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Non-canonical Hippo signaling in the regulation of leukocyte function

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Abstract

The mammalian sterile 20-like kinases are central constituents of the evolutionary ancient canonical Hippo pathway regulating cell proliferation and survival. However, perhaps surprisingly, MST1 deficiency in human patients leads to a severe combined immunodeficiency syndrome with features of autoimmune disease. In line with this, *Mst1*-deficient mice exhibit severe defects in lymphocyte and neutrophil functions as well as disturbed intracellular vesicle transport. These findings spurred research on the “non-canonical” functions of MST1 in leukocytes. Here, we summarize the latest findings on this topic and discuss MST1 as a critical regulator of various leukocyte functions.

Keywords

MST1; MST2; Hippo pathway; leukocyte; vesicle transport

The Hippo Pathway

Mammalian Sterile 20-like (MST) kinases are conserved homologues of yeast Sterile 20 (STE20) kinase and despite millions of years of divergent evolution, exert similar functions across eukaryotes. The MST family consists of five different proteins, which are conserved in all metazoans [1]. They can be divided into two subgroups: the first group containing MST1 (serine/threonine protein kinase 4 (STK4)) and MST2 (STK3); the second group containing MST3 (STK24), MST4 (STK26 or MASK) and MST5 (STK25 or YSK1) (Table 1) [2]. Cell division cycle 15 (Cdc15) in *S.cerevisiae* was the first MST kinase characterized [2, 3], later Hippo (Hpo) was discovered in *D.melanogaster* [4]. Both, Cdc15 and Hpo, are crucial in controlling cell proliferation and are homologs of mammalian MST1 and MST2, collectively called MST1/2 (Table1).

MST1/2 are involved in signalling pathways following along the canonical and non-canonical Hippo pathway. The canonical pathway is highly conserved between *D.melanogaster* and mammals and a master regulator of tissue growth. During development and within the canonical pathway MST1/2 seem to have redundant roles. In the canonical pathway, activated MST1/2 stimulate Large tumor suppressor kinases 1 and 2 (LATS1/2), which phosphorylate and inactivate the transcription factor Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), restricting YAP and TAZ localization to the cytoplasm. In contrast, activated YAP and TAZ move to the nucleus, acting as transcription factors by inducing the expression of various genes controlling growth, cell proliferation, organ size and cell- and tissue homeostasis (reviewed in [2, 5]) (Figure 1). Functions of MST1/2 other than growth and independent from LATS/YAP/TAZ are mediated via the non-canonical Hippo pathway which will be discussed in detail below.

Structurally, human MST1 and MST2 consist of 487 amino acids (AA) and 491 AA, respectively. They share 88% similarity and 76% identity in AA sequence and are more than 95% identical in their catalytic domains. MST1/2 have a N-terminal serine/threonine kinase domain, a coiled coil region, caspase 3 cleavage sites and a C-terminal SARAH domain [2]. The SARAH domain is a highly conserved dimerization domain, mediating homo- and heterodimerization, as well as ligand binding. Activation of MST1 and MST2 requires the phosphorylation at Thr183 (MST1) and Thr180 (MST2). This is facilitated by trans-autophosphorylation within the activation loop by the SARAH domain through the formation of antiparallel dimers (both MST1/2 homo- and heterodimers) [6]. Of note, the binding affinity of MST1/2 heterodimers is ~6-fold lower than for MST2 homodimers [7]. Both can be cleaved by caspase 3 (326 AA and 349 AA for MST1 and 322 AA for MST2) in response to apoptotic stimuli, separating the N-terminal kinase domain (37 kDa) and the C-terminal SARAH domain (18 kDa). The N-terminal part of MST1/2 has no regulatory inputs (apart from phosphatase-catalysed inactivation) and lacks a nuclear export signal [2]. In general, the regulation of MST1/2 is determined by the composition of MST1/2 complexes, which in turn is defined by the abundance and affinity of various non-catalytic SARAH domain-containing proteins in relation to MST1/2. The relative affinities and tissue specific abundance of SARAH domain-containing proteins in vivo has not been determined, yet.

