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For Antigen-Specific Effector or Foxp3 + Regulatory T Cell Fate, Cyclin-Dependent Kinases Hold the Trump Card

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18 Forkhead box p3⁺ (Foxp3⁺) regulatory T cells (Tregs) are indispensable for the immune
19 homeostasis and for maintaining the immune tolerance. This has been confirmed in several
20 studies that injection of T cells depleted of Tregs causes autoimmunity, rejection of grafts and
21 inflammatory disorders, whereas reconstitution with Tregs inhibits these afore-mentioned
22 pathogenic processes. Over the last two decades, intense efforts have been made to identify
23 subsets of Tregs, Treg differentiation process, and the molecular signatures and regulators that
24 determine Treg lineage specificity and stability.¹⁻⁶

25 Several lines of evidences clearly demonstrate that Foxp3⁺ Tregs are not homogenous
26 population and several subsets of Tregs like thymic Tregs (tTregs), *in vitro* generated Tregs
27 (iTregs), and peripherally induced Tregs (pTregs)) have been identified.⁵ In addition to Foxp3,
28 epigenetic factors and metabolic processes play a key role in maintaining Treg identity, function
29 and switching between effector T cells and Tregs.⁶⁻⁸ In fact, Tregs require phosphatase and tensin
30 homolog (PTEN) for their stability and for the maintenance of metabolic balance between
31 glycolysis and mitochondrial functions.⁹ A recent report advocates that mitochondrial complex
32 III is indispensable for the suppressive function of Tregs as loss of complex III in Tregs triggered
33 enhanced levels of metabolites 2-hydroxyglutarate (2-HG) and succinate that blocked the ten-
34 eleven translocation (TET) family of DNA demethylases resulting in DNA hypermethylation and
35 repression of several transcripts required for Treg functions.^{10,11}

36

37 The iTreg generation is also governed by several factors. Inhibition of C3aR
38 (complement component 3a receptor) and C5aR (complement component 5a receptor)-mediated
39 signaling in CD4⁺ T cells has been reported to reduce PI3K-Akt-mTOR pathway with a
40 concomittant enhancement in autocrine TGF- β signaling to promote Foxp3⁺ iTreg

41 generation.¹² A metabolic regulator hypoxia-inducible factor 1 (HIF-1) controls the balance
42 between effector Th17 cells and Tregs. Glutamine transamination, mainly catalysed by glutamic-
43 oxaloacetic transaminase 1, determines the fate of CD4⁺ T cell differentiation towards Th17 at
44 the expense of iTregs. Increased transamination leads to abundance of 2-HG that promotes
45 hypermethylation of Foxp3 gene locus and inhibition of its transcription.¹³

46 A recent study by Prof. Shimon Sakaguchi group aimed at identifying the molecular
47 regulator that controls the generation of antigen-specific Tregs.¹⁴ They reported that cyclin-
48 dependent kinase (CDK)8 and CDK19 (a homolog of CDK8 with 91% sequence homology) are
49 the key factors that control switching between antigen-specific effector and Foxp3⁺ Treg
50 polarization.¹⁴ CDK8 and CDK19 are collectively called as Mediator kinases. CDK8 in
51 association with cyclin C (CCNC), Mediator complex subunit 12 (MED12), and MED13 forms a
52 part of the mediator complex. These kinases in association with Mediator complex are implicated
53 in several cellular processes like transcription, cell signaling, metabolism and immunity to
54 infection (especially antiviral immunity). Contradictory and context dependent roles of CDK8/19
55 have been observed in cancer.^{15,16} During the innate immune responses, CDK8/19 consort with
56 NF- κ B to regulate respective inflammatory gene expression. Due to involvement of NF- κ B in the
57 inflammatory disorders, inhibition of associated factors (CDK8/19) represents an attractive
58 strategy to target inflammatory processes.¹⁷

59 Until now, many clinical studies have explored the therapeutic use of polyclonal Treg
60 therapy in autoimmune and inflammatory diseases. Clinical studies and experimental models
61 have also considered alternative strategy of expanding Tregs via IL-2-based therapies (low dose
62 IL-2 or specific targeting of IL-2 by monoclonal antibody), TGF- β , rapamycin, retinoic acid,
63 (aminooxy)acetic acid.^{13,18-20} Interestingly, Akamatsu et al. identified a novel molecule

64 AS2863619 (AS) that converts the naïve and effector/memory antigen-specific CD4⁺ and CD8⁺
65 T cells into Foxp3⁺ Tregs *in vitro* by an IL-2-dependent but TGF- β -independent mechanism
66 (Figure 1).¹⁴ However, AS-induced Tregs showed characteristics similar to that of TGF- β -
67 induced Tregs. Like in the case of TGF- β -induced Tregs, AS-induced Tregs lacked Treg-specific
68 DNA hypomethylation. Surprisingly, AS-transformed Tregs were not negatively regulated by the
69 inflammatory cytokines like IL-12 and IL-6.¹⁴ These data together provided an indicator that AS-
70 generated Tregs might have a antigen-specific beneficial effect in autoimmune diseases.

