

# Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3<sup>+</sup> regulatory T cells

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**Abstract** | Classical zinc-dependent histone deacetylases (HDACs) catalyse the removal of acetyl groups from histone tails and also from many non-histone proteins, including the transcription factor FOXP3, a key regulator of the development and function of regulatory T cells. Many HDAC inhibitors are in cancer clinical trials, but a subset of HDAC inhibitors has important anti-inflammatory or immunosuppressive effects that might be of therapeutic benefit in immuno-inflammatory disorders or post-transplantation. At least some of these effects result from the ability of HDAC inhibitors to enhance the production and suppressive functions of FOXP3<sup>+</sup> regulatory T cells. Understanding which HDACs contribute to the regulation of the functions of regulatory T cells may further stimulate the development of new class- or subclass-specific HDAC inhibitors with applications beyond oncology.

**Histone deacetylase (HDAC).** Enzyme that catalyses removal of acetyl groups from lysines in histone tails or non-histone proteins; such deacetylation usually decreases gene expression or protein function. Classical HDACs are those which are zinc-dependent and include HDACs 1–11. Sirtuins are a distinctly different family of HDACs (SIRT1–7) that are NAD-dependent and are mainly involved in cell metabolism.

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Control of autoimmunity and other undesired immune responses through manipulation of endogenous regulatory mechanisms has long been a dream of physicians and scientists. Recent insights into the role of epigenetics in the regulation of gene expression and cell function suggest new therapeutic opportunities, including regulation of chromatin remodelling and gene transcription by inhibition of histone deacetylases (HDACs) and DNA methyltransferases<sup>1–4</sup>. Moreover, although deacetylation of  $\epsilon$ -acetyl-lysine residues in the amino-terminal tail of core histones is a key function of several HDACs, HDACs also deacetylate many non-histone proteins<sup>1–5</sup>, including the forkhead transcription factor FOXP3, which is important in the development and regulation of regulatory T cells (T<sub>regs</sub>)<sup>6,7</sup>. Protein acetylation can affect DNA binding, either positively or negatively, protein–protein interactions and enhance protein stability<sup>4</sup>. Acetylation can promote the activation, nuclear translocation and DNA binding of transcription factors such as *STAT3*, *NF- $\kappa$ B* and *RUNX1*, and thereby promote expression of multiple genes, including pro-inflammatory cytokines and other mediators of inflammation and immunity.

The evidence of the anti-inflammatory effects of HDAC inhibitors (HDACIs) has been accruing for many years, but has not led to their development for immuno-inflammatory disorders by pharmaceutical companies. Despite their potency, existing HDACI drugs have toxicities or other limitations that have largely restricted their development to the potential treatment of patients with

malignancies. However, this assessment is changing as new insights into the roles of individual HDAC enzymes are emerging and new cellular targets are identified.

The 18 HDACs are classified structurally into class I (HDAC1, HDAC2, HDAC3, HDAC8), class IIa (HDAC4, HDAC5, HDAC7, HDAC9), class IIb (HDAC6, HDAC10), class III (SIRT1–7) and class IV (HDAC11) groups<sup>8,9</sup>. Class III HDACs or sirtuins act by a nicotinamide-dependent mechanism and are structurally and functionally distinct from class I, II and IV HDAC metalloenzymes. Activation of SIRT1, using resveratrol or newer analogues<sup>10</sup>, has antioxidant effects that might be therapeutically useful in metabolic, neurological and cardiac diseases<sup>11</sup>. However, little is yet known about the involvement of sirtuins in immune responses<sup>12</sup>. Similarly, there is only one study on HDAC11, the sole class IV member, showing that it inhibits expression of interleukin (IL)-10 by dendritic cells *in vitro*<sup>13</sup>. Hence, this Review focuses on the classical zinc-dependent class I and II HDACs.

As detailed below, class I HDACs are expressed in all cells and are essential for cell differentiation by contributing to a closed chromatin state and suppression of gene transcription; for example, a cell can become a lymphocyte or a myocyte by turning off genes that promote neuronal or endothelial differentiation, and class I HDACs have a major role in this suppression. Class II HDACs have more limited cellular expression and often control regulatory processes in a gradual or more subtle manner than their class I counterparts.

Much of the biology of HDACs has been unravelled through the use of HDACi. Surprisingly, given the key roles of class I HDACs in normal cells, these compounds have not proven overwhelmingly toxic. Rather, as the field has learnt to optimize their potency, pharmacokinetics and, increasingly, delineate their precise specificities, roles for selected HDACi in the control of inflammatory and immune processes have begun to be explored. These studies have shown potent *in vivo* effects of HDACi on the differentiation and activation of dendritic cells, T cells and other components of the immune response (for recent reviews, see REFS 9, 14–16).

Over the past decade, the recognition and characterization of  $T_{\text{regs}}$  that express FOXP3 in maintaining host homeostasis has captured the attention of many investigators. FOXP3<sup>+</sup>  $T_{\text{regs}}$  play a key part in limiting autoimmunity and maintaining peripheral tolerance, and mutations of FOXP3 lead to lethal autoimmunity in humans and mice<sup>17–21</sup>. From a therapeutic perspective, some groups are testing expansion of small numbers of  $T_{\text{regs}}$  before adoptive transfer back into an individual. However, repeated stimulation of expanded  $T_{\text{regs}}$  has been found to lead to loss of FOXP3 expression, especially when using  $T_{\text{regs}}$  generated *in vitro*<sup>22,23</sup>. An alternative approach has arisen from recent insights into the epigenetic regulation of FOXP3 (REFS 6, 7, 24, 25). Therapeutic manipulation of FOXP3 acetylation using HDACi can promote the development and suppressive functions of FOXP3<sup>+</sup>  $T_{\text{regs}}$ , with beneficial consequences in models of transplant rejection, colitis and arthritis<sup>7,26–28</sup>.

After summarizing the relevant background on HDAC complexes and HDACi, this Review will discuss the role of histone acetylation in inflammation and autoimmunity, and consider the therapeutic potential of HDACi as anti-inflammatory agents, including their use to promote FOXP3 acetylation and  $T_{\text{reg}}$ -mediated immunosuppression. The ongoing research at the intersections of epigenetics, pharmacology and  $T_{\text{reg}}$  biology may lead to new therapies for immuno-inflammatory disorders and transplant rejection.

### HDAC complexes

Deacetylation of  $\epsilon$ -acetyl-lysine residues in the N-terminal tail of core histones is a key function of class I HDACs, although new data indicate that they may also deacetylate 1,750 or more non-histone proteins<sup>5</sup>. HDACs do not have direct DNA binding activity, but function through interactions with other proteins in large molecular complexes.

**Class I HDAC complexes.** Class I HDAC proteins are present within intranuclear SIN3 or NURD complexes<sup>29</sup> and are ubiquitously expressed<sup>30</sup>. HDAC1 and HDAC2 in the SIN3 complex provide deacetylase activity, whereas retinoblastoma-associated proteins stabilize interactions with histones and the SIN3 and SAP30 proteins promote interactions with other molecules. The NURD complex has the same core components as the SIN3 complex, but has a different set of interacting proteins: namely the ATP-dependent chromatin remodelling protein Mi2

(also known as **CHD4**); one or more metastasis-associated proteins, for example, **MTA2**; and the methyl-binding domain protein 3 **MBD3** (REF. 29). NURD complexes promote histone deacetylation, like SIN3 complexes, but also regulate ATP-dependent chromatin remodelling, and act more globally through their recruitment to the tails of histone 3 and to sites of CpG-methylated DNA<sup>29</sup>.

HDAC3 is a transcriptional repressor of a specific set of genes involved in nuclear receptor signalling and functions as part of a complex that contains the silencing mediator of retinoid and thyroid hormone receptor (SMRT) and the nuclear hormone receptor corepressor (NCoR), and other proteins<sup>31,32</sup>. SMRT and NCoR repress transcription factors such as NF- $\kappa$ B and activator protein 1 (AP1; also known as **JUN**), and also bind to class II HDACs<sup>31,32</sup>.

