Antigen surface and multivalent antigen binds to

Antigen(Ag) –mediated crosslinking of the high-affinity immunoglobulin E (IgE)receptor (Fc? RI) on mast cells results in the degranulation and the release ofpre-stored granular mediators, followed by the production of many allergic andinflammatory cytokines and chemokines, which are key effectors in allergicdisorders, such as asthma and anaphylaxis. Previous studies have demonstratedthat ELKS, an active zone protein, involves in the neurotransmitter release inneuronal cells as well as exocytotic release in rat basophilic leukemia(RBL-2H3) cells. In this study, we generated conditional knockout(KO) mice forELKS to delete ELKS specifically in mast cells and showed that peritonealcell-derived mast cells (PCMCs) lacking ELKS exhibited significantly lessdegranulation in vitro while cytokineand chemokine production was slightly affected. Our finding suggests that ELKSis a positive regulator for mast cell degranulation.

Introduction Theprevalence of allergic diseases has been increasing continuously in thedeveloped countries over the past decades and approximately one third of thepopulation worldwide is affected by allergic diseases such as asthma, allergicrhinitis and dermatitis (ref). Besideshaving a role in innate and adaptive defense against pathogens, mast cells havelong been considered as the central effectors in allergic inflammation. Mastcells are granulated cells derived from the bone marrow and they localise attissues that are exposed to the external environment such as the skin and lung (ref).

Mast cells express the high-affinity IgE receptor Fc? RI on their surface andmultivalent antigen binds to Fc? RI-bound IgE causes receptor aggregation andthereby mast cell activation. Activated mast cells

degranulate within secondsto minutes after its exposure to antigen and release an array of pre-formed, granule-stored mediators including histamine and ?-hexosaminidase (ref). Severalhours after activation, mast cells also produce newly synthesized of lipidmediators such as leukotrienes and prostaglandins as well as de novo synthesisand secretion of cytokines and chemokines (for example interleukin (IL)-6, IL-4, IL-13, MCP-1) driven by transcription factors including Nuclear factorkappa B (NF-? B) (ref). The NF-? Bfamily is a group of evolutionarily conserved transcription factors that playan important role in cell survival, immunity and inflammatory responses. Inunstimulated cells, the most abundant NF-? B dimer, p50/p65, is bound by inhibitorsof ? B (I? Bs) and therefore retains in the cytoplasm and remains inactive (ref). The NF-? B pathway can be activated by a wide range of stimuli such as pathway lipopolysaccharide(LPS), tumour necrosis factor (TNF) and IL-1. After these inducers bind totheir corresponding receptors, the IKK complex that contains IKK?, IKK? and IKK?/NEMOis activated, leading to the phosphorylation, ubiquitination and degradation ofI? Bs. As a result, the p50/p65 dimer enters into the nucleus, causing thetranscription of many target genes that involve in inflammatory and immuneresponse as well as cell differentiation and survival (ref).

Apart from IKK?, IKK? and IKK?/NEMO, ELKS has been identified as a regulatory subunit within theIKK complex (ref). Theexocytotic machinery in mast cell degranulation and neurotransmitter release inneuronal cells share some similarities and both require the SNARE (soluble N-ethylmaleimide-sensitive factorattachment protein receptors) proteins (ref). In neuronal cells, ELKS, togetherwith several cytomatrix-at-the-active –zone (CAZ) -associated

structuralprotein (CAST) family members including Rab3 interacting molecule 1 (RIM1), Bassoon and Piccolo have been reported to be involved in the Ca2+ dependentexocytosis of neurotransmitters (ref). In addition, a study has demonstratedthat using siRNA to silence ELKS in rat basophilic leukemia (RBL-2H3) cells hasled to a decrease in mast cell degranulation, suggesting that ELKS also has arole in regulating the exocytosis of granular contents in mast cells (ref). Base onthe above, we would like to explore the role of ELKS in mast cell degranulationthrough the use of animal model and to decipher the role of ELKS in other mastcell functions. Therefore, the aims for this project are: 1.

