TCR/ITK Signaling in Type 1 Regulatory T cells

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Abstract

Type 1 regulatory T (Tr1) cells can modulate inflammation through multiple direct and indirect molecular and cellular mechanisms and have demonstrated potential for anti-inflammatory therapies. Tr1 cells do not express the master transcription factor of conventional regulatory T cells, Foxp3, but express high levels of the immunomodulatory cytokine, IL-10. IL-2-inducible T-cell kinase (ITK) is conserved between mouse and human and is highly expressed in T cells. ITK signaling downstream of the T-cell receptor (TCR) is critical for T-cell subset differentiation and function. Upon activation by TCR, ITK is critical for Ras activation, leading to downstream activation of MAPKs and upregulation of IRF4, which further enable Tr1 cell differentiation and suppressive function. We summarize here the structure, signaling pathway, and function of ITK in T-cell lineage designation, with an emphasis on Tr1 cell development and function.

Keywords

ITK · TCR · Tr1 cells · Ras · IRF4

7.1 Introduction

Regulatory T cells are critical in promoting self-tolerance and preventing immunopathology from excessive inflammation. Transcription factor Foxp3 is a well-recognized lineage specification factor of conventional T regulatory (Treg) cells. Foxp3$^+$ conventional Treg cells express Foxp3 and CD25 as identifying markers and are important immune regulators that promote self-tolerance and immune homeostasis in human and mouse (Bennett et al. 2001; Brunkow et al. 2001; Chatila et al. 2000; Fontenot et al. 2003; Gambineri et al. 2003; Hori et al. 2003; Khattri et al. 2003; Sakaguchi et al. 1995; Shevach et al. 2001; Wildin et al. 2001). Type 1 regulatory T (Tr1) cells, on the other hand, lack the expression of Foxp3 and CD25, but express high levels of the immunomodulatory cytokine IL-10, which can suppress inflammatory responses associated with type 2 cytokines (IL-4/5/13) and IL-17 responses

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(Gagliani et al. 2013; Gol-Ara et al. 2012; Huber et al. 2011; Okamura et al. 2012). Tr1 cells directly suppress the effector cell response through the secretion of IL-10, granzyme-dependent killing of antigen-presenting cells, and expression of coinhibitory receptors such as CTLA-4 and PD-1 (Akdis 2008; Haringer et al. 2009; Huber et al. 2011; Magnani et al. 2011). Indirect immune suppression by Tr1 cells has been shown as well, such as depletion of proinflammatory extracellular ATP by CD39 expressed on Tr1 cells (Mascanfroni et al. 2015).

Tr1 cells have been shown to suppress the development of diseases such as allergic asthma, colitis, and atopic dermatitis in animal models (Ahangarani et al. 2009; Groux et al. 1997; Volz et al. 2014). Antigen-specific tolerance correlates positively with Tr1 numbers during hematopoietic stem cell transplant and immunotherapy (Bohm et al. 2015; Serafini et al. 2009). Their ability to regulate inflammation and induce tolerance makes Tr1 cells a promising candidate for immunotherapies (Bohm et al. 1998; Gol-Ara et al. 2012; Mobs et al. 2010; Roncarolo et al. 2014; Volz et al. 2014; Zeng et al. 2015). However, this potent regulatory activity has the potential to "cut both ways" by hindering the protective response toward pathogens or tumors. Patients with chronic hepatitis C possess higher numbers of virus-specific Tr1 cells than patients who spontaneously clear the infection (Brady et al. 2003; MacDonald et al. 2002). In a mouse model of *Bordetella pertussis*, pathogen-specific Tr1 cells have been shown to suppress the protective Th1 response (McGuirk et al. 2002). Tr1 cells isolated from tumors display immunosuppressive functions ex vivo (Bergmann et al. 2008; Pedroza-Gonzalez et al. 2015). Thus, understanding the signaling pathways that drive Tr1 differentiation and function is of great interest. While much is known about signaling pathways involved in Treg cell development and function (Sakaguchi et al. 2008; Vignali et al. 2008), significantly less is known about the molecular mechanisms regulating Tr1 cell development and how they function against inflammation.

