Histone Deacetylation Inhibitors as Modulators of Regulatory T Cells

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Abstract: Regulatory T cells (Tregs) are important mediators of immunological self-tolerance and homeostasis. Being cluster of differentiation 4+ Forkhead box protein3+ (CD4+FOXP3+), these cells are a subset of CD4+ T lymphocytes and can originate from the thymus (tTregs) or from the periphery (pTregs). The malfunction of CD4+ Tregs is associated with autoimmune responses such as rheumatoid arthritis (RA), multiple sclerosis (MS), type 1 diabetes (T1D), inflammatory bowel diseases (IBD), psoriasis, systemic lupus erythematosus (SLE), and transplant rejection. Recent evidence supports an opposed role in sepsis. Therefore, maintaining functional Tregs is considered as a therapy regimen to prevent autoimmunity and allograft rejection, whereas blocking Treg differentiation might be favorable in sepsis patients. It has been shown that Tregs can be generated from conventional naïve T cells, called iTregs, due to their induced differentiation. Moreover, Tregs can be effectively expanded in vitro based on blood-derived tTregs. Taking into consideration that the suppressive role of Tregs has been mainly attributed to the expression and function of the transcription factor Foxp3, modulating its expression and binding to the promoter regions of target genes by altering the chromatin histone acetylation state may turn out beneficial. Hence, we discuss the role of histone deacetylation inhibitors as epigenetic modulators of Tregs in this review in detail.

Keywords: tolerance induction; epigenetics; Foxp3 expression; histone deacetylase inhibitor; sepsis; transplantation; autoimmunity; Treg

1. Introduction

Regulatory T cells (Tregs) are important to guarantee immunological self-tolerance and homeostasis. Since their first description in 1995 [1], several subpopulations of Tregs have been described to fulfill these requirements [2]. First, Tregs can originate from the thymus. Accordingly, these Tregs are named tTregs [3]. Second, Tregs can develop from effector T cells in the periphery and are thus designated as pTregs [4]. This usually happens upon the activation of post-naïve effectors with mainly oral antigens in the presence of specific cytokines. In the thymus, thymocytes are educated to self-antigenic peptides first in the cortex and then in the medulla with medium affinity, whereas thymocytes destined to become Tregs are educated to recognized self-antigenic peptides with high affinity, mainly in the cortical–medullary junction and the medulla, before being released to the periphery. To achieve this, self-reactive thymocytes are eliminated by negative selection [5]. However, although this mechanism is very effective, some self-reactive, and thus possibly autoimmunity-inducing T cells, escape this
machinery [6,7]. Therefore, a system must exist that restricts activity of these cells. This was proven by the classical thymectomy experiment in neonate mice, which showed T cell-dependent autoimmunity when the thymus was removed at day three after birth but not at day one or day seven [8–10]. These tTregs, which migrate to the periphery after day three, are essential for self-tolerance. Recent evidence identified thymocyte apoptosis, occurring after birth [11], as leading to the intrathymic release of transforming growth factor (TGF)-β as reason for the delayed tTregs export compared to cluster of differentiation (CD)4+ single positive (SP) thymocytes [12]. TGF-β initiates Foxp3 expression and tTregs development [12]. However, earlier data have shown normal tTregs development in mice deficient for TGF-β 1 but significantly reduced pTregs [13]. The expression of the transcription factor Foxp3 is a marker of Tregs. The activation of the corresponding gene locus is a multistep process [14,15]. It requires a high affinity binding of major histocompatibility complex (MHC)-self peptide complexes from thymic antigen-presenting cells (APCs) to the T cell receptor (TCR) and costimulatory signals as well as cytokine environments (IL-2) [16,17]. Foxp3 provokes the expression of target genes, which are important to trigger and maintain the immune suppressive Tregs phenotype, as shown by genome-wide analyses in mice and humans [18,19]. In mice, Foxp3 binding results in both the activation and repression of its target genes. This was determined by chromatin immunoprecipitation (ChIP) against epigenetic markers such as acetylated H3K9/14 (AcH3), tri-methyl H3K4 (Me3K4), and tri-methyl H3K27 (Me3K27). These data identified the cell surface molecules Il2ra (CD25), Ctl4a (CD152), Ntsε (CD73), and Icos (CD278) as well as the transcription factor Ikzf2 (Helios) to be Foxp3-dependently upregulated based on the chromatin markers AcH3 and Me3K4. In contrast, the phosphodiesterase Pde3b showed tri-methylation at H3K27, mandatory for its inhibited expression [20]. For the latter one, it was recently shown that Foxp3 also induces the microRNA-142-5p, which as an intracellular cAMP sensor leads to the posttranscriptional repression of the cAMP hydrolyzing enzyme Pde3b [21]. In human Tregs, a similar expression profile was observed, demonstrating the selective gene expression of IKZF2 (HELIOS) [22], IL2RA (CD25), and cytotoxic T lymphocyte associated protein 4 (CTLA4) (CD152) [23]. Moreover, the expression of the T cell survival factor IL-2 is Foxp3-dependently downregulated [24]. Further proof for the significance of Foxp3 in Treg differentiation and function came from studies analyzing mutations associated with the nonfunctional expression of Foxp3 in humans, causing IPEX syndrome (immune dysregulation polyendocrinopathy and enteropathy), which requires bone marrow transplantation in early childhood [25]. In mice, a lack of Foxp3 expression, as observed in scurfy mice, induces a similar phenotype [26]. The role of Foxp3 has been further corroborated in mice where the experimental depletion of Foxp3+ Tregs in healthy adult mice has been found to provoke autoimmunity and death [26,27].

