Review

The non-canonical Hippo/Mst pathway in lymphocyte development and functions

Xingrong Du1, Alan Yu2, and Wufan Tao1,*

1State Key Laboratory of Genetic Engineering and Institute of Developmental Biology and Molecular Medicine, Scholl of Life Sciences, Fudan University, Shanghai 200433, China, and 2University of Toronto, Toronto, Ontario M5S2J7, Canada

*Correspondence address. Tel: +86-21-65643948; Fax: +86-21-65642111; E-mail: wufan_tao@fudan.edu.cn

Received 22 September 2014; Accepted 23 October 2014

Abstract

The canonical Hippo/Mst pathway, originally discovered in Drosophila, is famous for its function in promoting apoptosis, inhibiting cell proliferation and tumorigenesis, and regulating tissue regeneration. However, emerging evidence shows that multiple non-canonical Hippo signaling pathways are also implicated in the regulation of various other biological processes. Recent studies have revealed that Mst1/2, the core kinases of Hippo/Mst pathway are required for T cell development, function, survival, trafficking, and homing, and also involved in regulation of autoimmunity. In this review, we discuss the roles of non-canonical Hippo/Mst signaling pathways in lymphocyte development and functions.

Key words: Mst1/2, lymphocyte, development, function

Introduction

The canonical Hippo/Mst pathway, which is evolutionally conserved from Drosophila to mammals, plays critical roles in organ size control during animal development and regeneration [1]. The Hippo/Mst pathway components were first identified in Drosophila by genetic screens. Mammalian sterile 20-like kinase 1 and 2 (Mst1/2), large tumor suppressor 1 and 2 (Lats1/2), Salvador (Salv, also known as WW45), Mps one binder 1A and B (Mob1A/B), and Yes-associated protein (Yap)/transcriptional coactivator with PDZ-binding motif (Taz) are the mammalian homologs of Drosophila hpo, wts, sav, mats, and yki, respectively [2]. Mst1/2 and Lats1/2 are the core kinases of the Hippo/Mst pathway. Mst1/2 proteins interact with Salv, a WW domain-containing protein, to phosphorylate and activate Lats1/2 kinases. Lats1/2 then phosphorylate and inactivate the transcriptional coactivator YAP/TAZ by sequestering phosphorylated YAP/TAZ in the cytoplasm by 14-3-3 proteins [1,2]. Inhibition or loss of function of Hippo/Mst pathway core components such as Mst1/2 and Lats1/2 results in the nuclear translocation of YAP/TAZ, and subsequently, YAP/TAZ in conjunction with TEADs (Scalloped orthologs) mediates transcription of target genes to promote cell proliferation and inhibit apoptosis. The above mentioned Mst–Lats–Yap signal pathway is a canonical signaling transduction process of the Hippo pathway in regulating cell proliferation, apoptosis, tumorigenesis, and tissue regeneration. Plenty of excellent reviews have already been published on this aspect [1–6].

In addition to their role in the regulation of cell proliferation and differentiation through YAP1, the Hippo pathway core components are also involved in multiple non-canonical Hippo signaling pathways, which are implicated in the regulation of other biological processes. Mst1 can also induce apoptosis in a number of different ways: by phosphorylating Foxo1/3 and enhancing their nuclear entry in primary granule neurons [7,8], by phosphorylating histone H2B [9], by promoting phosphorylation of Runx3 and Mst2–Sav1–Runx3 complex formation [10], or by antagonizing cell survival signals through interacting with AKT and suppressing its activation [11] in various tumor cell lines. MST1 can impair insulin secretion by phosphorylating and destabilizing the β-cell transcription factor PDX1 [12]. Mst2–Sav1 complex promotes adipocyte differentiation by stabilizing and activating PPARγ [13]. Mst1–Ndr1 signaling promotes stable kinctochore–microtubule attachment by restraining Aurora B activity and centrosome duplication, whereas the Mst1–Sav1 complex regulates centrosome disjunction via Nek2A [14–16]. Lats–Mob1 complex has an evolutionarily conserved role in mitotic exit and centrosome maintenance [17,18].
The immune system is a complex system of cells and biological reactions that constitute our body’s first line of defense for fighting off invaders and preventing its own cells from self-degeneration and mutation. The immune system is composed of both innate immunity and adaptive immunity in mammals. Several recent studies have revealed that non-canonical Hippo/Mst signaling, especially Mst1/2, play crucial roles in mammalian adaptive immunity. In contrast to the function that canonical Hippo/Mst signaling promotes apoptosis, Mst1/2 are required for T cell survival, normal development, and functions, but their roles in control of cell proliferation are controversial. In this review, we will summarize and discuss the emerging evidence showing that Mst1/2 regulate lymphocyte, especially T cell development, migration, and homing, and are essential for maintaining immunological self-tolerance and immune homeostasis.

