The role of Mammalian Sterile 20-like Kinase 1 in humoral immunity

By

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DEDICATIONS

To the loving memory of my Mom, Azam and my Dad, Hossein

To my brother, Omid

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ABSTRACT (EN)

The serine/threonine protein kinase mammalian sterile 20-like kinase 1 (Mst1) is one of the key components of the evolutionarily conserved Hippo pathway that regulates multiple biological functions such as organ size, cell survival, proliferation, differentiation, and migration. Since the discovery of a primary immunodeficiency caused by *MST1*-null mutations in humans, substantial efforts have been made to characterize the function of MST1 in the immune system. So far, most studies have focused on the cellular immune responses and overall provided conflicting results regarding the role of MST1 in protective immunity. Counterintuitively, MST1-deficient patients show mildly elevated class-switched antibody titers. However, the quality of these antibodies and whether they provide long-lasting protective immunity has not been addressed in the previous studies. Thus, the role of MST1 in humoral immune responses remains largely unknown. Here, I hypothesized that the lack of protective antibodies may underlie immunodeficiency in humans as well as in mice. To test this idea, I analyzed antibody responses both under steady state and upon immunization using Mst1-deficient mouse models.

Using a T-dependent immunization system to mimic a vaccination condition in humans, I demonstrated that young adult Mst1-deficient mice mounted early IgG1 responses to normal levels. However, antigen-specific IgG1 titers as well as bone marrow plasma cells were progressively lost similar to patients that failed to mount protective titers against protein vaccines administered at early ages of life. I provided evidence that this defect arises from the hematopoietic compartments as radio-resistant stromal tissues could support the long-lived antibody titers in bone marrow chimeras. Importantly, plasma cell production was clearly augmented at the early stages of germinal center reaction due to dysregulation of germinal center dynamics in the absence of Mst1. Thus, these findings indicate that overproduction of plasma cells and presumably accelerated turnover of bone marrow plasma cells may cause the premature loss of antigen-specific plasma cells in Mst1-deficient mice. My results suggest that long-lived high affinity humoral immune responses may be defective in MST1-deficienct patients.

In contrast to IgG1 responses, Mst1-deficient mice raised and maintained elevated levels of high affinity IgE upon aging or immunization consistent with the hyper IgE titers observed in some of the patients. For the first time, here I provided evidence that hyper IgE can be

pathogenic since immunized Mst1-deficient mice suffered from anaphylactic shock when re-challenged with the immunizing antigens. Despite that fact that there are no reports of allergy in MST1-deficient patients, my results suggest that these patients may be prone to allergic reactions if exposed to certain antigens. Although we do not understand the underling mechanisms of IgE dysregulation in Mst1-deficiency, selective deletion of Mst1 in $Foxp3^{+}$ Treg cells in our conditional knockout model demonstrated that Treg-specific Mst1-deficiency leads to pronounced hyper-IgE phenotypes. In addition, consistent with our expectations, my results demonstrated multi-organ autoimmune inflammation in Treg-specific Mst1-deficiency. Therefore, my work clearly indicates that Mst1 plays an important role in regulating IgE production through controlling the Treg cells.

Taken together, my work illustrates the pivotal role of Mst1 in maintaining long-lived humoral immunity and controlling potentially pathogenic IgE contents in the mammalian immune system. Accordingly, prophylactic IgG replacement therapy may benefit MST1-deficient human patients. Further studies on Mst1-dependent immune regulatory mechanisms should help understand clinical aspects of the disease as well as fundamental biology of the immune system.

ABSTRACT (FR)

La protéine Sérine/Thréonine kinase 1 mammalienne (Mst1) est l'une des protéines clés de la voie métabolique Hippo qui régule de multiples fonctions biologiques telles que la taille des organes, mais également la survie, la prolifération, la différenciation et la migration cellulaire. Depuis la découverte d'une immunodéficience primaire causée par des mutations nulles de *MST1* chez l'homme, la recherche scientifique dans ce domaine s'est focalisée sur la caractérisation de la fonction de MST1 au niveau du système immunitaire. Jusqu'à présent, la plupart des études se sont concentrées sur les réponses immunitaires cellulaires et ont globalement fourni des résultats contradictoires concernant le rôle de MST1 dans l'immunité protectrice. Étonnamment et contrairement à ce que l'on pouvait attendre, les patients déficients en protéine MST1 présentent des concentrations modérément élevées anticorps protecteurs de diverses classes. Cependant, la qualité de ces anticorps et l'évaluation de leur protection immunitaire à long terme n'ont pas été abordées dans les études précédentes. Ainsi, le rôle de Mst1 dans les réponses immunitaires humorales reste largement inconnu. Dans cette présente thèse de recherche, j'ai émis l'hypothèse que l'absence d'anticorps protecteurs peut induire une immunodéficience chez l'homme ainsi que chez la souris. Pour tester cette idée, j'ai analysé les réponses en terme de production d'anticorps, à la fois à en condition normale et après vaccination, en utilisant des modèles de souris déficientes en Mst1.

Afin d'imiter les conditions de vaccination chez l'homme, j'ai utilisé un système de vaccination lié aux cellules T chez des jeunes souris adultes déficientes en protéine Mst1. Dans un premier temps, j'ai démontré que ces souris présentent des réponses précoces d'IgG1. Cependant, les concentrations d'IgG1 ainsi que des cellules plasmatiques de la moelle osseuse ont été progressivement diminuées. Ce résultat est semblable à ce qui a été observé chez des patients n'ayant pas réussi à maintenir des concentrations suffisantes d'anticorps contre des vaccins administrés en jeune âge. Dans un second temps, j'ai fourni la preuve que ce défaut provient des compartiments hématopoïétiques. En effet, des tissus stromaux résistants aux radiations pourraient supporter les titres d'anticorps à vie longue dans les chimères de la moelle osseuse. Enfin, et c'est un élément important, la production de cellules plasmatiques a été clairement augmentée aux premiers stades de la réaction du centre germinatif en raison du dérèglement de la dynamique du centre germinal causé par l'absence de protéine Mst1.

L'ensemble de ces résultats indiquent que la surproduction de cellules plasmatiques et le renouvellement "vraisemblablement accéléré" des cellules plasmatiques de la moelle osseuse peuvent entraîner la perte prématurée de cellules plasmatiques spécifiques à l'antigène chez des souris déficientes en Mst1. Mes résultats suggèrent que les réponses immunitaires humorales de haute affinité à longue durée de vie peuvent être défectueuses chez les patients présentant une déficience en protéine MST1.

Contrairement aux réponses de production d'IgG1, les souris déficientes en protéine Mst1 ont produit et maintenu des niveaux élevés d'IgE de haute affinité lors de leur vieillissement ou après la vaccination. Ces observations sont à mettre en parallèle avec la présence de concentrations élevées d'hyper-IgE chez certains patients déficients en protéine MST1. Pour la première fois, mon étude a fourni des preuves que la production d'hyper-IgE pourrait être pathogénique (avoir un effet délétère) puisque les souris déficientes en Mst1 vaccinées ont souffert d'un choc anaphylactique lorsqu'elles ont été réexposées avec des antigènes immunisants. Bien qu'il n'y ait actuellement aucune mention spéciale d'allergie chez les patients déficients en protéine MST1, mes résultats suggèrent que ces patients peuvent être sujets à des réactions allergiques s'ils sont exposés à certains antigènes. Bien que nous ne comprenions pas les mécanismes de la dérégulation des IgE chez les patients atteint d'une déficience en protéine MST1, la délétion sélective de *Mst1* dans notre modèle de knock-out conditionnel au niveau des cellules T régulatrice Foxp3^{+} a conduit à des phénotypes de production d'hyper-IgE prononcés. En outre, et conformément à nos attentes, mes résultats ont démontré une inflammation auto-immune multi-organe chez ce modèle de souris à cellules T régulatrice déficiente en protéine Mst1. Par conséquent, mon travail indique clairement que Mst1 joue un rôle important dans la régulation de la production d'IgE en contrôlant les cellules T régulatrices.

L'ensemble de mes résultats illustre le rôle central de la protéine Mst1 au niveau du maintien à long terme de l'immunité humorale et du contrôle potentiel du contenu en IgE pathogénique au niveau du système immunitaire mammalien. En conséquence, une thérapie prophylactique en IgG pourrait s'avérer bénéfique pour les patients déficients en protéine MST1. Des études complémentaires sur les mécanismes ou les voies métaboliques liées à la protéine Mst1 pourraient aider à comprendre les aspects cliniques de cette maladie ou plus généralement à comprendre les aspects fondamentaux de la biologie du système immunitaire.

PREFACE

In accordance with McGill University's guidelines for thesis preparation, the candidate has chosen to present the results of her research in manuscript format. A general introduction is given in chapter I, and a final conclusion and summary is presented in chapter IV. A comprehensive review of relevant literature has been modified from manuscripts under revision or preparation, and all results described in chapters II and III have been submitted or will be used for grant proposal and future publication.

1. Bagherzadeh Yazdchi S, Witalis M, Meli A, Leung J, Li X, Panneton V, Chang J, Nutt S, Johnson R, Lim DS, Gu H, King I, Suh WK. (2018). Hippo pathway kinase Mst1 is required for long-lived humoral immunity. Manuscript under revision in *Journal of Immunology*

2. Bagherzadeh Yazdchi S, Witalis M, Nutt S, Johnson R, Lim DS, Suh WK. (2018). Dysregulation of immunoglobulin E responses in Mst1-deficient mice. Manuscript is prepared for grant proposal and future publication.