**Class IIa HDAC complexes.** In contrast to class I HDACs, expression of class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) varies with cell differentiation and by tissue; expression is notably high in brain, muscle and T lymphocytes<sup>9</sup>. Class IIa HDACs shuttle between the nucleus and cytoplasm as a result of activating stimuli, and are thought to largely lack deacetylase activity as determined using conventional substrates, although this assessment may change as alternative substrates are identified<sup>33</sup>.

The HDAC domains of HDAC4, HDAC5, HDAC7 and HDAC9 can bind the SMRT/NCoR complex and associated HDAC3 (REFS 31,32), and their N-terminal domains bind to the transcriptional repressor **BCL6** (REF. 34), which can also recruit SMRT/NCoR. Thus, much of the deacetylase activity associated with class IIa HDACs is attributed to HDAC3 (REFS 31–33). Additional repressor activity can arise from the binding of C-terminal-binding protein 1 (**CTBP1**)<sup>35</sup> and heterochromatin protein 1 (**HP1**; also known as **DEF1**)<sup>36</sup> to the N-terminal regions of class IIa HDACs. DEF1 binds methylated H3K9 and recruits the histone lysine methyltransferase SUV39H1, so that deacetylation of target genes can lead to the development of the repressive chromatin mark of methylated H3K9 and further gene silencing<sup>36</sup>. This ability of class IIa HDACs to repress genes independently of intrinsic deacetylase activity is exemplified by the HDAC9 isoform, myocyte enhancing factor 2 (**MEF2**)-interacting transcription repressor MITR, which lacks a catalytic domain but still represses MEF2 (REF. 37).

Class IIa HDACs have conserved N-terminal serines that are phosphorylated by enzymes such as Ca<sup>2+</sup>/calmodulin-dependent kinase (CaMK), protein kinase D, salt-inducible kinase and MAP/microtubule affinity-regulating kinase<sup>38,39</sup>. Upon phosphorylation, class IIa HDACs bind to export proteins such as 14-3-3 and move to the cytoplasm<sup>40</sup>, allowing derepression of target genes including MEF2, which binds class IIa HDACs through a highly conserved 17-amino acid N-terminal motif<sup>41</sup>. Once free of class IIa HDAC inhibition, MEF can be acetylated by the histone acetyltransferase (HAT) **p300** and activate transcription, or can undergo sumoylation

### FOXP3

Forkhead or winged helix transcription factor that is expressed primarily in regulatory T cells and controls their functions; its DNA binding and interactions with other proteins are promoted by acetylation and impaired by deacetylation. Mutations in FOXP3 are responsible for life-threatening autoimmunity in patients and experimental animals.

### Regulatory T cell

( $T_{\text{reg}}$ ). These cells express FOXP3 and dampen immune responses mediated by T and B cells. They may also modulate functions of cells of the innate immune system. They function by multiple mechanisms, including direct membrane–membrane effects, the release of soluble products and catabolism of essential amino acids.

### Histone acetyltransferase

(HAT). Enzymes that catalyse acetylation of key lysines in histone tails and various non-histone proteins; such acetylation usually promotes gene expression or protein function.

to maintain repression of target genes<sup>42,43</sup>. In the cytosol, phosphorylated class IIa HDACs are dephosphorylated by protein phosphatase 2A and recycled to the nucleus<sup>44</sup>.

**Class IIb HDAC complexes.** Both class IIb HDACs — HDAC6 and HDAC10 — are distinct from the other HDAC classes in that they have two catalytic domains and can be detected within the nucleus and the cytoplasm<sup>20,45</sup>. Little is known about the function of HDAC10, but HDAC6 has been found to deacetylate proteins within the cytoplasm and the nucleus, and to also have deacetylase-independent functions<sup>46</sup>.

### HDAC inhibitors

The first HDACIs were identified by their anticancer effects during drug screens, before the existence of HDACs was known<sup>47</sup>. Even today, crystallographic structures have only been reported for the catalytic domains of HDAC4 (REF. 48), HDAC7 (REF. 49) and HDAC8 (REFS 50,51). The 'classical' HDACIs acting on zinc-dependent HDACs (HDAC1–11) include short-chain fatty acids, such as sodium butyrate and valproic acid (VPA); hydroxamic acids, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA); benzamides, such as MS275; and cyclic tetrapeptides such as trapoxin and depsipeptide<sup>2,52–54</sup>. SAHA, also known as vorinostat (Zolinza; Merck) and approved as a monotherapy for cutaneous T cell lymphoma, is the only FDA-approved HDACI, although the approval of depsipeptide (also known as romidepsin) has been recommended for the same indication.

Agents with HDACI activity as well as other actions, such as butyrate and VPA, have long been used clinically in non-oncologic contexts. For example, butyrate is used as a therapy for inflammatory bowel disease, although whether its benefits are due to inhibition of HDAC activity is controversial<sup>55</sup>. Likewise, no data are available as to whether prolonged treatment of epileptic patients with VPA also protects against co-morbid immuno-inflammatory diseases through the inhibition of HDACs<sup>56</sup>.

Classical HDACIs are being tested in oncology trials due to their abilities to promote tumour cell-cycle arrest, differentiation and apoptosis<sup>57,58</sup>. These drugs have proven relatively non-toxic towards neighbouring normal cells, despite inducing hyperacetylation in both tumour and normal cells<sup>59</sup>. Given the paucity of knowledge of the functions of individual HDACs, an empirical one-size-fits-all strategy for the development of HDACs has largely prevailed. However, so-called 'pan-HDACIs' such as the hydroxamic acid compounds (for example, TSA, SAHA, M344, Scriptaid) may actually only block the HDAC activity of class I and class IIb HDACs, as class IIa HDACs largely lack detectable deacetylase activity<sup>9,60</sup>. By contrast, class I-selective HDACIs (for example, MS275 (REF. 61), 4-phenylimidazole<sup>62</sup> and MC1293 (REF. 63)), class II-selective HDACIs (such as MC1568 and MC1575 (REF. 64)) and HDAC isoform-specific inhibitors (for example, selective for HDAC4 (REF. 65), HDAC6 (REF. 66) or HDAC8 (REF. 67)) have been identified. Development of class I-specific HDACIs may

be particularly relevant in cancer<sup>15</sup>, but as will be discussed, anti-inflammatory and T<sub>H</sub>17-dependent mechanisms often involve targeting class II HDACs.

Classical HDACIs act by chelating or displacing the Zn<sup>2+</sup> present in the catalytic site, and their specificities arise from interactions with residues in the cap region that is adjacent to the catalytic site<sup>47–49</sup>. However, additional mechanisms of action of individual HDACIs are increasingly being recognized. For example, the short-chain fatty acid HDACI compounds VPA and butyrate, but not pan-HDACIs, selectively promote the proteasomal degradation and depletion of HDAC2 by induction of E2 and E3 ubiquitin ligases<sup>68</sup>. Likewise, two structurally distinct pan-HDACIs — SAHA and depsipeptide — promote the selective downregulation of HDAC7 gene transcription but by yet unknown mechanisms<sup>69</sup>. Lastly, selective downregulation of class IIa HDACs occurs in response to exposure to pan-HDACIs or class II-specific HDACIs, but not class I-specific HDACIs<sup>70</sup>. The downregulation of class IIa HDACs results from sumoylation and proteasomal degradation rather than inhibition of transcription, suggesting that pan-HDACIs can not only inhibit the action of class I HDACs by occupying their active sites, without altering protein expression, but that they may also decrease class II HDAC expression by promoting proteasomal degradation.

Given the poor catalytic activity of class IIa HDACs against standard substrates (acetylated lysine residues), various groups are investigating whether small-molecule inhibitors of protein–protein interactions may also function as atypical HDACIs. For example, HDAC4 and HDAC5 form hetero-oligomers, allowing CAMKII to promote the export of HDAC5 despite it lacking a CAMKII docking site, and pointing to the potential to maintain repression of MEF2 or other target genes by disrupting the interaction of HDAC4 and HDAC5 (REF. 71). Alternatively, disrupting the interaction of the MEF2-binding motif of class IIa HDACs with MEF2 might allow selective inhibition of some or all of the MEF2-dependent functions of class IIa HDACs<sup>41</sup>. Searches for inhibitors of such protein–protein interactions are typically more challenging to set up and run than standard high-throughput screens for enzyme inhibitors, but may prove a fertile area for future development as rationales for their use are established.

