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Role of Antiproliferative B Cell Translocation Gene-1 as an Apoptotic Sensitizer in Activation-Induced Cell Death of Brain Microglia

Heasuk Lee,* Sanghoon Cha,† Myung-Shik Lee,‡ Gyeong Jae Cho,* Wan Sung Choi,* and Kyoungho Suk2,8

Mouse brain microglial cells undergo apoptosis on exposure to inflammatory stimuli, which is considered as an autoregulatory mechanism to control their own activation. Here, we present evidence that an antiproliferative B cell translocation gene 1 (BTG1) constitutes a novel apoptotic pathway of LPS/IFN-γ-activated microglia. The expression of BTG1 was synergistically enhanced by LPS and IFN-γ in BV-2 mouse microglial cells as well as in primary microglia cultures. Levels of BTG1 expression inversely correlated with a proliferative capacity of the microglial cells. Tetracycline-based conditional expression of BTG1 not only suppressed microglial proliferation but also increased the sensitivity of microglial cells to NO-induced apoptosis, suggesting a novel mechanism of cooperation between LPS and IFN-γ in the induction of microglial apoptosis. An increase in BTG1 expression, however, did not affect microglial production of NO, TNF-α, or IL-1β, indicating that the antiproliferative BTG1 is important in the activation-induced apoptosis of microglia, but not in the activation itself. The synergistic action of LPS and IFN-γ in the microglial BTG1 induction and apoptosis was dependent on the Janus kinase/STAT1 pathway, but not IFN-regulatory factor-1, as demonstrated by a pharmacological inhibitor of Janus kinase (AG490), STAT1 dominant negative mutant, and IFN-regulatory factor-1-deficient mice. Taken together, antiproliferative BTG1 may participate in the activation-induced cell death of microglia by lowering the threshold for apoptosis; BTG1 increases the sensitivity of microglia to apoptotic action of autocrine cytotoxic mediator, NO. Our results point out an important link between the proliferative state of microglia and their sensitivity to apoptotic agents. The Journal of Immunology, 2003, 171: 5802–5811.

Microglia are a type of neuroglia that support, nurture, and protect the neurons, maintaining homeostasis of the fluid that bathes neurons. Although the ontogeny of microglial cells has long been debated, recent works using mAbs specific for microglial cells indicated that these cells are closely related to monocytes and macrophages (1). Microglia function as macrophages in the CNS; they migrate to areas of injured nervous tissue, and they engulf and destroy microbes and cellular debris (2). They also secrete inflammatory cytokines and toxic mediators, which may amplify the inflammatory responses (3, 4). Activation of microglial cells may be intended to protect neurons at first. However, activation of microglial cells and inflammatory products derived from them have also been implicated in the neuronal destruction commonly observed in various neurodegenerative diseases (4). From this point of view, one can speculate that the autoregulatory mechanisms that control the microglial activation may exist in vivo, and the failure of these autoregulatory mechanisms may be responsible for the deleterious effects of microglial activation. Accordingly, the elucidation of molecular mechanisms underlying the autoregulation of microglial activation may enhance our understanding of pathogenesis of neurodegenerative diseases. Recently, activated macrophages have been shown to undergo apoptosis (5–7). It has been suggested that the apoptosis of activated macrophages is one mechanism whereby an organism may regulate immune and inflammatory responses involving macrophages (7). We and others have demonstrated that a similar regulatory mechanism exists for microglial cells (8–10) and astrocytes (11) as well. We have previously shown that microglial cells and astrocytes undergo apoptosis upon inflammatory activation in a manner similar to that of activation-induced cell death (AICD)3 of lymphocytes, and NO acts as an autocrine cytotoxic mediator in this process (9, 11). Inflammatory stimuli played a dual role in the microglial apoptosis; they not only induced NO production through IFN-regulatory factor-1 (IRF-1) but also initiated the NO-independent apoptotic pathway via caspase-11 induction followed by caspase-3 activation (12). However, a role of the inflammatory stimuli in the microglial AICD other than the induction of NO production and caspase-11 expression has been also suggested, because pretreatment of microglia with LPS/IFN-γ marked increased the sensitivity of microglia to NO-induced apoptosis (12). Therefore, it is of great interest to

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3 Abbreviations used in this paper: AICD, activation-induced cell death; IRF-1, interferon-regulatory factor-1; JAK, Janus kinase; BTG1, B cell translocation gene 1; NMMA, N-nomethyl-L-arginine; SNAP, S-nitroso-L-acetylpenicillamine; SNP, sodium nitroprusside; AG490, α,α′-cyan(3,4-dihydroxy)-N-benzylcinchonamide.

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identify such LPS/IFN-γ-inducible apoptotic mediator(s) to understand the mechanism of microglial AICD to the fullest extent.

B cell translocation gene 1 (BTG1) was first identified as a translocation gene in a case of B cell chronic lymphocytic leukemia (13). BTG1 is constitutively expressed in quiescent cells, whereas its expression is down-regulated as the cells enter the growth cycle (14, 15). Experiments with the forced expression of the gene showed that BTG1 negatively regulates cell proliferation (16). The antiproliferative properties of BTG1 have been observed in many different cell types including PBMC (16), testis (17), and macrophages (18). Especially, in macrophages, we and others have found that the levels of BTG1 expression negatively correlate with cell proliferation (18) and that the gene expression colocalizes with an apoptotic marker (19). Moreover, overexpression of BTG1 in NIH3T3 cells inhibited the cell proliferation (16) and increased apoptotic frequency (19). These previous findings and the functional similarity between macrophages and brain microglia led us to speculate that BTG1 might play a role in both limiting cell proliferation and inducing apoptosis in microglia.