AUTHOUR'S CONTRIBUTIONS

Chapter II:

A version of chapter II is under revision for publication in *Journal of Immunology*. **Bagherzadeh Yazdchi S.**, Witalis M., Meli A., Leung J., Li X., Panneton V., Chang JS, Nutt S., Johnson R., Lim DS., Gu H., King I., Suh WK. (2018). Hippo pathway kinase Mst1 is required for long-lived humoral immunity.

Mariko Witalis performed Enzymes linked immunosorbent assay (ELISA) and some FACS analysis and genotyping and proof reading of manuscript and this thesis.

Alex Melli infected the mice with *Heligmosomoides polygyrus* and performed Enzymes linked immunosorbent assay (ELISA) in parasite infection model.

Joanne Leung performed genotyping, qPCR and intracellular staining for Mst1 analysis with flow cytometry.

Xin Li performed staining for BrdU labeling for pulse-chase experiment.

Vincent Panneton performed some genotyping and Immunofluorscence Microscopy.

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Dr. Stephen L. Nutts provided the BLIMP1-GFP mice and insightful advice.

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Dr. Hua Gu provided insightful advice.

Dr. Irah King provided advice and was involved in conception of the project regarding parasite infection model.

Dr. Woong-Kyung Suh provided valuable guidance to design experiments and interpret the results. He wrote most of the manuscript for chapter II, proofread and edited it and supervised the study. **I, Sahar Bagherzadeh Yazdchi** performed mice dissections and harvested organs and cells for experiments, did part of the Intraperitoneal injections and cheek bleeding, performed intensive genotyping, Flow cytometry, Immunohistochemistry, Enzymes linked immunosorbent assay (ELISA), Enzyme-Linked ImmunoSpot (ELISPOT), Immunofluorscence Confocal Microscopy, BrdU labelling pulse-chase assay and Migration Transwell assays. I also designed and performed experiments, analyzed and interpreted the data, and wrote part of the manuscript and prepared figures for chapter II.

Chapter III:

All results described in chapter III will be used for grant proposal and future publication.

Mariko Witalis performed Enzymes linked immunosorbent assay (ELISA) and proofread chapter III.

Dr. Dae-Sik Lim provided the Mst1 germline knockout and insightful advice.

Dr. Randy Johnson provided the Mst1 conditional knockout mice and insightful advice.

Dr. Woong-Kyung Suh provided valuable guidance to design experiments and interpret the results. He proofread and edited it and supervised the study.

I, Sahar Bagherzadeh Yazdchi performed mice dissections and harvested and prepared organ sections for hematoxylin and eosin (H&E) staining, did part of the intraperitoneal injections, performed intensive genotyping, Enzymes linked immunosorbent assay (ELISA) and monitored mice body temperature for evaluation of anaphylactic shock. I also designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript and prepared figures for chapter III.

The candidate was also involved in collaborations with other research projects, which resulted in the following publications:

1. Panneton V, **Bagherzadeh Yazdchi S**, Witalis M, Chang J, & Suh W K. (2018). **ICOS Signaling Controls Induction and Maintenance of Collagen-Induced Arthritis**. *The Journal of Immunology*, ji1701305.

2. Leconte J.*, **Bagherzadeh Yazdchi S***, Panneton V*, Suh WK. (2016). **Inducible costimulator (ICOS) potentiates TCR-induced calcium flux by augmenting PLCγ1 activation and actin remodeling**. *Molecular Immunology* 79: 38-46. * equal contribution.

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Chapter I

Introduction

1. Introduction

In 2003, *hpo* was identified by several groups as a gene encoding the Hippo (Hpo) serine/threonine kinase that restricts organ size in *Drosophila melanogaster*, thus mutations of which led to tissue outgrowth giving "hippopotamus"-like phenotypes (1-3). The core components of the Hippo signaling pathway were first identified as tumor suppressors in early genetic screens to identify mutations that restrict cell growth and organ size in *Drosophila*. As such, s*alvador* (*sav*) and *warts* (*wts*)/*large tumors* (*lats*) were identified among the few genes that regulate both proliferation and cell death, thus their mutations have been shown to promote proliferation and impair apoptosis. So far, there are more than 30 components discovered in this pathway, which are regulated through various internal and external signals (4). Consistent with a high level of evolutionary conservation from flies to mammals, components of Hippo pathway merged as key signaling molecules implicated in numerous diseases including cancer, endothelial malformations, and autoimmunity. Although its functional ortholog was discovered in flies, mammalian Hippo kinases belong to a group of "mammalian sterile 20-like kinases (Mst)", whose prototype yeast homologs are known to act downstream of pheromone-mediated mating pathway (5).

More recent evidence implicates that the Mst/Hippo pathway has gained new functions during evolution such that it interacts with mediators of other signaling networks and performs in a highly context-dependent manner. Indeed, Hippo signaling pathways in mammalian systems have been shown to mediate different physiological functions such as regulating organ size, cell proliferation, apoptosis, tumorigenesis and tissue regeneration (6). Extensive studies in mammalian cell lines and knockout mouse models provided insights into the molecular mechanisms of regulation, activation, and function of this pathway.

Importantly, the Mst/Hippo signaling pathway has most recently received the greatest clinical attention following the emergence of several reports of human patients harboring autosomal recessive null-mutations in *STK4* (also known as serine/threonine kinase-4) gene encoding MST1 protein. These patients manifest primary immunodeficiency suffering from recurrent infections before reaching 10 years of age. However, except for lymphopenia, specific immunological defects underlying weakened protective immunity remain largely unknown.

Given the intensity of studies performed over the past decade on Hippo signaling pathways and its relevance to human primary immunodeficiency as shown in MST1-deficient patients, this chapter will begin with the key concepts of the Mst/Hippo signaling pathway. Next, I will outline the previous findings in both mouse and human studies of MST1-deficiency. Lastly, I will bring up questions that shaped the rationale of my thesis work.

2. The Hippo Pathway

2.1 The Canonical Hippo Pathway

The canonical mammalian Hippo pathway has been known to be a kinase cascade in which the mammalian sterile 20-like kinase 1 (Mst1) and its homolog Mst2 (corresponding to Hippo (7) in *Drosophila*) initiate a cascade of downstream signaling events (**Fig 1**). Mst1/2 phosphorylate and activate "Large tumor suppressor 1/2" (Lats1/2; corresponding to Warts (Wts) in *Drosophila*). Association of Mst1/2 with WW-domain scaffolding protein WW45 (Salvador, corresponding to Sav in *Drosophila*) facilitates phosphorylation of Lats1/2. Moreover, phosphorylation of "Mps one binder kinase activator-like 1" (Mob1A/B, corresponding to Mats in *Drosophila*) by Mst1/2 also has been shown to facilitate Lats1/2 activation. Consequently, Lats1/2 phosphorylation leads to the negative regulation of two transcriptional coactivators, Yes-associated protein (YAP) and transcriptional coactivator TAZ; which are two homologs of *Drosophila* Yorkie [Yki]). In the active form, YAP and TAZ translocate and bind the TEAD transcription factor family [homologs of *Drosophila* Scalloped (8)] in the nucleus and stimulate the expression of a wide range of genes that are involved in cell proliferation, survival, and migration (9, 10). However, in both *Drosophila* and mammals, the Lats/Wts-mediated phosphorylation of YAP/Yki restrains these proteins in the cytoplasm through interaction with 14-3-3 protein and this leads to downregulation of downstream genes (11). Of note, Casein kinase 1δ/ε also phosphorylates YAP/TAZ in a process triggered by Lats-mediated phosphorylation and subsequently causes the ubiquitination and degradation of these proteins through recruitment of the ubiquitin ligase (12). Collectively, in the canonical Hippo signaling pathway, upstream sensors of cell-cell contact and hormonal signals lead to a cascade of protein phosphorylation that ultimately suppresses the key oncogenic transcription factor YAP thereby slowing down cell proliferation.

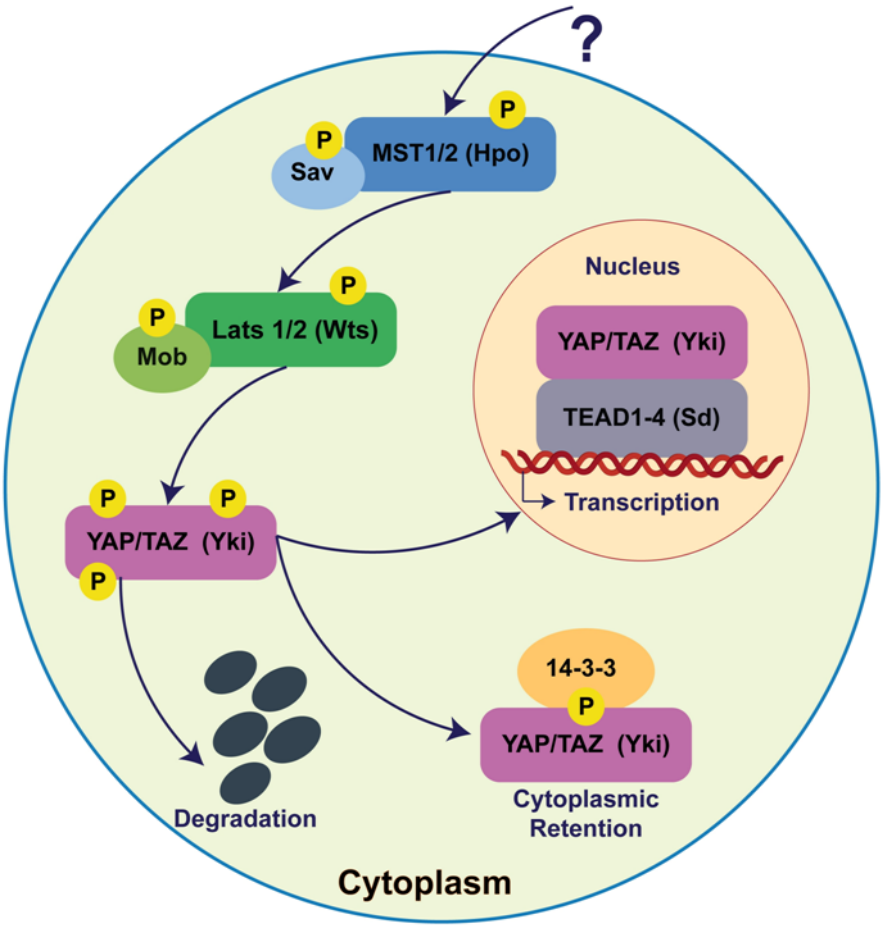

Figure 1. The canonical Hippo pathway. Following phosphorylation of Sav, Lats1/2, and Mob by MST1/2; YAP/TAZ get phosphorylated and thereby interact with 14-3-3 protein leading to their cytoplasmic retention. In addition, YAP/TAZ phosphorylation leads to protein degradation. On the other hand, dephosphorylation of YAP/TAZ enables them to enter the nucleus and induce gene transcription by interacting with transcription factors TEAD1–4. The *Drosophila* orthologs are shown in parentheses.