Members of the Tec family of nonreceptor tyrosine kinases are critical in signaling pathways of the immune system (August and Ragin 2012; Berg et al. 2005; Gilfillan and Rivera 2009). IL-2-inducible T-cell kinase (ITK) is the predominant Tec family kinase expressed in T cells and is a signaling mediator downstream of the T-cell receptor (TCR) (August et al. 2002). ITK plays major roles in modulating T-cell development, activation, differentiation, and function (Gomez-Rodriguez et al. 2014, 2016; Huang et al. 2014; Kannan et al. 2015; Schaeffer et al. 2000, 2001). The role of ITK in the differentiation and function of several T helper (Th) lineages is well studied (August and Ragin 2012; Gomez-Rodriguez et al. 2014; Huang et al. 2014; Kannan et al. 2013; Miller et al. 2004). However, it has only been recently explored in Tr1 cells (Huang et al. 2017). This chapter summarizes the role of ITK in TCR signaling and Th cell differentiation, with an emphasis on the role of ITK in Tr1 cell differentiation and function.

7.2 Structure of ITK

ITK is a member of the Tec family kinases and is expressed by mast cells and T cells, with a predominant preference in T cells (Andreotti et al. 2010; August et al. 2002). Tec family includes ITK, BTK, TEC, BMX, and TXK and is the second largest non-receptor protein-tyrosine kinase family (second to Src family) (Takesono et al. 2002). These kinases are classified based on the unique Tec-Homology (TH) domain, which is composed of a zinc-binding Btk-homology (BH) motif and/or proline-rich regions (PRR). ITK and its family members share a high homology in structure consisting of, from protein N terminal to C terminal, pleckstrin homology (PH), TH (BH + PRR), Src-homology (SH) 3, SH2, and kinase domains (Fig. 7.1a) (Felices et al. 2007). At the steady state in resting T cells, ITK normally resides in the cytosolic compartment, and the PH domain is critical in recruiting ITK to the plasma membrane upon TCR activation (August et al. 1997). The SH3 and SH2 domains are important for mediating protein–protein interactions between ITK and other components of the TCR signaling complex,

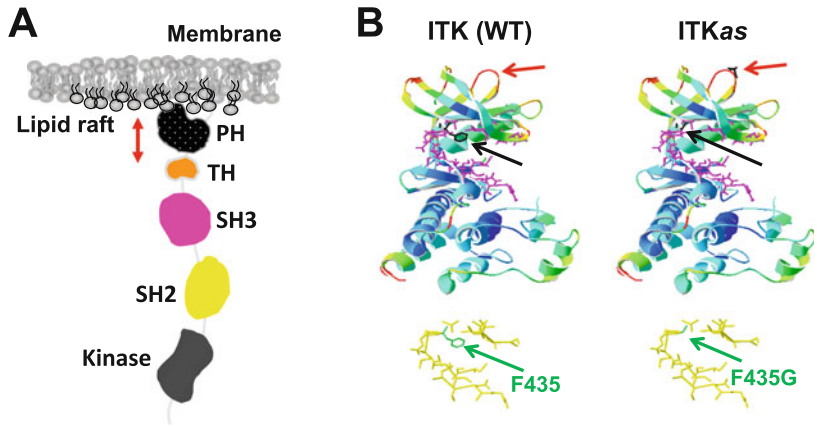

Fig. 7.1 Structure of ITK. (**a**) Schematic of structure of ITK: from N to C terminus, there are PH, TH, SH3, SH3, and kinase domains. (**b**) Structures of kinase domain of WT and allele sensitive (*as*) mutant of ITK (upper panel). ITK*as* harbors an F435G mutant (black and green arrows) in the ATP binding pocket (lower panel) of ITK kinase domain along with a ΔA429 (red arrows) to preserve the ITK kinase activity of ITK*as*. (Modified from the authors' previous publication (Kannan et al. 2015))

most notably SLP-76 (Berg et al. 2005; Bunnell et al. 2000; Su et al. 1999). In addition, these SH2 and SH3 protein–protein interactions are critical for kinase-independent functions in regulating actin cytoskeleton rearrangement (Dombroski et al. 2005; Grasis et al. 2003).

