Published in final edited form as:

J Leukoc Biol. 2019 August ; 106(2): 337–343. doi:10.1002/JLB.2RU1118-422R.

Sirtuin-1 in Immunotherapy: A Janus-headed Target

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Abstract

Sirtuin-1 (Sirt1), a member of the NAD-dependent sirtuin family of histone/protein deacetylases (HDAC), is an important target for immunotherapy due to its role in deacetylating the transcription factors Foxp3 and RORγt. Sirt1 inhibition can increase Foxp3 acetylation and promote the production and functions of $Foxp3^+$ T-regulatory (Treg) cells, whereas the acetylation of RORγt decreases its transcriptional activity DNA binding and decreases the differentiation of pro-inflammatory Th17 cells. Pharmacologic inhibitors of Sirt1 increase allograft survival and decrease autoimmune colitis and experimental allergic encephalomyelitis. However, in contrast to its role in T cells, Sirt1 has anti-inflammatory effects in myeloid cells, and, context dependent, in Th17 cells. Here, inhibition of Sirt1 can have pro-inflammatory effects. In addition to effects arising from the central role of Sirt1 in cellular metabolism and NAD-dependent reactions, such pro-inflammatory effects further complicate the potential of Sirt1 for therapeutic immunosuppression. This review aims to reconcile the opposing literature on pro- and anti-inflammatory effects of Sirt1, provides an overview of the role of Sir1 in the immune system, and discusses the pros and cons associated with inhibiting Sirt1 for control of pro-inflammation and immunologic responses.

Keywords

T cells; Foxp3; Treg; p65; Immunosuppression

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Author Contributions

Writing–original draft: S.C. and U.H.B.; Writing–editing: L.W. and W.W.H.

Conflict of Interest Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Introduction

Therapeutic immunosuppression is required for many different medical conditions, ranging from autoimmune diseases to transplantation. Current immunosuppressive drugs are limited by non-specificity and toxicities [1, 2], and developing less toxic and more precise drugs that suppress only the unwanted immune responses while preserving protective immunity is an important goal. Over the past 15 years, T-regulatory (Treg) cells have been recognized as a therapeutic target. Tregs express the transcription factor Forkhead box P3 (Foxp3) and are capable of restricting undesired immune responses against self-antigens, allergens, and commensal bacteria [3]. Tregs can be expanded *ex-vivo* and administered to patients, for example in the context of graft-versus-host disease [4]. However, this treatment approach is limited by Foxp3^{+} Treg instability once the expanded Tregs are adoptively transferred [5]. Pharmacologic interventions that favor stabilizing the Foxp3^{+} Treg phenotype and function *in vivo* are an unmet need, that could either supplement or substitute for adoptive Treg cell therapies. Foxp3 protein is post-translationally regulated by lysine acetylation through histone/protein deacetylases (HDACs) and histone acetyl transferases (HATs) [6]. Global HDAC inhibition [7-9] or deletion of HDAC6, 9, 11 and Sirtuin-1 [10-16] augments Treg function, whereas deleting HDAC3 or 5, Sirtuin-3, or targeting the HATs p300 or CBP impairs Treg [17-21].

Sirtuins are class III HDACs, and stand out from the other HDAC families by being dependent on nicotinamide adenine dinucleotide (NAD) as a co-factor (Figure 1). Sirtuins are highly conserved across eukaryotic species [22, 23]. Initial studies on Sirtuin-1 (Sirt1) in T cells utilized global Sirt1 deletion, and found that complete loss of Sirt1 led to profound autoimmunity [24]. To avoid confounding effects from Sirt1 deletion on thymic development, where Sirt1 plays a role on self-tolerance [25], we mated Sirt1$^{fl/fl}$ with CD4^{cre} and Foxp3^{cre} mice, generating mouse models bypassing the severe autoimmunity of global Sirt1 deletion, as well as using Sirt1 specific small molecule inhibitors [12]. We observed that Sirt1 deletion and pharmacologic inhibition augmented Foxp3^{+} Treg function [12-15]. In this review, we will discuss the role of Sirtuin-1 in the immune system and its mechanism of action. We will also address the practicality of therapeutically targeting Sirt1 for regulation of deleterious immune responses.