2.2 Mechanisms of Canonical Hippo Pathway Activation

Consistent with the role of Hippo signaling in suppressing tissue outgrowth, cell surface adhesion molecule Fat and cytoskeletal regulators such as Expanded have been shown to be involved in the activation of the Hippo pathway (13). However, the exact extracellular cues and molecular mechanisms leading to Hippo kinase activation remain to be elucidated. In an attempt to identify the immediate upstream players of Hippo kinase, Boggiano *et al.* showed that Tao-1, a sterile 20 family kinase, directly phosphorylates Hpo in *Drosophila*. Furthermore, they

confirmed that mammalian ortholog of Tao-1, TAOK3, was able to directly phosphorylate Mst1/2 and initiate the activation cascade of these kinases (Thr183 for Mst1 and Thr180 for Mst2)(14). In addition, it has been shown that Mst1/2 autophosphorylation enhanced by Mst1/2 dimerization can also initiate the activation process (15, 16). Therefore, activation of Mst1/2 can be independent of upstream kinase signaling cascade. More recent studies have shown the involvement of other kinases such as AKT and mTOR in phosphorylation and thereby modulation of Mst1/2 in multiple sites (17, 18). Given that Ras-association domain family (RASSF) proteins along with Mst and WW45 are known tumor suppressors which interact through their coiled-coil SARAH (Salvador/Rassf/Hippo) domain, it is known that RASSF1A and RASSF5 (NORE1) biochemically regulates Mst1 kinase. However, RASSF proteins play a dual function in regulating Mst1/2 depending on their activation status. Despite the known inhibitory function of RASSFs on Mst1/2 which prevents their dimerization and autophosphorylation, interaction of RASSFs with Mst1/2 in their active state sustains their kinase activity and thereby, recruits them to the membrane and induces apoptosis (19).

Notably, upon Fas-mediated apoptosis, Caspase 3/6/7 cleave both MAP4Ks (mitogen-activated protein kinase kinase kinase kinase) and Mst1/2. Following the Caspase-mediated cleavage, Mst1/2 kinases lose their coiled coil and SARAH domains which are required for interactions with other components of Hippo pathway such as Lats1/2 and WW45. It is therefore suggested that Caspase-mediated activation of Mst1/2 and MAP4Ks may have redundant functions (9, 20).

While our knowledge regarding upstream regulators of Hippo pathway is still limited, other studies investigated downstream targets of the Hippo pathway components in more detail. As such, one of the proteins that get recruited downstream of activated Mst1/2 is Mob1 which interacts with N-terminal regulatory domain of the kinases (9). Recently, it has been shown that following autophosphorylation and activation of Mst2, Mob1 protein gets recruited and then Mob1-phosphoMst2 complex binds to Lats1 which then leads to further phosphorylation of Mob1 and Lats1. Afterwards, Mob1 phosphorylation induces the dissociation of Lats1 and Mob1 from Mst2 leading to the activation of Lats1 (21). Moreover, along with Mst1/2, MAP4Ks can also directly phosphorylate and thereby activate Lats1/2 in an MST-independent pathway.

Further investigations are required to better understand the molecular mechanisms of activation in response to various upstream signals.

2.3 The Non-Canonical Hippo Pathways

In addition to the known Mst-Lats-Yap phosphorylation signaling cascade in regulating cell proliferation and differentiation, recent studies showed that the core components of the Hippo pathway are also involved in multiple non-canonical signaling networks in various biological processes. Accordingly, Mst1 and Mst2 have been shown to regulate apoptosis in a context-dependent manner. Apoptosis or programmed cell death plays a key role in cell development and homeostasis of multicellular organisms. Early studies in primary mammalian neurons showed that Mst1-mediated phosphorylation and activation of the Foxo1/3 forkhead transcription factors enhances their nuclear translocation and thereby induces cell death (22, 23). In another aspect, it has been shown that Mst1 gets cleaved and thereby activated by caspase-3. Therefore, activated Mst1 further phosphorylates the histone H2B at serine 14 (S14) and this post-translational modification of histones is suggested as part of apoptotic chromatin condensation mechanism (24). Yet another study has implicated a different aspect of a non-canonical signaling in which Mst1 initiates cell death in insulin producing β-cells. Given that the loss of these β-cells is the hallmark of diabetes mellitus, Mst1 has been recently identified as the key regulator of β-cell apoptosis and dysfunction. Indeed, under diabetogenic conditions, Mst1 activates the Bcl-2-like protein 11 (BIM)-dependent mitochondrial apoptotic pathway which further activates the caspase machinery. In addition, Mst1 has been shown to target β-cell function through phosphorylation of β-cell transcription factor PDX1 and promoting its degradation which impairs insulin secretion (25).

Moreover, the mammalian homolog of Sav (WW45) forms a complex with Mst2 and Runx3 both in *Drosophila* and human cells promoting apoptosis through non-canonical Hippo signaling (7). Runx family of transcription factors are known as key players of hematopoiesis, neurogenesis, bone development, and segmentation in vertebrate embryos. Particularly, Runx3 functions as a tumor suppressor in multiple cancers presumably through induction of apoptosis by upregulating the apoptotic regulator Bim (Bcl2L II) (26).

Mst1 has been shown to be capable of inducing apoptosis through suppression of AKT and its downstream survival signals in tumor cell lines (27). The AKT kinases are members of the AGC kinase subfamily (cytoplasmic serine/threonine protein kinase A, G and C) which mediate cell survival through antagonizing various apoptotic signals and become activated by phosphoinositide-3-kinase (PI3K). In this regard, it has been shown that Mst1 can directly bind and inhibit AKT and thereby perform a pro-apoptotic function.

Likewise, recent findings revealed a link between Mst1/2 and autophagy via yet another non-canonical pathway in which Mst1/2 directly phosphorylate autophagy mediator Beclin1, and therefore block autophagy in a mouse model of myocardial infarction (28). However, a more recent study by Deepti's group revealed an opposite function for Mst1 such that direct phosphorylation of autophagy mediator LC3 by Mst1 promoted autophagy (29). Therefore, it has been suggested that Mst1/2 kinases have multifaceted functions in autophagy which are possibly due to interactions with multiple substrates.

Taken collectively, these observations suggest that Mst1/2, as core components of the Hippo pathway participate in several non-traditional signaling cascades in order to mediate different physiological functions depending on the context. Therefore, it became clear that Mst1/2 participation in biological functions in mammalian cells is beyond the YAP/TAZ limitations. Of note, more recent studies have shown that the core components of the Hippo/MST pathway play crucial roles in adaptive immune responses through non-canonical pathways which will be discussed in more detail later in section 2.5 of this chapter.

2.4 Mst Kinase Family

Protein kinases and phosphatases are involved in well conserved catalytic signaling cascades that regulate cell growth and differentiation in all eukaryotes. Following the interactions of external stimuli with growth factors and some G protein-coupled receptors, members of the mitogen-activated protein kinase (MAPK) family, ERK1 and ERK2 get activated, leading to phosphorylation and thus activation of various substrates. Preliminary studies of yeast pheromone signaling pathway have revealed similar signal transduction mechanisms which demonstrated the structural homology between mammalian and yeast

pathways (30-32). Sterile 20 (Ste20) was the first component of the new family of serine/threonine protein kinases to be identified in the mating pathway of *S. cerevisiae*, downstream of pheromone-linked G protein. Later studies have identified several homologs to Ste20 in both mammals and yeast and these homologs have been mainly categorized into two classes; (1) the ones that are activated and thereby bind to the small GTPases namely Cdc42 and/or Rac1, (2) those that do not fall into the former class and are regulated in other manners. The former members including Ste20 have been shown to mediate variety of cytoskeleton effects in early studies whilst the later class members including the Sps1 in yeast and GC kinase in human have been shown to be involved in spore formation and stress-mediated kinase pathway. However, two other human protein kinases of the later class, Mst1 and Mst2 are known to mediate different functions depending on the context although details of all these mechanisms are not fully understood (33). Overall, the role of Ste20 in yeast is mostly distinct from those in flies and mammals.