### Anti-inflammatory effects of HDACIs

Inflammatory responses involve the orchestrated function of a host of bodily defences mobilized in response to injury and intended to result in healing and repair. However, as a result of defects in this response, failure of coordination or inability to clear the inflammatory stimulus, excessive and damaging inflammatory responses may develop and persist long after their normal utility has passed. Similarly, immune responses might be excessive or directed against self-antigens, leading to the development of autoimmune diseases.

The role of cytokines and other mediators in the development and persistence of inflammatory diseases is well established. More recently, recognition of a requirement for chromatin remodelling to promote transcription factor

binding and activation of cytokine and other genes has led researchers to consider HDACI use as a new approach to treat immuno-inflammatory disorders.

**Histone acetylation and inflammatory gene expression.** Eukaryotic DNA is tightly wrapped around an octamer of histone proteins and various post-translational modifications of the N-terminal tails of these histones, including methylation, acetylation, phosphorylation and ubiquitination, control the accessibility of transcriptional machinery to the DNA and hence gene expression. Lysine acetylation as a result of HAT activity or inhibition of HDAC function leads to recruitment of transcription factors, bromodomain-containing proteins that promote chromatin remodelling and activation of transcription, and RNA polymerase II<sup>72</sup>. The coordinated role of HATs and HDACs in regulating gene expression on a genome-wide scale was recently demonstrated<sup>45</sup>. However, as noted below, HDACI use is typically associated with repression of pro-inflammatory cytokine expression, which seems to conflict with this paradigm.

Resolution of this paradox probably involves several points. First, effective gene transcription is correlated with rapid acetylation and deacetylation rather than with sustained acetylation<sup>45,73</sup>, and HDACI use may upset this balance. Second, HDACI-induced chromatin remodelling may result in the recruitment of as many repressors as activators, or may lead to expression of pro-apoptotic molecules and induction of cytotoxicity<sup>74</sup>. Third, HDACIs affect the function of many non-histone proteins<sup>5</sup>, including FOXP3 (REF. 7) and potentially other inhibitory pathways. Indeed, phylogenetic analysis showed that, at least in bacteria, all four HDAC classes preceded the evolution of histone proteins, emphasizing the likely importance of non-histone proteins as targets of HDACIs<sup>75</sup>. Fourth, HDACIs have effects beyond promoting acetylation; for example, HDACIs can promote proteasomal degradation<sup>68–70</sup> or impair **AKT** activation by releasing protein phosphatase 1 from HDAC binding<sup>76</sup>. Until more insights are available, a need for cell-type and context-dependent analysis when assessing effects of HDACI use is paramount.

**Microarray studies.** Despite there being many clinical trials of HDACIs in oncology underway, little information is available concerning the effects of HDACIs on normal cells. In a pioneering study<sup>74</sup>, microarray and additional analyses of primary murine CD4<sup>+</sup> T cells undergoing activation induced by CD3/CD28 monoclonal antibodies showed that 5 nM concentrations of the pan-HDACI TsA induced mitochondria-dependent apoptosis of ~50% of cells and reversible G1 cell cycle arrest in the remainder. Whereas T cell production of IL-2 and the expression of many cell surface molecules were downregulated by TsA, microarray studies showed time- and stimulus-dependent effects of TsA on T cell gene expression. Surprisingly, TsA only modulated 2% of genes, and equal numbers of genes were downregulated as were upregulated<sup>74</sup>.

A subsequent analysis of the effects of another hydroxamate, LAQ824, on Toll-like receptor 4 (TLR4)-dependent activation of macrophages also showed that

pan-HDACI therapy up- or down-regulated expression of only about 5% of genes<sup>77</sup>. Interestingly, therapy with LAQ824 prevented development of T<sub>H</sub>1 but not T<sub>H</sub>2 T cell responses as a result of genes expressed by pan-HDACI-treated antigen-presenting cells. Together, these studies indicate that inhibition of HDAC activity alone is insufficient to drive broad gene expression, and point to the need for a careful understanding of the roles of multiple interacting pathways affecting chromatin remodelling and gene expression, before the results of HDACI therapy can confidently be predicted at the cellular level.

**In vitro and in vivo immune cell studies.** Many studies have documented multiple effects of HDACIs on cells of the innate and adaptive immune systems. However, these studies have typically used a 'black box' approach, whereby effects of the HDACIs *in vitro* or *in vivo* were delineated without analysis of the HDACs involved or of whether the HDACIs used were modulating non-histone proteins or chromatin remodelling. The broad effects of HDACIs on inflammatory and immune responses were primarily determined in rodents or using cultured human cells, as summarized in TABLE 1.

With regard to acute inflammation, treatment of mice with SAHA<sup>78</sup> or NVP-LAQ824 (REF. 77), two hydroxamic acid pan-HDACIs, suppressed lipopolysaccharide-induced production of the cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$  in dendritic cells both *in vitro* and *in vivo*<sup>77–79</sup>. Consistent with these findings, HDACI therapy is known to impair activation of NF- $\kappa$ B in dendritic cells and macrophages, although the mechanisms remain controversial<sup>14</sup>, and to impair their differentiation and maturation<sup>80,81</sup>. Recently, pan-HDACI exposure was shown to promote STAT3 acetylation<sup>82</sup> and indoleamine 2,3-dioxygenase (IDO) production by dendritic cells<sup>83</sup>. As IDO catabolizes tryptophan, an amino acid that is essential for T cell activation, induction of IDO blocks T cell activation and the development of murine graft-versus-host disease post-bone marrow transplantation<sup>83</sup>. *In vivo* use of TsA or SAHA also inhibited T cell cytokine production and proliferation<sup>74,84,85</sup> and promoted T cell energy<sup>86</sup>, in conjunction with altered chromatin remodelling at the IL-2 promoter and acetylation of key transcription factors, including NF- $\kappa$ B<sup>84,85</sup>.

With regard to chronic inflammation, TsA or SAHA decreased fibroblast proliferation and extracellular matrix production in murine models of fibrosis<sup>87–89</sup>, and both *in vitro* and *in vivo* TsA or SAHA blocked transforming growth factor beta (TGF- $\beta$ )-induced differentiation of fibroblasts into myofibroblasts and impaired epithelial–mesenchymal transformation<sup>89,90</sup>. Selective HDAC targeting might also be a useful strategy to limit inflammation, as in cultured epithelial cells, HDAC6 promotes TGF- $\beta$ -induced epithelial–mesenchymal transformation by deacetylating **SMAD3** or SMAD3-interacting proteins<sup>91</sup>, and *in vitro*, HDAC4 can inhibit expression of a TGF- $\beta$  repressor, TGIF<sup>92</sup>.

