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(54) Title: HISTONE DEACETYLASE INHIBITION ENHANCES ANTIMICROBIAL PEPTIDE BUT NOT INFLAMMATORY CYTOKINE EXPRESSION UPON BACTERIAL CHALLENGE

(57) Abstract: The present invention relates to methods and therapeutic uses of histone deacetylase (HDAC) inhibitors for increasing antimicrobial peptides (AMP), particularly AMP beta- defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor further increases AMP expression compared to the microbial organism alone. In this context, HDAC inhibitors may be used for treating infections, in particular infections caused by bacteria, and dysbiosis-driven diseases.



HISTONE DEACETYLASE INHIBITION ENHANCES ANTIMICROBIAL PEPTIDE BUT NOT INFLAMMATORY CYTOKINE EXPRESSION UPON BACTERIAL CHALLENGE

5 BACKGROUND OF THE INVENTION

Antimicrobial peptides (AMP) are ancient and conserved defense effectors of the host innate immunity. In the intestine, these peptides are produced and secreted by epithelial cells to protect the mucosa against colonization by pathogens, and to maintain gut homeostasis in presence of the microbiota. Genes encoding AMP are either expressed
10 constitutively, or are inducible, and their global regulation remains so far insufficiently known. In this study, we aimed to clarify AMP gene regulation by deciphering possible specific characteristics allowing their enhanced expression among innate immune genes in general, particularly those encoding *bona fide* pro-inflammatory mediators. Our emphasis was on possible epigenetic regulation of the gene encoding the AMP beta-defensin-2 (DEFB2) taken as a model of possibly specific induction, upon challenge
15 with a commensal bacterium, as compared to the pro-inflammatory cytokine interleukin-8 (IL8).

Antimicrobial peptides (AMP) exert antimicrobial, antifungal, antiviral and antiprotozoan activity. They are expressed at high concentrations at the intestinal
20 mucosal surface, where they play a crucial role in host intestinal homeostasis. Therefore, approaches aiming at boosting expression of AMP represent a future therapeutic strategy to treat infections and dysbiosis-driven diseases in humans at a time of increasing incidence of antibiotic resistance.

Antimicrobial peptides (AMP) are efficient defense components of the innate immunity.
25 They play a crucial role in the mucosal homeostasis and protection against microbes. In the intestine, AMP are produced and secreted by epithelial cells. Most genes encoding these defense peptides are inducible in response to various signals. Regulation of inducible genes encompasses genetic and epigenetic mechanisms taking place at the chromatin level. Among them, remodeling of chromatin between relatively “open” and
30 “closed” forms has a key role. Such remodeling results from the modification of

nucleosomal structures. Nucleosomes constitute the fundamental unit of chromatin. They comprise approximately two turns of DNA wound around a histone octamer. A range of modifications of the amino-terminal “tails” of histone proteins are involved in this process, including methylation, phosphorylation, or acetylation (1). These
5 modifications also occur within the “globular” domain of histones, which establish extensive contacts with DNA strands. The consequence of such modifications on gene expression depends on the amino acid residues targeted and their close environment. Perturbing the balance between these modifications leads to changes in gene expression (2). Recent publications provide evidence for the effect of histone modifications on
10 regulation of the innate immune response and expression of associated defense genes (3, 4).

Among histone modifications, acetylation and deacetylation play a crucial role in transcriptional regulation of genes (5). The acetylation status of histone proteins is determined by the opposing actions of histone acetyl-transferases (HAT) and histone
15 deacetylases (HDAC). HAT add acetyl groups to the ϵ -amino group of lysine residues of nucleosomal histones, while HDAC remove these acetyl groups. In most cases, a positive correlation can be established between the level of histone acetylation and transcriptional activity. Acetylation of histones by HAT promotes a relaxed structure of the chromatin by decreasing the positive charges interacting with negatively charged
20 DNA strands, thereby facilitating transcriptional activation. Conversely, HDAC act as transcriptional repressors, due to histone deacetylation, and consequently promote chromatin condensation. In humans, 18 HDAC have been identified and classified based on their homology to yeast HDAC (6). Most of them are zinc-dependent proteins and their enzymatic activity can be inhibited by compounds such as trichostatin A
25 (TSA) or suberoylanilide hydroxamic acid (SAHA) (7, 8). On the other hand, HAT have been classified by families, based on their cellular localization and primary structure homology, and include the well-known p300 family (9).

Despite their name, a large number of non-histone proteins have been identified as substrates for both HAT and HDAC. Many of these proteins are transcription factors
30 involved in the regulation of gene expression, including the transcription factor NF- κ B that regulates a wide range of genes involved in the host innate immune response (10, 11). Reversible acetylation of the p65 subunit regulates diverse functions of NF- κ B,

including DNA binding and transcriptional activity, as well as its ability to associate with the cytoplasmic inhibitor I κ B α (12). Seven acetylated lysines have been identified within p65 (residues K122, K123, K218, K221, K310, K314 and K315). The majority of these residues are acetylated by the HAT p300 (13). For instance, acetylation of K310 is required for full transcriptional activity of NF- κ B (14). Conversely, several HDAC, including HDAC1, HDAC3 and SIRT1, have been found to specifically deacetylate p65, thereby negatively regulating the transcriptional activity of NF- κ B (12).

Most genes involved in the innate immune response are inducible genes whose expression needs to be tightly regulated and rapidly and specifically activated in response to diverse stimuli (15). This is the case at the human intestinal mucosal surface. Intestinal epithelial cells, being a first line of interaction with microbes, are endowed with innate immune functions encompassing the balanced expression of an array of genes, including those encoding AMP and pro-inflammatory cytokines. These two groups of genes are in general considered to be synchronously expressed under the necessity to protect the epithelium against pathogenic microbes and keep commensal bacteria at bay, away from the epithelial surface. We hypothesized, however, that these two groups of genes might also obey to differential regulatory rules that do not necessarily imply their synchronous expression. For instance, in the case of the response to a pathogenic microbe that necessitates the mobilization of both arms of the innate immune system, or in the case of controlling and tolerating the microbiota that will rather mobilize expression of antimicrobial molecules (pathological *versus* physiological inflammation).

BRIEF SUMMARY OF THE INVENTION

Using an *in vitro* model of intestinal epithelial cells challenged with *Escherichia coli* K12, we showed that inhibition of histone deacetylases (HDAC) by trichostatin A dramatically enhanced the induction of DEFB2 expression, without affecting the expression of IL8. At the chromatin level, this mechanism was supported by an increased phosphorylation of histone H3 on serine S10, preferentially at the DEFB2 promoter. This process occurred through activation of the I κ B kinase (IKK) complex, which also led to activation of the NF- κ B transcription factor. In addition, we

demonstrated that NF- κ B was post-translationally modified by acetylation upon HDAC inhibition, partly by the histone acetyltransferase p300, and that both NF- κ B and p300 supported enhanced induction of DEFB2 expression. Finally, we identified a set of additional genes belonging to antimicrobial defense and epithelial restitution pathways that showed a similar pattern of epigenetic control of their transcriptional regulation. This work opens the way to use epigenetic pharmacology to achieve strong induction of epithelial antimicrobial defenses, as exemplified by the key human beta-defensin-2, while limiting the deleterious risk of an inflammatory response.

The invention thus encompasses methods for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. In one embodiment, the method comprises contacting the cell with a histone deacetylase (HDAC) inhibitor, wherein the HDAC inhibitor further increases the level of DEFB2 expression in the cell. The combination of the microbial organism, preferably a bacterium, and the HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to a histone deacetylase (HDAC) inhibitor, for use for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for preparing a drug for use for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The

HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to a pharmaceutical composition comprising a histone deacetylase (HDAC) inhibitor, for use for increasing antimicrobial peptides (AMP),
5 particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are
10 preferably increased without modifying pro-inflammatory cytokine expression.

In one embodiment, the invention encompasses a method for increasing the level of AMP expressed by a cell when said cell is contacted with a microbial organism said method comprising contacting the cell with a histone deacetylase (HDAC) inhibitor and
15 also contacting the cell with said microbial organism that increases the level of AMP, wherein the HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to a histone deacetylase (HDAC) inhibitor, for use for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the
20 cell.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for preparing a drug for use for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

25 The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to a pharmaceutical composition comprising a histone
30 deacetylase (HDAC) inhibitor, for use for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are preferably increased without modifying pro-inflammatory cytokine expression. Preferably, the AMP is beta-defensin-2 (DEFB2). Preferably, the microbial organism is a bacterium.

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In one embodiment, the invention encompasses a method for increasing AMP expression in a cell comprising contacting the cell with a microbial organism that increases the level of an AMP and contacting the cell with a histone deacetylase (HDAC) inhibitor, wherein the combination of the HDAC inhibitor and the microbial
10 organism increases the level of AMP expression in the cell more than the microbial organism alone.

The present invention also relates to a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP, for use for increasing AMP expression in a cell. The combination of the HDAC inhibitor and the
15 microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP for preparing a drug for use for increasing AMP expression in a cell. The combination of
20 the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP for increasing AMP expression in a cell. The combination of the HDAC inhibitor and the
25 microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

The present invention also relates to a pharmaceutical composition comprising a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP, for use for increasing AMP expression in a cell. The
30 combination of the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are preferably increased without modifying pro-inflammatory cytokine expression. Preferably, the AMP is beta-defensin-2 (DEFB2). Preferably, the microbial organism is a bacterium.

5

In one embodiment, the invention encompasses methods for increasing AMP expression in a cell comprising contacting the cell with microbial organism that increases the level of an AMP; and contacting the cell with a histone deacetylase (HDAC) inhibitor, wherein the HDAC inhibitor further increases the level of AMP expression in the cell.

10 The present invention also relates to a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP, for use for increasing AMP expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP for
15 preparing a drug for use for increasing AMP expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP for
20 increasing AMP expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to a pharmaceutical composition comprising a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP, for use for increasing AMP expression in a cell. The
25 HDAC inhibitor further increases the level of AMP expression in the cell.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are preferably increased without modifying pro-inflammatory cytokine expression. Preferably, the AMP is beta-defensin-2 (DEFB2). Preferably, the microbial organism is a bacterium. More preferably, the AMP is beta-defensin-2 (DEFB2) and the microbial
30 organism is a bacterium.

In preferred embodiments of the above methods, therapeutic uses and pharmaceutical compositions, the HDAC inhibitor is trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA).

In various embodiments, the HDAC inhibitor is selected from Panobinostat (LBH589),
5 Vorinostat (SAHA), Entinostat (MS-275), Belinostat (PXD101), Abexinostat (PCI-24781), Dacinostat (LAQ824), Quisinostat (JNJ-26481585), Mocetinostat (MGCD0103), Valproic acid, CUDC-101, Droxinostat, Pracinostat (SB939), PCI-34051, Givinostat (ITF2357), AR-42, Tubastatin A, CUDC-907, M344, Tacedinaline (CI996), Rocilinostat (ACY-1215), Abexinostat/PCI-24781, and Scriptaid.

10 In a preferred embodiment, the cell is contacted with a concentration of between 0.5 μ m and 50 μ m of the HDAC inhibitor.

Such methods may be performed in vitro, ex vivo or in vivo.

The present invention also relates to a histone deacetylase (HDAC) inhibitor, for use in
15 the treatment of infections and dysbiosis-driven diseases in humans.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for preparing a drug for use in the treatment of infections and dysbiosis-driven diseases in humans.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor
20 in the treatment of infections and dysbiosis-driven diseases in humans.

The present invention also relates to a pharmaceutical composition comprising a histone deacetylase (HDAC) inhibitor, for use in the treatment of infections and dysbiosis-driven diseases in humans.

The present invention also relates to a method of treating an infections or a dysbiosis-driven disease in a human subject in need thereof, comprising administering to said
25 human subject an efficient amount of a histone deacetylase (HDAC) inhibitor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. HDAC inhibition enhances expression of the beta-defensin DEFB2 upon bacterial challenge. (A) Transcriptional expression of genes encoding the beta-defensin
30 DEFB2 and the interleukin IL8 in cells challenged with the *Escherichia coli* K12 commensal strain. Values are presented on a logarithmic scale as the ratio of gene

expression in challenged cells compared to non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. **(B)** Transcriptional expression of the DEFB2 and IL8 genes in cells pretreated for 16 h with increasing concentrations of the HDAC inhibitor trichostatin A (0-50 μ M TSA), and then challenged for 2 h with *E. coli*. Values are presented on a logarithmic scale as the ratio of gene expression in pretreated and challenged cells (black bars), or pretreated and non-challenged cells (white bars), compared to non-treated and non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for TSA pretreated cells, challenged or not, compared to non-treated cells, challenged or not. **(C)** ELISA dosage of the DEFB2 and IL8 peptides in supernatants of cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not for 6 h with *E. coli*. Bacterial challenges were stopped by addition of gentamicin, and supernatants were collected 24 h after the beginning of the challenge. Experiments were performed at a MOI of 10 bacteria per cell. Values are presented on a logarithmic scale in picogram of peptide per milliliter. N = 3 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. Black bars: 5 μ M TSA pretreated cells; white bars: non-treated cells. Error bars represent the SD. *, P < 0,01 for TSA pretreated cells compared to non-treated cells.

Figure 2. HDAC inhibition activates the IKK complex and induces phosphorylation of the histone H3 protein. **(A)** Immunoblot analysis of histone H3 acetylation on lysine K9, K27 and K56, and phosphorylation on serine S10, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not with *E. coli*. After lysis of cells at the indicated time points, western-blot analysis was performed using antibodies directed against histone post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation. “P” prefix: phosphorylation. **(B)** Chromatin immunoprecipitation analysis of the phosphorylated H3S10 protein at the DEFB2 and IL8 promoters, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not for 1 h with *E. coli*. Experiments were performed at a MOI of 10 bacteria per cell. Enrichment in chromatin was detected using anti-P-H3S10 antibody or rabbit IgG as control, and

quantified by qRT-PCR using specific primers matching the DEFB2 or IL8 promoters. Values are presented as the percentage of signal relative to the input. N = 3 independent experiments. NC: non-challenged cells; NC+TSA: non-challenged cells pretreated with 5 μ M TSA; K12: cells challenged with the *E. coli* K12 strain; K12+TSA: cells pretreated with 5 μ M TSA and challenged with the *E. coli* K12 strain. Black bars: signal detected using the P-H3S10 antibody; white bars: signal detected using the IgG control. Error bars represent the SD. *, P < 0,05 for K12+TSA compared to K12. (C) Immunoblot analysis of the IKK α and IKK β proteins, the IKK α / β complex phosphorylation, and the I κ B α protein, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not with *E. coli*. After lysis of cells at the indicated time points, western-blot analysis was performed using antibodies directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. “P” prefix: phosphorylation.

Figure 3. NF- κ B is acetylated upon HDAC inhibition and mediates the enhanced induction of the DEFB2 gene expression. (A) Transcriptional expression of the DEFB2 and IL8 genes in cells transfected with siRNA silencing the p65 NF- κ B subunit, pretreated or not for 16 h with 5 μ M TSA, and finally challenged for 2 h with *E. coli*. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells, pretreated or not, compared to non-challenged and non-treated cells. Experiments were performed at MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for cells transfected with p65 siRNA, compared to cells transfected with scrambled siRNA. Black bars: p65 siRNA transfected cells; white bars: scrambled (SC) siRNA transfected cells. (B) Kinetic of DEFB2 and IL8 expression in cells inhibited for the NF- κ B pathway by treatment with BMS-345541, pretreated or not for 16 h with 5 μ M TSA, and then challenged with *E. coli*. Values are presented on a logarithmic scale as the ratio of gene expression in TSA pretreated and challenged cells (black lines), or non-treated and challenged cells (grey lines), compared to non-treated and non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. Dashed lines: with NF- κ B inhibitor BMS-345541; solid lines: without NF- κ B inhibitor BMS-345541. (C) Immunoblot analysis of the p65 protein, and the p65 K310 acetylated

protein, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not with *E. coli*. After lysis of cells at the indicated time points, western-blot were performed using antibodies directed against the protein with or without its modification mark. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation. **(D)** Chromatin immunoprecipitation analysis of the K310 acetylated p65 protein at the DEFB2 and IL8 promoters, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not for 1 h with *E. coli*. Experiments were performed at a MOI of 10 bacteria per cell. Enrichment in chromatin was detected using an anti-Ac-p65 antibody or rabbit IgG as control, and quantified by qRT-PCR using specific primers matching the DEFB2 or IL8 promoters. Values are presented as the percentage of signal relative to the input. N = 3 independent experiments. NC: non-challenged cells; NC+TSA: non-challenged cells pretreated with 5 μ M TSA; K12: cells challenged with the *E. coli* K12 strain; K12+TSA: cells pretreated with 5 μ M TSA and challenged with the *E. coli* K12 strain. Black bars: signal detected using the Ac-p65 antibody; white bars: signal detected using the IgG control. Error bars represent the SD. *, P < 0,05 for NC+TSA compared to NC, and K12+TSA compared to K12.

Figure 4. p300 acetylates NF- κ B and supports the enhanced induction of the DEFB2 gene expression upon HDAC inhibition. **(A)** Immunoblot analysis of the HAT p300, and p65 protein acetylation on the K310 lysine, in cells transfected with siRNA silencing p300, and challenged for 1 h with *E. coli* K12. After lysis of cells, western-blot were performed using specific antibodies directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain; SC: scrambled siRNA transfected cells; p300: p300 siRNA transfected cells. “Ac” prefix: acetylation. **(B)** Transcriptional expression of DEFB2 and IL8 in cells transfected with siRNA silencing p300, and challenged for 2 h with *E. coli*. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells. Experiments were performed at MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for cells transfected with p300 siRNA, compared to cells transfected with scrambled siRNA. Black bars: p300 siRNA transfected cells; white

bars: scrambled (SC) siRNA transfected cells. **(C)** Transcriptional expression of DEFB2 and IL8 in cells treated with increasing concentrations of the p300 inhibitor C646 (0-50 μ M C646), and challenged for 2 h with *E. coli*. Values are presented on a logarithmic scale, as the ratio of gene expression in challenged cells, compared to non-challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for C646 treated cells, compared to non-treated cells. **(D)** Chromatin immunoprecipitation analysis of p300 recruitment at the DEFB2 and IL8 promoters, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not for 1 h with *E. coli*. Experiments were performed at a MOI of 10 bacteria per cell. Enrichment in chromatin was detected using anti-p300 antibody or rabbit IgG as control, and quantified by qRT-PCR using specific primers matching the DEFB2 or IL8 promoters. Values are presented as the percentage of signal relative to the input. N = 3 independent experiments. NC: non-challenged cells; NC+TSA: non-challenged cells pretreated with 5 μ M TSA; K12: cells challenged with the *E. coli* K12 strain; K12+TSA: cells pretreated with 5 μ M TSA and challenged with the *E. coli* K12 strain. Black bars: signal detected using the p300 antibody; white bars: signal detected using the IgG control. Error bars represent the SD. *, P < 0,05 for K12+TSA compared to K12.

Figure 5. HDAC inhibition enhances expression of other genes from the antimicrobial defense and epithelial restitution pathways. **(A)** Transcriptional analysis of the whole genome of cells pretreated or not for 16 h with 5 μ M TSA, and then challenged for 2 h with the *E. coli* K12 strain. After RNA extraction, RNA sequencing was performed on each sample to determine the number of reads per gene. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells (X axis), against gene expression in TSA pretreated and challenged cells compared to non-treated and non-challenged cells (Y axis). Experiments were performed at a MOI of 10 bacteria per cell. **(B-C)** Transcriptional expression of gene clusters from the antimicrobial defense and epithelial restitution pathways **(B)**, and the pro-inflammatory pathway **(C)**. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells (white bars), or TSA pretreated and challenged cells compared to non-treated and non-challenged cells (black bars).

Figure 6. TSA-mediated HDAC inhibition enhances expression of AMP upon bacterial challenge.

(A) Transcriptional expression of the AMP DEFB3 and LL37 genes, and pro-inflammatory IL1B and CCL20 genes, in cells challenged with the *Escherichia coli* K12 commensal strain. Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells compared to non-challenged cells.

Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. **(B)** Cellular viability of cells challenged with *E. coli*. Lactate dehydrogenase assays were performed using supernatants of challenged cells. Values represent the percentage of cell death. N = 3 independent experiments.

Error bars represent the SD. **(C)** Transcriptional expression of DEFB3, LL37, IL1B and CCL20, in cells pretreated for 16 h with increasing concentrations of TSA (0-50 μ M TSA), and challenged for 2 h with *E. coli*. Values are presented on a logarithmic scale as the ratio of gene expression in pretreated and challenged cells (black bars), or pretreated and non-challenged cells (white bars), compared to non-treated and non-

challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for pretreated cells, challenged or not, compared to non-treated and non-challenged cells. **(D)** Cellular viability of cells pretreated for 16 h with increasing concentrations of TSA (0-50 μ M TSA), and then challenged for 2 h with *E. coli*. Lactate dehydrogenase assays were performed using supernatants from pretreated and challenged cells. Values represent the percentage of cell death. N = 3 independent experiments. Error bars represent the SD.

Figure 7. SAHA-mediated HDAC inhibition enhances expression of AMP upon bacterial challenge.

(A) Transcriptional expression of the AMP DEFB2, DEFB3, and LL37 genes, and the pro-inflammatory IL8, IL1B, and CCL20 genes, in cells pretreated for 16 h with increasing concentrations of SAHA (0-500 μ M SAHA), and then challenged for 2 h with the *E. coli* K12 strain. Values are presented on a logarithmic scale as the ratio of gene expression in pretreated and challenged cells (black bars), or pretreated and non-challenged cells (white bars), compared to non-treated and non-

challenged cells. Experiments were performed at a MOI of 10 bacteria per cell. N = 3 independent experiments. Error bars represent the SD. *, P < 0,01 for pretreated cells, challenged or not, compared to non-treated and non-challenged cells. **(B)** Cellular viability of cells pretreated for 16 h with increasing concentrations of SAHA (0-500 μ M

SAHA), and then challenged for 2 h with *E. coli*. Lactate dehydrogenase assays were performed using supernatants from pretreated and challenged cells. Values represent the percentage of cell death. N = 3 independent experiments. Error bars represent the SD. (C) ELISA dosage of the DEFB2 and IL8 peptides in supernatants of cells pretreated or not for 16 h with 5 μ M SAHA, and then challenged or not for 6 h with *E. coli*. Bacterial challenges were stopped by addition of gentamicin, and supernatants were collected 24 h after the beginning of the challenge. Experiments were performed at a MOI of 10 bacteria per cell. Values are presented on a logarithmic scale in picogram of peptide per milliliter. N = 3 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. Black bars: 5 μ M SAHA pretreated cells; white bars: non-treated cells. Error bars represent the SD. *, P < 0,01 for pretreated cells compared to non-treated cells.

Figure 8. HDAC inhibition modifies the acetylation pattern of histone H3 and H4 proteins.

(A) Immunoblot analysis of histone H3 protein acetylation marks on lysine residues K9+K14+K18+K23+K27, K4, K14, K18, and K23, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not with *E. coli*. After lysis of cells at the indicated time points, western-blot analysis was performed using specific antibodies directed against histone post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation. (B) Immunoblot analysis of histone H4 protein acetylation marks on lysine residues K5+K8+K12+K16, K5, K8, K12, and K16, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not with *E. coli*. After lysis of cells at the indicated time points, western-blot analysis was performed using specific antibodies directed against histone post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. “Ac” prefix: acetylation.

Figure 9. Phosphorylation status of the Erk, p38, and SAPK/JNK MAPK upon HDAC inhibition.

Immunoblot analysis of Erk1/2, phosphorylated Erk1/2, p38, phosphorylated p38, SAPK/JNK, and phosphorylated SAPK/JNK, in cells pretreated or not for 16 h with 5 μ M TSA, and then challenged or not with *E. coli*. After lysis of cells at the indicated time points, western-blot analysis was performed using specific antibodies

directed against proteins or post-translational modification marks. Experiments were performed at a MOI of 10 bacteria per cell. N = 2 independent experiments. NC: non-challenged cells; K12: cells challenged with the *E. coli* K12 strain. "P" prefix: phosphorylation.

