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# Gene expression signature-based approach identifies a pro-resolving mechanism of action for histone deacetylase inhibitors

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Despite several therapies being currently available to treat inflammatory diseases, new drugs to treat chronic conditions with less side effects and lower production costs are still needed. An innovative approach to drug discovery, the Connectivity Map (CMap), shows how integrating genome-wide gene expression data of drugs and diseases can accelerate this process. Comparison of genome-wide gene expression data generated with annexin A1 (AnxA1) with the CMap revealed significant alignment with gene profiles elicited by histone deacetylase inhibitors (HDACIs), what made us to hypothesize that AnxA1 might mediate the anti-inflammatory actions of HDACIs. Addition of HDACIs (valproic acid, sodium butyrate and thricostatin A) to mouse macrophages caused externalization of AnxA1 with concomitant inhibition of cytokine gene expression and release, events that occurred independently as this inhibition was retained in AnxA1 null macrophages. In contrast, novel AnxA1-mediated functions for HDACIs could be unveiled, including promotion of neutrophil apoptosis and macrophage phagocytosis, both steps crucial for effective resolution of inflammation. In a model of acute resolving inflammation, administration of valproic acid and sodium butyrate to mice at the peak of disease accelerated resolution processes in wild type, but much more modestly in AnxA1 null mice. Deeper analyses revealed a role for endogenous AnxA1 in the induction of neutrophil death *in vivo* by HDACIs. In summary, interrogation of the CMap revealed an unexpected association between HDACIs and AnxA1 that translated in mechanistic findings with particular impact on the processes that regulate the resolution of inflammation. We propose non-genomic modulation of AnxA1 in immune cells as a novel mechanism of action for HDACIs, which may underlie their reported efficacy in models of chronic inflammatory pathologies.

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Inflammation is a complex biological response to harmful stimuli such as pathogens or tissue injury in which several cells and chemical mediators take part to neutralize the injurious element and initiate the healing process. In the absence of inflammation, wound and infections would never heal. However an excessive or prolonged inflammatory response would also be detrimental, as it would cause excessive tissue damage and loss of function. Inflammation is then a protective response characterized by a tightly regulated balance between pro-inflammatory and pro-resolving mechanisms.[1,2] Neutrophil apoptosis is a crucial event for an effective resolution of inflammation, as it determines the number of activated neutrophils present in the inflammatory sites.[3,4] The processes of neutrophil apoptosis, and their subsequent phagocytic clearance by macrophages (efferocytosis), have been proposed as novel therapeutic targets to treat inflammatory diseases.[5,6] Therefore, new concepts regarding the management of inflammation are emerging from these novel approaches: not only is necessary modulation (yet not the abrogation) of pro-inflammatory events, but

promotion and enhancement of endogenous pro-resolving pathways, is required to achieve control of the inflammatory response with rapid restoration of tissue homeostasis.[7,8]

A novel approach to drug discovery, the Connectivity Map (CMap), proposed by Lamb *et al.*[9,10] and recently validated by Sirota *et al.*,[11] demonstrates how integrating genome-wide gene expression data of drugs and diseases can be used for compound repositioning (i.e., application of known drugs to new indications), or for discovery of new drugs, reducing costs and years required for their development. The CMap is a publicly available collection of genome-wide gene expression signatures of human cells treated with a number of bioactive small molecules, most of which are FDA-approved drugs. The most recent version contains 7056 gene expression profiles from 1309 bioactive compounds, representing 6100 individual treatments in five different human cell lines. This database can be queried with any gene-expression profile of interest (e.g., the expression profile of a compound, or a disease) and the results yield a list of the CMap drugs ranked, according to the similarity with the signature of interest. Hence, drugs with

negative score (opposite profiles) might have the potential as new treatments for specific diseases,[12–14] while drugs with positive score (similar profiles) could be useful for identification of novel actions of existing drugs[15] or to unravel drug mechanisms of action.[16–18]

Using the gene expression profile produced by the anti-inflammatory pro-resolving molecule annexin A1 (AnxA1),[19] we found very strong positive connections with several members of the histone deacetylase inhibitors (HDACIs) class of drugs. HDACIs are being actively investigated for the treatment of cancer owing to their pro-apoptotic effect,[20,21] although a substantial body of evidence indicates that this chemically-unrelated family of molecules affords potent anti-inflammatory properties[22,23] with potential therapeutic in pathologies including rheumatoid arthritis,[24,25] inflammatory bowel disease[26] and heart disease.[27,28] The mechanisms underlying these anti-inflammatory properties of HDACIs are largely not understood. In the present work, we performed a re-interpretation of genome-wide expression data using the CMap to reveal a mechanistic association between HDACIs and the pro-resolving molecule AnxA1.

## Results

### Query of the CMap database identifies a novel link between AnxA1 and HDACIs.
The gene expression signature of AnxA1 on HEK293 cells transfected with the receptor FPR2/ALX, consisting of 118 genes (described in[29]), was used to query the most recent version of the CMap database. Not too surprising in view of the anti-inflammatory nature of AnxA1, strong connections (positive score) were found with several NSAIDs and glucocorticoids, validating the use of the 118 genes signature to generate new predictions (score, cell line): paracetamol (0.933 in HL60 cells), aceclofenac (0.672, PC3; 0.509, MCF7), acetylsalicylic acid (0.307, MCF7), ketoprofen (0.733, HL60), diflunisal (0.629, HL60), naproxen (0.481, HL60), beclomethasone (0.995, HL60) or dexamethasone (0.742, HL60; 0.378, MCF7). More interestingly, consistent positive connections were found with several drugs of the HDACI family (Table 1). Several distinct hypotheses could be derived from these results, including the possibility that AnxA1 might have HDACI activity. However, as previous studies in the cancer field suggest that HDACIs engage, in a yet unclear manner, endogenous AnxA1,[30–32] we hypothesized that also the anti-inflammatory effects of HDACIs might be reliant, at least in part, on this pro-resolving mediator.

