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RESEARCH ARTICLE

AMPD3 is involved in anthrax LeTx-induced macrophage cell death

Sangun Lee[1,3], Yanhai Wang[1,2] ✉, Sung Ouk Kim[1,4], Jiahuai Han[2] ✉

[1] Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pine Road, La Jolla, CA 92037, USA
[2] The State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen 361005, China
[3] Current address Department of Biomedical Sciences, Tufts University, Cummings School of Veterinary Medicine, North Grafton, Massachusetts 01536, USA
[4] Current address Department of Microbiology and Immunology, Siebens-Drake Research Institute, University of Western Ontario, London, Ontario, Canada
✉ Correspondence: jhan@scripps.edu (J. Han), wangyh@xmu.edu.cn (Y. Wang)
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ABSTRACT

The responses of macrophages to *Bacillus anthracis* infection are important for the survival of the host, since macrophages are required for the germination of *B. anthracis* spores in lymph nodes, and macrophage death exacerbates anthrax lethal toxin (LeTx)-induced organ collapse. To elucidate the mechanism of macrophage cell death induced by LeTx, we performed a genetic screen to search for genes associated with LeTx-induced macrophage cell death. RAW 264.7 cells, a macrophage-like cell line sensitive to LeTx-induced death, were randomly mutated and LeTx-resistant mutant clones were selected. AMP deaminase 3 (AMPD3), an enzyme that converts AMP to IMP, was identified to be mutated in one of the resistant clones. The requirement of AMPD3 in LeTx-induced cell death of RAW 264.7 cells was confirmed by the restoration of LeTx sensitivity with ectopic reconstitution of AMPD3 expression. AMPD3 deficiency does not affect LeTx entering cells and the cleavage of mitogen-activated protein kinase kinase (MKK) by lethal factor inside cells, but does impair an unknown downstream event that is linked to cell death. Our data provides new information regarding LeTx-induced macrophage death and suggests that there is a key regulatory site downstream of or parallel to MKK cleavage that controls the cell death in LeTx-treated macrophages.

KEYWORDS AMP deaminase 3, anthrax lethal toxin, macrophage, cell death

INTRODUCTION

Bacillus anthracis is a Gram-positive, spore-forming bacterium that causes anthrax disease in humans and animals (Smith and Keppie, 1954). There are two major virulence factors for *B. anthracis*, the capsule which protects against phagocytosis and a tripartite toxin (Smith, 2000). The anthrax toxin is consisted of three subunits—lethal factor (LF), edema factor (EF), and protective antigen (PA). These three proteins are non-toxic individually, but assemble into toxic complexes on the surface of receptor-expressing host cells (Smith, 2002). PA is an 83-kDa pore-forming protein that binds to the anthrax receptors- tumor endothelial marker-8 (TEM-8) and capillary morphogenesis gene-2 (CMG-2) - on the surface of the target cell, allowing entry of the two enzymatic components of the toxin, LF and EF, into the cell cytoplasm (Scobie and Young, 2005). The toxin formed by PA and LF is named anthrax lethal toxin (LeTx), which can kill host macrophages or alter macrophage function depending on the genetic background (Park et al., 2002; Muehlbauer et al., 2007). LF functions as metalloprotease to cleave intracellular proteins such as mitogen-activated kinase kinases (MKK) (Duesbery et al., 1998; Vitale et al., 1998; Pellizzari et al., 1999; Vitale et al., 2000). Although LeTx has been considered to be a key factor in developing anthrax pathogenesis and killing the host, its mechanism is still poorly understood.

The lethality of different rats upon LeTx treatment correlates with macrophage sensitivity to LeTx (Friedlander, 1986; Friedlander et al., 1993). Although there is no correlation in lethality and macrophage sensitivity to LeTx in mice with different genetic backgrounds, LeTx-resistant

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macrophages can undergo apoptosis when the cells are treated with LeTx plus cytokines, such as tumor necrosis factor-α (TNFα) (Kim et al., 2003), supporting the idea that macrophage death is involved in the toxicity of LeTx *in vivo*. Studies suggest that at least three loci control murine sensitivity to LeTx, and macrophage sensitivity is one of the factors that determine susceptibility to LeTx in mice (McAllister et al., 2003). *Nalp1b* has been identified as the LeTx macrophage sensitivity locus (Moayeri et al., 2003; Moayeri et al., 2004). The genes involved in regulation of endocrine responses may also be important in LeTx-mediated killing of mice (Moayeri et al., 2005). Though it is only one of the determinants of LeTx-induced lethality of mice, LeTx-induced macrophage death is of significant importance since macrophages are essential for the pathogenesis of *B. anthracis*.

The construction of a novel phenotype and the identification of mutations that are responsible for the altered phenotype have led to some of the most important advances in biology. Retroviral insertion can create a single mutation in a cell and the retrovirus can be modified, offering a tag to find the mutated target gene with relatively less effort (Wang et al., 2001). Although retroviral insertional mutagenesis may be a powerful tool, the diploidity in eukaryotic cells is an obstacle yet to be solved. To overcome this problem, chemical mutagenesis could be applied in addition to retroviral insertional mutagenesis (Kim et al., 2007). In principle, chemical mutagenesis *in vitro* could allow a high rate of mutations by multiple applications and generate a pool of cells that can be regarded as 'quasi-haploid.' Then, the introduction of a retrovirus into the cells may make randomly disrupted genes that have been rendered haploid, permitting a rapid identification of each mutation that causes a phenotype of interest (Kim et al., 2007). We adapted the gene disruption system using chemical and retroviral insertional mutation in order to understand the molecular mechanism underlying LeTx-induced macrophage death. By this approach, we identified AMP deaminase 3 (AMPD3) as a gene involved in LeTx-induced macrophage death.