The C-terminal kinase domain has high levels of structural similarity between ITK and other Tec family members, making it challenging to identify small molecule inhibitors that would be specific for ITK over the other Tec kinases. The presence of unique gatekeeper residues in the kinase domain may enable the development of an allele-sensitive kinase domain that allows temporal inhibition of the kinase activity using small molecule inhibitors (Bishop et al. 1998). A bulky gatekeeper residue, F435, has been identified in ITK, and substitution of this residue with the short chain glycine (F435G) allows for bulkier ATP analogs to efficiently compete for the ATP binding and prevent activation of this allele sensitive mutant of ITK (ITK*as*) (Fig. 7.1b) (Shokat and Velleca 2002). These bulkier ATP analogs include modified derivatives of the Src-kinase inhibitor PP1 (1-(1,1-dimethylethyl)-3-(4-methylphenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine) such as 3MB-PP1 (1-(1,1-dimethylethyl)-3-[(3-methylphenyl)methyl]-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine), 1NM-PP1 (1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine), and 1-NA-PP1 (1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine), that have unique selectivity for kinases that are modified by substitution of similarly located gatekeeper residues in other kinases (Bishop et al. 2000).

7.3 TCR/ITK Signaling Pathway

The TCR is stimulated upon interaction with the peptide/MHC complex on antigen-presenting cells. This interaction results in the phosphorylation of Lck. Lck phosphorylates the ITAMs on the cytoplasmic CD3ζ chain which allows for recruitment and phosphorylation of ZAP-70. Activated ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76 which forms a crucial scaffold for the TCR signaling complex or signalosome (Werlen and Palmer 2002). Lck also phosphorylates phosphatidylinositol 3-kinase (PI3K) which generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) lipids in

the plasma membrane. ITK is recruited to the plasma membrane by PIP3 via the PH domain (August et al. 1997) where it is phosphorylated by Lck. This event allows ITK to interact with the TCR signalosome via phosphorylated tyrosine 145 on SLP-76 via its SH2 domain (Su et al. 1999). Once associated with the signalosome, ITK activates PLCγ-1 via phosphorylation. Activated PLCγ-1 converts membrane phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG) (Schaeffer et al. 1999). IP3 stimulates IP3R on the endoplasmic reticulum (ER), resulting in release of STIM1-mediated ER calcium (Ca^{2+}) stores into the cytoplasm. Depletion of ER Ca^{2+} stores results in an influx of extracellular Ca^{2+} through calcium release-activated channels such as Orai1 on the plasma membrane (Smith-Garvin et al. 2009). Increased intracellular Ca^{2+} levels activate calcineurin which dephosphorylates and promotes nuclear translocation of NFAT (Smith-Garvin et al. 2009). Production of DAG leads to the activation of Ras/Raf1/MAPK pathway (Smith-Garvin et al. 2009), which can further lead to activation and upregulation of transcription factor AP-1 and IRF family members (Huang et al. 2017; Schaeffer et al. 2001). DAG also recruits PKCθ to the plasma membrane where it regulates NF-κB activation and nuclear translocation (Smith-Garvin et al. 2009). This TCR/ITK signaling pathway is detailed in Fig. 7.2.