### HDACIs induce the release of AnxA1 by peritoneal macrophages and bone marrow neutrophils.
Neutrophils and macrophages contain high levels of AnxA1, which is normally stored inside their cytosol and it is released upon activation during ongoing inflammation. Drugs like glucocorticoids exert part of their non-genomic actions by inducing release of AnxA1. Thus, we tested if HDACIs could mobilize AnxA1 in murine peritoneal macrophages and bone marrow isolated neutrophils.

Western blotting analyses (Figure 1a) showed a concentration-dependent accumulation of AnxA1 in supernatants of

**Table 1** HDACIs drugs associated (positive score) with AnxA1

| Compound | Cell line | Score | n | P-value | Specificity |
|---|---|---|---|---|---|
| Trichostatin A | HL60 | 0.365 | 34 | 0.00022 | 0.4049 |
| | MCF7 | 0.621 | 92 | 0 | 0.2417 |
| | PC3 | 0.323 | 55 | 0.00004 | 0.8089 |
| | ssMCF7 | 0.884 | 1 | — | — |
| Valproic acid | HL60 | 0.393 | 14 | 0.01962 | 0.2349 |
| | MCF7 | 0.261 | 31 | 0.024 | 0.1521 |
| | PC3 | 0.214 | 10 | 0.67543 | 0.8269 |
| | SKMEL5 | −0.713 | 1 | — | — |
| | ssMCF7 | 0.693 | 1 | — | — |
| Vorinostat | HL60 | 0.707 | 3 | 0.0496 | 0.3824 |
| | MCF7 | 0.787 | 7 | 0.00002 | 0.2584 |
| | PC3 | 0.717 | 2 | 0.15935 | 0.4144 |
| Sodium phenylbutyrate | HL60 | −0.483 | 2 | — | — |
| | MCF7 | 0.511 | 3 | — | — |
| | PC3 | 0.735 | 1 | — | — |
| | SKMEL5 | 0.691 | 1 | — | — |
| MS-275 | PC3 | 0.749 | 2 | 0.12589 | 0.3896 |
| Resveratrol | HL60 | −0.776 | 1 | — | — |
| | MCF7 | 0.433 | 6 | 0.15166 | 0.6947 |
| | PC3 | 0.326 | 2 | 0.9557 | 0.9734 |
| Scriptaid | PC3 | 0.357 | 2 | 0.90994 | 0.9725 |
| Depudecin | MCF7 | 0.407 | 2 | 0.80309 | 0.7438 |
| Geldanamycin | HL60 | 0.355 | 3 | — | — |
| | MCF7 | 0.306 | 10 | 0.24961 | 0.743 |
| | PC3 | 0.579 | 2 | 0.35479 | 0.5424 |
| HC toxin | MCF7 | 0.813 | 1 | — | — |

Abbreviation: HDACIs, histone deacetylase inhibitors.

macrophages treated for 30 min with three distinct HDACIs, namely valproic acid (VPA), sodium butyrate (SB) or trichostatin A (TSA), before LPS stimulation for 4 h. Time-course studies revealed major differences at earlier time points, with maximal release of AnxA1 at 0.5–1 h after treatments (Figure 1b). The involvement of protein kinase C and protein phosphatase 2A, indicated to have a mechanistic role in AnxA1 release, was investigated using protein kinase C (PKC$_{19-36}$, 5 $\mu$M) and protein phosphatase 2A (okadaic acid, 10 nM) inhibitors. However, as shown in Supplementary Figure S1, we did not observe a common pattern of effect of these inhibitors on the HDACIs investigated.

In primary neutrophils, application of HDACIs did not augment AnxA1 release above levels observed in unstimulated cells (Figure 1c). However, when AnxA1 expression was analyzed at shorter incubation time, data were different. In fact, cell incubation with compounds for 10-min incubation with HDACIs induced AnxA1 release and, consequently, reduced cytosolic levels both in macrophages (Figure 1d) and neutrophils (Figure 1e). Collectively, these data indicate that HDACIs induce the release of pre-stored AnxA1.

### The anti-cytokine effect of HDACIs on macrophages is independent of AnxA1.
The increase of AnxA1 released in supernatants was mirrored by a dose–response decrease of IL-6 and TNF-$\alpha$ release by all three HDACIs (Supplementary
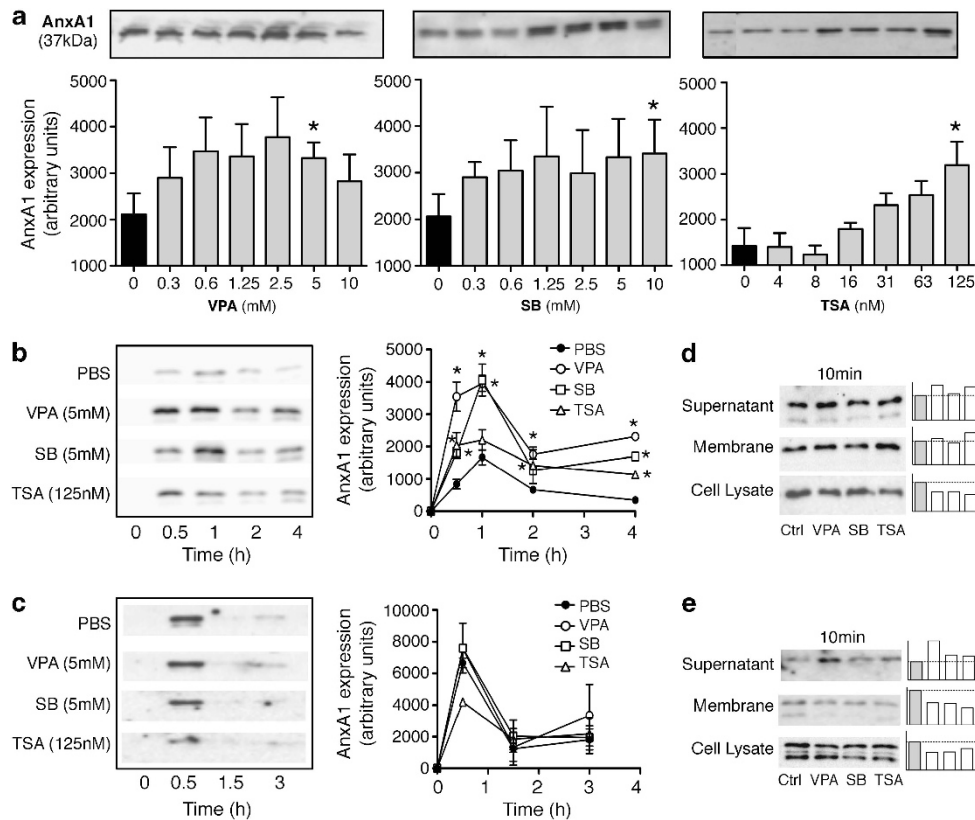
**Figure 1** AnxA1 release by murine macrophages and neutrophils. (**a**) Biogel-elicited peritoneal macrophages ($0.5 \times 10^6$ cells/well, in 400 $\mu$l final volume) were pre-treated with the reported concentrations of VPA, sodium butyrateSB and TSA for 30 min before stimulation with 75 ng/ml LPS for 4 h. Supernatant aliquots (20 $\mu$l) were analyzed by western blot. *$P < 0.05$, one-way ANOVA versus non treated cells, followed by Dunnett's correction. (**b**, **c**) AnxA1 release, as induced by 5 mM VPA or SB, or 125 nM TSA, from peritoneal macrophages ($0.5 \times 10^6$ cells/well (**b**)) and bone marrow neutrophils ($0.3 \times 10^6$ cells/well (**c**)) was assessed over time. *$P < 0.05$, two-way ANOVA versus PBS, followed by Bonferroni correction. (**d**, **e**) Modulation of AnxA1 localization in macrophages (**d**) and neutrophils (**e**) by the three HDACIs (using same concentrations as in panels **b** and **c**). Supernatants, membrane-bound and cytosolic AnxA1 expression was quantified, with the latter being normalized to $\beta$-actin levels. AnxA1 bands were quantified using ImageJ64 software. In all cases data are mean ± S.E.M. of at least three independent experiments, with blots displaying representative ones

