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A Quorum Sensing Signal Promotes Host Tolerance Training Through HDAC1-Mediated Epigenetic Reprogramming

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Abstract

The mechanisms by which pathogens evade elimination without affecting host fitness are not well understood. For the pathogen *Pseudomonas aeruginosa*, this evasion appears to be triggered by excretion of the quorum sensing (QS) molecule 2-aminoacetophenone (2-AA), which dampens host immune responses and modulates host metabolism, thereby enabling the bacteria to persist at a high burden level. Here, we examined how 2-AA trains host tissues to become tolerant to a high bacterial burden, without compromising host fitness. We found that 2-AA regulates histone deacetylase1 (HDAC1) expression and activity, resulting in hypoacetylation of lysine 18 of histone H3 (H3K18) at pro-inflammatory cytokine loci. Specifically, 2-AA induced reprogramming of immune cells occurs via alterations in histone acetylation of immune cytokines *in vivo* and *in vitro*. This host epigenetic reprograming, which was maintained for up to 7 days, dampened host responses to subsequent exposure to 2-AA or other pathogen-associated molecules. The process was found to involve a distinct molecular mechanism of host chromatin regulation. Inhibition of HDAC1 prevented the immunomodulatory effects of 2-AA. These observations provide the first mechanistic example of a QS molecule regulating a host epigenome to enable tolerance of infection. These insights have enormous potential for developing preventive treatments against bacterial infections.

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Author Contributions

AB, designed the study and did the experiments, performed data analysis and wrote the manuscript. AT, performed ChIP-PCR experiments and analysis of the data and wrote the manuscript. DM contributed to the *in vivo* experiments. KLJ supervised ChIP-PCR experiments. LGR designed the study and supervised the research and wrote the manuscript.

Pathogens have evolved mechanisms for evading eradication by their hosts' dynamic immune systems[1,2]. The establishment and maintenance of bacterial infections rely on the pathogen's ability to breach the host's innate immune responses[3]. Immune-driven resistance is the prevailing defense strategy against infection. However, organisms can also defend themselves through a different defense strategy, namely tolerance, defined as the ability to limit pathogen damage without controlling pathogen burden[4,5]. Tolerance is an evolutionarily conserved defense strategy shared by diverse plants and animals, including humans[6]. In tolerance (or resilience), a host copes with a pathogenic encounter without a reduction in fitness[4,6,7] and avoids harmful inflammatory responses that can occur with the immune-driven resistance strategy[8]. The resistance and tolerance defense strategies have different ecological and evolutionary consequences: resistance reduces pathogen presence, whereas tolerance allows pathogen prevalence. Our understanding of the biological mechanisms mediating the mutual pathogen-host adaptation and the causes and consequences of variation in host tolerance is limited.

Traditionally, immunological memory has been recognized as an attribute of the adaptive immune system, while the innate immune system has been regarded as perpetually naïve. However, the ability to respond more (or less) vigorously to a second pathogen encounter has been documented in organisms lacking an adaptive immune system (i.e. without T and B cells) and attributed to innate immune "training"[9,10]. Innate immune training is associated with epigenetic and metabolic reprogramming of innate immune cells[11,12]. However, the mechanisms by which such training can be triggered and its functional outcomes for the host and pathogen have not been resolved.

The small volatile bacterial quorum sensing (QS)-regulated molecule 2-aminoacetophenone (2-AA) acts as an immunomodulatory signal that enables the host to tolerate long-term bacterial presence[13]. Its impact on host metabolism likely also contributes to host tolerance[14]. In mice, 2-AA treatment improves host survival dramatically during *Pseudomonas aeruginosa (PA)* infection (90% vs. 10%), despite increases in bacterial load[13,15]. Hence, 2-AA appears to act as a critical mediator of host tolerance to pathogen burden. 2-AA is excreted liberally by *PA* and other pathogens[16-19] in infected tissues[16,20] and its synthesis is regulated by QS, a communication system governed by population density that is used by bacteria[21] to regulate virulence factors[22-24].

Our prior findings showing that 2-AA promotes bacterial phenotypes that favor a persistent bacterial presence[15,25] converge with emerging evidence suggesting that pathogens can repress host immunity through epigenetic reprogramming[9,11,26]. Epigenetic modifications are important mechanisms of gene regulation. Notably, mRNA transcription initiation and elongation are controlled by various posttranslational modifications of chromatin-associated histones, including acetylation and methylation of lysine residues on histone tails[27]. Bacteria can manipulate host immunity through effects on the enzymes that control epigenetic histone acetylation, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs)[28]. Here, we examined 2-AA regulation of host cell epigenetic programs and the effects of this regulation on host inflammatory responses and bacterial burden tolerance. We found that 2-AA acts as a host training molecule by reprogramming innate immune cells via HDAC activity and alterations in histone 3 acetylation on lysine 18 (H3K18ac). This new

understanding of mechanisms of host tolerance and training may provide avenues for the development of novel therapeutic interventions against bacterial infections.

Results

2-AA pretreatment attenuates pro-inflammatory responses in monocytes and promotes long-term immunosuppression

An initial exposure to 2-AA led to activation of the nuclear factor (NF)-κB pathway in THP-1 human monocytes, as evidenced by increased reporter activity (Fig. 1b and Supplementary Fig. 1a), as well as increased mRNA transcript and secreted protein levels of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β, as well as of monocyte chemotactic protein (MCP)-1 (Fig. 1c–h, Supplementary Fig. 1b–g). However, pretreatment of THP1 cells with 2-AA, followed by washing and 2-AA re-exposure (Fig. 1a), reduced NF-κB reporter activity (Fig. 1b) and attenuated transcription (Fig. 1c, d and e) and protein secretion (Fig. 1f, g and h) of TNF-α, IL-1β, and MCP-1. Because synthetic, endotoxin-free 2-AA was used (Supplementary Fig. 1h), the observed effects can be attributed to 2-AA rather than endotoxin-dependent tolerance. The immunomodulatory effects of 2-AA were recapitulated in primary human (Fig. 1i and j; Supplementary Fig. 2a–f) and mouse macrophages (Supplementary Fig. 2g–l), in which TNF-α, MCP-1, and IL-1β secretion were reduced upon 2-AA stimulation when cells were stimulated 6-h, 1 day, 3 days, or 7 days after 2-AA pretreatment (Fig. 1i, j and Supplementary Fig. 2a-l). These immunomodulatory effects also affected cells' subsequent responses to unrelated pathogen-associated molecular patterns. That is, compared to non-pretreated cells, 2-AA pretreated macrophages produced reduced levels of pro-inflammatory cytokines following lipopolysaccharide (LPS) or peptiodoglycan (PGN) stimulation (Fig. 1i and j).

2-AA pretreatment reduces H3K18ac at TNF-α promoter

Immunoblot analysis with a pan-acetyl antibody revealed that acetylation of the core histone H3 peaked 3 to 6-h after 2-AA exposure and then declined (Supplementary Fig. 3a), a pattern reminiscent of 2-AA-induced pro-inflammatory cytokine expression (Fig. 1c–h). Analysis of acetylation levels of purified histones from 2-AA stimulated cells with an H3 modification multiplex assay demonstrated preferential enhancement of the H3K18ac histone mark, but unaltered levels of H3K9ac, H3K9me3, H3K14ac, or H3K56ac (Supplementary Fig. 3b). Quantitation of global acetylation of H3K18 and H3K9 showed significantly elevated levels of H3K18ac, but not H3K9ac, 1-h after first-time 2-AA exposure in human monocytes (Supplementary Fig. 3c-d) or murine macrophages (Fig. 2a and 2b). Addition of the HDAC inhibitor trichostatin (TSA) restored H3K18 global acetylation levels in 2-AA pretreated cells as seen in the non-pretreated cells following 2-AA stimulation (Supplementary Fig. 3c).