AMP-deaminase (EC 3.5.4.6) catalyzes an irreversible reaction of deamination of AMP to IMP. The product of the reaction, IMP, is in the center of purine metabolism and may be further used for biosynthesis of guanine nucleotides (Morisaki and Morisaki, 2008). Tissue- and stage-specific isoforms of AMP-deaminase have been identified. Humans have three main AMP-deaminase isoforms [M (muscle), L (liver) and E (erythrocyte)] encoded respectively by *AMPD1*, *AMPD2*, and *AMPD3* (Bazan and Koch-Nolte, 1997). In the present study, we show that AMPD3 deficiency leads to resistance to LeTx-induced macrophage death. The role of AMPD3 in LeTx-induced macrophage death is not related to PA processing, LF delivery, or LF activity in the cells. AMPD3 deficiency-mediated LeTx resistance is not related to AMP activated protein kinase (AMPK) pathway, though it is associated with the increased AMP concentration resulting from the loss of AMPD3 activity.

RESULTS

Identification of AMP deaminase as a molecule involved in LeTx-induced cell death

As previously reported, a combination of chemical and insertional mutagenesis can be used for the mutagenic screen in cultured mammalian cells (Kim et al., 2007). We generated a pool of theoretically quasi-haploid RAW 264.7 cells, which have 99.5% of all genes in random mutation after eight rounds of N-ethyl-N-nitrosourea (ENU) treatment (Kim et al., 2007). Then, the retroviral insertion was introduced into the quasi-haploid cell pool to abolish gene expression or produce a truncated gene product. The retrovirus-infected cells were treated with blasticidin to select the retroviral gene-integrated clones. The murine macrophage cell line RAW 264.7 is sensitive to LeTx-induced cell death. The clones generated above were further screened for LeTx-resistant clones by treating the cells with LeTx (100 ng/mL PA plus 100 ng/mL LF). The LeTx-resistant clones were further analyzed to identify the mutated gene using 3′-rapid amplification of cDNA end (RACE) polymerase chain reaction (PCR). Clone 55111A is significantly resistant to LeTx, in comparison with parental wildtype RAW264.7 cells, when the cells were treated for four hours with 100 ng/mL PA plus different doses of LF or treated with 100 ng/mL PA plus 100 ng/mL LF for different periods of time (Fig. 1A). Sequencing the 3′-RACE-PCR product from clone 55111A revealed that one allele of AMPD3 has a retroviral insertion between exon 1 and exon 2 of AMPD3 (Fig. 1B), which should disrupt the AMPD3 gene expression from this allele. To determine whether AMPD3 can be expressed normally from the other allele, we analyzed AMPD3 mRNA in clone 55111A. We were able to amplify AMPD3 mRNA from both 55111A and wildtype cells by reverse transcription (RT)-PCR; however, two point mutations were found in AMPD3 cDNA isolated from 55111A but not wildtype cells (Fig. 1C). One of the point mutations converted threonine (T) 343 to isoleucine (I), while the other did not make any amino acid change (Fig. 1C). Molecular modeling suggests that T343 is located in a loop on AMPD3 surface, and is far away from the active site of AMPD3. Whether and how T343 to I mutation will affect the function of AMPD3 is unclear. Nevertheless, one allele of AMPD3 in clone 55111A was disrupted by retroviral insertion while the other had a point mutation, and thus the most important question at this stage is whether the resistance to LeTx of clone 5511A is due to AMPD3 deficiency.

To confirm the resistance to LeTx-induced cell death observed in the 55111A clone is due to the defect in the AMPD3 gene, we stably transfected 55111A cells with AMPD3 expression vector and control empty vector, respec-