Figure S2), which was not associated to apoptosis induction at the time point studied, as analyzed by AnxAV/PI staining. This indicated a genuine anti-inflammatory effect for the three compounds not consequent to cell damage or death. From this set of experiments, concentrations of 1.25–5 mM of VPA and SB, as well as 31.25–125 nM of TSA, were selected for subsequent analyses, as they afforded maximal AnxA1 release and cytokine reduction, without promoting apoptosis.

To assess whether the anti-cytokine effect was mediated by mobilization of endogenous AnxA1, we used peritoneal macrophages obtained from wild type (WT) and AnxA1 $-/-$ mice. However, the degree of inhibition of IL-6 and TNF-$\alpha$ attained by the HDACIs was identical in both macrophage genotypes (Figures 2a–b). The behavior of both cell types regarding gene expression was also very similar (Figure 2c) with no significant upregulation of AnxA1 gene expression in WT cells (Figure 2d). Together with results shown in Figure 1, these data suggest that HDACIs induce release of pre-stored AnxA1. In addition, the three HDACIs studied had the same effect on the expression of Il6 and Tnf genes (Figures 2e–f), as well as on the receptors Fpr1 and Fpr2 (Figures 2g–h), in both WT and AnxA1 $-/-$ macrophages. These data suggest that HDACIs reduce cytokine release on LPS-stimulated

macrophages by downregulating their gene expression, and this occurs in an AnxA1-independent manner.

**HDACIs promote apoptosis and phagocytosis via endogenous AnxA1.** One of the most important events required for the effective resolution of an acute inflammatory reaction is the induction of immune cell apoptosis with prompt removal of apoptotic cells.

Of interest, here is the notion that HDACIs can induce apoptosis on neutrophils,[33] and this effect contributes to their profile as anti-inflammatory compounds. To assess if AnxA1 mediated these actions, we used bone marrow cells from WT and AnxA1 $-/-$ mice, and incubated them with VPA, SB or TSA for 6 h. Apoptosis was studied specifically in the Ly6G$^+$ population (Figure 3a). HDACIs were able to induce apoptosis to a greater extent in WT compared with AnxA1 $-/-$ neutrophils as shown in Figure 3b, providing strong support to the positive role played by endogenous AnxA1 in HDACI-induced apoptosis of mouse neutrophils.