In this work, we have demonstrated that BTG1 exerts antiproliferative activity in microglia and that the antiproliferative BTG1 constitutes a novel apoptotic pathway of LPS/IFN-γ-activated microglia. LPS/IFN-γ-induced BTG1 participated in microglial apoptosis by increasing the sensitivity of microglia to NO, which acts as an autocrine apoptotic mediator in AICD of microglia. BTG1 induction, however, did not influence the production of inflammatory mediators from activated microglia. The enhancement of BTG1 expression by LPS/IFN-γ and the ensuing apoptosis-sensitizing effects in microglia were dependent on Janus kinase (JAK)/STAT1, but not IRF-1. Taken collectively, our results indicate that BTG1 may lower the threshold for microglial apoptosis by its antiproliferative activity, and this BTG1 action seems to be independent of the microglial activation process (at least with respect to the production of NO, TNF-α, and IL-1β).

Materials and Methods

Reagents

LPS, N-monomethyl-L-arginine (NMMA), S-nitroso-N-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), and tetracycline were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant mouse IFN-γ was purchased from R&D Systems (Minneapolis, MN). α-Cyano(3,4-dihydroxy)-N-benzylcinnamamide (AG490) was from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

Mice and cells

Mice with a targeted mutation in the IRF-1 gene (homozygous mice and their heterozygous littermates) were kindly provided by Dr. Y. C. Sung at Postech (Pohang, Korea) (20) and bred in a virus-free facility at the Sung Medical Center. The IRF-1 locus gene in a case of B cell chronic lymphocytic leukemia (13). BTG1 is constitutively expressed in quiescent cells, whereas its expression is down-regulated as the cells enter the growth cycle (14, 15). Experiments with the forced expression of the gene showed that BTG1 negatively regulates cell proliferation (16). The antiproliferative properties of BTG1 have been observed in many different cell types including PBMC (16), testis (17), and macrophages (18). Especially, in macrophages, we and others have found that the levels of BTG1 expression negatively correlate with cell proliferation (18) and that the gene expression colocalizes with an apoptotic marker (19). Moreover, overexpression of BTG1 in NIH3T3 cells inhibited the cell proliferation (16) and increased apoptotic frequency (19). These previous findings and the functional similarity between macrophages and brain microglia led us to speculate that BTG1 might play a role in both limiting cell proliferation and inducing apoptosis in microglia.

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cytometric analysis (FACSvantage; BD Biosciences, San Jose, CA). The percentage of cells in S phase was determined by a cell cycle analysis program, ModFitLT (Verity Software House, Topsham, ME). Apoptosis of microglial cells was also evaluated by annexin V-binding assay. In brief, cells were stained with annexin V-FITC, a fluorescent annexin V conjugate (Clontech), and then subjected to flow cytometric analysis.

Detection of DNA ladder and DNA fragmentation assay

Agarose gel electrophoresis of genomic DNA was conducted to detect a DNA ladder. For isolation of genomic DNA, BV-2 cells were lysed by incubation in the extraction buffer overnight at 55°C (10 mM Tris-HCl (pH 8.0), 0.1 M EDTA, 0.5% SDS, 100 mM NaCl), followed by phenol-chloroform extraction and ethanol precipitation. The final pellet was dissolved in distilled water containing 0.1 mg/ml RNase A. Isolated genomic DNA was electrophoresed on 1.5% agarose gel and stained with ethidium bromide to detect internucleosomal cleavage. For quantitative analysis of DNA fragmentation, the Cell Death Detection ELISA system (Roche Applied Science, Indianapolis, IN) was used. The assay is based on the quantitative sandwich enzyme immunoassay principle using mouse mAbs directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. After anhistidine Ab was fixed adsorptively on the wall of the microplate wells, the cytoplasmatic fractions of the samples were added. In the next step, anti-DNA Ab conjugated to peroxidase was added to detect released nucleosomes in the sample. After removal of unbound peroxidase conjugate, the amount of peroxidase retained in the immunocomplex was determined photometrically.

RNA analysis

Total RNA was extracted from BV-2 cells or primary microglial cells by a sequential addition of 4 M guanidinium thiocyanate, 2 M sodium acetate, and acid phenol-chloroform. Reverse transcription was conducted using Superscript (Life Technologies-BRL) and oligodeoxynucleotide primer. PCR amplification using primer sets specific for IRF-1 was conducted at 58°C annealing temperature for 30 cycles. PCR for BTG1 and β-actin was conducted at 55°C annealing temperature for 30 cycles. Nucleotide sequences of the primers were based on published cDNA sequences of mouse BTG1, β-actin, and IRF-1 (BTG1 forward, GAT TGG ACT GAG CAG AAG CAG; GAC CTA TAT GC; β-actin reverse, AAC GCA GCT CAG TAA CAG TC; IRF-1 forward, TCT GAG CAT AGT GAC GAC; IRF-1 reverse, GGT CAG AGA CCC AAA CTA TGG TCG). The lengths of PCR product for each primer set were 261 bp for BTG1, 287 bp for β-actin, and 426 bp for IRF-1, respectively. For Northern blot analysis, isolated total RNA (20 μg/sample) was electrophoresed on 1.5% formaldehyde-containing 0.7% agarose gel. Northern blotting was performed using a nylon membrane (Schleicher & Schuell, Keene, NH). After UV cross-linking, the membranes were hybridized with a BamHI fragment (511 bp) of BTG1 cDNA probe (10μM/cm). The membranes were then washed at 65°C, dried, and exposed to x-ray films.

