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

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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 methyl transferase (DNMT) inhibitor 5-Aza 2-deoxycytidine (Aza), the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), as well as the combination therapy of Aza and TSA (Aza+TSA) provides in the protection of ALI. In LPS-induced mouse ALI, post-treatment with a single dose of Aza+TSA showed substantial attenuation of adverse lung histopathological changes and inflammation. Importantly, these protective effects were due to substantial 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, and has a therapeutic potential for patients with sepsis.

KEY WORDS: Epigenetic modifiers, Acute lung injury, Histone deacetylase inhibitor, Sepsis, Inflammation and apoptosis

INTRODUCTION
In patients suffering sepsis, 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 et al., 2010). Lipopolysaccharides (LPSs) are bacterial cell wall toxins that are contributing agents implicated in ALI and the disease pathogenesis (Dellinger et al., 2008). Furthermore, the trigger of inflammatory gene expression is the direct contact of pathogens with cells of the immune system. This interaction is necessary for immune defense and is often harmful because of 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 et al., 2003; Jenuwein and Allis, 2001). We selected 5-Aza 2-deoxycytidine (Aza), an inhibitor of DNA methyl transferase (DNMT), and Trichostatin A (TSA), an inhibitor of histone deacetylases (HDACs), because of their well-established biological activities, and known safety and side effect profiles (Avila et al., 2007; Kaminskas et al., 2005). Aza is an effective drug for acute myelogenous leukemia and has the potential to be used in the treatment of other diseases (Christman, 2002; Kaminskas et al., 2005). TSA, a potent general inhibitor for HDACs, chelates the central zinc finger motif of HDACs, thereby inhibiting enzyme activity (Furumai et al., 2011; Suzuki and Miyata, 2005). It has been shown that TSA improved survival in a rat model of hemorrhagic shock (Lin et al., 2007). Recently, we have shown that combinatorial therapy of Aza and TSA reduces mortality and improves integrity of lung vascular cell in LPS-induced mouse ALI (Thangavel et al., 2014).

It is known that LPSs interact with the cell-surface protein Toll-like receptor 4 (TLR4) and that it is in contact with the intracellular connector protein MyD88 (Takeda et al., 2003). This LPS–TLR4–MyD88 signaling complex further triggers two important signaling cascades, the NF-κB as well as the mitogen-activated protein kinase (MAPK) pathways, both of which direct pro-inflammatory and anti-inflammatory responses. The MAPK pathway has a crucial role in innate immune response signaling (Dong et al., 2001, 2002). The three major families of MAPK pathway, which mediate innate immune response signaling, include ERK1 and ERK2 (also known as MAPK3 and MAPK1, respectively), p38MAPK (whose isoforms MAPK14, MAPK11, MAPK12 and MAPK13) and JNK (Blander and Medzhitov, 2004; Chang and Karin, 2001; Davis, 2000). A more recent 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 p38MAPK, 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; 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...
induce a protective mechanism through p38MAPK–HuR–TNFα and STAT3–Bcl2 signaling pathways during inflammatory and anti-inflammatory responses, respectively. These responses are currently not well understood. The challenge remains to identify the critical role of the epigenetic modifiers Aza+TSA (and the involvement of macrophages) in reducing inflammation related to pathologies during ALI. However, no publication has yet examined the efficacy of a nanomolar concentration of Aza+TSA in modulating cytokines and macrophages to quench inflammation and prevent mortality during sepsis. We chose to study lung injuries and inflammation because the lung is a main target for acute and chronic infections (Song et al., 2001).

The intent of this study was to identify the key modes of action in combinatorial treatment of the above mentioned 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 in mouse (Thangavel et al., 2014). Our current study extends our earlier study, by addressing the possibility that Aza+TSA-induced protection is 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 treatment for patients with sepsis and ALI.