T Cell Development in Thymus

The T cell is a critical component of the adaptive immune system. T cells recognize antigens and initiate the adaptive response. T cells are developed and matured in the thymus through a complicated selection process, which can be roughly divided into three stages depending on the surface expression of CD4 and CD8: double negative (DN, CD4−CD8−), double positive (DP, CD4+CD8+), and single positive (SP, CD4+CD8− or CD4−CD8+) stages. During this process, immature T cells first go through positive and then negative selection. Successful thymocytes become mature CD4 SP or CD8 SP cells. The expression of Mst1 and Mst2 genes can be detected in DP cells, and is much higher in SP cells [19]. The proportion and number of SP thymocytes are significantly increased in the thymus of Mst1−/− mice [20,21], while the number of peripheral T cells is dramatically decreased [20–23]. Further studies have revealed that Mst1 deficiency impairs negative selection of thymocytes probably due to inefficient migration and antigen recognition mediated by LFA-1 and ICAM-1 within the medulla [24]. Little to no effect is seen on positive selection as the trafficking patterns of DP and the number of immature positively selected CD69+ DP in Mst1−/− mice are comparable to those of wild type [24]. T cell development in Mst2−/− mice appears normal [19,23]. However, when both Mst1 and Mst2 are deleted, the severity of lymphopenia increases dramatically [19,23]. There is a significant decrease in the proportion, and the number of SP cells is significantly decreased in Mst1−/−; Mst2−/−; Lck-Cre DKO mice, in which the Cre transgene is driven by a proximal Lck promoter, compared with wild type and Mst1−/− mice (Du and Tao, unpublished data). These results suggest a redundant role of Mst1 and Mst2 in T cell development and homeostasis. It should be noted that SP cells have also been shown to be significantly increased in the thymus of Mst1−/−; Mst2−/−; Lck-Cre DKO mice, in which the Cre transgene is driven by a distal Lck promoter [19]. While the detailed molecular mechanism(s) by which Mst1 regulates T cell development remains to be elucidated, studies from Dr Tatsuo Kinashi’s lab have provided important clues. Mst1 proteins associate with and are activated by Rap1–RAPL complex, and co-localize with LFA-1 at the leading edge and in the immune synapse between T cells and APCs after TCR stimulation [26]. Recently, Ueda et al. reported that Mst1 deficiency or blocking the cell adhesion molecules LFA-1 and ICAM-1 results in inefficient migration and antigen recognition of CD4+ thymocytes within the medulla [24]. These results suggest that Mst1 plays a key role in regulating thymocyte self-antigen recognition in the medulla.

Development and Function of Regulatory T Cells

Recent studies have also shown that Mst1 and Mst2 play very important roles in the development and function of regulatory T cells (Tregs). The proportion and number of Tregs are significantly reduced in Mst1−/− thymus [24,25] and peripheral lymphoid organs from 1-week-old Mst1−/− mice [25]. However, upon maturation into adults, the proportion of Tregs is restored and even surpasses that of the wild-type mice [22,24,25]. This is probably due to enhanced proliferation resulting from T cell homeostasis in lymphopenic Mst1−/− mice [25]. Mst1 deficiency also impairs induction of Tregs from CD4+ naive T cells by TGF-β [25,27]. Functionally, the Mst1−/− Tregs fail to prevent the development of experimental colitis in vivo and antigen or TCR-induced proliferation of naive T cells in vitro [25,27]. Although Treg development is not affected in Mst2−/− mice, the proportion of Tregs in the thymus of Mst1/2 DKO mice are further decreased compared with that in Mst1−/− mice [25]. This demonstrates that Mst1 plays a dominant role in the development and function of Tregs, and Mst1 and Mst2 are functionally redundant in this process.