TCR signaling still occurs in the absence of ITK, but with attenuated strength (August and Ragin 2012; Schaeffer et al. 2001), which makes ITK more a signal amplifier rather than a signal switch during T-cell activation (August and Ragin 2012). In the absence of ITK, activation of the MAPK pathway and downstream AP-1 and IRF4 transcription factors is reduced, as well as the calcium flux and NFAT activity (Huang et al. 2017; Schaeffer et al. 2001); NF-κB activation is also impaired, but to a lesser extent than MAPK and NFAT pathways (Fowell et al. 1999; Schaeffer et al. 2001). The attenuation of these signaling pathways has consequences for T-cell subset differentiation and function.

7.4 Kinase-Independent ITK Function

ITK also has kinase-independent functions during TCR activation. Upon T-cell activation, ITK directly interacts with Vav, a regulator of actin polymerization, via the SH2 domain (Dombroski et al. 2005). In mouse models of *Itk* deficiency, T-cell-specific expression of ITK with mutations in the SH2 and SH3 domain display impaired actin polymerization along with reduced recruitment of Vav to the signaling complex during T-cell activation (Dombroski et al. 2005; Grasis et al. 2003). However, ITK kinase dead mutants do not show this deficiency, suggesting that ITK acts as part of a scaffold important for cytoskeleton reorganization independently of its kinase activity (Fig. 7.2) (Dombroski et al. 2005; Grasis et al. 2003; Hao et al. 2006; Qi et al. 2011; Sahu et al. 2008).

7.5 Function of ITK in T Helper Cell Designation

The role of ITK in T-cell subset differentiation and function has been well studied over the past two decades (Fig. 7.3). Naïve CD4 cells preferentially differentiate into IFN-γ-producing Th1 cells in the absence of ITK, partly due to its negative regulation of Tbet expression (Kannan et al. 2013; Miller et al. 2004). *Itk*-deficient mice have defects in Th2 differentiation and function and were reported to be resistant to developing allergic airway inflammation (Au-Yeung et al. 2006; Fowell et al. 1999; Kannan et al. 2013). ITK plays a crucial role in regulating the balance between Th17 and Treg cell differentiation in part via regulating sensitivity to IL-2 (Gomez-Rodriguez et al. 2014). Under Th17-polarizing conditions, ITK signals suppress Treg cell differentiation while promoting Th17 differentiation (Gomez-Rodriguez et al. 2014; Huang et al. 2014). Importantly, impaired calcium/NFAT signaling in $Itk^{-/-}$ $CD4^+$ cells results in decreased cytokine production by both Th17 and Th2 cells (Fowell et al. 1999; Gomez-Rodriguez et al. 2009). ITK has also been shown to be critical in Th9