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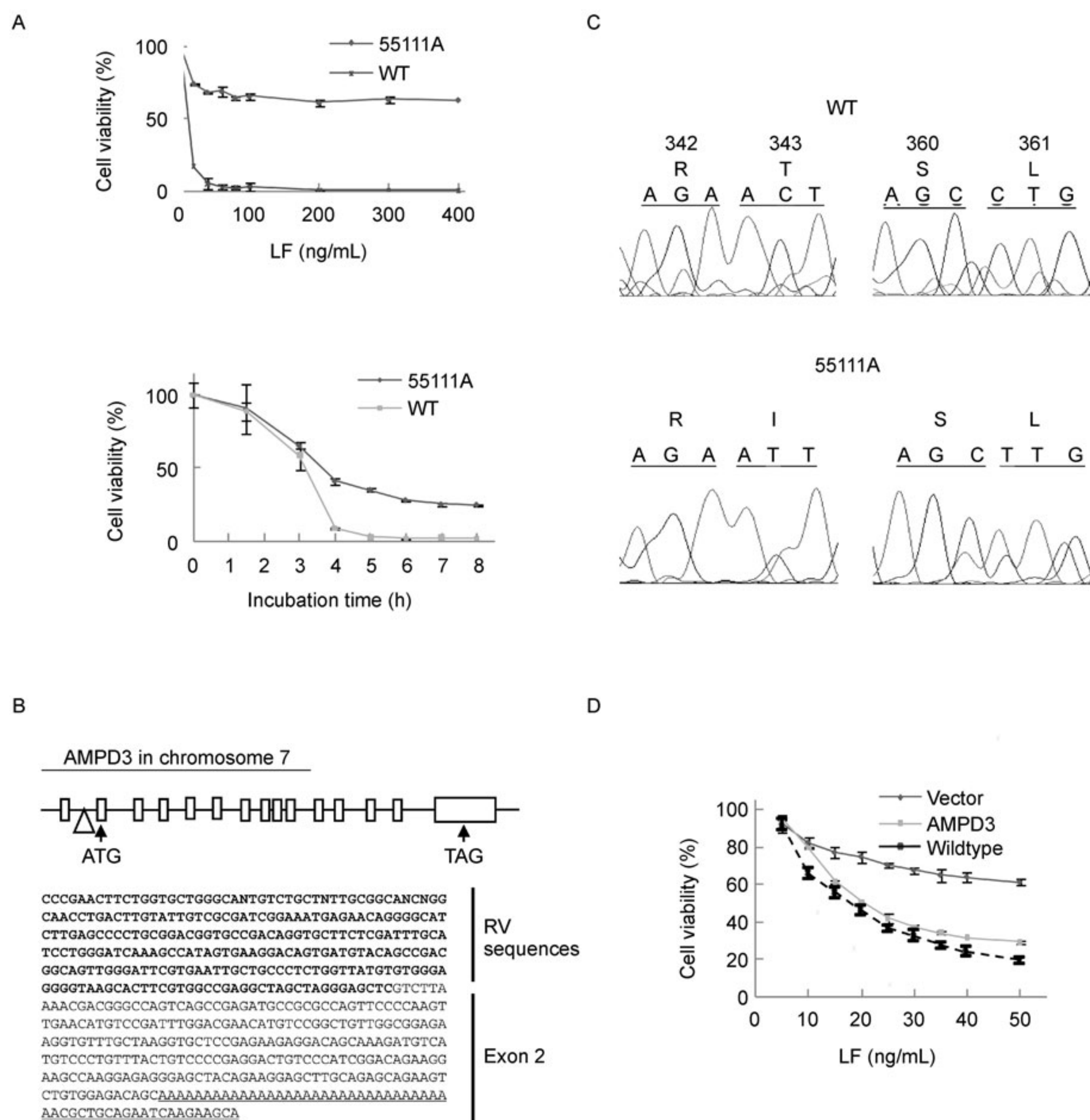

Figure 1. *Ampd3* deficiency leads to resistance to LeTx-induced death of RAW264.7 cells. (A) Cell viability of RAW264.7 wildtype and 55111A mutant cells were measured after the cells were treated with 100 ng/mL protective antigen (PA) plus lethal factor (LF) at different concentrations for 4 h or treated with 100 ng/mL PA plus 100 ng/mL LF for different periods of time. 55111A mutant showed resistance to LeTx-induced cell death in comparison with wildtype RAW264.7 cells. Means ± s.d. were based on 3 triplicated samples. (B) 3′ RACE was performed using cDNA from 55111A cells. Partial sequence of the 3′ RACE product is shown. The triangle indicates the retroviral insertion site located between exon 1 and exon 2 of *Ampd3* gene. Poly A sequence is underlined. RV, retrovirus. (C) RT-PCR was performed to amplify coding sequence of AMPD3 mRNA from wildtype and 55111A cells. Sequence of the RT-PCR products revealed point mutations in the AMPD3 cDNA from 55111A, but not wildtype cells. One point mutation resulted in a threonine 343 to isoleucine change. (D) AMPD3 expression vector or empty vector was stably transfected into 55111A cells. The cells were treated with 100 ng/mL PA plus different concentrations of LF for four hours. Cell viability is shown. Viability of wildtype cells is included for comparison. The reconstitution of AMPD3 expression in 55111A cells restores LeTx sensitivity.

tively. We then compared these two cell lines of their sensitivity to LeTx. As shown in Fig. 1D, the cells expressing AMPD3 are more sensitive to LeTx-induced cell death in comparison with vector-transfected cells. Over-expression of AMPD3 restored LeTx sensitivity to a level similar to wildtype RAW 264.7 cells. Thus, the resistance to LeTx-induced cell

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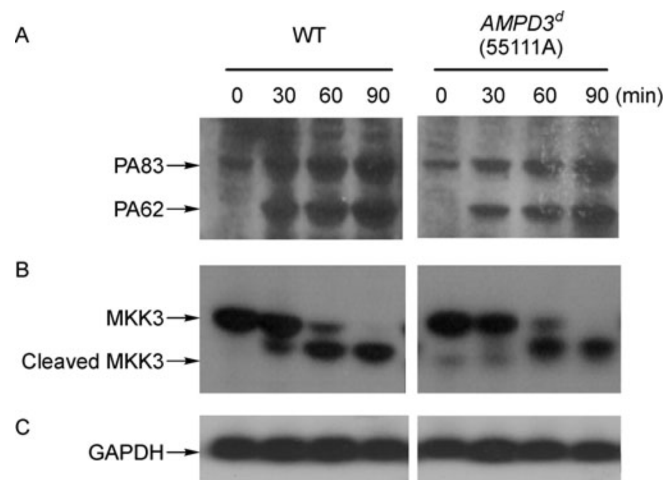

Figure 2. AMPD3 deficiency does not affect protective antigen (PA) processing and mitogen-activated kinase kinases (MKK) cleavage in LeTx-treated RAW264.7 cells. PA processing (A), MKK3 cleavage by lethal factor (LF) (B) and equal loading (C) were analyzed by immunoblotting using corresponding antibodies after wildtype and *AMPD3^d* cells were treated with LeTx (100 ng/mL PA plus 100 ng/mL LF) for different periods of time.

death of 55111A cells results from deficiency of AMPD3 expression, and this resistant phenotype can be rescued by reconstitution of AMPD3 expression.

