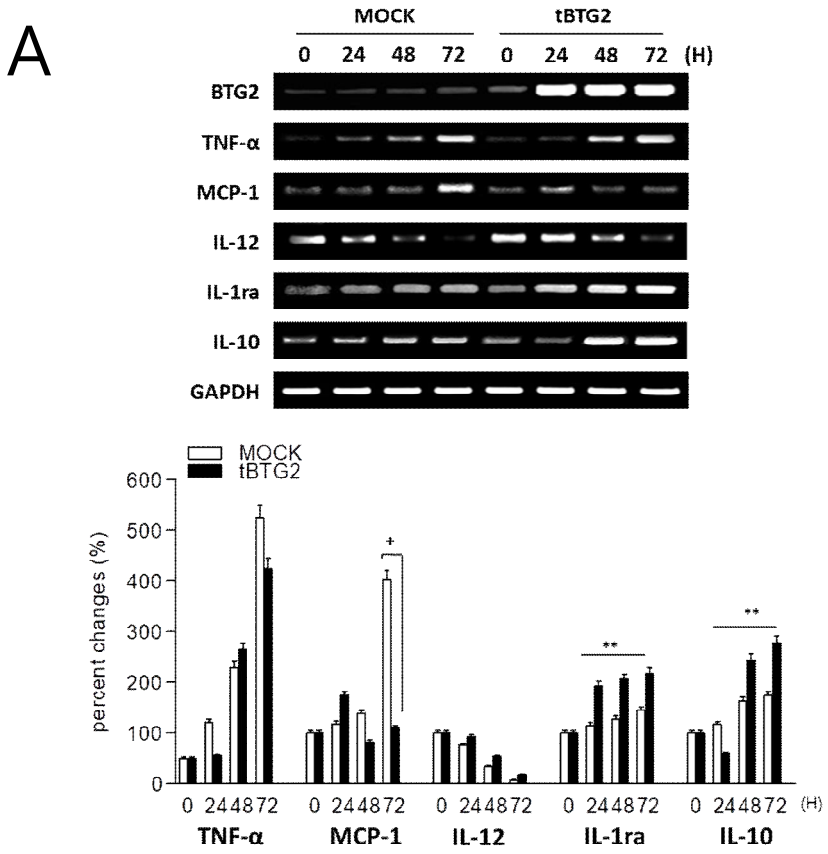

Figure 6. Pro- and anti-inflammatory cytokine mRNA levels in THP-1 monocytes

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock or MOCK infected (control) for 0, 24, 48, or 72 hours. Then, the monocytes were harvested, and mRNA levels were analyzed by RT-PCR (A). Data are mean ± SD values of triplicates and were analyzed with the unpaired *t*-test. ($^{+}p < 0.05$, $^{*}p < 0.05$ vs. the control)