Considering this important role of Tregs in maintaining immune self-tolerance, treatment with in vitro generated Tregs may be a therapeutic approach towards autoimmune-mediated diseases. Tregs can be generated ex vivo from conventional naïve T cells after TCR stimulation in the presence of TGF-β and IL-2. These cells are named iTregs according to their induced differentiation [28]. However, the stability of Foxp3 expression in these cells is much lower compared to tTregs or pTregs [29]. Therefore, treatment regimens to prolong and stabilize Foxp3 expression in iTregs are a topic of current research. In contrast, the role of Tregs in sepsis patients seems to be deleterious [30–34]. In this case, reducing the pTreg or tTreg number and function might prevent immunosuppression, consequently improving survival. Thus, epigenetic modifications are an interesting approach to cope with these opposite tasks.

2. Role of Tregs in Disease

2.1. Autoimmune Diseases

Based on Tregs’ role on the induction and maintenance of peripheral tolerance, Treg dysfunction is associated with severe autoimmune conditions. Disease patterns such as systemic lupus erythematosus (SLE) [35] and organ-specific autoimmune diseases, e.g., type 1 diabetes (T1D) [36] and psoriasis [37], have been attributed to a reduced number of Tregs or the failure of their function. Considering this
important role of Tregs, therapies have been developed to medicate autoimmune disorders (for reviews of single clinical trials, see [38,39]). Phase I clinical studies have been using autologous Tregs to treat SLE, pemphigus vulgaris, or T1D. Moreover, already published studies have supported an ameliorative impact of these treatment regimens for T1D, prolonging the survival of β-cells [40,41]. Beside these polyclonal Treg therapies, Treg-enhancing drugs are of interest. Among others, rapamycin-dependent mTOR inhibition was used to efficiently expand human Treg cells [42] and to treat SLE patients, with significant improvement of the clinical outcome [43]. Several other approaches have been shown to block mTOR activation in animal models and T cells derived from the blood of SLE patients, including the blocking of S1P receptors, antioxidants, and calmodulin kinase type II and type IV inhibitors [44–47]. Low-dose IL-2 treatment has also been identified for the treatment of patients with diseases associated with a decreased number of Tregs such as SLE [48–50]. Considering that IL-2 activates Tregs as well as Teff, a dose finding study was performed [51]. In this setting, clinical phase II trials are already running for the treatment of rheumatoid arthritis (RA), SLE, multiple sclerosis (MS), T1D, and amyotrophic lateral sclerosis (ALS).

2.2. Transplant Rejection

Patients with end-stage organ failure need organ transplantation as their therapy of choice. Moreover, an autologous bone marrow transfer is required in patients suffering from chemotherapeutic treatment or an allogenic transfer in patients with lymphoma or leukemia. As expected, Tregs are important mediators of graft tolerance induction following these transplantations. Based on their immunosuppressive function, an increased number of Tregs in the periphery and graft microenvironment has been attributed to confer graft tolerance, thus guaranteeing a long lasting life of solid organ transplants or transferred bone marrow [39,52,53]. Considering this important role of Tregs, in-man phase 1 and phase 2 clinical trials, as summarized in [53,54], have been performed [55–57] or registered. Briefly, liver transplantations were supported with Treg cell therapy, following Treg generation using autologous Tregs stimulated with irradiated donor PBMCs with inhibited costimulation, autologous donor antigen-expanded Tregs, or autologous, polyclonally-expanded Tregs. A similar setup was used to Treg-dependently assist liver transplantation. Additionally, in bone marrow transfer approaches, Tregs have been shown to suppress T cell alloreactions and to prevent graft-versus-host disease in mouse models [28,58–60] and in the human situation [61].

