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**References**

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Requirement of \textit{A1-a} for Bacillus Calmette-Guérin-Mediated Protection of Macrophages Against Nitric Oxide-Induced Apoptosis$^1$

Santhanam Kausalya, Robert Somogyi, Amos Orlofsky, and Michael B. Prystowsky$^2$

The role of apoptosis in regulating the course of intracellular microbial infection is not well understood. We studied the relationship between apoptotic regulation and bacillus Calmette-Guérin (BCG) treatment in murine peritoneal exudate macrophages (PEM) and the J774 macrophage cell line. In both PEM and J774 cells, mRNA expression of the anti-apoptotic gene, \textit{A1}, was selectively induced by BCG treatment as compared with other \textit{bcl2} family members (\textit{bcl-2}, \textit{bcl-xL}, \textit{bcl-xS}, \textit{bax}, \textit{bak}, \textit{bad}). In PEM, \textit{A1} expression was maximal by 8 h postinfection and was abrogated by the proteasomal inhibitor MG-132. The induction was independent of protein synthesis as well as the p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways and did not require live organism. Three genes encoding closely related isoforms of \textit{A1} were all expressed; however, the \textit{A1-a} isofrom displayed the greatest fold induction in PEM. BCG-induced \textit{A1} expression was associated with protection of host macrophages from NO-mediated apoptosis in both PEM and J774 cells. BCG-mediated protection was abrogated in PEM derived from \textit{A1-a}−/− mice, indicating a requirement of \textit{A1-a} for survival of inflammatory macrophages. \textit{The Journal of Immunology}, 2001, 166: 4721–4727.

Intracellular pathogens can modulate host cell apoptosis, with potentially important consequences for the course of infection. However, knowledge is limited regarding the apoptotic pathways regulated during intracellular infections. Macrophage apoptotic pathways are affected by a variety of mediators, including cytokines, NO, and NF-κB. In the case of several pathogens, including Salmonella, Shigella, Yersinia, and Legionella, the destruction of host macrophages by apoptosis is thought to favor pathogen dissemination. For \textit{Mycobacterium} spp., alternative arguments have been presented that killing of the host macrophages either favors the host by reducing the number of phagocytes available to support bacterial growth or facilitates the survival of the bacteria by allowing them to escape and multiply. Moreover, in the case of \textit{Mycobacterium bovis} bacillus Calmette-Guérin (BCG), the effect of the bacterium on host cell apoptosis is unclear. Klingler et al. have demonstrated apoptosis coupled with BCG killing in human peripheral blood monocytes, whereas prevention of apoptosis in human monocytes by BCG infection has also been reported. The consequences of BCG infection on host macrophages are of particular interest given the nonspecific protective effects of this organism against various infections and tumor cells.

Apoptosis can be either favored or inhibited by gene products of the \textit{bcl2} family. One of these family members, \textit{A1}, is a rapidly inducible gene that was initially characterized in murine macrophages and subsequently in human monocytes and endothelial cells. \textit{A1} functions to prevent apoptosis. Kremer et al. have observed increased \textit{A1} expression following BCG infection associated with the inhibition of apoptosis in resting human monocytes. Recently, expression of \textit{A1} in inflammatory macrophages and neutrophils elicited by infection with \textit{Toxoplasma gondii} has been reported from our laboratory. To gain additional insights into the effect of BCG treatment on macrophage apoptosis, we studied BCG treatment of murine peritoneal exudate macrophages derived from \textit{A1-a}+/+ and \textit{A1-a}−/− mice and the macrophage-like cell line J774. Our results demonstrate that BCG selectively induces \textit{A1} expression when compared with other \textit{bcl2} family members. Since NO has been associated with the induction of macrophage apoptosis in mycobacterial infection, we also examined NO-induced macrophage apoptosis during BCG infection. The results show that BCG protects macrophages from NO-induced apoptotic cell death in wild-type peritoneal exudate macrophages (PEM) and J774 cells but not in \textit{A1-a}−/− PEM, indicating the requirement of \textit{A1-a} induction in BCG-mediated survival of inflammatory macrophages.