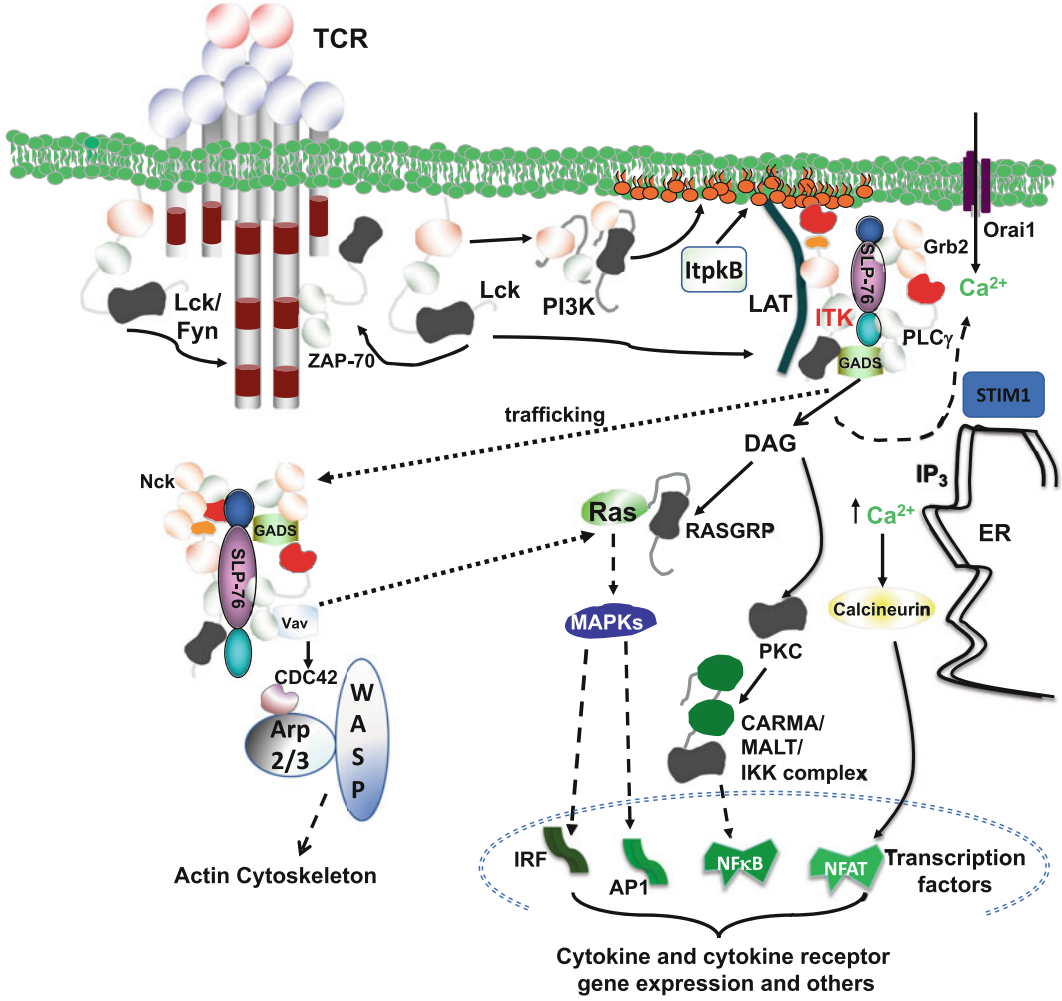

Fig. 7.2 Scheme of TCR signaling through ITK. (Modified from the authors' previous publication (Kannan et al. 2012))

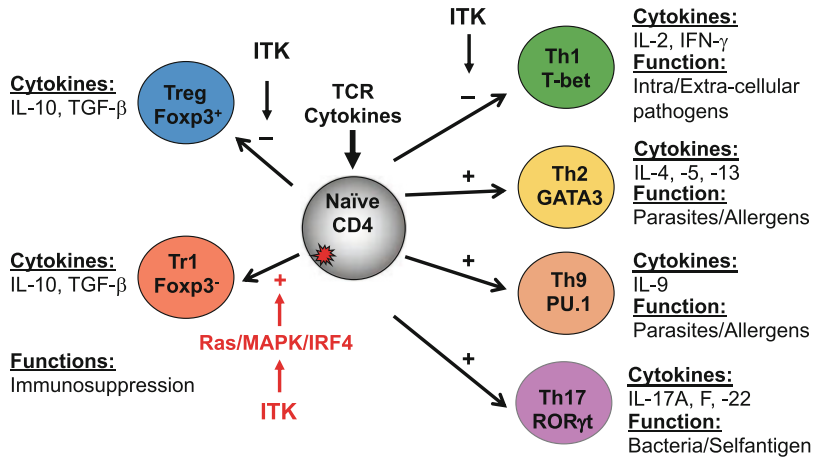

Fig. 7.3 ITK function in Th and regulatory T-cell differentiation and function. Note that ITK signals through Ras/MAPK/IRF4 are critical for Tr1 cell development and suppressive function

differentiation by regulating induction of IL-2/STAT5 signaling leading to upregulation of IRF4 (Gomez-Rodriguez et al. 2016). The roles of ITK in regulating cytokine production in Th1, Th2, and Th17 cells are dependent on the kinase activity of ITK (Kannan et al. 2015).