Loss of Hippo in *D.melanogaster* leads to unregulated cell proliferation and tissue overgrowth [4]. Surprisingly and in contrast to the *D.melanogaster* Hippo-mutant, knockout of both mammalian Hippo homologs, *Mst1* and *Mst2* (*Mst1*^{-/-}*Mst2*^{-/-}), in mice causes embryonic lethality, showing defects in placental development, vascular patterning, primitive haematopoiesis and regulation of cell proliferation and survival [8]. Hippo-like phenotypes emerge after tissue-specific MST1/2 deficiency, but only in some tissues. For example, *Mst1/2* conditional-deletion in the liver (adreno-cre; *Mst1*^{-/-}*Mst2*^{fl/fl} or *Mst1*^{-/-}*Mst2*^{fl/-} and albumin-cre; *Mst1*^{fl/fl}*Mst2*^{fl/fl}) leads to tissue overgrowth and tumorigenesis [9, 10]. Conditional deletion of *Mst1/2* in the intestine (villin-cre; *Mst1*^{-/-} *Mst2*^{fl/fl} mice) causes expansion of stem cell and progenitor cell compartments [11]. In contrast, skin-specific deletion of *Mst1/2* (K14-cre; *Mst1*^{-/-} *Mst2*^{fl/fl}) caused no abnormalities or hyperplasia [12].

Interestingly, *Mst1* and *Mst2* single knockout mice are viable and develop normally, revealing that these two kinases act at least in part in a redundant fashion. Unexpectedly, loss of MST1 in humans leads to a severe immunodeficiency syndrome as discussed below [13–17]. A similar phenotype is also seen in *Mst1*^{−/−} mice. In contrast, *Mst2*^{−/−} single knockout mice have normal leukocyte function and no obvious phenotype [8].

Emerging evidence implicates the regulation of a variety of biological processes via the non-canonical Hippo pathway. This is particularly true for leukocytes, where the MST1-dependent non-canonical Hippo pathway seems to play a critical role various leukocyte functions. This was mostly elucidated by studying *Mst1* and *Mst2* single- and double-knockout mice and been complemented by the recent identification of patients with MST1 deficiency.

Patients with *STK4* (*MST1*) deficiency

Recently, MST1 emerged as a critical regulator of leukocyte function and autoimmunity in humans. Five independent groups reported on autosomal recessive nonsense mutations in the gene serine/threonine protein kinase 4 (*STK4*) (which encodes for MST1) in human patients with a novel primary immunodeficiency disease (PID), now called *STK4* deficiency. One of the two first reports describes three patients from a consanguineous Iranian family [13]. Six patients were identified from three consanguineous unrelated Turkish families [16, 17] and four patients from two consanguineous families living in France [14] [15]. These 13 patients from 6 consanguineous unrelated families were diagnosed with *STK4* deficiency, resulting from 6 different loss of function mutations in *STK4* [13–17] (Figure 2, Table 2).

Patients with *STK4* deficiency usually presented in their first months of life with signs of immunodeficiency consisting in recurrent bacterial, viral and/or fungal infections including pulmonary infections and bronchiectasis, mucocutaneous candidiasis, skin abscesses and other superficial infections. Some patients also suffered from non-regressing cutaneous warts caused by multiple types of human papillomavirus infections. Two patients exhibited autoimmune haemolytic anaemia, one patient developed immune thrombocytopenia emphasizing the concomitant risk of developing autoimmune disease. Six patients, who all had a history of recurrent severe infections, received hematopoietic stem cell transplantation, which was successful in two patients. The other four patients died from transplantation related complications. One cardinal finding in all patients consisted in lymphopenia, mostly affecting naïve T cells. In addition, T-cell function tests revealed impaired T cell responses [17]. T cell proliferation assays using various stimuli including phytohemagglutinin, PMA, anti CD28, and anti CD3 consistently showed reduced proliferation of T cells in *STK4* deficient patients compared to control patients [13–17]. Reduced proliferative response was mostly explained by an increased rate of T cell apoptosis which was in line with elevated surface expression of FAS on T cells [17]. In addition, isolated T cells incubated with anti FAS led to an increased induction of apoptosis in *STK4*-deficient T cells as assessed by Annexin-V and propidium iodide staining [13]. Transwell assays using CCL19/21 in the bottom well revealed reduced chemotaxis of peripheral blood T cells from *STK4* patients which was accompanied by reduced expression of CCR7 and L-selectin in T cells [17]. Similar findings were reported in transwell assays using CXCL11 [15]. Furthermore,