Immunosuppressive effects from Sirt1 inhibition in T cells

Foxp3 protein expression is regulated not only by gene transcription and translation, but also heavily depends upon post-translational modifications, including acetylation, phosphorylation, ubiquitination, and poly(ADP)ribosylation [7, 26-31]. Sirt1 has a key role in binding and deacetylating Foxp3, which increases the rate of Foxp3 turnover since acetylated, but not deacetylated Foxp3 is resistant to K48 poly-ubiquitination [32, 33]. Three lysine acetylation sites in murine Foxp3 are susceptible to Sirt1 mediated deacetylation, K31, K262 and K267 [34]. Foxp3 acetylation is not just important for the protein stability of Foxp3, but also for its ability to bind to DNA and exert transcriptional control [6, 9]. In addition to the post-translational control of Foxp3, deletion or pharmacologic inhibition with EX-527, a Sirt1 inhibitor, leads to increased Foxp3 mRNA production [12, 13, 15]. Foxp3 mRNA transcription from Sirt1 targeting could be the result of chromatin accessibility

through histone acetylation, or Sirt1 affecting other regulators of Foxp3 gene expression. One such regulator is p65 (RelA), which is deacetylated by Sirt1 at lysine 310 (Figure 2) [35]. In our studies, we did not observe differences in chromatin accessibility with Sirt1 deletion but did find increased p65 K310 acetylation [12] as well as p65 nuclear translocation [13], consistent with the expected effects of Sirt1 deletion [35]. Although p65 often acts as a pro-inflammatory transcription factor, it can enhance Foxp3 gene expression together with c-Rel (like p65, a nuclear factor-κB (NF-κB) co-factor) and promote the formation of a Foxp3-specific enhanceosome, which increases Foxp3 gene expression (Figure 2) [36, 37]. Taken together, genetic deletion or pharmacologic inhibition of Sirt1 through EX-527 improves Foxp3^{+} Treg quantity and function through increased Foxp3 mRNA transcription and Foxp3 acetylation, which leads to decreased Foxp3 turnover from ubiquitination and poly(ADP)ribosylation, and increases the transcriptional efficiency of Foxp3. As a result, targeting Sirt1 increases both thymic and induced Foxp3^{+} Treg numbers and their suppressive functions.

In contrast to Treg, conventional T cells seem less Sirt1-dependent. While T cells from mice with germline Sirt1 deletion exhibit a phenotype of severe autoimmunity and co-stimulation independent activator protein-1 signaling [24], global Sirt1 deletion is detrimental to many different cell types causing a multitude of diseases, and only some strains are viable at all. For example, Sirt1 is known to control Aire, a key transcription factor in thymic T cell selection [25], which could explain the autoimmune phenotype seen in mice with global Sirt1 deletion. To assess the effects of Sirt1 deletion on CD4^{+} and CD8^{+} T cells independent of the effects of global Sirt1 deletion on thymic development, we used a CD4cre conditional Sirt1 knockout model [12]. We did not observe differences in effector and cytotoxic T cell function *in vitro*, or *in vivo*. Adoptive transfer of Sirt1$^{fl/fl}$CD4^{cre} conventional T cells in a graft-vs-host disease parent-to-F1 model did not show any differences in T cell proliferation, viability, or cytokine production [12]. However, Sirt1$^{fl/fl}$CD4^{cre} conventional T cells, when adoptively transferred to Rag1$^{-/-}$ recipients, did not induce colitis, and formed more Foxp3^{+} iTreg *in vivo* [15], matching the earlier observations by Kwon et al of increased Foxp3^{+} iTreg formation *in vitro* [34].

In addition to the beneficial effects of Sirt1 targeting on Foxp3^{+} iTreg formation, Lim et al. observed that Sirt1 can bind and deacetylate thymic retinoid acid receptor related orphan receptor gamma (RORγt, Figure 2) [38]. In contrast to Foxp3, acetylated RORγt has weaker transcriptional activity, leading to less IL-17 induction and IL-2 repression, and thus weakening the Th17 phenotype [38]. We later confirmed the observations by Lim et al. showing decreased Th17 polarization phenotype in Sirt1$^{fl/fl}$CD4^{cre} conventional CD4^{+} T cells [14]. That said, Sirt1 also has been observed to interfere with Th17 development via deacetylation of signal transducer and activator of transcription (STAT)-3, which is required for RORγt transcription [39]. A number of studies have indicated, that augmenting rather than inhibiting Sirt1 can impair Th17 development, especially in the context of autoimmune uveitis [40-42]. In conclusion, Foxp3, p65 and RORγt are T cell transcription factors in which Sirt1 targeting leads to net immunosuppressive effects in T cells, while STAT3 can augment Th17 development (Figure 2).