- 5 **Figure 10. Translocation of the NF- κ B p65 subunit in nuclei upon HDAC inhibition.** Detection (A) and quantification (B) of the p65-associated signal in nuclei by immunofluorescence experiments, in cells pretreated for 16 h with 5 μ M TSA, and then challenged for 30 minutes with *E. coli*. Experiments were performed at a MOI of 10 bacteria per cell. Results are representative of two independent experiments.
- 10 Magnification: $\times 40$. NC: non-challenged cells; NC+TSA: non-challenged cells pretreated with 5 μ M TSA; K12: cells challenged with the *E. coli* K12 strain; K12+TSA: cells treated with TSA and challenged with the *E. coli* K12 strain. Error bars represent the SD.

- 15 **Figure 11. Enhanced induction of the DEFB2 gene expression upon HDAC (TSA & SAHA) inhibition and E.coli K12 strain challenge.** Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells, pretreated or not, compared to non-challenged and non-treated cells.

- Figure 12. Enhanced induction of the DEFB2 gene expression upon HDAC (TSA & SAHA) inhibition and E.coli LF82 strain challenge.** Values are presented on a
- 20 logarithmic scale as the ratio of gene expression in challenged cells, pretreated or not, compared to non-challenged and non-treated cells.

- Figure 13. Enhanced induction of the DEFB2 gene expression upon HDAC inhibition (Abexinostat) and E.coli K12 and E.coli LF82 strain challenge.** Values are presented on a logarithmic scale as the ratio of gene expression in challenged cells,
- 25 pretreated or not, compared to non-challenged and non-treated cells.

- Figure 14. Potential of 24 HDACi to stimulate expression of the beta-defensin-2 antimicrobial peptide in TC7 cells. (A)** Transcriptional expression of HBD2 and IL8 genes at 24 and 48 hours post treatment. Values are presented on a logarithmic scale as the ratio of gene expression in treated cells compared to non-treated cells. **(B)** ELISA
- 30 dosage of HBD2 and IL8 peptides secreted in supernatant of cells treated with HDAC inhibitors for 48 hours. Values are presented on a linear scale in picogram of peptide per ml.

Figure 15. Potential of 24 HDACi to stimulate expression of additional antimicrobial peptide genes in TC7 cells. Transcriptional expression of genes encoding 4 antimicrobial peptides and 2 restitution effectors at 24 and 48 hours post treatment. Values are presented on a logarithmic scale as the ratio of gene expression in treated cells compared to non-treated cells.

Figure 16. Potential of 24 HDACi to stimulate expression of 12 beta-defensin genes in TC7 cells. Transcriptional expression of genes encoding 12 antimicrobial peptides at 48 hours post treatment. Values are presented on a logarithmic scale as the ratio of gene expression in treated cells compared to non-treated cells.

Figure 17. Potential of 24 HDACi to modulate expression of 6 pro-inflammatory genes in TC7 cells. Transcriptional expression of genes encoding 6 pro-inflammatory cytokines at 24 and 48 hours post treatment. Values are presented on a logarithmic scale as the ratio of gene expression in treated cells compared to non-treated cells.

Figure 18. Potential of 24 HDACi to stimulate expression of antimicrobial peptides in mICcl2 murine cells. Transcriptional expression of genes encoding 4 antimicrobial peptides and 4 pro-inflammatory cytokines at 24 hours post HDACi treatment. Values are presented on a logarithmic scale as the ratio of gene expression in treated cells compared to non-treated cells.

Figure 19. Effect of 4 HDACi on expression of the beta-defensin-2 antimicrobial peptide in TC7 cells upon an *Escherichia coli* K12 challenge. Transcriptional expression and ELISA dosage of the beta-defensin-2 in TC7 cells pre-treated for 16 hours with 5 μ M HDACi (from the hydroxamic and short chain fatty acid families), and challenged for 2 hours with *E. coli* K12 (multiplicity of infection of 10 bacteria per cell). Values are presented on a logarithmic scale. For transcriptional expression, data are presented as the ratio of gene expression in cells treated or not with HDACi, challenged or not with *E. coli*, compared to non-treated and non-challenged cells. For ELISA dosage, values are presented in picogram of peptide per ml of supernatant.

Figure 20. Effect of 10 additional HDACi on expression of the beta-defensin-2 antimicrobial peptide in TC7 cells upon an *Escherichia coli* K12 challenge. Transcriptional expression of the beta-defensin-2 in TC7 cells pre-treated for 16 hours with 5 μ M HDACi (from the benzamides, the hydroxamic and short chain fatty acid families), and challenged for 2 hours with *E. coli* K12 (multiplicity of infection of 10

bacteria per cell). Values are presented on a logarithmic scale as the ratio of gene expression in cells treated or not with HDACi, challenged or not with *E. coli*, compared to non-treated and non-challenged cells. NS: non-treated cells; NS+M: cells treated with 5 μ M HDACi; K12: cells challenged with *E. coli*; K12+M: cells treated with 5 μ M HDACi and challenged with *E. coli*.

Figure 21. Effect of 4 HDACi on expression of the interleukin-8 pro-inflammatory cytokine in TC7 cells upon an *Escherichia coli* K12 challenge. Transcriptional expression and ELISA dosage of the interleukin-8 in TC7 cells pre-treated for 16 hours with 5 μ M HDACi (from the hydroxamic and short chain fatty acid families), and challenged for 2 hours with *E. coli* K12 (multiplicity of infection of 10 bacteria per cell). Values are presented on a logarithmic scale. For transcriptional expression, data are presented as the ratio of gene expression in cells treated or not with HDACi, challenged or not with *E. coli*, compared to non-treated and non-challenged cells. For ELISA dosage, values are presented in picogram of peptide per ml of supernatant.

Figure 22. Effect of 10 additional HDACi on expression of the interleukin-8 pro-inflammatory cytokine in TC7 cells upon an *Escherichia coli* K12 challenge. Transcriptional expression of the interleukin-8 in TC7 cells pre-treated for 16 hours with 5 μ M HDACi (from the benzamides, the hydroxamic and short chain fatty acid families), and challenged for 2 hours with *E. coli* K12 (multiplicity of infection of 10 bacteria per cell). Values are presented on a logarithmic scale as the ratio of gene expression in cells treated or not with HDACi, challenged or not with *E. coli*, compared to non-treated and non-challenged cells. NS: non-treated cells; NS+M: cells treated with 5 μ M HDACi; K12: cells challenged with *E. coli*; K12+M: cells treated with 5 μ M HDACi and challenged with *E. coli*.

Figure 23. Effect of the Abexinostat HDACi on kinetic of expression of the beta-defensin-2 antimicrobial peptide gene upon an *Escherichia coli* challenge. Kinetic of expression of the HBD2 and IL8 genes in cells pretreated for 16 hours with 5 μ M HDACi, and then challenged with *E. coli* K12 (commensal) or *E. coli* LF82 (pathobiont). Values are presented on a logarithmic scale as the ratio of gene expression in cells pretreated or not with HDACi, challenged or not with *E. coli*, compare to non-treated and non-challenged cells.

Figure 24. Potential of the Trichostatin A HDACi to stimulate expression of several antimicrobial peptide genes in human colonic primary cells. Transcriptional expression of the HBD2, HBD3, and LL37 antimicrobial peptide genes, and IL1B, IL8, and TNF pro-inflammatory genes, in 6 days old human colonic organoids treated or not with 5 μ M TSA, and stimulated or not with 5 μ g/ml flagellin, for 24 h. Values are presented on a logarithmic scale as the ratio of gene expression in organoids treated or not with TSA, stimulated or not with flagellin, compared to non-treated and non-stimulated organoids. NS: non-stimulated organoids; NS+TSA: non-stimulated organoids treated with 5 μ M TSA; Flag: organoids stimulated with 5 μ g/ml flagellin; Flag+TSA: organoids treated with 5 μ M TSA and stimulated with 5 μ g/ml flagellin. *, $p < 0,05$ evaluated by Mann-Whitney U test.

DETAILED DESCRIPTION OF THE INVENTION

In an attempt to identify the additional regulatory circuit that may disconnect the expression of the two groups of antimicrobial and inflammatory genes when epithelial cells are engaged by a bacterium, we made the hypothesis that the expression of some of the genes encoding AMP, such as the beta-defensin-encoding gene DEFB2, may be “protected” by epigenetic mechanisms, in comparison to the *bona fide* pro-inflammatory genes. We accordingly conducted an in-depth analysis aiming to compare the epigenetic regulation of a restricted set of genes that are characteristic of the two groups, genes encoding inducible AMP (beta-defensins DEFB2, DEFB3, and cathelicidin LL37) and genes encoding pro-inflammatory cytokines (interleukins IL1B, IL8, and chemokine CCL20), in human intestinal epithelial cells. Using the *Escherichia coli* K12 commensal bacterium as an inducer, we reveal that expression of DEFB2 is greatly enhanced upon HDAC inhibition by TSA, while expression of IL8 is not modified. We investigate the molecular mechanism underlying this observation and identify an increased phosphorylation of the histone H3 protein on the serine S10 residue that occurs preferentially at the DEFB2 promoter. We show that this HDAC inhibition-dependent process happens through activation of the I κ B kinase (IKK) complex, which ultimately leads to activation of NF- κ B. Moreover, we demonstrate that NF- κ B is post-translationally acetylated on the p65 K310 lysine residue, partly by the HAT p300, and that both NF- κ B and p300 mediate the enhanced induction of DEFB2

expression. Finally, we identify other genes from the antimicrobial defense and epithelial restitution pathways that show a similar expression pattern upon HDAC inhibition. This work highlights the existence of a differential regulatory mechanism occurring in intestinal epithelial cells between antimicrobial and pro-inflammatory genes, which takes place at the chromatin level, through an acetylation-dependent process.

GENERAL DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

The term "a" and "an" refers to "one" or to "more than one" of the grammatical object of the article (i.e., at least one including 2, 3, 4, 5, etc) unless the context clearly dictates otherwise. By way of example, the term "a therapeutic vaccine" includes one therapeutic vaccine or a plurality of therapeutic vaccines, including mixtures thereof.

As used herein, when used to define products, compositions and methods, the term "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are open-ended and do not exclude additional, unrecited elements or method steps. Thus, a composition "comprises" the recited components when such components might be part of the final composition. "Consisting essentially of" means excluding other components or steps of any essential significance. Thus, a composition consisting essentially of the recited components would not exclude trace contaminants and pharmaceutically acceptable carriers. "Consisting of" means excluding more than trace elements of other components or steps.

In the present description, the term "treating" or "treatment" means an improvement of the patient's disease, which may be observed at the clinical, histological, biochemical level. In particular, any alleviation of a clinical, histological or biochemical symptom of the disease is included in the terms "treating" and "treatment". In the context of infections, "treating" or "treatment" thus notably relates to the fact to reduce the load of

microbial organism in the treated subject, or to reduce associated symptoms, such as fever and diarrhea. Treatment may require administration of a HDAC inhibitor more than once.

As used herein, a “therapeutically efficient amount” refers to an amount sufficient for the intended use. For the anti- HDAC inhibitor used according to the invention in the treatment of infections, it refers to an amount sufficient to reduce the load of microbial organism in the treated subject or associated symptoms such as fever and diarrhea.

METHODS, USES AND PHARMACEUTICAL COMPOSITIONS

The invention encompasses methods for increasing AMP, preferably beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP has already been increased by the presence of a microbial organism, preferably a bacterium. In preferred embodiments, the microbial organism increase the AMP level of a cell when the cell is contacted with the microbial organism.

In one embodiment, the method comprises contacting the cell with a histone deacetylase (HDAC) inhibitor, wherein the HDAC inhibitor further increases the level of an AMP, preferably DEFB2, expression in the cell.

The present invention also relates to a histone deacetylase (HDAC) inhibitor, for use for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor further increases the level of AMP (preferably DEFB2) expression in the cell, i.e. the combination of the microbial organism, preferably a bacterium, and the HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for preparing a drug for use for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor further increases the level of AMP (preferably DEFB2) expression in the cell, i.e. the combination of the microbial organism, preferably a bacterium, and the HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The
5 HDAC inhibitor further increases the level of AMP (preferably DEFB2) expression in the cell, i.e. the combination of the microbial organism, preferably a bacterium, and the HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

The present invention also relates to a pharmaceutical composition comprising a histone
10 deacetylase (HDAC) inhibitor, for use for increasing antimicrobial peptides (AMP), particularly AMP beta-defensin-2 (DEFB2), expression in a cell in which the level of the AMP (e.g., DEFB2) has already been increased by the presence of a microbial organism, preferably a bacterium. The HDAC inhibitor further increases the level of AMP (preferably DEFB2) expression in the cell, i.e. the combination of the microbial
15 organism, preferably a bacterium, and the HDAC inhibitor results in a higher level of AMP expression than the microbial organism alone.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are preferably increased without modifying pro-inflammatory cytokine expression. Preferably, the AMP is beta-defensin-2 (DEFB2). Preferably, the microbial organism is
20 a bacterium. Even more preferably, the AMP is beta-defensin-2 (DEFB2) and the microbial organism is a bacterium.

In one embodiment, the invention encompasses a method for increasing the level of
25 AMP expressed by a cell when said cell is contacted with a microbial organism said method comprising contacting the cell with a histone deacetylase (HDAC) inhibitor and also contacting the cell with a microbial organism that increases the level of AMP, wherein the HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to a histone deacetylase (HDAC) inhibitor, for use for
30 increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for preparing a drug for use for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

- 5 The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

- 10 The present invention also relates to a pharmaceutical composition comprising a histone deacetylase (HDAC) inhibitor, for use for increasing AMP expressed by a cell when said cell is contacted with a microbial organism. The HDAC inhibitor further increases the level of AMP expression in the cell.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are preferably increased without modifying pro-inflammatory cytokine expression.

- 15 Preferably, the AMP is beta-defensin-2 (DEFB2). Preferably, the microbial organism is a bacterium.

- 20 In one embodiment, the invention encompasses a method for increasing AMP expression in a cell comprising contacting the cell with a microbial organism that increases the level of an AMP and contacting the cell with a histone deacetylase (HDAC) inhibitor, wherein the combination of the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

- 25 The present invention also relates to a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP, for use for increasing AMP expression in a cell. The combination of the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

- 30 The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP for preparing a drug for use for increasing AMP expression in a cell. The combination of

the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP for increasing AMP expression in a cell. The combination of the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

The present invention also relates to a pharmaceutical composition comprising a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism that increases the level of an AMP, for use for increasing AMP expression in a cell. The combination of the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.

In all above methods, therapeutic uses and pharmaceutical compositions, AMP are preferably increased without modifying pro-inflammatory cytokine expression.

In some embodiments, the cell is contacted with the HDAC inhibitor in vitro. In some embodiments, the cell is contacted with the HDAC inhibitor in vivo. In some embodiments, the cell is contacted with the HDAC inhibitor ex vivo.

The invention further encompasses methods for increasing AMP, especially beta-defensin-2 (DEFB2), expression in a cell comprising contacting the cell with a microbial organism, preferably a bacterium that increases the level of the AMP; and further contacting the cell with a histone deacetylase (HDAC) inhibitor, wherein the HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism, preferably a bacterium that increases the level of an AMP, for use for increasing AMP, especially beta-defensin-2 (DEFB2), expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism, preferably a bacterium that increases the level of an AMP, for preparing a drug for use for increasing AMP, especially beta-defensin-2 (DEFB2), expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.

The present invention also relates to the use of a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism, preferably a bacterium that increases the level of an AMP, especially beta-defensin-2 (DEFB2), expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.

- 5 The present invention also relates to a pharmaceutical composition comprising a combination of a histone deacetylase (HDAC) inhibitor and a microbial organism, preferably a bacterium that increases the level of an AMP, for use for increasing AMP, especially beta-defensin-2 (DEFB2), expression in a cell. The HDAC inhibitor further increases the level of AMP expression in the cell.
- 10 In all above methods, therapeutic uses and pharmaceutical compositions, AMP (preferably DEFB2) is preferably increased without modifying pro-inflammatory cytokine expression.

- The present invention also relates to a histone deacetylase (HDAC) inhibitor, for use in
15 the treatment of infections and dysbiosis-driven diseases in humans.

The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor for preparing a drug for use in the treatment of infections and dysbiosis-driven diseases in humans.

- The present invention also relates to the use of a histone deacetylase (HDAC) inhibitor
20 in the treatment of infections and dysbiosis-driven diseases in humans.

The present invention also relates to a pharmaceutical composition comprising a histone deacetylase (HDAC) inhibitor, for use in the treatment of infections and dysbiosis-driven diseases in humans.

- The present invention also relates to a method of treating an infections or a dysbiosis-
25 driven disease in a human subject in need thereof, comprising administering to said human subject an efficient amount of a histone deacetylase (HDAC) inhibitor.

HDAC INHIBITORS

In all methods, uses and pharmaceutical compositions according to the invention, a HDAC inhibitor, or a combination of several HDAC inhibitors, is used.

- 30 The HDAC inhibitor, or at least one of the HDAC inhibitors, is preferably selected from the following HDAC inhibitor families:

- Hydroxamic acids, including but not limited to the following compounds: trichostatin A (TSA), Panobinostat (LBH589), Vorinostat (SAHA), Belinostat (PXD101), Abexinostat (PCI-24781), Dacinostat (LAQ824), Quisinostat (JNJ-26481585), Droxinostat, MC1568, Givinostat (ITF2357), Rocilinostat (ACY-1215),
- Benzamides, including but not limited to the following compounds: Tacedinaline (CI996), Entinostat (MS-275), Mocetinostat (MGCD0103),
- Short Chain Fatty Acids (SCFA), including but not limited to the following compounds: Valproic acid, Sodium butyrate, phenylbutyrate (S-HDAC-42, AR-42), Sodium phenylbutyrate (S4125),
- mercaptoketones, including but not limited to the following compounds: KD5170,
- cyclic tetrapeptides, including but not limited to the following compounds: Depsipeptide (Romidepsin), Apicidin.

Other HDAC inhibitors are not classified, such as Tubastatin A, CUDC-907, M344, and Scriptaid.

Preferably, the HDAC inhibitor, or at least one of the HDAC inhibitors, is selected from hydroxamic acids and benzamides, more preferably the HDAC inhibitor, or at least one of the HDAC inhibitors, is selected from hydroxamic acids.

The HDAC inhibitor can be selected from trichostatin A, Panobinostat (LBH589), Vorinostat (SAHA), Entinostat (MS-275), Belinostat (PXD101), Abexinostat (PCI-24781), Dacinostat (LAQ824), Quisinostat (JNJ-26481585), Mocetinostat (MGCD0103), Valproic acid, CUDC-101, Droxinostat, Pracinostat (SB939), PCI-34051, Givinostat (ITF2357), AR-42, Tubastatin A, CUDC-907, M344, Tacedinaline (CI996), Rocilinostat (ACY-1215), and Scriptaid. The HDAC inhibitor may also be MC1568 or Sodium phenylbutyrate (S4125, Selleckchem).

The HDAC inhibitor can comprises a combination of specific HDAC inhibitors.

Preferably, the HDAC inhibitor is not butyrate, in particular not Sodium butyrate, phenylbutyrate (S-HDAC-42, AR-42), or Sodium phenylbutyrate (S4125).

Preferably, the HDAC inhibitor is selected from trichostatin A, suberoylanilide hydroxamic acid (SAHA), and Abexinostat/PCI-24781.

Preferably, the HDAC inhibitor inhibits inflammatory cytokine expression.

In a preferred embodiment of methods, uses and pharmaceutical compositions according to the invention in which it is intended to increase AMP in a cell, in vitro, ex vivo or in vivo, the cell is contacted with a concentration of between 0.5 μ m-1 μ m, 0.5 μ m-5 μ m, 0.5 μ m-10 μ m, 0.5 μ m-50 μ m, 1 μ m-5 μ m, 1 μ m-10 μ m, 1 μ m-50 μ m, 5 μ m-10 μ m, 5 μ m-50 μ m, and 10 μ m-50 μ m of the HDAC inhibitor.

TARGETED CELLS

In all methods, uses and pharmaceutical compositions according to the invention in which it is intended to increase AMP in a cell, in vitro, ex vivo or in vivo, the targeted cell (in which it is intended to increase AMP) is preferably a human cell. In a preferred embodiment, the cell is an epithelial cell, preferably an intestinal, pulmonary, or skin epithelial cell. More preferably, the cell is a human intestinal epithelial cell.

INCREASED AMP

In all methods, uses and pharmaceutical compositions according to the invention in which it is intended to increase AMP in a cell, in vitro, ex vivo or in vivo, the AMP that is increased may be selected from many known AMP genes, including the human genes HBD2 (also referred to as DEFB2), HBD1 (also referred to as DEFB1), HBD3 (also referred to as DEFB3), LL37, PGLYRP4, DEFB110, DEFB111, DEFB114, DEFB115, DEFB116, DEFB125, DEFB126, DEFB127, DEFB129, and DEFB134.

Preferably, the AMP that is increased is beta-defensin-2 (DEFB2, also referred to as HBD2).

MICROBIAL ORGANISMS, INFECTIONS AND DYSBIOSIS-DRIVEN DISEASES

In all methods, uses and pharmaceutical compositions according to the invention in which it is intended to increase AMP in a cell, in vitro, ex vivo or in vivo, the microbial organism may be selected from the following types of microbial organisms: bacteria, viruses, fungi, and protozoans.

Preferably, the microbial organism is a bacterium. Preferably, the bacterium is not a *Shigella* bacterium. Preferably, the bacterium is an *Escherichia coli*, most preferably an *Escherichia coli* K12 (commensal strain) or an *Escherichia coli* LF82 (pathobiontic strain).

- 5 When the microbial organism is a virus, it is preferably not a human immunodeficiency virus (HIV) and also not a hepatitis virus.

When the microbial organism is a protozoan, it is preferably not a *Plasmodium* protozoan, in particular not a *Plasmodium falciparum* protozoan.

- 10 Similarly, the microbial organism causing infections that may be treated using a HDAC inhibitor in the context of the invention include bacteria, viruses, fungi, and protozoans. Preferably, the microbial organism is a bacterium. Preferably, the bacterium is not a *Shigella* bacterium. Preferably, the bacterium is an *Escherichia coli*, most preferably an *Escherichia coli* K12 (commensal strain) or an *Escherichia coli* LF82 (pathobiontic strain).
15 When the microbial organism is a virus, it is preferably not a human immunodeficiency virus (HIV) and also not a hepatitis virus. When the microbial organism is a protozoan, it is preferably not a *Plasmodium* protozoan, in particular not a *Plasmodium falciparum* protozoan.

- 20 In the context of the invention, dysbiosis-driven diseases refer to diseases reflecting mishandling of the microbiota by the host immune system and to clinical situations in which restoration of a normal microbiota is likely to be better achieved by strengthening natural homeostatic mechanisms than antibiotics.

Such diseases notably include:

- 25 • Hospital-acquired infections (HAI):

In modern hospital settings, invasive procedures, immunosuppressive/immunomodulatory therapies, and high-risk situations in intensive care units (ICU), create a major risk of hospital-acquired infections (HAI). Large multicentric studies in the USA and Europe have shown that at
30 least one HAI occurs in 27 % of ICU patients (i.e. 17 % pneumonia and 14.5 % bacteraemia/septicaemia), with severe sepsis and possibly death in 40 % of these

patients. Occurrence of HIA doubles the duration of hospitalization with a seemingly increase in costs.

This is particularly critical in the case of aggressive chemotherapies administered to treat solid tumors/lymphomas and leukemias (often in association with bone marrow transplantation). Deep bone marrow suppression, particularly severe leukopenia, put these patients at risk of bacterial translocation from the gut flora, a situation occurring in one third of them, often leading to severe sepsis and death.

In this critical period, patients are likely to be left essentially with their most primary mechanisms of innate defense like epithelial antimicrobial molecules/peptides. Boosting this compartment could provide an efficient mean to protect against bacterial translocation until functional recovery of the bone marrow. The availability of such molecule may also strengthen defenses at other mucosal surfaces, particularly the respiratory tract.