Enrichment of H3K18ac, but not H3K9ac or H3K9me3, specifically at the promoter locus of the gene that encodes the NF-κB–targeted cytokine *TNF-α*, was attenuated in 2-AA pretreatment in mouse macrophages (Fig. 2c, d, e, and f) and human monocytes (Supplementary Fig. 3e), compared to that in non-pretreated control cells. RNA polymerase

II occupancy, consistent with attenuation of transcription, was also higher at the *TNF-α* promoter in stimulated cells without 2-AA pretreatment than in those with pretreatment (Supplementary Fig. 3f).

2-AA pretreatment modulates acetylation activity

Induction of HAT activity was observed following 2-AA stimulation in human monocytes (Fig. 3a and Supplementary Fig. 4a) and mouse macrophages (Supplementary Fig. 4c and e), and this induction was reduced in 2-AA pretreated groups. Meanwhile, 2-AA pretreatment amplified 2-AA stimulation effects on HDAC activity (Fig. 3b, Supplementary Fig. 4b, d, and f). Hence, an initial exposure of 2-AA induced HAT activity whereas 2-AA re-exposure reduced HAT activity and resulted in increased and sustained HDAC activity, which suppresses pro-inflammatory gene expression.

To identify which HDACs repress pro-inflammatory gene expression in 2-AA pretreated cells, we employed a number of inhibitors with differing HDAC specificities. As shown in Figure 3b, 2-AA-induced HDAC activity in 2-AA pretreated cells could be blocked with TSA (inhibitor of Class I/II HDACs, i.e. HDAC 1-3 and 8/4, 5, 6, 7, 9, and 10), valproic acid (VPA; inhibitor of all Class I/II HDACs except HDAC 6 and 10)[29,30], and Ms275 (preferential inhibitor of HDAC1)[31], but not with TMP269 (inhibitor of Class IIa HDACs 5, 7, and 9)[32], RGFP966 (HDAC3 inhibitor)[33], PCI-34051 (HDAC8 inhibitor)[34], HPOB (HDAC6 inhibitor)[35], or nicotinamide (NIC; inhibitor of Class III HDACs, i.e. SIRT1-7)[36] (Supplementary Fig. 4f and 5). This selectivity profile points to the involvement of HDAC1 (Class I HDAC) in 2-AA promoted deacetylation.

2-AA pretreatment induces transcriptional repression through HDAC1

HDAC1 protein was highly accumulated in the nuclear fraction of 2-AA pretreated cells, whereas HDAC2 levels were unaltered relative to levels in non-pretreated controls (Fig. 3c and Supplementary Fig. 6a-e). HDAC1 enrichment was also increased at *TNF-α* promoters in the 2-AA pretreated cells compared to that in control cells (Fig. 3d and e, Supplementary Fig. 6f and g).

HDAC1 knockdown (KD) neutralizes 2-AA mediated immunomodulation

HDAC1 KD in murine macrophages by shRNA (Supplementary Fig. 6h) restored H3K18 acetylation (Fig. 4a and Supplementary Fig. 6i), TNF-α secretion (Fig. 4b), and NF-κB reporter activation (Fig. 4c) in 2-AA pretreated macrophages compared to untreated cells following 2-AA stimulation. Taken together, these data indicate that HDAC1 is involved in 2-AA induced immune tolerance training and regulation of H3K18 acetylation.

HDAC1 inhibition counteracts 2-AA immunomodulation *in vitro*

The Class I/II HDAC inhibitor TSA rescued H3K18ac levels (Supplementary Fig. 3c) and TNF-α secretion (Fig. 4b, 4d, Supplementary Fig. 7a) in 2-AA pretreated cells following 2-AA stimulation, but not in HDAC1 KD cells (Fig. 4b). Ms275 (preferential HDAC1 inhibitor) and VPA (Class I/II HDAC inhibitor) also restored TNF-α production in 2-AA pretreated THP1 cells (Fig. 4d and Supplementary Fig. 7a). Conversely, agents that inhibit HDACs other than HDAC1 (i.e. NIC, TMP269, RGFP966, PCI-34051, and HPOB) did not

rescue TNF-α secretion in 2-AA pretreated cells (Supplementary Fig. 7 j). TNF-α production in 2-AA pretreated cells was also unaffected by paragyline (histone demethylase inhibitor), 5'azacytidine (DNA methyltransferase inhibitor), and anacardic acid (HAT inhibitor) (Supplementary Fig. 7k). Taken together, we show that reversal of the immunomodulatory epigenetic effects of 2-AA pretreatment is achieved by HDAC1 inhibition.

HDAC1 pharmacological inhibition reduces 2-AA-mediated host tolerance *in vivo*

Daily administration of the Class I/II HDAC inhibitor TSA extinguished the survival outcome benefit of 2-AA pretreatment in burn-*PA* infection (BI) model mice dramatically ($p < 0.0001$; Fig. 5a). Furthermore, the TSA treatments reduced the survival of BI animals (12%) to a level even lower than that of untreated BI animals (56%)($p < 0.044$; Fig. 5a). The TSA treatment decreased bacterial burden ($p = 0.031$ vs. no TSA) in 2AA-pretreated BI animals (Fig. 5b); it had a weaker (non significant trend) effect on bacterial load in BI animals without 2-AA pretreatment ($p = 0.059$ vs. no TSA).

Biochemical analysis showed that 2-AA pretreatment of BI animals reduced H3K18 acetylation and HAT activity (Fig. 5c and d), while enhancing HDAC activity (Fig. 5e) and dampening pathogen-induced cytokine production (Fig. 5f and g). Even without exogenous 2-AA, in BI mice, H3K18 acetylation, HAT activity, and pro-inflammatory cytokine secretion fell gradually over time, while HDAC activity was increased and sustained (Fig. 5c-g). TSA administration restored global acetylation levels of H3K18ac (Fig. 5c) and HAT activity in the spleen (Fig. 5d), dampened HDAC activity (Fig. 5e), and increased serum levels of TNF-α and MCP-1 (Fig. 5f and g) in 2-AA pretreated BI animals. Hence, TSA treatment increased *PA* infection susceptibility while disrupting 2-AA mediated immunosuppression *in vivo*.

Discussion

Here, we uncovered mechanisms by which a pathogen-excreted molecule leads to mutual pathogen-host adaptation wherein the host immune response is trained to tolerate a high pathogen burden. We found that *PA*, which infects plants[37,38], insects[39], nematodes[40], mice[38] and humans[41], promotes host-tolerance training through epigenetic reprogramming via the QS molecule 2-AA. Although there are several examples of immunity repression via infection-induced host epigenetic reprogramming[26,28,42,43] and small molecules favoring acute infection have been identified[22], no pathogen-derived small molecule to our knowledge has been shown previously to alter immune responses toward tolerance to pathogen burden without compromising host survival.

