Epigenetic modifiers reduce Inflammation and modulate macrophage phenotype during endotoxemia-induced acute lung injury

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**Key words:** epigenetic modifiers, acute lung injury, Histone deacetylase inhibitor, sepsis, inflammation and apoptosis.

**ABSTRACT**

Acute lung injury (ALI) during sepsis is characterized by bilateral alveolar infiltrates, lung edema, and respiratory failure. Here, we examined the efficacy of DNA methyltransferase (DNMT) inhibitor Aza (5-Aza 2-deoxycytidine), histone deacetylase (HDAC) inhibitor TSA (Trichostatin A), and combination therapy (Aza+TSA) in protection of ALI. In LPS-induced mouse ALI, post-treatment with a single dose of Aza+TSA showed a substantial attenuation of adverse lung histopathological changes, and inflammations. Importantly, these protective effects were due to significant macrophage phenotypic changes observed in LPS-stimulated macrophages treated with Aza+TSA as compared with untreated LPS-induced macrophages or LPS-stimulated macrophages treated with either drug alone. Further, we observed significantly lower levels of pro-inflammatory molecules and higher levels of anti-inflammatory molecules in LPS-induced macrophages treated with Aza+TSA than in LPS-induced macrophages treated with either drug alone. The protection was ascribed to dual effects by an inhibition of MAPK-HuR-TNF and activation of STAT3-Bcl2 pathways. Combinatorial treatment with Aza+TSA reduces inflammation and promotes an anti-inflammatory M2 macrophage phenotype in ALI. This finding gives further evidence that the epigenetic treatment has a therapeutic potential for patients with sepsis.
INTRODUCTION

In septic patients, acute lung injury (ALI) is characterized by higher lung vascular permeability and severe lung infection and inflammation that typically lead to a gradual decline of lung function (Diaz, Brower, Calfee, & Matthay, 2010). Lipopolysaccharide (LPS), a bacterial cell wall toxin, is a contributing agent implicated in the ALI and the disease pathogenesis (Dellinger et al., 2008). Furthermore, the triggering of an inflammatory gene expression is caused by the direct contact of pathogens with cells of the immune system. This interaction is necessary for immune defense and is often harmful due to the exaggerated production of inflammatory proteins. Although antibiotics and volume replacement are the cornerstones for the treatment of sepsis (Dellinger et al., 2008), an unchecked inflammatory response limits their effectiveness. Therapies based on a new understanding of the disease pathogenesis are needed.

DNA methylation or demethylation and histone deacetylation or acetylation, are the two important epigenetic events that decide the fate of a cell and its gene expressions (Hashimshony, Zhang, Keshet, Bustin, & Cedar, 2003; Jenuwein & Allis, 2001). We selected 5-Aza 2-deoxycytidine (Aza), an inhibitor for DNA methyl transferase (DNMT), and Trichostatin A (TSA), an inhibitor for histone deacetylases (HDACs), because of their well-established biological activities, and known safety and side effect profiles (Avila et al., 2007; Kaminskas, Farrell, Wang, Sridhara, & Pazdur, 2005). Aza is an effective drug for acute myelogenous leukemia and has the potential to be repurposed (Christman, 2002; Kaminskas et al., 2005). TSA, a potent general inhibitor for HDACs, chelates the center zinc finger of the HDACs, thereby inhibiting enzyme activity (Furumai et al., 2011; Suzuki & Miyata, 2005). It has been
shown that TSA improved survival in the rat hemorrhagic shock model (T. Lin et al., 2007). Recently, we have shown that the combinatorial therapy with Aza and TSA reduces mortality and improves lung vascular cell integrity in LPS-induced mouse ALI (Thangavel et al., 2014).

It is known that the LPS interacts with the cell-surface protein TLR4, and that it is in contact with the intracellular connector protein, MyD88 (Takeda, Kaisho, & Akira, 2003). This LPS-TLR4-MyD88 signaling complex further triggers two important signaling cascades such as the NF-κB and the mitogen-activated protein kinase (MAPK) pathways, which directs the pro-inflammatory and anti-inflammatory responses. The MAPK pathway has a crucial role in innate immune response signaling (Dong, Davis, & Flavell, 2001, 2002). The three major families of MAPK pathway, which mediate innate immune response signaling, include MKK-3, -4, -6, MAPKp38, and JNK (Blander & Medzhitov, 2004; Chang & Karin, 2001; Davis, 2000). A newer KBH-A42 HDAC inhibitor has been recognized as an anti-inflammatory drug through its capability of reducing the production of TNFα and nitric oxide in LPS induced macrophages. This study has also shown that the substantial anti-inflammatory response of KBH-A42 is mediated through the phosphorylation of p38-MAPK, but not through the activation of ERK1/2 or JNK (Choi et al., 2008).

Macrophages, endothelial cells and neutrophils are crucial in regulating inflammation as well as lung vascular endothelial cell integrity in LPS-induced ALI (Di et al., 2012; Y. L. Wang et al., 2011). We chose to study the macrophages because of their role in orchestrating inflammation (Kochanek et al., 2012; Song et al., 2001). We focused on the combined treatment with epigenetic modifiers Aza and TSA (hereafter referred to as Aza+TSA) that mediate a protective mechanism of p38-MAPK-HuR-TNFα and STAT3-Bcl2 during inflammatory and anti-inflammatory responses,
respectively. These responses are currently not well understood. The challenge remains to identify the critical role of epigenetic modifiers Aza+TSA (and the involvement of macrophages) in reducing inflammation related to pathologies during ALI. However, to date, there is no literature examining the efficacy of the nanomolar concentration of Aza+TSA in modulating cytokines and macrophages to quench inflammation and prevent mortality during sepsis. We chose to study the lung injuries and inflammations because the lung is the major target for the acute and chronic infections (Song et al., 2001).