Western blot analysis

Cells were lysed in triple-detergent lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF). Protein concentration in cell lysates was determined using the Bio-Rad protein assay kit. An equal amount of protein for each sample was separated by 12% SDS-PAGE and transferred to Hybrid ECL nitrocellulose membranes (Amersham, Arlington Heights, IL). The membranes were blocked with 5% skim milk and sequentially incubated with rabbit polyclonal Abs (rabbit polyclonal anti-BTG1, Genemed Synthesis, South San Francisco, CA; rabbit polyclonal anti-human/mouse phospho-STAT1 (Tyrosine 701), Cell Signaling Technology, Beverly, MA; rabbit polyclonal anti-IRF-1, Santa Cruz Biotechnology, Santa Cruz, CA; mouse monoclonal anti-α-tubulin, Sigma-Aldrich) and HRP-conjugated secondary Abs (anti-rabbit or -mouse IgG, Amersham), followed by ECL detection (Amersham). Mouse BTG1-specific Ab was generated against two synthetic peptide sequences of mouse BTG1 protein (102-LTLWVD-PYEVSYRIGEDG-119 and 156-GRTSPSKNYNMM-167; Ref.25) by custom Ab service of Genemed Synthesis.

Transient transfection

BV-2 cells in six-well plates were cotransfected with 1 μg of mouse BTG1 cDNA under control of CMV promoter (pcDNA3-BTG1) together with 0.2 μg of lucZ gene (pCH110; Pharmacia, Peapack, NJ) using lipofectAMINE reagent (Life Technologies) according to the supplier’s instructions. At 48 h after the transfection, the cells were treated with NO donor. After another 24 h, the cells were fixed with 0.5% glutaraldehyde for 10 min at room temperature and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (1 mg/ml in 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM magnesium chloride at 37°C for the detection of blue cells expressing lucZ. Alternatively, in some experiments, BV-2 cells were cotransfected with a dominant negative mutant of STAT1 cDNA (provided by T. Hirano, Osaka University, Osaka, Japan) along with 0.2 μg of lucZ gene. At 48 h after the transfection, the cells were pretreated with 100 ng/ml LPS plus 10 U/ml IFN-γ for 16 h before the treatment with NO donor for another 24 h. The cells were then fixed and stained as described above. At least 250 blue cells were counted for each experiment, and transfection efficiency was 18–31%. The percentage of apoptotic cells was based on the morphology of blue cells coexpressing either BTG1 or dominant negative mutant of STAT1 and lucZ. Dark blue and condensed cells were considered to be apoptotic. For the analysis of BTG1 expression, the cells were cotransfected with a dominant negative STAT1 along with neo-resistant gene construct (pSV2neo). Seven days later, G418 (500 μg/ml)-resistant cells were selected, pooled, and then subjected to analysis of LPS/IFN-γ-induced BTG1 expression.

Statistical analysis

All data are means ± SEM from three or more independent experiments. Statistical comparison between different treatments was done by either Student’s t test or one-way ANOVA with Dunnett’s multiple comparison test using the GraphPad Prism program (GraphPad Software, San Diego, CA). Differences of p < 0.05 were considered statistically significant.

Results

Induction of BTG1 expression and microglial apoptosis by LPS/IFN-γ

Previously, we have observed that microglial cells activated by inflammatory stimuli such as LPS and IFN-γ undergo apoptosis as a possible autoregulatory mechanism for their own activation states (9), and IFR-1-mediated NO production and caspase-11 induction play a pivotal role in this process (12). Our previous work also suggested that LPS/IFN-γ pretreatment might lower the threshold for microglial apoptosis through the induction of inhibitors or pathways other than IFR-1 and caspase-11 (12). Thus, we initiated the current study by searching for LPS/IFN-γ-induced genes in BV-2 mouse microglial cells by PCR-based subtractive hybridization cloning in an attempt to better understand the mechanisms by which inflammatory stimuli induce the microglial apoptosis. One of the clones identified by subtractive hybridization was BTG1. BTG1 was first identified in 1991 by molecular cloning of chromosomal breakpoint in B cells (13). A negative correlation between BTG1 expression and cell proliferation has been observed in various cell types (16–18). In particular, BTG1 expression was associated with both apoptosis and inhibition of cell proliferation in macrophages (19), which are closely related to microglia. Thus, in the current work, we explored the possibility that BTG1 might negatively regulate proliferation of microglial cells as observed in macrophages and this antiproliferative action of BTG1 might be related with microglial apoptosis. Before testing this possibility, the enhancement of BTG1 expression by LPS/IFN-γ was first confirmed by Northern blot as well as Western blot analysis (Fig. 1, A–C). The expression of BTG1 at both mRNA and protein levels was markedly increased by LPS/IFN-γ treatment in BV-2 cells (Fig. 1, A and B) as well as mouse primary microglia cultures (Fig. 1C). However, either LPS or IFN-γ alone did not significantly affect BTG1 expression. An increase in the level of BTG1 expression by LPS/IFN-γ treatment correlated with microglial cell death; cotreatment with LPS plus IFN-γ significantly increased cell death of both BV-2 and primary microglia cultures compared with the single treatment of either LPS or IFN-γ alone (Fig. 1D). The reduction of cell viability was similarly observed by trypan blue.
Antiproliferative activity of BTG1 in microglia