- Inflammatory bowel diseases (IBD):

In IBD, such as Crohn's disease, a defect in production of antimicrobial peptides may play a significant role in letting some bacterial populations get too close to the epithelial surface, thus maintaining excessively strong pro-inflammatory signals. Restoring antimicrobial functions while maintaining inflammation in check would represent a therapeutic breakthrough.

- Recurrent/chronic pediatric enteric infections and pediatric environmental enteropathy (PEE):

Such diseases are marked by lingering "small bowel bacterial overgrowth" (SBBO) and represent a major cause of malnutrition and stunting with several epidemiological surveys showing association with psychomotor retardation.

Stimulation of mucosal antibacterial defenses is expected to help the host control such situations with a favorable cost/efficiency ratio.

Therefore, in the context of the invention, dysbiosis-driven diseases that may be treated using a HDAC inhibitor include:

- hospital-acquired infections (HAI), in particular in case of deep bone marrow suppression, particularly severe leukopenia;
- inflammatory bowel diseases (IBD), such as Crohn's disease; and

- recurrent/chronic pediatric enteric infections and pediatric environmental enteropathy (PEE), in particular those marked by lingering “small bowel bacterial overgrowth” (SBBO).

EXAMPLES

5 *EXAMPLE 1. HDAC inhibitors increase expression and secretion of AMP, in particular in response to microbial stimulation, without increasing expression of pro-inflammatory molecules*

Materials and Methods

Antibodies

- 10 We used the rabbit polyclonal antibodies anti-NF- κ B p65 subunit (SC-109, Santa Cruz; ab7970, Abcam), anti-NF- κ B p65 acetyl K310 (ab52175, Abcam), anti-I κ B α (SC-371, Santa Cruz), anti-IKK α (2682, Cell Signalling), anti-IKK β (2370, Cell Signalling), anti-phospho-IKK α (S176)/IKK β (S177) (2697, Cell Signalling), anti-histone H3 (ab1791, Abcam), anti-acetyl-histone H3 K9+14+18+23+27 (ab47915, Abcam), anti-acetyl-
- 15 histone H3 K4 (07-539, Millipore), anti-acetyl-histone H3 K9 (ab4441, Abcam), anti-acetyl-histone H3 K14 (07-353, Millipore), anti-acetyl-histone H3 K18 (ab1191, Abcam), anti-acetyl-histone H3 K23 (07-355, Millipore), anti-acetyl-histone H3 K27 (ab4729, Abcam), anti-acetyl-histone H3 K56 (07-677-I, Millipore), anti-histone H4 (ab7311, Abcam), anti-acetyl-histone H4 K5+8+12+16 (06-866, Millipore), anti-acetyl-
- 20 histone H4 K5 (07-327, Millipore), anti-acetyl-histone H4 K8 (07-328, Millipore), anti-acetyl-histone H4 K12 (07-595, Millipore), anti-phospho-histone H3 S10 (ab5176, Abcam), anti-SAPK/JNK (9252, Cell Signalling), anti-phospho-SAPK/JNK (T183/Y185) (4668, Cell Signalling), and anti-actin (A2066, Sigma). We used the rabbit monoclonal antibodies anti-p38 (8690, Cell Signalling), anti-phospho-p38 (T180/Y182)
- 25 (4511, Cell Signalling), anti-Erk1/2 (4695, Cell Signalling), anti-phospho-Erk1/2 (T202/Y204) (4370, Cell Signalling). We used the mouse monoclonal antibody anti-p300 (ab14984, Abcam), the sheep antibody anti-mouse-IgG-POX (NXA931, GE Healthcare), the goat antibody anti-rabbit-IgG-POX (GAR/IgG(H+L)/PO, Nordic

Immunology), and the Alexa Fluor 488 goat anti-rabbit-IgG (H+L) highly absorbed (A11034, Invitrogen).

Cell culture

The human intestinal epithelial cell line Caco-2, subclone TC7 (16), was cultured with
5 DME (Invitrogen) supplemented with 10% decompemented Fetal Bovine Serum (FBS, Invitrogen), 1% non-essential amino acids (NEAA, Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), at 37°C and 10% CO₂. Cells were split two times per week, using Versene solution (Invitrogen).

Bacterial challenges

10 The *Escherichia coli* K12 bacterial strain was grown in LB medium (Sigma) at 37°C. For challenge experiments, cells were grown at confluence in 6-well plates (1.5×10^6 cells/well) for 48 h at 37°C and 10% CO₂. Bacterial challenges were performed using supplemented DME without antibiotics with overnight bacterial cultures, at a multiplicity of infection (MOI) of 10 bacteria per cell, for indicated times. For
15 experiments involving pharmacological inhibitors, cells were pretreated overnight for 16 h and washed with supplemented DME without antibiotics.

Inhibitors

We used the pharmacological inhibitors trichostatin A (T1952, Sigma), suberoylanilide hydroxamic acid (SC-220139, Santa Cruz Biotechnology), pargyline hydrochloride
20 (P8013, Sigma), 5-azacitidine (A1287, Sigma), BMS-345541 (B9935, Sigma), and C646 (SML0002, Sigma).

Transfections

The RNAi Max reagent (Invitrogen) was used to transfect cells with a final concentration of 25 nM siGENOME SMARTpool siRNAs (Thermo Scientific)
25 silencing p65 (M-003533-02-0005), p300 (M-003486-04-0005), or with control scrambled siRNAs (D-001210-01-50). Transfections were performed in OptiMEM medium (Invitrogen) supplemented with 1% NEAA and 5% FBS. Knockdowns were assessed after 48 h using qRT-PCR and immunoblotting analysis.

qRT-PCR

RNA was isolated using the RNeasy Mini kit and the RNase free DNase kit (Qiagen). RT-PCR reactions were performed overnight using the SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT)18 primers (Thermo Scientific), as recommended by the suppliers. Gene-specific primers were designed and purchased from Sigma:

- (DEFB2 (also referred to as HBD2), GCCATGAGGGTCTTGTATCTC (SEQ ID NO:1) / TTAAGGCAGGTAACAGGATCG (SEQ ID NO:2);
- DEFB3, TTTGGTGCCTGTTCCAGGTCAT (SEQ ID NO:3) / GCCGCCTCTGACTCTGCAATAATA (SEQ ID NO:4);
- LL37, AAGGAAGCTGTGCTTCGTGCTA (SEQ ID NO:5) / AATCCTCTGGTGACTGCTGTGT (SEQ ID NO:6);
- IL1B, TACGATCACTGAACTGCACGCT (SEQ ID NO:7) / TCTTTCAACACGCAGGACAGGT (SEQ ID NO:8);
- IL8, AAGAAACCACCGGAAGGAACCA (SEQ ID NO:9) / ATTTCTGTGTTGGCGCAGTGTG (SEQ ID NO:10);
- CCL20, AAGAGTTTGCTCCTGGCTGCTT (SEQ ID NO:11) / GCAGTCAAAGTTGCTTGCTGCT (SEQ ID NO:12);
- B2M, ATTGCTATGTGTCTGGGTTTCA (SEQ ID NO:13) / AAGACAAGTCTGAATGCTCCAC (SEQ ID NO:14).

The qRT-PCR reactions were carried out in a 20 µl final volume containing 8 µl of cDNA (diluted at 1/100), 2 µl of primers (0,2 µM each), and 10 µl of Power SYBR Green mix (Applied Biosystems). Reactions were run on a QuantStudio 7 (Applied Biosystems) with recommended universal thermal cycling parameters. Each sample reaction was run in duplicate on the same plate. Relative gene expression quantification was performed using the comparative Ct method. Data were normalized to the β-2-microglobulin (B2M) housekeeping gene expression.

ELISA

We used the enzyme-linked immunosorbent assay (ELISA) kits for DEFB2 (900-K72, PeproTech), and IL8 (900-K18, PeproTech). Absorbance was measured on a M200PRO fluorimeter (Tecan).

Cytotoxicity measurement

Cytotoxic effect of bacterial challenges and pharmacological inhibitor treatments were evaluated by measurement of the lactate dehydrogenase (LDH) release, using the CytoTox 96 non-radioactive Cytotoxicity Assay (Promega).

5 *Immunoblotting*

Total cell lysates were harvested by removing growth medium and adding NP40 lysis buffer [25 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 1% NP40, 10% Glycerol, 150 mM NaCl] supplemented by a cocktail of protease inhibitors [Sodium Orthovanadate (Sigma), 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma), COMPLETE (Roche)]. Samples were diluted with sample
10 buffer [1M Tris HCl, 20% Glycerol, 6% SDS, 0.02% Bromophenol Blue, 10% β-Mercaptoethanol] and boiled for 5 min. Denatured proteins were loaded on 7.5%, 10% or 12% acrylamide Mini PROTEAN TGX precast gels (BioRad). Separated proteins were transferred onto a PVDF membrane using the iBlot Gel Transfer System
15 (Invitrogen). Membranes were blocked with 3% Albumin from Bovine Serum (BSA, Sigma) or 5% milk (Regilait), at room temperature, prior to incubation with primary antibodies overnight at 4°C, in 1% BSA or 5% milk. Incubation with the secondary horseradish peroxidase-conjugated IgG antibody was performed at room temperature. Blots were developed using the SuperSignal West Dura Extended Duration Substrate
20 solution (Thermo Scientific) and the ChemiDoc XRS System (BioRad).

Immunofluorescence experiments

Cell monolayers were fixed with 4% paraformaldehyde (Sigma). Cells were permeabilized for 10 minutes using permeabilization buffer [1× phosphate-buffered saline (PBS), 0,1% Triton], followed by an incubation step in saturation buffer [1× PBS,
25 3% BSA, 5% FBS] for 1 h at room temperature. Cells were incubated for 2 h at room temperature with the primary polyclonal antibody. Cells were washed 2 times with saturation buffer, incubated for 1 h at room temperature with the secondary antibody coupled to Alexa Fluor 488 (Invitrogen), washed 2 times with saturation buffer, and finally, incubated for few seconds with 4',6-diamidino-2-phenylindole (DAPI,
30 Invitrogen) solution (1× PBS). Coverslips were mounted using Prolong (Invitrogen),

and dried overnight before examination with an IX81 inverted microscope (Olympus). Quantification of p65 nuclear signal was performed on 5 acquisitions per condition with the Fiji image processing software, using the nuclei watershed separation algorithm.

Chromatin immunoprecipitation

5 Chromatin immunoprecipitations (ChIP) were performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signalling), using magnetic beads, as recommended by the supplier. Chromatin inputs corresponded to 5-10 µg DNA for each individual ChIP assay. The ChIP DNA fractions were quantified by qRT-PCR, on a QuantStudio 7 (Applied Biosystems), using the comparative Ct method. Gene-specific primers were
10 designed and purchased from Sigma (DEFB2, TTTGGCCAACCCTCCTATTTCCCT (SEQ ID NO:15) / ACCTCTGTAATGAGCATTGCACCC (SEQ ID NO:16); IL8, AGGACAAGAGCCAGGAAGAAACCA (SEQ ID NO:17) / AGAGCTGCAGAAATCAGGAAGGCT (SEQ ID NO:18)). All ChIP experiments were performed at 1 h post challenge due to the time existing between regulatory events
15 occurring at promoters, such as histone post-translational modifications, recruitment of transcription factors or co-regulators, and initiation of the gene transcription.

RNA sequencing

RNA libraries were created with Illumina TruSeq stranded PolyA+ mRNA kits and sequenced on 2 Illumina HiSeq lanes, 3 samples multiplexed per lane, with paired-end
20 50 base pair reads. Reads were mapped to human genome hg19 (GRCh37) using TopHat 2.0.11, with Gencode 19 human genes as a transcriptome guide and maximum of 50 multihits. Reads mapping to Gencode genes were counted with the featureCounts software of the Subread package. Read counts were normalized using the DESeq 1.14 estimateSizeFactors function. Genes having less than 10 reads in any condition
25 were filtered out, and remaining transcripts had a pseudocount of 5 added to down-weight poorly expressed transcripts in fold-change calculations.

Results and Discussion

HDAC inhibition enhances AMP expression in cells challenged with E. coli

To analyze the expression of inducible genes of the innate immune response, such as genes encoding AMP (DEFB2, DEFB3, LL37) and pro-inflammatory cytokines (IL1B, IL8, CCL20), we challenged human colonic epithelial cells Caco-2, subclone TC7 (16), with the commensal strain *Escherichia coli* K12. Experiments were performed on confluent cell monolayers using a ratio of ten bacteria per cell. RNA was extracted at different time points after challenge and analyzed by qRT-PCR. In non-challenged cells, we detected a basal expression level for all the studied genes. Alternatively, challenge of cells with *E. coli* was followed by strong transcriptional induction of the DEFB2, IL8, IL1B and CCL20 genes (Fig. 1A and Fig. 6A). In contrast, expression of DEFB3 and LL37 was not induced throughout the time course (Fig. 6A). As a control, cellular viability was not influenced by bacterial challenges, as assessed by lactate dehydrogenase assay (Fig. 6B). Collectively, these results demonstrate that most genes encoding AMP and pro-inflammatory cytokines are simultaneously expressed and induced in intestinal epithelial cells exposed to a commensal bacterium.

To investigate the role of epigenetic modifications in the mechanisms regulating expression of inducible AMP and pro-inflammatory genes, we tested the effect of inhibiting several chromatin-modifying enzymes. Dose response assays were carried out overnight on confluent cell monolayers using inhibitors of DNA methylation (azacytidine), histone demethylation (pargyline), or histone deacetylation (trichostatin A [TSA]; suberoylanilide hydroxamic acid [SAHA]). The inhibitor-containing medium was removed, and *E. coli* challenge was performed to induce expression of the studied genes. RNA was extracted at 2 h after challenge and analyzed by qRT-PCR. In these experimental conditions, we found that inhibition of DNA methylases or histone demethylases had no effect on the expression of AMP and pro-inflammatory genes, regardless of the inhibitor concentration used. In contrast, inhibition of histone deacetylases (HDAC) with TSA or SAHA had a significant impact on the induction of gene expression in a group-specific manner. Induction of the antimicrobial genes DEFB2 (Fig. 1B and Fig. 7A) and LL37 (Fig. 6C and Fig. 7A) was remarkably enhanced in a concentration dependent manner, whereas pro-inflammatory genes did

not respond to the inhibitors (Fig. 1B, Fig. 6C, and Fig. 7A). Quantitatively, bacterial-dependent induction of DEFB2 expression was close to 10,000 folds after pretreatment with 5 μ M TSA, compared to 100 folds without inhibitor (Fig. 1B). In contrast, induction of IL8 expression remained the same for all TSA concentrations (Fig. 1B).

- 5 Interestingly, this enhancement of AMP gene expression upon HDAC inhibition was also observed in non-challenged cells (Fig. 1B, Fig. 6C and Fig. 7A), in agreement with previous reports on stimulation of LL37 expression with sodium butyrate that has been shown to express HDAC inhibitory function (17). As a control, cellular viability was not affected by the inhibitors, except at the highest concentration (Fig. 6D and Fig. 7B).
- 10 Together, these results show that HDAC inhibition has a differential impact on the expression of AMP and pro-inflammatory genes in intestinal epithelial cells.

- To further characterize the enhanced induction of the DEFB2 gene expression upon HDAC inhibition, we investigated secretion of DEFB2 peptide. For this purpose, cells were pretreated with TSA or SAHA and challenged for 6 h with *E. coli*. Then, bacteria
- 15 were killed by gentamicin treatment, and ELISA assays were performed on cell supernatants collected 24 h later to quantify DEFB2 peptide secretion. In supernatants of non-challenged cells, the DEFB2 peptide was only detected after TSA or SAHA pretreatment, showing that the 10 folds induction of the DEFB2 gene expression observed subsequently to HDAC inhibition was followed by peptide production and
- 20 secretion (Fig. 1C and Fig. 7C). In supernatants of challenged cells, dosage of the DEFB2 peptide revealed a concentration reaching 10,000 pg/ml after pretreatment with TSA or SAHA, compared to 100 pg/ml for non-treated cells (Fig. 1C and Fig. 7C). In contrast, secretion of interleukin IL8 was similar in both conditions (Fig. 1C and Fig. 7C). Collectively, these results demonstrate that HDAC inhibition stimulates
- 25 transcription of AMP genes, as well as secretion of these defense peptides, without modification of pro-inflammatory cytokine expression, in intestinal epithelial cells.

HDAC inhibition activates the IKK complex and increases phosphorylation of histone H3 on the serine S10 at the DEFB2 promoter

- Based on these observations, we sought to determine the molecular mechanisms leading
- 30 to enhanced induction of DEFB2 expression upon HDAC inhibition. Several studies have suggested that accessibility of transcription factors to chromatin, especially on the

promoters of innate immunity genes, requires acetylation of histones H3 and H4 on several lysine residues, and phosphorylation of histone H3 at serine S10 (H3S10) (3, 18). We therefore investigated the consequence of HDAC inhibition on the occurrence of these histone marks. By immunoblot, we identified two classes of lysine residues that were differentially acetylated in an *E. coli*-independent manner, following TSA pretreatment. The first class included lysines from histones H3 (residues H3K4, H3K14, H3K18, and H3K23) or H4 (residues H4K5, H4K8, H4K12, and H4K16) that were constitutively acetylated and poorly affected by TSA (Fig. 8A and Fig. 8B). The second class encompassed lysines that were only weakly acetylated at basal state, but whose acetylation level was strongly enhanced after TSA pretreatment, such as residues H3K9, H3K27, and H3K56 (Fig. 2A). In addition, using an antibody against phosphorylated H3S10, we observed a strong increase of this phosphorylation mark following TSA pretreatment, in non-challenged and in challenged cells (Fig. 2A). Together, these results indicate that HDAC inhibition by TSA pretreatment has a differential impact on the acetylation status of histone lysine residues, and dramatically enhances the phosphorylation level of the H3S10 residue.

We next investigated whether HDAC inhibition increased the level of H3S10 phosphorylation, preferentially at the DEFB2 gene promoter. We pretreated cells overnight with 5 μ M TSA, followed by 1 h challenge with *E. coli*, and carried out chromatin immunoprecipitation assays using either antibody directed against phosphorylated H3S10 (P-H3S10), or an irrelevant immunoglobulin G of the same isotype as a control. The size of sonicated chromatin fragments was approximately 150-900 base pairs, corresponding to 1-5 nucleosomes. Data were analyzed by qRT-PCR, using the ribosomal protein RPL30 housekeeping gene as a control. In non-challenged cells, we observed a weak increase of the P-H3S10 signal at the DEFB2 promoter in TSA pretreated cells, compared to non-treated cells (Fig. 2B). Strikingly, in cells challenged with *E. coli*, we found a 2 folds enrichment of P-H3S10 at the DEFB2 promoter following TSA pretreatment, compared to non-treated and challenged cells (Fig. 2B). In contrast, the P-H3S10 signal remained unchanged in both conditions at the IL8 promoter (Fig. 2B). Collectively, these data show that the impact of HDAC inhibition on the level of H3S10 phosphorylation is gene-specific.

We then investigated the signaling pathway by which TSA-mediated HDAC inhibition induces phosphorylation of H3S10. We analyzed the canonical phosphorylation of histone H3 through activation of the MAPK signaling pathway. However, TSA failed to induce activation of Erk1/2, p38, or SAPK/JNK, as determined by phosphorylation level (Fig. 9). We therefore considered the alternative IKK α / β complex pathway. Indeed, several studies have demonstrated that IKK α functions as a H3S10 kinase regulating chromatin structure and facilitating gene expression (19, 20). By immunoblot, using an antibody detecting phosphorylated IKK α / β , we observed an increased phosphorylation of the IKK complex in non-challenged cells following TSA pretreatment (Fig. 2C). This TSA-dependent phosphorylation of IKK α / β was further enhanced in cells challenged with *E. coli* (Fig. 2C). Furthermore, the phosphorylated IKK complex phosphorylates the NF- κ B inhibitor I κ B α , resulting in its degradation. This process releases NF- κ B and allows its translocation into the nucleus (21). In agreement, following TSA pretreatment, we observed a strong decrease of the I κ B α protein by immunoblot (Fig. 2C) and an increase of the NF- κ B p65 subunit signal in nuclei by immunofluorescence (Fig. 10). Collectively, these results indicate that TSA-mediated HDAC inhibition promotes induction of H3S10 phosphorylation, preferentially at the DEFB2 promoter, and activates the NF- κ B pathway through the IKK complex.

NF- κ B is acetylated upon HDAC inhibition and mediates the enhanced induction of DEFB2 expression

A current model suggests that H3S10 phosphorylation accounts for a histone structure that favors the binding of chromatin-remodeling enzymes, thereby increasing promoter accessibility of NF- κ B on a specific set of innate immune genes (18). Both the DEFB2 and IL8 promoters harbor NF- κ B binding sites (22, 23). Therefore, we investigated its involvement in the enhanced induction of DEFB2 expression upon TSA-mediated HDAC inhibition. Using a pool of siRNA silencing expression of the NF- κ B p65 subunit, we found that transcription of DEFB2 and IL8 was dependent on NF- κ B upon challenge with *E. coli* in TSA pretreated and non-treated cells. In both conditions, with an efficacy of p65 silencing reaching 80%, expression of DEFB2 and IL8 was reduced at least 10 folds, compared to cells transfected with the scrambled siRNA negative

control (Fig. 3A). To confirm these results by an alternative approach, we tested the effect of the chemical inhibitor BMS-345541 on expression of DEFB2 and IL8. This compound specifically inhibits the NF- κ B pathway by targeting IKK complex activation (24). Without BMS-345541, DEFB2 expression was enhanced at least 10
5 folds in TSA pretreated cells, compared to non-treated cells, as soon as 1 h post bacterial challenge (Fig. 3B). In contrast, IL8 expression showed no difference between TSA pretreated cells and non-treated cells upon bacterial challenge, as previously observed (Fig. 3B and Fig. 1B). Strikingly, with BMS-345541, expression of DEFB2 and IL8 was completely abolished, both in TSA pretreated and non-treated cells (Fig.
10 3B). Collectively, these results demonstrate that NF- κ B controls the induction of DEFB2 and IL8 expression and mediates enhancement of DEFB2 expression upon TSA-mediated HDAC inhibition.

Recent studies indicate that post-translational modifications of NF- κ B, especially on the p65 subunit, play a critical role in fine-tuning its activity, adding another level of
15 complexity to the regulation by this transcription factor. Among them, reversible acetylation of p65 on the lysine K310 residue regulates several functions of NF- κ B, including its full transcriptional activity at the targeted promoters (12). To determine whether NF- κ B is acetylated upon TSA-mediated HDAC inhibition, we used an antibody detecting p65 K310 acetylation (Ac-p65). By immunoblot, we detected an
20 acetylation of p65 on K310 after TSA pretreatment (Fig. 3C). This acetylation mark was most visible within the first hour following pretreatment of cells, in an *E. coli*-independent manner. In addition, we investigated the specific recruitment of Ac-p65 at the DEFB2 and IL8 promoters by chromatin immunoprecipitation. In non-challenged cells, as well as in cells challenged for 1 h with *E. coli*, we found a 2 folds enrichment
25 of Ac-p65 at the DEFB2 promoter after TSA pretreatment (Fig. 3D). In contrast, the Ac-p65 signal did not significantly change upon TSA pretreatment at the IL8 promoter (Fig. 3D). Together, these data show that TSA-mediated HDAC inhibition leads to enhanced recruitment of the acetylated NF- κ B p65 subunit, preferentially at the DEFB2 promoter, compared to the IL8 promoter.

p300 acetylates NF- κ B and supports the enhanced induction of DEFB2 expression upon HDAC inhibition

It has recently been demonstrated in human embryonic kidney cells 293T that acetylation of the NF- κ B p65 subunit at the lysine K310 residue is performed by the HAT p300 (14, 25). We therefore investigated the involvement of the p300-mediated acetylation of p65 on DEFB2 expression. By immunoblot, using a pool of siRNA silencing expression of p300 with 85% efficiency, we found that p65 was less acetylated on the K310 lysine upon *E. coli* K12 challenge, compared to cells transfected with the scrambled siRNA negative control (Fig. 4A). As a control, the basal level of p65 acetylation at the K310 residue was found similar in non-challenged cells, for both silencing conditions (Fig. 4A). Together, these results confirm that p300 takes part in the acetylation process of the NF- κ B p65 subunit in intestinal epithelial cells.