The effects of HDACIs on antigen-presenting cell-induced T cell activation and differentiation are summarized in FIG. 1. In murine models of T cell-dependent

Table 1 | Effects of histone deacetylase inhibitors in inflammatory and autoimmune diseases

Disease model	Species	Inhibitor	Effects
Endotoxin	Human, mouse	SAHA	Reduced cytokine production (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IFN- $\gamma$ ) and lethality <i>in vivo</i> in mice <sup>77–79</sup> ; decreased cytokine production <i>in vitro</i> using human PBMC <sup>78</sup>
ConA hepatitis	Mouse	SAHA	Decreased liver injury <sup>78</sup>
Airway hypersensitivity	Mouse	TsA	Decreased allergen-induced airway inflammation in a model of asthma, with decreased airway hyperresponsiveness, mucus production, leukocyte infiltration and cytokine production <sup>93</sup>
Experimental autoimmune encephalomyelitis	Mouse	TsA	Decreased demyelination, leukocyte infiltration and T <sub>H</sub> 1 cytokine and chemokine production in relapsing phase of experimental autoimmune encephalomyelitis <sup>94</sup>
Stroke	Mouse	SAHA, TsA	Decreased infarct size post-middle cerebral artery occlusion <sup>103</sup>
Neurodegenerative diseases	Mouse	Butyrate <sup>106,107</sup> , SAHA <sup>105</sup>	Decreased disease progression in models of Huntington's chorea <sup>105</sup> , spinal and muscular dystrophy <sup>106</sup> , amyotrophic lateral sclerosis <sup>107</sup>
Colitis	Mouse	SAHA, TsA	Decreased weight loss, histological injury, leukocyte infiltration and cytokine production in dextran sodium sulphate- and 4,6-trinitrobenzene sulphonic acid-induced colitis and adoptive transfer models <sup>7,26,96</sup>
Glomerulonephritis	Mouse	SAHA, TsA	Decreased proteinuria in MRL/lpr model of systemic lupus erythematosus, and decreased leukocyte infiltration and production of inflammatory mediators <sup>95</sup>
Arthritis	Mouse, rat	FK228 <sup>97</sup> , SAHA and MS275 <sup>98</sup> , VPA <sup>19</sup>	Decreased joint swelling and bone and cartilage destruction in antibody- <sup>97</sup> and collagen-induced <sup>28,98</sup> models of arthritis, in conjunction with decreased overall leukocyte infiltration and T <sub>H</sub> 1 cytokine and chemokine production, but increased Foxp3 <sup>+</sup> T <sub>reg</sub> accumulation <sup>19</sup>
Graft-versus-host disease	Mouse	SAHA	Decreased graft-versus-host disease <sup>99</sup> and decreased CD3 monoclonal antibody-induced cytokine storm during bone marrow conditioning <sup>100</sup>
Transplant rejection	Mouse	SAHA, TsA,	Permanent engraftment of cardiac and islet allografts and Foxp3 <sup>+</sup> T <sub>reg</sub> -dependent tolerance induction <sup>7</sup>
Haemorrhage and resuscitation	Human, mouse, rat, pig	Butyrate, SAHA, TsA, VPA	Optimal protective effects with SAHA compared with TsA or VPA in a rat model <sup>109</sup> ; SAHA-induced suppression of TNF- $\alpha$ production in rats but variable effects in humans <sup>110</sup> ; VPA-associated increased expression of multiple protective genes in additional studies using pig <sup>111</sup> and rat <sup>112</sup> models, including BCL2 and HSP70 (REF. 111)

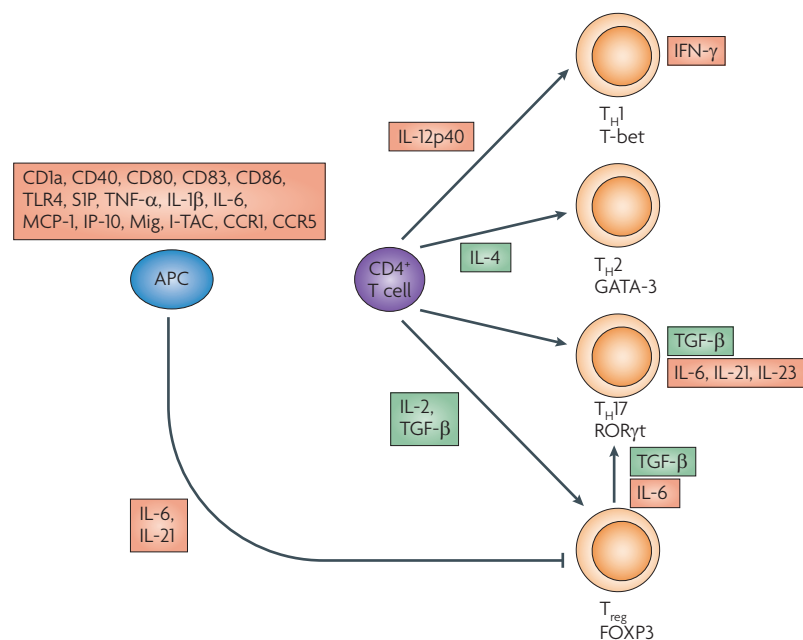
CD3, cluster of differentiation 3; HSP70, heat shock protein 70; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; SAHA, suberoylanilide hydroxamic acid; TNF, tumour necrosis factor; T<sub>reg</sub>, regulatory T cell; T<sub>H</sub>1, type 1 helper T cell; TsA, trichostatin A (TsA); VPA, valproic acid.

disease, therapy with TsA or SAHA decreased the severity of ConA-hepatitis<sup>78</sup>, T<sub>H</sub>2-associated lung airway hypersensitivity responses<sup>93</sup>; experimental allergic encephalomyelitis<sup>94</sup>; renal disease in MRL/lpr mice<sup>95</sup>; colitis<sup>96</sup>; arthritis<sup>97,98</sup>; graft-versus-host disease post-bone marrow transplantation<sup>99</sup> and the 'cytokine storm' induced by the CD3 monoclonal antibody therapy used in a bone marrow transplant conditioning regimen<sup>100</sup>. The underlying mechanisms responsible for the beneficial effects of pan-HDACI therapy in these models were usually attributed, by extrapolation from the cancer literature, to effects on effector T cell apoptosis<sup>96</sup> and no specific HDACs were identified as key targets for therapy.

T cell-independent and non-pro-apoptotic beneficial effects of pan-HDACI administration were noted in studies of organ ischaemia/reperfusion injury in mice, and class II HDACs proved especially useful, albeit with different HDACs being important in different models. TsA use prevented hypoxia-inducible factor 1 alpha (HIF1 $\alpha$ )-induced upregulation of a number of target genes during myocardial ischaemia and reperfusion, and limited myocardial infarct size by more than 50%<sup>101</sup>. Importantly, a therapeutic benefit was observed even when a single injection of HDACI was made one hour after ischaemia/reperfusion. Moreover, individual

knockdown studies in cultured myocytes pointed to a key role of HDAC4 in regulating myocyte expression of HIF1 $\alpha$ . Surprisingly little is known about the effects of HDACI therapy on renal ischaemia/reperfusion injury, but *in vitro* data using cultured tubular cells suggest that bone morphogenetic protein 7 (BMP7), which is normally inhibited by HDAC5, plays an important part in the regenerative response<sup>102</sup>. Within the central nervous system, pan-HDACI therapy proved beneficial in limiting the extent of acute brain injury in mice after a stroke, even when administered post-injury, through direct neuroprotective and anti-inflammatory effects<sup>103</sup>. Pan-HDACI use was also beneficial in models of neurodegenerative diseases involving oxidative stress<sup>104</sup>, slowing disease progression in models of Huntington's chorea<sup>105</sup>, spinal and muscular atrophy<sup>106</sup> and amyotrophic lateral sclerosis<sup>107</sup>. Inhibition of HDAC6 promotes acetylation of peroxiredoxins and might be responsible for much of the benefits of pan-HDACI or tubacin therapy in models of neurodegenerative diseases<sup>108</sup>.