RESULTS

Aza+TSA treatment does not affect cell viability

Previously, we examined the toxicity of Aza and TSA in mouse primary lung endothelial cells and identified that the combination of Aza+TSA at 50 nM Aza + 25 nM TSA, had no toxic effect and did not change cell viability (Thangavel et al., 2014). To identify a safe dosis of epigenetic modifiers Aza and TSA in mouse primary bone marrow-derived macrophages (BMDMs), we performed in vitro Trypan Blue dye exclusion viability assays and MTT assays (Promega, USA). For this, we cultured BMDMs in 12-well sterile tissue culture plates (supplementary material Fig. S1) and treated the cells with four different concentration combinations of Aza+TSA (10 nM+5 nM, 25 nM+12.5 nM, 50 nM+25 nM, and 100 nM+50 nM) for 48h and 72 h. After the treatments, the BMDMs were collected and subjected to 0.1% solution of Trypan Blue. Then, using a hemocytometer, the numbers of unstained viable and stained non-viable cells were counted; the data show that when BMDMs were treated with Aza+TSA at concentrations of up to 50 nM+25 nM, respectively, the cell viability was not affected. The effect of Aza+TSA on BMDM cell viability was further supported in MTT proliferation assays performed as per protocol provided by the manufacturer. Treated BMDMs showed normal ability to proliferate in the MTT assay. Thus, our observations suggest that a concentration of 50 nM Aza in combination with 25 nM TSA is safe and not affecting cell viability but, rather, cells are able to proliferate (Fig. 1A,B, *P*<0.05). Based on these data, we used a concentration of 50 nM Aza+25 nM TSA in all our in vitro experiments.

Aza+TSA treatment rescues 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 using flow cytometry. For this experiment, primary BMDMs (supplementary material Fig. S1) were treated with Aza (50 nM)+TSA (25 nM) in the presence or

Fig. 1. Effects of Aza+TSA treatment on BMDM viability, proliferation and apoptosis. (A) Trypan Blue exclusion assay shows that treatment of BMDMs with up to 50 nM Aza and 25 nM TSA had no significant effect on cell viability. (B) MTT assay showing normal ability of BMDMs to proliferate. (C) Anti-apoptotic effects of Aza+TSA on LPS-induced BMDMs. Cells were treated with Aza+TSA in the presence and absence of LPS (1 µg/ml), followed by staining with propidium iodide (PI) and annexin V conjugated to green-fluorescent FITC dye. After staining with both probes, 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%. However, LPS-induced apoptosis was significantly reduced (to 44%) after treatment with Aza+TSA. Numbers within parentheses indicate concentration in nM. Bars represents mean±s.e.m. of three triplicate experiments. A, Aza; T, TSA. *P<0.05 vs control.
absence of LPS (1 µg/ml) for 48 h. Then, cells were stained with PI for flow cytometry analysis, which showed that LPS-induced BMDMs have reduced viability; however, viability of LPS-treated cells was significantly rescued 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 (1 µg/ml) and treated them in parallel with Aza+TSA, Aza alone or TSA alone for 24 h. Then, these cells were harvested and mRNA expression of chemokines and cytokines was measured using quantitative real-time PCR (qRT-PCR) (SA Bioscience, USA) as described by the manufacturer and an earlier publication (Pal et al., 2012). BMDMs stimulated with LPS showed significantly increased gene expression of the pro-inflammatory chemokines CCL2, CCL3, CCL4, CCL5, CCL7, CD40, CXCL10 and CXCL12, and of cytokines IL6, IL1α, IL1β, IL1RN, IL18, lymphotoxin-β and TNFα (Fig. 2A,B). However, treatment with Aza+TSA abrogated the LPS-induced transcription of the above chemokines and cytokines (Fig. 2A,B). Expression levels of representative downregulated genes encoding TNFα, IL6 and IL1β, as observed in qRT-PCR array experiments, were independently confirmed by conventional qRT-PCR (Fig. 2C). To analyze the anti-inflammatory activity of Aza+TSA treatment, we performed conventional qRT-PCR for IL10 and IL10R. mRNA expression of IL10 and IL10R was higher in LPS-induced BMDMs or BMDMs treated with LPS+Aza alone or LPS+TSA alone (Fig 2D). Importantly, the LPS-induced BMDMs treated with Aza+TSA showed a downregulation in the expression of inflammatory cytokines and increased expression of the anti-inflammatory cytokine IL10 and its receptor IL10R (Fig. 2B,D).