At the transcriptional level, this observation was correlated with a 7 folds decreased induction of DEFB2 expression upon p300 silencing, compared to cells transfected with the scrambled siRNA negative control (Fig. 4B). To confirm these results by an alternative approach, we tested the effect of the chemical inhibitor C646 on the expression of DEFB2 and IL8. This compound is a specific inhibitor of p300 (26). Dose response assays were carried out and showed that induction of DEFB2 expression by bacterial challenge was impacted at a concentration of C646 as low as 25 μ M, and was reduced more than 10 folds at the highest dose, compared to non-treated cells (Fig. 4C). In contrast, expression of IL8 was much less affected by the decreased amount or activity of p300 (Fig. 4B and Fig. 4C). Next, recruitment of p300 at the DEFB2 and IL8 promoters was quantified by chromatin immunoprecipitation. In non-challenged cells, the p300-associated signal was measured quite similar in TSA pretreated cells, compared to non-treated cells, at both DEFB2 and IL8 promoters (Fig. 4D). Interestingly, in challenged cells, we found a 3 folds enhanced recruitment of p300 at the DEFB2 promoter after TSA pretreatment, compared to non-treated cells (Fig. 4D). In contrast, the p300-associated signal did not show significant differences between TSA pretreated and non-treated cells at the IL8 promoter (Fig. 4D). Collectively, these data demonstrate that p300 acetylates p65 and supports the enhanced induction of DEFB2 expression upon TSA-mediated HDAC inhibition, through its increased recruitment at the DEFB2 promoter.

Expression of additional genes from the antimicrobial defense and epithelial restitution is enhanced upon HDAC inhibition

We finally tested whether the enhanced induction of DEFB2 expression observed upon TSA-mediated HDAC inhibition truly reflected a differential regulatory mechanism existing between antimicrobial and pro-inflammatory genes. For this purpose, a transcriptomic analysis of cells pretreated or not with 5 μ M TSA and challenged for 2 h with *E. coli* was performed. RNA was extracted and analyzed by RNA sequencing, using Illumina technology. The fold-change calculations representative of the whole gene expression were determined and presented on a scatter-plot (Fig. 5A). We focused on the genes clustering in the innate immune response pathway for further investigation (Fig. 5B and Fig. 5C). We identified a total of 58 genes from this pathway whose expression was significantly modulated in cells upon bacterial challenge. Interestingly, in addition to DEFB2, we identified 18 other genes whose expression was enhanced in cells pretreated with TSA compared to non-treated cells (Fig. 5B). Those were genes belonging to the antimicrobial defense, such as PGLYRP1-4 encoding the four human peptidoglycan recognition proteins, which target the bacterial cell wall, PLA2G7 encoding the phospholipase A2 that hydrolyses bacterial membrane phospholipids and LCN2 encoding the lipocalin iron sequestration protein. Remarkably, besides these genes encoding antimicrobial defense molecules, we observed the enhanced expression of additional genes belonging to the epithelial restitution machinery upon HDAC inhibition. These encompassed TGFB2, TSLP, and IL7 (Fig. 5B). TGFB2 is a member of the transforming growth factor beta family of cytokines involved in the promotion of intestinal homeostasis. TSLP is a hemopoietic cytokine promoting T helper type 2 cell responses that are associated with immunity to moderate intestinal inflammation. IL7 is produced locally by intestinal epithelial cells, and may serve as a regulatory factor for intestinal mucosal lymphocytes. In comparison, the expression of genes belonging to the *bona fide* pro-inflammatory pathways was not significantly modified in challenged cells pretreated or not with TSA, as measured for IL1B and IL8 and most of the genes encoding pro-inflammatory cytokines (Fig. 5C). Collectively, these results show that TSA-mediated HDAC inhibition enhances expression of a rather restricted group of innate immunity genes encoding molecules involved in antimicrobial defenses and epithelial restitution, without modifying the pro-inflammatory gene pathways.

The human intestinal epithelium maintains a protective barrier function between the host and its microbiota. This barrier protects against invasion and systemic dissemination of colonizing microorganisms. Among the diverse actors that contribute to establishment and maintenance of epithelial homeostasis, AMP play a crucial regulatory role. They are ubiquitously expressed by epithelial cells throughout the intestinal tract and keep the resident and transient bacterial populations in check (27). These effectors participate in the innate immune response to commensals under steady-state conditions, a situation of tolerance actively maintained by commensals themselves (28). Epithelial cells orchestrate this dialogue mainly through signaling pathways, which result for one part in activation of histone modifying enzymes and remodeling complexes (29). These pathways induce the expression of hundreds of genes with different functions and therefore different regulatory requirements (30). Because these genes are induced by the same pathways, their expression must be regulated by gene-specific rather than signal-specific mechanisms. This is an essential adaptive element of the innate immune response that is based on epigenetic processes (31).

One link between bacterial sensing and its epigenetic consequences has been described for the MAPK cascades, whose activation leads to phosphorylation of H3S10. Both, the Erk and p38 kinases have been shown to induce phosphorylation of H3S10 at the promoter of activated genes (32). Here, we observed an increase of this histone mark in cells challenged with *E. coli*. Unexpectedly, we also found this increase in non-challenged cells, after TSA-mediated HDAC inhibition. This occurred without significant activation of Erk or p38. Therefore, we speculated that TSA-mediated HDAC inhibition was able to activate an alternative pathway inducing H3S10 phosphorylation. One potential candidate was the IKK pathway that can, on one hand, mediate this process through the IKK α kinase, and on the other hand, activate transcription of NF- κ B-responsive genes by phosphorylating and targeting its inhibitor Ikb α for proteasomal degradation (19, 20). In agreement, we observed a decrease of Ikb α protein levels in non-challenged cells after TSA pretreatment. The current evidence supports the hypothesis that phosphorylation of H3S10 is a predisposing mark for histone acetylation, a mark for active transcription (33). As such, we observed a strong increase in the acetylation of the H3K9, H3K27, and H3K56 residues after TSA-mediated HDAC inhibition, three hallmarks of active promoters. Therefore, it is

tempting to speculate about the role of all of these histone modification marks, and their combination. As we have shown that the increase of H3S10 phosphorylation happens preferentially at the DEFB2 promoter upon TSA-mediated HDAC inhibition, it would be of interest to determine whether these histone acetylation marks also occur
5 differently at the DEFB2 and IL8 promoters. This would demonstrate a correlation between their appearance and a gene-specific expression at varying degrees.

Phosphorylation of H3S10, as well as acetylation of H3 lysines, are highlighted in a current model as discrete modifications promoting chromatin remodeling at the promoter of specific innate immune genes, allowing a precise recruitment of NF- κ B
10 (18). Beside this chromatin-conditioned control, NF- κ B activity is also subjected to additional regulatory mechanisms. Among them, acetylation of NF- κ B subunits on specific lysine residues play distinct roles in regulating its DNA-binding ability and transcriptional activity (11). Interestingly, these post-translational modifications resemble not only those occurring on histone proteins, but also share the same key
15 enzymes, such as HAT and HDAC. Recent studies showed that TSA-mediated HDAC inhibition prolonged the presence of the p65 subunit in the nucleus of cells and enhanced its binding activity to DNA, possibly through its increased acetylation (34, 35). Here, we have observed a strong acetylation of p65 on the K310 lysine upon HDAC inhibition. Therefore, it is tempting to hypothesize that increased acetylation of
20 NF- κ B strengthens its nuclear location, and above all its activity. This would be in agreement with the enhanced expression of the DEFB2 gene observed at the basal state, as well as its increased induction in cells upon bacterial challenge, after TSA-mediated HDAC inhibition. Moreover, once it binds DNA, several studies have shown that NF- κ B can recruit HAT to targeted promoters where these enzymes change protein
25 acetylation profiles, functioning as co-activators for gene transcription (36, 37). Among them, p300 has been shown to interact with the transcriptional activation domain of p65. As we demonstrated that p300 supports induction of DEFB2 expression and takes part in acetylation of p65 at the K310 residue, it would be of interest to investigate whether p300 also acetylates other p65 lysines known to be involved in the NF- κ B function.
30 Conversely, looking for other HAT able to acetylate the K310 lysine of p65 would give a better idea about the specific role of this mark in the fine-tuning of the NF- κ B activity. This would give more insight into the role of p300, NF- κ B, and its acetylation status, in

the differential induction of the DEFB2 and IL8 gene expression observed upon HDAC inhibition.

Several HDAC inhibitors have already been evaluated as therapeutic compounds with activities in cancer therapies (38). Recently, molecules derived from those inhibitors
5 have been shown to have applications beyond cancer therapies, based on their additional properties (39). Apart from applications in oncology, a considerable research effort has been aimed at evaluating the potential of these inhibitory molecules as therapeutics for neurodegenerative disorders, cardiac hypertrophy, and asthma. However, except for HIV, hepatitis and malaria infection, the therapeutic potential of HDAC inhibitors has
10 not been largely investigated for treatment of infectious diseases. One example is the HDAC inhibitor sodium butyrate, which can stimulate expression of the endogenous AMP cathelicidin in the intestine and promote the clearance of the enteropathogen *Shigella* (40). Along the same line, we have demonstrated the property of the HDAC inhibitor TSA to enhance expression of the AMP beta-defensin-2 without modifying the
15 level of interleukin-8. This observation highlights the possibility to disconnect expression of AMP from pro-inflammatory cytokines, at the epigenetic level. In addition, we found that HDAC inhibition has a positive impact on expression of other genes from the epithelial defense and restitution pathways upon a bacterial challenge. This suggests a potential role of these inhibitors in immune therapies through epigenetic
20 pathways. Understanding the coordinated interplay between epigenetic regulation, gene expression, and environment, will allow translation of this fundamental knowledge into development of innovative pharmacoepigenetic treatments.

***EXAMPLE 2. Further HDAC inhibitors increase expression and secretion of AMP, in particular in response to microbial stimulation, without increasing
25 expression of pro-inflammatory molecules***

Materials and Methods

Chemicals

We used the pharmacological histone deacetylase inhibitors Panobinostat (S1030, Selleckchem), Trichostatin A (S1045, Selleckchem), Vorinostat (S1047, Selleckchem),
30 Entinostat (S1053, Selleckchem), Belinostat (S1085, Selleckchem), Abexinostat

(S1090, Selleckchem), Dacinostat (S1095, Selleckchem), Quisinostat (S1096, Selleckchem), Mocetinostat (S1122, Selleckchem), Valproic acid (S1168, Selleckchem), CUDC-101 (S1194, Selleckchem), Droxinostat (S1422, Selleckchem), MC1568 (S1484, Selleckchem), Pracinostat (S1515, Selleckchem), PCI-34051 (S2012, Selleckchem), Givinostat (S2170, Selleckchem), AR-42 (S2244, Selleckchem), Tubastatin A (S2627, Selleckchem), CUDC-907 (S2759, Selleckchem), M344 (S2779, Selleckchem), Tacedinaline (S2818, Selleckchem), Sodium phenylbutyrate (S4125, Selleckchem), Rocilinostat (S8001, Selleckchem), Scriptaid (S8043, Selleckchem).

Cell culture

The human colonic epithelial cell line Caco-2, subclone TC7, was cultured with DME (Invitrogen) supplemented with 10% decompemented FBS (Invitrogen), 1% nonessential amino acids (Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen), at 37°C and 10% CO₂. The murine intestinal cell line mICcl2, was cultured with DMEM/F12 (Invitrogen) supplemented with 2% decompemented FBS (Invitrogen), 10 ng/mL EGF (Sigma), 50 nM dexamethasone (Sigma), 1 nM tri-iodo-thyronine (Sigma), 20 mM HEPES (Invitrogen), 2 mM Glutamax (Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen), at 37°C and 10% CO₂. Cells were split two times per week using Versene or Trypsine solution (Invitrogen).

Bacterial challenge

The *Escherichia coli* K12 (commensal) or LF82 (pathobiont) bacterial strain were grown in LB medium (Sigma) at 37°C. For challenge experiments, cells were grown at confluence in 6-well plates (1.5×10^6 cells/well) for 48 h at 37°C and 10% CO₂. Bacterial challenges were performed using supplemented DME without antibiotics with overnight bacterial cultures, at a multiplicity of infection (MOI) of 10 bacteria per cell, for indicated times. For experiments involving pharmacological inhibitors, cells were pretreated overnight for 16 h and washed with supplemented DME without antibiotics.

qRT-PCR

RNA was isolated using the RNeasy Mini kit and the RNase free DNase kit (Qiagen). RT-PCR reactions were performed overnight using the SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT)18 primers (Thermo Scientific), as recommended by the suppliers. Gene-specific primers were designed and purchased from Sigma:

- HBD1 (also referred to as DEFB1), CAGGTGGTAACTTTCTCACAGG (SEQ ID NO:19) / AATAGAGACATTGCCCTCCACT (SEQ ID NO:20);
- HBD2 (also referred to as DEFB2), GCCATGAGGGTCTTGTATCTC (SEQ ID NO:1) / TTAAGGCAGGTAACAGGATCG (SEQ ID NO:2);
- HBD3 (also referred to as DEFB3), TTTGGTGCCTGTTCCAGGTCAT (SEQ ID NO:3) / GCCGCCTCTGACTCTGCAATAATA (SEQ ID NO:4);
- LL37, AAGGAAGCTGTGCTTCGTGCTA (SEQ ID NO:5) / AATCCTCTGGTGAAGTGTGCTGTG (SEQ ID NO:6);
- DEFB110, TTTACCAGCCAGAAGCAATTT (SEQ ID NO:21) / ATACGCAGCACTGACTTCTCC (SEQ ID NO:22);
- DEFB111, TGAGGAGAGAGTGCAGAATAGG (SEQ ID NO:23) / GCAATGAGTTCAGGTCTTATG (SEQ ID NO:24);
- DEFB113, TTGTCTTCACTGTGTCTTGTGG (SEQ ID NO:25) / CACGAACAAGCTGACATTCTC (SEQ ID NO:26);
- DEFB114, ACATGTACCTTGGTGAATGCTG (SEQ ID NO:27) / TCTTCATACAATTTCTCAGTGCAG (SEQ ID NO:28);
- DEFB115, ATCATGCAAAGAAATTGAGAGG (SEQ ID NO:29) / TTTGGTTCGTGAATGTGTGATAG (SEQ ID NO:30);
- DEFB116, TTGGAATCCATGTGAGCTTTAC (SEQ ID NO:31) / ATTTGGGCAGGTTAAGTATTGG (SEQ ID NO:32);
- DEFB125, TCTACCAATCCAATTTACCAG (SEQ ID NO:33) / AACTCTCTTGGCTGTTGTGTTG (SEQ ID NO:34);
- DEFB126, AAGTCCCTACTGTTACCCTTG (SEQ ID NO:35) / TTCAGGTTTGCACCTTCTTCTTG (SEQ ID NO:36);

- DEFB127, TTGCCTCAATATCAAGGAACTG (SEQ ID NO:37) /
TGAAGAGTCAGTGCAAGTGTTG (SEQ ID NO:38);
- DEFB128, TCTCATTATTCTGCTGTTTGAGG (SEQ ID NO:39) /
TCTCCTACCTTGCATTTCTTCC (SEQ ID NO:40);
- 5 • DEFB129, AAGCAGAAGAGCAGTAATGTGG (SEQ ID NO:41) /
ATGACTGATGACAGAGGAAACAG (SEQ ID NO:42);
- DEFB134, ATCAGAAATGCACAAGAAATGC (SEQ ID NO:43) /
GATTTCCCTTTGACACAGCACTC (SEQ ID NO:44);
- 10 • IL1B, TACGATCACTGAACTGCACGCT (SEQ ID NO:7) /
TCTTTCAACACGCAGGACAGGT (SEQ ID NO:8);
- IL6, CTTCCAATCTGGATTCAATGAG (SEQ ID NO:45) /
TGGCTTGTTCCCTCACTACTCTC (SEQ ID NO:46);
- IL8, AAGAAACCACCGGAAGGAACCA (SEQ ID NO:9) /
ATTTCTGTGTTGGCGCAGTGTG (SEQ ID NO:10);
- 15 • CXCL3, GTGGGAGACCATAATGTGTCAG (SEQ ID NO:47) /
TCCCTTACCCTAACAGTGATCC (SEQ ID NO:48);
- CCL2, TCGCTCAGCCAGATGCAATCAA (SEQ ID NO:49) /
TCTCCTTGGCCACAATGGTCTT (SEQ ID NO:50);
- TNFA, AAACAACCCTCAGACGCCACAT (SEQ ID NO:51) /
20 AGTGCTCATGGTGTCTTTCCA (SEQ ID NO:52);
- CCL20, AAGAGTTTGCTCCTGGCTGCTT (SEQ ID NO:11) /
GCAGTCAAAGTTGCTTGCTGCT (SEQ ID NO:12);
- TSLP, CCCAGGCTATTCGAAACTCAG (SEQ ID NO:53) /
CGCCACAATCCTTGTAATTGTG (SEQ ID NO:54);
- 25 • TGFB2, GGTGATTTCATCTACAACAGC (SEQ ID NO:55) /
AGTACTCTTCGTCGCTCCTCTC (SEQ ID NO:56);
- B2M, ATTGCTATGTGTCTGGGTTTCA (SEQ ID NO:13) /
AAGACAAGTCTGAATGCTCCAC (SEQ ID NO:14);
- Defb4, CCATGAAGATTCACAAATCAAAC (SEQ ID NO:57) /
30 TCTGCAAATGGTTGTTATAAAGG (SEQ ID NO:58);
- Camp, ACACCAATCTCTACCGTCTCCT (SEQ ID NO:59) /
TACAGTCTCCTTCACTCGGAAC (SEQ ID NO:60);

- Defb1, AATACAAATGCCTTCAACATGG (SEQ ID NO:61) /
TACAACAGTTGGGCTTATCTGG (SEQ ID NO:62);
 - RegIIIg, TCCTTTCTCAGGTGCAAGGTGA (SEQ ID NO:63) /
TTGGCAGGCCATATCTGCATCA (SEQ ID NO:64);
 - 5 • Il1b, AACTGGTACATCAGCACCTCAC (SEQ ID NO:65) /
CCAGCCCATACTTTAGGAAGAC (SEQ ID NO:66);
 - Il6, AATTCCTCTGGTCTTCTGGAG (SEQ ID NO:67) /
AAGGACTCTGGCTTTGTCTTTC (SEQ ID NO:68);
 - Cxcl1, TTCACCTCAAGAACATCCAGAG (SEQ ID NO:69) /
10 CTTGAGTGTGGCTATGACTTCG (SEQ ID NO:70);
 - Tnfa, CAGAACTCCAGAACATCTTGG (SEQ ID NO:71) /
CAGTGAGTGAAAGGGACAGAAC (SEQ ID NO:72) ;
 - B2m, GCCGAACATACTGAACTGCTAC (SEQ ID NO:73) /
CCTTGCTGAAGGACATATCTGA (SEQ ID NO:74).
- 15 The qRT-PCR reactions were carried out in a 20 µl final volume containing 8 µl of cDNA (diluted at 1/100), 2 µl of primers (0,2 µM each), and 10 µl of Power SYBR Green mix (Applied Biosystems). Reactions were run on a QuantStudio 7 (Applied Biosystems) with recommended universal thermal cycling parameters. Each sample reaction was run in duplicate on the same plate. Relative gene expression quantification
- 20 was performed using the comparative Ct method. Data were normalized to the β -2-microglobulin (B2M) housekeeping gene expression.

ELISA

We used the enzyme-linked immunosorbant assay (ELISA) kits for HBD2 (900-K72, PeproTech), and IL8 (900-K18, PeproTech). Absorbance was measured on a M200PRO
25 fluorimeter (Tecan).

Human colonic organoid culture

This study was approved by the Institut Pasteur's ethical and medical committee under the agreement N° 2012-37. Surgically resected human colonic tissues were obtained from the Henri Mondor Hospital. All samples were obtained from patients who
30 provided informed consent before surgery. Normal epithelia were isolated and cultured

according to the protocol described by Sato and colleagues, with minor modifications (41). Organoids were cultured with Advanced DMEM/F12 (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 2 mM GlutaMAX (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), 1× N2 and B27 supplements (Invitrogen), 1 mM N-acetyl-L-cysteine (Sigma), 10 µM Y-27632 (Sigma), 500 nM A83-01 (Tocris), 10 µM SB202190 (Sigma), 10 mM nicotinamide (Sigma), 10 nM gastrin I (Sigma), 100 ng/ml recombinant human Noggin (R&D Systems), 50 ng/ml recombinant human EGF (R&D Systems), 1 µg/ml recombinant human R-Spondin-1 (Peprotech), 100 ng/ml recombinant human Wnt-3A (R&D Systems), and 10% foetal bovine serum (Invitrogen), at 37°C and 5% CO₂. Organoids were cultured in 48-well plates, 100 crypts per well. RNA was isolated from 6 days old organoids treated or not with 5 µM TSA (Sigma), and stimulated or not with 5 µg/ml flagellin (InvivoGen), for 24 h, using the RNeasy Micro kit and the RNase free DNase kit (Qiagen). Gene expression was analyzed using TaqMan probes from Applied Biosystems: HBD2 (Hs00823638_m1), HBD3 (Hs00218678_m1), LL37 (Hs00189038_m1), IL1B (Hs01555410_m1), IL8 (Hs00174103_m1), and TNF (Hs01113624_g1). Data were normalized to the B2M (Hs00984230_m1) housekeeping gene expression.

Results and conclusions

HDACi are inducers of antimicrobial peptide expression in vitro.

To further investigate the role of acetylation in the mechanisms regulating expression of inducible antimicrobial peptides (AMP) and pro-inflammatory genes, we tested the effect of 24 histone deacetylase inhibitors (HDACi) on transcription of these genes in the human colonic TC7 cell line and the murine mICcl2 intestinal cell line. Dose-response assays (0,5-50 µM) were carried out on confluent cell monolayers, and RNA was extracted at 24 or 48 hours post-treatment and analysed by qRT-PCR. In these experimental conditions, we found that inhibition of histone deacetylases with most of HDACi was followed by induction of several AMP genes, in a concentration and time dependent manner, including the human genes HBD2 (Fig. AA), HBD1, HBD3, LL37, PGLYRP4 (Fig. B), DEFB110, DEFB111, DEFB114, DEFB115, DEFB116, DEFB125, DEFB126, DEFB127, DEFB129, DEFB134 (Fig. C), and the

murine genes Defb4 and Camp (Fig. E). Transcriptional expression of these genes was followed by detection of their product in the supernatant of cells, as exemplified by the concentration of the beta-defensin-2 antimicrobial peptide measured by ELISA dosage (Fig. AB). In contrast, expression of most of pro-inflammatory genes remained the same for all HDACi concentrations tested, including the human genes IL8 (Fig. AA), IL1B, CXCL3, CCL2, TNFA (Fig. D), and the murine genes Il1b and Tnfa (Fig. E).

Taken together, these results demonstrate that HDAC inhibitors stimulate transcription of AMP genes, as well as secretion of their product, without modification of pro-inflammatory cytokine expression, in human and murine intestinal epithelial cells.

HDACi are enhancers of antimicrobial peptide expression in vitro upon a bacterial challenge.

To analyse the role of acetylation modifications in the mechanisms regulating expression of antimicrobial peptide and pro-inflammatory genes upon a bacterial challenge, we tested the effect of inhibiting histone deacetylase enzymes, using a set of 14 HDACi belonging to the hydroxamic acid, benzamide and short chain fatty acid families. For this, TC7 confluent cell monolayers were pre-treated with 5 μ M of HDACi overnight, then the inhibitor-containing medium was removed and *E. coli* challenge was performed. RNA was extracted at 2 hours after challenge and analysed by qRT-PCR. In these experimental conditions, we found that inhibition of HDAC with most of HDACi had a significant impact on the induction of gene expression, and peptide secretion, in a group-specific manner. Induction of the beta-defensin-2 was remarkably enhanced at the gene and protein level (Fig. F, G), approximately 10-fold more compared to non-treated cells, whereas the interleukin-8 did not respond to the inhibitors (Fig. H, I).

Collectively, these data show that HDAC inhibitors enhance expression of AMP in human colonic epithelial cells, without modifying expression of pro-inflammatory cytokines, upon a bacterial challenge.

