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Akihiko Yoshimura *Editor*

Emerging Concepts Targeting Immune Checkpoints in Cancer and Autoimmunity

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Responsible series editor: Tasuku Honjo

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Editor
Akihiko Yoshimura
Department of Microbiology and
Immunology
Keio University School of Medicine
Tokyo
Japan

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Preface

The homeostasis of the immune system is maintained by positive and negative factors including effector/regulatory cells as well as positive intracellular signals/negative regulators. Autoimmune diseases and allergic diseases are states in which this balance inclines to the excessive "positive." In contrast, tumor microenvironment provides negative signals for immune systems, which suppress anti-tumor immunity. Those include regulatory T cells (Tregs) and myeloid-derived suppressor cell (MDSC) as well as immune regulatory molecules such as PD-L1, indoleamine 2,3-dioxygenase (IDO), TGF-β. Autoimmunity and anti-tumor immunity are two sides of the same coin. Indeed, knockout mice of various molecules so-called "immune checkpoints" often develop autoimmune diseases. Thus, the understanding of the mechanism of immunological balance is important for the treatment of both autoimmune diseases and cancer.

Typical positive and negative T cells of immune balance are effector T cells and regulatory T cells (Tregs), respectively. Major Treg cells developed in the thymus are called thymus-derived Treg (tTreg) cells. Treg cells are specified by an expression of the transcription factor Forkhead box P3 (Foxp3), which plays crucial roles in the differentiation, maintenance, and function of tTreg cells. Treg cells are believed to be involved in autoimmune diseases and allergy because Treg cells suppress excess immunity against a diverse range of antigens, including self-antigens, commensal bacteria-derived antigens, and environmental allergens. Tregs have been shown to be abundant in tumor tissues and suppress anti-tumor immunity.

In recent years, anti-tumor immunity has attracted attention not only by immunologists but also by cancer researchers. T-cell activation is initiated through antigen recognition by the T-cell receptor (TCR) and co-stimulatory signals such as CD28. On the other hand, the inhibitory signals for T-cell activation (i.e., immune checkpoints) are crucial for the maintenance of self-tolerance and prevention of autoimmunity as well as excess immune responses. The two immune checkpoint receptors, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4, also known as CD152) and programmed cell death protein 1 (PD1, also known as CD279), have been most actively studied in the context of clinical cancer immunotherapy. It has

been shown that PD1 recruits the tyrosine phosphatase, which inhibits TCR signaling, while CTLA4 inhibits CD28-mediated co-stimulatory signals. Antibodies against CTLA-4 and PD-1 have been shown to significantly improve survival in patients with metastatic cutaneous melanoma and other cancers. The action of PD-1 and CTLA-4 is now called "immune checkpoints," since these molecules are involved in T-cell exhaustion and anergy. Clinical efficacies of these antibodies proved that anti-tumor immunity can be enhanced by inhibiting immune checkpoints. However, PD-1 and CTLA4 are not the only molecules that negatively regulate T-cell activation. There are a number of cells and signals that suppress effector T-cell activation.

In addition to TCR and co-stimulatory signals, T-cell activation requires the third signal: signals from the cytokine receptors. For example, IL-2 is necessary for the proliferation of T cells, and IL-12 and IFNγ are important for Th1 differentiation and CTL activation. Various roles of IFNγ in anti-tumor immunity have been established. IL-15 has been shown to be necessary for memory T-cell survival. Thus, negative regulators of the cytokine signaling must be important immune checkpoint molecules that regulate anti-tumor immunity. The suppressors of cytokine signaling (SOCS) family proteins have been shown to negatively regulate cytokine signaling by binding to the receptors and/or JAK tyrosine kinases. Suppression of SOCS1 has been shown to cause autoimmunity and enhance anti-tumor immunity. Therefore, such negative regulators of cytokine signaling can be considered as the third immune checkpoint molecules.

Now we can extend the concept of immune checkpoints to "molecules and cells which negatively regulate T-cell activation." These molecules and cells must be involved in immune homeostasis and could be new targets of autoimmune diseases and cancer immunotherapy. This book is focusing on molecular and cellular biology of "extracellular" and "intracellular" immune checkpoint regulators. Such factors are regulatory T cells and tolerogenic dendritic cells, as well as signal inhibitors such as SOCS, tyrosine phosphatases, ubiquitin ligases, and miRNAs. I hope this CTMI volume promotes the understanding and application of "extended" immune checkpoint regulators.