different autoantibodies were detectable in STK4-deficient patients. Most prominently, two of the reported 13 patients developed autoimmune haemolytic anaemia, one patient suffered from immune thrombocytopenia [16, 17]. Some of the patients also had elevated autoantibodies against thyroglobulin, rheumatoid factor, granulocytes or PR3 [13, 14], although these patients did not develop symptoms of autoimmune disease. In addition, most of the patients did not develop appropriate antibody titers following the regular vaccination schedule, although this was not entirely consistent. Interestingly, almost all patients showed hypergammaglobulinemia. Besides the reported changes in adaptive immunity, some of the studies also reported on intermittent neutropenia. However, only one study performed additional analysis on neutrophil function [17]. Abdollahpour et al. investigated staurosporine-induced apoptosis in patient PMNs and found an increased rate of apoptosis in STK4-deficient PMNs compared to control PMNs. Furthermore, the same authors demonstrated a strong increase in valinomycin-induced loss of mitochondrial transmembrane potential and consecutive apoptosis in patient neutrophils suggesting that MST1 has a critical role in neutrophil function and might therefore contribute to the recurrent appearance of infections in STK4 deficient patients.

Finally, life expectancy was strongly reduced in STK4 deficient patients. Even in those patients who received hematopoietic stem cell transplantation, the majority of patients died due to transplantation-related complications, raising the question whether MST1 expressed in non-hematopoietic tissue might contribute to successful engraftment and restoration of immune function following hematopoietic stem cell transplantation. Of note, in some of the patients congenital malformations of the heart were documented [13].

Role of MST1/2 in lymphocytes

Similar to patients with STK4 loss of function mutation, *Mst1*^{-/-} mice displayed lymphopenia. They have a reduction in splenic white pulp, a decreased number of peripheral CD4⁺ and CD8⁺ T cells, B220⁺ B cells and absence of marginal zone B cells [18]. Global deletion of *Mst2* did not alter lymphocyte numbers. However additional elimination of *Mst2* in the hematopoietic lineage (*Mst1*^{-/-}*Mst2*^{fl/fl} *VavCre*) or in lymphocytes (*Mst1*^{fl/fl}*Mst2*^{-/-} *LckCre*) led to an aggravated phenotype shown by a dramatically increased severity of lymphopenia compared to *Mst1*^{-/-} mice. This indicates that MST2 can partially compensate for the loss of MST1 [19, 20]. Murine MST1 is most abundant in lymphoid organs and crucial in T cell development and function including selection in the thymus, adhesion, migration, apoptosis and lineage commitment [21]. The non-canonical Hippo pathway in lymphocyte development and function has been reviewed in detail before [21]. Therefore, we focus here only on the most recent findings concerning the functional role of MST1 in lymphocytes in humans and mice.

The role of MST1/2 in T cell development in the thymus

During development and maturation in the thymus, T cells undergo selection processes marked by 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⁺). Throughout this selection phase, immature T cells undergo first a positive followed by a

negative selection resulting in mature SP CD4⁺ and CD8⁺ T cells [21]. MST1/2 expression can be detected in DP T cells and increases in SP T cells [19]. In *Mst1*^{-/-} mice the number of SP T cells in the thymus is increased [22, 23], whereas the number of peripheral CD4⁺ and CD8⁺ T cells is strongly reduced [22–25]. The low number of peripheral T cells was mainly caused by defective thymic egress with a high rate of apoptosis being an additional factor [19]. This phenotype in the mouse equals the situation in human patients with *STK4* deficiency showing a severe loss of naïve T cells (both CD4⁺ and CD8⁺ T cells) in the periphery and an increased rate of cell death in activated T cells [17]. The exact mechanism leading to increased apoptosis of T cells is still controversial. Both, the egress phenotype and the reported inefficient negative selection can be explained by defective adhesion, migration and antigen recognition caused by defects in the β_2 integrin lymphocyte function-associated antigen 1 (LFA-1; $\alpha_L\beta_2$ integrin, CD11a/CD18) in MST1 deficient T cells [26], as further detailed below. Similar to *STK4*-deficient patients, *Mst1*^{-/-} mice are prone to autoimmune diseases. Young as well as aged *Mst1*^{-/-} mice have increased lymphocyte infiltration, autoantibodies and over-activation of T cells [20, 26] whereas *Mst2*^{-/-} mice show normal T cell development. *Mst1*^{fl/fl}*Mst2*^{-/-}*LckCre* double knockout (DKO) mice display a more severe phenotype with an even stronger decrease in the number of SP T cells in the periphery than in *Mst1*^{-/-} mice [19, 20] suggesting that MST2 can compensate for the loss of MST1 at least to some extent.

MST1/2 controls T cell migration via vesicle trafficking of LFA-1

MST1 is critical for lymphocyte trafficking by controlling the proper intracellular handling of LFA-1, a major adhesion molecule involved in T cell trafficking [21]. Katagiri *et al.* provided first evidence, showing that knockdown of MST1 in T cells affected LFA-1 activation/clustering, T cell polarization and adhesion in response to chemokine or TCR stimulation [27] which was later confirmed in *Mst1*^{-/-} T cells [23, 24, 28].