HDACi impact kinetic of antimicrobial peptide gene expression in vitro upon a bacterial challenge.

To study the role of acetylation in the mechanisms regulating kinetic of antimicrobial peptide and pro-inflammatory gene expression, we tested the effect of the Abexinostat HDACi on transcription of the HBD2 and IL8 genes in human colonic TC7 cells upon a bacterial challenge with the *E. coli* strains K12 (commensal) and LF82 (pathobiont). Cell monolayers were pre-treated with 5 μ M of HDACi over-night, then the inhibitor-containing medium was removed and *E. coli* challenge was performed. RNA was extracted at different time points after challenge and analysed by qRT-PCR. In these experimental conditions, we found that inhibition of HDAC with Abexinostat had a significant impact on the kinetic of expression of the HBD2 gene (Fig. J). Induction of HBD2 was higher and faster in pre-treated and challenged cells, compared to non-treated and challenged cells. In contrast, kinetic of IL8 gene expression was not modified by the HDACi treatment.

Taken together, these results highlight that inhibition of histone deacetylases by HDACi trigger modifications that enhance induction of AMP genes and increase their kinetic of expression, in human colonic epithelial cells.

Expression of AMP genes is enhanced upon HDAC inhibition ex vivo in human colonic primary cells.

We investigated the relevance of our finding in human colonic primary cells, using *ex vivo* cultured organoids of crypts from normal colon tissues. We analyzed expression of a set of genes from the antimicrobial defense and pro-inflammatory pathways in 6 days old organoids treated or not with 5 μ M TSA, and stimulated or not with 5 μ g/ml flagellin to mimic a bacterial challenge, for 24 h (Fig. K). Crypts were isolated from normal human colons and embedded in matrigel in the presence of growth factors to induce the formation of three-dimensional structures termed “organoids”. Organoids recapitulate an intact architecture, harboring an internal lumen, stem cells, which are located in surface protusions that correspond to novel crypts, and the different epithelial lineages, including colonocytes. RNA was extracted and analyzed by qRT-PCR, using TaqMan assays. In non-treated and non-stimulated organoids, we detected a basal expression level for all the studied genes. Treatment of organoids with TSA alone

was followed by transcriptional induction of the HBD2, HBD3, and LL37 antimicrobial genes, with ratios ranging from 5- to 11-fold. In contrast, expression of the IL1B, IL8, and TNF pro-inflammatory genes was decreased. Besides, stimulating organoids with flagellin alone induced expression of all genes from the two classes. Strikingly, stimulating organoids with flagellin upon inhibition of histone deacetylases with TSA had a differential impact on the gene expression in a group-specific manner. Induction of HBD2, HBD3, and LL37 antimicrobial genes was enhanced, whereas expression of pro-inflammatory genes was either decreased for IL1B and IL8, or not modified in the case of TNF.

Collectively, these data validate the existence of specific epigenetic regulations allowing the enhancement of antimicrobial peptide gene expression among genes from the innate immune response, particularly in contrast to those encoding pro-inflammatory mediators, in human colonic primary cells.

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- 5

CLAIMS

1. A method of increasing expression in a cell in which the level of an antimicrobial peptide (AMP) has already been increased by the presence of a microbial organism comprising contacting the cell with a histone deacetylase (HDAC) inhibitor,
5 wherein the HDAC inhibitor further increases the level of AMP expression in the cell.
2. A method of increasing AMP expression in a cell comprising:
 - a) contacting the cell with a microbial organism that increases the level of an AMP; and
 - b) contacting the cell of a) with a histone deacetylase (HDAC) inhibitor,
10 wherein the combination of the HDAC inhibitor and the microbial organism increases the level of AMP expression in the cell more than the microbial organism alone.
3. The method of claim 1 or claim 2, wherein the AMP is beta-defensin-2 (DEFB2).
15
4. The method of any one of claims 1 to 3, wherein the microbial organism is a bacterium.
5. The method of any one of claims 1 to 4, wherein the HDAC inhibitor is selected
20 from hydroxamic acids, benzamides, short chain fatty acids, mercaptoketones, and cyclic tetrapeptides.
6. The method of any one of claims 1 to 5, wherein the HDAC inhibitor is trichostatin A or suberoylanilide hydroxamic acid.
25
7. The method of any one of claims 1 to 5, wherein the HDAC inhibitor is selected from Panobinostat (LBH589), Vorinostat (SAHA), Entinostat (MS-275), Belinostat (PXD101), Abexinostat (PCI-24781), Dacinostat (LAQ824), Quisinostat (JNJ-26481585), Mocetinostat (MGCD0103), Valproic acid, CUDC-101, Droxinostat, Pracinostat (SB939), PCI-34051, Givinostat (ITF2357), AR-42, Tubastatin A, CUDC-30 Pracinostat (SB939), PCI-34051, Givinostat (ITF2357), AR-42, Tubastatin A, CUDC-907, M344, Tacedinaline (CI996), Rocilinostat (ACY-1215), Abexinostat/PCI-24781, Scriptaid, MC1568, and Sodium phenylbutyrate (S4125).

8. The method of any one of claims 1 to 7, wherein the cell is contacted with a concentration of between 0.5 μ m and 50 μ m of the HDAC inhibitor.

5 9. A histone deacetylase (HDAC) inhibitor, for use for increasing antimicrobial peptides (AMP) expression in a cell in which the level of the AMP has already been increased by the presence of a microbial organism.

10. A histone deacetylase (HDAC) inhibitor, for use for increasing AMP expressed by a
10 cell when said cell is contacted with a microbial organism.

11. A combination of a histone deacetylase (HDAC) inhibitor and a microbial
organism that increases the level of an AMP, for use for increasing AMP expression in
a cell.
15

12. The HDAC inhibitor or combination for use according to any one of claims 9 to
11, wherein the AMP is beta-defensin-2 (DEFB2).

13. The HDAC inhibitor or combination for use according to any one of claims 9 to
20 12, wherein the microbial organism is a bacterium.

14. The HDAC inhibitor or combination for use according to any one of claims 9 to
13, wherein the HDAC inhibitor is selected from hydroxamic acids, benzamides, short
chain fatty acids, mercaptoketones, and cyclic tetrapeptides.

25 15. The HDAC inhibitor or combination for use according to any one of claims 9 to
13, wherein the HDAC inhibitor is trichostatin A or suberoylanilide hydroxamic acid.

16. The HDAC inhibitor or combination for use according to any one of claims 9 to
30 13, wherein the HDAC inhibitor is selected from Panobinostat (LBH589), Vorinostat
(SAHA), Entinostat (MS-275), Belinostat (PXD101), Abexinostat (PCI-24781),
Dacinostat (LAQ824), Quisinostat (JNJ-26481585), Mocetinostat (MGCD0103),

Valproic acid, CUDC-101, Droxinostat, Pracinostat (SB939), PCI-34051, Givinostat (ITF2357), AR-42, Tubastatin A, CUDC-907, M344, Tacedinaline (CI996), Rocilinostat (ACY-1215), Abexinostat/PCI-24781, and Scriptaid.

- 5 17. A histone deacetylase (HDAC) inhibitor, for use in the treatment of infections and dysbiosis-driven diseases in humans.

18. The HDAC inhibitor for use according to claim 17, wherein the infection is caused by a bacterium.

10

19. The HDAC inhibitor for use according to claim 18, wherein said bacterium is an *Escherichia coli*, preferably an *Escherichia coli* K12 (commensal strain) or an *Escherichia coli* LF82 (pathobiontic strain).

- 15 20. The HDAC inhibitor for use according to any one of claims 17 to 19, wherein said HDAC inhibitor is selected from hydroxamic acids, benzamides, short chain fatty acids, mercaptoketones, and cyclic tetrapeptides.

- 20 21. The HDAC inhibitor for use according to any one of claims 17 to 19, wherein said HDAC inhibitor is trichostatin A or suberoylanilide hydroxamic acid.

22. The HDAC inhibitor for use according to any one of claims 17 to 19, wherein said HDAC inhibitor is selected from Panobinostat (LBH589), Vorinostat (SAHA), Entinostat (MS-275), Belinostat (PXD101), Abexinostat (PCI-24781), Dacinostat
25 (LAQ824), Quisinostat (JNJ-26481585), Mocetinostat (MGCD0103), Valproic acid, CUDC-101, Droxinostat, Pracinostat (SB939), PCI-34051, Givinostat (ITF2357), AR-42, Tubastatin A, CUDC-907, M344, Tacedinaline (CI996), Rocilinostat (ACY-1215), Abexinostat/PCI-24781, and Scriptaid.

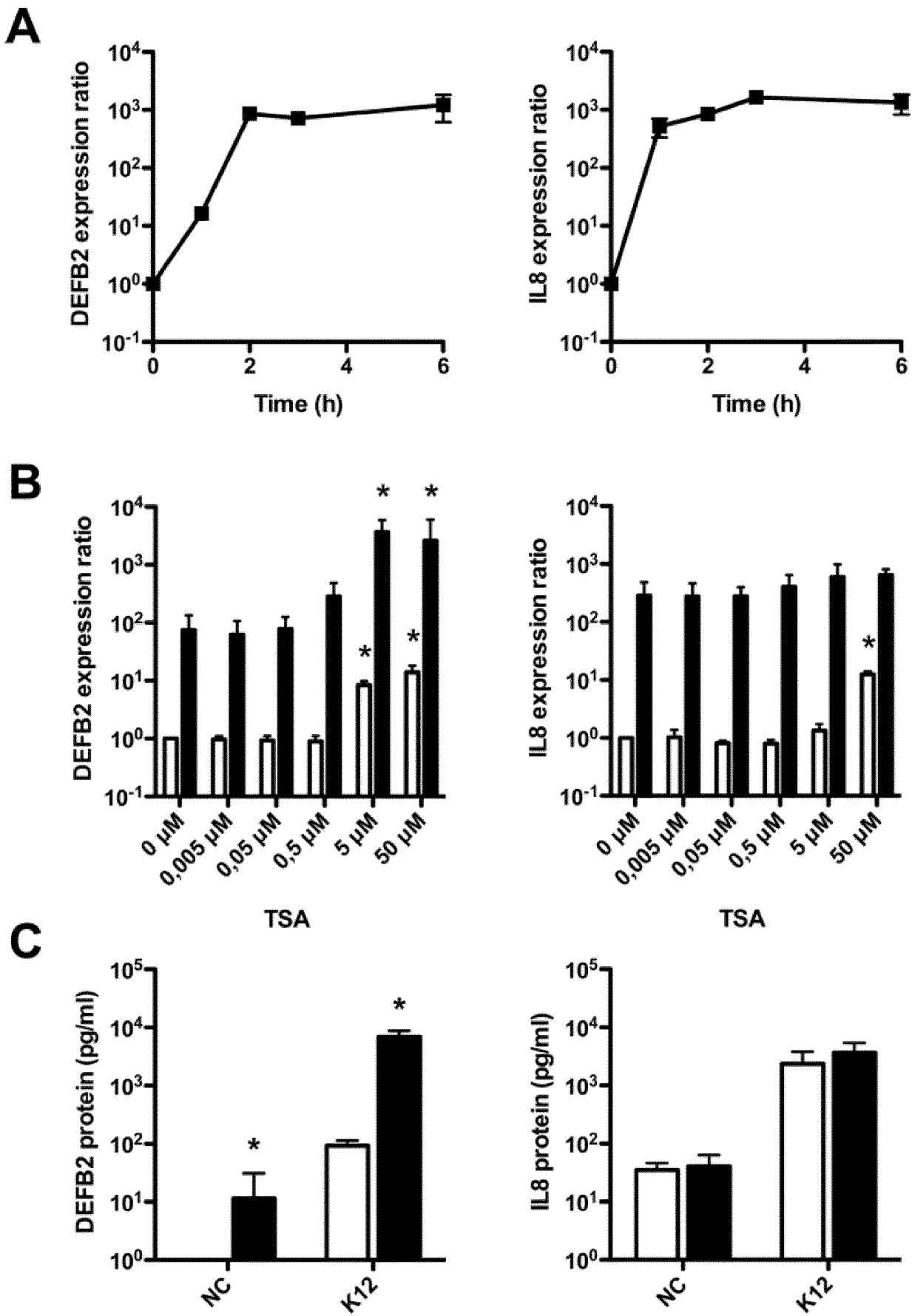


Figure 1

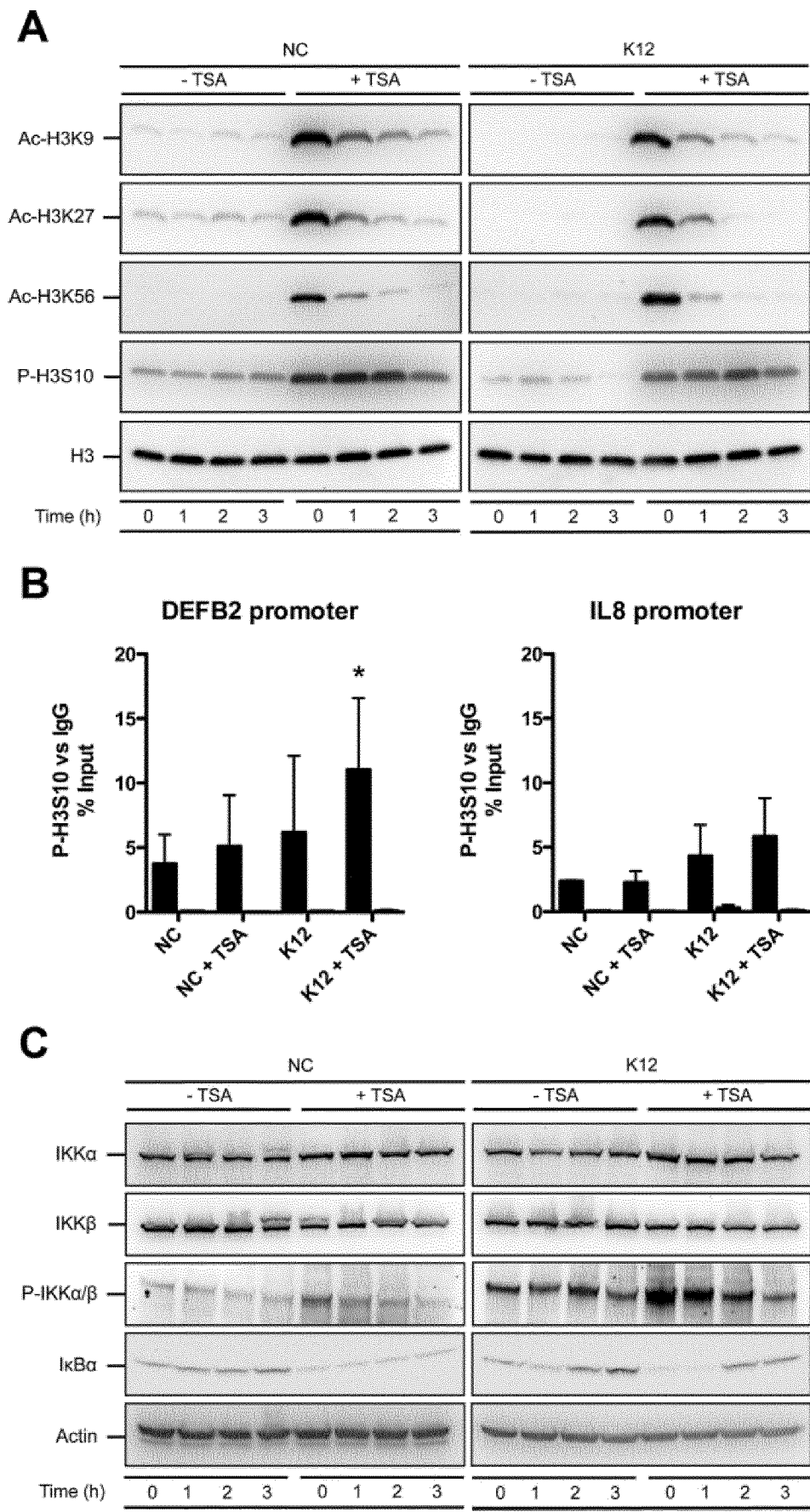


Figure 2

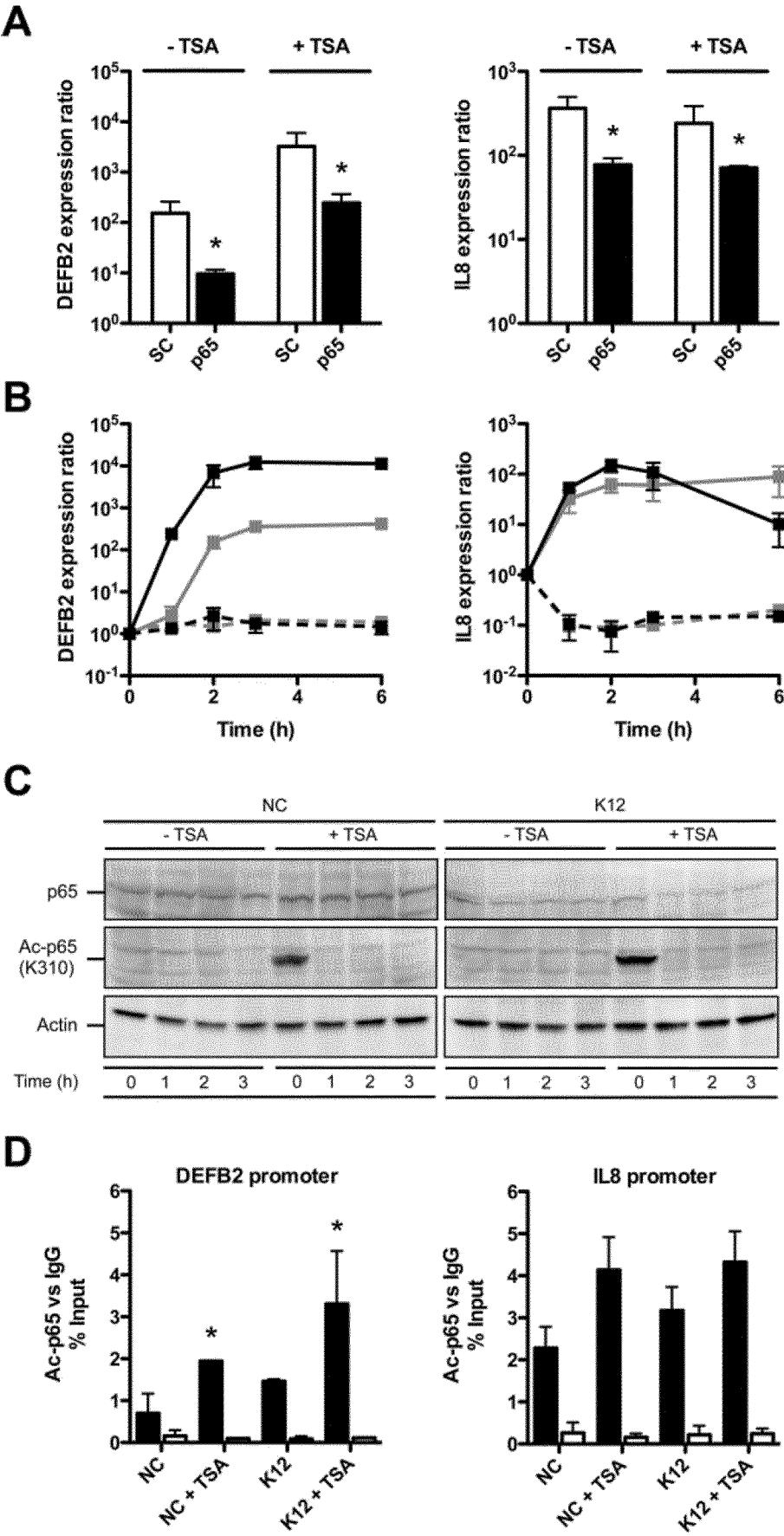


Figure 3

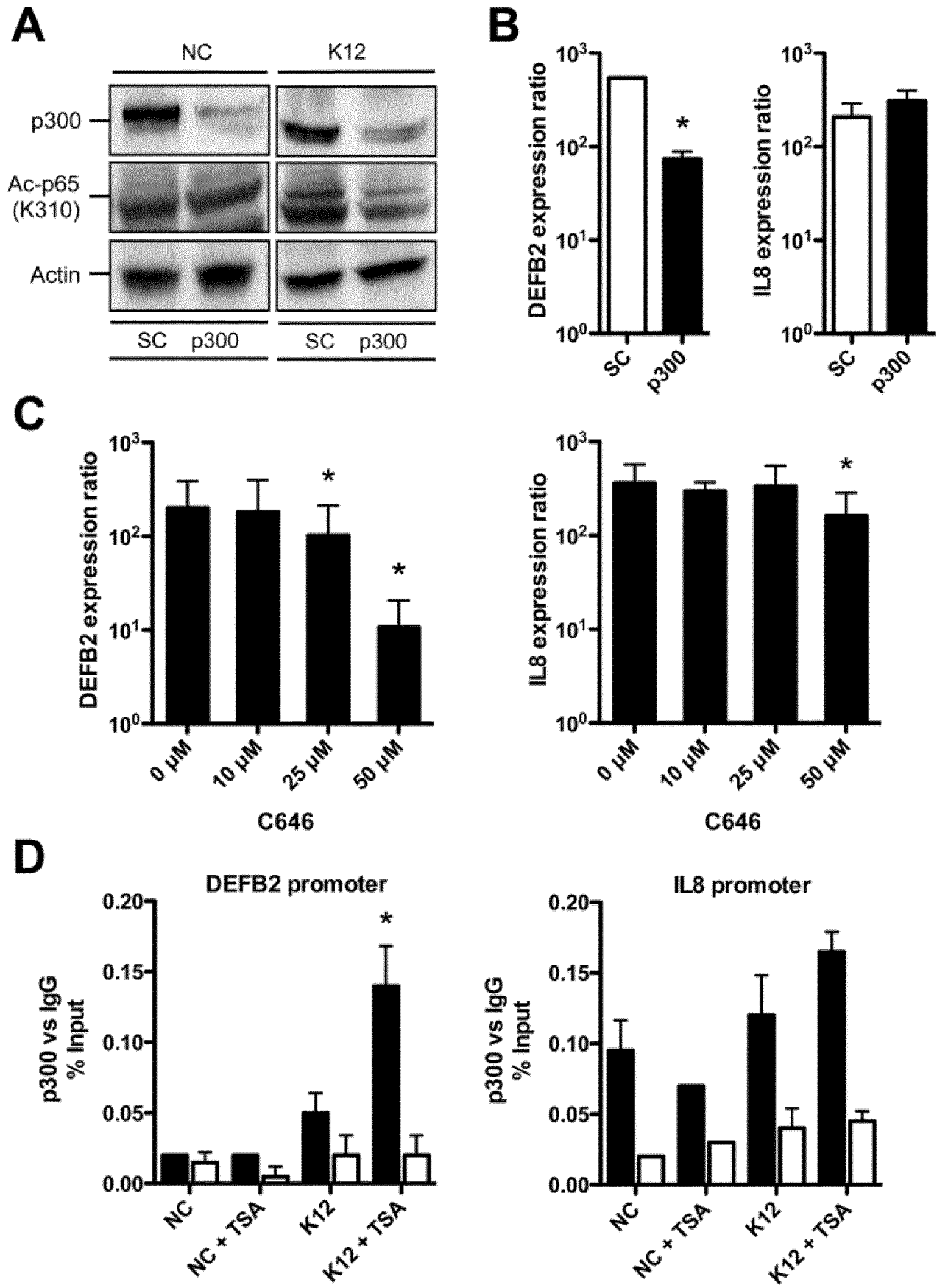


Figure 4

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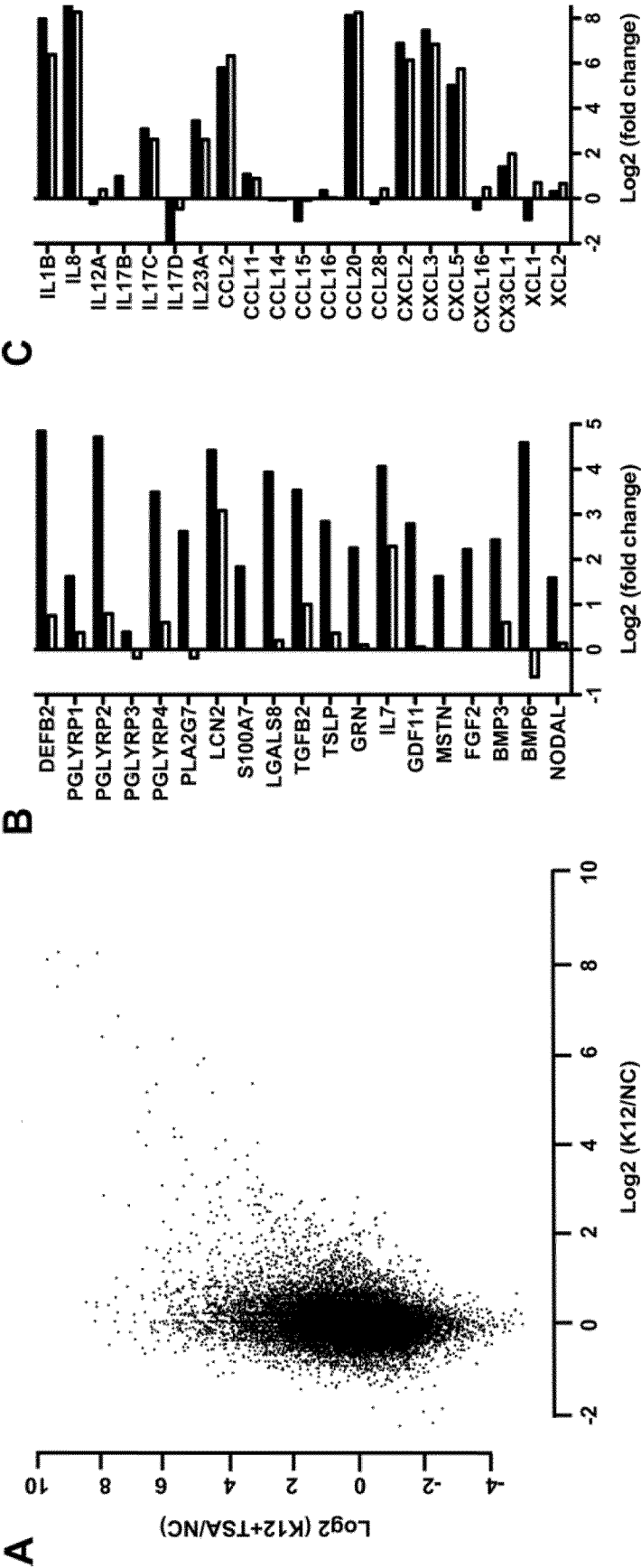