We report four principal results demonstrating the effects of 2-AA on host epigenetic reprogramming (Fig. 5h). Firstly, 2-AA promoted long-lasting effects that impacted inflammatory responses non-specifically. Secondly, reduction of histone acetylation in 2-AA pretreated cells attenuated transcription of pro-inflammatory cytokines *in vitro* and *in vivo*. Thirdly, HDAC1 expression and activity were elevated in 2-AA pretreated cells. Finally, HDAC1 inhibition counteracted 2-AA's immunomodulatory effects *in vitro* and *in vivo*. These results show that 2-AA triggers epigenetic modulation that switches innate immune

cells from a transcriptionally permissive to a more silent chromatin state that in turn promotes pathogen survival while augmenting host tolerance.

Previous cell culture studies have pointed to histone deacetylation as a mechanism of host immunomodulation during infection with other pathogens[28,42,44,45]. However, the *in vivo* relevance of these studies for both host and pathogen, as well as the bacterial component mediating the effect, were unknown. Consistent in principle with our findings, Eskandarian and colleagues found that *Listeria monocytogenes* infection—which under normal circumstances triggers H3K18 deacetylation—was reduced in mice when a critical deacetylase was knocked out[45].

Our findings suggest that 2-AA trains innate immune cells to limit pathogen-induced inflammation via histone deacetylation. This phenomenon differs from prior reports of so-called immunity/memory training, such as that seen with Bacillus Calmette-Guerin vaccination, *Candida albicans* infection, or fungal cell wall component exposure, which induce histone acetylation and provide non-specific protection through an augmentation of pro-inflammatory responses and clearance[9,10,46]. On the contrary, 2-AA dampens inflammatory responses and allows maintenance of a high bacterial burden, adaptations that benefit the pathogen as well as the host. Interestingly, it has been reported that nasal treatment with live attenuated *Bordetella pertussis* offers long-term protection against influenza viruses by limiting virus-induced inflammation in an antigen-independent manner, without affecting viral load[47].

The mechanism mediated by 2-AA is also distinct from previously described effects of other bacterial molecules that dampen inflammatory responses while promoting bacterial clearance[48,49]. That is, 2-AA triggers immunomodulation that does not promote clearance. Although both dampen immune responses, the infection outcomes are opposite. The particular HDACs involved also differ between 2-AA and LPS effects[50].

Based on our results, we propose a model (Fig. 5h) in which acute 2-AA stimulation results in increased HAT activity, resulting in histone acetylation at pro-inflammatory cytokine promoter loci that potentiate transcription, whereas prolonged exposure to 2-AA induces deacetylation by HDAC1, leading to a loss of active transcription, thus promoting immunosuppression. The CCAAT/enhancer-binding protein (C/EBP)β is activated following 2-AA treatment in mouse macrophages, which inhibits transcription[13], and histone deacetylation is initiated by HDAC recruitment through C/EBPβ at specific cytokine promoter sites during persistent mycobacterial infection[28]. Our results suggest that the presently observed host tolerance to infection is a result of sustained deacetylation leading to long-lasting epigenetic alterations that favor long-term bacterial presence and host tolerance of a bacterial burden.

Collectively, our results show that the QS derived molecule, 2-AA, modulates both the source pathogen and its host. In the pathogen, 2-AA permits survival and may promote persistence via the silencing of acute virulence functions[15,25]. In the host, 2-AA triggers immunomodulation that prevents an overly robust inflammatory response, thus enabling the host and pathogen to coexist. Based on our findings, we propose that 2-AA is a critical

mediator of the intricate interplay between host and pathogen. Given that QS molecules are widely conserved across bacterial genera and the common occurrence of polymicrobial settings, it is important to note that 2-AA impacts other Gram-negative microbes[25]. Elucidation of patho-epigenetic changes is critical for understanding host tolerance to commensal microbes and may guide the development of treatments that may improve resilience to pathogen damage in patients with serious infections.

Methods

Ethics statement

The animal protocol was approved by Institutional Animal-Care and Use Committee (IACUC) of Massachusetts General Hospital (Permit: 2006N000093). No randomization or exclusion of data points was used.

Cell culture

All cells were maintained in 5% CO_2 at 37°C. THP-1 Blue cells (human monocytic leukemia line with an NF-κB–inducible reporter (Invivogen) and RAW264.7 cells carrying the NF-κB luciferase plasmid (IMGENEX, USA) were maintained in RPMI 1640 medium (Invitrogen) and Iscove's modified Dulbecco's medium (IMDM, Invitrogen), respectively. The media were supplemented with 10% heat-inactivated FBS (endotoxin-free Certified FBS; Invitrogen), 2% penicillin/streptomycin, 2 mM L-glutamine, and 10 mM HEPES (all from Gibco). The cells were seeded in T-75 tissue culture flasks (Falcon, USA) and used between passages 2 and 3.The THP-1 and RAW264.7 cell lines were extensively validated by the Invivogen and IMAGENEX respectively. For quality control, we have tested the cells for mycoplasma with PlasmoTest™ kit (Invivogen).

Preparation of human macrophages from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from buffy coats of healthy donors (from MGH blood components lab) by Lymphoprep (Stemcell tech) density gradient centrifugation and purified by adherence [51]. The cells were allowed to differentiate in complete XVIVO medium containing 100ng/mL human M-CSF (Peprotec) for 7 days at 37°C, 5% CO_2. On day 4, cultures were supplemented with one volume of complete XVIVO medium containing 100ng/mL human M-CSF. Macrophages were seeded onto 24 well (1.5×10^5 cells/well) tissue culture plates for the treatments.

Preparation of Bone marrow derived mouse macrophages (BMDM)

Bone marrow was flushed from mouse tibias and femurs and cells (previously spun at 1500 rpm for 10min to remove debris) seeded at a density of 50×10^6 cells (yield from 1 mouse)/non treated 10cm dish with complete DMEM/F12 (Gibco) medium containing 5 ng/mL of IL-1 (Peprotec) and 5ng/mL CSF-1 (Peprotec) and incubated at 37°C, 5% CO_2. After 24-h, non-adherent cells were seeded into 24-well (1.5×10^5 cells/well) tissue culture dish with fresh complete medium containing 5 ng/ml of IL-1 and 5ng/mL CSF-1.Cells were matured over 5 days.

2-AA

Chemically synthesized 2-AA was purchased form Sigma-Aldrich (titration by $HClO_4$, 98.0–102.0%; purity (GC) > 98.0%). The exact amount of 2-AA in human tissues has not been quantified due the compound's high volatility. The basis for selecting the 2-AA concentrations in our studies is based on the assessment of 2-AA levels in bacterial cultures of several clinical *Pseudomonas aeruginosa* isolates from burned patients. LC/MS found that 2-AA levels were in the range of 37-225 μM. The concentration used in our murine studies, namely 50 μM (6.75 mg/kg), is at the low end of this range, and thus should be considered a reasonable approximation of "physiological" levels. Because 2-AA is a highly volatile molecule and tolerization assays of human monocytes and murine macrophages run for ~ 24-h in microtiters (prone to evaporation), we use 1-2 (200–400 μM) and 1.8-3.5 (400–800 μM) orders of magnitude higher concentrations in our culture studies than that measured in bacterial cultures, respectively.

Endotoxin test for 2-AA preparation

2-AA was serially diluted in PBS. For positive control, LPS was used. The endotoxin unit was measured using the *Limulus* Amebocyte Lysate (LAL) technique with LAL chromogenic Endotoxin Quantitation Kit (Thermo-scientific) according to the manufacturer's instructions.