Foxo1 and Foxox3 (Foxo1/3) are transcriptional factors that can directly bind to the Foxp3 promoter and enhance Foxp3 expression. The Foxo1/3 protein levels are dramatically decreased in Mst1-deficient T cells from human patients [28,29] and mice generated independently by Tao’s and Lin’s labs [23,25]. Foxp3 expression is also significantly reduced in Mst1−/− Tregs [25]. However, neither the Foxo1/3 protein levels nor Foxp3 expression are significantly altered in the conventional T cells and Tregs of Mst1−/− mice generated by Kinashi’s lab [24,27]. The reason for this discrepancy is unknown. Mst1 regulates Treg development and function by directly or indirectly stabilizing Foxo proteins through phosphorylation of Foxo1/3 and inhibition of TCR/CD28-induced Akt activation [25]. These results suggest that reduced Foxo1/3 expression and Foxp3 induction in Mst1−/− Tregs could be one of the mechanisms underlying the impaired Treg development and function in Mst1−/− mice. Using two-photon imaging, Tomiyama et al. [27] observed that Mst1−/− Tregs had impairments in their interactions with antigen-loaded dendritic cells (DCs). This inefficient conjugate formation between Mst1−/− Treg and DCs leads to failure of down-regulation of co-stimulatory molecules such as CD86 in DCs, finally resulting in impaired contact-dependent suppressor functions of Mst1−/− Tregs. LFA-1 appears to be the downstream molecule affected in this process. This study provides another explanation for the impaired suppression function of Mst1−/− Tregs. However, because conventional Mst1 deficient and/or T-cell-specific Mst1/2 DKO mice were used for all the studies mentioned above, the effect of other thymic cells on Treg development cannot be ruled out. Further studies using Treg-specific Mst1 deficient and/or Mst1/2 DKO mice are needed to investigate the intrinsic roles of Mst1/2 in Treg development and function.

T Cell Activation, Apoptosis, and Survival

Mst1-deficient mice display a severe reduction of peripheral T cells with lower percentages of CD62Lhigh CD44low naive T cells and higher percentages of CD62Llow CD44high effector/memory T cells [22,25], which may result in homeostasis-driven proliferation [25]. This lymphopenic mutant phenotype is much more severe in Mst1/2 double deficient mice [19,25] and also phenocopied in human patients with Mst1 mutations [28,29]. The lack of peripheral T cells in Mst1-deficient mice and human patients is probably due to enhanced activation-induced cell death (AICD) and impaired thymocyte egress.
The results reported so far about apoptosis, proliferation, and cytokine production of Mst1-deficient T cells vary dramatically and are sometimes contradictory. This is probably due to different methods used for generating the Mst1-deficient mice such as gene trapping versus gene targeting, and differences in genetic background of the mutant mice used by different research groups.

There is enhanced apoptosis of peripheral T cells in Mst1−/− mice [20,21]. The studies have also observed that JNK over-activation led to their enhanced apoptosis. Moreover, there was partially blocked at G1/S transition [30]. Again, lower expression of IL-2 and higher expression of CD279 in Mst1−/− mice were found to be required for integrin LFA-1 activation/cluster- ing, T cell polarization and adhesion in a Mst1-knockdown cell line following chemokine or TCR stimulation [26]. Later, this phenotype was confirmed by using T cells from Mst1 deficient mice [21,22,31]. Consistent with the function of Mst1 in regulating T cell polarization and adhesion, Mst1-deficient mice also displayed inefficient migration and antigen recognition of CD4+ thymocytes within the medulla [24], impaired thymocyte egress [19–21], decreased lymphocyte homing [20–22] and defective lymphocyte migration in a traditional transwell assay [20,22,31] or on immobilized chemokines, over lymph node-derived stromal cells or within explanted lymph nodes [21]. Mst1-deficient DCs are also impaired in trafficking from skin to draining lymph nodes [21].