The "STE" superfamily of kinases are named after yeast Sterile 20 (Ste20) protein kinase (5). The STE superfamily contains several subfamilies, one of which is "mammalian Sterile 20-like" (Mst) family with 5 Mst kinases in mammals; Mst1 (a.k.a STK4), Mst2 (a.k.a STK3), Mst3 (a.k.a STK24), Mst4 (a.k.a STK26), YSK1 (a.k.a STK25). Importantly, the Mst kinases are divided into two structurally distinct groups; p21-activated kinase (PAK) and the germinal center kinase (GCK). The latter is further subdivided into eight subfamilies, all of which contain a conserved N-terminal kinase domain and a diversified C-terminal domain (34). It is shown that GCKII subgroup includes Mst1 and Mst2 whereas Mst3 and Mst4/YSK1 are considered in GCKIII subgroup with distinct biological functions despite being related to the former subgroup. Compared to Mst1/2, the regulation and activation mechanisms of Mst3, Mst4, and YSK1 are less clearly understood. Studies using mammalian cell cultures revealed that they mediate cell polarity as part of another complex called PP2A, and Hippo signaling pathway is not involved in this regard.

Following the pioneering studies, numerous studies have been done using conditional mouse knockouts of Mst1 and Mst2 which reinforced Mst kinases as key players of the Hippo signaling pathway to mediate cell survival, proliferation, differentiation, and migration (35). Accordingly, the Mst1/Mst2/Lats signaling network has been shown to be critical in cell

proliferation and cancer development. The Mst1/2 proteins contain a short N-terminal catalytic domain, an autoinhibitory segment, a dimerization domain and a nuclear localization motif at the non-catalytic, C-terminal part (8, 33). The SARAH domain located in the C-terminus of these proteins facilitates autophosphorylation and thus initiates activation processes via formation of antiparallel homodimers. Consequently, activated Mst1 and Mst2 homodimers are known to dissociate and thus become targeted to various substrates or subcellular locations mainly through interactions with Sav1 and RASSFs proteins (35).

2.5 Biological Functions of Mst1/2 in Mammalian Cells

2.5.1 Regulation of Organ Size

It is known that in mammalian cells, Mst1 functions in a highly context-dependent manner and thereby Mst1-deficiency in different tissues results in a wide spectrum of defects such as survival, proliferation, differentiation, migration. One of the key concepts in developmental biology is the regulation of organ size during normal development. For this, contact inhibition is known as a key player to ensure that cell proliferation is seized once cells are confluent. Therefore, modulation or loss of this regulation mechanism was suggested to be the cause of cancer development in a majority of human cancers (36). Indeed, the Hippo signaling pathway has been identified as a network of tumor suppressor genes in flies which regulates organ size. Therefore, Hippo pathway mutants have been shown to result in overgrowth of imaginal discs and adult organs (37). Hence, it was expected that the Hippo signaling pathway was required for the regulation of organ size in mammalian cells and thereby suppression of tumor formation. Later, more evidence revealed the Hippo pathway to be the main signaling pathway that mediates cellular responses to various physiological stimuli (9). In this regard, Mst1 and Mst2 conditional knockout mice were generated and it has been shown that Mst1 and Mst2 cooperate to suppress proliferation of mature hepatocytes and thereby inhibit tumor formation in mouse liver. Therefore, YAP protein accumulation in nucleus of hepatocytes in knockout mice suggested that the canonical Hippo signaling pathway operates in the similar setting as limiting cellular proliferation by sensing neighboring cells (38). In accordance with mouse studies, elevated nuclear localization of YAP protein has been reported in most human hepatocellular carcinomas indicating suppressed Hippo pathway signaling (39).

2.5.2 Cell Death and Cell Proliferation

Another key player that affects cell survival and homeostasis is oxidative stress. Initial studies have shown that oxidative stress modulates gene expression profiles which then leads to either programmed cell death or cell recovery (40). Protein kinases have been reported to be crucial in transferring external signals to the nucleus and likewise, regulate the cellular responses to oxidative stress (41, 42). Among the key players involved in this process, FOXO transcription factors are known to trigger apoptosis or mediate adaptive responses following exposure to oxidative stress in mammalian cells. Therefore, underlying regulation mechanisms of FOXO proteins have been extensively studied. Results of these studies implicated that FOXO proteins are directly phosphorylated by AKT at distinct sites which consequently leads to their cytoplasmic sequestration and inhibits their function. In addition, it has been shown that the PI3K/ AKT kinase cascade activated by insulin-like growth factors inhibits FOXO protein activity (43, 44). However, further light was shed by the discovery of FOXO transcription factors as targets of the Mst kinase family which has been shown to be conserved during evolution. Accordingly, it was demonstrated that Mst1 gets activated upon oxidative stress in mammalian primary neurons and activated Mst1 phosphorylates FOXO1/3a proteins at their conserved sites disrupting their interaction with 14-3-3 proteins. These Mst1-mediated events lead to dissociation of FOXO1/3a proteins from 14-3-3 proteins and through their nuclear translocation induces neuronal cell death (22). Likewise, *in vitro* studies of primary culture neurons and rat hippocampal neurons demonstrated that oxidative stress mediated tyrosine phosphorylation of Mst1 activates the Mst1-FOXO3 signaling pathway and thereby induces cell death. Collectively, these data indicate that the Mst1-FOXO signaling pathway is central in cellular responsiveness to oxidative stress and therefore may contribute to pathological complications such as neurodegenerative diseases in mammalian central nervous system (10). Indeed, the Mst1-Foxo3a pathway promotes β-amyloid-induced neuronal cell death implicating its role in Alzheimer's disease (45). Similarly, another group reported that loss of Mst1 causes enhanced proliferation and increased survival of skin fibroblast cells reprogramming to induced pluripotent stem cells (iPSC). In this setting, they showed that ablation of Mst1 activated YAP protein and further YAP nuclear translocation induced proliferation and thereby reduced apoptosis (46).

Despite the fact that Mst1 was first identified to have anti-proliferative and pro-apoptotic functions and therefore induced apoptosis in early tumorigenesis studies, later studies noted that it can also play an anti-apoptotic role in lymphocytes. Both murine studies and human reports of Mst1-deficiency indicated that Mst1 is expressed most abundantly in lymphoid tissue (47, 48). In light of studies on mouse models of Mst1-deficiency, Avruch's group showed that despite normal T cell development, mature naïve T cell numbers were lower than normal whilst effector/memory T cell numbers were intact. Consistent with previous findings, Mst1-null naïve T cells exhibited increased proliferation upon receptor stimulation *in vitro*. However, freshly isolated Mst1-deficient T cells had higher rates of apoptosis along with reduction of cell cycle inhibitory proteins MOBKL1A/B which are among the known targets of Mst1 and the only ones to be affected in this study. Therefore, these observations suggested that Mst1/2-mediated phosphorylation of MOBKL1A/B suppresses the proliferative responses upon TCR stimulation in naïve T cells. However, analysis of apoptosis rate in Mst1-null naïve T cells versus mature effector/memory T cells *in vitro* and *in vivo* generated controversial data. In contrast to earlier findings that overexpression of Mst1 promotes apoptosis (49); in this study it was observed that Mst1-null naïve T cells exhibited higher rates of apoptosis. Collectively, these observations suggested that Mst1 can exert an anti-apoptotic function in T cell subsets (48).

Further studies shed more light in regard to new features of Mst1 kinase activity in the immune system. Maintenance of T cell populations is critical for host defense. Of note, both extracellular and intracellular signaling mechanisms such as Fas, TNF and ROS (reactive oxygen species) are known to trigger apoptosis and participate in these regulation mechanisms. In sharp contrast to neuronal cells, Mst1-FOXO signaling in T cells play anti-apoptotic roles (50). Mechanistically, Mst1-mediated stabilization and nuclear transportation of FOXO1/3a promotes expression of superoxide dismutase and catalase which protect naïve T cells from ROS-induced cell death.

2.5.3 Cell Polarization, Adhesion, and Migration

In addition to cellular proliferation and death, Mst1 regulates LFA1 clustering. Given the importance of immune cell trafficking and its dynamics during the immune response, studies have shown that integrins, particularly those expressed by leukocytes such as LFA1 (lymphocyte

function-associated antigen 1), VLA4 (very late antigen 4) and α4β7-integrin play important roles in cell adhesion and migration (51, 52). During this coordinated process, cell polarization and regulated adhesion leads to cytoskeletal reorganization via vesicle transport and integrin recycling (51). In this regard, it is known that protein and lipid kinases, small GTPases of the Ras and Rho families, and their regulators and adaptor proteins modulate the integrin mediated adhesive functions (52, 53). T cell receptor stimulation leads to activation of the small GTPase Rap1, which is known to be required for transmigration of lymphocytes through endothelial cells and during immunological synapse formation with antigen-presenting cells. Consequently, Rap1 interacts with RAPL (also known as Nore1B) effector protein which then mediates spatial distribution of integrin LFA1. Further investigations in regard to downstream molecular mechanisms identified Mst1 kinase as the downstream effector molecule of RAPL. Collectively, these observations implied a new role for Mst1 in the regulation of lymphocyte polarization and adhesion through LFA1 clustering downstream of Rap1-RAPL interaction (54). Accordingly, it has been shown that Mst1 is required for LFA1-dependent thymocyte migration and antigen recognition during negative selection of thymocytes (55).

Other studies further revealed that Mst1 is required for efficient thymocyte egress (56) as well as lymphocyte homing within lymph nodes (57). In an effort to further investigate the biological roles of Mst1, Dong's group generated Mst1 knockout mice. Despite the observed lymphopenia in their model, the apoptosis rate of freshly isolated activated T-cells from lymph nodes was shown to be only marginally increased in Mst1-deficient mice. Given the significance of CCL19 and CCL21 chemokines that mediate thymocyte migration during developmental stages of T cells in thymus and their known involvement in thymus egress (58, 59), it has been suggested that reduction of mature peripheral thymocytes was due to the defective responses to chemokines as evidenced by lower chemotactic responses of Mst1-deficient thymocytes (60). Similarly, Mst1 mediates trafficking of dendritic cells (DC) from skin to the draining lymph nodes as stable adhesion to ICAM and fibronectin was impaired in Mst1-deficient DCs (57). Collectively, these results show that Mst1 plays critical roles in immune cell polarity, adhesion, interstitial motility, and migration through non-canonical pathway.