2-AA cell treatment

THP-1 cells were plated at a density of 10^5/mL in 24 well plates and grown overnight at 37 °C in 5% CO_2. Cells in the treatment groups were pretreated with 200 μM 2-AA for 24-h; treated and non-treated cells were washed with 1X phosphate buffered saline (PBS) and kept in fresh medium. Cells were stimulated with 200 μM 2-AA for the durations indicated in the figures. Similarly, murine macrophage RAW264.7 cells were pretreated (or not) with 800 μM 2-AA for 48-h then stimulated with 400 μM 2-AA.

For memory experiments, human and mouse primary macrophages were used. Human macrophages (10^5/mL) were pretreated with 400 μM 2-AA for 24-h; treated and non-treated cells were washed out and after 1 day, 3 days or 7 days, the cells were stimulated with 200 μM 2-AA for 6-h. Mouse primary macrophages (10^5/mL) were pretreated with 800 μM 2-AA for 48-h; treated and non-treated cells were washed out and after 1 day, 3 days or 7 days, the cells were stimulated with 400 μM 2-AA for 6-h.

SEAP assay

THP-1 Blue cells express a secreted embryonic alkaline phosphatase (SEAP) under the control of NF-κB transcription factors. To quantify secreted SEAP, the culture supernatant was incubated with QUANTI-Blue colorimetric assay reagent (Invivogen) according to the manufacturer's instructions. All assays were run in triplicate.

Luciferase assay

After treatment, Raw 264.7 cells were washed with PBS and then lysed in the buffer provided with the Luciferase Assay Kit (Promega) as described earlier [13].

RNA isolation and quantitative RT-PCR

Total RNA from approximately 1.2×10^6 cells was extracted with the RNeasy Mini Kit (Qiagen). cDNAs were prepared with the RETROscript™ Kit (Ambion® Life Technologies), according to the manufacturer's protocol. Real-time PCR was performed using the Brilliant II SYBR green super mix (Agilent) and primer sets specific for human or murine *TNF-α*, *IL-β*, *MCP-1*, and *GAPDH* and *β-actin* (Supplementary Table 1). Expression of cytokines was normalized to *GAPDH* or β-actin with the ΔCt Method and relative expression was calculated with non-pretreated and unstimulated cells as references. The assay was conducted in triplicate; means and standard deviations were calculated for each group.

Histone extraction and acetylation analysis

Histones were isolated from cells and tissues as described previously[52]. To study the histone modifications, 5 μg of isolated histones were analyzed by immunoblotting using acetyl-pan-H3 (cat no: 06-599), acetyl-pan-H4 (cat no: 04-557) (Millipore), acetyl-Histone H3 (Lys18) (cat no: 9675) and H3 (cat no: 4499) (Cell Signaling Technology).

Quantification of global histone H3 acetylation

Quantification of global level of lysinie-specific histone acetylation was done using Global Acetyl Histone H3-K9/K18 colorimetric assay kits (Abnova) according to manufacturer's protocol.

Preparation of nuclear fraction

Nuclear extracts were obtained from cells at experimentally indicated time points with a Nuclear Extract kit (Active Motif, Carlsbad, CA) as described earlier [13].

H3 Modification Multiplex Assay

The binding specificity of purified histone to modified H3 residues was analyzed by using Histone H3 Modification Multiplex kit (Epigentek) according to manufacturer's protocol.

HAT activity assay

HAT activity was measured in nuclear lysates using a non-radioactive HAT fluorescent assay kit (Active Motif), according to the manufacturer's instructions. The fluorescence intensity was measured using Tecan plate reader (excitation wave length 360-390nm, emission wavelength 450-470nm). HAT activity was expressed as arbitrary fluorescent units (AFU).

HDAC activity assay

Nuclear fraction were used to measure deacetylase activity with a non-isotopic assay that used a fluorescent derivative of epsilon-acetyl lysine (HDAC Fluorescent Activity Assay Kit, Active Motif), according to the manufacturer's instructions. The fluorescence intensity was measured using Tecan plate reader (excitation wave length 340-360nm, emission wavelength 440-460nm). HDAC activity was expressed in arbitrary fluorescent units (AFU).

Quantitative Analysis of HDAC1 and HDAC2

The protein content of HDAC1 and HDAC2 was measured in nuclear lysate using colorimetric EpiQuick HDAC1 and HDAC2 assay kits (Epigentek) according to the manufacturer's protocol. Absorbance was read at 450 nm. Results were calculated using a standard curve and expressed as nanogram/mg of protein. The assay was conducted in triplicate.

Chromatin Immunoprecipitation (ChIP)

Cells were washed in 1X PBS, cross-linked in 1% methanol-free formaldehyde for 10 min, then glycine was added to a final concentration of 0.125M for 5min at RT. Using the *tru*ChIP™ High Cell Chromatin Shearing kit (Covaris), cells were prepared for sonication according to the manufacturer's protocol. Approximately 1×10^7 cells were placed in a 12×12-mm tube and subjected to genomic DNA shearing with the Covaris S220 sonicator for 8min (140 peak power, 5 duty factor, and 200 cycles/burst). ChIP was conducted with the Magna ChIP™ A/G Kit (Millipore) according to the manufacturer's protocol. Briefly, chromatin from approximately 1.2×10^6 cells was incubated overnight at 4°C with 4μg of anti-acetyl H3K18, acetyl H3K9, H3K9me^3, HDAC1 (Abcam) or anti-RNA polymerase II phospho S CTD (Millipore) ChIP-grade antibodies and 20 μl of A/G magnetic beads. The beads were washed serially (5 min each) with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and TE buffer from the kit at 4°C. Chromatin was eluted with elution buffer containing Proteinase K at 62°C for 4-h, then incubated at 95°C for 10min. DNA was isolated by column purification. Real-time PCR was performed with the Brilliant II SYBR green super mix (Agilent) and primer sets to amplify various regions of *TNF-α* and *β-actin* loci (Supplementary Table 1). Normalized values were calculated by the percent-input method. *β-actin* percent input normalized values are shown. The assay was conducted in triplicate; means are reported with standard deviations.

HDAC1 Knock Down by short hairpin (shRNA)

HDAC1 (sc-29344-V) and non-targeting control (sc-108080) shRNA lentivirus clones were purchased from Santa Cruz Biotechnology. Murine macrophage Raw264.7 cells were infected with lentiviral shRNA. After selection with puromycin, a pool of infected cells was expanded and efficacy of HDAC1 KD was validated by Western blot analyses.

Western blot

Cellular extracts were prepared in RIPA buffer and analyzed by standard immunoblot techniques. The primary antibodies used were specific for HDAC-1(cat no.5356) (Cell Signaling Technology), and mouse anti-β-actin (cat no: sc-47778) (Santa Cruz Biotechnology). 20μg of proteins were subjected to immunoblot analysis, fractionized by polyacrylamide gel electrophoresis (PAGE) and transferred to membrane. The bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) reaction, according to the manufacturer's instructions.

Measurement of TNF-α, IL-β, and MCP-1 by enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-β, and MCP-1 protein levels in culture supernatants of 2-AA pre-treated and non pretreated cells were measured by ELISA using the Quantikine human/mouse TNF-α, IL-β, and MCP-1 kits (R & D Systems) according to the manufacturer's instructions. Mouse serum was isolated and cytokine secretion assayed by ELISA using the Quantikine TNF-α and MCP-1 kit (R & D Systems) according to the manufacturer's instructions.