2.3. Sepsis

Sepsis is a syndrome where T cell depletion and, consequently, an inappropriate immune response to the recurring initial infection or acquired second infection is one characteristic [62]. Consequently, it is of interest to follow the fate of Tregs during sepsis initiation and progression. As shown recently by Carvelli et al. [32], the number of Tregs was decreased in patients with septic shock, which is in some discrepancy to previous reports that have shown an increased number of Treg cells in these patients as one reason for long-term immune-suppression [33,34]. Based on these contradictory data, the role of Tregs in sepsis needs further evaluation. Thus, the different stages, i.e., infection, organ dysfunction caused by an inappropriate immune response, septic shock, and finally sepsis survivors, must be carefully examined to draw any conclusion whether Tregs are important to block an overwhelming immune response or whether these cells are crucial for the resolution of inflammation. In both situations, excessive Tregs might be detrimental. Therefore, the pharmaceutical or immunological fine tuning of Tregs will be advantageous to intervene with the respective prevailing pro- vs. anti-inflammatory responses. This has also been shown for the role of Tregs in resolving lung injury [63]. In this animal approach, mice were treated with lipopolysaccharide (LPS) or recombinant high-mobility-group-protein B1 (HMGB1), which is a key mediator during inflammation to induce acute lung injury (ALI). Tregs were modulated with myeloid-specific β-catenin and phosphatase and tensin homologue (PTEN)-knockout mice. As shown in Table 1, several rodent studies have shown an altered number of Tregs following polymicrobial sepsis by cecal ligation
and puncture (CLP). In these studies, various mouse (BALB/c, C57BL/6, FVB/N, ICR, NMRI) and rat strains (Fischer, Wistar, Wistar Hannover, Sprague Dawley) have been used, applying different severities of polymicrobial sepsis. This can be achieved by the diameter of the needle and the number of cecum perforations [64,65]. Most studies have demonstrated an increase of T_{reg} in spleen or blood, independently from the execution of the model. This is in line with the assumption that T_{reg} are generally involved in downregulating the immune response, thus contributing to an immunosuppressed phenotype during sepsis. Interestingly, our own data support this notion. We found that a prevention of CLP-dependent liver damage was associated with a decreased number of liver localized T_{reg} [66]. Thus, reducing the T_{reg} count might be a prerequisite for improving sepsis outcome by restoring a functional T cell response.

Table 1. Polymicrobial sepsis induced by a cecal ligation and puncture (CLP) operation in mice and rats increased regulatory T cell (T_{reg}) count in the blood and spleen. Different mouse and rat strains have been used. The severity of the model is affected by the needle diameter, the number of punctures, and the ligation length [64,65]. (Ø, diameter; CLP, cecal ligation and puncture; f, female; G, gauge; m, male; MLN, mesenteric lymph nodes; and PC, peritoneal cavity.)

| Strain          | Sex | Weight [g] | Age [weeks] | Ligation                      | CLP Needle Ø | Perforation | Duration [h] | Organ       | Ref. |
|-----------------|-----|------------|-------------|--------------------------------|---------------|-------------|--------------|-------------|------|
| **BALB/c**      | m   | 20 ± 2     | 6–8         | 1/3, 2/3, 3/3                  | 23G           | single      | 24 h         | ↑ (spleen)  | [68] |
|                 | m   | 20–25      | 8           | 50%                            | 21G           | once        | 15 d         | ↑ (spleen)  | [69] |
|                 | m   | 18–22      | -           | 50%                            | 21G           | twice       | 24 + 48 h    | ↑ (spleen)  | [70] |
|                 | m   | 20 ± 1     | 6–8         | below the ileocecal valve      | 18G           | once        | 1/2/3/4 d    | ↑ (blood)   | [71] |
| **C57BL/6**     | m/f | 25         | 8           | cecum ligated at its base      | 18G           | twice       | ↑ (blood)    | [72] |
|                 | f   | -          | 6–8         | 50%                            | 27G           | twice       | 3/7 d        | ↑ (spleen)  | [73] |
|                 | m   | 25–27      | -           | 23G                            | -             | 48 h        | ↑ (spleen)   | [74] |
|                 | m   | 25–35      | -           | 50%                            | 18G           | twice       | 24 h         | ↑ (spleen)  | [75] |
|                 | m   | 20–25      | 7–9         | 30% of its length              | 21G           | once        | 5 d          | ↑ (spleen)  | [76] |
|                 | m   | 20–25      | 8–10        | 1.5 cm from the tip            | 22G           | twice       | 20 h         | ↑ (spleen)  | [77] |
| **ICR**         | m/f | 6–8        | 6–8         | below the ileocecal valve      | 21G           | nine        | 24 h         | ↑ (PC, MLN) | [78] |
|                 | m   | 22–25      | 6–8         | 22G                            | once          | 3 d         | ↑ (spleen)   | [79] |
|                 | m   | 20–25      | -           | 75%                            | 21G           | once        | 24 h         | ↑ (spleen)  | [80] |
|                 | m   | 20–25      | -           | 75%                            | 21G           | twice       | 24 h         | ↑ (spleen)  | [81] |
| **NMRI**        | m   | 22–30      | 8–10        | below the ileocecal valve      | 22G           | twice       | 24 h         | ↑ (spleen)  | [82] |
| **FVB/N**       | m   | 25         | 8           | at its base                    | 21G           | once        | 1/3 d        | ↑ (MLN)     | [83] |
|                 | m   | -          | 7           | 1 cm from the apex             | 18G           | twice       | 16 h         | ↑ (spleen)  | [84] |
| **9xNFAT luc**  | f   | -          | 8–12        | 30%                            | 27G           | once        | 24 + 48 h    | ↑ (spleen)  | [85] |
| **m**           | -   | -          | 6–8         | 75%                            | 21G           | twice       | 24 h         | ↑ (spleen)  | [86] |
| **ICR**         | m   | 30–35      | 6–8         | 50%                            | 23G           | twice       | 24 + 72 h    | ↑ (blood)   | [89] |
| **NMRI**        | m   | 27–29      | -           | at its distal site             | 20G           | twice       | 26 h         | ↑ (spleen)  | [90] |
|                 | m   | 20–30      | -           | 30%                            | 27G           | once        | 1/2/3 d      | ↑ (spleen)  | [91] |
Table 1. Cont.