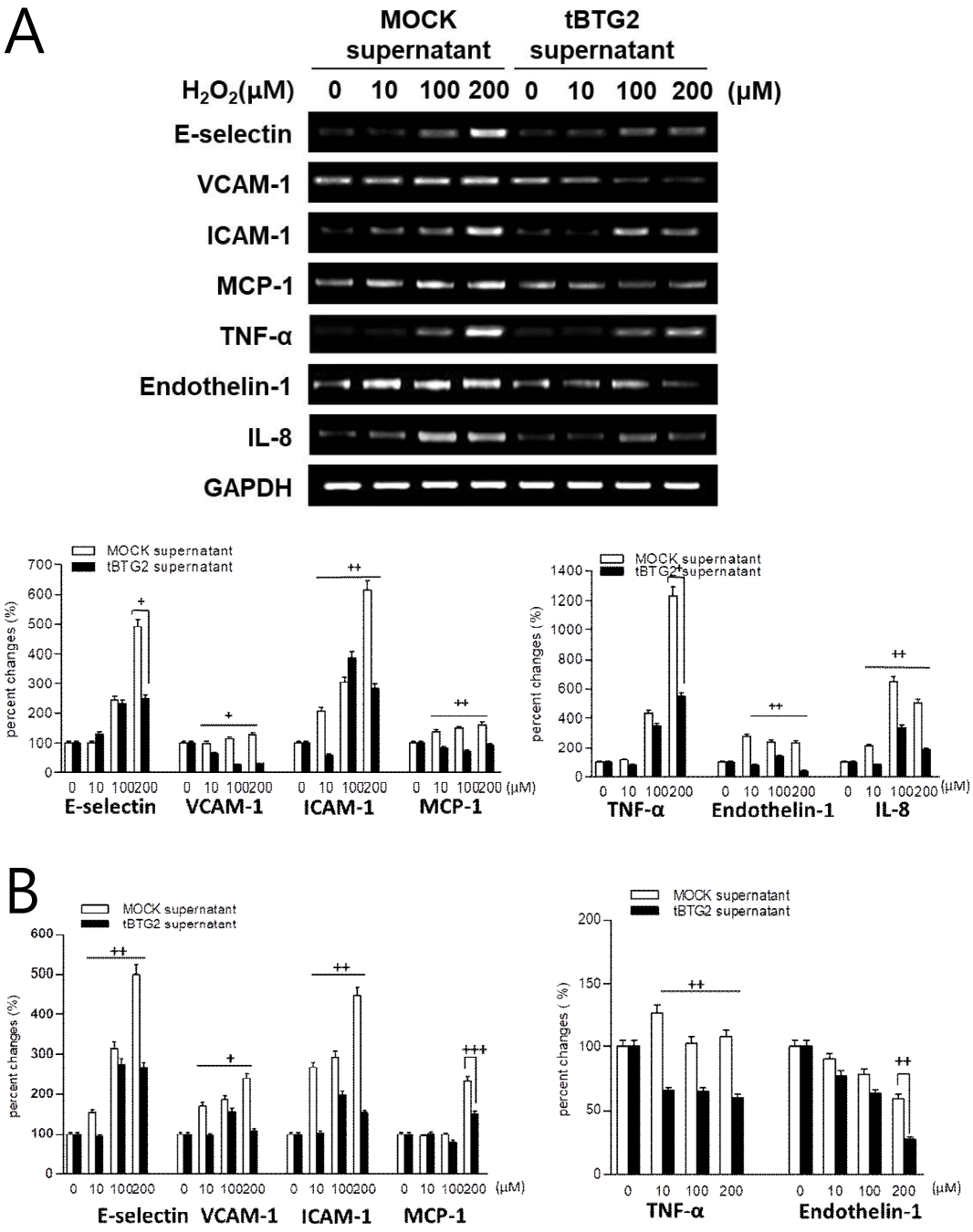

Figure 7. Expression of cell surface proteins and pro and anti-inflammatory cytokines mRNA levels in human endothelial cells

Human THP-1 monocytes were infected with 50 λ of lentiviral BTG2 stock or MOCK infected (control) for 72 hours. Next, endothelial cells were incubated with the culture supernatant from BTG2-infected or MOCK-infected THP-1 monocytes for 24 hours. Then,

the endothelial cells were treated with H_2O_2 for 2 hours at 37°C. The endothelial cells were harvested, and the mRNA levels were analyzed by RT-PCR (A) and real-time PCR (B). Data are the mean ± SD values of triplicates and were analyzed with the unpaired *t*-test.
($^{+}p < 0.05$, vs. the control)

Discussion

Our overall research aim is to inhibit the differentiation of monocytes and their transmigration into inflammatory lesions to slow the progression of atherosclerosis. At inflammatory lesions, monocytes can secrete anti-inflammatory cytokines, such as IL-10 and IL-1ra, which also have positive effects on other cells, such as endothelial cells. In the present study, the change of monocyte activity is found to be promoted by BTG2. BTG2 overexpression in monocytes reduced their differentiation into macrophages via downregulation of CD14 and CD36 and inhibited their transmigration into endothelial cells. Thus, expression of BTG2 in monocytes should slow the progression of atherosclerosis by decreasing the number of monocytes entering the atherosclerotic plaque. Thus, we suggest that overexpression of BTG2 in monocytes may be more positive in inhibiting the progression of atherosclerosis.

Circulating monocytes infiltrate atherosclerotic plaques and are transformed into macrophages. The macrophages in atherosclerotic plaque lesions have two phenotypes. M1 (or pro-inflammatory) macrophages and M2 (or anti-inflammatory) macrophages, which are able to transform in response to stimulation by bacteria, viruses, cytokines, chemokines, and small bioactive molecules. [26, 27] In a previous study, it was shown that inhibiting M2 macrophage activation aggravates cardiac dysfunction and plaque rupture following myocardial infarction in a mouse model. [28] In addition, upregulation of STAT6, a critical member of the Signal Transducer and Activator of Transcription (STAT) family that is involved in regulating cell differentiation and cytokine production, promoted M2 polarization and suppressed oxidized LDL-induced cell apoptosis, indicating that STAT6 upregulation contributes to the stability of atherosclerotic plaques by modulating macrophage differentiation. [27] The results of our study show that BTG2 overexpression in monocytes dramatically increased STAT6 (Figure 3). We proposed that BTG2 overexpression monocytes will promote their differentiation into M2-subtype macrophages, which will contribute to the recovery of atherosclerosis by increasing the expression of anti-

inflammatory cytokines, such as IL-10 and IL-1ra (Figure 6).