The intent of this study was to identify the key modes of action in combinatory treatment of these epigenetic modifiers in reducing inflammation-related pathologies during sepsis-induced ALI. We have already shown that combinatorial treatment with Aza+TSA significantly reduced mortality in the ALI model (Thangavel et al., 2014). This study extends our earlier study in order to address the possibility that Aza+TSA-induced protection may be due to reduced inflammation and stimulates the M2 anti-inflammatory macrophage phenotype in the lung during endotoxemia-induced sepsis. This study further supports the potential for the use of epigenetic modifiers as therapeutic treatments for patients with sepsis and ALI.
RESULTS

Aza+TSA treatment does not affect cell viability

In our previous study, we examined the toxicity of Aza and TSA in primary mouse lung endothelial cells and identified that the combinations of Aza and TSA treated with the concentrations of 50 nM and 25 nM respectively, had no toxic effect and no change in cell viability (Thangavel et al., 2014). To identify a safe dose of these epigenetic modifiers Aza and TSA in primary mouse bone marrow-derived macrophages (BMDMs), we performed in vitro trypan blue dye exclusion viability assay and MTT assay (Promega, USA). For this, we have cultured BMDMs in twelve-well sterile tissue culture plates (Fig. S1) and treated with four different combination of Aza Aza TSA for 48 and 72 hours. After the treatments, the BMDMs were collected and subjected to a 0.1% Trypan blue solution. Then using a hemocytometer the number of unstained viable and stained non-viable cells was counted. The data show that the BMDMs treated with Aza and TSA concentrations up to 50 nM and 25 nM respectively did not affect the cell viability. The effect of Aza+TSA on BMDM cell viability was further supported MTT proliferation assay and was performed as per the protocol provided by the manufacturer. The treated BMDMs showed the normal ability to proliferate in the MTT assay. Thus, our observations suggest that the concentrations of 50 nM of Aza and 25 nM of TSA are safe and are not affecting the cell viability, but rather cells are able to proliferate (Fig. 1A, B, p<0.05). Based on these data, we used the concentrations of 50 nM of Aza and 25 nM of TSA in all of our in vitro experiments.
Aza+TSA treatment rescues the cell viability of LPS-induced BMDMs

To show the effect of Aza+TSA on the viability of LPS-induced BMDMs, we performed propidium iodide (PI) and Annexin V staining and analyzed the results by flow cytometer. For this experiment, primary BMDMs (Supplementary Fig. S1) were treated with Aza (50 nM)+TSA (25 nM) in the presence and absence of LPS (1µg/ml) for 48 hours. Then the cells were stained with PI for flow cytometer analysis. The flow cytometer analysis data showed that LPS-induced BMDMs have reduced viability, which was significantly protected following Aza+TSA treatment (Fig. 1C, D).

The immunomodulatory effect of Aza+TSA-treatment on LPS induced BMDMs

To evaluate the in vitro immunomodulatory effect of Aza+TSA on LPS-induced chemokines and cytokines in BMDMs, we cultured the BMDMs in the presence and absence of LPS (1µg/ml) and treated them for 24 hours with either Aza+TSA or Aza alone or with TSA alone. Then these cells were harvested and measured via PCR array for the mRNA expression of chemokines and cytokines (SA Bioscience, USA) as described by the manufacturer’s instructions as well as an earlier publication (Pal et al., 2012). BMDMs stimulated with LPS showed significantly increased expression of proinflammatory chemokines CCL2, CCL3, CCL4, CCL5, CCL7, CD40, CXCL10, and CXCL12 gene transcripts, and cytokines IL6, IL1α, IL1β, IL1RN, IL18, Lymphotxin–β, and TNFα gene transcripts. Whereas, the treatment with Aza+TSA abrogated the LPS-induced increase in gene expression of both chemokines and cytokines (Fig. 2A, B). The expression levels of the representative down-regulated genes TNFα, IL6, and IL1β observed in PCR array experiments were independently confirmed by real-time RT-PCR (Fig. 2C). To analyze the anti-inflammatory activity of Aza+TSA treatment, we performed qRT-PCR for IL10 and IL10R. The mRNA
expressions of IL10 and IL10R were higher in LPS-induced BMDMs treated with Aza+TSA than in untreated LPS-induced BMDMs or LPS-induced BMDMs treated with Aza alone or TSA alone (Fig. 2D).

Aza+TSA induces more immuno-plastic M2 macrophages

To understand the effect of molecular changes that occur during Aza+TSA treatment on LPS-induced macrophage subtypes, we cultured the BMDMs in the presence and absence of LPS (1µg/ml) and treated them with Aza+TSA for 24 hours. These cells were then harvested, stained for CD14 and CD40 for M1 subtype and CD23 and CD124 for M2 subtype, and analyzed as well as quantified by flow cytometer (Kigerl et al., 2009). Our flow cytometer data show a significant decreased expression of CD14 and CD40 (Fig. 3A) and increased expression of CD23 and CD124 (Fig. 3B) in Aza+TSA-treated cells compared with untreated LPS-induced cells or LPS-induced cells treated with either drug alone (Fig. 3C). To confirm the immuno-plasticity, we used LPS-induced BMDMs treated for 24 hours with either Aza alone or TSA alone or with a combination of Aza+TSA. Then the Western analysis for M1 marker NOS2 and M2 marker CD206 was performed. Our data showed lesser expression of NOS2 in LPS-challenged macrophages treated with Aza+TSA than in the cells treated with either Aza or TSA alone (Fig. 3D). Likewise, the Western analysis data for CD206 showed higher protein expression in Aza+TSA- treated cells than in the untreated cells and in the cells treated with either drug alone (Fig. 3E). The data supports LPS-stimulated macrophages treated with Aza+TSA show more M2 macrophages, which secrete anti-inflammatory cytokines.
**Aza+TSA treatment suppresses HuR expression and p38 MAPK activation**