Lastly, surgically-oriented studies have examined the balance between HAT and HDAC functions in various organs, and the effects of HDACI therapy following haemorrhage and resuscitation in mice, rats, pigs and with human cells<sup>109–112</sup>. TsA or SAHA administration



**Figure 1 | Main anti-inflammatory effects of histone deacetylase inhibitors (HDACIs) in leukocytes.** Key pathways for differentiation of activated CD4<sup>+</sup> T cells are shown, along with lineage-specific transcription factors (T-bet, GATA-3, RORγt and FOXP3). HDACI use decreases the production of proteins shown in red boxes. Many anti-inflammatory effects of HDACIs are mediated through effects on antigen-presenting cells (APCs), including dendritic cells and monocytes. HDACI-induced suppression of the production of multiple cytokines impairs the differentiation of CD4<sup>+</sup> T cells into T<sub>H</sub>1 cells characterized by T-bet expression and T<sub>H</sub>17 cells characterized by RORγt expression. However, HDACI treatment does not impair development of T<sub>H</sub>2 cells expressing GATA-3 or regulatory T cells (T<sub>reg</sub>) expressing FOXP3. HDACI use also prevents the conversion of T<sub>reg</sub>s into T<sub>H</sub>17 cells. The effects of HDACIs on other leukocytic cell types, including granulocytes, natural killer cells and non-malignant B cells, are poorly understood. CCR, C-C receptor; CD, cluster of differentiation; IFN, interferon; IL, interleukin; IP-10, inducible protein-10; I-TAC, inducible-T-cell activating chemokine; MCP, monocyte chemoattractant protein; Mig, monokine induced by interferon γ; S1P, sphingosine-1-phosphate; TGF, transforming growth factor; TNF, tumour necrosis factor; TLR, Toll-like receptor.

proved beneficial even in the absence of conventional fluid replacement, and was associated with patterns of increased acetylation that varied between organs. Optimal effects of pan-HDACI use were seen in conjunction with hyperacetylation, especially of histone 3 and a series of non-histone proteins, and increased DNA binding of acetylated β-catenin and increased expression of multiple protective genes, including *BCL2* and heat shock protein 70 (*HSP70*) (REFS 109–112). In addition, HDACI use attenuated organ injury, and inhibited expression of a variety of pro-inflammatory cytokines.

**Clinical trials.** Collectively, these studies indicate that HDACIs can act through multiple mechanisms that encompass effects on chromatin remodelling and also increased acetylation of non-histone proteins. However, most studies were performed with HDACIs that are not being developed for clinical application as anti-inflammatory agents, including TsA, which is relatively toxic; SAHA and depsipeptide, which have been developed for treatment of cutaneous T cell lymphoma and

potentially other oncological applications; or agents with weak potency, such as butyrate and VPA. An exception is the benzamide HDACI MS275 that is in Phase II trials for cancer, but which is also proposed for development, after further optimization is achieved, for the treatment of psoriasis and rheumatoid arthritis (TABLE 2).

At least an additional seven companies are developing HDACIs for treatment of inflammation and/or autoimmunity, with most attention being given to identification of hydroxamic acid derivatives. Interestingly, even within this class of compounds, some pan-HDACIs and class I HDACIs have been identified, as well as agents with individual HDAC selectivity. The latter include hydroxamates selective for HDAC6 (class IIb) or HDAC8 (class I), indicating the robust range of potential inhibitory actions of this broad class of compounds. One such hydroxamate, ITF2357 (also known as givinostat) has shown clinical benefit and safety in Phase II trials in children with active systemic onset juvenile idiopathic arthritis<sup>113</sup>. Givinostat significantly reduced the systemic feature score and number of painful joints.

**HDACIs and FOXP3<sup>+</sup> T<sub>reg</sub>s**

Over the last decade, roles for FOXP3<sup>+</sup> T<sub>reg</sub>s in maintaining immune homeostasis have been identified and this cell type has emerged as a prime target for therapeutic manipulation in new efforts to curb autoimmunity and transplant rejection<sup>23</sup>. HDACI therapy has been shown to increase the production and suppressive functions of T<sub>reg</sub>s *in vitro* and *in vivo*, leading to the concept that HDACI use might provide a pharmacological means to exploit the actions of this recently recognized cell type. This section summarizes the biology of FOXP3<sup>+</sup> T<sub>reg</sub>s and discusses the broad effects of HDACIs on T<sub>reg</sub>s, including whether all or just selected HDACs should be targeted.

**Defects in FOXP3<sup>+</sup> T<sub>reg</sub> cells induce inflammatory and autoimmune diseases.**

Thymectomy of neonatal mice has been shown to lead to autoimmune gastritis; however, disease was prevented by adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells from normal mice<sup>114</sup>. In 2001, it was reported that 5–10% of mammalian CD4<sup>+</sup> T cells express an X-linked gene encoding FOXP3, which serves as a lineage specification factor for the development and function of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>s<sup>17,18</sup>. The dominant role of T<sub>reg</sub>s in maintaining immune homeostasis is illustrated by the fatal autoimmune disease found in patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, and in mice lacking functional Foxp3 (REFS 17–21).

Naturally occurring T<sub>reg</sub>s arise during thymic development and enter the periphery, surviving for up to 70 days<sup>115</sup>. Whereas replacement of dying peripheral T<sub>reg</sub>s in juveniles occurs by thymic emigration, in adults, peripheral conversion of CD4<sup>+</sup>CD25<sup>+</sup> cells into CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>s<sup>116,117</sup> and extrathymic development probably takes place in gut-associated lymphoid tissues under the trophic effects of gut metabolites<sup>118,119–121</sup>. FOXP3 is considered key to T<sub>reg</sub> development<sup>19</sup>, given its ability to modulate the expression of hundreds of genes when transfected into T cells. As T<sub>reg</sub>s cannot produce IL-2, they need a local source of IL-2 and

Table 2 | Histone deacetylase inhibitors in development for the treatment of inflammatory and autoimmune diseases

Company (and partner)	Compound(s); structural group	Target effects	Indication(s)	Development stage*
Acetylon	Multiple hydroxamic acid derivatives	HDAC6-selective	Rheumatoid arthritis	Discovery
Chipscreen Biosciences	CS0240-CS0600	Series of subclass-selective HDACIs	Neurodegenerative diseases, viral infections, immuno-modulation	Preclinical
Chroma Therapeutics (and GlaxoSmithKline)	CHR3996; hydroxamic acid	Orally active class I-selective HDACI that blocks macrophage production of TNF- $\alpha$	Inflammatory disease, for example, rheumatoid arthritis	Discovery
Envivo Pharmaceuticals (and Methylgene)	EVP-0334	HDACI that blocks TNF- $\alpha$ production	Neurodegenerative diseases	Phase I
Italfarmaco	ITF2357 (Givinostat); hydroxamic acid	Orally active pan-HDACI that reduces the production and activity of multiple pro-inflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-12, IL-18 and IFN- $\gamma$ )	Inflammatory diseases, including juvenile arthritis <sup>113</sup>	Phase II
Karus Therapeutics and EOS Pharmaceutical Corporation	OS-HDI and other depsipeptide derivatives	OS-HDI is an HDAC1-selective inhibitor	Rheumatoid arthritis, psoriasis and transplantation	Preclinical
Pharmacyclics	PCI-34051; hydroxamic acid	HDAC8-selective inhibitor that blocks production of multiple cytokines (IL-1 $\beta$ , IL-18)	Psoriasis and rheumatoid arthritis	Preclinical
Syndax	SYDX-275 (etinostat, MS275); benzamide	HDAC1–3 inhibition	Psoriasis and rheumatoid arthritis <sup>98</sup>	Preclinical

\*Information on company website. HDAC, histone deacetylase; HDACI, histone deacetylase inhibitor; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

must express an IL-2 receptor (CD25). Their development is facilitated by exposure to TGF- $\beta$ <sup>116</sup>, retinoic acid<sup>119,120</sup> and by T cell receptor activation, and is impaired by exposure to IL-6 and IL-21 (REFS 122–124).

T<sub>regs</sub> exert suppressive effects on adjacent cells through multiple mechanisms, only some of which seem to be active using *in vitro* assays of T<sub>reg</sub> function<sup>125</sup>. For example, T<sub>regs</sub> are the main source of IL-10 in the colon, yet splenic or lymph node T<sub>regs</sub>, which are typically used to obtain cells for T<sub>reg</sub> assays, produce little IL-10 (REF. 126). T<sub>regs</sub> express inhibitory surface proteins, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA4), tumour necrosis factor receptor superfamily member 18 (TNFR18) and tumour necrosis factor ligand superfamily member 14 (TNF14), and secrete inhibitory cytokines such as IL-10, TGF- $\beta$  and IL-35 (REFS 125–128). They also express membrane ecto-ATPases such as CD39 and CD73 that may contribute to the inhibition of immune activation by affecting local adenosine levels<sup>129,130</sup>. Consumption of essential amino acids may be an important mechanism of action of FOXP3<sup>+</sup> T<sub>regs</sub> *in vivo* through competition with other immune cells<sup>131</sup>.