BTG2-overexpressing monocytes showed inhibited expression of CD14 and CD36, two surface proteins associated with monocyte differentiation (Figure 4). Human blood monocytes are classified into at least three types 1) classical $CD14^{++}$ $CD16^{-}$ monocytes are high level expression of the CD14 cell surface receptor, 2) non-classical $CD14^{+}CD16^{++}$ monocytes are low level expression of CD14 and additional co-expression of the CD16 receptor, and 3) intermediate $CD14^{++}CD16^{+}$ monocytes with high level expression of CD14 and low level expression of CD16. The classical monocytes present especially at the region of infection or disease, [29-31] and these monocytes respond to several inflammatory signals, by differentiating into macrophages. [29] Our results demonstrated that BTG2-overexpressing monocytes show decreased mRNA and protein (Figure 4) expression of CD14. Decreased CD14 expression in monocytes may inhibit their transformation into macrophages. We propose that this inhibition will have a positive effect on atherosclerosis, a typical inflammatory disease.

CD36 is an important factor in the differentiation of monocytes into macrophages. [32] CD36 is also an oxidized LDL receptor that promotes the differentiation of macrophages into foam cells after ingestion of large amounts of oxidized LDL. [4, 7, 32] Based on previous studies, CD36 may promote the development of atherosclerosis, and our results suggest that BTG2-overexpressing monocytes may reduce atherosclerosis by lowering the expression level of CD36 (Figure 4).

In this study, we confirmed that BTG2 induces anti-inflammatory responses in endothelial cells following damage by oxidants, such as hydrogen peroxide, via cytokines and chemokines, such as IL-10 and IL-1ra secreted from BTG2-overexpressing monocytes (Figure 6, 7). However, we could not confirm whether the endothelial cells were changed by any other cytokine or chemokine. Alternatively, a previous study showed that IL-10 protects against oxidant-induced inflammation. [33, 34] Injection of superoxides, such as LPS and NO, into $IL\text{-}10^{-/-}$ and $IL\text{-}10^{+/+}$ mice cause acetylcholine dysfunction in the blood vessels of

IL-$10^{-/-}$ mice but not IL-$10^{+/+}$ mice. Thus, in IL-$10^{+/+}$ mice, the endothelium is protected during acute inflammation. [34] We suggest that the large amount of IL-10 secreted from BTG2-overexpressing monocytes protected the endothelial cells from oxidants, such as hydrogen peroxide.

BTG2 has mainly been studied in cancers, including breast, bladder, kidney, and brain cancer. [35, 36] In cancer, the most important role of BTG2 is the downregulation of carcinogenesis and migration via regulation of oxidative stress. In addition, BTG2 regulates the cell cycle at the G1/S [21, 22] and G2 / M phases [23, 24] in cancer cells with inactive pRB or p53-dependant. Expression of BTG2 inhibits G1/S progression by regulating cyclin D1 transcription independent of pRb, and promotes G2/M phase arrest and apoptosis in a p53-dependent manner. Previous studies showed that BTG2 expression was lower in muscle-invasive bladder cancer (MIBC) than in non-muscle-invasive bladder cancer (NMIBC). [37] In the progression of MIBC, Sp1 increases BTG2 expression, inhibiting the expression of DNMT-1 and slowing the progression of bladder cancer.

Furthermore, in previous studies, it was shown that tumor grade, size, and cyclin D1 protein overexpression were decreased by BTG2 expression in breast cancer. [38, 39] In addition, the migration of mammary epithelial cells was increased through activation of the human epidermal growth factor receptor (HER) pathway in BTG2-knockout mice. [40] Furthermore, signaling downstream of the $BTG2^{/TIS21/Pc3}$ gene involved in the regulation of cancer cell invasion and migration is mediated by Akt1 activation. $BTG2^{/TIS21/Pc3}$-mediated reduction of cancer cell invasion and motility may inhibit cell growth. In addition, the $BTG2^{/TIS21/Pc3}$-Akt1 signal also inhibited the transcription of Nox4 by Sp1, leading to downregulation of cytosolic ROS generation in breast cancer cells. [41] Previous, BTG2 has mainly been studied in cancers, in the present study, we overexpressed BTG2 in monocytes under atherosclerotic condition and confirmed how it affects the progression of atherosclerosis.

Previous in vivo and in vitro studies have shown that p53 induced by C-reactive protein (CRP) in macrophages upregulates BTG2 and subsequently promotes apoptosis and inhibits

proliferation. [25] CD32, a FC gamma receptor Ⅱ, directly activated by CRP, subsequently activating Nox2, which leads to the production of ROS in monocytes/macrophages. Then, p53 is activated and induces upregulation of BTG2. CRP-induced BTG2 inhibits proliferation and promotes apoptosis in macrophages. In the present study, we demonstrated that overexpression of BTG2 in monocytes inhibited differentiation, by downregulating CD14 and CD36 (Figure 4), as well as MCP-1-induced transmigration (Figure 5). In addition, the level of anti-inflammatory cytokines was increased by BTG2 overexpression in monocytes (Figure 6).