On the basis of the known antiproliferative properties of BTG1 in macrophages and other cell types, we next sought to determine whether BTG1 negatively regulates microglial proliferation. Because the antiproliferative nature of BTG1 may impede the generation of stable transfectants that constitutively overexpress the gene, we used a tetracycline-based conditional expression system to control the expression of BTG1 in BV-2 cells. A synthetic inducible expression system on the basis of the tetracycline repressor of E. coli (23) was constructed to reversibly control the expression of BTG1 in the microglial cells (Fig. 2A). BV-2/tet3 cells, a derivative of the BV-2 cell line with tetracycline-controlled expression of BTG1, were generated. In BV-2/tet3, transfected BTG1 gene expression is turned on in the absence of tetracycline in the cultivating medium. A low constitutive expression of BTG1 was detected in BV-2/tet3, and this was enhanced 5.2-fold by the removal of tetracycline (Fig. 2B). The proliferative capacity of BV-2/tet3 cells in the presence or absence of tetracycline was then compared to evaluate the potential antiproliferative activity of BTG1 in microglia. Enhancement of BTG1 expression by the removal of tetracycline suppressed the proliferation of BV-2/tet3 cells (Fig. 3, A and B) and led to the reduction in the percentage of S phase cells (Fig. 3C). Induction of BTG1 expression per se, however, did not significantly affect cell viability, as demonstrated by trypan blue staining (data not shown). Similarly, treatment of parental BV-2 cells with LPS/IFN-γ that increased BTG1 expression also attenuated the proliferative capacity of the cells as evidenced by the reduction of cells in S phase (24.5% in untreated cells, 11.6% in LPS/IFN-γ-treated cells). These results again support the antiproliferative activity of BTG1 in microglia. Because LPS/IFN-γ treatment itself causes cytotoxicity by inducing NO production, the experiments were done in the presence of NMMA, an inhibitor of NO synthesis. The percentage of microglia in S phase of cell cycle was assessed by DNA ploidy analysis of viable cells.

Role of BTG1 in microglial apoptosis

Previously, BTG1 has been shown to inhibit cell proliferation and induce apoptosis when overexpressed in NIH3T3 cells (19). Moreover, current results indicated that BTG1 was induced by LPS/IFN-γ and that BTG1 inhibited microglial proliferation. On the basis of these results, we next asked whether BTG1 plays any role in the apoptosis of microglia following inflammatory activation. First of all, we investigated a possible correlation between BTG1 expression and the propensity of microglial cells to undergo apoptosis on exposure to NO, an apoptosis-inducing agent in microglia. Augmentation of BTG1 expression by the removal of tetracycline from the cultivating medium increased the sensitivity of BV-2/tet3 cells to the toxicity of the NO donor, SNAP, at a low concentration (0.2 mM; Fig. 4). Similar results were obtained by another NO donor, SNP (data not shown). Previously, we have

FIGURE 1. BTG1 induction and microglial cell death by LPS/IFN-γ. After treatment of BV-2 cells with LPS (100 ng/ml) and IFN-γ (10 U/ml) either alone or in combination, BTG1 expression at mRNA (4 h after treatment) or protein level (8 h after treatment) was assessed by Northern blot analysis (A) or Western blot analysis (B), respectively. Only the combination of LPS and IFN-γ strongly increased BTG1 expression. C, Enhancement of BTG1 expression by LPS/IFN-γ (4 h after treatment) was similarly observed in mouse primary microglia cultures by RT-PCR. Ethidium bromide staining of rRNA of 28S and 18S or detection of α-tubulin or β-actin was done to confirm the equal loading and integrity of the samples. The amplified products were not observed in RT-PCR without reverse transcriptase (data not shown). D, When BV-2 cells or primary microglia cultures were treated with LPS and IFN-γ alone or in combination for either 24 h (BV-2 cells) or 72 h (primary microglia cultures), >50% cytotoxicity was observed only after LPS plus IFN-γ treatment. Viability of untreated cells was set to 100%. Results are mean ± SEM of three independent experiments. *, Statistically significant differences from untreated control (p < 0.05).