Additional functional studies using *Mst1*-deficient mice showed that MST1 is involved in lymphocyte trafficking by controlling surface mobilization and localization of LFA-1 at the plasma membrane [29]. After chemokine stimulation, MST1 is activated and associates with the cytoplasmic tail of the α_L subunit of LFA-1 via Rap1-RAPL [27, 30]. Then, MST1 activates Rab13 by promoting phosphorylation of DENND1C, a guanine nucleotide exchange factor (GEF) for Rab13. MST1 together with activated Rab13 leads to the translocation of LFA-1 containing vesicles along actin filaments in a myosin Va-dependent manner [29]. In addition, MST1 promotes polymerization of F-actin, by phosphorylating VASP, which is critical for Rab13-dependent mobilization of LFA-1 containing vesicles [21, 29]. Mou *et al.* further demonstrated that MST1 and MST2 are required for chemokine induced T cell migration and actin polarization by regulating Rho GTPase activation through enhancing Mob1 phosphorylation and its association with DOCK8, a Rac1GEF [19] (Figure 3a).

Role of MST1/2 and TAZ in T_{reg} cell development and function

MST1/2 in lymphocytes is not only important during negative selection in the thymus and localization of LFA-1 to the plasma membrane. Recently, it has been demonstrated that MST1/2/TAZ contribute to the development and function of T_{reg} cells. *Mst1*^{-/-} mice have a

reduced number of T_{reg} cells in infancy, which is restored in adulthood [20, 24, 26]. Again, loss of MST2 does not affect T_{reg} cell development, but loss of both, MST1 and MST2 (*Mst1^{fl/fl}Mst2^{-/-}-LckCre*) aggravates the phenotype seen in *Mst1* deficient mice [20]. T cells, isolated from *STK4*-deficient patients, show decreased levels of Foxo1 and Foxo3, critical transcription factors for the activation of Foxp3, suggesting that MST1 regulates T_{reg} biology also in the human system [13, 17]. By using multi-photon microscopy, impaired interactions between T_{reg} and antigen-loaded dendritic cells have been observed in the absence of MST1. This would lead to an impaired LFA-1-mediated suppressor function of *Mst1^{-/-}* T_{reg} cells [31]. However, due to the use of either global *Mst1^{-/-}* mice or lymphocyte specific DKO mice (*Mst1^{fl/fl}Mst2^{-/-}-LckCre*), an impact of other lymphocyte subsets cannot be excluded.

Recently a study by Geng et al. demonstrated that MST1/2 and TAZ are involved in T_H17 - T_{reg} cell balance. By using *Mst1^{fl/fl}Mst2^{fl/fl}* bred with *Ox40Cre* mice, a marker of activated T cells (*Ox40Cre*), the authors observed normal T cell development and normal numbers of peripheral T cells. But, they found that TAZ, a downstream target of MST1/2 in the canonical Hippo pathway, alters the interaction between ROR γ t and Foxp3 thereby promoting the differentiation into T_H17 over T_{reg} cells. The expression of the transcription factor TAZ is strongly induced after simultaneous stimulation of TGF- β and IL-6/IL-23, which activate SMAD3 and STAT3. TAZ thus favours the expression of IL17a and thereby the differentiation into T_H17 cells in several ways: First, TAZ acts as a transcriptional co-activator of ROR γ t, promoting the expression of IL17a. Second, TAZ promotes the degradation of Foxp3, a critical factor for T_{reg} cell differentiation. And third, binding of TAZ to ROR γ t and Foxp3 blocks their interaction, preventing Foxp3-mediated inhibition of ROR γ t. Furthermore, TAZ activity can be inhibited by the interaction with MST1/2 as well as TEAD1 (Figure 3b) [32]. Taken together these findings demonstrate that the fine-tuned balance between MST1/2 and TAZ activity is critical for T_H17 and T_{reg} cell differentiation and maintenance [17, 33].

Role of MST1/2 in myeloid cells

Evidence that MST1 might play a role in myeloid cell function came from the first studies in *STK4*-deficient patients reporting increased susceptibility to bacterial infections paired with intermittent neutropenia and an elevated rate of neutrophil apoptosis, as mentioned earlier [13]. Another study showed a high rate of infections in *Mst1^{-/-}Mst2^{fl/fl} Vav-Cre* mice also suggesting functional deficits in the myeloid lineage in the absence of MST1 in the mouse [19].