On a similar vein, to study if HDACIs influence the phagocytic properties of macrophages, WT and AnxA1 $-/-$ cells were incubated with fluorescent zymosan at a 1 : 10 ratio (macrophage to zymosan) after 30-min pre-treatment with
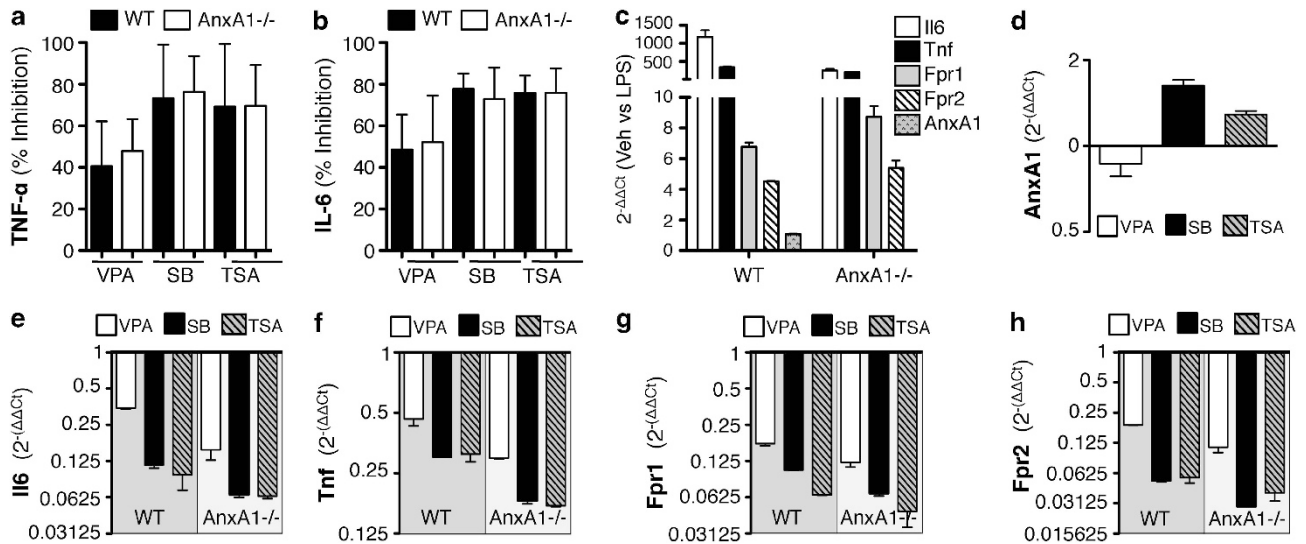
**Figure 2** Effect of HDACIs on cytokine release and gene expression on WT and AnxA1 − / − peritoneal macrophages. Macrophages (0.5 × 10^6 cells/well) were pre-treated with 2.5 mM VPA, 2.5 mM sodium butyrateSB or 62.5 nM TSA for 30 min before stimulation with 75 ng/ml LPS for 4 h. Levels of IL-6 (**a**) and TNF-α (**b**) in supernatants were analyzed by ELISA. (**c–d**) gene expression was quantified by real-time PCR using Power SYBR Green PCR Master Mix, and the ABI Prism 7900HT Sequence Detection System. Fold change was calculated as 2^−ΔΔCt using *Gapdh* as endogenous control. Quantification was calculated as vehicle *versus* LPS-treated cells (**c**) or HDACIs-treated *versus* vehicle-treated cells (**d–h**). Data are mean ± S.E.M. of three independent experiments conducted with cells prepared from distinct mice (2–3 mice each time)
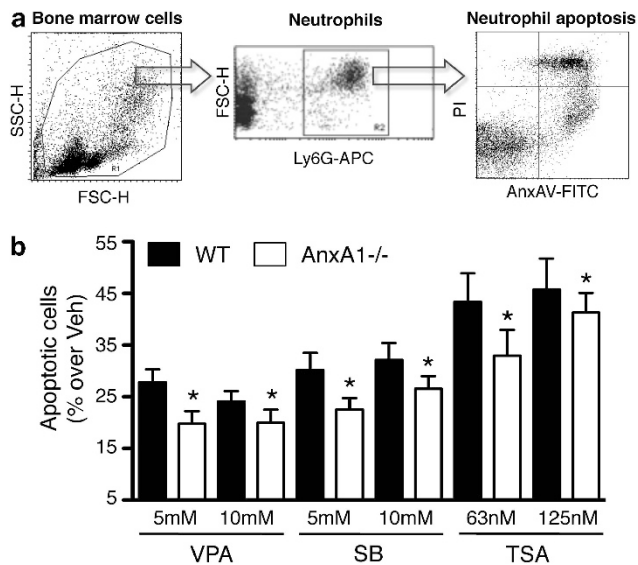


**Figure 3** HDACIs regulation of neutrophil apoptosis. (**a**) Apoptosis was assessed by flow cytometry using triple staining with Ly6G-APC antibody to detect neutrophil population and FITC-AnxAV/PI staining, to detect apoptosis on Ly6G+ cells. (**b**) Bone marrow cells from WT and AnxA1 − / − mice were incubated for 6 h in vehicle, or treated with the reported concentrations of VPA, SB, TSA. Data are reported as increase of total apoptosis (AnxAV+) over vehicle-treated cells and represent the cumulative data of three independent experiments, each one with two mice. *$P < 0.05$, one-way ANOVA WT *versus* AnxA1 − / −, followed by Dunnett's correction

HDACIs. Efferocytosis was studied in a similar way using CFSE-labeled apoptotic neutrophils, at a 1 : 2 ratio (macrophage to neutrophil). As shown in Figure 4, the enhancing effects of HDACIs on phagocytosis of zymosan (Figure 4a), as well as efferocytosis (Figure 4b) evident in WT cells, were

abrogated in AnxA1 − / − macrophages. The latter protocol was used to explore the involvement of receptors that could mediate the effect of endogenous AnxA1 in these experimental settings. Thus, VPA and TSA were added to the cells in the presence of WRW₄ peptide (FPR2-selective antagonist) or cyclosporin H (FPR1-selective antagonist). These data indicated, again, differences in the mechanisms of action of the distinct HDACIs. While the effect of VPA was prevented by both antagonists, TSA pro-efferocytic effect was only affected by the FPR1 antagonist cyclosporin H (Figure 4c).

Collectively, these data describe novel non-genomic actions of HDACIs, promotion of apoptotic and phagocytosis, centred on endogenous AnxA1. As these two processes are fundamental in the resolution of inflammation, we then tested if HDACIs could accelerate the resolution of ongoing inflammatory reaction.

**AnxA1 mediate the pro-resolution actions of HDACIs during ongoing inflammation.** Zymosan-induced peritonitis was selected as a model amply applied to study the resolution processes. A first experiment was conducted with VPA in WT and AnxA1 − / − mice, which were pre-treated for 30 min with vehicle/drug before zymosan (1 mg/mouse) injection. Supplementary Figure S3 shows a modest but significant reduction on neutrophil infiltration, only in WT mice, as assessed 4 h after post-zymosan. In view of the *in vitro* pro-resolving mechanisms activated by HDACIs, we hypothesized higher pharmacological efficacy when given at the peak of neutrophilic inflammation.