71 Mass spectrometry analyses of mouse T lymphoma cells with or without treatment of AS
72 identified CDK8 and CDK19 as the target proteins for AS.¹⁴ In addition, inhibition of CDK8
73 and CDK19 was correlated with the enhanced Foxp3 expression. Further, RNA interference
74 studies and retroviral overexpression studies confirmed the repressive role of CDK8/19 on
75 Foxp3⁺ T cells. CDK8/19 inactivates the signal transducer and activator of transcription 5
76 (STAT5) by phosphorylating serine at PSP (pro-ser-pro) motif, thereby hindering the Foxp3
77 expression. Immunoprecipitation studies to identify the downstream signaling confirmed that AS
78 suppresses STAT5 serine phosphorylation while promoting the C-terminal domain tyrosine
79 phosphorylation. Data from whole-genome chromatin immunoprecipitation sequencing revealed
80 that AS enhances the binding of STAT5 mainly to the CNS0 region of Foxp3 and to a minor
81 extent Foxp3 promoter and CNS2 region to induce Foxp3⁺ Tregs.¹⁴ Notably, AS enhanced the
82 expression of several genes that are critical for Treg functions including *Il2ra*, *Tnfrsf18*, *Foxo1*,
83 *Ccr4*, and *Icos* (Figure 1).

84 Could AS induce Foxp3 in the antigen specific T cells *in vivo* ? To explore this authors
85 used DO11.10 TCR transgenic mice on the RAG2-deficient background.¹⁴ These mice lack
86 tTregs and hence pTreg generation following immunization with model antigen (ovalbumin;

87 OVA) could be monitored. In line with *in vitro* results, authors found that AS induced Foxp3⁺ T
88 cells when T cell receptor (TCR) is activated by the antigen interaction, but not naïve T cells.
89 Based on the expression of killer cell lectin like receptor G1 (KLRG1) and Treg-specific DNA
90 demethylation, authors confirmed the complete differentiation of Tregs induced by AS.¹⁴

91 Preclinical studies in mouse models revealed the therapeutic effect of AS in allergy and
92 autoimmune disease. AS treatment in the aforementioned mouse models reduced the skin
93 hypersensitivity reactions, OVA-induced delayed-type hypersensitivity reaction, spontaneous
94 diabetes, and experimental allergic encephalomyelitis by increasing the Foxp3⁺ T and KLRG1⁺
95 cells and reciprocally reducing the effector T cell populations.¹⁴ However, AS-induced pTregs
96 were relatively less activated as compared to tTregs as assessed by the expression pattern of
97 CD25, Glucocorticoid-Induced tumor necrosis factor receptor-related (GITR), Cytotoxic T-
98 lymphocyte antigen-4 (CTLA-4). All these data together suggested that AS could suppress both
99 acute and chronic immune responses by promoting the generation of antigen-specific pTregs.

100 This study thus identifies a novel molecular regulator that determines the balance
101 between antigen-specific effector T cells and Foxp3⁺ Tregs. Further, authors have also identified
102 a small molecule (AS) that could promote the generation of antigen-specific pTregs and protect
103 the mice from allergic and autoimmune diseases. Devoid of toxicity is one the great virtues of
104 this small molecule though CDK8/19 inhibitors have shown unexpected toxicity in other animal
105 species (rats and dogs).^{15,21} This work also attracts the focus of chemists to prepare structural
106 mimics and analogs. Further work is necessary regarding the relative stability of AS-induced
107 pTregs *in vivo*. Whether long-term (chronic) inflammatory microenvironment affects the efficacy
108 of AS-induced Tregs need to be determined.^{22,23}

109 Since targeting CDK8/19 by AS induces antigen-specific pTregs, possible systemic
110 immunosuppression by the polyclonal Treg therapy could be avoided. If successful, therapeutic
111 use of AS would be more economical than conventional polyclonal Treg therapy. However,
112 caution should be exercised as depending on the availability of antigens, AS could also convert
113 effector T cells specific for the pathogens, tumors or vaccine antigens into pTregs and as a
114 consequence might reduce protective immune responses.

115

116 **CONFLICT OF INTEREST**

117 Authors declare no conflict of interests.

118

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122

123 **AUTHOR CONTRIBUTIONS**

124 SRB and JB performed literature search, analyses and drafted the manuscript

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Figure Legend

Figure 1. Cyclin-dependent kinases check the switching of antigen-specific effector T cells into Foxp3+ regulatory T cells. TCR signaling induces cyclin-dependent kinase 8 and its paralog 19 (CDK8/19). CDK8/19 inactivates the IL-2 induced signal transducer and activator of transcription 5 (STAT5) by phosphorylating serine at PSP (pro-ser-pro) motif, thereby hindering the Foxp3 expression. In contrast, inhibition of CDK8/19 by AS2863619 (4-[1-(2-methyl-1H-benzimidazol-5-yl)-1H-imidazo[4,5-c]pyridin-2-yl]-1,2,5-oxadiazol-3-amine dihydrochloride) converts the antigen-specific effector/memory T cells into Foxp3+ regulatory T cells. Mechanistically, AS2863619 keeps the active state of STAT5 (phosphorylation at tyrosine in the C-terminal (C-ter) domain), which binds to regulatory regions of the Foxp3 locus majorily at conserved non-coding sequence (CNS)0, and to a lesser extent to Foxp3 promoter and CNS2 region to induce *Foxp3* and enhanced expression of numerous genes critical for the Treg functions (*Il2ra*, *Tnfrsf18*, *Foxo1*, *Ccr4*, and *Icos*). Although not observed under *in vitro* conditions, *in vivo* AS2863619-induced Tregs also displayed stable Treg-specific demethylation features at *Foxp3* and *Helios* gene loci. Moreover, AS treatment rendered protective effects in the mouse models of allergy and autoimmune diseases by increasing the Foxp3+ T cells and reciprocally reducing the effector T cell populations.