Aza+TSA induces more immunoplastic M2 macrophages

To understand the effect of molecular changes that occur during Aza+TSA treatment on LPS-induced macrophage subtypes, we cultured BMDMs in the presence or absence of LPS (1 µg/ml) and treated them with Aza+TSA, Aza alone or TSA alone for 24 h. Then, the cells were harvested, stained for CD14 and CD40 in the M1 subtype, and for CD23 and CD124 in the M2 subtype, analyzed and quantified by using flow cytometry (Kigerl et al., 2009). Our flow cytometry data show significantly 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 immunoplasticity, we used LPS-induced BMDMs treated for 24 h with either Aza or TSA, or a combination of Aza+TSA. Then, the western analysis for the M1 marker nitric oxide synthase 2 (NOS2) and the M2 marker CD206 was performed. Our data showed lesser expression of NOS2 in LPS-challenged macrophages treated with Aza+TSA than in cells treated with either Aza or TSA alone (Fig. 3D). Likewise, western analysis for CD206 showed increased

Fig. 2. The immunomodulatory effect of Aza+TSA on LPS-induced BMDMs. BMDMs were cultured in the presence or absence (control) of LPS (1 µg/ml), and treated in parallel for 24 h with Aza alone, TSA alone or Aza+TSA, and analyzed for mRNA expression of chemokines and cytokines. (A,B) qRT-PCR array data showing LPS-induced mRNA expression of (A) pro-inflammatory chemokines (ccl2, ccl3, ccl4, ccl5, ccl7, CD40, CXCL10 and CXCL12) and (B) cytokines (IL1α, IL1β, IL1RN, IL18, lymphotoxin-β and TNFα). mRNA levels were significantly reduced upon treatment with Aza+TSA. Bars in A and B represent mean±s.e.m. of two duplicate experiments. (C,D) qPCR array results were further validated by qRT-PCR, showing that Aza+TSA treatment significantly reduced TNFα, IL1β and IL6 (C) and IL10 and IL10R (D) gene expression compared with no treatment or treatment with Aza alone or TSA alone in BMDMs that had been challenged with LPS (L+A and L+T, respectively). Bars in C and D represent mean±s.e.m. of three replicate experiments. *P<0.5 (LPS vs LPS+TSA, and TSA vs LPS+Aza+TSA); †P<0.01 (LPS vs LPS+Aza+TSA). A, Aza; T, TSA; L, LPS.
protein expression in Aza+TSA-treated cells compared with untreated cells and with cells treated with either drug alone (Fig. 3E). The data indicate that LPS-stimulated macrophages that were treated with Aza+TSA have a higher number of M2 macrophages, which secrete anti-inflammatory cytokines.

**Aza+TSA treatment suppresses HuR expression and p38MAPK activation**

We and others have shown previously that members of the MAPK family are involved in modulating mRNA stability and/or translation of several pro-inflammatory genes (Kochanek et al., 2012; Rajasingh et al., 2006). Studies have shown that activation (i.e. phosphorylation) of p38MAPK and its downstream target is provided through activation of the RNA-stabilizing protein HuR, a protein that triggers the stabilization of mRNA encoding key inflammatory mediators, such as IL6, IL8 and TNFα mRNA (Neininger et al., 2002; Rajasingh et al., 2006). Thus, p38MAPK-induced activation appears essential for HuR translocation to the cytoplasm and for the stabilization of target mRNAs (Winzen et al., 1999). In our previous study, we found that in a mouse model of myocardial infarction, HuR protein expression was upregulated, and was associated with increased myocardial inflammatory response and cardiomyocyte cell death, which led to cardiac dysfunction and remodeling (Krishnamurthy et al., 2009). Thus, members of the MAPK family 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).

In vitro LPS-induced BMDMs treated with Aza+TSA showed a decrease of pro-inflammatory chemokine and cytokines mRNA transcripts, including TNFα (Fig. 2). Furthermore, to examine the significance of the HuR–p38MAPK signalling cascade during the Aza+TSA-mediated mechanism that protect against inflammation, we performed western analysis of the TNFα-stabilizing protein HuR and phosphorylated p38MAPK (P-P38 in Fig. 4C,D) in LPS-induced BMDMs. BMDMs were cultured in the presence or absence of LPS and treated in parallel with Aza+TSA, Aza alone or TSA alone for 24 h. An ELISA assay was performed by using a collected culture supernatant to estimate the amount of secreted TNFα, and the cells were used to analyse HuR protein expression.
levels by western blotting. The assay showed decreased levels of secreted pro-inflammatory cytokine TNFα in LPS-induced BMDMs treated with Aza+TSA when compared with LPS-induced BMDMs treated with either drug alone (Fig. 4A). To examine the Aza+TSA-mediated mechanism that protect against inflammation, we performed western analysis for the 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 control (Fig. 4B). Furthermore, to analyze the role of members of the MAPK family in the Aza+TSA-treatment mediated protective effect, we cultured BMDMs in the presence or absence of LPS, and treated them for 30 min with Aza+TSA, with Aza or with TSA alone. Then, the cells were subjected to protein analysis for phosphorylated p38MAPK. The western analysis data showed increased activation of p38MAPK in LPS-induced BMDMs that was significantly inhibited in response to treatment with Aza+TSA as compared with treatment with either drug alone (Fig. 4C; P<0.01).