Anti-inflammatory effects from Sirt1 augmentation

The immunosuppressive effects of Sirt1 deletion in T cells indicate that Sirt1 inhibition could be a promising option for immunosuppressive therapy. While this is the case with the Sirt1 inhibitor EX-527 extending allograft survival and alleviating colitis [12-15], there are a number of studies in which Sirt1 deletion or inhibition led to pro-inflammatory changes [43, 44], and in which Sirt1 activators like resveratrol or SRT1720 act in an anti-inflammatory fashion [45, 46]. Beyond the above mentioned ambivalent role of Sirt1 increasing Th17 differentiation [39-42], a common denominator of many inflammatory conditions is the prominent role of Sirt1 in myeloid cells.

As noted above, Sirt1 binds and deacetylates the p65 subunit of NF-κB (Figure 2). Schug et al. observed that deletion of Sirt1 in myeloid cells results in hyperacetylated NF-κB, causing increased transcriptional activation of genes that promote an inflammatory response [44]. This occurs through lysine K310, the same residue found to be more acetylated in Sirt1-deficeint T cells [12]. However, unlike in Treg cells, in myeloid cells, p65 K310 acetylation translates into the transcription of inflammatory response mRNAs controlled by NF-κB [47]. Thus, loss of Sirt1 in macrophages induces production of NF-κB-dependent pro-inflammatory cytokines such as TNF-α and IL-1β [43]. As a result, Sirt1's ability to target acetylation of NF-κB allows regulation of the inflammatory response, and Sirt1 inhibition can lead to chronic inflammation through its direct deacetylation of p65 [48].

Beyond p65, c-Jun (a member of the activator protein-1 complex) is an additional Sirt1 target relevant to myeloid cells. Sirt1 deacetylation of the c-Jun can reduce inflammation in peritoneal macrophages by decreasing cyclooxygenase-2 (COX2) mRNA expression and prostaglandin E_2 production [49]. Decreased prostaglandin E_2 production attenuates macrophage and tumoricidal functions, in another example of the anti-inflammatory effects of Sirt1 in myeloid cells.

Sirt1 and cellular metabolism

In contrast to the effects of Sirt on Foxp3, p65, Parp1, and p53, the key metabolic regulators transcription factor PGC1α (peroxisome proliferators-activated receptor-γ coactivator 1α) and FoxO1 are activated and stabilized by Sirt1-mediated deacetylation (Figure 1) [50, 51], whereas their acetylation by the HATs, GCN5 and p300 respectively [50, 51], impairs their transcriptional activity [52]. For these reasons, Sirt1 mediated deacetylation has marked effects on cellular metabolism and mitochondrial biogenesis.

$Foxp3^+$ Treg rely on oxidative phosphorylation for their energy production [53, 54]. Perhaps unsurprisingly, $Foxp3^+$ Treg deficient in PGC1α or FoxO1 show weaker suppressive function [19, 55] and phenotypic stability [56, 57]. Given the role of Sirt1 deacetylation in enhancing PGC1α and FoxO1, we had expected that Sirtuin-1 knockout Treg would have dysfunctional mitochondria and impaired oxidative phosphorylation. However, this was not observed [19, 58], leading us to speculate that the effect of absent Sirt1 on transcription factors such as Foxp3 [58-60] may overrule the potential impairment of mitochondrial biogenesis and function resulting from PGC1α acetylation. HATs that acetylate and

inactivate PGC1α (GCN5) and FoxO1 (p300) both also acetylate (and activate) Foxp3, mirroring the role of Sirt1, with deletion of either GCN5 or p300 impairing $Foxp3^+$ Treg [20, 61].