\textbf{Materials and Methods}

\textit{Cells, cell lines, and culture conditions}

\textit{J774.A1}, a murine macrophage-like cell line derived from BALB/c mice, was obtained from American Type Culture Collection (Manassas, VA). It was maintained in DMEM supplemented with 10% FCS, 1% glutamine, 1% nonessential amino acids, and 1% penicillin-streptomycin mixture (Life Technologies, Gaithersburg, MD). PEM were obtained by harvesting peritoneal exudate cells from female BALB/c mice (6–8 wk old) after inducing inflammation by injecting 1 ml of sterile 3\% thioglycolate broth. Mice were sacrificed 3–7 day later and the peritoneal cavity was washed with 4 ml of HBSS containing 0.2% BSA. Peritoneal lavage was washed twice with DMEM and cultured in DMEM supplemented with 10% FCS (complete (C)-DMEM) for 60 min. The resulting adherent macrophages were washed twice and cultured in C-DMEM under different conditions. S-nitroso-N-acetylpenicillamine (SNAP; Sigma, St. Louis, MO) was prepared as a 1 M stock in DMSO and stored frozen.
Bacterial culture

*M. bovis* BCG was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% albumin dextrone complex (OADC; Becton Dickinson, Cockeysville, MD) and 0.5% glycerol in a rotating flask. Stock cultures of mid-log phase bacilli were aliquoted in equal volumes of 1% Tween 80 in PBS and 10% glycerol, and stored at −80°C until further use. The growth of the cultures was determined by plating serial dilutions of bacteria onto petri dishes containing Middlebrook 7H10 solid medium containing OADC and 10% glycerol.

Treatment of cells with live or heat-killed BCG

Frozen aliquots of bacilli were thawed, resuspended in C-DMEM, and sonicated (5 × 15-s cycles, 500 W). J774 were infected with BCG, in vitro, at a multiplicity of 25–30 viable organisms per macrophage. Cells were precultured with 100 U/ml murine recombinant IFN-γ for 16 h before BCG infection where indicated. BCG were killed by heating at 70°C for 2 h. Where indicated, cultures were treated immediately before infection with either the proteasomal inhibitor MG-132 (Sigma), the p38 mitogen-activated protein kinase inhibitor SB203580 (SmithKline Beecham, Philadelphia, PA), the phosphatidylinositol 3-kinase inhibitor LY294002 (Calbiochem, La Jolla, CA), or the protein synthesis inhibitor cycloheximide (Sigma).

RNA preparation

Total cellular RNA was prepared by using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions.

RNAse Protection Assay (RPA)

Relative levels of mRNA for the *bcl2* family members were determined by using Riboquant, a multiprobe RPA system (PharMingen, San Diego, CA). Total RNA (10 μg) from each condition was hybridized overnight at 56°C to a mAPO-2 (mouse apoptosis) probe set containing [α-32P]UTP-labeled antisense RNA transcripts. Free probe and ssRNA were digested with RNase A. The protected mRNAs were purified and resolved on a 5% de-naturing polyacrylamide gel. The resulting protected bands were analyzed by autoradiography and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). RNA loading was normalized to the protected fragments of the housekeeping gene L32.

RT-PCR

RT-PCR to detect A1 expression in J774 cells and PEM was performed by reverse transcribing 3 μg of total RNA using oligo(dT) and superscript II RT (Life Technologies). PCR was conducted in 100 μl using 1.5 mM MgCl2, 0.25 mM dNTP, 5U/ml Taq DNA polymerase, 10% of the RT product and 15–20 pmol each of 5’ and 3’ primers. PCR was performed in a DNA thermal cycler as follows: 94°C for 4 min, followed by 35 amplification cycles (94°C for 15 s, 55°C for 30 s, 72°C for 1.8 min). Sense and antisense primer sequences were as follows: 5’-AAAT TCC AAC AGC CTC CA and 3’-GGA ATT TAT CTG CAA CTA TCG TGG. The resulting PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