To confirm the protective effect of Aza+TSA via MAPK signaling pathways, western analysis was performed of cultured LPS-induced BMDMs that had been treated for 30 min with Aza+TSA in the presence or absence of the specific MAPK inhibitor SB202190 – that selectively inhibits kinase activity of phosphorylated p38MAPK in macrophages (Karahashi et al., 2000). Western analysis showed that additional treatment with SB202190 reversed the suppressive effect of Aza+TSA in LPS-induced p38MAPK activation in BMDMs (Fig. 4D). Our finding suggests that treatment with Aza+TSA acts through the p38MAPK pathway by binding to the phosphorylated sites of p38MAPK. Therefore, when SB202190 was used together with Aza+TSA, the level of p38MAPK phosphorylation was increased (Fig. 4D in lane 8) compared with when Aza+TSA was used alone (Fig. 4D, lane 7). Overall, these data suggest that LPS increases the secretion of TNFα, inducing apoptosis in BMDMs, whereas additional treatment with Aza+TSA suppresses activation of the LPS-induced TNFα-stabilizing proteins HuR and p38MAPK, indicating its role in protection from endotoxemia-induced injury.

**Treatment with Aza+TSA 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 Bcl2 overexpression protects...
mice suffering sepsis from systemic inflammation (Iwata et al., 2003). We, therefore, hypothesized that the Aza+TSA-mediated survival of macrophages during ALI is essentially provided following 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 of active STAT3 and Bcl2 proteins. For this, BMDMs were cultured in the presence and absence of LPS and then treated with Aza+TSA for 30 min or 24 h. The data showed that the LPS-stimulated BMDMs treated with Aza+TSA showed increased phosphorylation of STAT3 compared with LPS-stimulated BMDMs treated with either Aza or TSA alone. *P<0.05 (LPS vs LPS+TSA); †P<0.01 (LPS vs LPS+Aza+TSA). (B) Expression of Bcl2 protein also significantly increased in LPS-stimulated 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) LPS-stimulated BMDMs treated with Aza+TSA were highly acetylated compared with untreated BMDMs, or BMDMs treated with either Aza alone or TSA alone for 24 h. *P<0.05 (LPS vs LPS+Aza, and LPS+TSA); †P<0.01 (LPS vs LPS+Aza+TSA). (D) ChIP data show that H3 in the STAT3 promoter region is highly acetylated in LPS-stimulated BMDMs compared with untreated BMDMs. Total STAT3, H3 and β-actin were used as loading controls. Band intensities were quantified using ImageJ. The ratios of band intensity (Bcl2 to β-actin, p-STAT3 to STAT3, AceH3K9 to H3) were calculated by using the value of the band intensity of experimental protein divided by its respective loading control. Data are expressed as means±s.e.m. of three replicated experiments.

DNA methylation/histone deacetylation or DNA demethylation/histone acetylation, respectively (Trojer and Reinberg, 2006). Here, we evaluated the epigenetic modification in the STAT3 promoter region after treatment with Aza+TSA in LPS-treated BMDMs. For this experiment, BMDMs were cultured with or without LPS and then treated for 24 h with the combination of Aza+TSA or with Aza alone or TSA alone. Western blot analysis showed that protein levels of histone 3 (H3) acetylated at lysine residue 9 (aceH3K9) were significantly increased in BMDMs treated either with Aza or TSA alone, or the combination of Aza+TSA when compared to untreated controls (Fig. 5C). Our ChIP data showed that the STAT3 promoter was highly acetylated at H3 (aceH3K9) in LPS-stimulated BMDMs treated with Aza+TSA compared with untreated BMDMs (Fig. 5D), suggesting an euchromatic state of STAT3.