We and others have shown that members of the MAPK family are involved in modulating mRNA stability/translation of several proinflammatory genes (Kochanek et al., 2012; Rajasingh et al., 2006). Studies have shown that the activations of P38 MAPK through the expression of target genes are implicated via RNA stabilizing protein HuR. HuR triggered stabilization of mRNA coding for key inflammatory mediators, including IL6, IL8, and TNFα mRNA (Neininger et al., 2002; Rajasingh et al., 2006). Thus, P38 MAPK-induced activation appears essential for HuR translocation and stabilization of target mRNAs (Winzen et al., 1999). In our previous study, we found that in a mouse model of myocardial infarction, HuR expression was up regulated and was associated with increased myocardial inflammatory response and cardiomyocyte cell death, leading to cardiac dysfunction and remodeling (Krishnamurthy et al., 2009). Thus, MAPK and HuR proteins play an important role during inflammatory responses. To show the protective mechanism of Aza+TSA in LPS-challenged macrophages, we have performed the following experiments as described in detail earlier (Rajasingh et al., 2006). The in vitro LPS-induced BMDMs treated with Aza+TSA showed decreased mRNA transcripts of pro-inflammatory chemokines and cytokines, including TNFα (Fig. 2). Furthermore, to examine the significance of HuR-p38MAPK cascade during Aza+TSA-mediated protective inflammatory mechanisms, we performed Western analysis for TNFα stabilizing protein HuR and p-P38 in LPS-induced BMDMs. BMDMs were cultured in the presence and absence of LPS and treated for 24 hours with either Aza+TSA or Aza alone or with TSA alone. ELISA was performed in a collected culture supernatant to estimate the secretory level of TNFα and the cells were used for the expression of
HuR protein by Western analysis. ELISA data showed that the LPS-induced BMDMs treated with Aza+TSA had decreased pro-inflammatory cytokine TNFα secretion compared with LPS-induced BMDMs treated with either drug alone (Fig. 4A). To examine the Aza+TSA-mediated protective mechanism of inflammation, we performed Western analysis for TNFα stabilizing protein HuR. We observed a significant decrease in HuR protein in LPS-stimulated BMDMs treated with Aza+TSA compared with the untreated LPS-stimulated BMDMs or LPS-stimulated BMDMs treated with Aza or TSA alone, suggesting the role of HuR protein in the protection of LPS-induced inflammation (Fig. 4B). Furthermore, to analyze the role of MAPK in the Aza+TSA-treatment mediated protective effect, we cultured BMDMs in the presence and absence of LPS and treated them for 30 minutes with either Aza+TSA or Aza or with TSA alone. Then the cells were subjected to protein analysis for activated p38. The Western analysis data showed an increased activation of p38 in LPS-induced BMDMs, which was significantly inhibited by Aza+TSA treatment compared with treatment with either drug alone (Fig. 4C p<0.01).

To confirm the protective effect of Aza+TSA in mediating through MAPK, Western analysis was performed on LPS-challenged BMDMs cultured for 30 minutes with the combination of Aza+TSA in the presence and absence of specific MAPK inhibitor SB202190, which selectively inhibits the kinase activity of phosphorylated P38 in macrophages (Karahashi, Nagata, Ishii, & Amano, 2000). The Western analysis data showed that the treatment with MAPK inhibitor reversed the suppressive effect of Aza+TSA in LPS-induced p-38 activation in BMDMs (Fig 4D). Our finding suggests that treatment with Aza+TSA acts via the P38 MAPK pathway by binding to the phosphorylated sites of P38. Therefore, when the selective inhibitor (SB202190) was used together with Aza+TSA, there was higher phosphorylation of P38 (Fig. 4D in
than when Aza+TSA was used alone (Fig. 4D, lane 7). Overall, these data suggest that LPS increases TNFα secretion, inducing BMDMs towards apoptosis, whereas treatment with Aza+TSA suppresses LPS-induced TNFα stabilizing protein HuR and p38 MAPK activation, indicating its role in protection from endotoxemia-induced injury.

**Aza+TSA treatment activates STAT3-Bcl2 signaling in LPS-induced BMDMs**

Recently, STAT3 has been shown to be necessary to control systemic inflammation (Jacoby et al., 2003; Sander et al., 2010). A previous study has also shown that the Bcl2 over-expression provides this protection in septic mice (A. Iwata et al., 2003). We hypothesized that the Aza+TSA-mediated survival of macrophages during ALI is essentially through the activation of STAT3 and Bcl2. To evaluate the role of the STAT3-Bcl2 cascade during Aza+TSA-mediated protective anti-inflammatory mechanisms, we performed Western analysis for active STAT3 and Bcl2 proteins. For this experiment, the BMDMs were cultured in the presence and absence of LPS and treated with Aza+TSA for 30 minutes or 24 hours. The data showed that the LPS-stimulated BMDMs treated with Aza+TSA increased phosphorylation of STAT3 compared with the LPS-stimulated BMDMs treated for 30 minutes with either Aza or TSA alone (Fig. 5A). Likewise, the expression of Bcl2 protein also significantly increased in LPS-challenged BMDMs treated with Aza+TSA as compared to the BMDMs treated either with Aza or TSA alone for 24 hours (Fig. 5B). This data suggest that the stimulation of STAT3-Bcl2 signaling pathway might be responsible for the protective effect in LPS-induced mice treated with Aza+TSA.
Acetylation of STAT3 promoter region histone 3 in LPS-challenged BMDMs treated with Aza+TSA

It has been revealed that the epigenetic modifications that led to posttranscriptional changes in gene suppression or activation are reliant on the combination of either DNA methylation/histone deacetylation or DNA demethylation/histone acetylation, respectively (Trojer & Reinberg, 2006). In this current study, we evaluated the epigenetic modification in the STAT3 promoter region after treatment with Aza+TSA in LPS-induced BMDMs. For this experiment, the BMDMs were cultured with LPS or without LPS and treated for 24 hours with the combination of Aza+TSA or with Aza alone or TSA alone. The Western analysis data showed that the expression of acetylated H3 lysine9 (aceH3K9) protein was significantly increased in BMDMs treated either with Aza alone or with TSA alone or in combination of Aza+TSA when compared to untreated controls (Fig. 5C). Our ChIP data show that the STAT3 promoter was highly acetylated at H3 (aceH3) in LPS-challenged BMDMs treated with Aza+TSA compared with untreated BMDMs (Fig. 5D), suggesting the euchromatin state of STAT3.