Tokyo, Japan Akihiko Yoshimura

Contents

Part I
Immune Checkpoint Cells

Regulatory T Cells: Molecular and Cellular Basis for Immunoregulation

Yosuke Togashi and Hiroyoshi Nishikawa

Abstract $CD4^+$ regulatory T cells (Tregs) are a highly immune-suppressive subset of $CD4^+$ T cells, characterized by expression of the master regulatory transcription factor FOXP3. Tregs are proven to play central roles in the maintenance of self-tolerance in healthy individuals. Tregs are involved in maintaining immune homeostasis: they protect hosts from developing autoimmune diseases and allergy, whereas in malignancies, they promote tumor progression by suppressing anti-tumor immunity. Elucidating factors influencing Treg homeostasis and function have important implications for understanding disease pathogenesis and identifying therapeutic opportunities. Thus, the manipulating Tregs for up- or down-regulation of their suppressive function is a new therapeutic strategy for treating various diseases including autoimmune disorders and cancer. This review will focus on recent advances in how Tregs integrate extracellular and intracellular signals to control their survival and stability. Deeper mechanistic understanding of disease-specific Treg development, maintenance, and function could make disease-specific Treg-targeted therapy more effective, resulting in an increase of efficacy and decrease of side effects related to manipulating Tregs.

Contents

Y. Togashi · H. Nishikawa
Division of Cancer Immunology, Research Institute/EPOC, National Cancer Center, Tokyo, Japan

H. Nishikawa (✉)
Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Japan
e-mail: hnishika@east.ncc.go.jp

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1 Introduction

$CD4^+$ regulatory T cells (Tregs) are a highly immune-suppressive subset of $CD4^+$ T cells, characterized by expression of the master regulatory transcription factor FOXP3 (Sakaguchi et al. 1995; Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). Tregs were originally identified as $CD4^+CD25^+$ T cells by Sakaguchi et al. (1995) and are proven to play central roles in the maintenance of self-tolerance in healthy individuals (Sakaguchi et al. 2010; Wing and Sakaguchi 2010). Mutations of the human FOXP3 result in impaired development or dysfunction of Tregs and, consequently, the occurrence of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome accompanying severe autoimmune diseases, inflammatory bowel disease, and allergy (IPEX syndrome) (Bennett et al. 2001). Likewise, mice that carry a mutation or genetic deletion of FOXP3, called Scurfy mice are deficient in Tregs and develop fatal systemic autoimmunity (Brunkow et al. 2001; Fontenot et al. 2003). In addition, forced expression of FOXP3 is able to confer Treg-like suppressive activity on naive conventional T cells (Tconvs) (Fontenot et al. 2003; Hori et al. 2003). FOXP3 has therefore been considered as a lineage-specifying transcription factor of Tregs or a master regulator of its functions.

Tregs are involved in maintaining immune homeostasis: they protect hosts from developing autoimmune diseases and allergy, whereas in malignancies, they promote tumor progression by suppressing anti-tumor immunity (Onizuka et al. 1999; Shimizu et al. 1999; Sakaguchi et al. 2010; Wing and Sakaguchi 2010). Elucidating factors influencing Treg homeostasis and function have important implications for understanding disease pathogenesis and identifying therapeutic opportunities. Thus, manipulating Tregs for up- or down-regulation of their suppressive function is a new therapeutic strategy for treating various diseases including autoimmune disorders and cancer. This review will focus on recent advances in how Tregs integrate extracellular and intracellular signals to control their survival and stability. We will discuss how these new insights can be utilized for the development of new approaches to promote and stabilize Tregs in many illnesses.