**FOXP3 structure–function relationships.** FOX family members show only low-affinity binding for DNA in the absence of dimerization or co-factor binding<sup>132</sup>. Insights into FOXP3 interactions are largely derived from over-expression in cells that do not necessarily express the same regulatory molecules as T<sub>regs</sub>. Bearing in mind these caveats, studies show that FOXP3 forms homo-oligomers at the proline-rich N-terminal repressor domain, the zinc-finger leucine zipper domain and the C-terminal forkhead domain<sup>133,134</sup>, and forms heterodimers with FOXP1 (REF. 133). FOXP3 also interacts with multiple

transcription factors, including NFAT<sup>135,136</sup>, NF- $\kappa$ B<sup>135,137</sup>, RUNX1<sup>138</sup>, JUN<sup>139</sup> and CREB<sup>137</sup>. FOXP3 alters the binding of these transcription factors to the promoters of target genes either by binding to immediately adjacent critical residues, or by binding to the transcription factors themselves, as with NFAT and NF- $\kappa$ B<sup>135,136</sup>. NFAT and NF- $\kappa$ B interact with the N-terminal domain of FOXP3 (REFS 135,136), whereas the zinc-finger leucine zipper domain is important for protein–protein interactions<sup>122</sup>, and the C-terminal domain promotes nuclear localization and DNA binding<sup>125</sup>. Thus, FOXP3 functions as a transcription repressor, and probably as a transcriptional activator, through the formation of both DNA–protein and protein–protein interactions.

**Post-translational modifications of FOXP3.** The binding of human<sup>6</sup> and murine<sup>7</sup> FOXP3 to chromatin and its effects on gene expression in T<sub>regs</sub> are enhanced by FOXP3 acetylation. Moreover, biochemical fractionation shows that FOXP3 exists as part of one or more supra-molecular complexes in T<sub>regs</sub><sup>133,140</sup>. A higher-molecular-weight complex has been shown to contain FOXP3 and several chromatin remodelling enzymes (for example, SMCA4, ATP-dependent DNA helicases and MBD3)<sup>133,140</sup>, and a lower-molecular-weight complex contained FOXP3 and additional transcription factors, including FOXP1 and NFATc2. In addition, co-immunoprecipitation studies showed a physical association between FOXP3, a histone acetyltransferase (for example, TIP60) and one or more deacetylases (for example, HDAC7, HDAC9)<sup>6</sup>, as well as between FOXP3 and HSP70 (REF. 26). These data indicate the potential to regulate FOXP3-dependent T<sub>reg</sub> functions through modulation of the acetylation of FOXP3.

**FOXP3<sup>+</sup> T<sub>reg</sub> cells as a target of HDACs.** Promoting FOXP3 acetylation seems to be a useful strategy for decreasing autoimmunity and transplant rejection, and can be achieved pharmacologically with HDACI therapy<sup>7,27,141</sup>. The main classes of HDACs have recently been assessed for effects on FOXP3<sup>+</sup> T<sub>reg</sub> function *in vitro*<sup>142</sup>. Pan-HDACIs of the hydroxamic acid series, including TsA, SAHA, M344 and Scriptaid, boosted T<sub>reg</sub> suppression *in vitro* when used at nanomolar concentrations, and the short-chain fatty acids, phenylbutyrate and VPA, enhanced T<sub>reg</sub> function when used at micromolar and millimolar levels, respectively. Hydroxamates also increased FOXP3 mRNA expression and promoted peripheral conversion upon adoptive transfer of T cells into immunodeficient mice<sup>7</sup>. By contrast, quinolone (NSC3852) and benzamide (MS275, MS1293) compounds had no effect on FOXP3 expression or FOXP3<sup>+</sup> T<sub>reg</sub> function when assayed at micromolar levels. Small-molecule inhibitors showing efficacy *in vitro* also increased T<sub>reg</sub> function when injected into normal mice. Comparison of the effects of the pan-HDACI compounds, TsA and SAHA, with the class I HDACI MS275 in colitis models also showed these differences in modulation of T<sub>reg</sub> function, and lack of HDACI efficacy in T<sub>reg</sub>-depleted mice<sup>26</sup>. TsA was shown to prevent the differentiation of FOXP3<sup>+</sup> T<sub>regs</sub> into T<sub>H</sub>17 cells<sup>143</sup>, and pan-HDACI therapy enhanced T<sub>reg</sub> function and decreased inflammatory responses in arthritis<sup>27</sup> and renal transplant rejection<sup>144</sup>.

It should be noted that although class I-specific HDACs fail to enhance T<sub>reg</sub> function in our studies, these agents still have important dose-dependent anti-inflammatory effects *in vivo*. For example, in collagen-induced arthritis, SAHA was far less efficacious than MS275 in decreasing joint destruction<sup>98</sup>. By contrast, TsA was highly effective in a second model of adjuvant-arthritis<sup>145</sup>. Whether this reflects different drug potencies, different sets of genes being induced in each model or other factors, is unknown<sup>16</sup>. In any case, the finding that pan-HDACIs but not class I-selective HDACs enhance T<sub>reg</sub> production and function points to a role for class II HDACs in the regulation of T<sub>reg</sub> biology. Moreover, although there is biochemical evidence of HDAC7 and HDAC9 in association with FOXP3 in large supra-molecular complexes, given the typically weak deacetylase activity of such class IIa HDACs, the pharmacological evidence points to the potential value of targeting class IIb HDACs.

**Class IIa-specific HDAC targeting.** HDAC class IIa-selective inhibitors have been reported but are not widely available and there are little data on their efficacy *in vivo*<sup>64,146–149</sup>. As noted, class IIa HDACs are considered enzymatically inactive when tested in isolation and their catalytic activity after immunoprecipitation may be due to co-precipitated HDAC3 (REF. 31, 150). This assessment becomes more nuanced in the light of studies showing basal activity against standard acetyl-lysine substrates but increased activity upon mutation of a key histidine to tyrosine in the active site<sup>32,48</sup>, and proposals that these HDACs might be active against other substrates. For example, HDAC9 was recently shown to deacetylate the transcription factor USF1 and thereby regulate

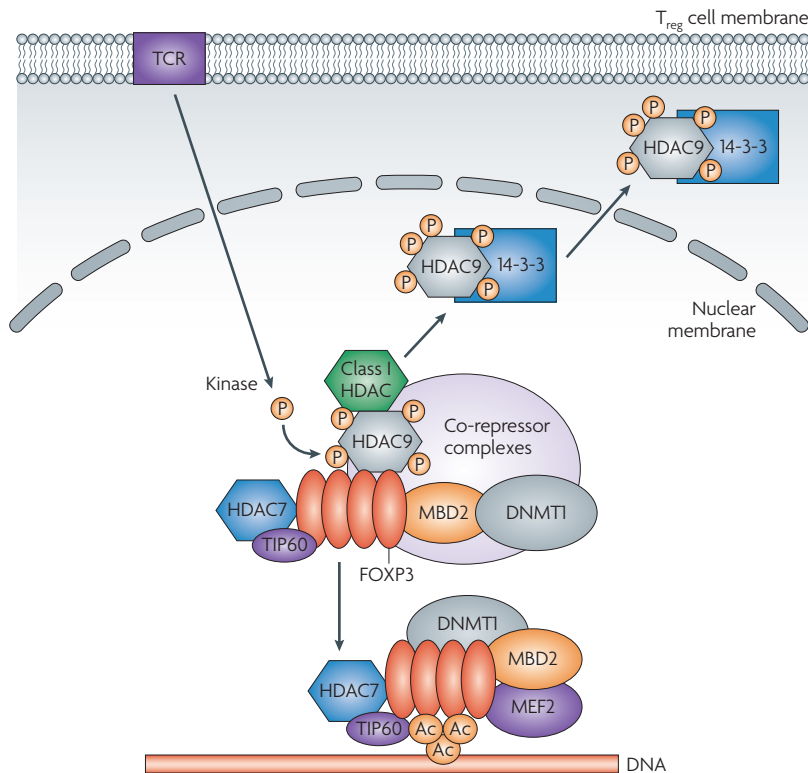
activation of fatty acid synthase<sup>151</sup>. Class IIa-selective HDACs might thereby act only when the inhibitor is complexed to a class I HDAC, interfere with class I or class IIa complex formation, or block activity of class IIa HDACs against non-standard substrates.