2.6 Clinical Findings of MST1-deficiency in Human Patients

Initial studies of the Hippo signaling pathway provided evidence that Mst1/2 and Lats1/2 function as tumor suppressors and loss of these key components leads to activation of YAP/TAZ oncogenic factors and thereby hyperproliferation and cancer (61, 62). In particular, Mst1 has been shown to be significant in prognosis of cancer in patients with MMR (mismatch repair) deficient colorectal cancer (63) and breast cancer (64). However, it was only until recently that human patients with MST1-deficiency were identified and reported. In 2012, two separate studies described a novel phenotype in human patients harboring autosomal recessive null-mutations in *STK4* gene encoding MST1. The MST1 loss-of-function mutations were nonsense mutations in the kinase or regulatory domains of MST1 (47, 65). These patients suffered from primary immunodeficiency associated with T and B cell lymphopenia and thereby featured recurrent bacterial, viral and fungal infections at early ages (1-10 years). Moreover, MST1-deficient patients manifested reduced naïve T-cell numbers and defective T-cell survival as evidenced by increased apoptosis in Fas-mediated cell death assays (47). Collectively, the key features in human patients namely lymphopenia and increased apoptosis of naïve T-cells were consistent with findings in Mst1-deficient mice (48, 60). Consistent with several murine studies of Mst1-deficiency, reduced levels of FOXO1 transcription factors, IL-7R, CCR7, CD62L, and transcription factor Krüppel-like factor 2 (KLF2) have been reported in MST1-deficient human patients (47, 66, 67).

Another feature of MST1-deficiency is the presence of autoantibodies and autoimmune phenotype in some of the patients (47, 65) consistent with mouse models of Mst1-deficiency (68, 69). Importantly, regulation and fine-tuning of immune responses is the key factor of efficient protection. In this regard, regulatory T cells (Tregs) have been identified as key regulators of the immune system to maintain dominant tolerance and thereby prevent autoimmune diseases (70). In light of mouse studies, it has been shown that Mst1 is critical for development and function of regulatory T cell (Treg). Indeed, Mst1-null young mice have been reported with reduced number of Tregs both in the thymus and periphery (70). In addition, it has been shown that TGF-β-mediated differentiation of naïve T cell into Tregs is defective in Mst1-deficient mice (71). Given previous findings that lineage specific transcription factor FOXP3 can directly be

upregulated through FOXO1/3 transcriptional factors and further observations in human patients with reduced FOXO1/3 protein levels in MST1-deficient T cells, one would expect that loss of MST1 in T cells and particularly in Tregs is the cause of defective Treg function making the patients prone to autoimmune diseases. However, further investigation is required to better understand the consequences of MST1-deficiency in human patients.

Despite the impaired adaptive immunity reflected by recurrent episodes of viral infections with HSV, HPV and cutaneous molluscum contagiosum, serological profile of the patients showed detectable antibody titers and almost all patients had higher titers of IgG and IgA. Given that patients had no signs of active infection, these antibody titers could be the result of vaccination or earlier infections.

Similarly, another study of inherited MST1-deficiency demonstrated profound T cell deficiency which was featured in susceptibility to viral, bacterial and fungal infections in a 19-year-old patient (72). MST1-deficiency was caused by a nonsense mutation in the kinase domain, upstream of previously known mutations in human patients. Notably, in contrast with previous reports, T cells were hypoproliferative following TCR stimulation and were defective in recall responses to antigens despite normal immunization against tetanus toxoid and PPD. Consistent with previous findings; the patient had elevated levels of serum IgA and IgE with slightly higher IgG titers but surprisingly was incapable of developing antibody responses to several immunizations.

Collectively, these observations indicate that T cell lymphopenia is a probable cause of immunodeficiency in MST1-deficient patients. However, it needs to be explained why patients experience recurrent infections despite the elevated levels of serum antibody titers.

3. Humoral Immune Response

The immune system is a highly sophisticated multilayer defense system that mounts protection against pathogens through 3 major mechanisms: (1) physical and chemical external barrier, (2) innate and (3) adaptive immunity. The innate immune responses are the first line of defense against pathogens that have entered the body. However, the innate immunity is quick but lacks memory for future encounters. Adaptive immunity is slower but capable of establishing a

state of “memory” which is supposed to efficiently protect the body against re-infection of the same agent (73). The lymphoid organs of the immune system are divided into primary organs including the bone marrow and thymus, which generate lymphocytes and secondary organs including lymph nodes, spleen and the mucosal and gut-associated lymphoid tissues where the adaptive responses initiate. Adaptive immunity is further subdivided into 2 major arms namely cell-mediated and humoral immunity. Lymphocytes (T and B cells), the key players of adaptive immunity, are generated from bone-marrow hematopoietic precursors and migrate to the secondary lymphoid tissues to further differentiate into mature effector cells. Adaptive immune cells contain multiple lineages amongst which the mature B lymphocytes (B cells) are responsible to produce antibody responses in a T-dependent (TD) or T-independent (TI) manner. The heterogeneous mature B-cell repertoire contains multiple subsets of B-cells including B1-cells, marginal zone (MZ) and follicular (FO) B-cells which have distinct functions and originate from different progenitors (74).

In TI immune responses, B cells with MZ and B1 phenotype initiate the early wave of antibody following antigen encounter and this microenvironment is known to be critical when dealing with bacterial and viral pathogens. B1 cells have been speculated to associate with antigens within mucosal lymphoid tissue, whereas MZ B cells deal with blood-borne pathogens due to their specific localization in the spleen. Therefore, splenic MZ B cells are known to rapidly differentiate into plasma cells (PC) that are short-lived by nature and can give rise to multivalent, cross-reactive antibodies with low affinity. In contrast, TD responses are mainly mediated by FO B cells and require professional help of T follicular helper (Tfh) cells through germinal center (GC) reaction. Consequently, FO B cells differentiate into antibody secreting PCs which produce class switched, high affinity antibodies of different Ig isotypes that are long-lived by nature. Given the pivotal significance of B cells and their antibody products in humoral immunity as part of the adaptive immune responses in collaboration with T cells, here I will describe the key steps of B and T cell biology and antibody production and will further expand the topic over PC development, maturation and longevity.

3.1 B Lymphocyte Biology

3.1.1 Early Development in the Bone Marrow

B cell development initiates in the bone marrow from pluripotent hematopoietic stem cells (HSCs) which are generated in the fetal liver and later seed the bone marrow. In the current view, HSCs give rise to multi-potent common lymphoid progenitors (CLPs) which can further differentiate into T, B and innate lymphoid cells and some dendritic cell (DCs) lineages. Importantly, fetal-liver derived stem cells are known to give rise to B1 cells whereas the majority of circulating B cells including FO and MZ B cells are derived from bone marrow stem cells. The key features of CLPs are the cell surface expression of c-Kit^+ and IL-$7Ra^+$ while they lack the surface expression of multiple lineage markers including B220, CD4, CD8, CD11b, GR1 and Ter119 (75). B lineage differentiation can be fractionated into multiple sequential stages called Hardy's fractions A to D following his pioneering work. These stages segregate according to immunoglobulin (Ig) gene segment rearrangement status (76). Accordingly, EBF, E2A and Pax-5 transcription factors commit the fate of developing lymphocytes into B lineage by upregulating genes facilitating B cell differentiation such as recombination activation genes 1 and 2 (Rag1/2).

Early developmental stages initiate with Ig gene segments rearrangement under a process called V(D)J recombination which is responsible for the generation of diverse repertoire. As such, the Ig heavy chain (H-chain) D_H, and J_H gene segments rearrange and start the pro-B cell stage which is followed by a second rearrangement that joins the V_H to the DJ segment. The pre-B cell stage initiates when the functional μ-H-chain gene segments are rearranged and is followed by expression of the surrogate light chain and signal-transducing components Ig-α and Ig-β, which subsequently form the so-called pre-B-cell antigen receptor (pre-BCR) complex. This shuts down the machinery that mediates rearrangement of the H-chain in a process called allelic exclusion and thereby initiates light chain (L-chain) gene segments rearrangement (77). If the newly formed Ig L-chain variable (VL) regions fit with pre-existing H-chain variable (VH) regions, then immature B cells expressing surface IgM arise. In addition, the newly formed immature B cells may undergo BCR-mediated negative selection process through which B cells expressing high-affinity autoreactive BCRs die by apoptosis. Then, positively selected immature B cells with low self-reactivity leave the bone marrow and seed the gut-and lung-associated lymphoid tissue pools as progenitors of B1 type B cells whereas immature B cells incapable of recognizing autoantigens will enter peripheral pools in the spleen and lymph nodes to later shape the conventional pools of FO and MZ B cells (78).

3.1.2 Migration of Newly Generated B Cells into Secondary Lymphoid Organs

Following positive selection and successful surface expression of the BCR, immature B cells emigrate from the bone marrow, circulating through the bloodstream targeting secondary lymphoid organs such as spleen, lymph nodes, tonsils, Peyer's patches and mucosal tissues to home inside B cell follicles. G protein coupled proteins have been shown to mediate this migratory network. Consistently, studies of sphingosine-1-phosphate (S1P) receptor deficient mice implicated that immature B cell emigration from bone marrow is partly mediated with S1P receptor 1 (79). In the spleen, immature B cells reside inside niches and form MZ and FO B cell compartments. Of note, Cannabinoid receptor 2 (CB2) is another mediator involved in positioning of cells into splenic MZ in mice and it has been shown that CB2 positively regulates the TI immune responses (80).