A1 isoform diagnostic

To distinguish A1 isoforms a, b, and d, RT-PCR products positive for A1 expression were digested with *BglII* and *NsiI* restriction enzymes for 2 h at 37°C as described by Hatakeyama et al. (19). The digested products were separated by electrophoresis on 4% NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME). The gel was stained with Sybr gold (Molecular Probes, Eugene, OR) for 45 min and scanned in a FluorImager (Molecular Dynamics). The restriction digest results in bands of 743 bp (corresponding to A1-b), 602 bp (corresponding to A1-d), and 471 bp (corresponding to A1-a). The relative intensities of the isoform-specific bands were quantified by using ImageQuant software (Molecular Dynamics), and the expression of each isoform relative to L32 was calculated according to the following expression: intensity of a, b, or d/(2) × total A1 expression (from RPA results).

Immunocytochemistry

For immunostaining experiments, cells were cultured in 8-well plastic chambered slides (Nunc, Naperville, IL) for the appropriate length of time. Cells were washed with sterile PBS twice and fixed with cold 4% paraformaldehyde for 20 min at room temperature. The slides were washed twice with PBS and stained for nicked DNA by TUNEL using a fluorescein kiti from Boehringer Mannheim (Indianapolis, IN) according to the manufacturer’s protocol. Stained slides were mounted with ProLong anti-fade (Molecular Probes) and examined for fluorescence at ×400. A total of 200 cells was counted and the percentage of apoptotic cells was determined.

Production of A1-a−/− mice

Full details on the construction and characterization of A1-a−/− mice will be reported elsewhere (A. Orlofsky, L. M. Weiss, and M. B. Prystowsky, manuscript in preparation). Briefly, a targeting construct was designed that deletes a portion of exon 1 of A1-a (including the translational start site) as well as 389 bp of 5′ flank. Recombinant WW6 ES cells were injected into blastocysts in the Transgenic and Gene Targeting Core Facility at Albert Einstein College of Medicine. Chimeras were mated with C57BL/6 mice. Transgenic and nontransgenic progeny were separately intercrossed to generate parallel A1-a−/− and A1-a+/− lines. Isoform-specific RT-PCR analysis showed that the A1-a band failed to appear upon LPS treatment of bone marrow-derived macrophages from gene-targeted mice (data not shown).

Results

Selective induction of A1 in macrophages

mRNA levels of *bcl2* family members were determined by RPA in PEM or J774 cells treated with BCG for 8–48 h. The effect of pretreatment with IFN-γ was also examined. In PEM, A1 was the most inducible *bcl2* member upon stimulation with either BCG or BCG + IFN-γ at all time points tested (Fig. 1). Maximal A1 levels were comparable to those of the housekeeping genes (L32 and GAPDH) as demonstrated by the density of the bands in the RPA (Fig. 1A). Induction of A1 was maximal (∼4-fold) at 8 h after BCG treatment with levels decreasing minimally from 16 through 48 h (Fig. 1B). We found minimal induction of A1 at 2 h (data not shown). The proapoptotic genes *bcl-xx*, *bak*, and *bad* were expressed at low levels and induction of *bax* was minimal (Fig. 1B). IFN-γ alone did not induce A1 expression in PEM. However, the combination of the cytokine with BCG resulted in a synergistic increase in A1 expression at 8 h as well as modest responses for several other family members (Fig. 1C).

As with PEM, A1 was the most inducible *Bcl2* family member in J774 cells (Fig. 2). A1 expression in J774 peaked at 8–16 h, decreased by 24 h, and was comparable to control levels by 48 h, in contrast to the high expression maintained in PEM at 48 h (Fig. 2A). Addition of BCG alone to the J774 cells resulted in maximal A1 induction of 11-fold at 16 h (Fig. 2A). In contrast to PEM, there was >5-fold increase in A1 expression upon treatment with IFN-γ alone at 8 h (Fig. 2B). Combined treatment with IFN-γ + BCG led to a 17-fold increase in A1 expression. These results are consistent with our previous findings that A1 is an early response gene that is rapidly induced during macrophage activation (14). Although there is no marked induction among the other *Bcl2* family members in J774 cells, the death-promoting gene, *bax*, is constitutively expressed at high levels (Fig. 2). Low levels of *bcl-xx*, *bak*, and *bad* were also constitutively expressed in J774 (Fig. 2).