FIGURE 2. Construction of tetracycline-regulated BTG1 expression system in microglia. A, BTG1 cDNA fragment containing the entire coding region was cloned into the EcoRI/SpeI site of pTet-Splice plasmid under the control of the Tet promoter to generate pTet-Splice/BTG1 (see Materials and Methods). B, After BV-2 cells were cotransfected with pTet-Splice/BTG1, pTet-tTA, and pSV2neo, stable transfectants were selected in the presence of G418 and tetracycline. In a selected clone, BV-2/tet3, BTG1 induction was evaluated by removal of tetracycline from the growth medium. Western blot detection of BTG1 protein and densitometric analysis indicated ∼5-fold increase in BTG1 protein level in the cells grown in the absence of tetracycline (∼tet) compared with the cells grown in the presence of tetracycline (∼tet). BTG1 protein level was normalized to α-tubulin. Results are representative of three independent experiments.
determined that SNAP or SNP (0.2 mM) alone does not induce a significant change in the viability of BV-2 microglial cells (12). Thus, an increase in *BTG1* expression seems to render microglial cells sensitive to an otherwise nontoxic dose of NO donors. Cytotoxicity was based on the number of apoptotic cells assessed by flow cytometric analysis of annexin V-binding cells (Fig. 4A) or quantification of DNA fragmentation using specific Ab against apoptosis (Fig. 4B). When *BTG1* expression was induced in BV-2/tet3 by tetracycline removal from the growth medium, the proliferative capacity of the cells was decreased as determined by MTT assay (A) or phase contrast microscopy of the cells at day 3 during the MTT assay (×200; B). Results are mean ± SEM of three independent experiments. *Statistically significant differences between the cells grown in the presence (+ tet) and those grown in the absence (− tet) of tetracycline (p < 0.05). BTG1 induction (− tet) also led to the reduction in S phase cells from 26.9% to 15.4% as determined by flow cytometric analysis of DNA ploidy and cell cycle analysis program at day 3 (C). Results are representative of four independent experiments. x-axis, DNA content; y-axis, cell number.

**FIGURE 3.** Antiproliferative activity of *BTG1* in microglia. Proliferative capacity of BV-2/tet3 cells was compared before and after removal of tetracycline. When *BTG1* expression was induced in BV-2/tet3 by tetracycline removal from the growth medium, the proliferative capacity of the cells was decreased as determined by MTT assay (A) or phase contrast microscopy of the cells at day 3 during the MTT assay (×200; B). Results are mean ± SEM of three independent experiments. *Statistically significant differences between the cells grown in the presence (+ tet) and those grown in the absence (− tet) of tetracycline (p < 0.05). BTG1 induction (− tet) also led to the reduction in S phase cells from 26.9% to 15.4% as determined by flow cytometric analysis of DNA ploidy and cell cycle analysis program at day 3 (C). Results are representative of four independent experiments. x-axis, DNA content; y-axis, cell number.

Role of *BTG1* in inflammatory activation of microglia

Because LPS or IFN-γ is one of the major activators of macrophages and microglial cells in the inflammatory responses (25), we next asked whether LPS/IFN-γ-induced *BTG1* plays a role in the inflammatory activation processes of microglia. When the production of inflammatory mediators such as TNF-α, IL-1β, and NO was compared among BV-2/tet3 microglial cells with different levels of *BTG1* expression (with or without tetracycline in culture medium), no significant difference was found (Fig. 5). The results indicate that *BTG1* is involved in the apoptosis of activated microglial cells, but not activation processes (at least in terms of NO and proinflammatory cytokine production).

**Involvement of JAK/STAT1 pathway in LPS/IFN-γ action in microglia**

To delineate the signaling pathway of LPS/IFN-γ-induced *BTG1* expression and microglial apoptosis, the possibility of involvement of various signaling components was investigated with a focus on the JAK/STAT pathway and IRF-1. IFN-γ, but not LPS, induced activation of STAT1 in BV-2 microglial cells (Fig. 6A, left). Phosphorylation of STAT1 started at 30 min and peaked at 1 h after stimulation with IFN-γ. A similar pattern of STAT1 phosphorylation was observed after LPS/IFN-γ treatment (Fig. 6A, right). Inhibition of STAT1 pathway by the transfection of a dominant negative mutant of STAT1 partially abolished the apoptosis-sensitizing effect of LPS/IFN-γ treatment (Fig. 6B). Compared with the empty vector-transfected cells, transfection of the dominant negative mutant of STAT1 attenuated the effect of LPS/IFN-γ; i.e., LPS/IFN-γ pretreatment sensitized microglia to NO donor-induced apoptosis in the empty vector-transfected cells, whereas in the BV-2 cells transfected with dominant negative mutant of STAT1 the sensitizing effect of LPS/IFN-γ on NO-induced apoptosis was significantly reduced. The percentage of apoptosis of transfected cells was evaluated by separately counting dark blue condensed apoptotic cells and healthy blue cells coexpressing lacZ. To further investigate the role of the JAK/STAT1 pathway in the LPS/IFN-γ-induced *BTG1* expression and microglial apoptosis, a JAK inhibitor (AG490) was used. A specific inhibition of JAK by AG490 diminished LPS/IFN-γ-induced *BTG1* expression (Fig. 6C), supporting the theory that the JAK/STAT1 pathway mediates LPS/IFN-γ-induced *BTG1* expression and the subsequent apoptosis sensitization in microglia. Treatment of the cells with AG490 also diminished LPS/IFN-γ-induced NO production as well as cell death (Fig. 7A). The AG490 inhibition of *BTG1* expression was, however, not due to its inhibitory effect on NO production, because a similar inhibition of NO production by NOS inhibitor (NMMA) did not affect *BTG1* expression (Fig. 7). A definitive evidence for the involvement of the STAT1 pathway in LPS/IFN-γ-induced *BTG1* expression was obtained by transfection of dominant negative mutant of STAT1. Compared with empty vector-transfected cells, dominant negative STAT1 transfectants showed a marked decrease in *BTG1* induction after LPS/IFN-γ treatment (Fig. 7B). Because IRF-1 has been previously shown to
be induced by the JAK/STAT1 pathway and to play an important role in cellular apoptosis (12, 26, 27), we next examined the role of IRF-1 as a downstream mediator of LPS/IFN-γ action in microglia. IRF-1 protein expression was induced by IFN-γ as early as 2 h after the treatment in BV-2 cells, and gene induction was also achieved by treatment with LPS alone or LPS plus IFN-γ (Fig. 8, A and B). However, LPS/IFN-γ-induced BTG1 expression was not affected by IRF-1 gene deficiency, because a similar enhancement of BTG1 expression by LPS/IFN-γ was observed in primary microglial cultures from both wild-type and IRF-1-deficient mice (Fig. 8C). Taken collectively, our results suggest that LPS/IFN-γ action on BTG1 expression and their effect on microglial apoptosis might be mediated through JAK/STAT1, but not IRF-1. Although IRF-1 is a critical mediator of IFN-γ action in both apoptosis and cell cycle control (28), IRF-1 does not appear to be involved in LPS/IFN-γ-mediated BTG1 induction or the ensuing proliferation inhibition in microglia.