While lymphopenia and defective T cell function may explain a major part of the clinical manifestations, the impaired responses to acute infections implied additional defects in myeloid cell function [13, 14, 16, 17, 34–36]. However, LFA-1-mediated functions, like slow neutrophil rolling and adhesion, were not affected in MST1-deficient mice [35]. In contrast, *Mst1*-deficient neutrophils show a severe extravasation defect with neutrophils trapped within post capillary venules, between the endothelial layer and the vascular basement membrane (BM), failing to penetrate the BM and thus being unable to extravasate to the site of inflammation. This will impair efficient host defence during bacterial infection,

as has been described for other diseases with primary defects in leukocyte recruitment including LAD-III [35][61].

Role of MST1-dependent vesicle transport during neutrophil extravasation

Neutrophil granules contain a variety of cargo and membrane associated molecules, including adhesion molecules and receptors, that are rapidly upregulated during an acute inflammatory response [37]. A precise regulation of exocytosis is critical as many of their cargoes, while necessary to respond to infections, are concurrently inducers of inflammation. Furthermore, the adhesion molecule presentation at the plasma membrane must be regulated in a timely manner because early activation can induce overshooting inflammation while delayed activation would prevent neutrophil migration and therefore hamper neutrophil function. Neutrophil transmigration across the venular wall still remains incompletely understood. However, recent reports have highlighted an important role of $\beta 1$ and $\beta 3$ integrins, two laminin binding integrins [38]. After passing the endothelial cell layer, neutrophils need to interact with the BM to fully extravasate. In particular, VLA-3 ($\alpha 3\beta 1$, CD49c/CD29) is necessary for neutrophil extravasation in TNF- α and fMLP-induced transmigration *in vivo* [40]. VLA-3 also supports neutrophil swarming and aggregation in a fungal infection model [41]. In addition, another member of the $\beta 1$ integrin family, VLA-6 ($\alpha 6\beta 1$, CD49f/CD29), was proposed as a key adhesion molecule for neutrophil infiltration into tissue [42]. Using blocking antibodies against VLA-6 in a mouse intravital microscopy model suggest a role for VLA-6 in IL-1 β , but not TNF- α dependent neutrophil extravasation [43]. Interestingly, when using neutrophils deficient in the TNF- α receptor, blocking of PECAM-1 traps neutrophils between the endothelial layer and the BM [44]. Of note, this difference might be explained by the fact, that the release of vesicles is regulated by a binary signal, one being adhesion dependent and the other involving the ligation and activation of immune receptors [45, 46]. Recent work from our laboratory has emphasized a central role of MST1 in the upregulation of VLA-3 and VLA-6 on the plasma membrane caused by granule mobilization. This enables the penetration of the basement membrane and completes extravasation into tissue *in vivo* [35].

In addition, *Mst1*^{-/-} neutrophils showed defective mobilization of Rab27a-positive vesicles to the surface, connecting MST1 function with the neutrophil secretory machinery. Rab27a is a master regulator of neutrophil exocytosis and is present in neutrophil primary and secondary granules [47]. Its deficiency has not only been associated with defective neutrophil exocytosis [48] but also with impaired NK and T-cell degranulation [49]. As a consequence, and similar to *STK4*-deficiency (*MST1*-deficiency), genetic loss of function mutations in *Rab27a* cause an immunodeficiency disorder in humans called Griscelli syndrome type 2 (GS2) [50, 51]. Different from patients with *STK4*-deficiency, patients with GS2 are clinically characterized by partial albinism, hemophagocytic lymphohistiocytosis, recurrent infections, neurological abnormalities, uncontrolled T cell activation as well as macrophage activation syndrome [52]. The pigmentation defect is caused by abnormal melanosome transport [53]. The susceptibility to viral and sometimes bacterial infections in GS2 is associated with defective NK and CTL function [54] and neutrophil abnormalities [48, 55]. Although neutropenia is common to both GS2 and *STK4*-deficiencies, most features are unique in GS2 including the development of an accelerated phase with a

massive lymphohistiocytic infiltration affecting liver, spleen (hepatosplenomegaly) and central nervous system. The accelerated phase is usually triggered by viral infections including Epstein-Barr virus, hepatitis A and herpes virus and, much less frequently reported, bacterial infections [56].