Aza+TSA-treated mice show decreased activity of myeloperoxidase and circulatory pro-inflammatory cytokines in ALI

In our earlier report, ALI was induced in C57BL/6J mice by intraperitoneal injection of LPS at 40 μg/g body weight (BW). One hour after LPS administration, mice were treated intraperitoneally with vehicle, with TSA or Aza alone, or Aza+TSA. Mice treated with TSA alone had a mortality rate of 80%, whereas those treated with Aza+TSA had a mortality rate of 50% (Fig. 5C). In our current study, Aza+TSA-treated mice showed a significant decrease in myeloperoxidase activity compared with LPS-treated mice (Fig. 5D). In addition, Aza+TSA-treated mice showed a significant decrease in the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, compared with LPS-treated mice (Fig. 5E). These results suggest that Aza+TSA treatment is an effective strategy for the treatment of ALI induced by LPS.
with Aza+TSA displayed a strikingly lower mortality rate of 20%. Mice treated with Aza+TSA also showed a reduced level of neutrophils in their airspace and prolonged survival time during the observed period of two weeks (Thangavel et al., 2014). On day 2, hematoxylin and eosin (H&E) staining of lung tissues showed consistently less recruitment of LPS-induced neutrophils into the airspace in mice treated with Aza+TSA compared with untreated mice (Fig. 6A). We also observed a reduction in the number of lung polymorphic nuclear neutrophils (PMNs) in bronchoalveolar lavage fluid (BALF) of the Aza+TSA-treated 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 (Thangavel et al., 2014). 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 pro-inflammatory cytokines in LPS-challenged mice treated with Aza+TSA was measured in ELISA assays. We observed lower levels of pro-inflammatory cytokines IL1\(\beta\) (Fig. 6D), TNF\(\alpha\) (Fig. 6E) and IL-6 (Fig. 6F), and higher levels of the anti-inflammatory cytokine IL-10 (Fig. 6G) in the blood of LPS-treated mice post-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 numbers of M2 macrophages in lung tissue**

Immunofluorescence staining was performed on day-2 lung tissue sections to determine the immune plasticity of macrophages that are present in LPS-challenged ALI mice after treatment with Aza+TSA. The lung tissue sections were stained for the macrophage marker proteins CD68 (pan-macrophage marker, green) and Arg1 (M2-specific marker, red) (Fig. 7, top panel). Our immunofluorescence images showed fewer cells that stain double-positive for CD68 and Arg1 (yellow) in the LPS-treated mice lung tissue, but staining for CD68 and Arg1 together was significantly increased in LPS-treated ALI mice post-treated with Aza+TSA, as compared with untreated mice and mice treated with Aza or TSA alone (Fig. 7, bottom panel). These in vivo data indicate that Aza+TSA treatment modulates lung macrophages towards an anti-inflammatory M2 subtype in lung tissue.

**Aza+TSA-treated ALI mice show decreased numbers of M1 macrophages in lung tissue**

As described above for Arg1, we immunofluorescently stained day-2 lung tissue sections for the general macrophage marker protein CD68 (pan-macrophage marker, green) and NOS2 (M1-specific marker, red) (Fig. 8, top panel). Our data showed that Aza or TSA treatment consistently decreased the number of M1 macrophages in LPS-induced (yellow). Also, staining for CD68 and NOS together (double positive cells,
yellow fluorescence in the merged image) was 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. 8, bottom panel). These in vivo data indicate that Aza+TSA treatment decreases the LPS-induced inflammatory M1 subtype in lung tissue.

DISCUSSION

Sepsis-induced ALI is an inflammatory disorder with limited treatment options that affects millions of people worldwide (Nemeth et al., 2010; Wheeler and Bernard, 2007). A curative treatment regime is desperately needed. The available studies on epigenetic modifiers in several animal models have shown different results; i.e. effects of epigenetic modifiers varied from having no effect to being beneficial in attenuating inflammatory cytokines and chemokines as well as inflammatory injury to the airway, digestive tract and joints (Choi et al., 2008; Iwata et al., 2002; Rahman, 2002; Zhang et al., 2010). The variability in results might be because previous studies have focused on effects pre-treatment with HDAC inhibitors has, rather than studying their therapeutic potential. To our knowledge, this is the first study that examines the efficacy of a combinatorial post-treatment with Aza+TSA in inhibiting inflammation and apoptosis, and modulating the macrophage phenotype during sepsis. Furthermore, our study addresses the incompletely understood molecular mechanisms of these combined approaches, which regulate inflammatory response, cell proliferation and survival of mice following inflammation.