3.1.3 Transitional 1 and 2 (T1 and T2) B Cells

Upon arrival to secondary lymphoid tissues, immature B cells undergo multiple stages of maturation. The newly generated immature B cells in spleen and bone marrow are called transitional 1 (T1) B cells that express $B220^{+}IgM^{hi}CD21^{lo}CD23^{-}CD93^{+}$ and yet need to acquire the ability to recirculate. Inside the follicles, T1 B cells mature into transitional 2 (T2) B cells characterized by surface expression of $B220^{+}IgM^{hi}CD21^{lo}CD23^{+}CD93^{+}$(80). Of note, there is yet another checkpoint screening for these transitional B cells which leads to anergy or apoptosis of potentially autoreactive B cells in the pool (78).

3.1.4 Marginal Zone B Cells

In the final stages of development within the spleen, immature B cells differentiate into naïve, MZ and FO B cells. Despite that MZ B cells have been identified in both mice and humans which express IgM and CD21 coreceptors, they are known to occupy different compartments. Indeed, MZ B cells are found only within the spleen in mice whereas in humans they can occupy both the spleen and lymph nodes. Given the limited diversity of MZ B cells and their primary localization around splenic marginal sinuses at the interface between the red pulp and the white pulp, these cells are considered as the first line of defense against blood-borne pathogens that get activated within the first 3 days of exposure and thereby rapidly differentiate

into short-lived IgM-secreting plasma cells (81). Splenic MZ B cells are characterized to be $B220^{+}IgM^{hi}IgD^{low}CD21^{hi}CD1d^{hi}CD23^{-}$ and during differentiation they gain the ability to self-renew and thereby have an unlimited lifespan. However, the mechanisms of differentiation and final development are yet to be understood in more detail (82). Importantly, the Notch signaling pathway has been shown to play an indispensable role in the commitment of immature B cell in MZ B cells fate (83). Likewise, it has been shown that the BCR strength is also partly involved in the fate decision between MZ and FO B cells. Accordingly, studies have revealed that weak BCR signaling leads to MZ B cell fate whereas stronger signals favor FO B cell differentiation (82).

3.1.5 Follicular B Cells

Upon arrival, the majority of mature B cells home within the secondary lymphoid organs inside compartments called B cell follicles and therefore are called FO B cells. Given that B cell follicles are located in the close vicinity of T cell zones; this special arrangement favors further migration of T and B cells towards each other and thereby suits their involvement in TD immune responses. In addition, FO B cells can recirculate within the bone marrow and position themselves adjacent to bone marrow sinuses and thereby they are capable to mount TI responses. FO B cells are phenotypically $IgM^{lo/hi}$ IgD^{hi} and are known to have broad BCR repertoire which enables them to recognize a wild spectrum of pathogens (82).

3.1.6 Surviving in the Periphery

During initial stages of development, tonic BCR signaling is sufficient to assure survival of newly generated B cells whereas following the generation of T2 B cells, other mediators are also required for this process. Given the role of TNF superfamily in the regulation of immune response by promoting either apoptosis or proliferation or both in lymphocytes (84), the TNF family ligand BAFF (a.k.a. TNFSF13B, TALL-1, THANK, BLyS) is found in high concentrations within the lymphoid follicles and thereby is a key regulator of peripheral B cell populations. Accordingly, BAFF ligand has been speculated as a survival factor for newly generated B cells and mature follicular B cells by triggering signals through its receptor BAFFR. The significance of BAFF-mediated survival of B cells is seen in mice deficient for BAFF or

BAFFR, as these animals showed a marked reduction in B cell numbers following a blockage of developmental stages in T2 stage (85).

Similarly, human patients harboring mutations blocking B cell responsiveness to BAFF suffer from common variable immunodeficiency (CVID) with strong B cell lymphopenia, reduction of MZ and switched memory B cells, and relative increase in transitional B cell numbers (86). Likewise, it has been proposed that BAFF may correlate to pathogenesis of autoimmunity by increasing the survival of autoreactive B cells following the observation of elevated levels of this ligand in patients with systemic lupus erythematosus (SLE) (87). Moreover, BAFF has been shown to have a second distinct function to mediate MZ B cell development. In addition to BAFFR, studies have identified other receptors for BAFF such as transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) and B-cell maturation factor (BCMA) (88). Despite the increased expression of BAFFR upon transition of B cells into MZ and FO cells, it has been shown that long-lived PCs only express BCMA instead of BAFFR (89) whereas MZ and memory B cells express TACI (90).

3.2 The Germinal Center Reaction

As the key feature of humoral immune responses, antibody affinity progressively increases through a process called somatic hypermutation (SHM) of the antigen-binding variable regions of Ig genes. This process is mediated by activation-induced cytidine deaminase (AID) and leads to the generation of a pool of mutated B cells that are then selected depending on their affinity. Consequently, high affinity selected B cells undergo further proliferation and differentiate into antibody secreting plasma cells (PCs) and memory B (Bmem) cells. All of these processes take place inside micro-anatomical structures called germinal centers (GCs) which arise within B cell follicles in the secondary lymphoid organs including spleen, lymph nodes (LNs) and Peyer's patches (PPs) (**Fig. 2**) (91). Indeed, upon antigen stimulation during TD immune responses, activated T and B cells interact with each other inside GCs which sequentially give rise to PCs and Bmem cells (78). GCs emerge in the spleen and LNs within the first week of antigen stimulation, reach their peak at days 10-14 and stay detectable up to 95 days post-exposure, whereas PPs feature an ongoing GC reaction due to exposure to gut microflora. Therefore, GCs progressively decline as a result of limited antigen exposure and enhanced

differentiation of PCs (91). In this section, I will describe the molecular mechanisms of GC development and the key players in this process which give rise to antibody secreting PCs and Bmem cells and finally I will explain how antibody is produced and provide long-lived humoral immunity.

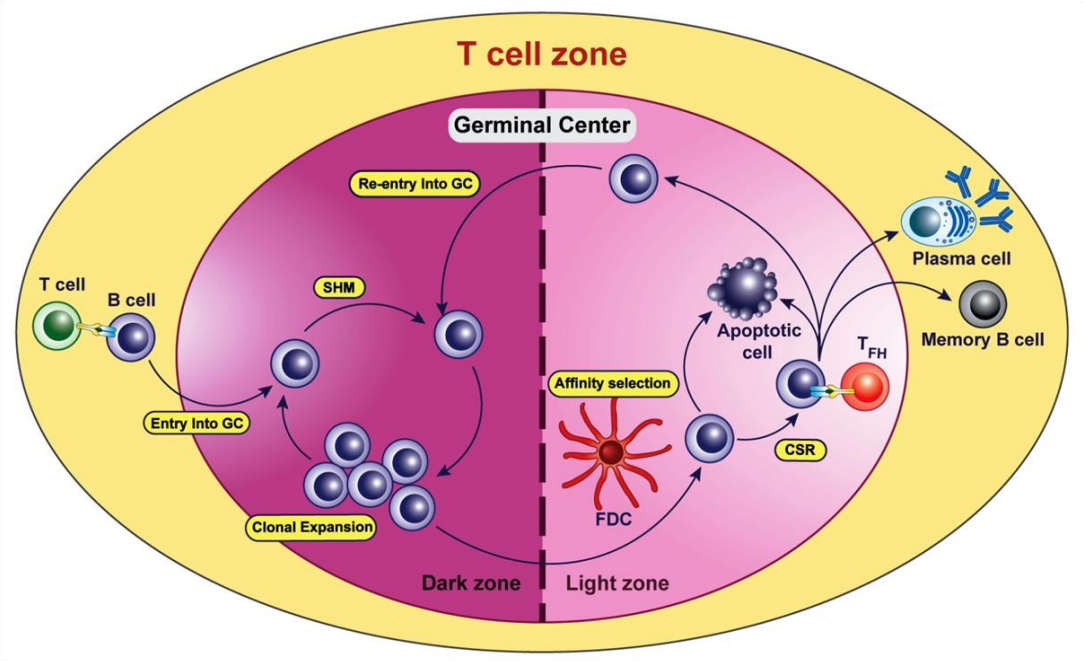

Figure 2. Schematic view of the germinal center. Following the interaction of T cells with B cells at the T cell-B cell border, T cells provide co-stimulatory signals to antigen presenting B cells. Selected cells then undergo somatic hypermutation (SHM) upon entering the GC dark zone thus upregulating components of the SHM machinery, including activation-induced deaminase (AID). Next, the B cells migrate to the light zone following several cycles of proliferation and SHM. In the light zone, the mutated BCRs encounter antigens on the follicular dendritic cells (FDCs). The low affinity interactions lead to cell death and only those with higher affinities will survive and therefore need to compete for available T cells help in favor of B cells with higher affinity. The remaining B cells will then take one of three fates: they can migrate back into the dark zone and continue to proliferate and undergo SHM, they can leave the GC either as PCs or memory B cells.