Despite these different manifestations, MST1 and Rab27a functions seem to cross-talk in neutrophils. In human neutrophils, MST1 co-fractionates with the small GTPase Rab27a at low density and high density vesicles, suggesting that they may colocalize at more than one granule subset, most likely gelatinase and azurophilic granules [35], two type of granules whose degranulation is regulated by Rab27a [57]. Rab27a and MST1 translocate to the plasma membrane in response to PECAM-1/ICAM-1/CXCL1 but translocation of Rab27a is defective in *Mst1*^{-/-} neutrophils, indicating that MST1 regulates Rab27a-positive vesicle trafficking [35]. Two Rab27a effectors have been identified so far as regulators of Rab27a in neutrophils: JFC1/Slp1 and Munc13-4 [57]. The upregulation of VLA-3 and VLA-6 was defective in *JFC1*^{-/-} (*Syt11*^{-/-}) neutrophils, but not in *Munc13-4*-deficient neutrophils [35] suggesting that MST1 (directly or indirectly) regulates JFC1 dependent activation of Rab27a (Figure 4a).

Regulation of ROS production and bactericidal activity by MST1/2 in phagocytic cells

Increasing evidence supports a role of MST1/2 in the regulation of reactive oxygen species (ROS) generation. Earlier work focused on MST1/2 in ROS-induced apoptosis. In fact, in mammalian neurons MST1/2 was found to mediate oxidative stress dependent apoptosis by inducing the phosphorylation of FOXO3 [58].

Although earlier reports indicate that MST1/2 mediates oxidative stress induced apoptosis by several mechanisms in other cell types, little is known about a possible role of MST1/2 itself in the regulation of ROS in innate immune cells. In this regard, a recent report using *Mst1*^{-/-}*Mst2*^{fl/fl} *Vav*-Cre mice elucidated that MST1/2 induces the bactericidal activity of innate immune cells by a mechanism involving the production of intra-phagosomal ROS [34]. Intriguingly, MST1/2 regulation of phagosomal ROS is not mediated by the NADPH oxidase complex but by the juxtaposition of mitochondria in proximity to bacteria-loaded phagosomes (Figure 4b). Macrophages and neutrophils lacking MST1/2 seem to phagocytose less bacteria, and bacteria survived for longer periods of time in their phagosomes. TLR agonists induce ROS production in both macrophages and neutrophils. In macrophages isolated from *Mst1*^{-/-}*Mst2*^{fl/fl} *Vav*-Cre mice, the effect of LPS on ROS induction was decreased. In addition, basal ROS production was elevated in neutrophils of those mice, when measured with the fluorogenic probe 2',7'-dichloro-fluorescein diacetate (CM-H2DCFDA), a probe that reacts with hydroxyl, peroxy and carbonate radicals, but reacts weakly with superoxide, the main oxidative radical in these cells. These results seem to highlight differences between neutrophils and macrophages and suggest that the mechanisms mediating juxtapositioning of mitochondria at the phagosome to induce ROS in neutrophils may require further investigation.

Concluding Remarks and Future Perspectives

Through its non-canonical pathways, the Hippo homologue MST1 (and to a lesser degree MST2) has recently emerged as an important regulator of leukocyte functions. This has been learnt from *Mst1*^{-/-} and *Mst2*^{-/-} mice as well as from conditional *Mst1* and *Mst2* double deficient mice which aggravated the phenotype of *Mst1*^{-/-} mice. Finally, these findings are complemented by the growing number of studies from patients with *Stk4*-deficiency. These patients show a similar phenotype as *Mst1*^{-/-} mice although one major difference exists in the rate and early onset of severe infections observed in patients with *Stk4*-deficiency compared to *Mst1*^{-/-} mice. The difference might at least in part be explained by the fact that mice are bred and held under specific pathogen-free (SPF) conditions which reduces the risk for infections [59].

There is now growing evidence that MST1 has a strong and cell type specific influence on recruitment functions in immune cells. In neutrophils, MST1 is critical for triggering Rab27/JFC1-dependent mobilization of VLA-3, VLA-6 and NE-containing vesicles to the neutrophil surface, a prerequisite for the penetration of the vascular basement membrane. In T-cells, MST1 controls LFA-1 dependent adhesion and migration via mediating Rab13/DENND1C-dependent mobilization of LFA-1 containing vesicles to the surface. These findings point towards a regulatory role of MST1 on vesicle trafficking by acting on distinct Rab-GEFs in a cell type specific fashion. Future studies are needed to further elucidate the molecular mechanisms of the interplay between MST1 and Rabs/Rab-GEFs in neutrophils and T-cells. Furthermore, it would be very interesting to expand these studies to other immune cells including monocytes, macrophages, and dendritic cells, where vesicle trafficking is also critical for their immune functions. These studies will certainly shine new light on the diverse and cell type specific functions of MST1 in immunity and further establish the importance of MST1 in immune cell regulation.