**Discussion**

AICD is an autoregulatory mechanism by which the immune system removes unwanted activated immune cells after making appropriate use of them. Although AICD has been first identified in lymphocytes (29, 30), recent works indicated that both microglial cells (9) and astrocytes (11) in the CNS might be under the control of a similar regulatory mechanism. In contrast to AICD of T lymphocytes where Fas-Fas ligand interaction plays a central role, neither Fas-Fas ligand interaction nor TNF-α is important in AICD of microglial cells. Instead, NO produced by activated microglial cells themselves is the major cytotoxic mediator (9). However, the presence of NO-independent cytotoxic mechanism has been also reported. Our previous work indicated that inflammatory stimuli play a dual role in AICD of microglial cells (12) and astrocytes (31). They induce not only the indirect apoptotic pathway via production of NO, but also the direct apoptotic pathway through caspase-11 induction. Whereas caspase-11 induction and its activation were required for the NO-independent apoptotic pathway, IRF-1 and NF-κB were involved in NO-dependent apoptosis of microglial cells mainly by mediating NO synthesis (via inducible NO synthase induction). Up-regulated caspase-11 is autoactivated in microglia and triggers an activation cascade of downstream caspases, which ultimately leads to cellular apoptosis. Meanwhile, NO donor-induced apoptosis appears to directly activate downstream executioner caspases in microglia.

Inflammatory stimuli such as LPS and IFN-γ are more than just cellular activators to microglial cells. They not only activate the cells to produce inflammatory mediator such as TNF-α and NO but also induce autoregulatory apoptosis. Inflammatory stimuli appear to induce or activate a specific group of genes and signaling pathways, some of which are commonly involved in both activation and apoptosis. Our previous work suggested that caspase-11 may be an example of such genes (12). Induction and activation of
mediators induced by LPS/IFN-γ, IL-1β, and NO from BV-2/tet3 cells cultured in the presence (+ tet) or absence (− tet) of tetracycline by specific ELISA or Griess reaction. An increase in BTG1 expression by removal of tetracycline from growth medium did not significantly affect LPS-induced production of TNF-α, IL-1β, or NO (100 ng/ml LPS, 24 h treatment). Results are mean ± SEM of three independent experiments. No statistically significant difference was found between + tet and − tet for any of the inflammatory mediators tested.

caspase-11 are involved in the production of proinflammatory cytokines such as IL-1β and IL-18 by activating the processing enzyme (caspase-1) required for the conversion of procytokines to their mature form (32, 33). Caspase-11 induction also causes microglial apoptosis. Therefore, inflammatory stimuli seem to activate microglial cells to produce various inflammatory mediators and concomitantly activate a built-in autoregulatory apoptosis mechanism. The role of inflammatory stimuli in microglial AICD was not limited to the NO production or caspase-11 induction. Our previous findings that pretreatment of microglia with inflammatory stimuli enhanced their sensitivity to exogenous NO independently of caspase-11 suggested the presence of yet other mechanisms whereby the inflammatory stimuli affect microglial AICD process (12).

Here, we present evidence that BTG1 constitutes a novel apoptotic pathway during AICD of microglia. Because LPS/IFN-γ has been previously shown to increase the sensitivity of microglia to NO-induced apoptosis (12), we hypothesized that LPS/IFN-γ may lower the threshold for microglial apoptosis through the expression of inducible mediators. We now report that BTG1 is one of such mediators induced by LPS/IFN-γ. This is supported by our results that 1) LPS/IFN-γ synergistically augmented BTG1 expression in microglia, 2) BTG1 inhibited microglial proliferation, and 3) conditional expression of BTG1 increased the sensitivity of microglial cells to NO-induced apoptosis. Thus, the LPS/IFN-γ-mediated increase in BTG1 expression may prevent microglia from entering cell cycle, and this antiproliferative action of BTG1 may render the cells more sensitive to apoptotic signals. Our current results support the model of microglial apoptosis as a self-regulatory mechanism that has been previously proposed (8, 9). Activation of microglia may lead to their proliferation under certain conditions. However, overactivating signals that are strong enough to enhance BTG1 expression, e.g., LPS plus IFN-γ, may retard microglial proliferation, making them ready to be eliminated by apoptosis. NO that has already been produced by activated microglia in the same microenvironment could be an ideal apoptotic signal to remove these overactivated microglia with a high BTG1 expression. Thus, overactivation of microglia may mark themselves with a high BTG1 expression for the apoptotic elimination by NO.