AMPD3 mutation has no effect on PA processing or LT activity in cells

The first step in LeTx targeting cells is the binding of PA to the cellular receptor. PA then undergoes proteolysis and binds LF to form LeTx. LeTx is endocytosed by the cell and after acidification of the endosome LF translocates into the cell cytosol (Banks et al., 2005). To investigate how the AMPD3-deficient clone acquires LeTx resistance, we measured PA processing after its binding to cells. As shown in Fig. 2A, PA was cleaved in both wildtype and AMPD3-deficient (*AMPD3^d*) 55111A cells after incubation with 100 ng PA plus 100 ng LF for 0–90 min, indicating that AMPD3 deficiency did not affect PA binding and cleavage by furin. We then examined whether LF was delivered into the cytosol and whether LF protease activity was affected in *AMPD3^d* cells by measuring the MKK3 cleavage in the cells after the cells were incubated with LeTx. As shown in Fig. 2B, MKK3 cleavage by LF had the same efficiency in wildtype and *AMPD3^d* cells. Collectively, these data demonstrate that AMPD3 deficiency-mediated resistance to LeTx-induced cell death in RAW264.7 cells is not associated with LeTx processing, entering, or its protease activity.

The AMPK pathway has no role in AMPD3 deficiency-mediated LeTx resistance

The function of AMPD3 is to convert AMP to IMP by deamination of an adenosine residue (Fig. 3A). Therefore, depletion of AMPD should increase the concentration of AMP in cells. Since AMP concentration regulates AMPK activity, we addressed whether the AMP-activated protein kinase AMPK has any role in AMPD3 deficiency-mediated resistance to LeTx-induced cell death. AICAR is an AMPK activator. We tested whether activation of AMPK has any effect on LeTx-induced death of RAW 264.7 cells by using AICAR to activate AMPK, and found that including AICAR did not affect LeTx-induced RAW 264.7 cell death (Fig. 3B). We also examined whether LeTx treatment had an effect on AMPK activation and found that AMPK activity was not affected in either wildtype or *AMPD3^d* cells (Fig. 3C), consistent with AICAR data showing that AMPK has no role in LeTx-induced cell death. The mammalian target of rapamycin (mTOR) functions downstream of AMPK to play a central role in regulating cellular energy homeostasis. We tested whether mTOR plays any role in LeTx-induced cell death and found that rapamycin did not affect LeTx-induced cell death in either wildtype or *AMPD3^d* cells (Fig. 3D). Actually, LeTx treatment slightly inhibited mTOR complex 1 activity since S6K1 activity was reduced following times of LeTx treatment in both wildtype and *AMPD3^d* cells (Fig. 3E). Equal numbers of live cells were loaded in each lane and MKK3 cleavage was analyzed to show the activity of LeTx (Fig. 3E). Collectively, our data excluded the involvement of AMPK and mTOR in AMPD3 deficiency-mediated LeTx-resistance.

AMPD3 deficiency-mediated increase in AMP concentration is at least partially responsible for LeTx resistance

Although AMPK has no role in AMPD3 deficiency-induced RAW 264.7 cell death, it cannot be ruled out that AMP has a role in LeTx-resistance in *AMPD3^d* cells. To test whether AMP

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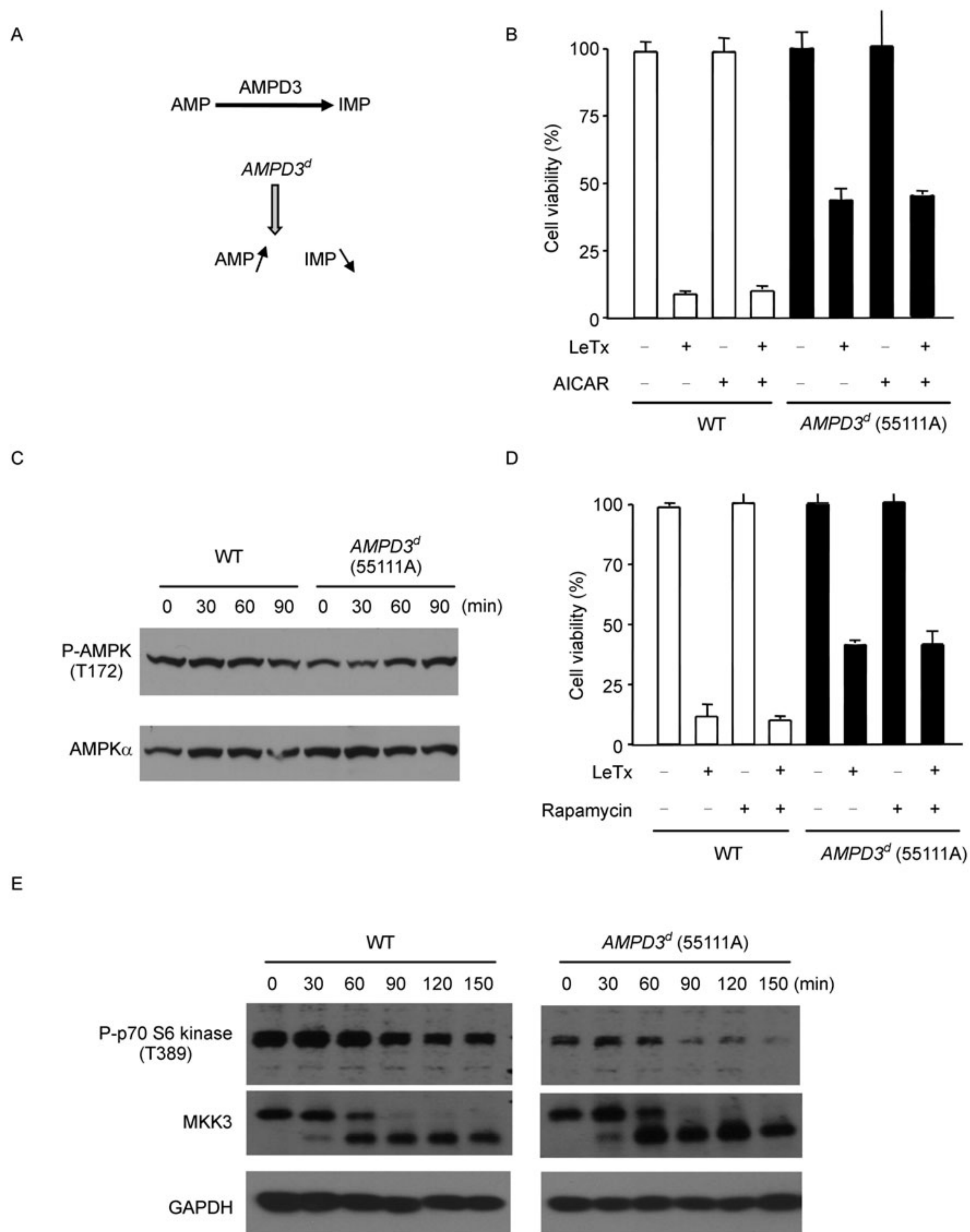