High concentrations of chemotherapeutic agents, such as Aza or TSA, induce apoptosis in already transformed cells (Kaminskas et al., 2005; Sassi et al., 2014). Our observations indicate that treatment with nanomolar concentrations of Aza or TSA had no negative effect on cell viability but, rather, both agents enhanced cell proliferation. We understand there is some concern about the use of BMDMs in order to characterize these mechanisms because a recent study has shown that, during perinatal development, alveolar macrophages do not originate from blood monocytes but from fetal monocytes (Schneider et al., 2014). That study mainly focuses on alveolar macrophages during normal development, rather than on a diseased tissue model. However, during ALI, the number of residual alveolar macrophages is not high enough to combat inflammation and, therefore, under the influence of colony stimulating factor (CSF), the mobilization of monocytes and/or macrophages from bone marrow to the injured alveolar site would be necessary 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 show that treatment with Aza+TSA significantly reduced LPS-induced apoptosis of BMDMs and improved cell survival. Other studies have shown that macrophages actively participate in the production of pro-inflammatory and inflammatory factors in response to LPS stimulation (Choi et al., 2008; Iwata et al., 2002; Nicodeme et al., 2010; Rahman, 2002; Zhang et al., 2010), and a recent study has shown the use 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 at nanomolar concentration 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 putative anti-inflammatory treatment that 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 and/or translation of several genes (Kochanek et al., 2012; Rajasingh et al., 2006). Our data show that treatment with Aza+TSA abrogated the LPS–TLR4–MAPK pathway, which initiated an inflammatory cascade, and is known to potentiate apoptotic signals within the macrophages. This response was mediated primarily by a reduction in phosphorylated (i.e. activated) p38MAPK, as well as by a decrease of TNFα protein levels, possibly through the binding of TNFα mRNA to HuR (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 could be reversed by the MAPK inhibitor SB202190, 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 combinatorial treatment modality with distinct mechanistic effects at the level of the RNA transcripts of genes encoding inflammatory proteins.

Studies have shown that the increased phosphorylation of p38MAPK and reduced phosphorylation of STAT3 are the two important factors that play a role signaling pathways associated with apoptosis during inflammation in macrophages and endothelial cells (Rajasingh et al., 2006; Wang et al., 2005). In our present data, BMDMs exposed to LPS showed a significant increase in phosphorylated p38MAPK and reduction in phosphorylated STAT3, which was ameliorated following treatment with Aza+TSA. These data highlight the dual protective mechanism that is 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 (Lin et al., 2008) play a pivotal role in the pathogenesis of ALI, and plasticity of M1 and M2 macrophages takes place because of bacterial products, which – in turn – result in the inflammation of lung tissue. 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 as M1 (inflammatory) and M2 (anti-inflammatory) macrophages. To understand the molecular changes that occur during treatment with Aza+TSA, we characterized M1 and M2 macrophages in LPS-induced BMDMs, and found significantly reduced expression of the M1 markers CD40, CD14 and NOS2, and increased expression of the M2 surface markers CD23, CD124 and

Fig. 8. Treatment with Aza+TSA shows decreased M1 macrophages in ALI mice lung tissue pre-treated with LPS. (Top panel) Immunofluorescence staining for the macrophage marker proteins CD68 (pan marker, green) and NOS2 (M1-specific marker, red), showing that treatment with Aza+TSA consistently decreased LPS-induced colocalization of NOS2 and CD68, indicating a total decrease of M1 macrophages (yellow). Nuclei were stained with DAPI (blue) colocalization of M1 macrophages (yellow). (Bottom panel) Quantification of CD68–NOS2 double-positive cells in lung tissue. *P<0.01 (LPS vs LPS+Aza+TSA). A, Aza; T, TSA; L, LPS.
CD206 in treated versus untreated cells. These findings further support the previous observations of 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 mechanisms underlying M1 and M2 plasticity of macrophages upon treatment with Aza+TSA.