3.2.1 Germinal Center Anatomy

GCs form inside the B follicles of secondary lymphoid organs and are scattered by a network of stromal cells known as follicular dendritic cells (FDCs). FDCs play distinct roles in both primary and secondary follicles. Within the primary follicles that lack GCs, FDCs help B cells to aggregate into clusters whereas in secondary follicles containing GCs, these cells are known to be responsible for long-term retention of antigens in the form of immune complexes and thereby favor the affinity check of SHM-modified BCRs during the selection process in GCs (92) and moreover support GC B cell survival (93). GC formation initiates by priming of naïve B cell with antigens and migration of these cells towards the T cell zone in the T: B border and upon receiving co-stimulatory signals from antigen-specific $CD4^+$ helper T cells. This migration is mediated through upregulation of T cell zone homing receptors namely CCR7 and Epstein-Barr virus-induced G-protein coupled receptor (EBI)2 (94). Upon this interaction, responding B cells undergo an intense proliferation due to induced CD40 signaling and locate preferentially in the outer B cell follicles. Consequently, a fraction of these cells will aggregate in tight clusters in the center of follicles adjacent to the FDC network thereby giving rise to early GCs. Of note, several G-protein coupled receptors (GPCR) contribute to retention of GC B cells and their cognate helper T cells inside the newly formed GCs. As such, the (EBI)2 which attracts naïve B cells towards the outer follicle has been shown to be downregulated in GC B cells (95). Furthermore, S1P2 (one of the five receptors for S1P) is upregulated and thereby as the second mediator prevents the migration of GC B and helper T cells towards outer follicles (96).

Early studies over 70 years ago characterized the organization of mature GCs with 2 compartments or "zones": the light zone (LZ) and the dark zone (DZ) based on their histological appearance. The DZ is adjacent to the T cell zone in spleen and lymph nodes where FDCs are largely absent whereas the LZ is proximal to the capsule in LN or marginal zones in the spleen. However, in the current view these histological differences are known to be associated with distinct functions (97). The highly proliferative B cells located in the DZ are classically called "centroblasts" and strongly express chemokine receptor CXCR4 that interacts with the CXCL12 chemokine (the ligand for CXCR4) expressed by DZ reticular cells and thereby holds these B cells specifically inside the DZ and away from FDC network (97). Given the high expression of AID and the error-prone DNA polymerase eta (Polη) in DZ B cells, the DZ is referred to as the

site of Ig SHM. In contrast, the LZ contains less proliferative GC B cells classically known as "centrocytes" along with antigen-coated FDCs and antigen-specific T helper cells that favor the BCR-driven selection of GC B cells and further differentiation of these cells into high affinity SHM-variants (91). Importantly, highly proliferative centroblasts are known to continuously renew the LZ centrocyte pool. Moreover, it is suggested that a fraction of high affinity B cells within the LZ are capable to re-enter the DZ in order to undergo further rounds of proliferation and SHM which supports the degree of affinity maturation observed *in vivo* (98).

3.2.2 Germinal Center Dynamics

For the sake of simplicity, GC dynamics can be subdivided into 3 main stages as (1) priming of both B and T cells, (2) effector function and (3) GC formation. Within the few days of an immune response, naïve B cells encounter antigens through different mechanisms depending on the size and nature of the antigen. Accordingly, small antigens are known to directly enter B follicles via simple diffusion whereas bigger antigens need to be captured and presented to B cells by subcapsular sinus macrophages (99). Alternatively, FDCs present antibody-bound antigens which deposit on their surface as immune complexes. Likewise, antigen-specific naïve $CD4^+$ helper T cells are activated via antigen presenting dendritic cells (DCs) within T cell zones.

Following priming of T cells, a fraction of these cells initiate early stages of differentiation by upregulating the expression of factors associated with T follicular helper (Tfh) cells including transcriptional repressor BCL6, the B cell follicle homing receptor CXCR5, and the costimulatory receptor PD1 whereas other factors related to other Th cell effector subsets get suppressed (100). These DC-activated CD4 T cells which all express BCL6 are referred as pre-Tfh cells and migrate towards B cell follicles (Tfh cells will be discussed in more detail later in this chapter). On the other hand, naïve B cell priming through BCR antigen stimulation leads to the migration of these cells towards the T:B interface and this B cell engagement is known to induce the second wave of BCL6 expression in a fraction of pre-Tfh cells and provide further stimuli for the full differentiation of these cells (101).

The second stage of GC reaction includes full differentiation of Tfh cells, which initiates following the migration of antigen-specific B cells and pre-Tfh cells to the T: B border. Here, B

cells act as antigen presenting cells (APCs) for pre-Tfh cells (102) and following this cognate interaction, the Tfh cells signal a subset of high affinity B cells to migrate back to the B follicles and thereby seed the GCs (103) while in return, stimuli from highly proliferative cognate B cells induce migration of fully differentiated Tfh cells into B follicles. Importantly, it has been indicated that at the interface of T cell zone and B follicles, a subset of B cells differentiate into short-lived plasmablasts (PB) which then migrate into extrafollicular foci and thereby bypass the GCs (104). Likewise, it has been shown that $CD38^{+}GL7^{+}$ B cell precursors are capable of direct differentiation into either IgM^{+} or isotype-switched Ig^{+} memory B cells in a GC-independent manner or alternatively differentiate into GC B cells (105).

The final stage involves GC formation during which the centroblasts upregulate expression of genes coding for AID that is known to introduce point mutations in the BCR variable and switch region thereby leading to SHM as well as class switch recombination (CSR), respectively. In the current view, it is proposed that clonal expansion and SHM of centroblasts give rise to daughter cells that further differentiate into centrocytes and home into LZ. Following exposure of these cells with antigens through FDCs or other means, the LZ centrocytes act as APCs for Tfh cells via major histocompatibility complex-class II (MHC-II) and in return only B cells with highest affinity BCRs will receive stimulatory signals including CD40L, IL21 and IL4 from Tfh cells (106). Therefore, B cells with low affinity undergo apoptosis due to lack of survival signals. The remaining B cells that managed to survive will either migrate back to the DZ to further undergo another round of clonal expansion and SHM, differentiate into antibody producing PCs or differentiate into Bmem cells (107). Collectively, cycles of clonal expansion following SHM and affinity selection during the GC reaction guarantee the long-lasting protection by producing high affinity antibody secreting PCs and long-lived Bmem cells.

3.2.3 Post-GC Fate Decision

One of the key functions of the immune system is to protect against reinfection. Successful humoral memory is achieved through two different layers of defense. During constitutive humoral memory, protective antibodies are secreted from PCs at the site of reinfection providing the first line of defense. Additionally, the reactive humoral immunity becomes functional when the constitutive immunity is insufficient. Indeed, memory B cells that

previously experienced antigens reactivate and produce antibodies providing rapid and stronger antibody responses. Therefore, antibody secreting PCs and Bmem cells as long-lived effector cells are the key components of immunological memory (108). As mentioned earlier, following GC reactions, B cells with higher affinity are preferentially recruited to the pool of long-lived PCs whereas lower affinity B cells can either commit to more rounds of affinity maturation and selection within the DZ or give rise to the memory B cell pool. However, the underlying mechanisms that lead to these commitments are not fully understood. Here, I will review the key points that lead to post-GC fate decision.

3.2.3.1 Memory B cell formation

In TD immune responses, the majority of Bmem cells are known to arise following GC reaction. However, several recent studies provided new evidence regarding the existence of unswitched IgM^+ Bmem cells alongside GC-dependent switched Bmem cells, thus suggesting a GC-independent origin of these cells. In the current view, it is suggested that long-term Tfh-B cell interactions provide the adequate signals that leads to GC formation whereas shorter conjugate interactions are more likely to prone the B cells into the GC-independent memory B cell formation. Likewise, it has been shown that receiving CD40 signal alone from T cells triggers the memory formation pathway (105). Interestingly, it has been shown that IgG^+ memory B cells have lower expression levels of pro-apoptotic factor BIM along with higher expression of pro-survival factor BCL-2 in comparison with activated B cells. In addition, studies in IL21R-deficient mice revealed a reduced GC reaction while antigen-specific Bmem cells were increased, thus suggesting a negative regulatory function of IL21 on Bmem commitment within GCs (109). In the classical view, it was therefore suggested that differentiation of B cells into Bmem happens in a more likely stochastic manner and the survival advantage is the key requirement given that no deterministic transcription factor was found to be associated with Bmem fate (110).

However, in 2016, results of two separate groups identified Bach2 to be a key transcription repressor regulating the memory differentiation of lymphocytes in response to antigen. Their observations shed more light on the possible molecular mechanisms of Bach2's function; however, the details of this regulation remain to be elucidated. Accordingly, weaker help signals

from T cells via CD40L have been speculated to favor the sustained high expression of Bach2 in GC B cells which further promotes the entrance of Bmem pool (111, 112). Of note, Bach2 has been previously known to mediate CSR and SHM in GC B cells (113). Overall, new evidence suggested that the Bach2 gradient is a key factor that at a low level promotes CSR and SHM whereas at higher levels supports Bmem fate (114).

3.2.3.2 Plasma cell formation

The vast amount of knowledge has been generated during the past decades regarding the underlying mechanisms of antibody secreting PC differentiation. Current evidence suggests that regulation of the developmental program leading to PC differentiation is mainly guided by transcriptional repressions (**Fig. 3**). As such, three major transcription factors including interferon regulatory factor 4 (IRF4), B-lymphocyte-induced maturation protein 1 (BLIMP1) and X-box binding protein (XBP-1) have been identified as key factors in PC differentiation. On the other hand, the differentiation program gets repressed by a group of factors expressed in activated B cells such as Pax5, Mitf, Bach2, and BCL6. Therefore, the mutually antagonistic interactions between these transcription factors are the key to set the ultimate outcome of differentiation. Pax5 is known to suppress the gene expression profiles related to stem cells and non-B lineage as well as *Prdm1* (gene encoding Blimp-1) and *Xbp1* that are involved in PC differentiation. Likewise, Bach2 and BCL6 have been shown to suppress *Prdm1* whereas Mitf mainly suppresses yet another regulator of PC differentiation, *Irf4* (115).