Figure 5

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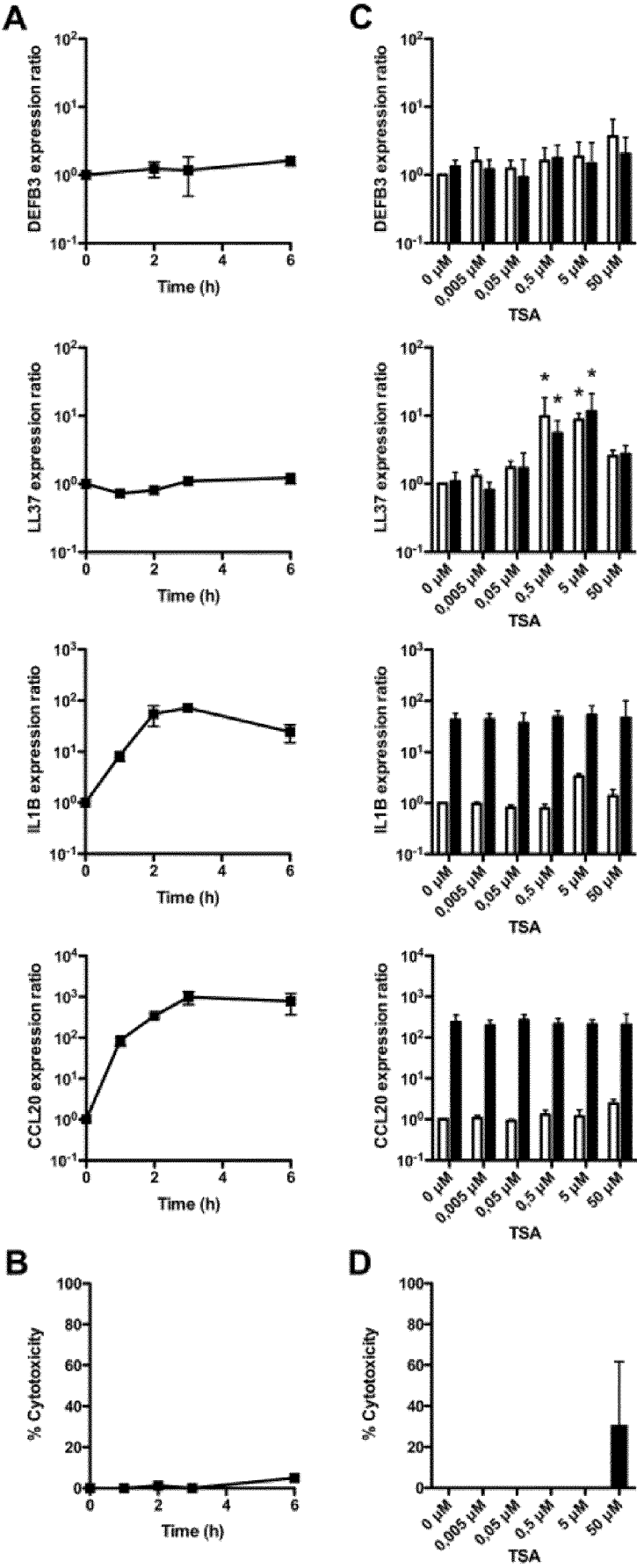


Figure 6

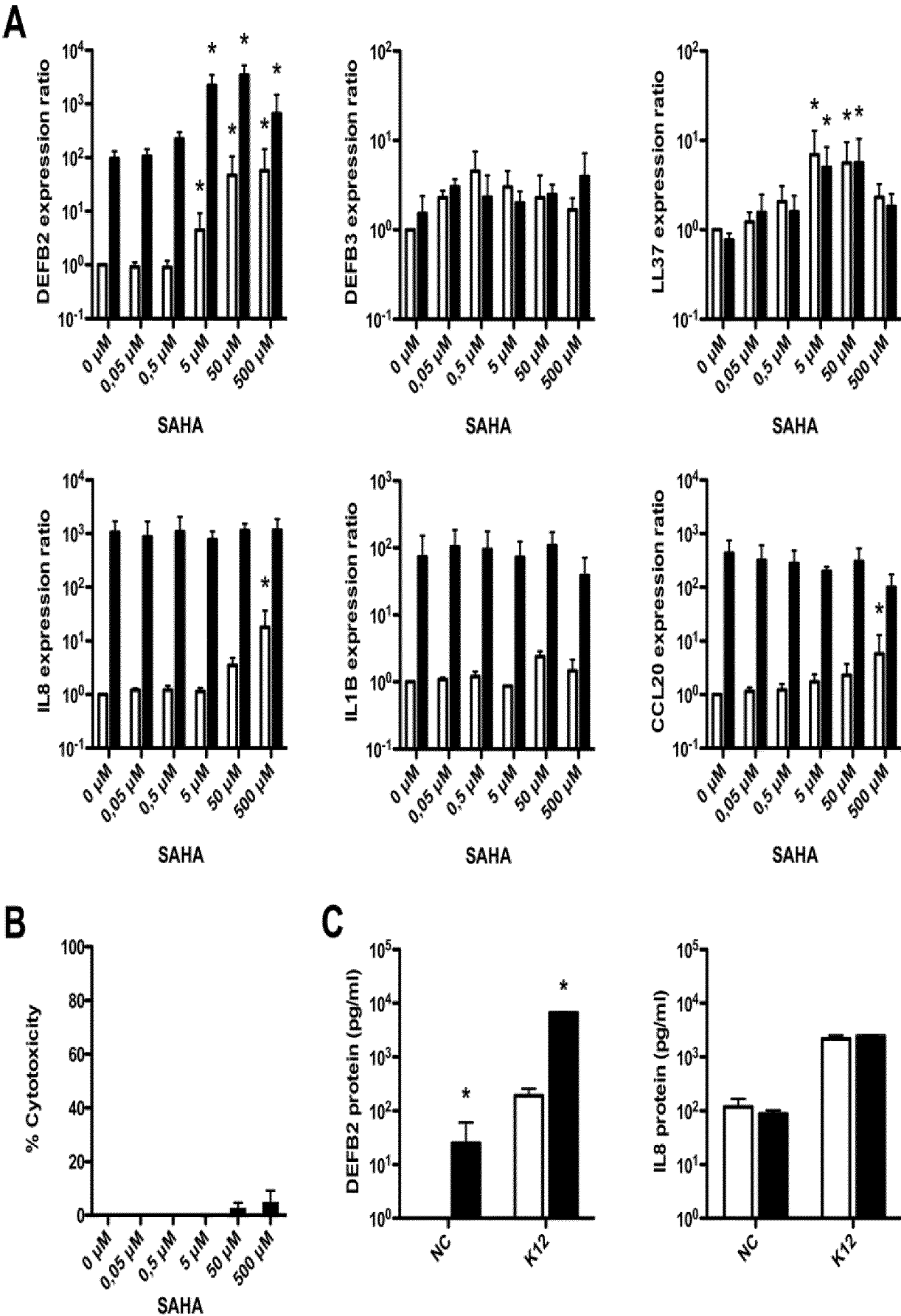


Figure 7

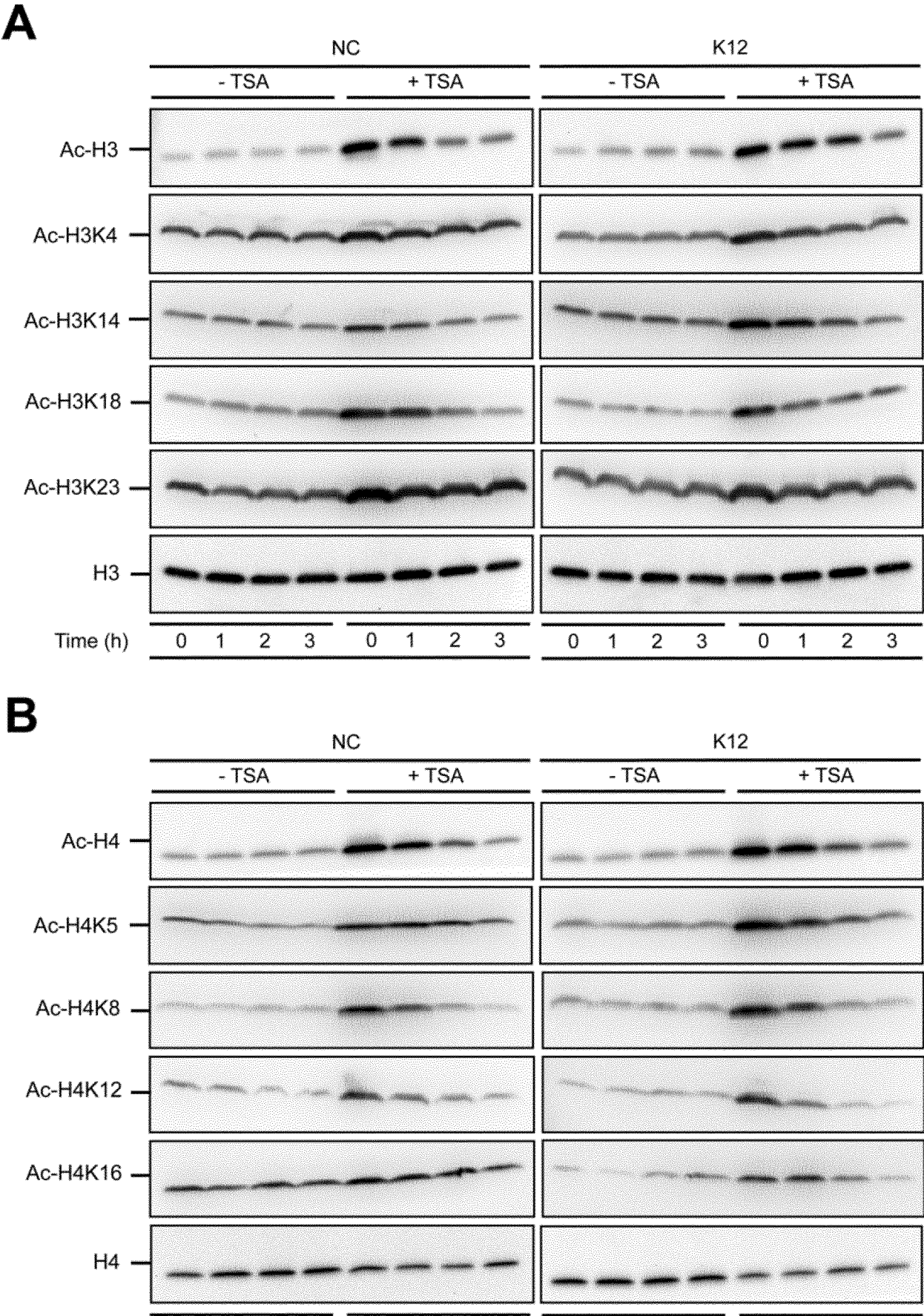


Figure 8

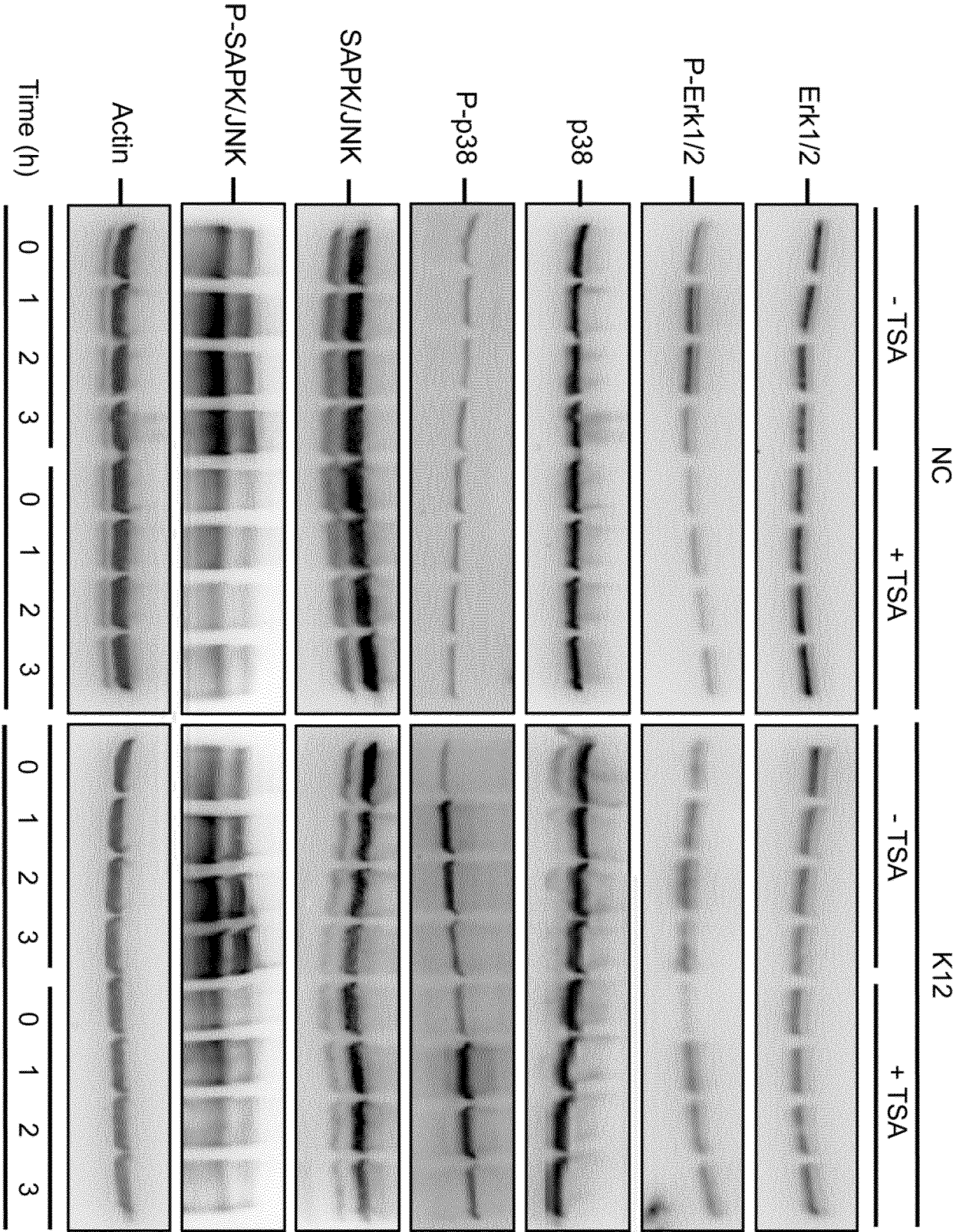
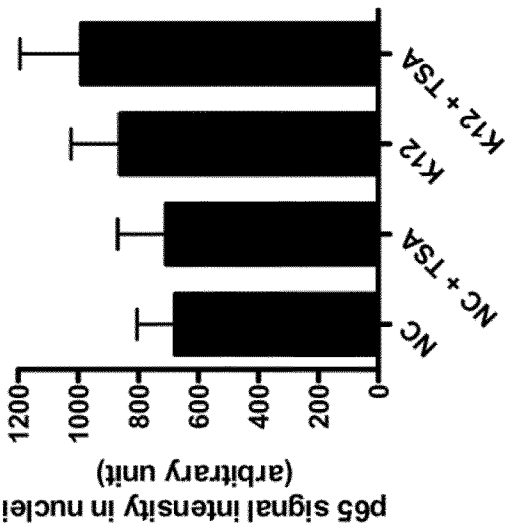


Figure 9

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B



A

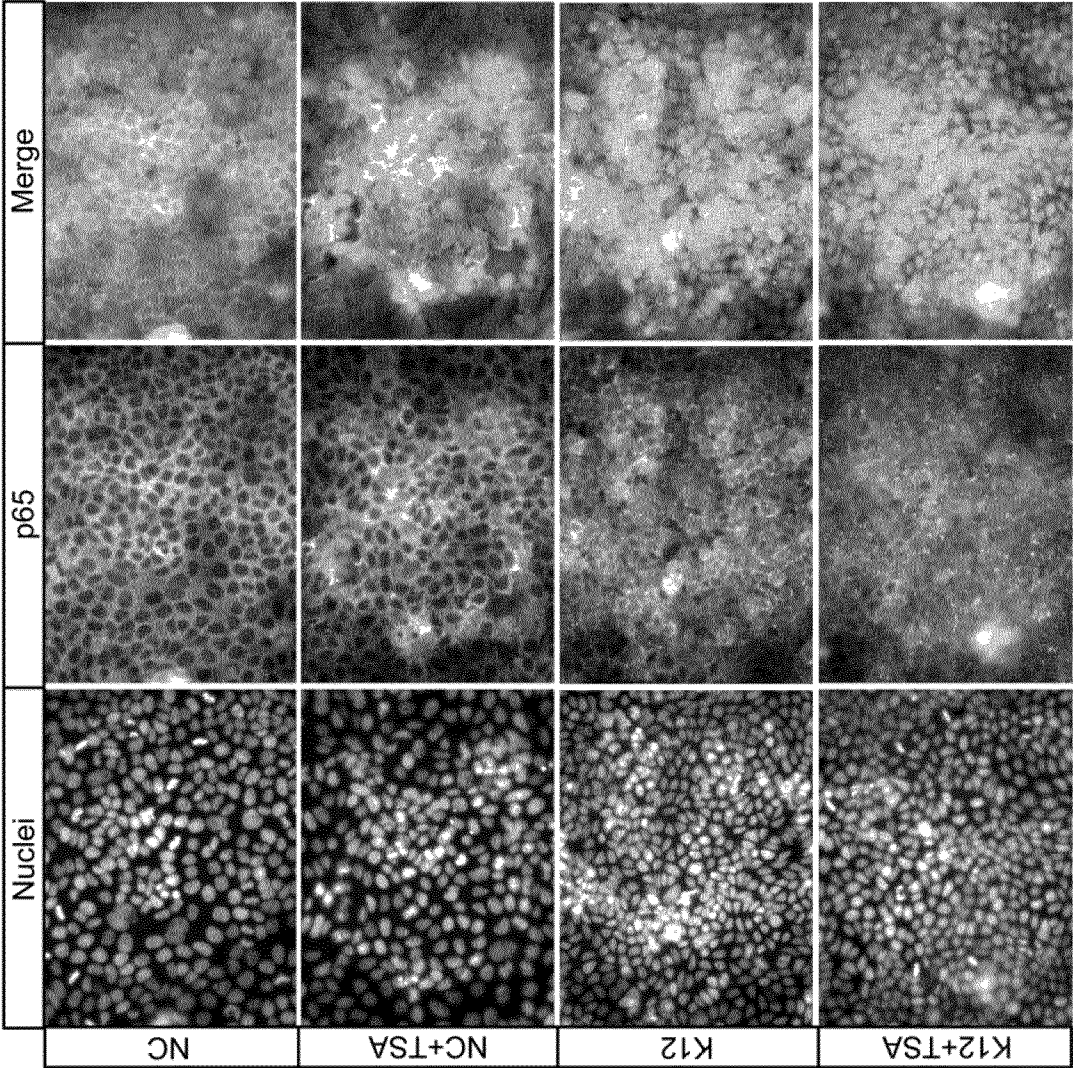


Figure 10

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E. coli K12 : commensal strain
Cell challenge : 2 hours
Multiplicity of infection : 10 bacteria per cell

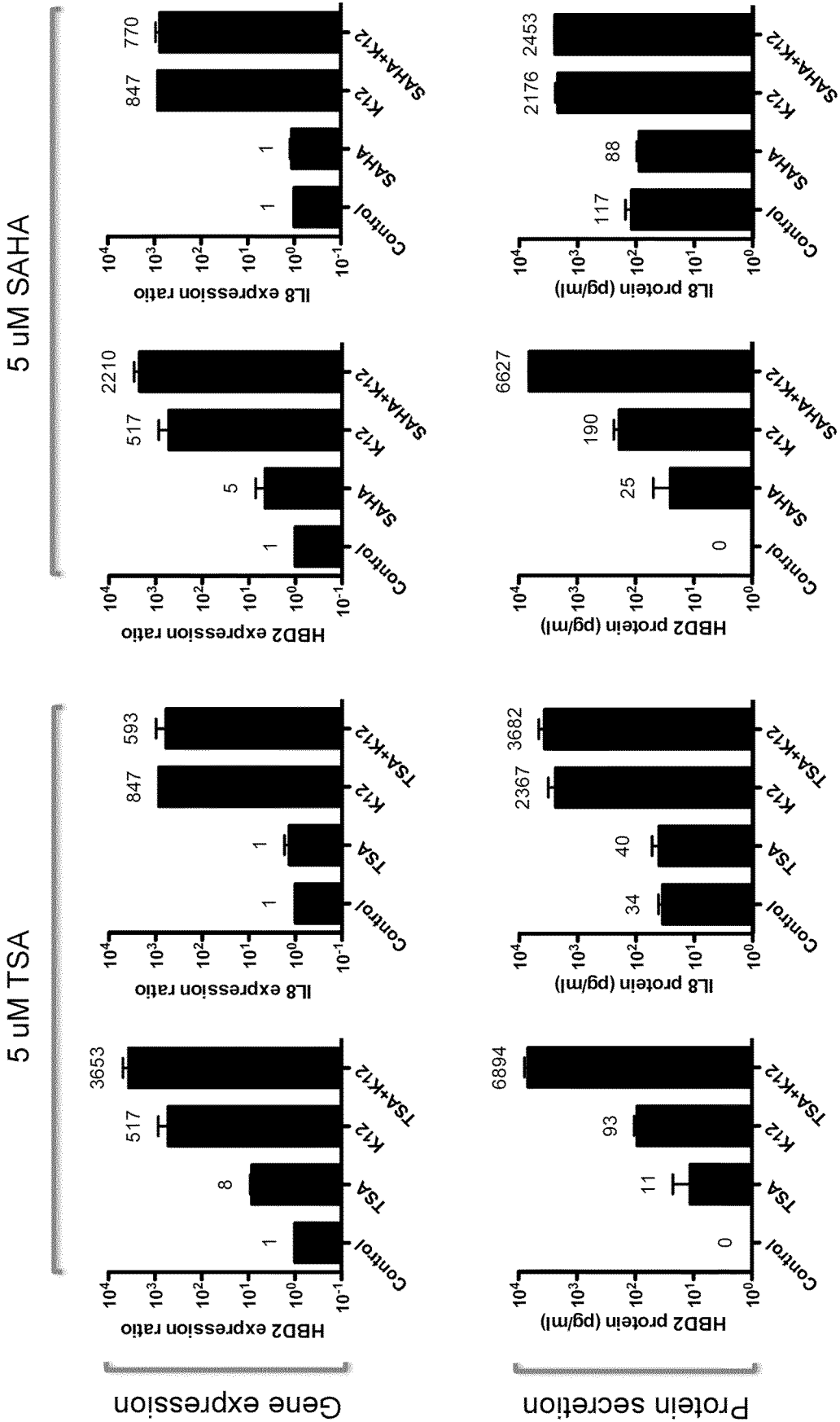


Figure 11

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E. coli LF82 : pathobiontic strain
Cell infection : 2 hours
Multiplicity of infection : 10 bacteria per cell