An additional metabolic (and immunologic) effect of Sirt1 targeting can arise from NAD^+ availability. Sirt1 consumes NAD^+ during its deacetylation reaction (Figure 1). Mice deficient in the enzyme PARP-1, which also utilizes NAD, have increased Sirt1 activation, improved metabolism and are leaner [62]. Hence, loss of Sirt1 activity may affect the NAD^+ pool or other NAD consuming enzymes, which can have its own effects on the cellular redox state and immune function [30, 58, 63].

Sirt1 in other organ systems

Sirt1 is an important regulator of cellular metabolism, autophagy and chromatin accessibility, and is associated with a wide variety of pathophysiologic processes, including longevity, aging, obesity, heart disease, and cancer [64, 65]. The effects of targeting Sirt1 are thus very widely spread and hard to accurately predict. For example, with regard to Sirt1 and cancer, Sirt1 can both suppress or favor the formation of tumors, depending upon its targets in individual signaling pathways or in specific cancers, such as p53, p65, or metabolic and autophagy regulatory circuits [66]. This is especially relevant when Sirt1 inhibition is considered. For example, when using the Sirt1 inhibitor EX-527 in MHC-mismatched murine renal allografts, we noted that prolongation of allograft survival and function was present, though to a lesser extent than in mice with conditional deletion of Sirt in their T cells ($Sirt1^{fl/fl}CD4^{cre}$), and was worsened upon increasing the dose of EX-527 from 1 to 10 mg/kg/d [14]. In addition to pro-inflammatory effects from innate immune cells [44, 67], Sirt1 can aid protecting from ischemic injury [68, 69], which is practically unavoidable during transplantation, and thus, the immunosuppressive treatment with EX-527 may augment such injury. Furthermore, Sirt1 has been shown to alleviate lipopolysaccharide-induced injury, and mitigate oxidative stress arising in diabetic nephropathy [70-72], all of which would be common exposures in kidney transplant recipients. These broad side effects make Sirt1 targeting for immunosuppression less practical [6].

The role of other sirtuins in in the immune system

In addition to Sirt1, other class III histone/protein deacetylases (Sirt2-7) have effects on immune responses. While all sirtuins share the NAD-dependent deacetylation reaction (Figure 1), each of these sirtuins has individual binding partners, and different subcellular localizations that affect their role in the immune system. Sirt1, Sirt6, and Sirt7 are predominantly nuclear, Sirt2 is present in the cytosol, while Sirt3-5 are located in the mitochondria. Sirt2, like Sirt1, regulates p65 K310 acetylation [73]. Sirt2 did not have any notable effect on T-effector or Treg cell functions *in vitro* [6], perhaps because of its cytosolic localization. In myeloid cells, Sirt2 deletion improved bacterial phagocytosis and augmented host responses against staphylococcal infections [74]. On the flip side, $Sirt2^{-/-}$ mice had increased susceptibility to dextran sodium sulfate induced colitis [75]. Sirt3 deletion did not have any overt effect on myeloid and lymphocyte development [19, 76], however, $Sirt3^{-/-}$ Treg function was noted to be impaired [19]. The role of Sirt4 in the

immune system is relatively unknown, through Sirt4 has been suggested to be important to recover monocyte function during sepsis [77]. Sirt5 has an interesting effect on inflammation by competing with Sirt2 and blocking deacetylation of p65 at K310, the same amino acid targeted by Sirt1, in turn causing cytokine production and inducing an inflammatory response *in vivo* [78]. In fact, Sirt5 had notably opposing expression levels and effects when compared to Sirt1 and Sirt2 on inflammation arising from peritoneal macrophages [78]. While reduction of Sirt1/2 expression increased IL-6 mRNA expression, Sirt5 targeting also downregulated IL-6 mRNA. Accordingly, $Sirt5^{-/-}$ mice show a reduced inflammatory cytokine response during sepsis [78]. Sirt6 is important for TNF-α production [79] and dendritic cell function [80]. $Sirt7^{-/-}$ mice show a shortened lifespan and due to cardiac failure, cardiac myocyte apoptosis mediated by p53 hyperacetylation, with myocardial infiltrates of granulocytes and T cells [81]. In addition to cardiac myocyte apoptosis, these findings also raised the question if Sirt7 may have immunoregulatory functions. However, Burg et al. did not observe Sirt7 to affect outcomes in experimental autoimmune encephalomyelitis aside from a minor reduction in $CD8^+$ T cell IFN-γ production [82].