| Strain          | Sex | Weight [g] | Age [weeks] | Ligation | Needle Ø | Perfo-Ration | Dura-tion | Organ       | Ref.   |
|-----------------|-----|------------|-------------|----------|----------|--------------|-----------|-------------|--------|
| Fischer         | m   | 104        | 70% of its length | 18G      | twice    | 20 h         | ↑ (spleen) | [92]        |
| Wistar          | m   | 250–300    | 50%         | 18G      | twice    | 18 h         | ↑ (blood)  | [93]        |
| Wistar Hannover | m   | 200–250    | 8           | below the ileocecal valve | 18G | twice | 24 h | ↑ (MLN) [94] |
| Sprague Dawley  | m   | 350–400    | -           | distal ligation | 18G | twice | 3 d | ↑ (blood) [95] |
| Sprague Dawley  | m   | 320–350    | -           | distal ligation | 18G | twice | 3 d | ↑ (spleen) [96] |
| Sprague Dawley  | m   | 400–450    | -           | distal ligation | 18G | twice | 72 h | ↑ (blood + spleen) [97] |

3. HAT and HDAC Activities in T<sub>reg</sub> Differentiation

Epigenetic, i.e., reversible modifications of chromatin that do not alter the DNA sequence, can be achieved by inhibiting histone deacetylase (HDACs) to maintain chromatin histone acetylation, consequently keeping genomic DNA accessible for the binding of transcription factors and the RNA polymerase. This finally allows for the increased expression of genes known to be involved in T<sub>reg</sub> differentiation and function.

3.1. Foxp3

Foxp3 is a member of the Forkhead box protein (Foxp) subfamily of transcription factors. Due to its function as a master regulator of T<sub>reg</sub> and pT<sub>reg</sub> differentiation and immunosuppressive performance, understanding Foxp3’s transcriptional, translational, and post-translational regulation is important [14,98,99]. Therefore first, the gene structure is crucial [100,101]. The expression of the Foxp3 gene is mediated by five control elements. Starting 5’, the first conserved non-coding sequence (CNS) 0 [102], which is the binding site for the special AT-rich sequence-binding protein (SATB) 1, a super-enhancer that enables T<sub>reg</sub>-lineage-specific gene induction was recently identified [102,103]. Following CNS0, the promoter region [104,105] and three further CNS (CNS1–3) are located. CNS1, located in intron 1, is associated with TGF-β inducibility (TGF-β sensor) [105]. CNS2, also located in intron 1, is the so called T<sub>reg</sub>-cell-specific demethylation region (TSDR) [106,107] and CNS3, localized in intron 3, named the Foxp3 pioneer element, is known to confer the NF-κB inducibility of Foxp3 [108].