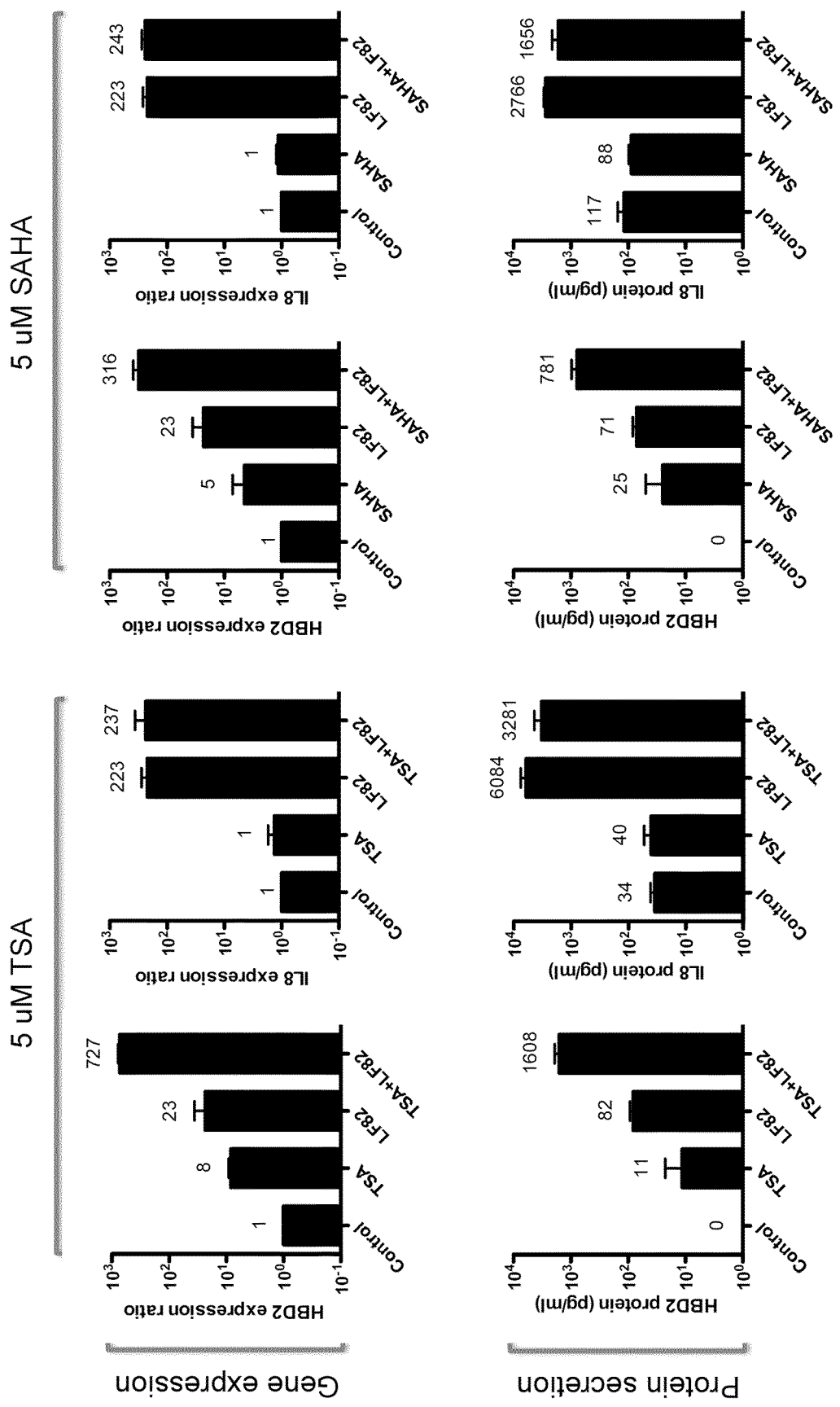


Figure 12

HDACi : Abexinostat (PCI-24781)
Cell challenge : 2 hours
Multiplicity of infection : 10 bacteria per cell