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Highlights

- MST1 controls the localisation of integrins via Rab GTPases in T-cells and neutrophils
- In T-cells, MST1 regulates LFA-1 dependent adhesion and migration via Rab13/DENND1C
- MST1 in neutrophils is important for extravasation by regulating VLA-3 and VLA-6 localisation via Rab27a/JFC1

Text Box 1 –**Leukocyte recruitment**

Leukocyte recruitment is a critical immunological process which enables the extravasation of circulating leukocytes into tissues. The recruitment process is characterized by adhesion and activation events which occur in a cascade-like fashion and includes leukocyte rolling, firm arrest, postarrest modifications and transmigration into tissue [60, 61]. Leukocyte rolling is mediated through binding of selectins to carbohydrate-based moieties on selectin ligands. While leukocyte rolling in non-lymphoid tissues is mostly dependent on endothelium-expressed P-selectin and E-selectin interacting with P-selectin glycoprotein ligand-1 (PSGL-1) and other less defined selectin ligands (e.g. CD44, ESL-1, L-selectin) on leukocytes, lymphocyte rolling on high endothelial venules (HEVs) in lymphoid tissue is reversed in orientation with L-selectin expressed on leukocytes interacting with L-selectin ligands called peripheral node addressins (PNAd)s which can be found on HEVs [62]. During rolling, leukocytes have ample of time to screen the endothelial surface for activation signals including chemokines, and DAMP and PAMP molecules. Together with selectins, these surface factors trigger the activation of leukocyte-expressed integrins such as beta2 integrins LFA-1 and Mac-1 as well as alpha4 integrins VLA4 and VLA7 which bind to integrin ligands on the endothelium (ICAM-1, VCAM-1 and others). This mediates further slowing down of the rolling leukocyte and eventual firm arrest on the endothelium [61]. Additional signaling events triggered by interactions between leukocyte integrins and their respective ligands on the endothelium then prepare leukocytes for extravasation into tissue. These postarrest modifications include intravascular leukocyte flattening and spreading followed by crawling along the endothelium in search for an appropriate spot for transmigration into tissue. Transmigration per se also proceeds in a cascade-like fashion and involves additional sets of adhesion relevant surface molecules such as JAM family members, PECAM-1, VE-cadherin, and CD99. Transendothelial migration can occur through a para- or transcellular route depending on the leukocyte subset and the tissue context [63]. To fully transmigrate, leukocytes have to penetrate the basement membrane, which requires the expression of beta1 integrins on leukocytes [60].

Text Box 2 –**Granule subsets in neutrophils**

Neutrophil immune functions strongly depend on the capacity to synthesize and store pre-formed pro-inflammatory mediators in specialized intracellular vesicles [64]. Defects can result in severe immunodeficiencies as seen in patients with neutrophil-specific granule deficiencies [65].

Circulating mature neutrophils lack any proliferative capacity. Structurally, they possess a characteristic multi-lobed nucleus, very few mitochondria, a small Golgi apparatus, and a highly granular cytosol, that is packed with vesicles. In contrast to other cells, the protein content of neutrophil granules is not sorted, but sequentially formed during neutrophil differentiation in the bone marrow (sequential synthesis model) [66]. Neutrophils consist of at least four types of granules: azurophilic (primary) granules containing e.g. myeloperoxidases (MPO) and neutrophil elastase (NE); specific (secondary) granules containing e.g. lactoferrin; gelatinase (tertiary) granules containing e.g. matrix metalloproteinases 9 (MMP9); and secretory vesicles. Azurophilic as well as specific granules can be further subdivided due to their specific protein composition [67]. Azurophilic granules are heterogeneous both based on physical properties and their ability to undergo exocytosis or engage in phagosomal maturation, properties specified by the presence or absence of Rab27a in the granule membrane, respectively [37]. Of note, in mature neutrophils secretory vesicles are formed by endocytosis [68].

In general, secretory vesicles and gelatinase granules can be rapidly transported to the plasma membrane (for example $\beta 2$ integrins) upon moderate activation. A stronger activation of neutrophils is required to release azurophilic and specific granules [69, 70]. This hierarchy of granule release corresponds to the different roles of their secreted proteins during adhesion, migration, chemotaxis, phagocytosis and ROS production [47]. During transendothelial migration (TEM), close to 100% of secretory vesicles and 38% of gelatinase granules undergo exocytosis. For specific and azurophilic granules, exocytosis during TEM occurs in only 22% and 7%, respectively [45, 71].