In summary, our results indicate that BTG2 is an anti-proliferation gene. Overexpression of BTG2 in monocytes can inhibit their stimulation and reduce their transmigration into endothelial cells. In addition to suppressing their own stimulation, BTG2-overexpressing monocytes also actively secrete anti-inflammatory cytokines, which protect other cells such as vascular endothelial cells from oxidant damage. Based on these results, BTG2 may be a new target for slowing the progression of atherosclerosis, a typical inflammatory disease.

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국문요약

배경 - 죽상동맥경화증은 동맥 혈관벽 내의 염증 반응으로 인한 대표적인 염증 질환이다. 단핵구는 죽상경화의 발전에 중요한 역할을 한다. 혈액 속을 돌아다니던 단핵구는 염증반응에 의해 혈관 벽에 점증(recruitment) 되고, 혈관벽 내로 침투(infiltration) 된다. 혈관벽 내로 들어온 단핵구들은 대식세포(macrophages)로 분화하여 oxidized LDL 에 대한 식균 작용(scavenging)을 하게 된다. 분화 된 단핵구는 TNF-α 와 같은 전-염증성 사이토카인 (pro-inflammatory cytokines)을 분비한다. 과도한 oxidized LDL 의 흡수는 대식세포가 foam cells 로 분화되어, 동맥 혈관벽에 죽상경화반(atherotic plaque)을 형성한다.

BTG2 (B cell translocation gene 2)는 anti-proliferative (APRO) 유전자로 알려져 있으며, 비장, 위, 흉선, 폐와 같은 다양한 조직에서 발현된다. BTG2 의 중요한 역할은 위암, 유방암 등을 일으키는 다양한 암세포의 성장과 증식, 침입을 억제할 뿐만 아니라 세포사멸을 촉진시킨다. 따라서 본 연구에서는 죽상동맥경화증의 주요 원인인 단핵구의 염증 유발을 억제하는 BTG2 의 역할을 증명하는 실험을 수행하였다.

방법 및 결과 - 단핵구에서 BTG2 의 과발현이 세포 분화에서 중요한 전사 인자(transcription factor)인 STAT6 를 증가시키는 것을 RT-PCR 과 immunoblotting 으로 증명하였다. 또한, BTG2 과발현에 따른 CD14 와 CD36 이 억제되는 것을 RT-PCR 과 flow cytometry 로 확인하여, 단핵구의 분화를 억제한다는 것을 증명하였다. BTG2 가 과발현 된 단핵구 표면에 존재하는 CD49 와 같은 integrin 의 발현을 억제한다는 것을 RT-PCR 을 이용하여 확인하였다. 또한, MCP-1 에 의한 단핵구의 이동(transmigration)이 억제된다는 것을 transmigration assay 로 증명하였다. IL-10 과 IL-1ra 같은 항-염증성 사이토카인(anti-inflammatory cytokines)의 발현은 증가했으며, TNF-α, MCP-1, IL-12 와 같은 전-염증성 사이토카인(pro- inflammatory cytokines)의 발현 감소를 통해 BTG2 의 과발현이 염증성 사이토카인의 분비에 영향을 준다는 것을 확인하였다. BTG2 가 과발현 된 단핵구의 배양액으로 내피 세포를 배양하여 산화 스트레스를 주었을 때, Endothelin-1, TNF-α, IL-8 과 같은 전-염증성 사이토카인과 E-selectin, VCAM-1 및 ICAM-1 과 같은 표면 단백질의 발현이 감소되었다는 것을 RT-PCR 과 Realtime PCR 을 이용하여 확인하였다.

결론 - BTG2 가 과발현 될 경우 단핵구가 염증성 대식세포로 분화되는 것을 억제할 수 있고, MCP-1 에 의한 이동을 억제할 수 있다. 또한 단핵구의 pro-atherogenic 활성 유도를 억제해준다. TNF-α, MCP-1, IL-12 와 같은 전-염증성 사이토카인의 분비를 억제하며, IL-10, IL-1ra 와 같은 항-염증성 사이토카인의 분비를 증가시킨다. 이는 내피세포와 같은 주변 세포에 긍정적인 영향을 주어 산화 스트레스에 의해 손상을 보호해 줄 것이다. 본 연구 결과를 통해서, 대표적인 염증 질환 중 하나인 죽상동맥경화증의 진행을 지연시키는 것에 기여할 것이다.

중심단어 - B cell Translocation Gene 2 (BTG2), 단핵구/대식세포, 동맥내피세포, 죽상동맥경화증, 염증 질환, Inflammatory cytokines, Monocyte chemoattractant protein-1 (MCP-1), C-C chemokine receptor type 2 (CCR2), Integrin