In vivo experiments of using the general HDAC inhibitor TSA have shown its role in anti-inflammatory activities through the suppression of cytokines and reactive oxidative species (Blanchard and Chipoy, 2005), and the attenuation of macrophage infiltration (Hsing et al., 2012). By contrast, HDAC inhibitors have shown enhanced synthesis of IL-8 in lung epithelial cells following stimulation with LPS (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, because alveolar macrophages and neutrophils are the primary initiators of a pro-inflammatory milieu in ALI. Hence, one may speculate that other epigenetic modifications that are targeted, such as DNA methylation, orchestrate the expression of inflammatory genes. We have already shown that, by using a sepsis mouse model subjected to pre-treated with LPS, additional treatment of those mice with a single dose of Aza+TSA prevents lung injury and resulted in a significantly reduced mortality rate (20%) compared with 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 the significant attenuation of adverse lung histopathological changes and inflammation. Moreover, our in vitro data showed that Aza+TSA treatment significantly suppresses LPS-induced inflammation and the reprogramming of macrophages towards an anti-inflammatory M2 subtype. We attribute the above effects to the dual effect of Aza+TSA – which is mediated through inhibition of the TNFα- and mRNA-stabilizing protein HuR and phosphorylated p38MAPK – as well as to the activation of STAT3–Bcl2 pathways.

Unlike previous studies, in which pre-treatment with a single agent, such as TSA, showed limited efficacy and marginal benefits towards mortality outcome, the combinatorial regimen of Aza+TSA – which targets dual-epigenetic modifications with a multi-targeted downstream effect – provides a higher and longer-lasting survival rate in vivo. This knowledge might further facilitate the repurposing of clinically available epigenetic cancer drugs in studies that investigate sepsis, and aid the development of novel therapeutic approaches to prevent and reverse persistent lung injury. Even though many animal models are currently available, the LPS-injection model is well-established in investigating sepsis and appropriate for this study. Therefore, our study further supports the repurposing of drugs – e.g. therapy that uses Aza+TSA in combination, concluding that this is a save approach in treating sepsis, thereby improving and prolonging the lives of millions of people.

**MATERIALS AND METHODS**

**Antibodies and reagents**

We used antibodies (Abs) against phosphorylated p38MAPK, total p38MAPK, phosphorylated STAT3, total STAT3, histone 3 (H3) acetylated at lysine residue 9 (aceH3K9), total H3 and aceH3 (Cell Signaling Technology), β-actin, HuR and Bcl2 (Santa Cruz Biotechnology, Inc.). Secondary Abs were horseradish peroxidase (HRP)-conjugated donkey anti-mouse, anti-rabbit and anti-goat (Santa Cruz Biotechnology, Inc.); TRITC-, FITC- or Cy5-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 or IRDye800-conjugated anti-goat IgG (Rockland, PA), and DAPI (Life-Tech). Also were used antibodies against CD11b, CD14, CD23, CD124, CD40, CD206 NOS2 (e-Bioscience) annexin V, the MTT assay kit (Cell Proliferation assay kit, Promega, USA), inflammatory PCR array kit, ELISA kits (Millipore) and the specific p38MAPK inhibitor SB 203580 (Calbiochem), Lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (Sigma Aldrich, USA).

**Mice**

C57 black 6 (C57BL/6J) male mice were obtained from Jackson Laboratory. All experiments were conducted in accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD), and 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 BMDM culture**

Primary mouse bone-marrow-derived macrophages (BMDMs) were obtained from C57BL/6J 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 DMEM supplemented with 10% FBS, together with 10% L929-cell-conditioned medium on 10-cm cell culture dishes. After 7 days of culture, the cells were stained with antibody against 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 (supplementary material Fig. S1) was evaluated in Trypan-Blue-exclusion and 3,4,5-dimethylthiazol-2-yl 2,5-diphenyl tetrazolium bromide (MTT) assays as described by us earlier (Rajasinh et al., 2011; Thangavel et al., 2014). Briefly, 10⁴ BMDMs were cultured in 96-well culture plates (200 μl/well) in DMEM containing 10% FBS in the presence and absence of various concentrations of Aza+TSA for 48 h at 37°C at 5% CO₂. The cells were manually counted under the phase-contrast microscope by using a hemocytometer. Effects of Aza+TSA on BMDM proliferation were determined in MTT assays according to the manufacturer’s instructions (Cell Proliferation Assay kit, Promega).