We do not know how antiproliferative activity of BTG1 mediates or enhances apoptotic action of NO. In many cases, blockade of cell cycle by anticancer drugs leads to cellular apoptosis. It is well documented that cell cycle control and apoptosis are closely linked intracellular events (34, 35). Apoptosis could be viewed as a safeguard mechanism that allows the elimination of cells the physiological cell cycle of which has been blocked. In this respect, BTG1-mediated inhibition of cellular proliferation may trigger this type of safeguard mechanism, thereby rendering microglial cells extremely sensitive to incoming apoptogenic signals. BTG1 has been shown to interact with transcription factors such as CAF1 (36, 37) and Hoxb9 (38) that control the expression of proliferation-regulatory genes. BTG1 also forms a complex with PRMT1 that methylates various intracellular proteins, thereby modulating its methyltransferase activity (39). BTG1 may exert its antiproliferative activity through the interaction with these proteins. The apoptosis-sensitizing effect of BTG1 demonstrated in the current

FIGURE 5. BTG1 is not involved in the inflammatory activation of microglia. Role of BTG1 in the inflammatory activation of microglia was investigated by comparing the production of inflammatory mediators such as TNF-α, IL-1β, and NO from BV-2/tet3 cells cultured in the presence (+ tet) or absence (− tet) of tetracycline by specific ELISA or Griess reaction. An increase in BTG1 expression by removal of tetracycline from growth medium did not significantly affect LPS-induced production of TNF-α, IL-1β, or NO (100 ng/ml LPS, 24 h treatment). Results are mean ± SEM of three independent experiments. No statistically significant difference was found between + tet and − tet for any of the inflammatory mediators tested.

FIGURE 6. Essential role of JAK/STAT1 signaling in LPS/IFN-γ-mediated apoptosis sensitization and BTG1 induction in microglia. A, STAT1 was activated by IFN-γ (10 U/ml) or combination of LPS (100 ng/ml) and IFN-γ, but not by LPS alone, as measured by Western blot detection of phosphorylated STAT1 protein (at Tyr701) at the indicated time points in parental BV-2 cells. B, Inhibition of STAT1 signaling by transient transfection of the dominant negative mutant of STAT1 cDNA (DN STAT1) suppressed LPS/IFN-γ-induced sensitization of microglia to apoptogenic action of SNAP. LPS/IFN-γ pretreatment rendered BV-2 microglial cells sensitive to otherwise nontoxic dose of SNAP (0.2 mM, 24 h treatment). However, STAT1 inhibition partially abolished the apoptosis-sensitizing effect of LPS/IFN-γ. LPS/IFN-γ pretreatment (16 h) was done in the presence of a NOS inhibitor, NMMMA (0.5 mM) to eliminate confounding effects of LPS/IFN-γ-mediated inducible NO synthase induction and resulting NO production. The percentage of apoptosis was determined by differential counting of healthy blue cells and apoptotic blue cells coexpressing lacZ (pCH110). Empty vector indicates pcDNA3. Results are mean ± SEM of three independent experiments. +, Statistically significant differences (p < 0.05). C, Treatment of BV-2 cells with JAK inhibitor AG490 (10 μg/ml), attenuated LPS/IFN-γ-induced BTG1 protein expression (100 ng/ml LPS, 10 U/ml IFN-γ, 8 h treatment). The numbers indicate a fold induction of BTG1 expression normalized to α-tubulin as determined by densitometric analysis of Western blot results.
FIGURE 7. Attenuation of LPS/IFN-γ-induced microglial cell death and NO production by AG490. A, Treatment of BV-2 cells with AG490 (10 μg/ml) attenuated LPS/IFN-γ-induced microglial NO production (left) and cell death (right) as assessed by MTT assays and Griess reaction at 24 h. NMMA (0.5 mM) was used for comparison. Results are mean ± SEM of three independent experiments. *, Statistically significant differences from LPS/IFN-γ. RT-PCR was performed to evaluate BTG1 or β-actin messages after the stimulation for 4 h.

FIGURE 8. No role of IRF-1 in LPS/IFN-γ-induced BTG1 induction. Expression of IRF-1 protein was induced by both IFN-γ (10 U/ml) and LPS (100 ng/ml) in BV-2 cells as determined by Western blot analysis (A and B). IRF-1 or α-tubulin protein was detected at the indicated time points after IFN-γ treatment (A) or 4 h after treatment with the indicated stimuli (B). A similar level of BTG1 message induction by LPS/IFN-γ was observed in primary microglial cells from wild-type mice (IRF-1+/+) and IRF-1 deficient mice (IRF-1−/-; C). The absence of an IRF-1 gene did not affect LPS/IFN-γ induction of BTG1 messages in microglia as assessed by RT-PCR (4 h treatment with the stimuli). The absence of IRF-1 transcripts in microglia from IRF-1-deficient mice was confirmed by RT-PCR (D). Mouse β-actin was used as an internal control in RT-PCR. The amplified product was not observed in RT-PCR without reverse transcriptase (data not shown). Results are representative of three independent experiments.