All these five elements are mainly characterized by the existence of CpG islands (promoter, CNS2) and histones, which can be acetylated (promoter, CNS0-3) or show permissive methylation (CNS3) [18,102]. Thus, these regulatory structures are targets for epigenetic modifications, altering the accessibility of the Foxp3 gene [109]. In naïve CD4<sup>+</sup> T cells, CpGs are heavily methylated (Figure 1A), silencing the Foxp3 gene [18]. Especially, the promoter region is the target of protein inhibitor of activated STAT (signal transducer and activator of transcription) (PIAS1), a SUMO E3 ligase, which restricts T<sub>reg</sub> differentiation by recruiting DNA methyltransferases and heterochromatin protein 1 to the Foxp3 promoter [110]. Following DNA demethylation, histone acetylation, and permissive methylation, Foxp3 expression and, consequently, T<sub>reg</sub> differentiation are induced by the activation of transcription factors in response to T cell receptor (TCR) engagement, CD28 co-stimulation, IL-2 treatment, and TGF-β addition. Established transcription factors are activator protein 1 (AP-1) (promoter) [111], cAMP-responsive element binding protein (CREB) (CNS2) [105], Ets-1 (CNS2) [112,113], FoxO1 (promoter) [114], nuclear factor of activated T cells (NFAT) (promoter, CNS1) [18,111], nuclear receptor 4a (NR4a) (promoter) [115], c-Rel (CNS3) [108], retinoid x receptor/retinoid acid receptor (RXR/RAR) (promoter, CNS1) [116,117], Runt-related transcription factor 1 (RUNX) (promoter, CNS2) [118], STAT3/5 (promoter, CNS2) [119], and SMAD2/3/4 (CNS1) [120,121] (Figure 1B).
The Foxp3 protein contains four domains, including a repressor domain at the N-terminal end (responsible for transcriptional repression), a zinc finger domain with a so-far unclear function, a leucine zipper domain (important for dimerization), and, finally at its C-terminus, the Forkhead domain, which is important for DNA-binding (Figure 1C). It has been established that the repressor domain located at the N-terminus of Foxp3 is associated with the downregulation of the expression of HIF1α, RORγt, RORα, and Eos. Thus, among others, differentiation towards a Th17 phenotype is prevented [121].
When expressed, Foxp3 can form heterodimers with FoxO1, keeping Foxp3 in an inactive state. It can transiently homodimerize, which enables its regulation, or stably consequently leading to the expression or repression of target genes (Figure 1D). Moreover, transient Foxp3 homodimers may combine as clusters. Foxp3 can additionally bind roughly 700 different proteins, which is important to activate or repress the expression of target genes. Stable Foxp3 coiled-coil-mediated homodimerization is essential for Treg function [122]. As shown in Figure 2A, Foxp3 associates with histone acetylases (HATs) (e.g., p300 or HIV-Tat-interactive protein (TIP60) [123]), leading to Foxp3 (hyper)acetylation, which increases Foxp3 stability as well as HDACs (e.g., silent information regulator 1 (SIRT1) or HDAC5 [124,125]), which reciprocally deacetylate Foxp3, making it more susceptible for proteasomal degradation [126]. Foxp3 lysine residues identified to be affected are shown in Figure 2B. Foxp3 expression and function are also regulated by the histone H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2), which is not expressed in naïve Tregs and is upregulated in CD28-activated Tregs, provoking a stable Treg phenotype by allowing for Foxp3 expression and stabilization [127,128]. Consequently, EZH2-specific inhibitors as well as specifically disrupting EZH2 in Tregs, reduced Foxp3 expression, and concomitantly attenuated the immune suppressive Treg phenotype [127]. However, Foxp3 binding to EZH2 seems to inactivate Foxp3 [129]. Correspondingly, histone demethylases are involved in Foxp3 regulation. There, Jumonji domain-containing 3 (Jmjd3) is the most prominent demethylase, responsible for H3K27me2 and H3K4me3 demethylation, provoking Foxp3 expression and, accordingly, promoting Treg differentiation [130]. Interestingly, in an acute lung injury (ALI) model in mice, the expression of JMJD3 is downregulated in Tregs isolated from the lungs [131]. These data support the notion of an organ and microenvironment specificity of Tregs.

Figure 2. HAT and HDAC binding to Foxp3. (A) Amino groups located at the ε-CH2 group of lysines can be acetylated by histone acetylases (HATs) such as p300 or TIP60 leading to acetylysine, which enhances Foxp3 stability by preventing its proteasomal degradation. Reciprocally histone deacetylases (HDACs) such as Sirt1 deacetylate lysines of Foxp3, which are acetylated at the amino-group next to the ε-C atom. Deacetylated Foxp3 is prone to proteasomal degradation [124]. (B) Lysines of Foxp3, which have been identified as targets for acetylation [122,125,132,133].