To generate the mast cell specific ELKSknockout mouse – Mcpt5-Cre ELKS Strain2. To study the role of ELKS in mast celldegranulation in vitro3. To study the role of ELKS in de novosynthesis of cytokines and chemokines in mast cells in vitro4. To investigate if ELKS have a role in early intracellularsignaling in mast cells5. To examine the localization of ELKS in mastcells6. To study the role of ELKS in mast celldegranulation in vivoGeneration of mast cell specific ELKS knockoutmice (ELKS Mcpt5-Cre Mice) Since wewould like to study the specific role of ELKS in mast cell and whole bodyknockout of ELKS in mouse has resulted in embryonic lethality (Liu et al.

, 2014; Wu et al., 2010), ELKS conditional knockout mice were generated usingCre-LoxP system. Mice with their ELKS alleles floxed with LoxP sequence (ELKSf/f) was first crossed with Mcpt5-Cre mice that express Cre recombinaseselectively in connective tissue mast cells (Ref.). Then, ELKS f/f mice wascrossed with ELKS f/f Mcpt5-Cre mice and the number of ELKS f/f

and ELKS f/fMcpt5-Cre pups in F2 progeny was similar, which matched the expected Mendelianratio (Table 1). Cells wereextracted from the peritoneal cavity of wild-type (WT) and ELKS Mcpt-Creknockout (KO) mice and there are similar population of mast cells in theperitoneal lavage cells between WT and KO mice (Fig.). These cells are then culturedfor 21 days in the presence of interleukin (IL)-3 and stem cell factor (SCF).

The surface expression levels of mast cell-specific markers Fc? RI and c-Kit onKO PCMCs were similar to that of WT PCMCs (Fig.). Similarly, the generation ofBMMCs in the presence of IL-3 and SCF was not affected by ELKS deficiency asboth WT and ELKS KO BMMCs had comparable levels of Fc? RIand c-Kit surface expression (Fig.). Therefore, ELKS is not required for mastcell development.

Next, themRNA and protein level expression of ELKS in PMMCs and BMMCs from ELKS f/f mice(WT) and ELKS f/f Mcpt5-Cre mice (KO) were quantified at mRNA and proteinlevels using real-time PCR and Western blot respectively. Deletion of ELKS inPMMCs at mRNA and protein levels were confirmed as shown in Fig. . However, as statedin previous literature that the efficacy of Cre/Lox recombination in BMMCs forMcpt-Cre strain is not 100%, the deletion of ELKS in BMMCs from ELKS f/fMcpt5-Cre mice was not complete (Fig.). Therefore, we only used PCMCs fromthese mice for later experiments. ELKS regulates mast cell degranulation in vitro Mast cellsdegranulate rapidly after being stimulated through the Fc? RI. To determine if ELKS plays a role in such mast cell function, WT and ELKS KO PCMCswere first sensitised with anti-DNP-IgE antibodyand then stimulated withDNP-BSA and the release of granular-stored enzyme, ?-hexosaminidase wasmeasured.

Release of ?-hexosaminidasewas optimal at a dose of antigen at 10ng/mL in WT PCMCs (Fig.) andELKS-deficient PCMCs had significantly lower release of ?-hexosaminidasecompared to WT PMMCs upon Fc? RI activation (Fig.).

Likewise, less surfaceexposure of CD107a was detected in KO PCMCs than WT PCMCs following IgE/Ag stimulation(Fig.). Hence, these data indicated that ELKS-deficient mast cells have adeficit in their capacity to degranulate invitro. ELKS is required for cytokine production frommast cells Engagementof the Fc? RI receptor can also result in denovo synthesis of various cytokines and chemokines that charaterises thelate-phase pro-inflammatory response. Therefore, we analysed gene expression ofa selection of pro-inflammatory and Th2-related cytokines and chemokinesincluding TNF?, IL-6, CCL1, IL-1?, IL-33, GM-CSF, MCP-1 andIL-13. WT and KO PCMCs were sensitised with anti – DNP IgE overnight andstimulated with DNP-BSA for 1. 5h.