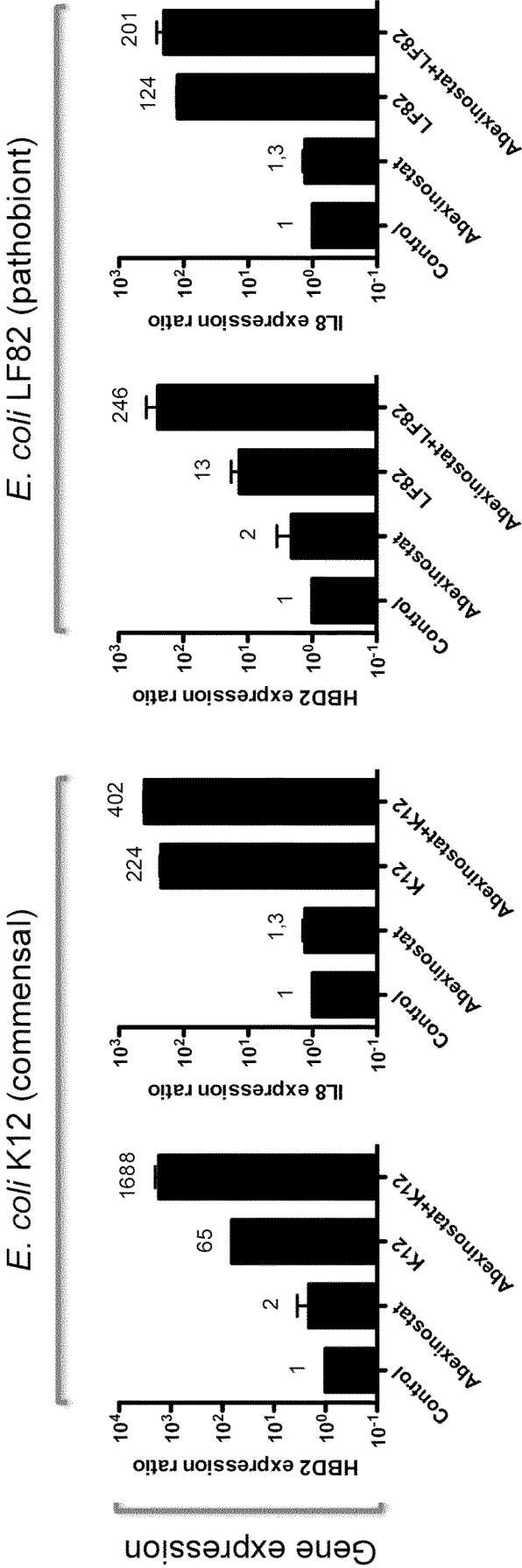


Figure 13



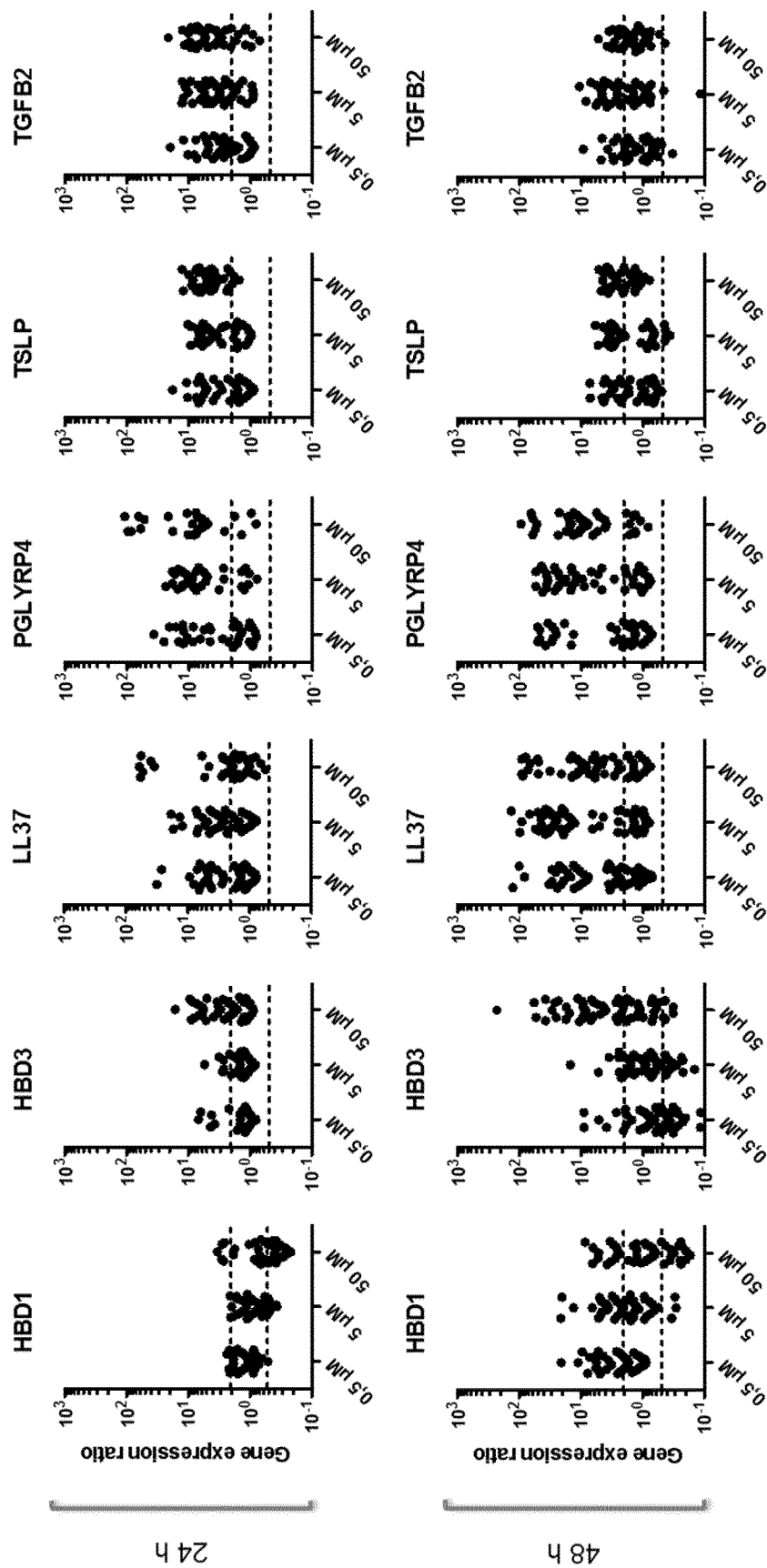


Figure 15

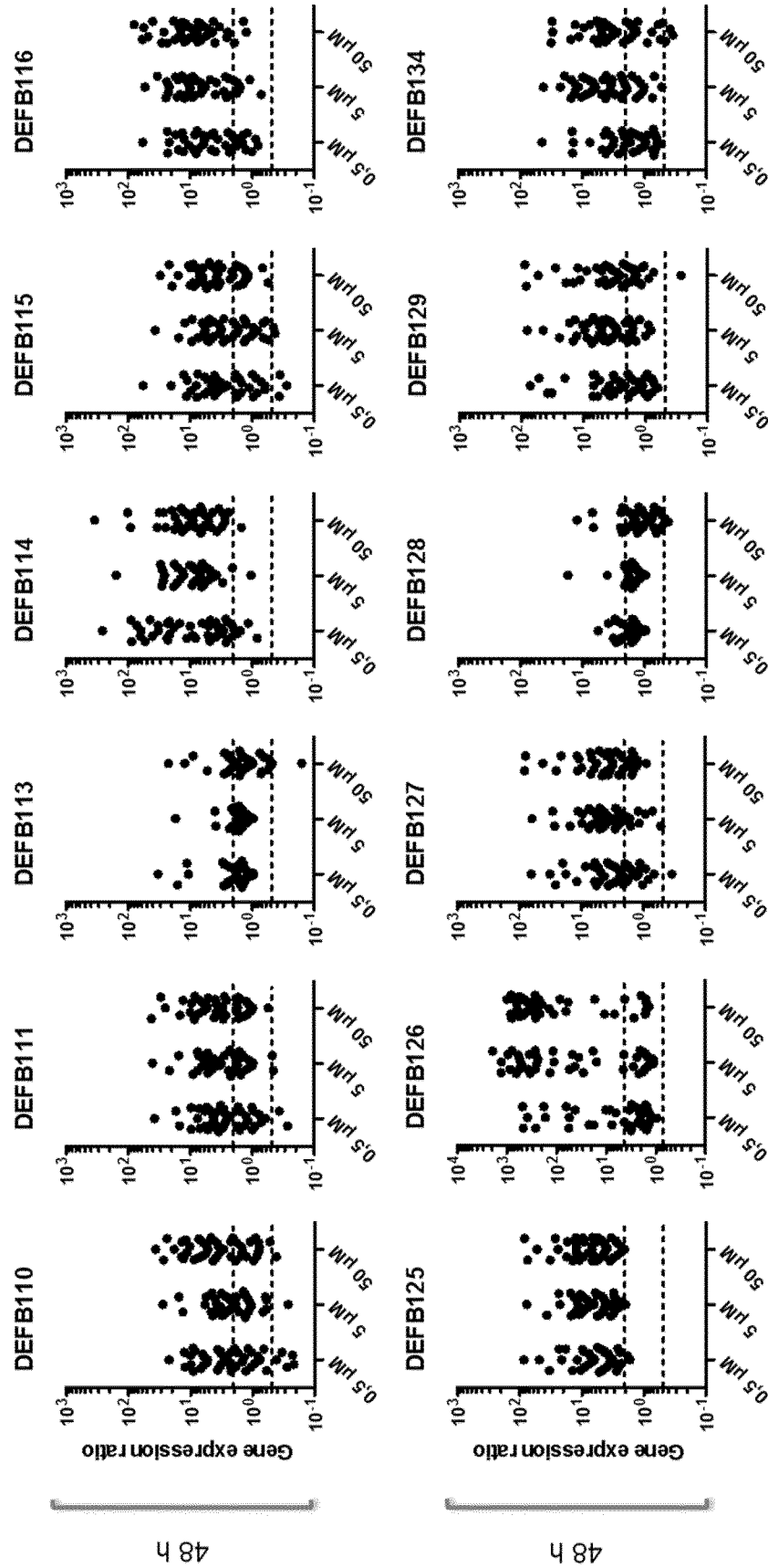


Figure 16

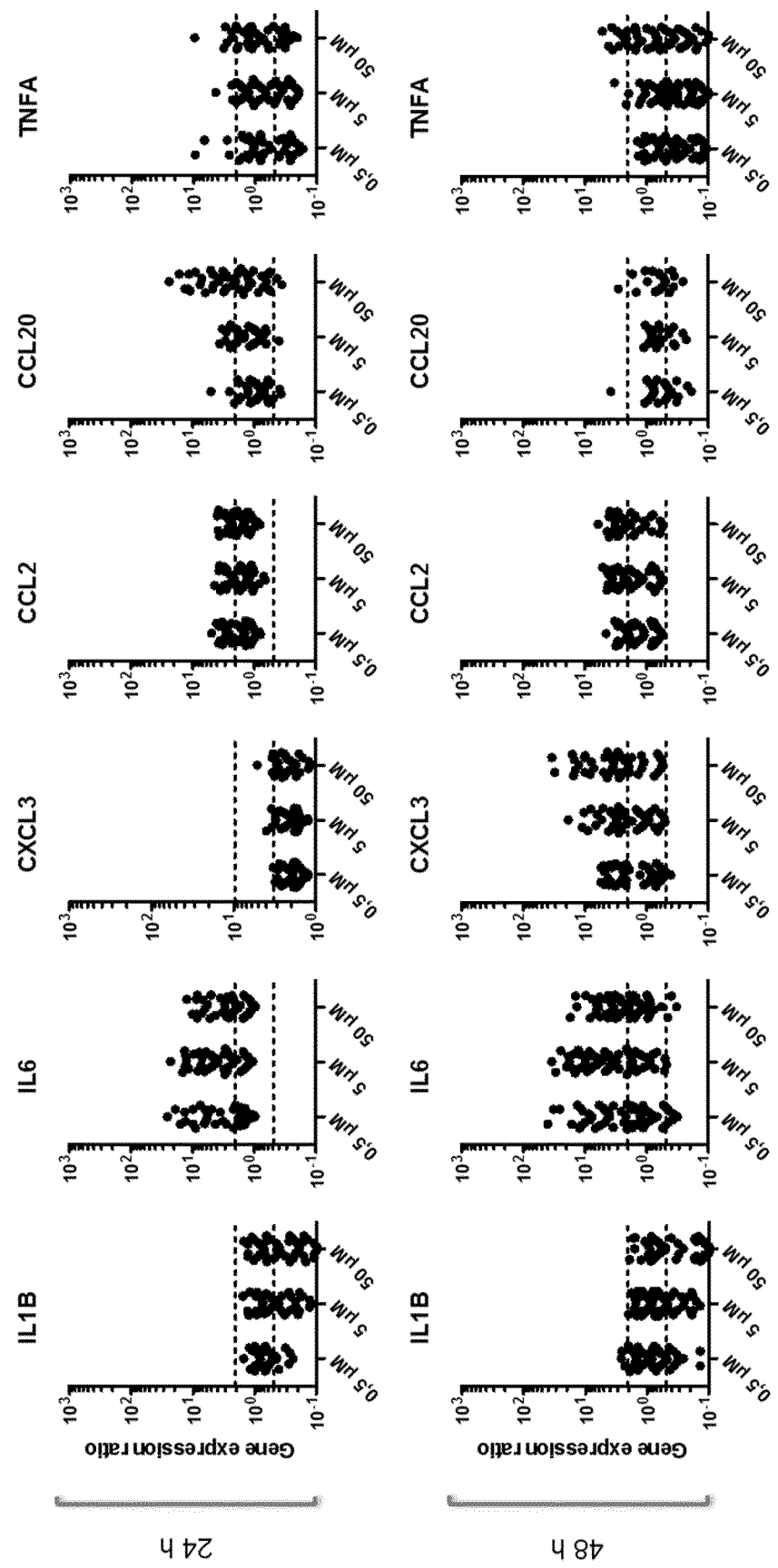


Figure 17

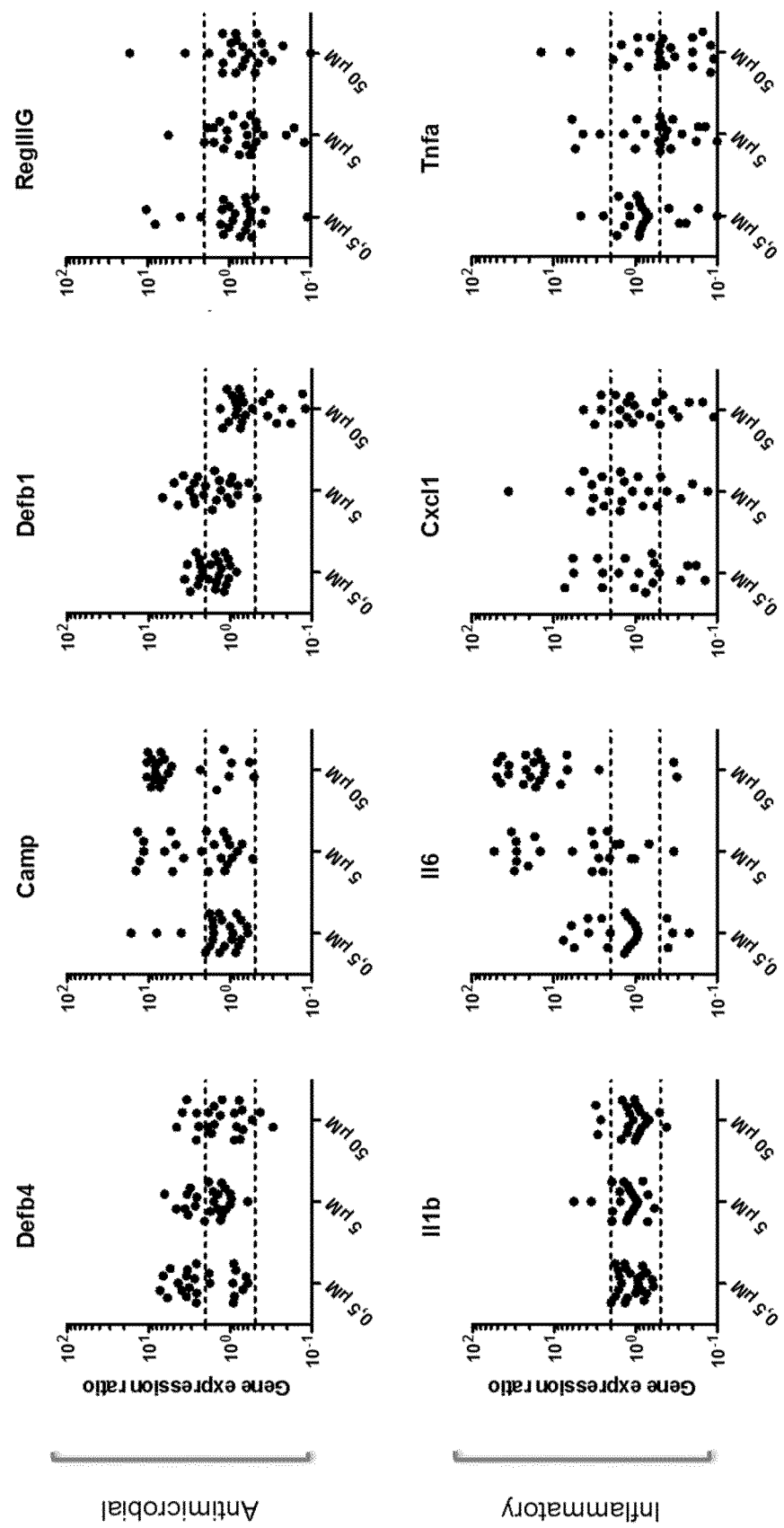


Figure 18

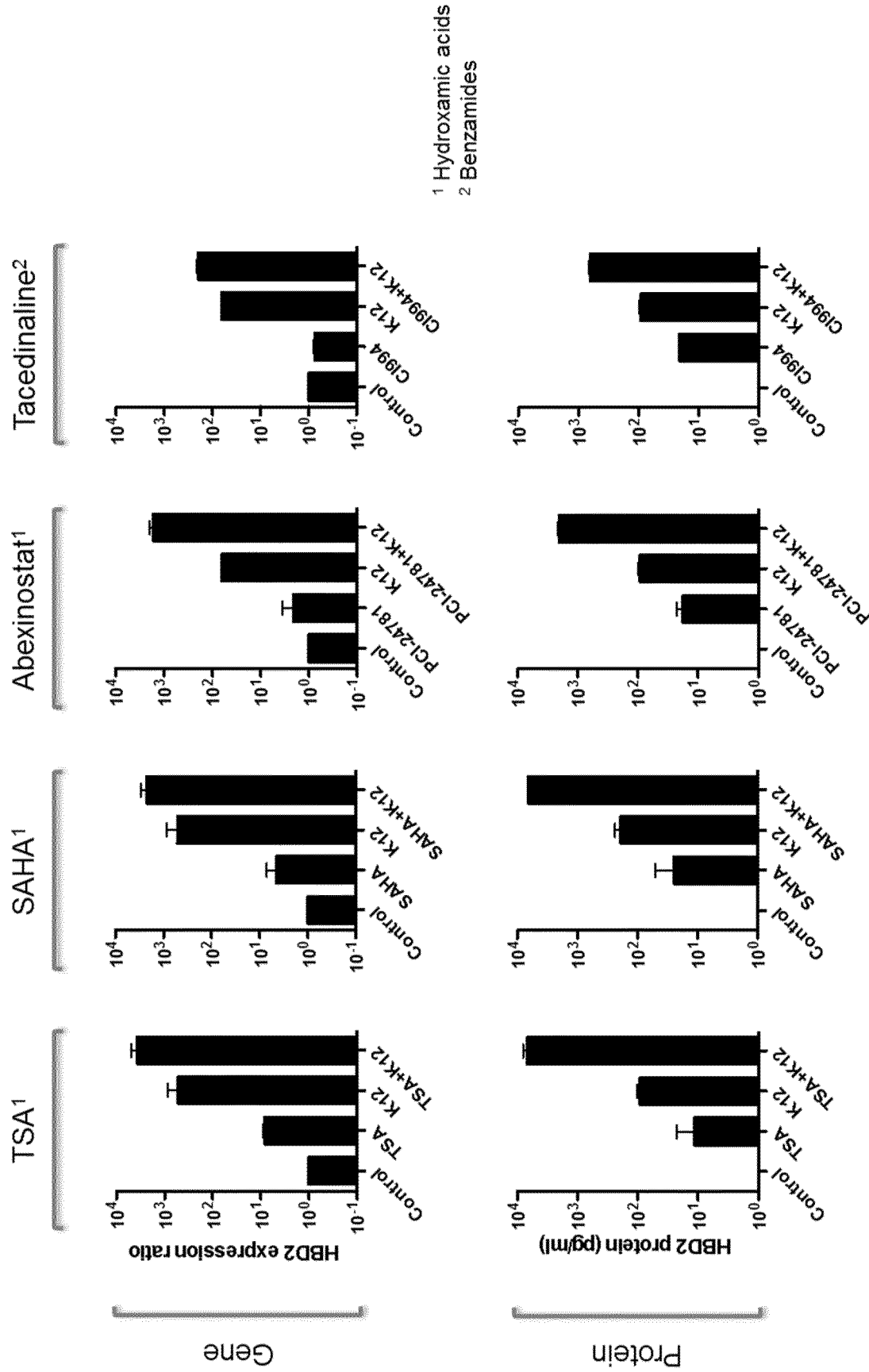


Figure 19

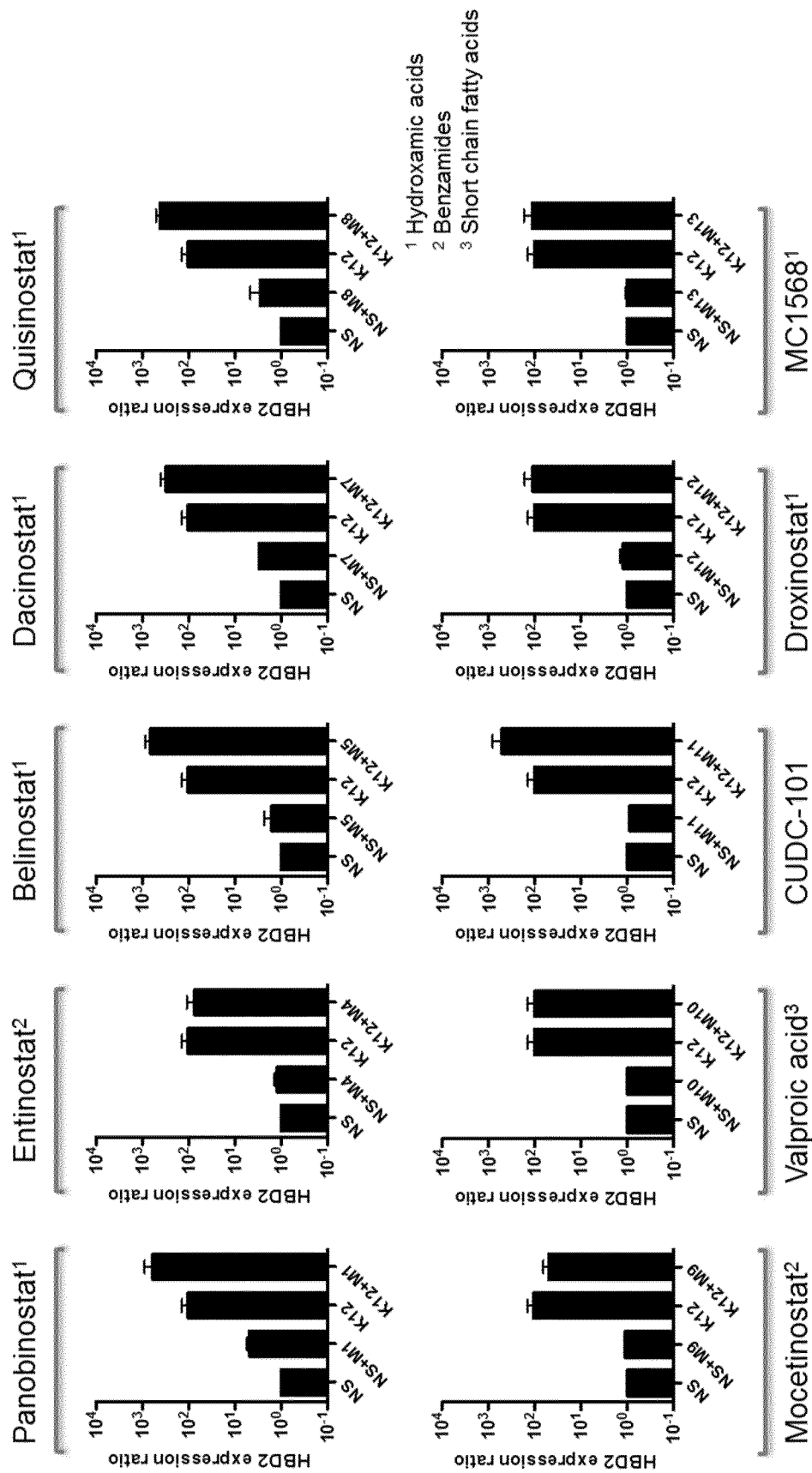


Figure 20

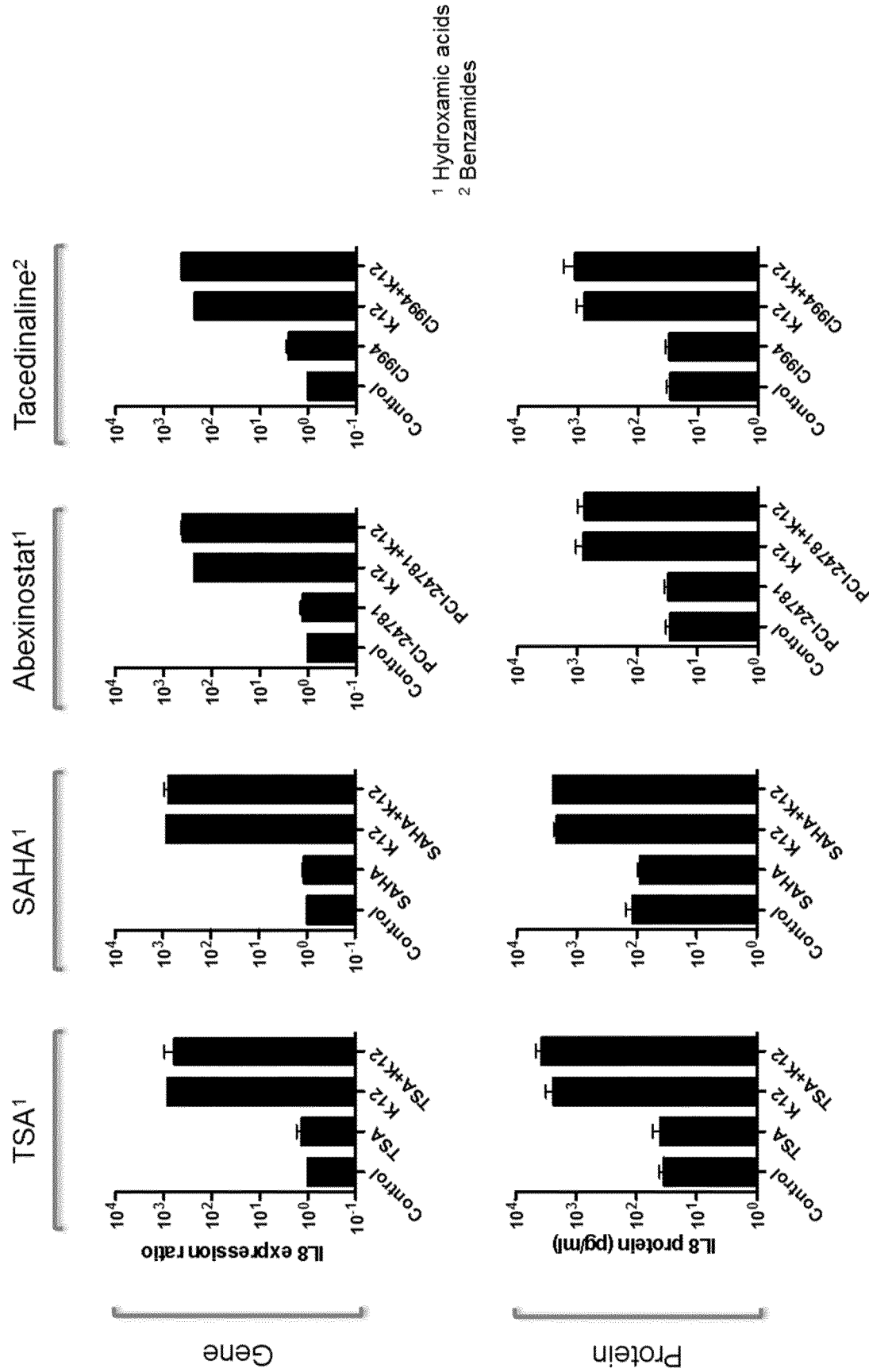


Figure 21

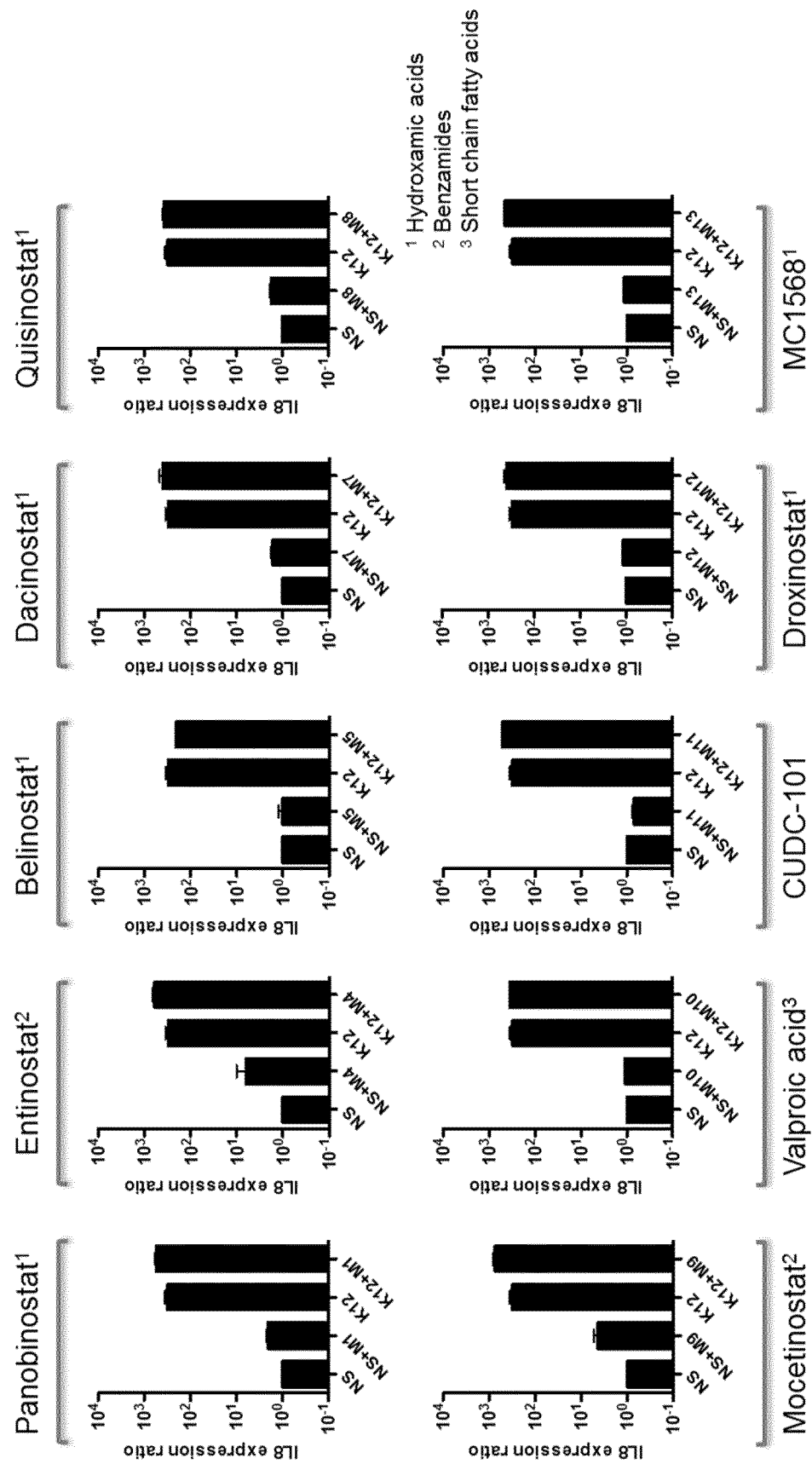


Figure 22

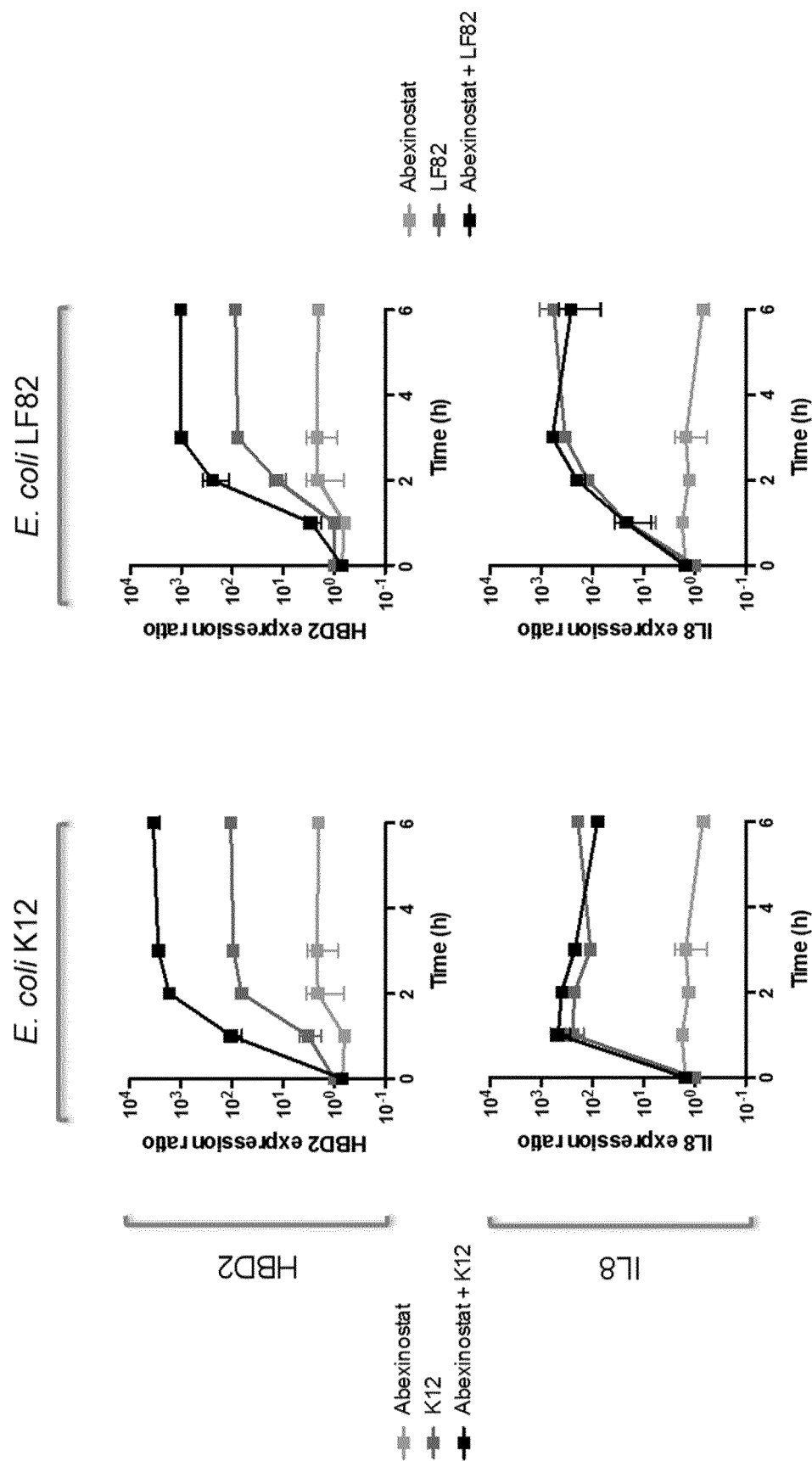


Figure 23

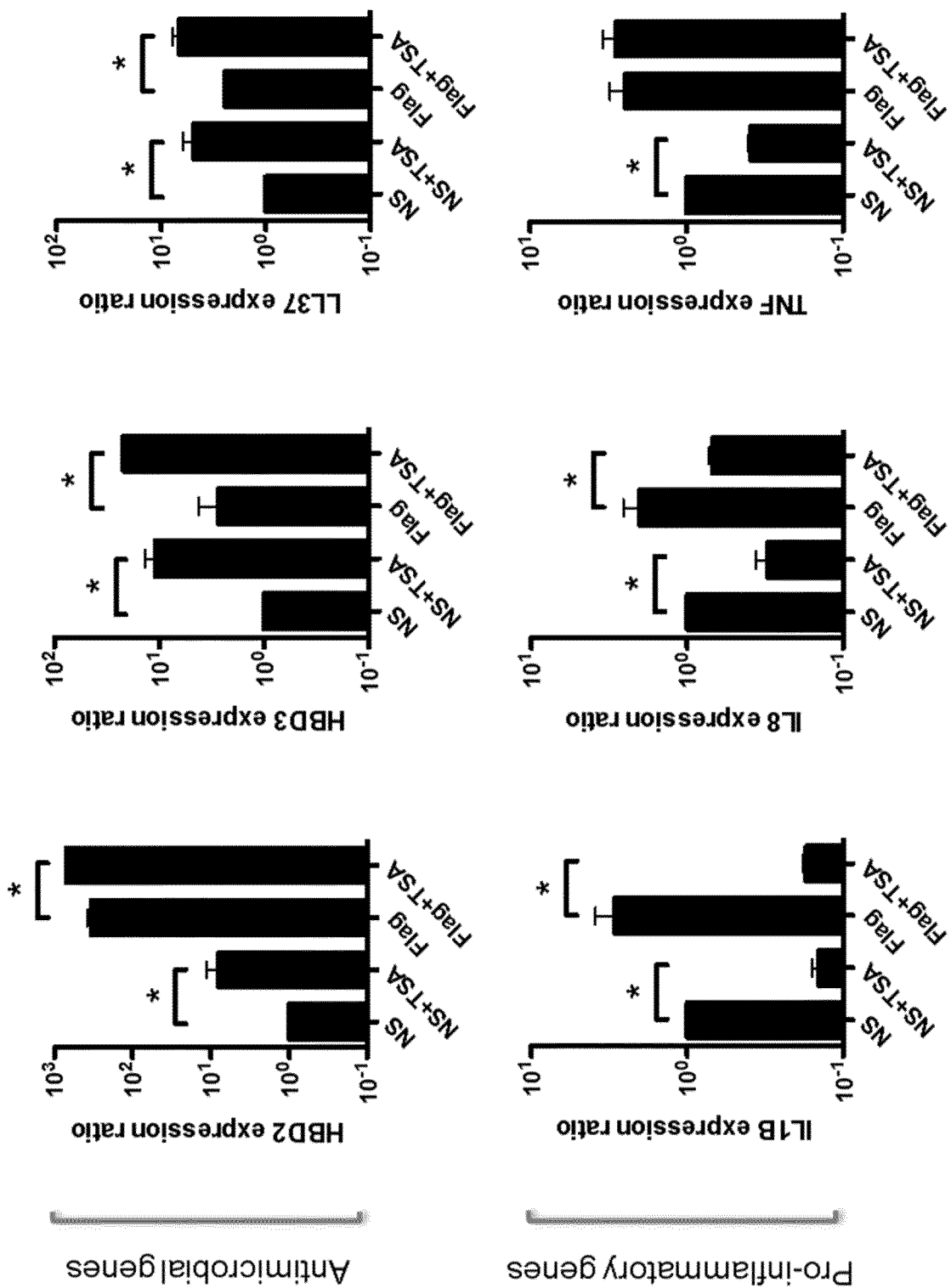


Figure 24

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/066622A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K45/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YIN L ET AL: "Epigenetic regulation of human [beta]-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria.", MUCOSAL IMMUNOLOGY JUL 2011, vol. 4, no. 4, July 2011 (2011-07), pages 409-419, XP002761695, ISSN: 1935-3456 abstract ----- -/--	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 September 2016

Date of mailing of the international search report

23/09/2016

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Fax: (+31-70) 340-3016

Authorized officer

Weinberg, Suzanna

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/066622

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YEDERY ROSHAN D ET AL: "Augmentation of Cationic Antimicrobial Peptide Production with Histone Deacetylase Inhibitors as a Novel Epigenetic Therapy for Bacterial Infections.", ANTIBIOTICS (BASEL, SWITZERLAND) 2015, vol. 4, no. 1, 12 January 2015 (2015-01-12), pages 44-61, XP002761693, ISSN: 2079-6382	1-22
Y	the whole document	1-22
X	RAQIB RUBHANA ET AL: "Efficacy of sodium butyrate adjunct therapy in shigellosis: a randomized, double-blind, placebo-controlled clinical trial.", BMC INFECTIOUS DISEASES 2012, vol. 12, 111, 10 May 2012 (2012-05-10), pages 1-11, XP002761694, ISSN: 1471-2334 abstract page 2, left-hand column	17,18,20
X	AL-MAMUN ABDULLAH ET AL: "Treatment with phenylbutyrate in a pre-clinical trial reduces diarrhea due to enteropathogenic Escherichia coli: link to cathelicidin induction.", MICROBES AND INFECTION / INSTITUT PASTEUR NOV 2013, vol. 15, no. 13, November 2013 (2013-11), pages 939-950, XP002761711, ISSN: 1769-714X abstract	1-22
Y	FAHEY J W ET AL: "Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[<i>a</i>]pyrene-induced stomach tumors", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 99, no. 11, 28 May 2002 (2002-05-28), pages 7610-7615, XP008086721, ISSN: 0027-8424, DOI: 10.1073/PNAS.112203099 the whole document	1-22
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/066622

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	FISCHER NATALIE ET AL: "Histone deacetylase inhibition enhances antimicrobial peptide but not inflammatory cytokine expression upon bacterial challenge.", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 24 MAY 2016, vol. 113, no. 21, 24 May 2016 (2016-05-24) , pages E2993-E3001, XP002761696, ISSN: 1091-6490 the whole document -----	1-22