7.6 TCR/ITK → Ras/MAPK/IRF4 Pathway in Tr1 Cells

Naïve $CD4^+$ cells can differentiate into Tr1 cells upon TCR stimulation in the presence of IL-27 (Fitzgerald et al. 2007). Th17 cells are also capable of transdifferentiating into Tr1 cells upon resolution of inflammation (Gagliani et al. 2015). IL-27 signals through STAT1 and STAT3 to induce a network of transcription factors, including IRF4, Blimp-1, Ahr, and c-Maf, that controls Tr1 differentiation (Apetoh et al. 2010; Cretney et al. 2011; Pot et al. 2011; Stumhofer et al. 2007). The absence of ITK or ITK kinase activity results in severely impaired upregulation of IL-10, Tr1 cell associated surface markers, and transcription factors under Tr1 cell-inducing conditions both in vivo and in vitro. Indeed, anti-CD3 antibody treatment induces significantly less Tr1 cells in $Itk^{-/-}$ compared to WT mice. The absence of ITK also reduced the development of Tr1 cells in models of parasitic and viral infections. Inhibition of ITK kinase activity significantly reduced Th17 to Tr1 cell trans differentiation in vitro. Furthermore, ITK kinase activity is critical for upregulating the expression of transcription factors IRF4, Ahr, and Blimp-1, all of which are involved in regulating Tr1 cell development, in both mouse and human, although there are some nuances between mouse and human (e.g., Maf appears to be downstream of ITK in mice, but not human Tr1 cell differentiation). Downstream of TCR/ITK, Ras/MAPK pathway is upregulated, further leading to upregulation of IRF4 expression. Using ITK*as*-expressing Tr1 cells co-cultured with TCR-activated WT responder T cells, ITK*as* kinase activity was shown to be critical in the suppressive function of Tr1 cells against responder T-cell expansion. This reduction in suppression coincided with a decrease in IL-10 production. Importantly, $Itk^{-/-}$ Tr1 cell differentiation could be rescued when IRF4 was reintroduced via retroviral transduction, and the resultant IRF4-expressing $Itk^{-/-}$ Tr1 cells were functionally suppressive. Taken together, these data show that the TCR/ITK → Ras/MAPK/IRF4 signaling pathway is critical in both Tr1 differentiation and suppressive function (Fig. 7.3) (Huang et al. 2017).

7.7 Conclusions

Regulatory T cells serve to protect against autoimmunity and restrict immunopathology but may also prevent eradication of pathogens or tumors. While the TCR/ITK → Ras/MAPK/IRF4 pathway is crucial for Tr1 cell differentiation and function, the exact mechanism of Ras activation and signaling leading to upregulation of IRF4 has not yet been determined. Proof-of-concept clinical trials with Tr1 cells have demonstrated the safety and feasibility of this approach and indicated some preclinical benefits in graft-versus-host disease during hematopoietic stem cell transplantation (Bacchetta et al. 2014). In mouse models, IL-10-producing T cells exhibited anti-inflammatory effects in host immune rejection to organ transplants (Mfarrej et al. 2017), inflammatory bowel diseases (Clemente-Casares et al. 2016; Desreumaux et al. 2012), allergic asthma (Tousa et al. 2017), food allergy (Pellerin et al. 2018), dermatitis (Volz et al. 2014), type 1 diabetes (Clemente-Casares et al. 2016), autoimmune encephalitis (Clemente-Casares et al. 2016), arthritis (Clemente-Casares et al. 2016), acute immunopathology due to infection (Huang et al. 2017), and other related inflammatory conditions, as well as in shaping vaccine-induced immune responses (Ndure and Flanagan 2014). A more complete understanding of the signaling pathways that promote Tr1 cell differentiation and function may allow for the development of improved strategies to modulate the immune responses under different disease conditions.

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