2 Development and Maintenance of Tregs

Tregs are separated into natural/thymic and peripheral/induced Tregs based on the sites where they are generated (Sakaguchi et al. 2010, Adeegbe and Nishikawa 2013). $FOXP3^+$ natural Tregs are generated in the thymus as an antigen-primed and functionally mature T cell subpopulation specialized for immune suppression (natural/thymic Tregs; nTregs). Some of $FOXP3^+$ Tregs also differentiate from Tconvs in the periphery under certain conditions (peripheral/induced Tregs; iTregs). The main task of $FOXP3^+$ nTregs is to migrate to inflammatory sites and suppress various effector lymphocytes, especially helper T (Th) cell subsets and $CD8^+$ cytotoxic T cells (Chaudhry et al. 2009; Koch et al. 2009; Chung et al. 2011; Linterman et al. 2011). nTregs reportedly express high levels of Helios (a member of the Ikaros transcription factor family) and Neuropilin-1 (a type-1 transmembrane protein). In contrast, iTregs that develop in the periphery often lack or have a low-level expression of these molecules. According to data from animal models, these iTregs are readily converted from Tconvs by in vitro stimulation with TGF-β or retinoic acid (Coombes et al. 2007). However, in humans, $FOXP3^+$ T cells induced from Tconvs by in vitro TCR stimulation with TGF-β fail to gain suppressive function and rather produce pro-inflammatory cytokines (Walker et al. 2005; Tran et al. 2007). At present, the function of iTregs such as TGF-β-induced ones in humans is obscure though there are some reports showing that several cytokines or a specific microbiota environment can induce Tregs with a suppressive function from $CD4^+CD25^-$ T cells (Ellis et al. 2012; Atarashi et al. 2013; Hsu et al. 2015). Yet it remains to be determined whether these peripherally induced $FOXP3^+$ Tregs are functionally stable in vivo.

2.1 TCR, CD28, and IL-2

nTreg development is initiated by TCR signal followed by a sequential activation of CD25 (IL-2 receptor α chain) expression, IL-2 signal, and FOXP3 expression (Lio and Hsieh 2008; Weissler and Caton 2014). Although not fully clarified in humans, nTregs stem from self-reactive thymocytes present in the thymus (Sakaguchi et al. 2010). A fraction of $CD4^+CD8^-$ thymocytes receive T cell receptor (TCR) stimulation by complexes of major histocompatibility complex (MHC) plus self-peptide and acquire expression of CD25, through which IL-2 signals are delivered via STAT5, resulting in expression FOXP3 and differentiation into Tregs (Jordan et al. 2001; Boyman and Sprent 2012; Malchow et al. 2013). nTreg development can be enhanced through the constitutive activation of STAT5 and directly binds cis elements in the FOXP3 promoter and enhancer to stabilize FOXP3 expression (Burchill et al. 2008). In addition to induction of CD25, TCR and CD28 signal also contribute to establishing and stabilizing the Treg lineage commitment in the thymus by inducing epigenetic and differentiation events in Tregs (Salomon et al.

2000; Tai et al. 2013; Zhang et al. 2013; Franckaert et al. 2015). Thus, antigen and IL-2 signal provided through TCR, CD28, and CD25 are essential for Treg lineage commitment in the thymus.

In the periphery, mature Treg survival for their homeostasis and function depends on TCR, CD28, and CD25, but their roles appear to be distinct from those in the thymus. Tregs proliferate more than Tconvs in steady state in a CD28 dependent fashion, suggesting that Tregs continuously recognize cognate antigens driving their cell cycle progression (Tang et al. 2003; Walker et al. 2003). Indeed, analysis of Treg subsets in the periphery shows that continuous stimulation through the TCR is required to maintain this population (Levine et al. 2014; Vahl et al. 2014). TCR-deficient Tregs proliferated less and expressed fewer effector molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), IL-10, and Ebi3.

Proliferating Tregs have a tendency to lose their FOXP3 expression and lineage stability in vitro and in vivo in lymphopenic hosts (Hoffmann et al. 2006; Zhou et al. 2009; Rubtsov et al. 2010). The conserved noncoding sequence 2 (CNS2) enhancer element, also known as Treg-specific demethylation region, is crucial for safeguarding lineage stability of proliferating Tregs (Feng et al. 2014; Li et al. 2014). However, stimulation via TCR with limited IL-2 leads to a loss of FOXP3 expression in Tregs with intact CNS2. CNS2 harbors binding sites for both the TCR-triggered transcription factor nuclear factor of activated T cells (NFAT) and IL-2-induced transcription factor STAT5, providing a transcriptional basis for Treg stability by coordinating TCR and IL-2 signal. Interestingly, forced expression of constitutively active STAT5 prevented the loss of FOXP3 in CNS2-deleted Tregs, demonstrating that STAT5 can stabilize FOXP3 expression independent of CNS2 (Feng et al. 2014). This may be explained by the NFAT-mediated looping between CNS2 and the FOXP3 promoter, also having NFAT and STAT5 binding sites (Li et al. 2014). Together, TCR-mediated signals are important for mature Treg function but pose a threat to their stability unless they are balanced by the IL-2 signal.