Figure 3. AMP activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) have no role in AMPD3 deficiency-mediated resistance to LeTx. (A) A diagram of possible consequence of AMPD3 deficiency. (B) Wildtype and *AMPD3^d* cells were treated with or without LeTx (100 ng/mL PA plus 100 ng/mL LF) in the presence or absence of AICAR (0.5 mmol/L) for 4 h. Cell viability was measured. (C) Wildtype and *AMPD3^d* cells were treated with LeTx (100 ng/mL PA plus 100 ng/mL LF) for different periods of time. Phospho-AMPK (P-AMPK) and AMPKα subunit were analyzed by immunoblotting with corresponding antibodies. (D) Wildtype and *AMPD3^d* cells were treated with or without LeTx (100 ng/mL PA plus 100 ng/mL LF) in the presence or absence of rapamycin (20 nmol/L) for 4 h. Cell viability was measured and shown. (E) Wildtype and *AMPD3^d* cells were treated with LeTx (100 ng/mL PA plus 100 ng/mL LF) for different periods of time. Phospho-S6 kinase 1 (P-p70S6 kinase 1), MKK3, and GAPDH were analyzed by immunoblotting with corresponding antibodies.

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concentration had an effect on LeTx-induced RAW 264.7 cell death, we included 50 mmol/L AMP in the culture medium and then treated the cells with LeTx. cAMP was used as control for AMP. Although AMP and cAMP are charged molecules that cannot freely pass through the cell membrane, they can enter the cells by channels or unknown cell surface receptors when high concentrations of these molecules are present in the medium. We found that including AMP in the medium significantly reduced LeTx-induced cell death (Fig. 4A). The inhibition was clearly seen when 10–30 ng/mL LF was used for up to 12 h. Including cAMP had little or no effect on LeTx-induced RAW 264.7 cell death, suggesting that the inhibitory effect of AMP is specific. We then tested whether addition of AMP can function after LeTx treatment by adding AMP at 0, 80, 100, and 120 min after LeTx treatment. As anticipated, adding AMP at time 0 inhibited cell death when the cells were treated with LeTx for four hours (Fig. 4B, upper-left panel). Interestingly, adding AMP at 80 min and 100 min was still able to inhibit LeTx-induced cell death (Fig. 4B, upper-right and lower-left panels). Addition of AMP at 120 min after LeTx treatment began to have no effect on LeTx-induced cell death (Fig. 4B, lower-right panel). Because LeTx-mediated MKK cleavage completed around 90 min (Fig. 2B) and LeTx-induced cell death was initiated during one to four hours (Fig. 1A), AMP-mediated inhibition of LeTx-induced cell death is likely to occur at a stage after LF-mediated MKK cleavage.

DISCUSSION

LeTx is known as a main player in anthrax pathogenesis and is able to induce death of macrophages from certain mouse strains (Moayeri and Leppla, 2009). Although LF functions as a metalloprotease to cleave MKKs in cytosol, the precise mechanism to induce cell death is not clear yet (Duesbery et al., 1998; Vitale et al., 1998; Pellizzari et al., 1999; Vitale et al., 2000). In this study, we found that AMPD3 is involved in LeTx-induced macrophage death. Our data suggest that AMP concentration plays a role in AMPD3 deficiency-mediated resistance to LeTx-induced cell death, though the molecular mechanisms still await further investigation.

Macrophages are important in the pathogenesis of *B. anthracis* since spores of the bacteria are taken up in macrophages adjacent to the epithelial route of entry, and then transported by macrophages to the lymph nodes where germination of the spores occurs (Guidi-Rontani et al., 2001). Although only the macrophages from select inbred mouse strains undergo rapid lysis in response to LeTx, it is known that a strong IL-1β and cytokine burst can result from macrophage lysis and can exacerbate LeTx-induced vascular collapse (Moayeri et al., 2004). Therefore, macrophage death has an effect on the pathogenesis of *B. anthracis* if it occurs.