3.2. Cytotoxic T Lymphocyte-Associated Protein 4 (CTLA4 or CD152)

Considering a connection of the suppressive effect conferred by Tregs and their CTLA4 expression [134,135], mechanistic insights into the regulation of CTLA4 expression are important. CTLA4 is a co-inhibitor that is generally upregulated upon antigen stimulation via the TCR to prevent an uncontrolled immune response [136,137], allowing for the fine tuning or consequently shutdown of the immune response as an immune checkpoint [138]. This is achieved by its higher affinity to the co-activators CD80/86 (B7-1/-2) expressed on antigen-presenting cells such as macrophages (MΦ) and dendritic cells (DC) compared to the co-activator CD28. Besides this role, CTLA4 is constitutively expressed on Tregs [139,140], contributing to the immunosuppressive phenotype of these cells [141]. Moreover, it has been shown that the transgenic expression of CTLA4 is one prerequisite of converting a conventional to a regulatory T cell [142]. When expressed on the T cell surface, CTLA4 binds to CD80/CD86 on antigen-presenting cells (APC) with a higher affinity than CD28, downregulating
CD80/86 on DC to decrease the potency of APC to activate T cells [135]. From this data, it is obvious to assume that altering CTLA4 expression, i.e., to enhance or downregulate its expression, will be an appropriate treatment regime in autoimmune diseases to enhance $T_{reg}$-dependent tolerance induction, e.g., to prevent cardiac allograft rejection [143], or to inhibit this immune-suppressive reaction to enhance anti-tumor immunity, e.g., by using CTLA4-neutralizing antibodies [144]. The HDAC canonical pan-inhibitor SAHA (suberoylanilide hydroxamic acid, Vorinostat), inhibiting HDAC1-9 with similar potency, has been shown to enhance CTLA4 expression in $T_{reg}$s [143]. This upregulation was even enhanced when tacrolimus was added to inhibit calcineurin in parallel. Additionally, SAHA promoted selectively effector T cell apoptosis, which is consequently associated with an increased $T_{reg}$ proportion. This combined setting might be a therapeutic concept in preventing allograft rejection.

3.3. HDACs and HDACi as a Starting Point for Altering $T_{reg}$ Function

Thus far, 18 HDAC enzymes have been described. Eleven are Zn$^{2+}$-dependent (HDAC1-11) and seven need NAD$^+$ (Sirt1-7) for their activity. Though there have already been several clinical trials using HDAC inhibitors (HDACi) for treatment in oncology, none have been initiated for the therapy of autoimmune diseases. Based on the use of HDACi to change the epigenetic structure of $T_{reg}$-lineage-dependent genes, experiments using pan-HDACi have been performed. It has been shown that the differentiation of human CD25$^{high}$Foxp3$^+$ $T_{reg}$s into IL-17 producing cells can be prevented by the HDACi trichostatin A (TSA) [145]. TSA inhibits, similarly to SAHA, HDAC1-9 without any preference [146]. Taking this unspecific inhibition into consideration, it is difficult to provide any data on the role of a single HDAC, apart from mouse knockout studies or HDACs, where specific inhibitors already are at hand. As shown in Table 2, $T_{reg}$s express class I HDACs 1, 2, 3, and 8 [147], class IIa HDACs 5, 7, and 9 [148], class IIb HDACs 6 and 10 [149], unrelated class III SIRTs 1, 2, 3, and 4 [150], and class IV HDAC 11 [151].

Table 2. HDAC isoforms expressed in $T_{reg}$s promoting/attenuating their function. (C, colitis; CAT, cardiac allograft transplantation; CF, cystic fibrosis; C/JIA, collagen/juvenile-induced arthritis; CLP, cecal ligation and puncture; MCAO, mouse transient middle cerebral artery occlusion.)

| Class | Isoform | Localization | Effect of HDAC Targeting | Specific HDACi | HDAC-Foxp3 Interaction | Models | Ref. |
|-------|---------|--------------|--------------------------|----------------|------------------------|--------|------|
| I     | HDAC1   | nucleus      | ↓                        | no             | inhibits HDAC1         | CAT, C | [152,153]|
|       | HDAC2   | nucleus      | ↑                        | in progress    | associates with Foxp3  | CAT, C | [153,154]|
|       | HDAC3   | nucleus/cytosol | ↓                        | no             | destabilizes Foxp3     | CAT, C | [155] |
|       | HDAC8   | nucleus      | ↓                        | no             | ?                      | CAT    | [155,156]|
|       | HDAC5   | nucleus/cytosol | ↓                        | available    | ?                      | CAT    | [157] |
| IIa   | HDAC7   | nucleus/cytosol | ↓                        | no             | forms a transcriptional complex with Foxp3 | thymic positive and negative T cell selection | [158,159]|
|       | HDAC9   | nucleus/cytosol | ↑                        | no             | destabilizes Foxp3     | C      | [132,160,161]|
| IIb   | HDAC6   | nucleus/cytosol | ↑                        | available     | destabilizes Foxp3     | CF, CIA, JIA, lupus prone mice | [160,162–166]|
|       | HDAC10  | nucleus/cytosol | ↑                        | in progress   | destabilizes Foxp3, represses Foxp3 transcription | CAT, C | [167–169]|

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Table 2. Cont.