Aza+TSA treated mice show decreased myeloperoxidase (MPO) activity and circulatory proinflammatory cytokines in ALI

In our earlier report, ALI was induced in C57BL/6J mice by intraperitoneal injection of LPS (40 μg/g BW). One hour after LPS administration, the mice were treated intraperitoneally either with vehicle or with TSA or Aza alone, or in combination of Aza+TSA. The mice post-treated with TSA alone had a 80% mortality rate, whereas those treated with Aza+TSA displayed a strikingly lower mortality rate of 20%. The
mice treated with Aza+TSA also had reduced neutrophils in the airspace and prolonged survival time during the observed period of two weeks (Thangavel et al., 2014). On day two, the H&E staining of lung tissues showed consistently lesser recruitment of LPS-induced neutrophils into the airspace in mice treated with Aza+TSA compared with untreated mice (Fig. 6A). We also observed reduction in lung polymorphic nuclear neutrophils (PMN) in bronchoalveolar lavage fluid (BALF) from the Aza+TSA treatment group compared with untreated ALI mice and ALI mice treated with either of the drugs alone (Fig. 6B). Measurement of lung myeloperoxidase (MPO) content is a well-established parameter for studying neutrophil sequestration in the lungs. Samples of lung for MPO analysis were frozen in liquid nitrogen immediately after removal from the mouse. Tissue MPO levels were determined as described earlier [49]. The LPS-induced MPO activity was significantly reduced in mice treated with Aza+TSA compared with untreated mice (Fig. 6C, p<0.01). The release of blood proinflammatory cytokines in LPS-challenged mice treated with Aza+TSA was measured by ELISA. We observed lesser levels of proinflammatory cytokines IL1β (Fig. 6D), TNFα (Fig. 6E), and IL-6 (Fig. 6F) and greater levels of anti-inflammatory cytokine IL-10 (Fig. 6G) in the blood of LPS-induced mice treated with Aza+TSA as compared with untreated ALI mice and ALI mice treated with Aza or TSA alone.

**Aza+TSA-treated ALI mice show increased M2 macrophages in lung tissues**

Immunofluorescence staining was performed on the day-two lung tissue section to determine the immune-plasticity of macrophages that are present in the LPS-challenged ALI mice after treatment with Aza+TSA. The lung tissue sections were stained with macrophage markers CD68 (pan marker-green) and Arg1 (M2 specific marker-red). The macrophages, which expressed CD68, were green and the
macrophages, which express Arg1 were red in color. Our immunofluorescence images showed fewer number of CD68+ and Arg1+ double positive cells (M2 subtype, represents in yellow) in the LPS-induced mice lung tissue, and this was significantly increased in the ALI mice treated with Aza+TSA than in untreated mice and mice treated with Aza alone and TSA alone (Fig. 7A, B). These in vivo data indicate that the Aza+TSA treatment modulates the lung macrophages towards an anti-inflammatory M2 subtype.

**Aza+TSA-treated ALI mice show decreased M1 macrophages in lung tissues**

As with Arg1, we performed immunofluorescence staining of day-two lung tissues for the macrophage markers CD68 (green) and NOS2 (M1 specific marker-red). Our immunofluorescence staining data showed that Aza+TSA treatment consistently decreased LPS-induced co-localization of M1 macrophages (yellow). Also, CD68 plus NOS double positive cells (yellow fluorescence in the merged image) were observed less in ALI mice treated with Aza+TSA than in untreated mice and in mice treated with either Aza alone or TSA alone (Fig. 8A, B). These in vivo data indicated that the Aza+TSA treatment decreases the LPS-induced inflammatory M1 subtype in the lung tissue.
DISCUSSION

Sepsis-induced acute lung injury (ALI) is an inflammatory disorder with limited treatment options that affects millions of people worldwide (Nemeth, Mayer, & Mezey, 2010; Wheeler & Bernard, 2007). A curative treatment regime is desperately needed. The available studies on epigenetic modifiers in several animal models have shown varying results. Effects of epigenetic modifiers have ranged from beneficial to none in attenuating inflammatory cytokines and chemokines as well as inflammatory injury to the airway, digestive tract, and joints (Choi et al., 2008; K. Iwata et al., 2002; Rahman, 2002; Zhang, Jin, Wang, Jiang, & Wan, 2010). The variability in results may be because previous studies have focused on the effects of pretreatment with HDAC inhibitors rather than studying the therapeutic potential. To best of our knowledge, this is the first study to examine the efficacy of a combinatory post-treatment with Aza+TSA in inhibiting inflammation and apoptosis and modulating macrophage phenotype during sepsis. Furthermore, our study addresses the incompletely understood molecular mechanism of these combined approaches that regulate inflammatory response, cell proliferation, and the survival of mice following inflammation.

High concentrations of chemotherapeutic agents such as Aza and TSA induce apoptosis in already transformed cells (Kaminskas et al., 2005; Sassi et al., 2014). Our observations indicated that treatment with nanomolar concentration of Aza and TSA had no apoptotic effect on cell viability, and rather both agents enhanced cell proliferation. We understand there is some concern about using BMDMs to characterize these mechanisms because of a recent study showing that during
perinatal development, the alveolar macrophages do not originate from blood monocytes but from fetal monocytes (Schneider et al., 2014). This study mainly focuses on alveolar macrophages during normal development rather than on a diseased model. However, during ALI, the residential alveolar macrophages are not enough to combat inflammation. Therefore, there would be a mobilization of monocytes/macrophages from bone marrow under the influence of colony stimulating factor (CSF) to the injured alveolar site to compensate for the requirements of macrophages (O'Dea et al., 2009). Thus, studying the mechanism of BMDMs in a mouse model of ALI is relevant. Our data also showed that treatment with Aza+TSA significantly reduced LPS-induced apoptosis of BMDMs and improved cell survival. Other studies showed that macrophages actively participate in the production of pro-inflammatory and inflammatory factors in response to LPS stimulation (Choi et al., 2008; K. Iwata et al., 2002; Nicodeme et al., 2010; Rahman, 2002; Zhang et al., 2010). A recent study has shown the utility of synthetic histone mimics in abrogating the expression of key LPS-inducible cytokines and chemokines, and modulating innate immunological responses (Nicodeme et al., 2010). Our data suggest that combinatorial Aza+TSA therapy in nanomolar concentrations is more efficacious than therapy with either drug alone in preventing the release of LPS-induced chemokines and cytokines from macrophages. Thus, Aza+TSA might be considered as a putative anti-inflammatory drug, which modulates LPS-stimulated inflammatory responses and activates an array of key anti-inflammatory signaling pathways. Recent data indicate that members of the MAPK family are involved in modulating mRNA stability/translation of several genes (Kochanek et al., 2012; Rajasingh et al., 2006). Our data showed that treatment with Aza+TSA abrogated the LPS-TLR4-
MAPK pathway and initiated an inflammatory cascade, which is known to potentiate apoptotic signals within the macrophages. This event was mediated primarily by a reduction in activated phosphorylated p38 MAPK as well as lowering the levels of TNFα might be through the HuR binding of TNFα mRNA (Rajasingh et al., 2006). Thus LPS-challenged BMDMs treated with Aza+TSA effectively promote a more cytoprotective outcome than LPS-challenged BMDMs treated with either of these drugs alone. Furthermore, the Aza+TSA-mediated inhibitory effect was reversed by a MAPK inhibitor, further demonstrating the central role of the MAPK pathway in augmenting a pro-apoptotic signal and in suggesting a putative drug target. This finding further confirms the efficiency of a combinatory treatment modality with distinct mechanistic effects at the level of the inflammatory RNA transcripts.