Pharmacological inhibitors

For the HDAC inhibition assay, cells were pretreated with TSA (5nM, Sigma-Aldrich). For the TNF-α secretion assay, human THP1 or murine macrophage RAW264.7 cells were treated with TSA, VPA (0.1 μM), Ms275 (5 μM), NIC (0.5 μM) HPOB (0.5 μM) (Sigma-Aldrich), TMP269 (0.1 μM), PCI-34051 (0.1 μM), RGFP966 (0.5 μM) (Selleckchem), paragyline (3 μM), 5'azacytidine (10 μM) or anacardic acid (10 μM) (Cayman Chemical). The concentration of HDAC inhibitors used did not induce cytotoxicity (Supplementary Fig. 5 a-h). It is important to note that at high concentrations, HDAC inhibitors blocked TNF-α secretion (Supplementary Fig. 7b-i) [30].

MTT assay for cell cytotoxicity

The cytotoxicity of cells treated with TSA (Sigma-Aldrich), NIC, Ms275, HPOB (Sigma-Aldrich), TMP269, RGFP966, PCI-34051 (Selleckchem) or VPA (Cayman Chemical) was measured by MTT assay as described previously [13].

Bacterial strains and growth conditions

A *P. aeruginosa* strain known as Rif^R human clinical isolate UCBPP-PA14 (a.k.a. PA14) was used [37,38]. They were grown at 37 °C in Luria-Bertani (LB) broth under shaking and aeration or on LB agar plates containing appropriate antibiotics. Over day PA14 cultures were grown in LB from single colony to $O.D._{600nm}$ 1.5 and diluted 1:50,000,000 in fresh LB media and grown overnight to $O.D._{600nm}$ 3.

Mice survival and assessment of bacterial burden

A thermal injury mouse model [37] was used to assess 2-AA effects on survival and bacterial burden in 6-wk-old CD1 male mice (Charles River; Boston, MA). Four days prior to thermal injury and infection mice were injected intravenously (IV) with 2-AA (6.75 mg/kg). Following administration of anesthesia, a full-thickness thermal burn injury involving 5–8% of the total body surface area was produced on the shaved mouse abdomen dermis, and an inoculum of 1×10^4 PA14 cells in 100 μl of MgSO4 (10 mM) was injected intradermally into the burn eschar. Immediately after BI, a group of mice received intraperitoneally (IP) TSA (0.5 mg/kg,) once per day for up to 10days. Mice survival was assessed over the course of 11 days.

Because of the aggravated mortality rates as a result of TSA treatment daily for 10 days, CFU assessment for all groups was performed at 5 days instead of 10 days post-infection. CFU counts were assessed in muscle samples obtained from underneath the burn eschar and site of bacterial cell inoculation. Samples were homogenized in 1 mL of PBS, diluted and

plated on LB-agar plates containing rifampicin (50 mg/L). For animal experiments, the sample size analysis was performed to ensure that our sample size was more than sufficient for necessary power of 0.8 using the PASS software, version 12 (NCSS, LLC, Kaysville, UT, USA). Investigators were not blinded to experimental conditions, and no randomization or exclusion of data points was used.

Cytokines, histone acetylation and HAT/HDAC assays

Spleen and blood samples were collected from all the above mice groups at 1 day, 5 days and 10 days post-BI for cytokines, histone acetylation and HAT/HDAC assays.

Statistical analysis

Wherever applicable, at least three independent experiments were performed, and the data were analyzed using the Student's *t* test or a one-way analysis of variance (ANOVA) with Tukey's HSD Post-hoc test, as appropriate. Animal data were analyzed using the Kaplan-Meier survivability test. Bacterial CFU counts were analyzed using the Kruskal-Wallis non-parametric test with Dunn's post-test. For all experiments P values < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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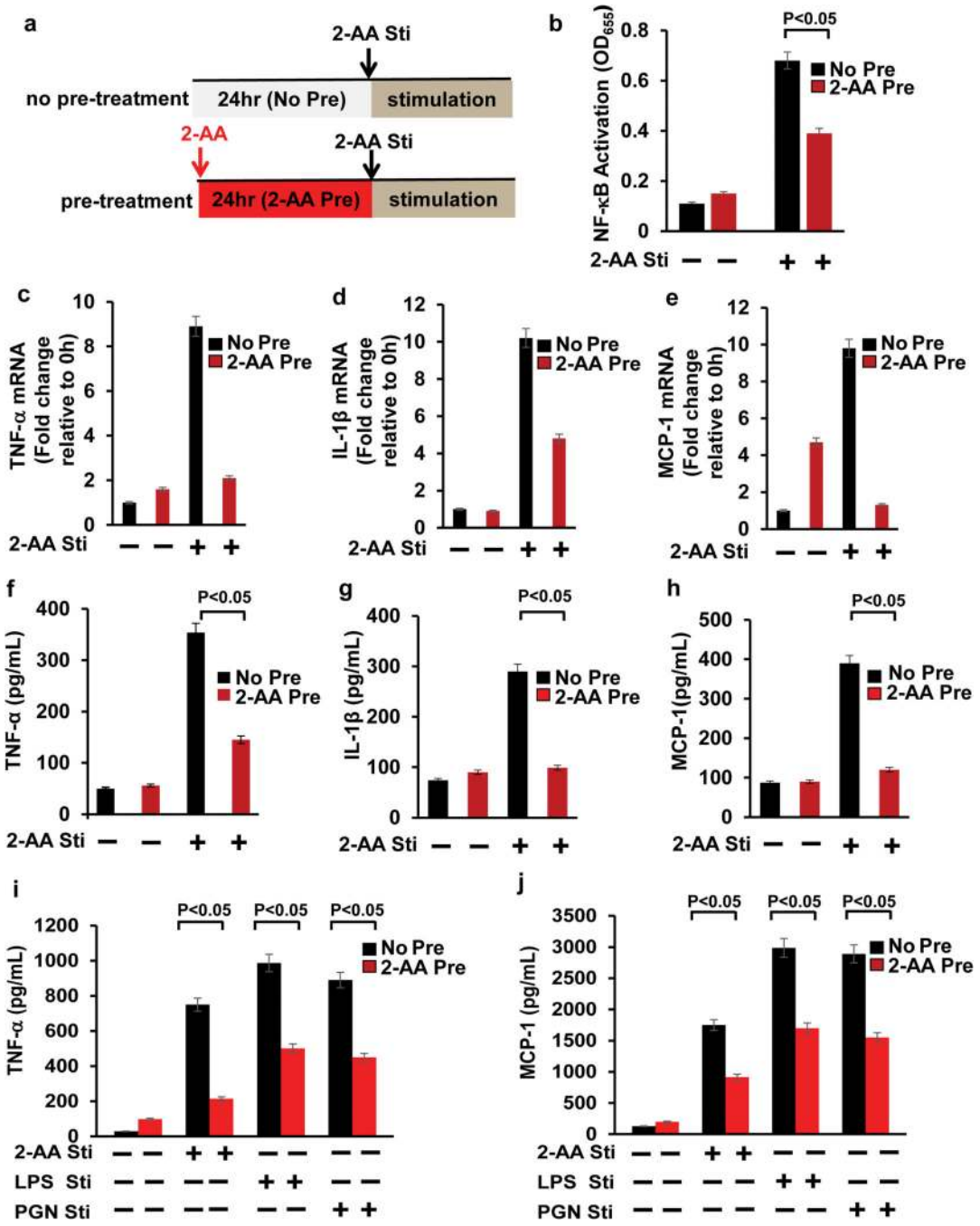