Drugs were then given 12 h after zymosan injection, and mice monitored for further 14 h. Vehicle-treated WT and AnxA1 − / − mice showed similar inflammatory cell profiles (Figure 5a), with slightly less neutrophils in AnxA1 − / − mice, although among normal values expected for this model.
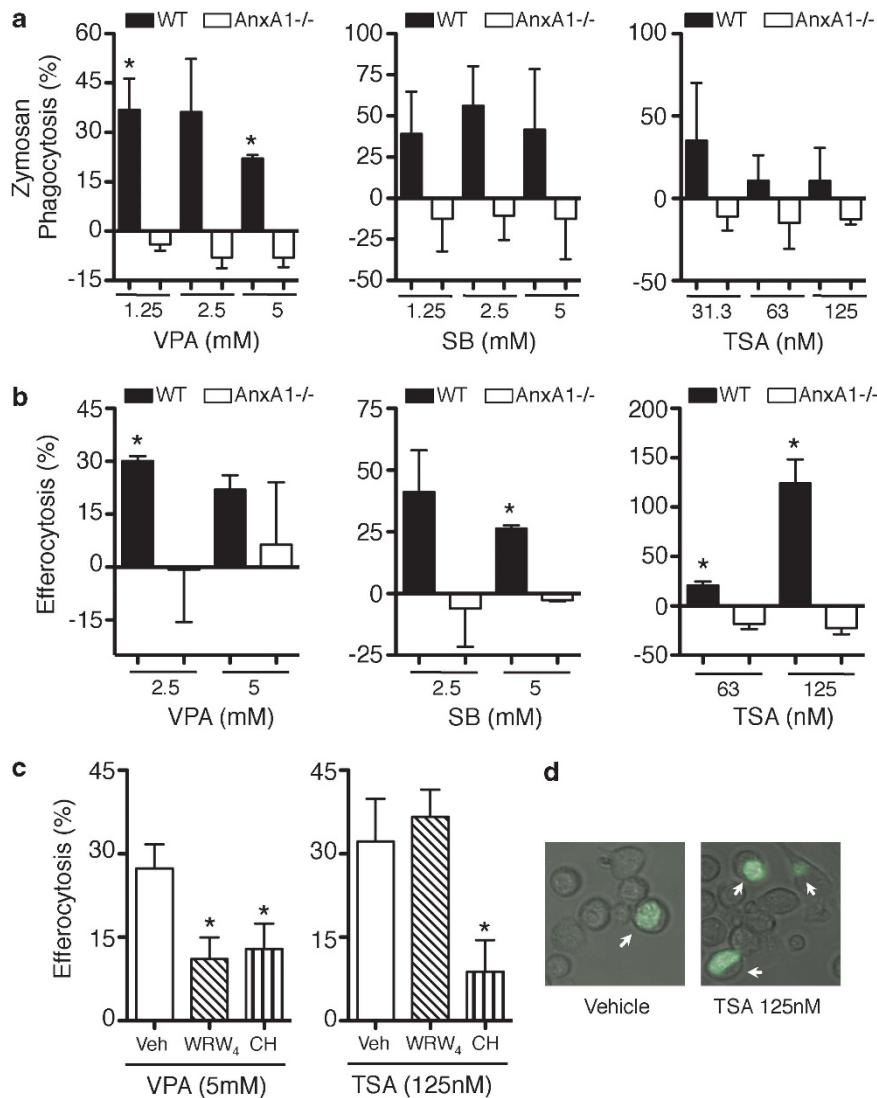
**Figure 4** HDACIs induce macrophage phagocytosis and efferocytosis. Mouse peritoneal macrophages ($10^5$ cells/well) obtained from wild-type (WT, white bars) or AnxA1 − / − (closed bars) mice were treated with the reported concentrations of VPA, SB, TSA or vehicle for 30 min before the addition of FITC labeled zymosan at 1 : 10 ratio for 15 min (**a**) or CFSE-labeled apoptotic human neutrophils at 1 : 2 ratio for 1 h (**b**). In other settings, macrophage incubation with CFSE-labeled apoptotic human neutrophils was conducted in the presence of a FPR1 antagonist (cyclosporin H, 10 $\mu$M) or a FPR2 antagonist (WRW$_4$, 10 $\mu$M) (**c**). Plates were washed three times with PBS and fluorescence measured in a NOVOstar plate reader. Data are mean ± S.E.M. of $n = 3$–4 mice and it is expressed as percentage of increase with respect to vehicle-treated cells. *$P < 0.05$, $t$-test WT versus AnxA1 − / − . Basal values for zymosan phagocytosis in untreated cells were 40 ± 6.24% for WT and 31.5 ± 3.4% for AnxA1 − / − cells. The basal values for apoptotic neutrophil phagocytosis were 12.3 ± 1.59% and 8 ± 1.16 for WT and AnxA1 − / − cells, respectively. (**d**) Representative images of macrophages engulfing CFSE-labeled apoptotic neutrophils (green)

However, although the number of apoptotic macrophages was identical, there was an important increase in the number of apoptotic neutrophils in knockout mice compared with WT (Figure 5b), suggesting perhaps a defect in apoptotic neutrophil clearance in vivo.

The three HDACIs were able to significantly reduce the total cells, neutrophils and monocytes/macrophages numbers in WT mice but not in AnxA1 − / − (Figure 5c), indicating that, also in vivo, AnxA1 is crucial for the anti-inflammatory actions of HDACIs. VPA and, even more, SB provided consistent modulation of these parameters, whereas TSA did not produce a significant effect on any of them (Figure 5c). It is plausible that over a 12-h period, pharmacokinetic

characteristics associated to each compound could underlie these differences. More importantly, further information was acquired by analysis of cell apoptosis: both VPA and SB—that inhibited immune cell values in WT mice—augmented the extent of cell apoptosis selectively for neutrophils and not macrophages (Figure 5d). Such a pronounced effect determined during the dynamics of an ongoing resolving inflammatory reaction was absent in AnxA1 − / − mice.