Real-time PCR analysis demonstrated that ELKS-deficientmast cells have slight increase in mRNA expressions for TNF?, IL-6, CCL1, IL-1? and IL-33 compared to WT mast cells. Collectively, theseresults suggest that ELKS is playing an additional role in Fc? RI-mediatedcytokine and chemokine synthesis in mast cells besides degranulation. ELKS is not required for early signaling NF-? B andMAP kinase cascades orchestrate the production of cytokines from mast cellsfollowing Ag-induced IgE-Fc? RI aggregation and ELKS is part of the NF-? B signalingpathway. Hence, we next examinedwhether ELKS is required for early signaling pathways in mast cells.

WT and KOmast cells were again sensitised with anti-DNP IgE and then stimulated withDNP-BSA. KO mast cells have reduced I? B? mRNA expression compare to WT mastcells upon IgE-Ag stimulation (Fig.). There was no difference in p-pERK andp-p38 between WT and KO mast cells (Fig.). Discussion In thepresent study, we have generated conditional knockout mice for ELKS inconnective tissue mast cells and have demonstrated that ELKS deletion in mastcells causes reduced degranulation. Mast cells from KO mice also produced moreinflammatory cytokines and chemokines upon IgE-induced activation compare tothose from WT mice. We have also shown that loss of ELKS has resulted in less I? B?.

Collectively, our data has reconfirmed the role of ELKS in positive regulationof exocytosis. Previousstudies have implicated the involvement of different IKK complex subunitswithin the NF-? B signaling pathway in mast cell functions. I? B kinase ? (IKK?)was shown to be critical for mast cell degranulation as fetal liver-derivedmast cells from IKK?-deficient mice had impaired degranulation upon IgE-Agstimulation (ref.).

However, another study by Peschke et al. (2014) found that therewas unaffected degranulation but impaired production of cytokine in peritoneal mastcells generated from mice with connective tissue mast cell-specific IKK? deletion. In the same study by Peschke et al. (2014), they have also reported that activatedperitoneal NEMO/IKK? KO mast cells had impaired cytokine production. Inaddition, several lines of evidence suggest that ELKS, a regulatory subunit ofthe IKK complex, is a positive regulator for exocytosis. A study by Inoue etat. (2006) has shown that ELKS regulates Ca2+ dependent exocytosisin PC12 cells (ref.

) while another study by Ohara-Imaizumi et al. (2005) hasdemonstrated that there was a decrease in insulin exocytosis after silencingELKS with RNA interference (RNAi) in MIN6? cells (ref). In addition, anotherstudy has demonstrated that knockdown and overexpression of ELKS in RBL-2H3cells have resulted in a decrease and increase in their exocytotic activityrespectively (ref.). Therefore, our data showing less ?-hexosaminidase releasefrom KO PCMCs than WT PCMCs after stiumation (Fig.) further supported the roleof ELKS in positively regulating degranulation in mast cells through the use ofanimal model generated by the Cre/LoxP system. ELKS isconsidered to be an essential regulatory subunit within the IKK complex as knockingdown ELKS by RNAi inhibited expression of I? B? (ref.

). Here, we have shown thatKO mast cells have less I? B? mRNA expression. Furthermore, we have demonstratedthat the gene expression for some pro-inflammatory cytokines and chemokines arehigher in activated KO mast cells than in activated WT mast cells (Fig.), suggesting that ELKS might have an additional role in cytokine and chemokineproduction in mast cells. However, more biological repeats are needed toconfirm this result and secreted cytokines and chemokines should also bemeasured in the future experiments.

Taken together, components within the IKK complex, including ELKS, could contribute todifferent mast cell functions and our work will provide further insight intohow ELKS regulate mast cell functions and thereby extend our understanding inthe molecular mechanisms for allergic and anaphylactic

disorders and toidentify potential therapeutic targets for allergic inflammation.