Figure 1. 2-AA pretreatment modulates activation of NF-κB and cytokines, and promotes long-term immunosuppression

(a) Experimental design: Human THP-1 monocytes were left untreated (No Pre) or pretreated with 2AA for 24-h (2-AA Pre), and then stimulated (Sti) with 2-AA. **(b)** SEAP assay analyzing NF-κB reporter activation in No Pre and 2-AA Pre cells after 2AA stimulation (4-h). (N = 3; means ± SDs; $p < 0.05$, Student's *t* test). **(c-e)** Real-time PCR analysis of *Tnf-α* (1-h), *IL-1β* (1-h) and *Mcp-1* (3-h) mRNA in No Pre and 2-AA Pre cells following 2AA stimulation. Transcript levels were normalized to *β-actin* (N = 3; means ±

SDs). **(f-h)** ELISA of pro-inflammatory cytokines in supernatants of No Pre and 2-AA Pre cells following 6-h 2-AA stimulation (N = 3; means ± SDs; $p < 0.05$, one-way ANOVA). **(i-j)** ELISA of TNF-α and MCP-1 in supernatants of No Pre and 2-AA Pre primary human macrophages, which were washed out and after 7 days and stimulated with 2-AA, LPS, or PGN for 6-h (N=3, means ± SD, $p < 0.05$, one-way ANOVA). Data are representative of three independent experiments.

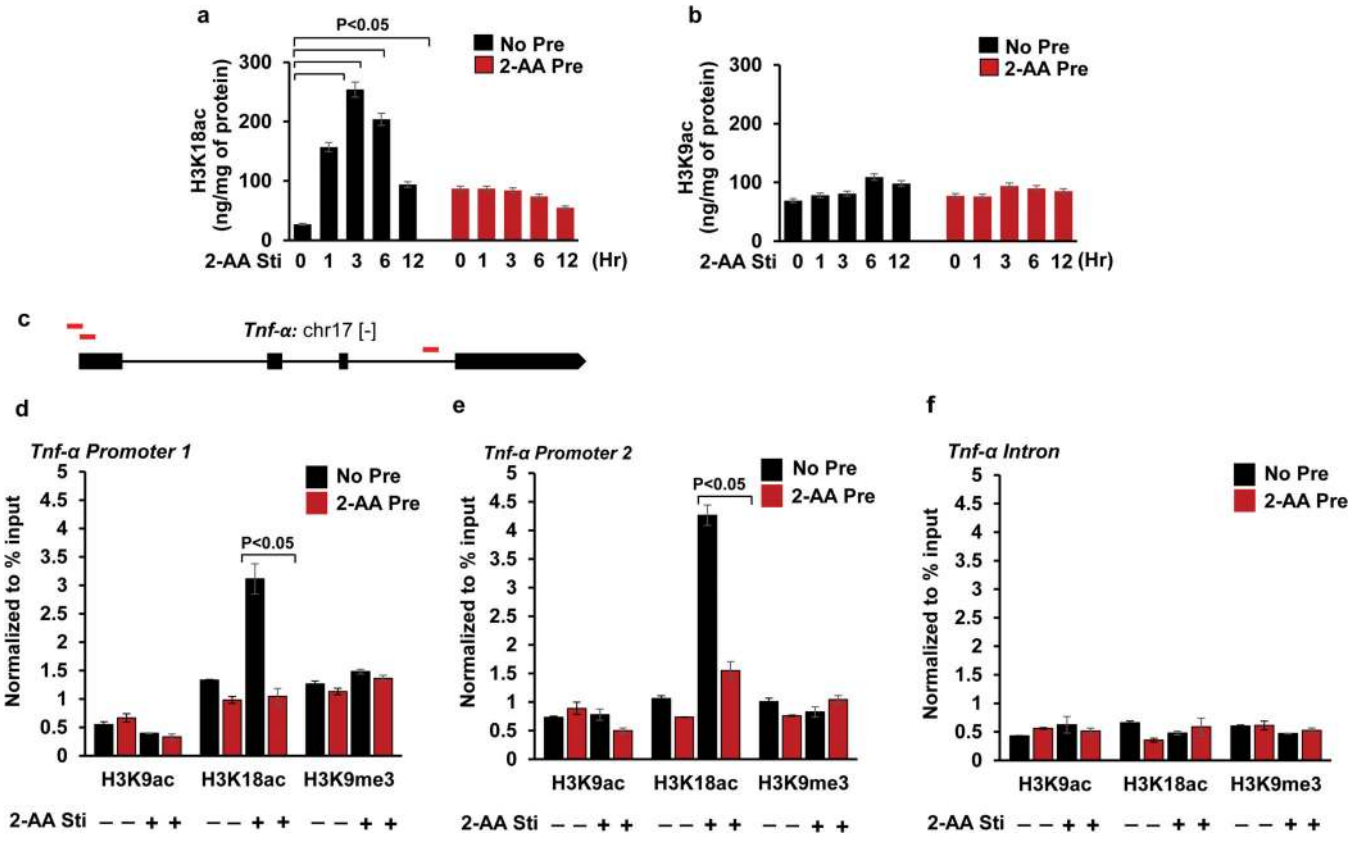

Figure 2. 2-AA pretreatment modulates histone acetylation

(**a-b**) Global acetylation levels of H3K18 and H3K9 in mouse macrophages in 2-AA pretreated and non-pretreated cells following 2-AA stimulation. (**c**) Red lines denote promoter 1, promoter 2, and intron primer target at *Tnf-α* locus. (**d-f**) ChIP assay of H3K18ac, H3K9ac, and H3K9me3 at the *Tnf-α* promoter in 2-AA pretreated or non-pretreated RAW264.7 cells following 3-h 2AA stimulation, assessed by real-time PCR with primers covering promoter sites and intronic region (as in **c**) of *Tnf-α* (**d-e**). The intronic region was unaffected **(f)**. (N = 3; means ± SDs; $p < 0.05$, Student's *t* test). Data are representative of three independent experiments.