So far, only genetic evidence is available as to the value of selective class IIa targeting to promote T<sub>reg</sub> function *in vitro* and *in vivo*. A model of class IIa HDAC function in T<sub>regs</sub> is shown in FIG. 2. HDAC9-deficient mice are immunocompetent and FOXP3<sup>+</sup> T<sub>regs</sub> isolated from these mice show increased suppressive functions *in vitro* and *in vivo*, including in models of inflammatory bowel disease<sup>7,26</sup>. HDAC9<sup>-/-</sup> T<sub>regs</sub> have elevated levels of HSP70 and are resistant to pro-apoptotic conditions<sup>26</sup>, such that the development of small molecules to block HDAC9 or promote HSP70 expression in T<sub>regs</sub> could be valuable.

Preliminary data also showed that HDAC7 targeting enhances T<sub>reg</sub> suppression *in vitro* and *in vivo*. In biochemical studies with human<sup>6</sup> and murine<sup>152</sup> T<sub>regs</sub>, immunoprecipitation of FOXP3 co-precipitated HDAC7. Deletion of HDAC7 by mating mice expressing floxed HDAC7 and CD4-Cre transgenes did not markedly affect peripheral CD4, CD8 or T<sub>reg</sub> numbers, but enhanced T<sub>reg</sub> suppression *in vitro*. Increased T<sub>reg</sub> function was not associated with significant changes in expression (as assessed by microarray or quantitative PCR) of other HDACs or repression of IL-2, IL-6 or IFN- $\gamma$ , but was associated with increased expression of IL-10 and CTLA4. In addition, whereas C57BL/6 mice rejected BALB/c cardiac allografts within 14 days despite use of low-dose rapamycin for the same period, similarly treated HDAC7-deficient hosts accepted their cardiac allografts indefinitely (>100 days) and without development of chronic rejection. The presence or absence of HDAC7 as a key feature of T<sub>reg</sub> function was confirmed in immunodeficient recipients of cardiac allografts adoptively transferred with varying ratios of wild-type or HDAC7<sup>-/-</sup> T<sub>reg</sub> to wild-type T effector cells.

Lastly, although HDAC7 displays only minor catalytic activity using standard assays, the catalytic domain is likely to be important for recruiting repressive SMRT/NCoR/HDAC3 complexes. HDAC7 dominant-negative mice bearing a transgene with four point mutations in the catalytic domain of HDAC7 under the control of the lymphocyte-specific protein tyrosine kinase (Lck) promoter showed essentially normal peripheral CD4, CD8 and T<sub>reg</sub> numbers, but had enhanced T<sub>reg</sub> suppression *in vitro*. By contrast to wild-type controls, these mice accepted fully major histocompatibility complex (MHC)-disparate cardiac allografts long-term when treated for 14 days with low-doses of rapamycin<sup>152</sup>. Hence, HDAC7 functions as an important brake on normal FOXP3-dependent T<sub>reg</sub> function, and HDAC7 targeting might be of considerable therapeutic utility in autoimmunity and transplantation.

**Class IIb-specific HDAC targeting.** Ongoing analysis of HDAC expression and functions in FOXP3<sup>+</sup> T<sub>reg</sub> cells showed TCR-activated T<sub>regs</sub> had 5–6 fold more HDAC6 mRNA than corresponding resting T<sub>reg</sub> or non-T<sub>reg</sub> cells<sup>153</sup>. In various cell types, HDAC6 deacetylates  $\alpha$ -tubulin, cortactin and HSP90, abrogates formation of the



**Figure 2 | HDAC/FOXP3 complex in regulatory T cells.** Within the nucleus, the forkhead transcription factor FOXP3 undergoes homo- and hetero-oligomerization (the latter with FOXP1) and recruits the histone acetyl transferase (HAT) TIP60 and the class IIa histone deacetylase (HDAC) HDAC7 to a region between 100 and 200 amino acids from the amino-terminus. FOXP3 binding to DNA is impaired by its interaction with a second class IIa HDAC, HDAC9, that acts as a scaffold for assembly of a complex that includes DNA methyltransferase 1 (DNMT1), a class I HDAC (for example, HDAC3), methyl-binding domain 2 (MBD2) and co-repressor complexes, for example, silencing mediator of retinoid and thyroid hormone receptors/nuclear hormone receptor co-repressor (SMRT/NCOR), T cell receptor (TCR) stimulation activates one or more kinases that phosphorylate class II HDACs; candidate kinases include protein kinase D (PKD), calcium/calmodulin-dependent kinase (CaMK) and salt-inducible kinase (SIK). Phosphorylation of HDAC9 acts as an ‘off’ switch, as phosphorylated HDAC9 undergoes a conformational change and dissociates from FOXP3, removing inhibitory complexes and leading to de-repression of FOXP3 and its interaction with other proteins (for example, myocyte enhancing factor 2 (MEF2)) and DNA. Phosphorylated HDAC9 is exported from the nucleus in conjunction with the exportin 14-3-3. In the cytoplasm, phosphorylated HDAC9 is either degraded by the proteasome or might be dephosphorylated and re-enter the nucleus to re-establish binding to FOXP3, limiting its action again (‘on’ switch). Upon removal of HDAC9 and its associated inhibitory complexes, FOXP3 is acetylated by one or more HATs, for example, PCAF and p300, and binds to the promoter regions of target genes in the DNA. Ac denotes acetylation; P denotes phosphorylation.

**Aggresome**

An intracellular inclusion body that stores misfolded intracellular proteins and recruits motor proteins to transport misfolded or aggregated proteins to chaperones and proteasomes for subsequent destruction.

aggresome, and blocks the unfolded protein response, although nothing is known regarding these pathways in  $T_{reg}$ . An HDAC6-specific inhibitor, tubacin (but not the control compound niltubacin)<sup>66</sup>, increased  $T_{reg}$  suppressive function *in vitro*, in association with increased expression of CTLA, IL-10, TNFR18, programmed cell death protein 1 (PDCD1) and other  $T_{reg}$ -associated genes ( $p < 0.05$ ), and increased  $T_{reg}$  FOXP3 protein (but not mRNA) expression. Tubacin enhanced the conversion of  $CD4^+ CD25^-$  cells into  $CD4^+ FOXP3^+ T_{reg}$  *in vitro*, and globally decreased cytokine production, with the exception of IL-10 and IL-17

mRNA. Comparable and dose-dependent effects were seen using the HSP90 inhibitor, geldanamycin, suggesting that the effects of HDAC6 inhibition were mediated, at least in part, by blocking the chaperone effect of HSP90.