Studies have shown that the increased p38 MAPK activation and reduced STAT3 phosphorylation are the two important pathways that play a role in apoptosis during inflammation in macrophages and endothelial cells (Rajasingh et al., 2006; Y. Y. Wang et al., 2005). In our present data, the BMDMs exposed to LPS showed a significant increase in activated p38 MAPK and reduced phosphorylated STAT3, which was ameliorated by treatment with Aza+TSA. These data indicate the dual protective mechanisms that were attributed to combinatorial Aza+TSA treatment.

Macrophages maintain organ homeostasis and retain a functional dichotomy because they exist in a dynamic microenvironment. Lung alveolar macrophages (K. L. Lin, Suzuki, Nakano, Ramsburg, & Gunn, 2008) play a pivotal role in the pathogenesis of ALI, and the M1 and M2 plasticity takes place because of the bacterial products, which in turn result in an inflamed lung. M2 macrophages are involved in tissue repair and remodeling (Ishii et al., 2009; Wynn, 2004). The genes expressed by the macrophages can be phenotypically characterized by the M1 and
M2 macrophages. To understand the molecular changes that occur during Aza+TSA treatment, we characterized the M1 and M2 macrophages in LPS-induced BMDMs and found significant reduction in M1 markers CD40, CD14, and NOS2 and increased expression of M2 surface markers CD23, CD124, and CD206 in the treated versus untreated cells. These findings further support the previous observations of a distinct epigenetic reprogramming that is switched on in favor of an anti-inflammatory subtype of M2 macrophages, which may prove beneficial in arresting the inflammatory cytokine response in sepsis (Kambara et al., 2015). Future experiments are required to define the molecular mechanism of the M1 and M2 plasticity of macrophages upon treatment with Aza+TSA.

In vivo experiments on a general HDAC inhibitor, TSA have shown its role in anti-inflammatory activities via the suppression of cytokines and reactive oxidative species (Blanchard & Chipoy, 2005), and attenuating macrophage infiltration (Hsing et al., 2012). In contrast, HDAC inhibitors have shown enhanced IL-8 production in lung epithelial cells stimulated by LPS (K. Iwata et al., 2002) or TNFα (Rahman, 2002). This discrepancy can be attributed to the presence of cell-specific HDAC isoforms and more importantly to the cell type, as alveolar macrophages and neutrophils are the primary initiators of a pro-inflammatory milieu in ALI. Hence one may speculate that other epigenetic modifications that must be targeted, such as DNA methylation orchestrating the expression of inflammatory genes. We have already shown in an LPS-induced sepsis mouse model that post-treatment of mice with a single dose of Aza+TSA prevents lung injury and affords a significantly reduced mortality rate (20%) than no treatment (100%) or treatment with either Aza or TSA alone (80%) (Thangavel et al., 2014). Our present data show that these protective effects are mediated by a significant attenuation of adverse lung
histopathological changes, and inflammation. Moreover, the in vitro data have shown that Aza+TSA treatment significantly suppresses LPS-induced inflammation and the reprogramming of macrophages towards an anti-inflammatory M2 subtype. These effects above were attributed to the dual effects of Aza+TSA mediated by inhibition of TNFα mRNA-stabilizing protein HuR and activated p38 MAPK, as well as to the activation of STAT3-Bcl2 pathways.

Unlike in previous studies where pre-treatment with a single agent such as TSA has shown limited efficacy with a marginal benefit towards mortality outcomes, a combinatory regimen of Aza+TSA targeting dual-epigenetic modifications with a multi-targeted downstream effect provides a higher and longer survival rate in-vivo on follow-up. This knowledge might further facilitate repurposing clinically available epigenetic cancer drugs for studies related to sepsis and aid in formulating novel therapeutic approaches to prevent and reverse the persistent lung injury. Even though many animal models are present, the LPS-injection model is a well-established animal model of sepsis and an appropriate one for this study. Therefore, this study further support and concludes that repurposing combination therapy with Aza+TSA is safe in sepsis that could improve and prolong the lives of millions of people.
MATERIALS AND METHODS

Antibodies and reagents

We used antibodies for phospho p38-MAPK, total P38-MAPK, p-STAT3, total-STAT3, AceH3K9, total-H3, aceH3 (Cell Signaling Technology), β-actin, HuR, and Bcl2 (Santa Cruz Biotechnology, Inc.). Secondary Abs were HRP-conjugated donkey anti–mouse, anti–rabbit, and anti–goat (Santa Cruz Biotechnology, Inc); TRITC-, FITC-, and Cy-5-conjugated donkey anti–mouse, anti–goat, and anti–rabbit (Jackson ImmunoResearch Laboratories, Inc.), IRDye680 conjugated anti-mouse IgG and IRDye680 conjugated anti-goat IgG, IRDye800 conjugated anti-rabbit IgG and IRDye800 conjugated anti-goat IgG (Rockland, PA), and DAPI (Life-Tech); Antibodies CD11b, CD14, CD23, CD124, CD40, CD206, NOS2 (e-Bioscience) Annexin V, MTT assay (Cell Proliferation assay kit, Promega, USA), inflammatory PCR array kit, ELISA kits (Millipore), and MAPK-inhibitor SB 203580 (Calbiochem).

Mice

C57 black 6 (C57BL6) male mice were obtained from Jackson Laboratory. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD) and were approved by the institutional animal care and use committees of the University of Kansas Medical Center and the University of Illinois at Chicago. All experiments were performed on 8- to 10-week-old mice.

Primary mouse bone marrow-derived macrophages (BMDMs) culture

BMDM culture was performed in C57BL6 mice as described earlier (Beardmore et al., 2005). Briefly, bone marrow derived mononuclear cells isolated from the tibias
and femurs of mice were cultured in supplemented 10% FBS/DMEM medium along with 10% L929 cell conditioned medium on 10 cm cell culture dishes. After 7 days of culture, the cells were stained with CD11b and analyzed by flow cytometry. A phase contrast microscopic image was taken to show the uniform morphology.