Due to high cytotoxicity, the release of neutrophilic granules is strongly regulated by binary signals that involve adhesion-dependent mechanisms ($\beta 2$ -integrin or actin depolymerization) as well as ligation and activation of immune receptors [45]. The Src kinases Fgr and Hck mediate both the adhesion-dependent signaling cascade and the immune receptor mediated activation [72]. Current evidence favours the existence of an activation threshold, accomplished by binary signals, which induce degranulation or downstream pathways. This can induce an increase in intracellular Ca^{2+} and subsequently the hierarchical release of neutrophil granules in the order of secretory vesicles > gelatinase granules > specific granules > primary granules [73].

Canonical Hippo pathway

Figure 1. Canonical Hippo pathway

The key steps of the canonical Hippo pathway are displayed which regulate growth and proliferation as well as cell differentiation. Activated MST1/2 stimulate LATS1/2, which phosphorylate and inactivate the transcription factor Yes-associated protein (YAP) and transcriptional co-activator PDZ-binding motif (TAZ), restricting their localization to the cytoplasm. Activated YAP and TAZ move to the nucleus and induce the expression of various genes controlling cell growth and proliferation [28, 29].

Figure 2. Schematic representation of human mutations in MST1 (STK4)

MST1 (STK4) is a protein of 487 amino acids (AA) with a N-terminal kinase domain (30–281 AA), a coiled coil region (290–310 AA), caspase 3 cleavage sites (326, 349 AA) and a C-terminal SARAH domain (432–480AA) for ligand binding [19, 37]. So far, 6 different nonsense mutations have been identified, leading to a complete loss of MST1 function.

Non-canonical Hippo pathway

Figure 3. The non-canonical, cell type-specific Hippo pathway in lymphocytes

Lymphocyte specific functions via non-canonical pathways involving MST1/2 are shown.

(a) Successful T cell migration requires the transport of Rab13 positive, LFA-1 containing vesicles to the leading edge, which is dependent on the phosphorylation of Rab13-GEF DENND1C by MST1 [40]. (b) The balance between Treg cells and TH17 cells is controlled by TAZ. Activation of SMAD3 and STAT3 induces TAZ expression, which favours TH17 cell development by preventing the binding of Foxp3 to ROR γ t promoting ROR γ t dependent gene transcription. In the absence of STAT3 activation, MST1 and TEAD prevent TAZ-mediated inhibition of Foxp3, favouring Treg cell development [41]

Non-canonical Hippo pathway

Figure 4. The non-canonical, cell type-specific Hippo pathway in myeloid cells

Myeloid cell specific functions via non-canonical pathways involving MST1/2 are shown.

(a) Education by the endothelium (mediated via interactions with GPCR, LFA-1 and PECAM-1) induces MST1 and JFC1 dependent trafficking of Rab27a positive vesicles containing VLA-3, VLA-6 and NE. This mobilization is a prerequisite to overcome the basement membrane and successfully extravasate from postcapillary venules into inflamed tissue [14]. (b) Phagocytosis of bacteria leads to TLR/MyD88 mediated phosphorylation of MST1/2, increasing mitochondrial ROS (mROS) production [38]. *MST1 also mediates LC3 phosphorylation to regulate autophagy-mediated bacterial clearance in non-phagocytic cells [39].

Table1

Nomenclature of MST kinases in different organisms

Organism	MST1/MST2 sub-family		MST3/MST4/MST5 sub-family		
S. cerevisiae	Cdc15		Kic1/Sps1		
D. melanogaster	Hippo		GckIII		
Mammals	MST1/STK4	MST2/STK3	MST3/STK24	MST4/MASK	MST5/STK25/YSK1

Table2*STK4* mutations revealed in patients

DNA sequence change	Amino acid change	Commonly used nomenclature	Site of mutation	Type of mutation	Reference
c.58-61delATAG	p.Glu22X	E22X	Exon 2	Nonsense	[16]
c.C>T	p.Arg115X	R115X	Exon 4	Nonsense	[14]
c.349C>T	p.Arg117X	R117X	Exon 4	Nonsense	[17]
c.442C>T	p.Arg148X	R148X		Nonsense	[15]
c.750G>A	p.Trp250X	W250X	Exon 7	Nonsense	[13]
c.1103delT	p.369X	369X		Nonsense	[17]