Integrin LFA-1, also known as CD11a/CD18 and integrin αLβ2, is a major adhesion molecule involved in T cell trafficking. LFA-1 clusters at the leading edge of polarized cells stimulated with chemokine or anti-CD3 in intracellular trafficking-dependent manner and associates with RAPL through the cytoplasmic region of the α chain [32]. RAPL or Mst1-deficient T cells exhibit a defect of LFA-1 clustering at the leading edge [21,33]. Rap1–RAPL promotes LFA-1 integrin clustering and T cell polarization by recruiting and activating Mst1 after chemokine and TCR stimulations. Thus, the Rap1–RAPL–Mst1 pathway has been proposed to play an important role in controlling T cell trafficking [21,26]. Similar to RAPL or Mst1-deficient mice, lymphocyte trafficking in Rab13-deficient mice is also impaired [34]. After chemokine stimulation, Mst1 activates Rab13 by promoting the phosphorylation of DENND1C, a guanine nucleotide exchange factor for Rab13. Mst1 then forms complexes with activated Rab13 to facilitate LFA-1 delivery to the leading edge of lymphocytes along actin filaments in a myosin Va-dependent manner [34]. At the same time, Mst1 promotes F-actin polymerization through enhancing the phosphorylation of VASP, which is crucial for the Rab13-dependent vesicular transport of LFA-1 [34]. Mst1 has also been found to regulate the anterior and posterior distribution of low and higher affinity LFA-1 on the membrane of migrating T cells by modulating the activity of Myosin Ila [31], another molecular motor protein that moves along actin filaments. Therefore, after chemokine or anti-CD3 stimulation, Mst1 kinase is activated by RAP1–RAPL and promotes the Rab13-dependent vesicular transport of LFA-1 along actin filaments to the leading edge of polarized cells by activating Rab13 and promoting F-actin polymerization. In addition, Mou et al. [19] found that chemokines and SIP chemoattractant-induced activation of the Rho family small GTPase and polarization of the actin cytoskeleton were abolished in the Mst1/Mst2-deficient 5P thymocytes. Mst1/Mst2 are required for CCL19 induced phosphorylation of Mob1A/B and Dock8 recruitment by phosphorylated Mob1A/B in thymocytes. Mst1 and Mst2 kinases regulate T cell migration and actin polarization by control of Rho GTPase activation through promoting Mob1 phosphorylation and its association with Dock8, a Rac1 GEF [19].

T Cell Migration and Homing

In lymphocytes, Mst1 is required for the proper organization of integrins in the plasma membrane at the leading edge of migrating cells, which is critical for lymphocyte trafficking. The first evidence that Mst1 regulates T cell trafficking was demonstrated in a study where Mst1 was found to be required for integrin LFA-1 activation/cluster- ing, T cell polarization and adhesion in a Mst1-knockdown cell line following chemokine or TCR stimulation [26]. Later, this phenotype was confirmed by using T cells from Mst1 deficient mice [21,22,31]. Consistent with the function of Mst1 in regulating T cell polarization and adhesion, Mst1-deficient mice also displayed inefficient migration and antigen recognition of CD4+ thymocytes within the medulla [24], impaired thymocyte egress [19–21], decreased lymphocyte homing [20–22] and defective lymphocyte migration in a traditional transwell assay [20,22,31] or on immobilized chemokines, over lymph node-derived stromal cells or within explanted lymph nodes [21]. Mst1-deficient DCs are also impaired in trafficking from skin to draining lymph nodes [21].
**B Cell**

In contrast to the extensive researches on the roles of Mst1 and Mst2 in T cell development and function, there are few studies focusing on the function of Mst1 and Mst2 in B cells. The number of peripheral B cells is reduced in Mst1-deficient mice [20–22]. Splenic marginal zone B cells are dramatically reduced and B cell adhesion and trafficking are impaired in Mst1-deficient mice [21,22]. A similar mechanism of T cell trafficking was proposed to explain the defects of B cell adhesion and trafficking [21]. When stimulated by BCR, the response of Mst1-deficient B cells was reported to be similar to controls [21]. However, Salojin et al. [30] found that Mst1-deficient B cells showed decreased responsiveness to B cell mitogens in vitro and displayed deficient antigen-specific IgE production in vivo when compared with the controls. More experiments are needed to further explore and confirm the function of Mst1 and Mst2 in B cells.

**Eosinophil Apoptosis**

Eosinophils and neutrophils are two types of innate immune cells that play important roles in fighting multicellular parasites and infections. They are also involved in the pathogenesis of allergies and asthma. Both Mst1 and Mst2 kinases are expressed in eosinophils, but not in neutrophils. However, only Mst1 is activated by caspase-mediated cleavage during spontaneous or Fas-induced apoptosis of human eosinophils, suggesting that Mst1, but not Mst2, plays a role in the regulation of eosinophil apoptosis [35]. However, whether Mst1 can affect eosinophil development and function has not been investigated yet.