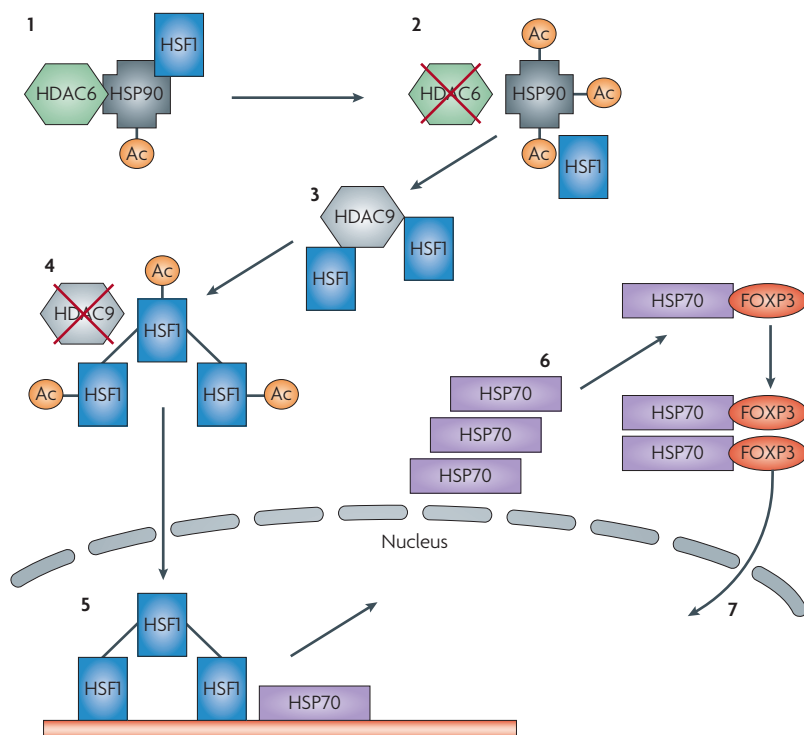
Use of tubacin *in vivo* significantly decreased the severity of colitis in two murine inflammatory bowel disease models, dextran sodium sulphate-induced colitis and the  $CD4^+ CD62L^{high}$  adoptive transfer model of colitis, as assessed by clinical and histological criteria. In addition, 14 days combined use of tubacin and a sub-therapeutic dosage of rapamycin led to significantly prolonged cardiac allograft survival compared with use of either agent alone<sup>153</sup>. Hence, use of a selective HDACI has important therapeutic effects, including enhancing the production and suppressive function of  $T_{reg}$ . Ongoing studies are directed towards unravelling the interactions of HDAC6-dependent pathways and  $T_{reg}$  functions, as the current data indicate the importance of understanding the functions of HDACs to the development of new ways to regulate host immune responses. A model of FOXP3–HDAC6–HDAC9 interactions is shown in FIG. 3.

**Challenges for  $T_{reg}$ -based therapies.** Extrapolation of data from mice to humans always has limitations, including with  $T_{reg}$  biology. For example, the cytokine IL-35 is constitutively expressed by murine  $Foxp3^+ T_{reg}$  and promotes their suppressive function, but it is not expressed in corresponding human  $T_{reg}$ <sup>154</sup>. Likewise, the conversion of murine T cells to  $T_{reg}$  that occurs on activation and culture in the presence of IL-2 and TGF- $\beta$  does not occur reliably in human cells<sup>155</sup>. Moreover, it may not always be desirable to try and increase  $T_{reg}$  suppression, given data from the cancer field showing that the recruitment and function of  $T_{reg}$  may counteract host anti-tumour immune responses<sup>156</sup>. Lastly, T cells may develop increased resistance to  $T_{reg}$ -mediated suppression, as seen clinically<sup>157</sup> and in murine models of type 1 diabetes<sup>158</sup>. These considerations reinforce the need to develop effective strategies to control  $T_{reg}$  functions, to validate their use with human  $T_{reg}$ , and to judiciously pick applications for their use.

**Challenges and future directions**

Available data indicate that HDACIs can have important anti-inflammatory and immunosuppressive effects through direct effects on cells of the innate and adaptive immune systems, including  $FOXP3^+ T_{reg}$  cells. However, the momentum in drug discovery is only gradually moving towards development of HDACIs that might selectively block individual HDAC isoforms. This hesitation reflects the existing one-size-fits-all standard in the field, at least from an oncological perspective, difficulties in developing screens to identify individual HDAC blocking agents, and the as yet fragmentary biology available to stimulate these efforts. This article emphasizes the potential for these agents, especially as they might harness the functions of  $FOXP3^+ T_{reg}$ . However, additional challenges remain.

**Potential adverse effects of HDACIs.** There is a potential for several serious adverse effects of HDACIs, which may limit their use beyond cancer. Use of several classes of HDACI in preclinical studies and clinical trials in



**Figure 3 | HDAC6 and HDAC9 as synergistic targets for T<sub>reg</sub>-based histone deacetylase inhibitor (HDACI) therapy.** In the presence of functional HDAC6, heat shock protein 90 (HSP90) exists in a state of basal acetylation and binds multiple client proteins, including heat shock factor HSF1 (1). With inhibition of HDAC6, HSP90 acetylation is markedly enhanced by the actions of one or more unknown histone acetyltransferases and client proteins are released. Whereas many client proteins undergo ubiquitination and proteasomal degradation, others, including HSF1, are functional (2). HDAC9 and SIRT1 may bind HSF1 and differentially maintain its deacetylation (3). Inhibition of HDAC9 promotes HSF1 acetylation, probably by p300, and its trimerization (4). Trimeric HSF1 undergoes phosphorylation by POLO1 or other kinases and nuclear translocation to induce the expression of other heat shock proteins such as HSP70 and HSP27 (5). HSF1-induced HSP70 binds to and serves as a chaperone for many proteins, including FOXP3 (6). HSP70 may promote the maturation of newly synthesized FOXP3 and possibly nuclear translocation and DNA interactions (7). Ac denotes acetylation.

patients with various tumours was linked with increased electrocardiographic abnormalities, including QT interval prolongation<sup>159</sup>. This is a serious adverse drug reaction as it can potentially lead to ventricular arrhythmia and sudden death. Whether this is a class effect that is common to all HDACIs is currently unclear. In addition, clinical use of members of any of the four main classes of HDACI is accompanied by dose-limiting rates of nausea, vomiting and fatigue, and in some cases, bone marrow suppression, although the mechanisms involved are unclear<sup>160–163</sup>. At least theoretically, HDACIs may also reduce the anti-inflammatory effects of corticosteroids in patients with severe chronic obstructive airway disease

by blocking the deacetylating activity of HDAC2 on NF-κB<sup>164,165</sup>, and could promote reactivation of latent viruses (for example, HIV)<sup>166</sup>. Interestingly, the re-activation of HIV in infected T cells may provide a mechanism for the purging of latently infected T lymphocytes in HIV patients and hence may prove of benefit if appropriately monitored. Lastly, selective targeting of HDAC9 might promote cardiac hypertrophy as seen in aging HDAC9<sup>-/-</sup> mice, although this phenotype was not reproduced by long-term pan-HDACI therapy in wild-type mice<sup>37</sup>.

**How many HDACs should be targeted?** A one-size-fits-all approach has largely prevailed with HDACI therapy in oncology settings, but as noted here, there are data indicating that a more selective approach might be useful, at least in non-oncology settings. Given that pan-HDACIs are effective anti-inflammatory agents in some models in which class I-selective agents are ineffective, coupled with the weak deacetylase activity of HDAC class IIa members, a focus on targeting HDAC class IIb members is logical. Moreover, initial data from pharmacological and genetic targeting of HDAC6 is encouraging, although more data from additional models are needed. Lastly, selective targeting of HDAC class IIa members may prove useful in enhancing T<sub>reg</sub> functions, if selective small molecules are identified.

**HDACIs in combination with other agents.** In addition, taking a cue from oncology studies<sup>167</sup>, assessment of potentially synergistic interactions of HDACIs with other compounds needs to be explored in inflammatory and immune models. We have noted marked synergy in suppressing transplant rejection and promoting tolerance induction in experimental models by combining pan-HDACI therapy with subtherapeutic regimens of rapamycin<sup>7</sup> or DNA methyltransferase inhibitors<sup>168</sup>, and are currently exploring the use of pan-HDACIs and proteasome or HSP90 inhibitors.

**Future directions.** It remains to be determined whether HDACIs can restore optimal regulation clinically by enhancing FOXP3 functions and/or by direct effects on effector T cells, dendritic cells and other cellular components of the inflammatory cascade. Ongoing studies are directed towards the characterization of key pathways that can control FOXP3-dependent T<sub>reg</sub> functions, with the intent of identifying and testing selective inhibitors of these pathways. Although the field is at an early stage, the ongoing analysis of HDAC structure–function relationships and the development of new and selective HDACIs may provide a major application of HDACIs and associated agents in the treatment of immuno-inflammatory disorders.

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#### DATABASES

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