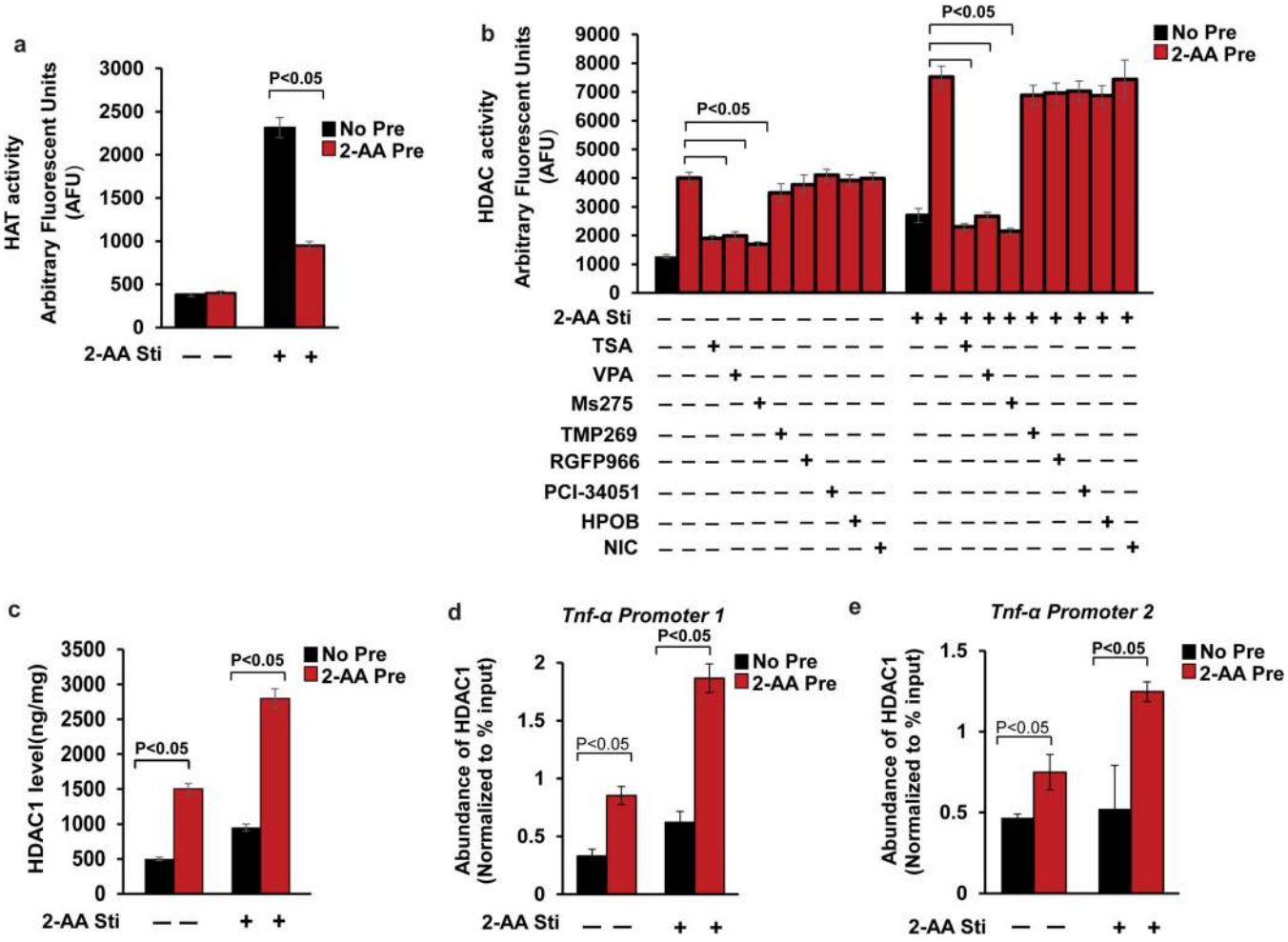

Figure 3. 2-AA pretreatment modulates the HAT/HDAC activity and promotes HDAC1 accumulation

(**a**) HAT activity in nuclear lysate of 2-AA pretreated THP-1 cells following 1-h 2-AA stimulation (N = 3; means ± SDs; $p < 0.05$, Student's *t* test). (**b**) Nuclear HDAC activity in 2-AA pretreated and non-pretreated THP-1 cells following 2AA stimulation +/−TSA, +/−VPA, +/− Ms275, +/− TMP269, +/− RGFP966, +/−PCI-34051, +/− HPOB or +/− NIC for 6-h, (N = 3; means ± SDs; $p < 0.05$, one-way ANOVA). (**c**) Quantitation of nuclear HDAC1 in 2AA-pretreated and nonpretreated THP-1 cells following 3-h 2-AA stimulation. (N = 3; means ± SDs; $p < 0.05$, Student's *t* test). (**d-e**) ChIP assay of HDAC1 abundance at *Tnf-α* promoter in 2-AA pretreated or non-pretreated RAW264.7 cells following 3-h 2-AA stimulation, assessed by real-time PCR with primers covering H3K18ac enriched site in *Tnf-α* promoter region (N = 3; means ± SDs; $p < 0.05$, Student's *t* test). Data are representative of three independent experiments.

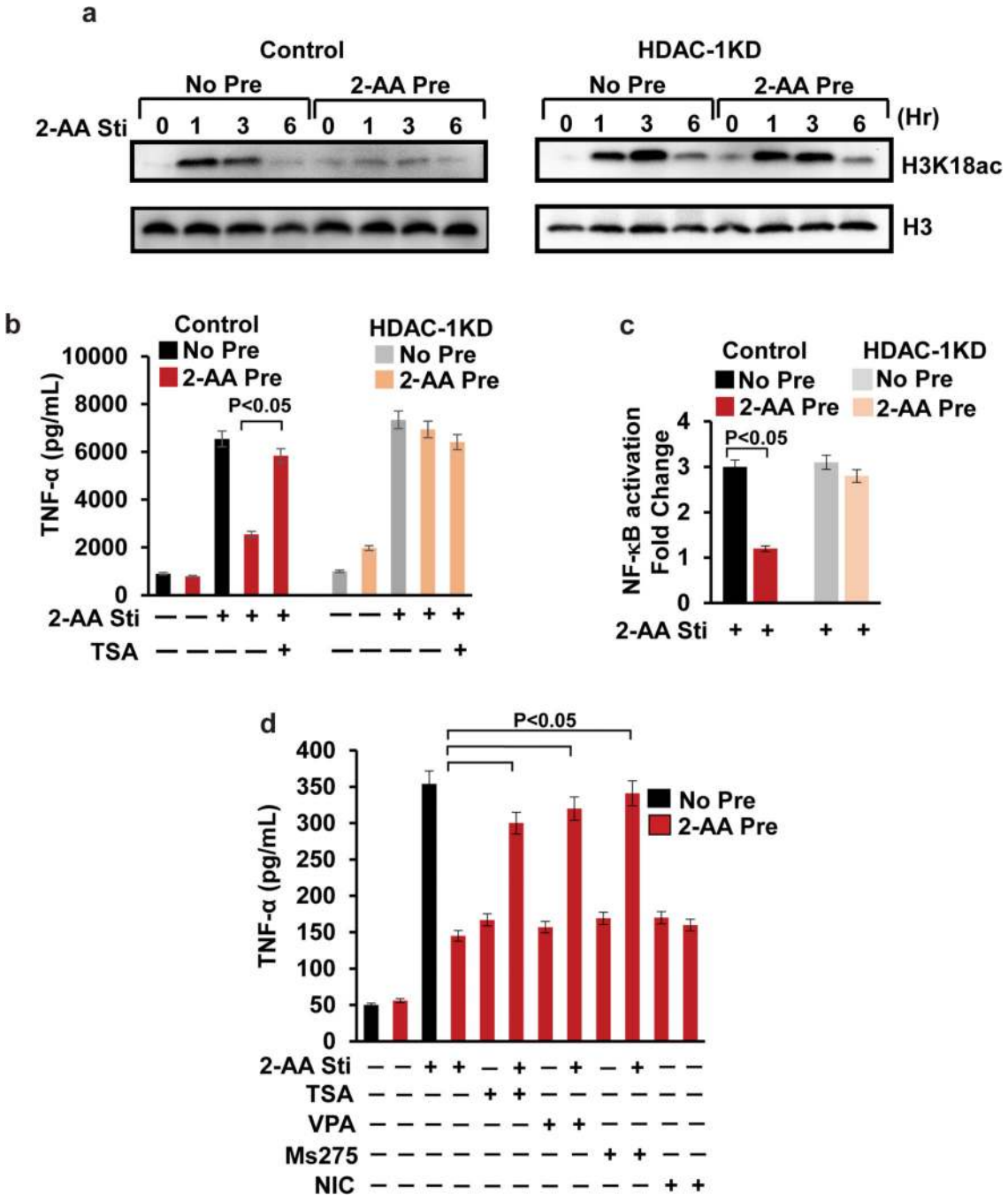

Figure 4. HDAC1 inhibition reinstates histone acetylation and NF-κB activation, and modulates cytokine secretion