**Cell viability and proliferation assays**

The in vitro viability and proliferation of the Aza+TSA treated primary mouse BMDMs (Fig. S1) was evaluated by Trypan-blue exclusion assays and 3,4,5-(dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) as described by us earlier (Rajasingh et al., 2011; Thangavel et al., 2014). Briefly, 10^4 BMDMs were cultured in 96-well culture plate (200 μl/well) in DMEM containing 10% FBS in the presence and absence of various concentrations of Aza+TSA for 48 hours at 37°C at 5% CO_2. The cells were manually counted by a hemocytometer under the phase-contrast microscope. Effects of Aza+TSA on BMDM proliferation were determined by 3, 4, 5-(dimethylthiazol-2-yl) 2, 5- diphenyl tetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Cell Proliferation Assay kit, Promega).

**Flow cytometer analysis**

Cells from a six-well plate were harvested and washed twice in phosphate-buffered saline (PBS), counted, and resuspended in FACS buffer (1% BSA in PBS containing 0.01% NaN3). For flow cytometer phenotypic analysis, cells (1 x 10^6 cells/stain) were initially incubated with 10% mouse serum for 20 minutes at 4°C. Subsequently, cells were incubated with the appropriately labeled primary antibodies for 1 hr. Then the cells were washed with washing buffer three times and incubated 20 minutes in an appropriate secondary antibody. All incubations were performed on ice. Appropriate isotype controls were used in all cases. Finally, the cells were washed three times
with FACS buffer, resuspended in 0.5 ml PBS, and analyzed by flow cytometer (FACSCalibur, BD Biosciences) using Cell Quest software. Data were analyzed by using FlowJo software (Tree Star, Ashland, OR). Anti-CD11b was used for characterizing macrophages, anti-CD23 and anti-CD124 were used for quantifying M2, and anti-CD14 and anti-CD40 were used for quantifying M1 subtype.

**Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)-array**

We performed PCR array for inflammatory cytokines in LPS-induced macrophages treated with Aza and TSA using Mouse Inflammatory Response and Autoimmunity PCR array kit (SA Bioscience, USA) as described by the manufacturer’s instructions as well as a previous publication (Pal et al., 2012). Briefly, first-strand cDNA was synthesized from 100ng of RNA using the RT2 First Strand Kit. A total volume of 25 µl of PCR reaction mixture, which included 12.5 µl of RT2 Real-Time SYBR Green/ROX PCR master mix, 11.5 µl of nuclease-free water, and 1 µl of template cDNA, was loaded in each well of the RT2 Profiler PCR array. PCR amplification was performed in an ABI ViiA7 real-time PCR machine (Applied Biosystems). Data were imported into RT2 Profiler PCR array data analysis, version 3.5 to detect the alterations of gene expression. Ct values were normalized to housekeeping genes. Results were expressed as fold change in expression, and values were calculated as a ratio of induced expression-to-control expression.
Quantitative RT-PCR

The LPS-induced cells treated and untreated with Aza+TSA from a six-well plate were washed once in PBS and harvested for qRT-PCR analysis as described by us earlier (Rajasingh et al., 2008; Rajasingh et al., 2011). Briefly, total cellular RNA was obtained for qRT-PCR analysis to determine mRNA expression of mouse cytokine inflammatory markers TNFα, IL1β, IL6, IL10, and IL10R. The relative mRNA expression of target genes was normalized to endogenous 18S control gene (Applied Biosystems). The primers are summarized in online Table 1. Results were expressed as fold change in expression, and values were calculated as ratio of induced expression-to-control expression.

Western blot analysis

Western blot analysis of p-P38 MAPK, HuR, p-STAT3, and Bcl2 proteins was performed as described earlier (Thangavel et al., 2014). BMDM lysates were resolved by SDS-PAGE on a 4–12% gradient or 10% separating gel under reducing conditions and transferred to Duralose membrane. Membranes were blocked with 5% dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 for 1 h. Membranes were incubated with indicated primary antibody (diluted in blocking buffer) overnight. All primary antibodies were used at the dilution of 1:1000. Following three washes, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or with mouse antibody or donkey antibody. Protein bands for STAT3, Bcl2, and AceH3K9 were detected by the enhanced chemiluminescence method. All the other proteins (NOS2, CD206, p-P38, and HuR) were detected by a LiCor Odyssey scanner system. Band intensities were quantified
using ImageJ (National Institutes of Health). The ratio of band intensity was calculated by using the value of band intensity of experimental protein divided by its respective loading control. Data are expressed as means ± SEM of three replicated experiments.

**Enzyme-Linked Immunosorbent Assay**

Enzyme-linked immunosorbent assays (ELISA) were performed on supernatants of lung homogenates and cell culture supernatants using kits for mouse-specific TNFα, IL1β, IL6, IL10, and MPO (ELISA Kit, Millipore), according to the manufacturer’s instructions.

**Immunofluorescence staining**

Protein expression was evaluated by immunofluorescence staining as described by us earlier (Rajasingh et al., 2008; Rajasingh et al., 2011). Briefly, cultured cells or lung tissue was rinsed once with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma) in PBS for 30 minutes, then rinsed three times with PBS, permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 5 minutes, washed twice with PBS, and incubated overnight at 4°C with primary antibodies (against Arginase 1, NOS2) diluted with 1% FBS in PBS. After 3 washes with PBS, cells were incubated with specific secondary antibodies for 1 hour at 37°C, and cells were rinsed three times with PBS, stained with DAPI to visualize cell nuclei, rinsed 3 times with PBS, dried, and mounted in Vectashield mounting medium for fluorescent imaging. All immunofluorescence staining was photographed using either confocal and immunofluorescence microscope.
Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays for the STAT3 promoter region in LPS-induced BMDMs treated or untreated with Aza+TSA were performed according to the manufacturer’s protocol (Millipore, USA), also described in our recent publication.(Rajasingh et al., 2008; Rajasingh et al., 2011) Briefly, approximately 5 × 10^6 cells in two-10 cm culture plates for each sample were incubated with 1% formaldehyde diluted in culture medium for 10 min at 37°C. The samples were then lysed in SDS lysis buffer containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Next, the samples were sonicated on ice to shear DNA to lengths between 200 and 1000 bp and incubated with primary antibodies to acetylated histone 3 (aceH3) overnight at 4°C on a shaker plate. Then samples were incubated with Salmon Sperm DNA/Protein A agarose slurry for 1 h at 4°C on a shaker plate. After 4 washes with washing buffers, the pelleted protein A agarose/antibody/histone complex was incubated with elution buffer (1% SDS and 0.1 M NaHCO₃) for 15 min at room temperature. Then the samples were incubated with 0.2 M NaCl for 4 h at 65°C. After DNA was recovered by phenol/chloroform extraction and ethanol precipitation, PCR was performed using STAT3 promoter forward 5′-GGTGACACCTGGGGACCGCCTAAG-3′ and reverse 5′-AAAAACGCCTCTAGGAGAGAAGGCG-3′ primers from the region of -140 to -470. Aliquots of samples before immunoprecipitation (input) were analyzed by PCR to quantify the amount of DNA present in different samples. The relative DNA levels in each sample were normalized to its input DNA levels (Rajasingh et al., 2008; Rajasingh et al., 2011).
**Induction of acute lung injury (ALI)**