## Discussion

Gene expression data has traditionally been used to identify a number of genes or pathways associated with a disease or
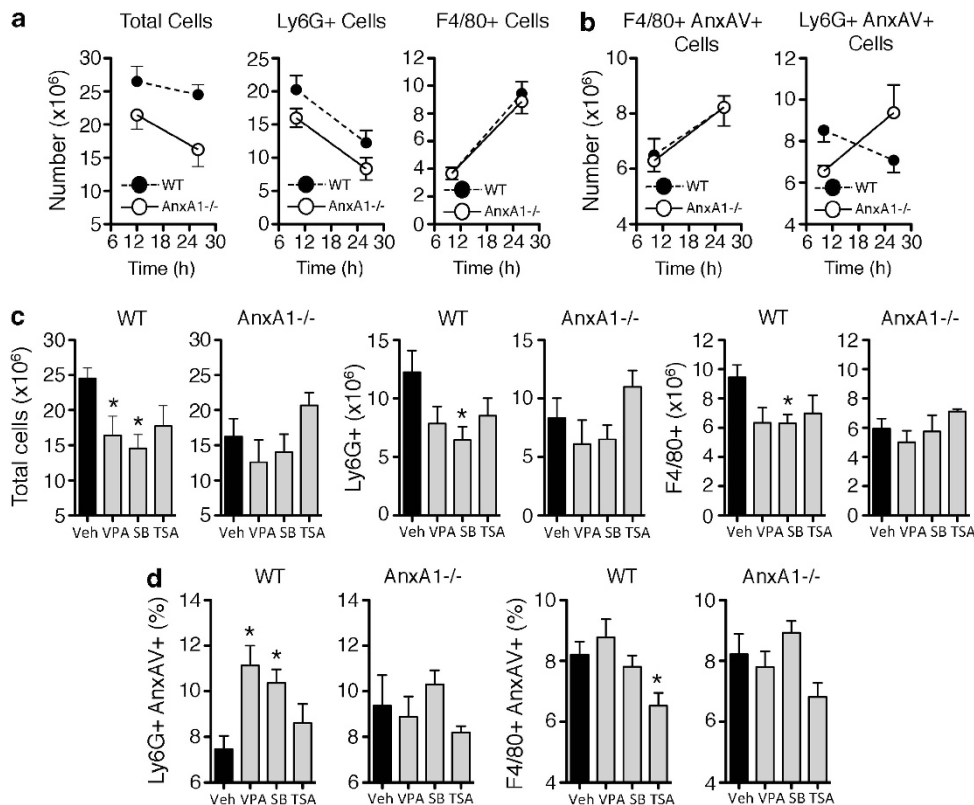
**Figure 5** Pro-resolving actions of HDACIs on the zymosan-induced peritonitis model. (**a**) Peritonitis was induced in WT and AnxA1 − / − mice with 1 mg zymosan A per mouse and peritoneal cavities were analyzed for cell infiltration after 12 and 26 h: total cells, neutrophils (Ly6G + ) and monocytes/macrophages (F4/80 + ). (**b**) Apoptosis was measured in neutrophils and monocytes/macrophages by double staining with AnxAV/PI. (**c–d**) Mice were treated with valproic acid (VPA, 300 mg/kg), sodium butyrate (SB, 300 mg/kg), trichostatin A (TSA, 1 mg/kg) or vehicle 12 h after zymosan injection and peritoneal cell numbers (**c**) and apoptosis (**d**) analyzed at 26 h. Data are mean ± SEM of five mice per group. *$P < 0.05$, one-way ANOVA *versus* vehicle, followed by Dunnett's correction

drug application. Novel approaches include the use of gene expression data for class discovery,[34] to generate prognosis predictors in cancer[35,36] and, more recently, to find 'connections' between drug–disease or drug–drug pairs using the CMap.[9] Drug repositioning strategies, which aim to identify if drugs used for one disease could be applied to another, can benefit from this new approach.[11,12,14]

The mechanisms of action of many drugs already on the market are not always completely understood. The CMap strategy can be of great help to this purpose, as gene expression signature of a particular drug can be used to query the database and explore connections with existing drugs. As an example, using this strategy, Hieronymus *et al.*[16] found that the natural compounds celastrol and gedunin act as HSP90 inhibitors .

AnxA1 is a 37-kDa glucocorticoid-regulated protein with multiple actions on the events that regulate the immune responses.[19] To unravel new connections between AnxA1 and other molecules, we queried the CMap with the previously reported gene expression signature produced by AnxA1 in FPR2-transfected HEK293 cells.[29] Interestingly, we found very robust positive connections with multiple HDACIs, which act by inhibiting HDAC enzyme activity altering the balance in favor of acetylated histones, leading to an increase in transcription of specific genes. Although HDACIs present an important potential as anti-inflammatory drugs, little is known

about the actual mechanisms of action they activate to exert these bioactions. The strong connection found between AnxA1 and HDACIs (see Table 1), together with previous reports showing an association between the pro-apoptotic effect of HDACIs and this protein in cancer cells,[30–32] made us hypothesize that AnxA1 might be mediating at least some of the aspects of the complex anti-inflammatory machinery activated by HDACIs. Conversely, HDACIs might be not solely anti-inflammatory drugs, but by engaging the AnxA1 pathway they can exert pro-resolving effects.

The three pan-inhibitors of histone de-acetylases promoted a time and concentration-dependent increase of AnxA1 release, mirrored by a reduction in TNF-$\alpha$ and IL-6 on LPS-stimulated cells. However, cytokine reduction by HDACIs is consequent to direct gene expression modulation[37] hence is AnxA1 independent, as demonstrated with WT and AnxA1 − / − cells. HDACIs also induce the release of AnxA1 by neutrophils, and this response was faster, peaking at 10 min. It is possible that release of functional AnxA1 from macrophages and neutrophils promoted by HDACIs would follow different pathways, as macrophages are more 'long-acting' cells, in contrast to neutrophils, which generally can react very rapidly by mobilizing their granules. Endogenous AnxA1 is emerging as an important effector of resolution. It induces neutrophil apoptosis,[38] can promote phagocytosis[39] and efferocytosis,[40] and it acts as a fail-safe mechanism when

released from secondary necrotic cells.[41] It was therefore natural to assess the ability of HDACIs in promoting phagocytosis.

In macrophages, VPA, SB and TSA mobilized AnxA1 and increased the engulfment of zymosan particles or apoptotic neutrophils. Removal of apoptotic cells is a pivotal step toward resolution, preventing secondary necrosis, which would further ignite the inflammatory response[42]; therefore, these results, coupled to the ability of HDACIs to promote neutrophil apoptosis in WT but not AnxA1 −/− cells, indicate that processes central to inflammatory resolution[8,43] can be promoted by HDACIs through mobilization of endogenous AnxA1.