Identification of *AMPD3* as a gene involved in LeTx-induced macrophage death suggests that nucleotide metabolism can affect LeTx-mediated cellular changes. One of the consequences of AMPD3 deficiency is accumulation of AMP. The finding that AMPD3 mutation produced no effect on AMPK activation suggests that AMPD3 deficiency-mediated accumulation of AMP did not affect the AMP: ATP ratio, which could be because the amount of AMP accumulation was too small to affect this ratio or because the cell increased ATP to balance the AMP: ATP ratio. The supply of high concentrations of AMP in RAW 264.7 cell culture brought a resistance against LeTx, which suggests that AMP inside the cells did influence LeTx sensitivity, though we were unable to measure the intracellular AMP concentration because of high level contamination of extracellular AMP in this situation. The inhibitory effect of AMP on LeTx-induced cell death also rules out the possibility that decrease of IMP resulting from AMPD3 deficiency plays a role in AMPD3 deficiency-mediated resistance to LeTx. It is very interesting to see that the AMP-mediated LeTx resistance can be induced 100 min after LeTx treatment, indicating that the inhibition occurs at a later time in LeTx-induced death process. We do not know whether LeTx treatment alters AMPD3 activity, but the effect of AMP addition after 120 min of LeTx treatment suggested that the effect of AMPD3 is on the later event(s) of LeTx-induced cell death. It is clear that AMPD3 deficiency did not affect LeTx-mediated MKK cleavage, suggesting another cellular change in LeTx-treated cells was affected. Taken together, these data indicate that there are many unknown events in LeTx-stimulated macrophages. Our study provides new information for understanding the mechanisms underlying LeTx-induced macrophage death.

MATERIALS AND METHODS

Mutagenesis with N-nitroso-N-ethylurea

Chemical mutagenesis was performed as previously described (Kim et al., 2007). Briefly, N-nitroso-N-ethylurea (ENU; Sigma, St. Louis, MO, USA), O^6-benzylguanine (O^6-BG), and 6-thioguanine (6-TG) were purchased from Sigma (St. Louis, MO). ENU was dissolved in equal portions of 95% ethanol and PCS buffer (50 mmol/L sodium citrate, 100 mmol/L sodium phosphate, pH 5.0) and O^6-BG was dissolved in dimethyl sulphoxide (DMSO). ~50% confluent cells in a 10 mm dish were pre-treated with O^6-BG (10 μmol/L) for 12 h and then treated with ENU (0.35 mg/mL) for 2 h in the presence of O^6-BG. The cells were washed and incubated with fresh medium containing O^6-BG for 24 h for recovery. Then the cells were plated onto 10 mm dishes at ~1000 cells per dish. Four dishes were used to measure mutation frequency and the other two were used for the next round of mutagenesis. To detect *Hprt*-null mutants, individual clones were picked from the 100 mm dish into 96-well plates. After culturing these clones for 24 h, 6-TG (10 μmol/L) was added into the medium and the resistant clones were scored one week later.

Mutagenesis with retrovirus

The retrovirus preparation and infection using the pDisrup 8 retroviral

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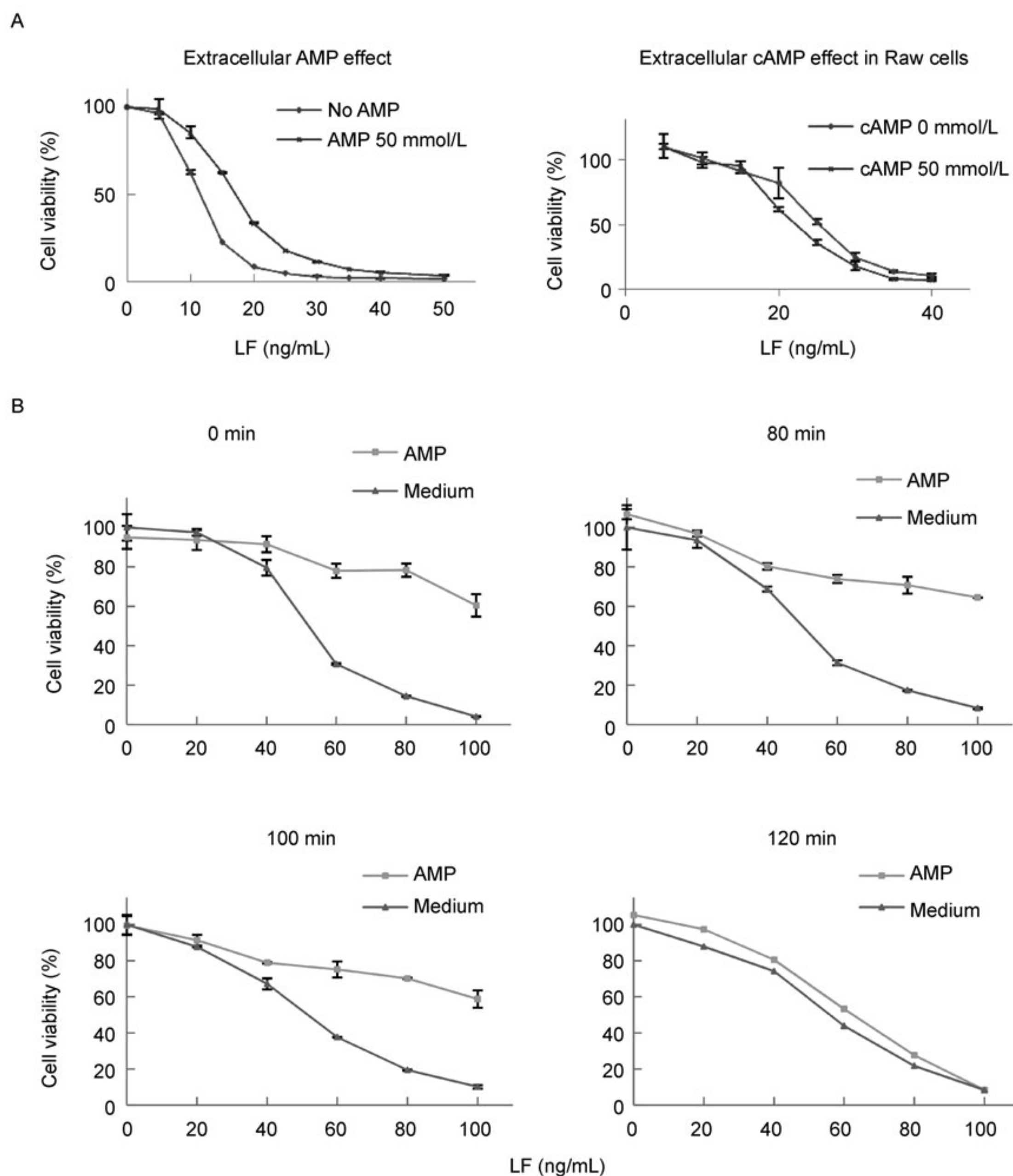