Acute lung injury was induced in C57BL/6J mice by intraperitoneal injection of LPS (40 μg/g BW). One hour after LPS administration, the mice received vehicle or TSA (1 μg/g) or Aza (1 μg/g) or Aza+TSA. The histological changes and gene expression were evaluated in the lung.

**Acknowledgments**

The electron microscopy research laboratory of the Kansas University Medical Center was used for electron microscopy analyses.

**Author Contributions:** J.T. conception and design, collection and/or assembly of data, data analysis and interpretation; S.S. collection and/or assembly of data, manuscript writing; S.R. collection and/or assembly of data; Y.T.X. conception and design; B.D. financial support, administrative support, manuscript writing; J.R. conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing.

**Competing Interests**

The authors declare no competing or financial interests.

**Funding**

This work was supported, in part, by American Heart Association Grant - Jon Holden DeHaan Foundation 10SDG2630181 and National Institutes of Health (NIH) grant R21HL97349 (to JR) and R01HL117730 (to BD).
Footnote
The work presented here was partly done while the investigators JR and JT were at the Department of Pharmacology, University of Illinois at Chicago.

Supplementary materials
Supplementary material available online
Figure 1
Effects of treatment with Aza+TSA on BMDM viability, proliferation, and anti-apoptosis. A: Trypan blue exclusion assay shows that treatment with up to 50 nM Aza and 25 nM TSA had no significant effect on cell viability. *p< 0.05 vs. control. B: The MTT assay shows normal ability to proliferate. *p< 0.05 vs. control. C: The anti-apoptotic effects of Aza+TSA on LPS-induced BMDMs. BMDMs were treated with Aza+TSA in the presence and absence of LPS (1µg/ml), followed by staining with propidium iodide (PI), and Annexin V conjugated to green-fluorescent FITC dye. After staining with both probes, the apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence as analyzed by flow cytometry. D: Graphic representation of flow cytometry (11.5%...
LPS vs. 56% LPS+Aza+TSA treatment, †p< 0.01); After LPS treatment, apoptosis increased to 88.5%, whereas it was significantly reduced to 44% after treatment with Aza+TSA (LPS vs. LPS+Aza+TSA treatment, †p< 0.01). Numbers within the parentheses indicate the concentration in nM. Each bar represents mean ± SEM of three triplicate experiments. A, Aza; T, TSA.
Figure 2
The immunomodulatory effect of Aza+TSA on LPS-induced BMDMs. The BMDMs were cultured in the presence and absence of LPS (1µg/ml), treated for 24 h with Aza alone, TSA alone, or Aza+TSA, and analyzed for mRNA expression. A: RT-PCR array data show that the LPS-induced mRNA expression of pro-inflammatory chemokines (ccl2, ccl3, ccl4, ccl5, ccl7, CD40, cxcl10, and cxcl12), †p< 0.01 LPS vs. LPS+Aza+TSA and B: cytokines (IL1-α, IL1β, IL1R, IL6, IL18, ITGβ2, lymphotoxin-β, and TNFα) were significantly reduced by treatment with Aza+TSA. Each bar in A and B represents mean ± SEM of two duplicate experiments. †p< 0.01 LPS vs. LPS+Aza+TSA. The PCR-array results are further validated by qRT-PCR. C: The qRT-PCR data show that Aza+TSA treatment significantly reduced the TNFα, IL1β,
and IL6 gene expression compared with no treatment or treatment with Aza alone or TSA alone in BMDMs challenged with LPS, *p < 0.5 LPS vs. LPS+TSA; †p< 0.01 LPS vs. LPS+Aza+TSA. D: The fold mRNA gene expression of IL10 and IL10R in LPS-induced BMDMs treated with Aza+TSA compared with either alone. *p<0.5 LPS vs. LPS+TSA; *p<0.5 TSA vs. LPS+Aza+TSA; †p<0.01 LPS vs. LPS+Aza+TSA. A, Aza; T, TSA; L, LPS. Each bar in C and D represents mean ± SEM of three triplicate experiments.
Figure 3

Aza+TSA treatment promotes more anti-inflammatory M2 macrophages. A: The flow cytometry data show significantly reduced expression of M1 markers CD14 and CD40 in LPS-induced BMDMs treated with Aza+TSA compared with untreated LPS-stimulated cells and control cells. B: The flow cytometry data show significantly increased expression of M2 markers CD23 and CD124 in LPS-induced BMDMs treated with Aza+TSA compared with untreated BMDMs or BMDMs treated with Aza alone or TSA alone. C: Quantification of flow cytometry data from A and B. *p<0.5 LPS vs. LPS+TSA, LPS vs. LPS+Aza+TSA, and LPS+TSA vs. LPS+Aza+TSA; †p<0.01 LPS vs. LPS+Aza+TSA and ‡p<0.01 LPS vs. Aza+TSA. D: Western analysis
data showed that the LPS-challenged BMDMs treated with Aza+TSA have lesser M1 macrophage protein NOS2 expression than the cells treated with Aza alone and TSA alone. E: The same NOS2 blot reprobed with M2 marker CD206 showed higher protein expression in Aza+TSA treated cells than in untreated cells and cells treated with Aza or TSA alone. A representative Western blot is shown. β-actin was used as a loading control. Bands intensities were quantified using ImageJ. The ratio of band intensity was calculated by using the value of band intensity of experimental protein divided by its respective loading control. Data are expressed as means ± SEM of three replicated experiments (C, D and E). *p < 0.5 LPS vs. LPS+TSA; †p< 0.01 LPS vs. LPS+Aza+TSA. A, Aza; T, TSA; L, LPS.
Figure 4