AnxA1 can engage receptors of the FPR family expressed by macrophages and neutrophils. In our settings, use of selective antagonists[44] indicated a role for FPR2 (also referred to as FPR2/ALX) in the actions of VPA, whereas both VPA and TSA can operate through FPR1. Although full-length AnxA1 does not bind to human FPR1, N-terminal-derived peptides do (e.g., peptide Ac2-26[45]); we postulate that cell incubation with both HDACIs might lead to the activation of a proteolytic activity that, downstream, would cause generation of FPR1 agonists from the externalized AnxA1. While this hypothesis needs to be tested further, the data generated with these antagonists are coherent with a model whereby—once externalized—endogenous AnxA1 would act in a juxtacrine/paracrine fashion on FPRs to augment phagocytosis. A recent study, conducted with macrophages, confirmed the central role of FPR2 in this process when elicited by either lipoxin $A_4$ or AnxA1-derived peptides.[46]

These in vitro data, and the implications they induce, were translated in vivo by testing whether resolution of an ongoing inflammatory reaction could be accelerated upon HDACIs application. The zymosan-induced peritonitis model was deemed ideal, as used to detail the dynamics of resolution of acute inflammation[47] and characterized by marked AnxA1 gene expression in extravasated cells.[48] The model responds to treatments that modulate cell apoptosis and/or phagocytosis as shown, for example, upon application of resolvin E1[49]; the same processes are activated by phosphodiesterase inhibitors in active resolving inflammation induced by LPS.[50]

In these settings, SB, and to a lesser extent VPA, displayed significant inhibitory properties, whereas TSA produced milder effects. As HDACIs comprise very different chemical entities, it is not surprising that they may only share in part other biological properties. The variable in vivo efficacy could also be linked to the distinct pharmacokinetics characteristics of each molecule administered during ongoing inflammation.

Comparison of the extent of cell infiltration post-zymosan injection in WT and AnxA1 −/− did not reveal major differences, except for the number of apoptotic neutrophils quantified in the peritoneum at the 26-h time point, which is probably secondary to a defect in efferocytosis, as discussed above. Therapeutic delivery of SB reduced the total number of cells, as well as neutrophils and monocytes/macrophages numbers in WT mice, but not in AnxA1 −/−: this clear cut result supports the mechanistic link between this effector of endogenous resolution and HDACIs.

Analysis of neutrophil apoptosis indicates that the actions of HDACIs might be related to the induction of this process, through AnxA1 release and engagement of its receptors, which would plausibly couple with enhanced efferocytosis. A recent study conducted with a model of experimental pleurisy revealed fundamental actions of endogenous and intact AnxA1 in controlling acute inflammation by promoting neutrophil apoptosis.[51] Furthermore, Pupjalis et al.[52] reported that AnxA1 released from apoptotic neutrophils has an anti-inflammatory role by reducing pro-inflammatory cytokines release from monocytes via FPRs. Our present findings support the hypothesis that the efficacy of HDACIs in models of acute and chronic inflammation, especially if characterized by a neutrophilic component, could be consequent to potentiation of AnxA1-mediated pro-resolution properties.[53]

In conclusion, we report how integrative genome-wide computational approaches can be useful for the discovery of drug mechanisms of action. Our results add new knowledge to the complex anti-inflammatory mechanisms of action of HDACIs, showing that the mediator AnxA1 has non-redundant function especially for their pro-resolving effects, some of which described here for the first time (e.g., pro-phagocytic properties).

## Materials and Methods

**In silico prediction and hypothesis generation.** We previously reported the gene expression signature of AnxA1 on FPR2-transfected HEK293 cells (Renshaw D et al,[29] GEO ID: GSE14807). Briefly, cells were treated with 0.5 $\mu$M AnxA1 for 4 h and gene expression analyzed using the whole-genome microarrays Human Genome U133 Plus 2.0 Arrays (Affymetrix, Inc. Santa Clara, CA, USA). After normalization, data analysis and filtering, we identified 118 differentially expressed probes. This AnxA1 gene expression signature was compared with the >7000 gene expression profiles obtained from 1309 small molecules studied in different cultured human cell lines (mainly MCF-7, PC3 and HL60) contained in the new version (build 02) of the Connectivity Map (CMap, http://www.broadinstitute.org/cmap/). The similarity between the gene expression profile of interest and those contained in the CMap database is determined by the connectivity score, ranging from −1 to +1. Considering the hypothesis that if a drug has a gene expression signature that is similar to another drug could potentially be used to identify novel mechanisms of action, we made our predictions on the basis of positive connectivity score values and considering specificity (an estimation of the connectivity between a compound in the CMap database and gene expression signatures extracted from The Molecular Signatures database (MSigDB)) and P-values.

**Animals.** Male age-matched 7–8-week old mice were maintained on the standard chow pellet diet and had free access to water, with a 12-h light–dark cycle. AnxA1-deficient (AnxA1 −/−, on a C57BL/6J background)[54] and wild-type C57BL/6J mice were purchased from Charles River (Edinburgh, UK). All animal studies were approved by and performed under the guidelines of the Ethical Committee for the Use of Animals, Barts and The London School of Medicine and Home Office regulations (Scientific Procedures Act, 1986).

**Compounds.** The HDAC inhibitors valproic acid (VPA), trichostatin A (TSA) and sodium butyrate (SB) were purchased from Sigma-Aldrich, Poole, UK. Cyclosporin H (a selective FPR1 antagonist) was purchased from Enzo Life Sciences, Exeter, UK. The selective FPR2 antagonist WRW$_4$ was purchased from Calbiochem, Nottingham, UK. The PKC inhibitor (PKC$_{19-36}$) and the protein phosphatase 2 inhibitor (okadaic acid) were purchased from Tocris Bioscience, Bristol, UK.

**Zymosan-induced peritonitis.** Peritonitis was induced by injection of 1 mg zymosan A (Sigma-Aldrich) i.p. in 0.5 ml PBS. After 12 h, mice ($n = 5$ per group) were treated with compound/vehicle and sacrificed 14 h later (time point 26-h post-zymosan) by $CO_2$ exposure. Peritoneal cavities were washed with 4 ml of ice-cold

PBS containing 3 mM EDTA and 25 U/ml heparin. Cells were counted on a Neubauer haemocytometer using Turk's solution and analyzed by flow cytometry (BD FACSCalibur, Oxford, UK), following staining along canonical protocols with the specific antibodies: FITC-Ly6G/Gr1, APC-F4/80, isotype controls and blocking antibody anti-mouse CD16/32, all from eBioscience (San Diego, CA, USA) and used according to the manufacturer's instructions.