**Immune System-related Disease**

The Mst1 and Mst2 kinases emerge as critical regulators of lymphocyte function and autoimmunity. Loss-of-function mutations of Mst1 in human patients have been identified in clinical studies [28,29,36]. Mst1 deficiency in humans leads to T cell lymphopenia with a low proportion of naive T cells and high proportion of effector T cells, impairment of T cell response to stimulation with anti-CD3, various mitogens and recall antigens [28]. It is also associated with neutropenia and heart malformations in some patients [29]. Mst1-deficient patients also display recurrent pulmonary infections, susceptibility to candidiasis and non-regressing cutaneous warts caused by multiple types of human papillomavirus infections [28,29,36]. These phenotypes have not been reported or observed in Mst1-deficient mice, probably as a result of the mice under SPF conditions. Autoimmune antibodies, which usually occur in autoimmune diseases, are also detectable in Mst1-deficient patients [28,29]. The protein levels of FOXO1 and FOXO3, IL-7 receptor and BCL2 are significantly lower in T cells from Mst1-deficient patients than those in the control [28,29]. Conversely, FAS expression and the FAS-mediating apoptotic pathway are up-regulated [28].

Similar to Mst1-deficient patients, Mst1-deficient mice, generated separately by Dr Kinashi’s lab and Dr Tao’s lab, have been shown to be prone to autoimmune diseases [24,25]. Lymphocyte infiltration, T cell over-activation, and autoantibody production are observed in young [25] and aged Mst1-deficient mice [24,25]. The autoimmune phenotypes were more severe when both Mst1 and Mst2 genes were deleted, indicating that the functions of Mst1 and Mst2 are redundant in preventing autoimmunity, although Mst2 does not display any autoimmune phenotypes [25]. Although Mst1−/− bone marrow is sufficient for inducing colitis as well as over-activation of naive T cells and splenomegaly in recipient mice, these Mst1-deficiency-mediated mutant phenotypes are all suppressed in recipients with co-transplanted wt Tregs [25]. Mst1−/− Tregs fail to inhibit colitis induced by wt naïve T cells transplanted into recipient mice [27]. These results suggested that the impaired function of Mst1−/− Tregs is a major cause of autoimmunity in Mst1−/− mice. However, it has also been reported that deletion of Mst1 in mice reduces the severity of experimental autoimmune encephalomyelitis (EAE) with a lower number of infiltrated CD4 T cells in the spinal cord and protects the mice from the development of collagen-induced arthritis (CIA) [30]. It was found that the severity of EAE was alleviated in mice treated with an Mst1 inhibitor [30]. One explanation is that Mst1-deficient mice are prone to autoimmune diseases in normal conditions but are protected from autoimmune EAE and CIA induction. Further detailed studies are required to determine the exact role of Mst1 in autoimmunity.

In addition to the role of Mst1/2 in the regulation of autoimmune diseases, Mst1 is also implicated in preventing leukemia. Mst1-null mice are highly susceptible to the development of ENU-induced T-ALL [37], probably through its role in the maintenance of chromosome integrity [38].

**Concluding Remarks**

Recent studies about the roles of non-canonical Hippo/Mst signaling in lymphocyte development and functions have revealed that at least Mst1/2, core components of Hippo/Mst pathway, can play important but different or opposite roles in a cell or tissue-dependent manner. Mst1/2 are required for T cell survival, whereas the canonical Hippo/Mst signaling promotes apoptosis of non-lymphocytes. Until now, no spontaneous leukemia or lymphomas has been reported in mice with Mst1/2 double mutations in T cell lineage, while spontaneous hepatomas [39,40] or colon adenomas [41] are easily observed in mice with liver cell-specific or intestinal epithelium-specific deletion of Mst1/2. The effect of Mst1/2 genes on proliferation of lymphocytes is also controversial. Mst1-deficient mice generated in some labs were shown to be prone to autoimmune diseases [24,25] but the mice generated by Salojin et al. [30] displayed resistance to EAE and CIA. More investigations are desperately needed to resolve these discrepancies and to explore the underlying molecular mechanisms. Little is known about the molecular mechanisms by which Mst1/2 regulates T cell and Treg development. Furthermore, nothing is known about the roles of Hippo/Mst signaling in the development and function of B cells and myeloid cells. Answers to these challenging questions will advance our understanding of the regulation and function of the non-canonical Hippo pathway.

**Funding**

This work was supported by the grants from the National Basic Research Program of China (Nos. 2013CB945300, 2011CB510102), the National Natural Science Foundation of China (Nos. 31171406, 30971666), and the Key Projects Grant for Basic Research (No. 12JC1400600) from the Science and Technology Committee of Shanghai Municipality.

**References**