2.2 PI3K-Akt-mTOR

Phosphatidylinositide 3 kinase (PI3K), protein kinase B (Akt), and mammalian target of rapamycin (mTOR) form an intracellular signal hub common to the TCR, CD28, and IL-2 receptor. PI3K is directly activated when these receptors are engaged, leading to initial activation of Akt by the PH-domain containing protein PDK1 through phosphorylation of threonine 308. Akt is fully activated by additional phosphorylation on serine 473 by the mTOR complex 2 (mTORC2). Akt has many cellular targets; the Forkhead box O (Foxo) transcription factors and mTORC1 are most relevant to Treg biology. Foxo family transcription factors are crucial for Treg lineage commitment (Harada et al. 2010; Ouyang et al. 2012; Samstein et al. 2012) and are inhibited by Akt. mTORC1 coordinates anabolic

activities in cells and inactivates mTORC2, limiting further Akt activation. In the thymus, Treg development is enhanced by mutating the p110d catalytic subunit of PI3K (Patton et al. 2006) and is repressed by forced expression of a constitutively active Akt (Haxhinasto et al. 2008), demonstrating a negative role of the PI3K axis on nTreg development. However, deletion of mTOR (thus inactivating both mTORC1 and 2) or individual deletion of mTORC1 or 2 in T cells does not alter thymic development (Delgoffe et al. 2009), suggesting that the negative effect of PI3K and Akt on nTreg development is mTOR independent and mainly due to their role in Foxo1 inactivation.

Activation of PI3K is naturally antagonized by phosphatase and tensin homolog (PTEN). PTEN expression is progressively inhibited by stronger TCR stimulation, permitting efficient T cell activation and effector differentiation, an effect mediated by IL-2-inducible T cell kinase (Itk) (Gomez-Rodriguez et al. 2014). Thus, T cells with Itk deficiency fail to down regulate PTEN after activation and favor FOXP3 induction. In committed Tregs, the PI3K-Akt-mTOR signal axis continues to be repressed by high expression of PTEN. Treg-specific deletion of PTEN disrupted Treg homeostasis, function, and stability (Huynh et al. 2015; Shrestha et al. 2015). These PTEN-deficient Tregs lost both FOXP3 and CD25 expression but had a significant increase of mTORC2, but not mTORC1 activities. Additional deletion of mTORC2 in Tregs largely rescues the phenotype in mice with Treg-specific deletion of PTEN, demonstrating the normal function of PTEN in mature Tregs is to keep mTORC2 in check. In fact, the intact mTORC1 function is required for Treg function because mice with selective deletion of mTORC1 in Tregs die of multi-organ autoimmune diseases similar to FOXP3-deficient mice (Zeng et al. 2013). Mechanistically, mTOR is found to control Treg function in part by regulating metabolic programming. T cells rely on mitochondrial oxidative phosphorylation at steady state and switch to glycolysis after activation, a process essential for effector T cell differentiation (Wang and Green 2012). In contrast, Tregs preferentially use oxidative metabolism even after activation. An emerging concept is that metabolic input can also dictate T cell fate decision (Wang and Green 2012). PTEN-deficient Tregs show exaggerated glycolysis that is thought to contribute to Treg instability (Huynh et al. 2015; Shrestha et al. 2015). Additionally, functional defects in mTORC1-deficient Tregs are associated with disrupted lipid biosynthesis (Zeng et al. 2013). Thus, the impact of PI3K-Akt-mTOR axis on mature Treg function is controversial, while excessive activation of this pathway is clearly detrimental to Treg function as observed in PTEN-deficient Tregs; complete blockade of PI3K impairs Treg function as well (Patton et al. 2006; Patton et al. 2011).

2.3 Epigenetics

Epigenetic modifications, which include histone modifications, DNA methylation, microRNAs, nucleosome positioning, chromatin interaction, and chromosome