(a) Immunoblot analysis of H3K18 acetylation in 2-AA pretreated vector control RAW264.7 and HDAC1 KD cells following 2-AA stimulation. Blots are representative of three independent experiments. (**b**) ELISA of TNF-α secretion in culture supernatants of 2-AA pretreated and nonpretreated vector control RAW264.7 and HDAC1 KD cells following 2-AA stimulation +/− TSA for 6-h. **(c)** SEAP assay of NF-κB activation in 2-AA pretreated or non-pretreated vector control RAW264.7 and HDAC1 KD cells following 4-h 2-AA

stimulation. **(d)** ELISA of TNF-α levels in culture supernatants of THP-1 cells pre-exposed (or not) to 2-AA with TSA, VPA, Ms275, or NIC following 6-h 2-AA stimulation. Data are representative of three independent experiments (b-d; N = 3; means ± SDs; $p < 0.05$, one-way ANOVA)

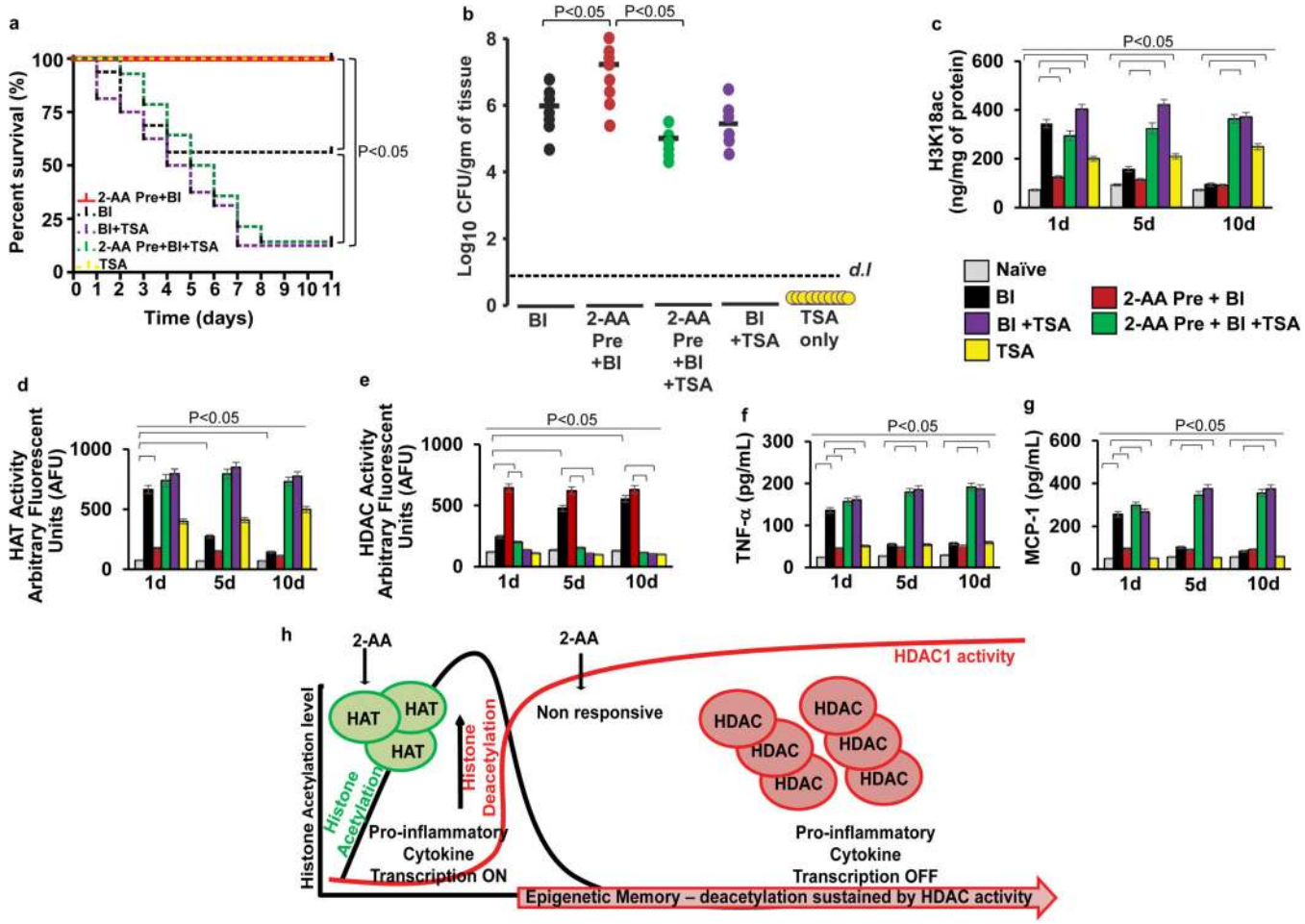

Figure 5. HDAC1 is important for 2-AA mediated host tolerance training *in vivo*

(**a**) Kaplan-Meier survival curves of BI mice infected with the *PA* clinical isolate PA14 (dotted black, N= 16), BI mice exposed to 2-AA 4 days prior to BI (red, N= 14), BI mice treated with 2-AA 4 days before BI and TSA after BI (dotted green, N = 14), and BI mice treated with TSA following BI (dotted purple, N = 16). Non-BI TSA-only treated mice (dotted yellow, N=10) served as a negative control group. All TSA-treated animals received TSA daily for 10 days. **(b)** Bacterial burden (as CFUs) in muscle 5 days posttreatment in BI mice that received 2-AA treatment 4 days prior to BI with (green, N = 6) or without TSA (red, N= 7); non-pretreated BI minus TSA (black N = 6), or plus TSA following BI (purple, N = 7). The TSA-only treatment group (yellow, N = 10) served as a negative control group. The significance between the infection groups was assessed by the Kruskal-Wallis test ($p < 0.05$). Each circle represents an individual mouse; small horizontal lines represent the median of log values. Dotted line represents the detection limit (d.l.). **(c)** Analysis of global level of H3K18 acetylation in the spleens of naïve, BI only, BI with TSA, 2-AA pretreated BI without TSA treatment, and 2-AA pretreated BI groups 1 day, 5 days, and 10 days post-BI. (**d-e**) Nuclear HAT (**d**) and HDAC (**e**) activity in spleens of 2-AA pretreated and non-pretreated BI mice with or without TSA 1 day, 5 days, and 10 days post-BI. **(f-g)** ELISA of TNF-α and MCP-1 in serum from 2-AA pretreated and non-pretreated BI mice with or without TSA. TSA-only treatment served as the negative control condition. Data are

representative of two independent experiments (**c-g**, N = 3 mice/group, means ± SDs; $p < 0.05$, Student's *t* test). **(h)** A schematic representation of 2-AA mediated reprogramming of innate immunity. Following cell stimulation, increased HAT activity initiates histone acetylation, potentiating transcription of pro-inflammatory genes; subsequent histone deacetylation prevents transcription. Upon subsequent stimulation, cells remain unresponsive as a result of sustained 2-AA induced deacetylation by HDAC1.