Concluding Remarks

Pharmacologic Sirt1 inhibition can produce immunosuppressive effects, but these are context dependent and vary upon the cells type(s) involved in the immune response that is being targeted. This is in addition to other non-immune effects of Sirt1 inhibition. Together, these limitations make Sirt1, in contrast to other HDACs, such as HDAC6, less suitable as a pharmacologic target to achieve immunosuppression.

Acknowledgments

Funding

Supported by grants from the National Institutes of Health to U.H.B. (AI095353), W.W.H. (AI073489 and AI095276), as well as the Laffey McHugh foundation and American Society of Nephrology (to U.H.B.).

Abbreviations

COX	Cyclooxygenase
Foxp3	Forkhead box P3
NAD	Nicotinamide adenine dinucleotide
HAT	Histone/protein acetyltransferase
HDAC	Histone/protein deacetylase
NF-κB	Nuclear factor-κB
PGC1α	Peroxisome proliferators-activated receptor-γ coactivator 1α
RORγt	Thymic retinoid acid receptor related orphan receptor gamma
Sirt	Sirtuin

STAT	signal transducer and activator of transcription
Treg	Regulatory T cells

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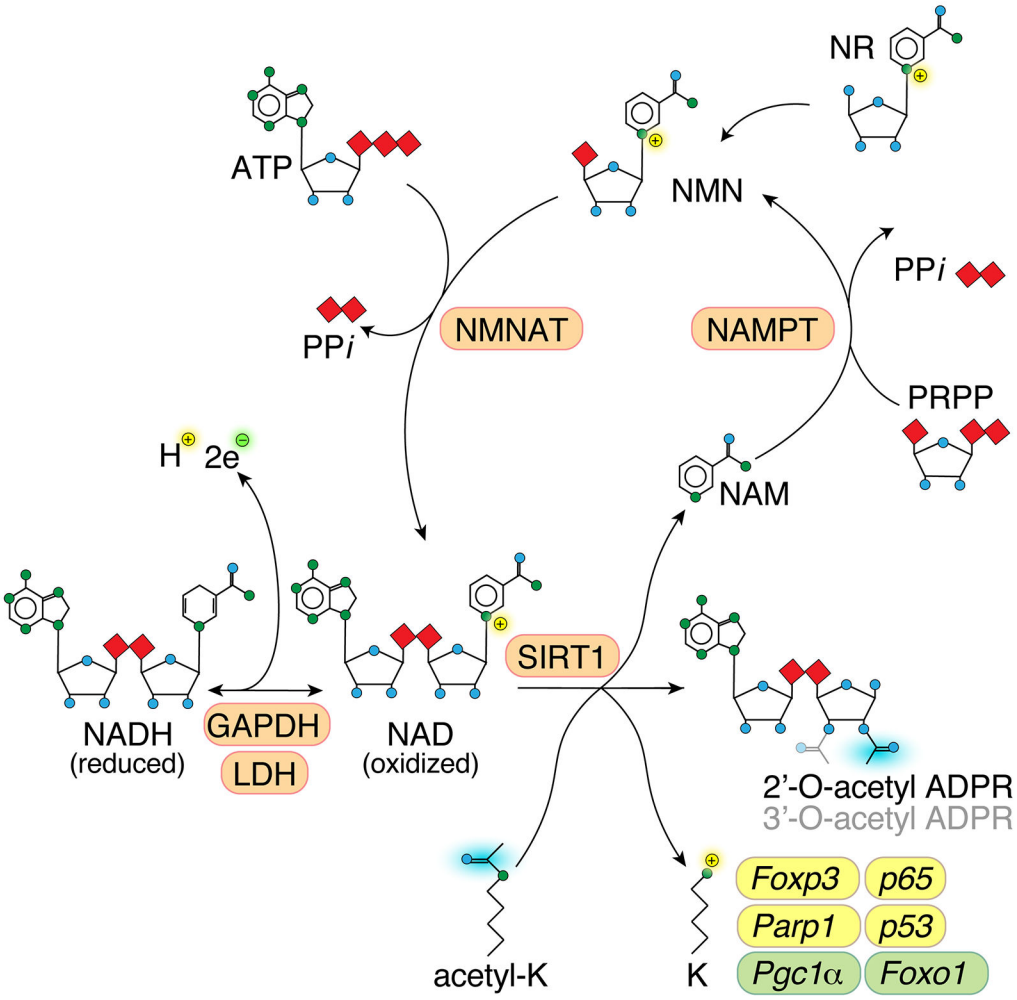