The study may not be solely due to its antiproliferative activity. Preliminary results in our laboratory indicated that cell cycle-inhibitory drugs did sensitize microglia to NO-induced apoptosis; however, their sensitizing activity was much less effective than the forced expression of BTG1. The results suggest that antiproliferative BTG1 may have an additional function, which is uniquely involved in the NO-induced apoptosis. However, a firm conclusion on the role of BTG1 cannot be drawn with these experiments, because the cell cycle-inhibitory drugs alone may cause cell death (depending on the concentrations used) as opposed to BTG1 expression, which does not affect the cell viability by itself. Further works are required to resolve this issue and to see whether BTG1 induction can sensitize microglia to other apoptosis-inducing agents besides NO.

Role of BTG1 in cell cycle control has been previously documented (14). Studies with PHA-activated PBL and serum stimulation of growth-arrested NIH3T3 cells showed that BTG1 expression negatively correlates with the percentage of cells entering S phase (16). Involvement of BTG1 in cellular apoptosis has been also suggested by a previous report, in which the forced expression of BTG1 in NIH3T3 cells increased the frequency of apoptosis and BTG1 expression colocalized not only with cells positive for markers of apoptosis but also with macrophage-rich regions in Watanabe heritable hyperlipidemic rabbits (19). In addition, we have previously demonstrated a negative correlation between BTG1 expression and proliferation of mouse peritoneal macrophages as well as the RAW264.7 macrophage-like cell line (18). Based on functional similarities between macrophages and microglia, anti-proliferative action of BTG1 currently observed in microglia is not greatly unexpected. Furthermore, our results on the role of BTG1 in microglial apoptosis are in agreement with the previous studies that demonstrated the apoptosis-related role of BTG1 in NIH3T3 cells (19).

In this work, we focused on the effects of LPS and IFN-γ as representative inflammatory stimuli. Other known microglial activators include thrombin (40), chromogranin A (10), gangliosides (41), and amyloid β peptide (42). These agents induce the inflammatory activation of microglia. Many of these inflammatory stimuli initiate common intracellular signaling pathways such as NF-κB activation, p38 MAPK, and/or ERK activation. In particular, gangliosides uniquely evoke activation of JAK/STAT signaling pathways in microglia (43). Considering the similarity between gangliosides and IFN-γ in the initiation of the JAK/STAT pathway, the two stimuli may share BTG1 as a common downstream mediator to induce microglial apoptosis. Moreover, because LPS is a canonical activator of NF-κB (44), there might be other unexpected overlaps in signal transduction pathways among these various microglial activators. All these activators may potentially induce microglial apoptosis,
and it will be interesting to examine whether they are capable of inducing BTG1 expression either alone or in various combinations in the process of AICD of microglia. The mechanism of LPS/IFN-γ-induced AICD of microglial cells proposed in the current studies may well be applicable to other inflammatory stimuli. Either LPS or IFN-γ alone was not sufficient for the strong induction of BTG1 expression; a marked induction was achieved only by cotreatment. Moreover, in contrast to IFN-γ, LPS failed to activate STAT1 signaling. Thus, the JAK/STAT1 signaling pathway may be necessary, but not sufficient, for LPS/IFN-γ-induced BTG1 expression. It is speculated that IFN-γ uses the JAK/STAT1 signaling pathway, whereas LPS may trigger other signaling events such as NF-κB or mitogen-activated protein kinase pathways. Cooperation between these signaling events seems to be required for the efficient BTG1 induction and the subsequent apoptosis sensitization in microglia. We and others (26, 27, 45–48) have previously reported that STAT1/IRF-1 pathway plays a central role in cellular apoptosis induced by inflammatory cytokines. IRF-1 is a member of a family of IFN-γ-inducible transcription factors. IRF-1, as one of the genes the transcription of which is up-regulated by STAT1, is known to mediate apoptosis as well as cell cycle arrest in IFN-γ responses (49). Although caspase induction has been suggested as a possible downstream event following IRF-1 induction in IFN-γ-induced apoptosis (47), studies using IRF-1-deficient mice have shown that p53 and p21 are associated with IRF-1 action in IFN-γ-induced cell cycle arrest (50). IRF-1 was also an important player in microglial apoptosis by mediating NO production (12, 51). However, the absence of IRF-1 gene did not affect the inducibility of caspase-11 (12) or BTG1 (Fig. 8). Although we have not tested the induction or activation of other caspases, apoptotic or cell cycle mediators in IRF-1-deficient microglial cells, a role of IRF-1 in microglial apoptosis seems to be related to NO production rather than caspase induction or cell cycle control.

There is now growing evidence that toxic mediators produced by activated microglial cells might be involved in the pathogenesis of various neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and HIV-associated dementia (3, 4, 52). Thus, in CNS, the production of toxic inflammatory mediators by activated microglial cells must be strictly regulated to avoid harmful effects. Because the apoptosis of activated microglia is believed to be one of the autoregulatory mechanisms for microglial activation, the elucidation of microglial AICD mechanism and the better understanding of associated apoptosis pathways will lead to a novel way of controlling microglial activation. This will in turn allow us an opportunity for the therapeutic interventions against the neurodegenerative diseases, in which overactivation of microglia plays a pathogenic role.

In conclusion, we identified BTG1 as an important constituent in microglial AICD process. In the activation-induced apoptosis of microglial cells, inflammatory stimuli (LPS/IFN-γ) play a multiple role. They induce not only direct apoptotic mediators (NO, caspase-11) but also BTG1 expression to sensitize the target cells to an apoptotic mediator, NO. This BTG1 action appears to be related to its antiproliferative activity.

References