We next investigated further the signaling requirements for the induction of A1 by BCG in PEM. As shown in Fig. 3, heat-killed BCG, which has been shown to prevent spontaneous monocyte apoptosis as effectively as the live organism (9), was equivalent to live BCG with respect to its ability to induce A1. MG-132, a proteasomal inhibitor that blocks NF-κB signaling, abolished the A1 signal. Similar inhibition is observed in LPS-treated macrophages (data not shown). In contrast, neither LY294002, an inhibitor of PI-3K, nor SB203580, a p38 mitogen-activated protein kinase inhibitor, affected A1 expression. Finally, induction of A1 RNA accumulation in the presence of BCG + cycloheximide was greater than that in the presence of cycloheximide alone, indicating that the BCG-mediated signal is...
FIGURE 1. Selective induction of A1 in PEM after BCG treatment. Peritoneal lavages from BALB/c mice were harvested 72 h after thioglycolate injections. Peritoneal exudate cells were washed and allowed to adhere for 1 h to obtain PEM that were then treated for various times with medium (C-MEM), IFN-γ (100 U/ml), IFN-γ (100 U/ml) + BCG (multiplicity of infection (MOI) of 25–30 organisms per macrophage) or BCG alone. A, PEM were harvested at the indicated time points (8, 16, 24, or 48 h), and RNA was extracted and subjected to RPA using a bcl2 multiprobe set. 0, 0-h control (harvested immediately after macrophage adherence), M, medium, I, IFN-γ, IB, IFN-γ + BCG, B, BCG. The data presented are representative of two experiments. B, The gel from A was quantitated by PhosphorImager analysis and the kinetics of bcl2-related gene expression was calculated relative to the housekeeping gene L32. C, PhosphorImager analysis from A was used to assess the interaction of BCG and IFN-γ.
independent of protein synthesis. The induction of A1 by cycloheximide alone, a characteristic of a number of early response genes, has been previously observed (14).

**BCG protects J774 cells from apoptotic killing**

We next asked whether BCG-mediated A1 induction in macrophages correlated with cell survival following treatment with SNAP, a donor of NO. The TUNEL assay was conducted to detect apoptotic cells. For all conditions used in Figs. 1 and 2 (IFN-γ, IFN-γ + BCG, BCG), there were at most 3–4% apoptotic cells in the absence of SNAP (data not shown). J774 cells were pretreated with BCG for 2 h and incubated with $10^{-7}$ M SNAP for 18 h. SNAP treatment increased the proportion of apoptotic cells to $40 \pm 5\%$ (Fig. 4, C and D), compared with $3 \pm 1\%$ in controls (Fig. 4, A and B). Infection with BCG before treatment with SNAP reduced apoptosis to control levels (Fig. 4, G and H). Thus, BCG protects J774 cells from SNAP-mediated apoptosis. Furthermore, the morphology of J774 cells treated with BCG with or without SNAP is similar (compare Fig. 4, E and G).