| Class | Isoform | Localization | Effect of HDAC Targeting | Specific HDACi Interaction | Models | Ref. |
|-------|---------|--------------|--------------------------|---------------------------|--------|------|
| III   | SIRT1   | nucleus      | ↑ available              | destabilizes Foxp3        | CLP, heterotrophic cardiac and ortho-tropic renal allograft, C | [87,126,160,169,170] |
|       | SIRT2   | cytosol      | ↑ no                     | destabilizes Foxp3        | MCAO   | [171] |
|       | SIRT3   | mito         | ↓ no                     |                          | CAT    | [172] |
|       | SIRT4   | mito         | ↑ no                     | inhibits Foxp3 expression | mouse spinal cord compression injury | [173] |
| IV    | HDAC11  | nucleus      | ↑ available              | destabilizes Foxp3        | CAT    | [158] |

According to the diverse roles of HDACs in T<sub>reg</sub> immunology (see Graphical Abstract), corresponding HDAC inhibitors might be used to reduce or enhance T<sub>reg</sub> cell number and function. Obviously, the expression of Foxp3 is an essential element in T<sub>reg</sub> differentiation. Its transcription is inhibited by HDAC10 [167]. HDAC10 deletion in mice has been shown to enhance Foxp3 stability and increase H2K4Me3-activating marks on the Foxp3 promoter and CNS2 region [167]. Moreover, SIRT2 [171] and SIRT4 [173] downregulate protein Foxp3 expression by a so far unknown mechanism. Blocking these three HDACs will likely increase Foxp3 expression and concomitantly start and enhance T<sub>reg</sub> differentiation. HDAC7 associates with NR4a and Foxp3, being involved in the Foxp3-dependent repression of target genes. Following protein kinase D-dependent phosphorylation, HDAC7 is exported from the nucleus, consequently allowing for gene expression [174]. HDAC3, 6, 9, 11, and SIRT1 have been established to deacetylate Foxp3, which target it for proteasomal degradation [155,158,160,175]. Moreover, HDAC9 inhibits the expression of PPARgamma coactivator 1 alpha (PGC1α), an important factor in inducing proteins of the oxidative phosphorylation (OXPHOS)-system, important for the mitochondrial-dependent energy supply of the cells [172]. Finally, HDAC1 has been attributed to block the activity of the transcription factor RUNX, mandatory to maintain CD4<sup>+</sup> T cell integrity [152,176,177].

In various models, the role of HDAC inhibition or deletion has been determined. Briefly, the inhibition of class I HDACs in models of cardiac allograft transplantation (CAT) or colitis has shown an enhanced T<sub>reg</sub> function following HDAC2 deletion, thus preventing HDAC2 association with Foxp3 [153,154], whereas the blockage of HDAC1, 3, and 8 has been shown to provoke an attenuated T<sub>reg</sub> number and function by destabilizing Foxp3 [152,153,155,156]. Blocking class IIa HDACs attenuates T<sub>reg</sub> function following HDAC5 and 7 inhibition in CAT and positive and negative selection in the thymus [157–159], whereas blocked HDAC9 enhances T<sub>reg</sub> function by Foxp3 stabilization in a colitis model [152,160,161]. Interestingly, class IIb HDACs are only involved Foxp3 destabilization, as shown in cystic fibrosis, collagen-induced arthritis, juvenile idiopathic arthritis, and lupus prone mice, as well as in cardiac allograft transplantation and colitis. Thus, their inhibition enhances and restores T<sub>reg</sub>-dependent effects [160,162–169]. Members of the sirtuin-family of HDACs (class III HDACs) are important regulators of the inflammatory stress response in immune and non-immune cells linking inflammation and metabolism [178,179]. Therefore, their role in T<sub>reg</sub> cell differentiation is mainly characterized by a Foxp3 destabilizing effect in murine sepsis (cecal-ligation and puncture), heterotrophic cardiac and orthotrophic renal allograft transplantation, colitis [55,87,126,160,169–171,180], and by preventing Foxp3 expression in a mouse model of transient middle cerebral artery occlusion [173]. In contrast, SIRT3 is important for the metabolic adaption of T<sub>regs</sub>, which makes it necessary for their function. Thus, SIRT3 inhibition is associated with a reduced number of T<sub>regs</sub> in cardiac allograft transplantation [172]. Lastly, intervening with the function of HDAC11, the only class IV
HDAC, has been shown to result in Foxp3 stabilization, enhancing T_{reg} function in cardiac allograft transplantation [158].

4. Concluding Remarks

Considering these various effects of HDACs related to the epigenetic regulation of genes that are important for T_{reg} differentiation and maintenance, the development of a therapy setting including HDAC-specific inhibitors is a promising task.