Figure 4. AMP can inhibit LeTx-induced cell death. (A) RAW264.7 cells were treated with 100 ng/mL PA plus different concentrations of LF in the presence or absence of AMP (50 mmol/L) or cAMP (50 mmol/L). Cell viability was measured after 12 h treatment. (B) RAW264.7 cells were treated with 100 ng/mL PA plus different concentrations of LF. AMP was added at 0, 80, 100, or 120 min after LeTx addition. Cell viability was measured four hours after LeTx treatment.

vector was performed as previously described (Wang et al., 2001). Briefly, pDisrup 8 recombinant retroviruses were generated in amphotropic packaging cells using the calcium phosphate method of transfection. Viruses were produced at 32°C, and a virus-containing medium was collected 24 h posttransfection and filtered through a 0.45-μm filter. A pool of the chemically mutated theoretical haploid RAW 264.7 cells was plated in six-well plates at a density of 5×10^5 cells/well. Retroviral infection was performed by replacing the medium with 2 mL of pDisrup 8 virus (containing 4 μg of Polybrene per mL), followed by centrifugation of the six-well plates at 2500 rpm for 30 min at RT. The infection efficiency was estimated by paralleled experiments using a retrovirus to transiently express green fluorescent protein. Blasticidin (10 μg/mL; Invivogen, San Diego, CA) was used to select the resistant clones.

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3'-RACE PCR

The portion of the endogenous gene that was fused with the *bsd* (blasticidin S deaminase) gene was amplified by the 3' rapid amplification of cDNA ends (RACE) technique. Total RNA was isolated, and reverse transcription was performed with the primer 5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC$[T]_{17}$-3'. A nested PCR was performed with the resulting reverse transcription product with the following primers: P1/Q1 (5'-AAAGCGATAGTGAAG-GACAGTGA-3' and 5'-CCAGTGAGCAGAGTGACG-3') and P2/Q2 (5'-TGCTGCCCTCTGGTTATGTGTGG-3' and 5'-GAGGACTC-GAGCTCAAGC-3'). P1 and P2 are located on the *bsd* gene, while Q1 and Q2 are on the anchor sequence of QT. The PCR products of 3'-RACE and RT-PCR were directly sequenced.

Cell culture and viability assay

A single clone of RAW 264.7 cells was isolated and used as parental cells for the mutagenesis. The mutated cells generated at every experimental step were stored at very early passages so that we always had original mutants, as spontaneous reversions occur during cell culture. Components of lethal toxin, lethal factor (LF), and protective antigen (PA), were obtained from List Biological Laboratories (Campbell, CA). The extent of LeTx-induced cell death was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipehnyltetrazolium bromide; Sigma) assay and propidium iodide (Molecular probes Inc., Eugene, OR) exclusions (Kim et al., 2003). For the MTT assay, cells were prepared to be about 10^5 cells per well in 96-well plates a day before toxin treatment. LeTx was applied with various incubation times and various doses, depending on the experimental design. LeTx-containing medium was removed from wells and fresh medium containing MTT (0.5 mg/mL) was added. After 2 h of incubation, the medium was removed and a solubilization solution (10% Triton-X 100 and 0.1 mol/L HCl in anhydrous isopropanol) was added to dissolve MTT formazan crystals. The absorbance was measured at 570 nm. For PI staining, cells were harvested by a cell scraper and centrifuged after LeTx treatment. The cell pellet was resuspended with PBS containing PI (1 µg/mL) and the level of PI incorporation was quantified by flow cytometer.

Reconstitution of AMPD3

In order to reconstitute the *AMPD3* gene in the mutant cells, the full length of cDNA in the pCMV-SPORT6 vector was purchased from Open Biosystems (MMM1013-7511114; www.openbiosystems.com). The vector was cotransfected into AMPD3 mutant with pRS vector containing a hygromycin-resistant gene. The hygromycin-resistant cells were selected and tested for LeTx toxicity.