Aza+TSA treatment suppresses HuR expression and p38 MAPK activation. A: ELISA data showed that the LPS-induced BMDMs treated with Aza+TSA had decreased TNFα. *p < 0.5 LPS vs. TSA and TSA vs. LPS+Aza+TSA; †p < 0.01 LPS vs. LPS+Aza+TSA. B: The Western analysis showed a significant decrease in HuR protein in LPS-induced BMDMs treated with Aza+TSA compared with the untreated LPS control. *p < 0.5 LPS vs. TSA; †p < 0.01 LPS vs. LPS+Aza+TSA. C: The Western analysis data showed an increased activation of p38 in LPS-induced BMDMs, which was significantly inhibited by Aza+TSA treatment compared with treatment with Aza or TSA alone in 30 minutes of cell culture *p < 0.5 LPS vs. LPS+Aza+TSA; †p < 0.01 LPS+Aza vs. LPS+Aza+TSA, and LPS+TSA vs. LPS+Aza+TSA. D: Treatment with
MAPK inhibitor (SB 202190) reverses the suppressive effect of Aza+TSA in LPS-induced p-38 activation in BMDMs. \textsuperscript{†}p < 0.01 LPS vs. LPS+Aza+TSA and LPS+Aza+TSA vs. LPS+Aza+TSA+ SB202190. A representative Western blot is shown. Total p38 and \( \beta \)-actin were used as a loading control. Bands intensities were quantified using ImageJ. The ratio of band intensity was calculated by using the value of band intensity of experimental protein divided by its respective loading control. Data are expressed as means ± SEM of three replicated experiments.
Figure 5

Aza+TSA treatment targets STAT3-Bcl2 signaling in BMDMs. Western analysis was performed on BMDMs cultured in the presence and absence of LPS and treated with Aza+TSA for 30 min. A: The LPS-challenged BMDMs treated with Aza+TSA showed higher phosphorylation of STAT3 than in LPS-challenged BMDMs treated with either Aza or TSA alone. *p<0.5 LPS vs. LPS+TSA; †p<0.01 LPS vs. LPS+Aza+TSA. B: The expression of Bcl2 protein also significantly increased in LPS-challenged BMDMs treated with Aza+TSA compared with cells treated with either Aza alone or TSA alone. *p<0.05 LPS vs. LPS+Aza; †p<0.01 LPS vs. LPS+Aza+TSA. C: The Western
analysis data showed that LPS-challenged BMDMs treated with Aza+TSA were highly acetylated compared with untreated BMDMs or BMDMs treated with either Aza alone or TSA alone for 24 hours. *p<0.05 LPS vs. LPS+Aza and LPS+TSA; †p<0.01 LPS vs. LPS+Aza+TSA; D: The ChIP data show that H3 in the STAT3 promoter region was highly acetylated in LPS-challenged BMDMs compared with untreated BMDMs. Total STAT3, H3, and β-actin were used as a loading control. Bands intensities were quantified using ImageJ. The ratio of band intensity was calculated by using the value of band intensity of experimental protein divided by its respective loading control. Data are expressed as means ± SEM of three replicated experiments.
Figure 6

Aza+TSA treated mice show decreased myeloperoxidase (MPO) activity and circulatory proinflammatory cytokines in ALI. The mice challenged with LPS (40 μg/g BW) and treated with Aza alone (1 μg/g BW) or TSA alone (1 μg/g BW) or treated with the combination of A+T. All mice were observed for two weeks (n=5 mice/group). A: Representative microscopic H&E staining images of lung sections exposed to LPS with or without treatment. Magnification is 600X. (B) Aza+TSA treatment decreases the lung PMN in BALF; *p<0.05 LPS vs. LPS+TSA; †p<0.01 LPS vs. LPS+Aza+TSA. (C) The lung tissue neutrophil sequestration was measured by MPO activity. Aza+TSA treatment reduces the lung MPO activity; *p<0.05 LPS vs.
LPS+TSA; *p<0.05 LPS vs. LPS+Aza+TSA. (D) Blood levels of cytokines IL1β, *p<0.05 LPS vs. LPS+TSA; †p<0.01 LPS vs. LPS+Aza+TSA, (E) TNF-α, *p<0.05 LPS vs. LPS+TSA; †p<0.01 LPS vs. LPS+Aza+TSA, (F) IL-6, †p<0.01 LPS vs. LPS+Aza+TSA and (G) IL-10 †p<0.01 LPS vs. LPS+Aza+TSA. Each bar represents the mean ± SEM of three replicated experiments. A, Aza; T, TSA; L, LPS.
Figure 7

Treatment with Aza+TSA shows increased M2 macrophages in ALI mice lung tissues. A: Immunofluorescence staining of macrophage markers CD68 (pan marker-green) and Arg1 (M2 specific marker-red). The data showed that Aza+TSA treatment consistently increased LPS-induced co-localization of M2 macrophages (yellow). B: Quantification of CD68/Arg1 double positive cells in the lung tissue. †p< 0.01 LPS vs. LPS+Aza+TSA. A, Aza; T, TSA; L, LPS.
Figure 8

Treatment with Aza+TSA shows decreased M1 macrophages in ALI mice lung tissues. A: Immunofluorescence staining of macrophage markers CD68 (pan marker-green) and NOS2 (M1 specific marker-red). Our data showed that treatment with Aza+TSA consistently decreased LPS-induced co-localization of M2 macrophages (yellow). B: Quantification of CD68/NOS2 double positive cells in the lung tissue. †p< 0.01 LPS vs. LPS+Aza+TSA. A, Aza; T, TSA; L, LPS.
References


Jacoby, J. J., Kalinowski, A., Liu, M. G., Zhang, S. S., Gao, Q., Chai, G. X., Ji, L.,


treatment attenuates MAP kinase pathway activation and pulmonary inflammation following hemorrhagic shock in a rodent model. *J Surg Res, 176*(1), 185-194. doi: 10.1016/j.jss.2011.06.007


anatomical ischemic tissue repair. Circ Res, 102(11), e107-117. doi: 10.1161/circresaha.108.176115


protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J, 18*(18), 4969-4980. doi: 10.1093/emboj/18.18.4969