**Regulation of A1 isoforms in BCG-infected macrophage**

Recent studies have indicated that there are four A1 genes in mice encoding four isoforms named A1-a, A1-b, A1-c, and A1-d, of which A1-c is likely a pseudogene (19). The relative proportions of

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**FIGURE 2.** A. Kinetics of bcl2 family member expression in BCG-treated J774 cells. J774 cells were treated with BCG at a MOI of 25–30 organisms per macrophage. Cells were harvested at indicated time points for RNA extraction. RPA was conducted with the bcl2 multiprobe set, analyzed, and expressed relative to L32. The data presented are representative of two experiments. RPA results were quantified using ImageQuant software. Control cells (C) were treated with medium for 8 h. B. The same data set was used to compare expression after treatment with IFN-γ, IFN-γ + BCG, or BCG for 8 h.
A1 isoforms during BCG treatment were quantified by RT-PCR followed by diagnostic restriction digestion that yields isoform-specific bands (Figs. 5 and 6A). This analysis was combined with the previous RPA data (as described in Materials and Methods) and the results are expressed in terms of isoform contributions in Figs. 5B and 6B. The sum of the A1-a, -b, and -d control levels shown in Figs. 5B and 6B is set to one. In PEM, significant increases in all three isoforms at the 8-h time point occurred following treatment with IFN-γ + BCG (A1-a = 42-fold, A1-d = 20-fold, and A1-b = 8-fold) or BCG treatment alone (A1-a = 20-fold, A1-d = 10-fold, and A1-b = 4-fold) (Fig. 5). The results indicate that A1-d displayed the greatest fold induction in PEM following BCG treatment. In J774 macrophages (Fig. 6), A1-b showed the greatest increase; e.g., at 8 h, A1-b was induced by IFN-γ (11-fold), IFN-γ + BCG (38-fold), and BCG (5-fold). A1-a and A1-d also increased but to a lesser extent.

**FIGURE 3.** Signaling requirements for BCG induction of A1 mRNA in PEM. PEM were harvested 7 days after thioglycolate injection, adhered for 2 h, and either harvested immediately (0) or treated for 7 h as indicated with medium (M), live or killed BCG (MOI = 30), 10 μg/ml cycloheximide (CH), 30 μM MG-132 (MG), 100 nM SB203580 (SB), or 10 μM LY294002 (LY). A1 expression relative to L2 was quantitated by RPA analysis and fold induction was calculated relative to untreated cells (0).

**FIGURE 4.** BCG protection of J774 macrophages against NO-mediated apoptosis. J774 cells were grown in eight-well chamber slides and treated with BCG at an MOI of 25–30 bacilli per macrophage. Cells were washed in PBS, fixed with 4% paraformaldehyde, and stained by TUNEL assay. A total of 200 cells was counted for each condition and percentage apoptosis was determined. A, C, E, and G are the respective phase microscopy images of the fields shown in B, D, F, and H, and A and B represent untreated (control) J774 cells that have been maintained in culture for the same period of time as the experimental conditions. C and D indicate cells treated with 10⁻³ M SNAP for 16 h. E and F represent J774 cells treated with BCG for 18 h. G and H are cells treated with BCG for 2 h before incubation with SNAP for 16 h.

Pathogens that induce apoptosis in host cells include *Shigella flexneri* (5), *Bordetella pertussis* (20), *Listeria monocytogenes* (21), and *Salmonella typhimurium* (4). Alternatively, other pathogens may suppress host cell death to facilitate successful replication and survival within the host cellular milieu. *Leishmania donovani* is one such parasite that inhibits macrophage apoptosis by inducing the synthesis of anti-apoptotic cytokines by host macrophages (22). In the present study, we report that prevention of NO-induced macrophage apoptosis by *M. bovis* BCG requires up-regulation of anti-apoptotic A1-a gene expression.

Apoptosis is a genetically controlled pathway, and the bel2 family represents a multigene family with genes for both cell survival and death. Among these, bel-w, bel2, A1, bel-xl, and mcl-1 prevent apoptosis, whereas bax, bak, bad, and bik and others are apoptosis-promoting proteins (for review, see Ref. 23). In the present study, A1 is the most inducible family member during BCG infection in both PEM and J774 cells. Previous reports have indicated that BCG infection of monocytes can lead to up-regulation of A1 (9) or