Cell lysate preparation and Western blot analysis

Extraction of total cell lysates and Western blot analysis were performed to evaluate PA processing, MKK3 cleavage, and further intracellular signaling. Briefly, after cells were treated properly, cells were lysed in ice-cold RIPA cell lysis buffer (10 mmol/L $NaPO_4$, pH 7.2, 0.3 mol/L NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, and 2 mmol/L EDTA) with a protease inhibitor cocktail or sample buffer. Cell extracts were obtained by centrifuging the homogenate at 13,000 × g for 10 min. After quantification, the extracts were electronically resolved in SDS-PAGE gel and transferred onto nitrocellulose or PVDF membranes. Membranes were subsequently blocked with 5% skim milk for 1 h, reacted with proper primary and secondary antibodies, and developed using the Supersignal west femto maximum sensitivity substrate (Pierce, Rockford, IL) system.

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ABBREVIATIONS

AMPD3,AMP deaminase 3; AMPK, AMP activated protein kinase; LeTx, anthrax lethal toxin; MKK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; RACE, 3′-rapid amplification of cDNA end

REFERENCES

Banks, D.J., Barnajian, M., Maldonado-Arocho, F.J., Sanchez, A.M., and Bradley, K.A. (2005). Anthrax toxin receptor 2 mediates Bacillus anthracis killing of macrophages following spore challenge. Cell Microbiol 7, 1173–1185.

Bazan, J.F., and Koch-Nolte, F. (1997). Sequence and structural links between distant ADP-ribosyltransferase families. Adv Exp Med Biol 419, 99–107.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K. R., Copeland, T.D., *et al.* (1998). Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280, 734–737.

Friedlander, A.M. (1986). Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J Biol Chem 261, 7123–7126.

Friedlander, A.M., Bhatnagar, R., Leppla, S.H., Johnson, L., and Singh, Y. (1993). Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect Immun 61, 245–252.

Guidi-Rontani, C., Levy, M., Ohayon, H., and Mock, M. (2001). Fate of germinated Bacillus anthracis spores in primary murine macrophages. Mol Microbiol 42, 931–938.

Kim, S.O., Ha, S.D., Lee, S., Stanton, S., Beutler, B., and Han, J. (2007). Mutagenesis by retroviral insertion in chemical mutagen-generated quasi-haploid mammalian cells. Biotechniques 42, 493–501.

Kim, S.O., Jing, Q., Hoebe, K., Beutler, B., Duesbery, N.S., and Han, J. (2003). Sensitizing anthrax lethal toxin-resistant macrophages to lethal toxin-induced killing by tumor necrosis factor-alpha. J Biol Chem 278, 7413–7421.

McAllister, R.D., Singh, Y., Du Bois, W.D., Potter, M., Boehm, T., Meeker, N.D., Fillmore, P.D., Anderson, L.M., Poynter, M.E., and Teuscher, C. (2003). Susceptibility to anthrax lethal toxin is controlled by three linked quantitative trait loci. Am J Pathol 163, 1735–1741.

Moayeri, M., Haines, D., Young, H.A., and Leppla, S.H. (2003). Bacillus anthracis lethal toxin induces TNF-independent hypoxia-mediated toxicity in mice. J Clin Invest 112, 670–682.

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Moayeri, M., and Leppla, S.H. (2009). Cellular and systemic effects of anthrax lethal toxin and edema toxin. Mol Aspects Med 30, 439–455.

Moayeri, M., Martinez, N.W., Wiggins, J., Young, H.A., and Leppla, S. H. (2004). Mouse susceptibility to anthrax lethal toxin is influenced by genetic factors in addition to those controlling macrophage sensitivity. Infect Immun 72, 4439–4447.

Moayeri, M., Webster, J.I., Wiggins, J.F., Leppla, S.H., and Sternberg, E.M. (2005). Endocrine perturbation increases susceptibility of mice to anthrax lethal toxin. Infect Immun 73, 4238–4244.

Morisaki, H., and Morisaki, T. (2008). AMPD genes and urate metabolism. Nippon Rinsho 66, 771–777.

Muehlbauer, S.M., Evering, T.H., Bonuccelli, G., Squires, R.C., Ashton, A.W., Porcelli, S.A., Lisanti, M.P., and Brojatsch, J. (2007). Anthrax lethal toxin kills macrophages in a strain-specific manner by apoptosis or caspase-1-mediated necrosis. Cell Cycle 6, 758–766.

Park, J.M., Greten, F.R., Li, Z.W., and Karin, M. (2002). Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science 297, 2048–2051.

Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M., and Montecucco, C. (1999). Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNgamma-induced release of NO and TNFalpha. FEBS Lett 462, 199–204.

Scobie, H.M., and Young, J.A. (2005). Interactions between anthrax toxin receptors and protective antigen. Curr Opin Microbiol 8, 106–112.

Smith, H. (2000). Discovery of the anthrax toxin: the beginning of in vivo studies on pathogenic bacteria. Trends Microbiol 8, 199–200.

Smith, H. (2002). Discovery of the anthrax toxin: the beginning of studies of virulence determinants regulated in vivo. Int J Med Microbiol 291, 411–417.

Smith, H., and Keppie, J. (1954). Observations on experimental anthrax: demonstration of a specific lethal factor produced in vivo by Bacillus anthracis. Nature 173, 869–870.

Vitale, G., Bernardi, L., Napolitani, G., Mock, M., and Montecucco, C. (2000). Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. Biochem J 352, 739–745.

Vitale, G., Pellizzari, R., Recchi, C., Napolitani, G., Mock, M., and Montecucco, C. (1998). Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. Biochem Biophys Res Commun 248, 706–711.

Wang, X., Ono, K., Kim, S.O., Kravchenko, V., Lin, S.C., and Han, J. (2001). Metaxin is required for tumor necrosis factorinduced cell death. EMBO Rep 2, 628–633.

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