**Flow cytometer analysis**

Cells from six-well plates were harvested and washed twice in phosphate-buffered saline (PBS), counted and resuspended in FACs buffer (1% BSA in PBS containing 0.01% Na3) for flow cytometric phenotypic analysis. cells (1×10⁶ 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 h. Then, the cells were washed with PBS three times and incubated 20 min with 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 cytometry (FACSCalibur, BD Biosciences) using Cell Quest software. Data were analyzed by using FlowJo software (Tree Star, Ashland, OR). Anti-CD11b was used to characterize macrophages, anti-CD23 and anti-CD124 were used to quantify M2, and anti-CD41 and anti-CD40 were used to quantify the M1 subtype.

**Quantitative real-time PCR array**

We performed quantitative real-time PCR (qRT-PCR) arrays for inflammatory cytokines in LPS-induced macrophages treated with Aza+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 100 ng 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 AB1 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 the 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 (Rajasigh et al., 2008, 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 supplementary material Table S1. Results were expressed as fold change in expression, and values were calculated as the ratio of induced expression to control expression.

**Western blot analysis**

Western blot analyses of phosphorylated p38MAPK, HuR, phosphorylated STAT3 and Bcl2 proteins were 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 a Duralose membrane. Membranes were blocked with 5% dry milk or 10% PBS, 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 HRP-conjugated secondary antibodies. Protein bands of STAT3, Bcl2 and aceH3K9 were detected by using enhanced chemiluminescence. All the other proteins (NOS2, CD206, phosphorylated p38MAPK, HuR) were detected by using 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±s.e.m. of three replicated experiments.

**ELISA**

Enzyme-linked immunosorbent assays (ELISAs) 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 (Rajasigh et al., 2008, 2011). Briefly, cultured cells or lung tissue was rinsed once with PBS and fixed with 4% paraformaldehyde (Sigma) in PBS for 30 min, then rinsed three times with PBS, permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 5 min, washed twice with PBS, and incubated overnight at 4°C with primary antibodies (against Arg1, NOS2) diluted with 1% FBS in PBS. After three washes with PBS, cells were incubated with specific secondary antibodies for 1 h at 37°C, and cells were rinsed three times with PBS, stained with DAPI to visualize cell nuclei, rinsed three times with PBS, dried and mounted in Vectashield mounting medium for fluorescent imaging. All immunofluorescence staining was photographed under either a confocal or an immunofluorescence microscope.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays of 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 (Rajasigh et al., 2008, 2011). Briefly, ∼5×10⁶ cells in 2–10 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 into fragments with lengths between 200 and 1000 bp, and incubated with primary antibodies against 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 four washes with washing buffer, the pelleted complex of protein-A–agarose, antibody and histone 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′-GTTGACACCTTGAGGACCGCTAAG-3′ and reverse 5′-AAAAAC-GCTCTTAGGAGAAGCG-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 (Rajasigh et al., 2008, 2011).

**Induction of ALI**

Acute lung injury (ALI) 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 BW) alone or Aza (1 μg/g BW) alone, or Aza+TSA (1 μg/g BW + 1 μg/g BW) in combination. The histological changes and gene expression were evaluated in the lung.

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**Competing interests**

The authors declare no competing or financial interests.

**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. B.B.: 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.

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**Supplementary material**

Supplementary material available online at http://jcs.biologists.orglookup/suppl/doi:10.1242/jcs.170258/-/DC1

**References**


Fig. S1

Bone-marrow-derived macrophages as seen under phase contrast microscopy and confirmed by flow cytometric characterization for CD11b+ cells
**Table S1.** Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>F: CAGCCGGAAGACAATAACTG</td>
<td>R: CCGCAGCTCTAGGAGCATGT</td>
</tr>
<tr>
<td>IL-10R</td>
<td>F: TGTCTGTATGCAAAGCTTGAAAT</td>
<td>R: GTCTGTGCCCGCTTTTTCCTCA</td>
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<tr>
<td>TNF-α</td>
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<tr>
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<td>R: TCCATTGAGGTGAGAGCTTTC</td>
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