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Review

Review

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

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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 kinetochore–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-deterioration 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*, the core components of Hippo/Mst pathway 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 [19,25]. However, when both *Mst1* and *Mst2* are deleted, the severity of lymphopenia increases dramatically [19,25]. There is a significant decrease in the proportion, and the number of SP cells is significantly decreased in $Mst1^{fl/fl}$; $Mst2^{-/-}$; *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 thyme of $Mst1^{-/-}$; $Mst2^{fl/fl}$; *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 $CD4^+$ naïve 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 naïve 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 Foxo3 (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 Lim'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 $CD62^{high}$ $CD44^{low}$ naïve T cells and higher percentages of $CD62L^{low}CD44^{high}$ 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

[20,21]. The results reported so far about apoptosis, proliferation, and cytokine production of *Mst1*-deficient peripheral 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 [22,23,30] and human patients [28,29]. Zhou *et al*. [22] reported that ongoing apoptosis of peripheral *Mst1*-deficient T cells was enhanced. However, it was not observed in *Mst1*$^{-/-}$ mice generated by other groups [20,30]. Ongoing apoptosis of peripheral *Mst1*$^{-/-}$ T cells is significantly increased only when CD62^{high} CD44^{low} naïve T cells and CD62L^{low}CD44^{high} effector/memory T cells are analyzed separately [20]. Apoptosis is significantly increased when *Mst*$^{-/-}$ T cells are cultured *in vitro* with CD3/CD28 activation [30] or oxidative stresses [23]. Furthermore, increased apoptotic cells were found to be restricted to *Mst1*$^{-/-}$ Th1 cells [30]. The exact mechanism behind this enhanced apoptosis is still controversial. Zhou *et al*. [22] found that the JNK activation was increased in *Mst1*-deficient T cells and suggested that JNK over-activation led to their enhanced apoptosis. Choi *et al*. [23] reported that *Mst1* enhanced T cell survival and reduced apoptosis by promoting transcription of *Sod2* and *Cat* (catalase) genes and protecting against excessive ROS accumulation through increasing Foxo1/3 stability and nuclear entry. More recently, Salojin *et al*. [30] proposed that lower expression of IL-2 and higher expression of CD279 (programmed cell death protein 1, PD-1) in *Mst1*$^{-/-}$ naïve T cells might be one of mechanisms underlying enhanced AICD and reduced cell proliferation of *Mst1*-deficient Th1 cells.

It has also been reported that the *Mst1*-deficient naïve T cells exhibit markedly increased proliferation in response to TCR stimulation when cultured *in vitro* [22]. However, this has not been successfully confirmed. In contrast, proliferation of naïve T cells was shown to be markedly reduced in the absence of *Mst1* after activation with CD3 mAb, Con A, and PMA/ionomycin, and cell cycle progression was partially blocked at G1/S transition [30]. Again, lower expression of IL-2 and higher expression of CD279 in *Mst1*$^{-/-}$ naïve T cells were proposed to be responsible for this cell cycle blockage [30]. However, it is important to note that the mammalian *Lats1/2* and *Yap1* do not participate in the proliferative response of naïve T cells to TCR/CD28 co-stimulation [22].

Cytokines production is generally closely associated with T cell activation. However, results of cytokine production in T cells from *Mst1* deficient mice generated by different research groups were not consistent. Previous reports showed that the production of cytokines such as IL-2, IFN-γ, IL4, and IL-17A [22] and the proportion of cytokine-producing cells [25] are increased in *Mst1*$^{-/-}$ mice, suggesting hyperactivation of *Mst1* deficient peripheral T cells. It has also been recently reported that *Mst1* deficiency leads to altered distribution of peripheral T cell subsets and reduced *in vitro* T cell activation [30]. The production of Th1 cell-specific cytokines including IL-2, IFN-γ and TNF-β is inhibited and the levels of Th2 cell-specific cytokines such as IL-4, IL-5, IL-10 and IL-13 are elevated in the cultures of *Mst1*$^{-/-}$ T cells stimulated by CD3 or Con A. The reduction in Th1 cytokines and Th2 skewing in CD3/CD28-activated *Mst1*$^{-/-}$ T cell cultures are attributed to the reduced number of T cells that produce Th1 cytokines and deficient Th1 cytokine synthesis [30]. This discrepancy may be due to differences in the T cell development and cytokine balance of various *Mst1*$^{-/-}$ mice with different genetic backgrounds.

In summary, the *Mst1* gene is required for survival maintenance and apoptosis inhibition of T cells. This function is opposite to that of canonical Hippo pathway demonstrated in *Drosophila* and some mammalian cells such as liver epithelial and cultured neuronal cells. However, more experiments under same conditions using different *Mst1* deficient mice generated by different labs are needed to verify the effect of *Mst1* deficiency on T cell proliferation.

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/clustering, 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 $\alpha_L\beta_2$, 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 an intracellular trafficking-dependent manner and associates with RAPL through the cytoplasmic region of the α_L 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 IIa [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 S1P chemoattractant-induced activation of the Rho family small GTPase and polarization of the actin cytoskeleton were abolished in the *Mst1/Mst2*-deficient SP 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 Rac1GEF [19].

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 naïve 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 naïve 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 autoimmune diseases 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 linage, 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.

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