**Discussion**

Bacterial and parasitic microbes have evolved a variety of strategies to evade the host immune system and survive within the host. One strategy may involve the killing of immune cells that are infected, leading to dissemination and survival of the pathogens.
Bcl-xL (10). The current study is the first to directly compare the regulation of these two genes following BCG infection. Among proapoptotic family members, we observed constitutive expression of \(bax\), consistent with previous observations in monocytes (10) where \(bax\) protein is constitutively expressed by BCG-infected human peripheral blood monocytes. \(bax\) heterodimerizes with anti-apoptotic \(bcl2\) members, including A1, and in turn accelerates programmed cell death (24). Orlofsky et al. (17) showed parallel increases in expression of A1 and \(bax\) in \(T. gondii\)-elicited PEM. The investigation of potential interactions between A1 and \(bax\) may be important for understanding pathogen-mediated regulation of apoptosis in host macrophages.

The mouse genome contains four closely related A1 genes, designated as A1-a, -b, -c, and -d, which demonstrate a high degree of nucleotide and amino acid homology (19). All of the functional studies demonstrating the anti-apoptotic activity of mouse A1 have been conducted with A1-a (16, 25–27). It is therefore important to determine whether up-regulation of A1 expression includes up-regulation of this isoform. In PEM, we found up-regulation of A1-a, A1-b, and A1-d as a result of BCG treatment, with maximal fold induction of A1-a (this analysis presumes the absence of A1-c, which may be nonfunctional (19)). Lack of BCG-mediated protection in A1-a/2 mice indicates that A1-a is a critical survival factor in inflammatory macrophages. A recent study described BCG-induced apoptosis in human alveolar macrophages (28). We assessed the outcome of BCG infection in cultured macrophages. Apoptosis was not significantly induced in

significant apoptosis was reported by Klingler et al. (10) following BCG infection in human peripheral blood monocytes. A recent study described BCG-induced apoptosis in human alveolar macrophages (28). We assessed the outcome of BCG infection in cultured macrophages. Apoptosis was not significantly induced in

THE EFFECT OF BCG ON APOTOPSIS HAS BEEN CONTROVERSIAL. IT HAS BEEN REPORTED THAT HUMAN PERIPHERAL BLOOD MONOCYTES INFECTED WITH BCG ARE PROTECTED FROM APOTOPSIS (9). IN CONTRAST,
BGC-treated J774 cells at all time points tested. We further examined the effect of BGC infection on apoptosis induced by SNAP, a long-lasting S-nitrosothiols derivative and a stable donor of NO (29). Regulation of host apoptosis and control of mycobacterial growth by NO has been demonstrated (30). Previous studies have also shown that NO-induced apoptosis can be inhibited by Bcl2 induction in neurons and tumor cells (31, 32). BGC infection of J774 cells and heat-killed BCG treatment of PEM protected Bcl2 cells from apoptotic death induced by SNAP in the current study (Figs. 4, A–H, and 7). These results indicate that BGC treatment can prevent macrophage apoptosis. The lack of protection in AI-α−/− mice indicates that AI-α is an integral part of the protective mechanism.

It is likely that other factors regulated during mycobacterial infection may interact with AI-α or independently affect host cell survival. Kremer et al. (9) have demonstrated that BCG infection impairs IL-10 secretion, thereby preventing apoptosis in human monocytes. IL-10 is an immunosuppressive cytokine reported to induce apoptosis of human monocytes (9, 33). The ability of BCG to prime monocytes to secrete TNF-α, a proinflammatory cytokine, may also prevent human monocytes from undergoing apoptotic cell death (9, 34). In contrast, Mycobacterium tuberculosis-induced cell death in murine macrophages is promoted by TNF-α and inhibited by IL-10 (35). It is thus conceivable that AI functions downstream of a cytokine-driven sequence of events following infection. However, our results indicate that AI induction by BCG is a protein synthesis-independent event that is likely to be mediated directly by BCG-mediated signaling, perhaps via NF-kB. Therefore, an alternative model is that multiple pathways modulate the survival of infected macrophages: one acting directly through BCG-triggered signaling and resulting in AI induction, and a second acting indirectly through BCG-stimulated cytokine expression.

References