**Primary peritoneal macrophages.** Mice were injected i.p. with 1 ml of 2% biogel (Bio-Rad, Hemel Hempstead, UK); 4 days later, the peritoneal cells were collected by lavage using 4 ml of 3 mM EDTA in PBS and plated in 24-well plates at a density of $0.5 \times 10^6$ cells/well, in RPMI-1640-containing 10% FCS and 50 mg/ml gentamicin. After 2 h of incubation, non-adherent cells were removed leaving adherent macrophages ($>90\%$ enriched population). Compounds or vehicle were added in 1%FCS medium for 30 min before stimulation with 75 ng/ml LPS (Sigma-Aldrich). Supernatants (for western blot and ELISA determinations) and cell pellets (for RNA extraction) were collected after 4 h and kept frozen at $-80\,^\circ$C until used.

**Bone-marrow neutrophil isolation.** WT and AnxA1$-/-$ mice were killed, and the femurs were excised. The epiphyses were removed, and bone marrow was flushed from the bone using HBSS buffer using a syringe equipped with a 23-gauge needle. Cell suspensions were then filtered using a 70 $\mu$m mesh nylon strainer. Neutrophils were separated using the EasySep negative selection mouse neutrophil enrichment kit (STEMCELL Technologies, Grenoble, France). Neutrophils were then plated in 96-well plates in RPMI-1640-containing 10% FBS and treated for 6 h with compounds or vehicle.

**Western blot analysis.** Cell supernatants (400 $\mu$l) were collected after corresponding treatments. To collect the membrane-bound fraction, cells were washed with $Ca^{2+}$-containing PBS and then incubated for 2 min with 200 $\mu$l of 1 mM EDTA in $Ca^{2+}$-free PBS. The recovered supernatants contained the membrane-bound proteins. Finally, cells were lysed with 200 $\mu$l of RIPA buffer (Thermo Scientific, Leicestershire, UK) containing protease inhibitors cocktail (Calbiochem).

Samples were subjected to standard SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (Millipore, Watford, UK). These were incubated with rabbit anti-AnxA1 (1 : 5000; Invitrogen, Paisley, UK) for 2 h and horseradish peroxidase-conjugated anti-rabbit IgG for 1.5 h (1 : 2000; Dako, Cambridge, UK). Cell lysates membranes were re-blotted with anti-$\beta$-actin antibody (1 : 10 000, SIGMA, Dorset, UK). Proteins were detected using an enhanced chemiluminescence detection kit and visualized on Hyperfilm (GE Healthcare).

**Neutrophil isolation from human blood.** Experiments using healthy volunteers were approved by the local research ethics committee (P/00/029 East London and The City Local Research Ethics Committee 1). Informed written consent was provided, according to the Declaration of Helsinki. Blood was collected into 3.2% sodium citrate and diluted 1 : 1 in RPMI-1640 before separation through a double-density gradient using Histopaque 10771 and 11191 (Sigma-Aldrich). After polymorphonuclear cells and PBMCs isolation and washing, contaminating erythrocytes were removed by hypotonic lysis. polymorphonuclear cells were re-suspended at a concentration of $4 \times 10^6$ cells/ml in 10% FCS-containing medium and incubated overnight at $37\,^\circ$C, 5%$CO_2$ to let neutrophils undergo spontaneous apoptosis.

**ELISA determinations and enzyme immunoassay.** Levels of IL-1$\beta$, TNF-$\alpha$ and IL-6 were quantified in the supernatants from cultured macrophages using ELISA Ready-SET-Go (eBioscience, Hatfield, UK), according to the manufacturer's instructions.

**RNA extraction, cDNA synthesis and real-time PCR.** Total RNA was extracted using the RNeasy Mini Kit and genomic DNA removed by on-column digestion with RNase-Free DNase Set, following the manufacturer's instructions (QIAGEN, Crawley, UK). cDNA was synthetized using 1 $\mu$g of pooled RNA from three or more samples with the SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed in duplicates, with 200 ng of cDNA per well, 1$\mu$l primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), using the ABI Prism 7900HT Sequence Detection System. Quantitect primers (QIAGEN) used are the following: *Gapdh* (QT01658692), *Il6* (QT00098875), *Tnf* (QT00104006), *Fpr1* (QT01165899), *Fpr2* (QT00171514) and

*AnxA1* (QT00145915). Dissociation step was always included to confirm the absence of unspecific products. Fold change was calculated as $2^{-\Delta\Delta Ct}$ using *Gapdh* as reference gene.

**Assays of phagocytosis and efferocytosis.** Primary peritoneal macrophages were cultured in 1% FCS medium-containing compounds/vehicle for 30 min before the addition of FITC-zymosan A (Invitrogen) at 1 : 10 ratio (macrophage to zymosan) for 15 min, or CFSE-labeled apoptotic neutrophils (1 : 2, macrophage to neutrophil) for 1 h. Cells were washed three times with ice-cold PBS and fluorescence analyzed using a NOVOstar reader (BMG Labtech, Ortenberg, Germany).

**Assessment of apoptosis.** Cells were stained with APC-Ly6G/Gr1 (1 : 100, eBioscience) to detect neutrophils population and FITC-AnxAV and propidium iodide (PI) using the apoptosis detection kit from BD Pharmingen (Oxford, UK), following the manufacturer's instructions and analyzed by flow cytometry (BD FACSCalibur).

**Statistics.** Experiments were done at least in triplicates and repeated 3–5 times. Experiments involving human donors were repeated at least two times. The Student's *t* test or one-way ANOVA, followed by Dunnett's multiple-comparison tests were applied as appropriate considering $P$-values $<0.05$ significance.

## Conflict of Interest
The authors declare no conflict of interest.

## Author contributions
TMM planned and designed study, performed research, analyzed and interpreted data and wrote manuscript (including first draft). JD designed study, interpreted data and wrote manuscript. MP designed study, interpreted data, wrote manuscript and obtained funding.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)