Based on the already established methods to generate and expand polyclonal, antigen-specific, or engineered T_{regs} ex vivo for adoptive cell therapy (for review see [52]), these can be treated with specific HDAC inhibitors to enhance Foxp3 expression, which will consequently induce and maintain an immunosuppressive T_{reg} phenotype. After these T_{regs} have been infused back, the limiting factor is the half-life of transferred T_{regs}. This is especially important in patients with autoimmune diseases, where a permanent T_{reg}-based immunosuppression is required. This holds true as well for patients following solid organ transplantation. Bone marrow transfer includes the risk of graft-versus-host disease, which exists temporarily and does not demand a very long T_{reg} life. However, in sepsis patients, the situation is completely different. Because here T_{regs} are mainly deleterious and contribute to an immunosuppressive state that is linked to an inappropriate immune response that finally causes a fatal outcome, the number of these cells should be reduced or their immunosuppressive phenotype should be immediately mitigated. This possibly can be achieved by HDAC inhibition, provoking Foxp3 destabilization or reduced expression. However, it should be taken into account, that, if the HDAC inhibitor is applied in an unspecific formulation to the sepsis patient, it will operate in all cells that express the corresponding HDAC. Therefore, putative side effects have to be carefully proven before. Moreover, a T_{reg} cell-specific HDAC-inhibitor formulation might be a chance to circumvent these side effect studies.

As discussed for autoimmune diseases, transplant rejection and sepsis, adequately altering the generation and number of T_{regs}, i.e., increasing or decreasing their count, is associated with an improved outcome. To achieve this successfully, clinical trials are mandatory in the near future to clarify the role of epigenetics, especially during sepsis initiation and progression. Moreover, the development of HDAC-specific inhibitors is important to allow for the fine tuning of chromatin histone acetylation. Considering the expression and activity of the transcription factor Foxp3 as the main mediator for T_{reg} cell differentiation and function, its epigenetic modulation might be an appropriate target to reduce or enhance T_{reg} function according to the disease state.

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Abbreviations

Ø diameter
Ac acetylation
ALI acute lung injury
ALS amyotrophic lateral sclerosis
AP1 activator protein 1
APC antigen presenting cell
AS ankylosis spondylitis
C colitis
| Acronym | Description |
|---------|-------------|
| CAT     | cardiac allograft transplantation |
| CD      | cluster of differentiation |
| CF      | cystic fibrosis |
| ChIP    | chromatin immunoprecipitation |
| CIA     | collagen-induced arthritis |
| CLP     | cecal ligation and puncture |
| CNS     | conserved non-coding region |
| CREB    | cAMP-responsive element binding protein |
| CTLA4   | cytotoxic T lymphocyte associated protein 4 |
| ETS     | E26-AMV virus oncogene cellular homologue |
| f       | female |
| Foxp3   | Forkhead-box-protein P3 |
| g       | gauge |
| H       | histone |
| HAT     | histone acetyltransferase |
| HDAC    | histone deacetylase |
| HIF     | hypoxia-inducible factor |
| HMGB1   | high-mobility-group-protein B1 |
| HP1     | heterochromatin protein-1 |
| i       | in vitro |
| IBD     | inflammatory bowel disease |
| ICOS    | inducible T-cell costimulatory |
| Ikzf2   | ICAROS family zinc finger 2 |
| IPEX    | immune dysregulation polyendocrinopathy and enteropathy |
| K       | lysine |
| LPS     | lipopolysaccharide |
| m       | male |
| Me      | methylation |
| MHC     | major histocompatibility complex |
| MLN     | mesenteric lymph nodes |
| MS      | multiple sclerosis |
| NFAT    | nuclear factor of activated T cells |
| NRa4    | nuclear receptor 4a |
| Nt5e    | ecto-5'-nucleotidase |
| OXPHOS  | oxidative phosphorylation |
| p       | periphery |
| PC      | peritoneal cavity |
| PGC1α   | PPARgamma coactivator alpha |
| PIAS    | protein inhibitor of activated STAT |
| PSO     | psoriasis |
| PTEN    | phosphatase and tensin homologue |
| RA      | rheumatoid arthritis |
| Pde3b   | phosphodiesterase 3b |
| RAR     | retinoid acid receptor |
| RUNX    | Runt-related transcription factor 1 |
| RXR     | retinoid X receptor |
| SAHA    | suberoylanilide hydroxamic acid |
| Satb1   | special AT-rich sequence binding protein |
| SIRT    | silent information regulator |
| Smad    | small mothers against decapentaplegic |
| SP      | single positive |
| SSc     | systemic sclerosis |
| STAT    | signal transducer and activator of transcription |
| sumo    | small ubiquitin-like modifier |
t thymus
T1D type 1 diabetes
TCR T cell receptor
TIP60 HIV-Tat-interactive protein
TGF-β transforming growth factor-beta
TLR toll-like receptor
Treg regulatory T cell
TSDR Treg-cell specific region

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