Figure 1: Sirtuin-1 reaction and NAD recycling

Schematic showing Sirt1 reaction and NAD recycling. Sirt1 deacetylates ε-amino groups of lysine side chains in histones and many non-histone proteins, creating a positive charge. The acetylgroup is transferred to NAD, which has nicotinamide cleaved off, generating 2'- or 3'-O-acetyl ADP-ribose [23]. Sirt1 can use oxidized NAD, but not reduced NADH as co-factor. Nicotinamide is recycled to NAD through NAMPT and NMNAT. Several Sirt1 client proteins relevant to the immune system are indicated, which are either activated and stabilized (yellow) or destabilized (green) when Sirt1-dependent deacetylation is impaired. Abbreviations: NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; ATP, adenosine triphosphate; ADPR, adenosine diphosphate riboside; PRPP, phosphoribosyl pyrophosphate; PP*i*, inorganic pyrophosphate; K, lysine; NAMPT, Nicotinamide phosphoribosyltransferase; NMNAT, nicotinamide mononucleotide adenylyltransferase.

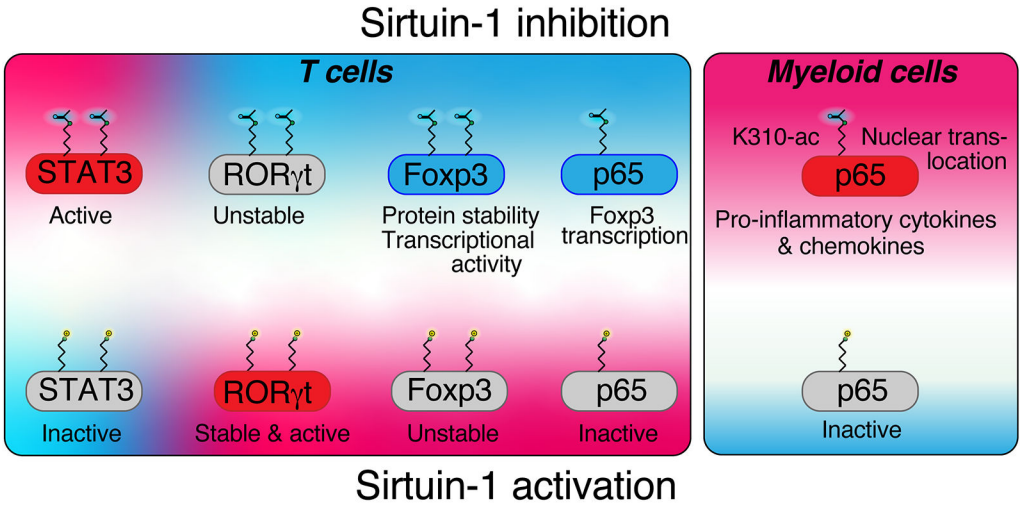

Figure 2: Sirtuin-1 targeting in T cells and myeloid cells

Sirtuin-1 targeting has contrasting effects in myeloid and in T cells: In myeloid cells, p65 acetylation at lysine 310 leads to increased transcription of pro-inflammatory cytokines. In T cells, Foxp3 is stabilized by Sirtuin-1 inhibition due to increased lysine acetylation, which increases transcriptional activity and reduces proteasomal turnover. Foxp3 transcription is augmented by p65. In contrast to Foxp3, Rorγt is stabilized by Sirtuin-1 deacetylation, and thus, impaired with Sirtuin-1 inhibition, which aids in tilting the balance between pro-inflammatory Th17 and suppressive Treg cells. STAT3 can also be deacetylated (and destabilized) by Sirt1, which may explain that in some models, especially autoimmune uveitis, Sirt1 activation can suppress Th17 differentiation.