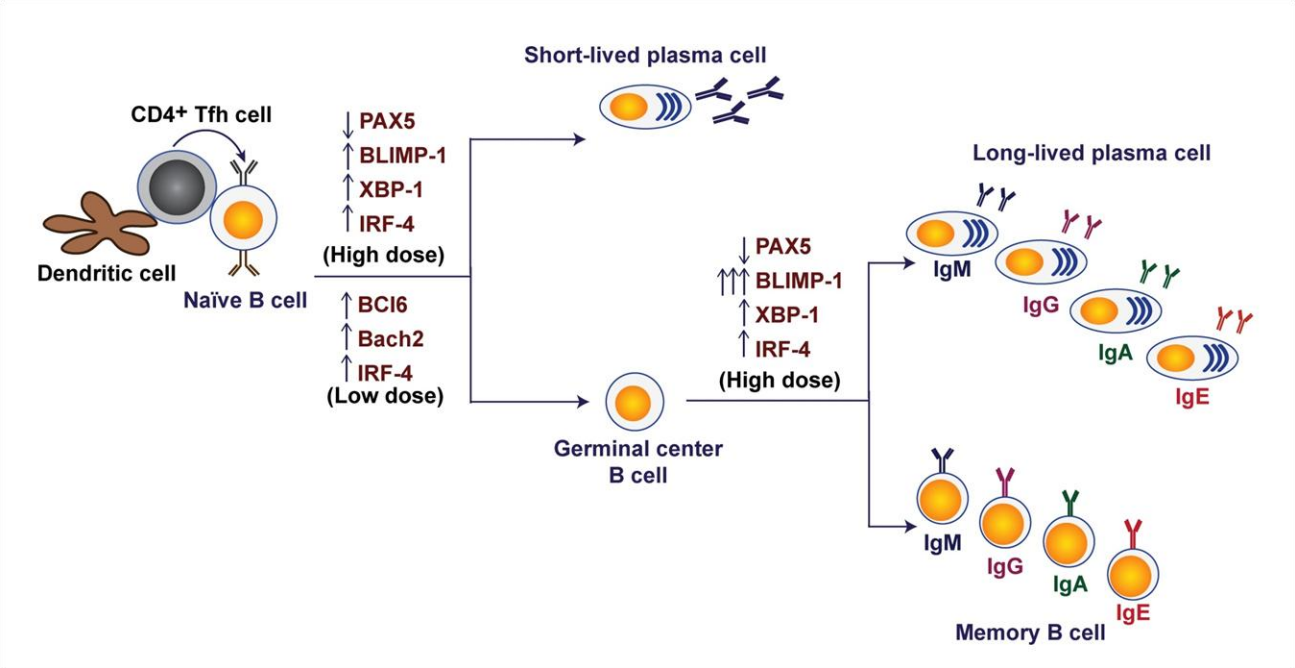

Figure 3. T cell dependent B cell differentiation. The differentiation of naïve B cells to these distinct effector fates is controlled by the balanced expression and regulated function of various transcription factors, including (but not exclusively) PAX5, BCL-6, BLIMP-1, XBP-1, and IRF4.

Importantly, IRF4 is known for its unique involvement in both GC B cells and PCs regulatory network. Indeed, it has been shown that IRF4 functions in a dose-dependent manner such that lower expression levels in GC B cells favors GC fate and CSR (116). However, higher levels of IRF4 expression induces the PC fate through suppression of BCL6 and activation of BLIMP1-related transcription factor termed zinc finger and BTB domain-containing protein 20 (Zbtb20), which are the known mediators of PC differentiation (117).

BLIMP1 is yet another transcriptional repressor which suppresses the B cell-linage genes such as Pax5, Bach2 and Bcl-6 and is extensively expressed in both PBs and PCs. However, its expression does not follow a similar pattern in these cells and details of its mechanism of function in PC differentiation and maturation remains elusive. Significantly, some studies have used BLIMP1-GFP knockin mice in order to track the quantitative changes of BLIMP1 expression level by monitoring GFP levels in order to define various stages in development of antibody secreting cells (ASCs). They have linked the intermediate BLIMP1 expression levels to short-lived plasmablasts and higher expression levels to long-lived PCs (118). Likewise, the

early work of Stephen Nutt's group using BLIMP1-GFP reporter mice revealed the expression of GFP within all ASCs. Their further analysis of ASCs that were generated post-immunization indicated that BLIMP1 expression level can be used as a developmental marker as its increasing expression level defines stages of PC differentiation. However, despite the fact that BLIMP1 was expressed in ASCs isolated from both humans and mice, B cells at early stages of development lacked BLIMP1 (119). Later studies of the same group have speculated that BLIMP1 is required for full PC differentiation rather than PC fate commitment. They suggested a transient early stage for ASC differentiation which gives rise to the cells called "preplasmablasts" following either GC reaction or extrafollicular pathway. This transient stage has been shown to be BLIMP1-independent in which preplasmablasts were capable of secreting low levels of antibodies (115). Studies of BLIMP1-deficient mice by Shapiro-Shelef's group revealed that serum antibody levels were reduced and antibody responses following immunization were severely impaired. However, inactivation of *Prdm1* in previously formed PCs caused the complete loss of this lineage (120).

Another transcription factor that is strongly induced in ASCs is XBP-1 which is known to directly control the secretory pathway in these cells. Studies have shown that loss of Pax5-mediated gene suppression along with posttranscriptional control via unfold protein response (UPR) induces XBP-1 expression in ASCs. Notably, UPR is a known highly coordinated response which is involved in the folding, processing, export and degradation of all proteins under both stressed and normal conditions (121) and therefore, plays a critical role in PCs that secrete large amounts of immunoglobulin proteins.

3.3 Longevity of Plasma Cells

Over the past 50 years, PC longevity and its role in the maintenance of long-term serum antibody levels have been a matter of debate. It has long been believed that PCs could not have significant functions in long-term antibody production due to their short-lived nature. Therefore, Bmem cells were initially considered to be the only key mediators of immunological memory for a long time. In 1998, a new theory has emerged that proposed existence of the long-lived PCs and their critical role in long lasting humoral immunity. In light of early studies, it has been shown that the majority of PCs generated within the first 2 weeks following vaccination were

short-lived with a half-life of a few days (122). Interestingly, long term monitoring of PCs, revealed a biphasic response in PCs (123), such that at early time points the majority of PCs disappeared rapidly whereas at later time points the PCs declined more slowly and yet were detectable six months post vaccination. Likewise, monitoring the ^{3}H-labeled PCs in other studies further confirmed the existence of the long-lived PCs (124).

Currently, several models of humoral immunity are available which describe the duration of these responses. As such, Bmem-dependent responses are known to rely on the activation and differentiation of these memory phenotype B cells into antibody secreting PCs. Both antigen-specific and non-antigen-specific stimulation have been shown to mediate these activations. On the other hand, there are two other models that are independent of Bmem cells, in which PCs and Bmem cells are considered as two independent and distinct populations. Thus, in the later models, competitions for the limited space in immunological niches as well as some intrinsic factors have been suggested to determine the lifespan of PCs (125). Accordingly, experimental evidence over the past years have described that the microenvironment has a huge impact on PC longevity. Survival niches are mainly located in the splenic red pulp, bone marrow and inflamed tissues. Therefore, migration of newly generated PCs into the survival niches which are mainly located in the BM, is considered a key step to ensure persistent antibody responses (126). Accordingly, chemokines and their receptors have been identified to mediate the cell movement in both primary and secondary lymphoid organs through the coordinated changes in receptor expression and chemokine responsiveness. More than 40 members of most secreted proteins have been identified in the chemokine family. Of note, all the chemokine receptors are heterotrimeric G-protein–coupled receptors. In this regard, studies of CXCR4-deficient fetal liver chimeras indicated that CXCR4 is an important mediator in positioning of PCs in the spleen as well as homing of these cells in the BM niches. Notably, CXCR4 is known to pair with its ligand, CXCL12 that is mainly expressed in BM, splenic red pulp, and lymph node medullary cords. Hence, in this model it has been suggested that at the late stages of PC development, reduced responsiveness to CXCL13, CCL19, and CCL21 directs PCs out of B and T zones, while the CXCR4/CXCL12 interaction mediates splenic red pulp localization and further BM homing (127).

In light of other studies, it has been shown that primary immunization with Ag together with type I interferons induced long-term Ab production whereas Ag alone could only mount short-term Ab responses (128). In addition, several intrinsic mediators including Blimp-1, BCL-6, BSAP and XBP-1 have been identified related to PC longevity. Likewise, the anti-apoptotic molecule A1 is yet another candidate that has been suggested to induce PC survival (129) following receiving potential signals within survival niches. However, more recent evidence regarding the generation of PCs with a short-lived nature following extrafollicular reactions suggested the external interactions and microenvironment rather than intrinsic properties to be more important in this aspect. As mentioned earlier, TD help is required to promote the generation of long-lived PCs. Overall, it has been implicated that various signals from the microenvironment can affect the potentiated cells and thereby determine the PC lifespan. Another mechanism of survival has been discovered following studies of CD28-deficiency in which CD28-mediated activation of nuclear factor κB (NFκB) in bone marrow PCs lead to elevated expression of pro-survival factors (130).

Given the complex nature of the bone marrow compartment and thus limitations of experimental designs, the majority of current knowledge in this accord is obtained through *in vitro* experiments. Therefore, synergistic effects of various cytokines and adhesion molecules have been identified to mediate PC survival. As such, IL5, IL6, Tumor necrosis factor (TNFα), B-cell activation factor (BAFF) and proliferation-inducing ligand (APRIL) as well as bone marrow stromal cells have been shown to prolong the PC lifespan. Similarly, interaction of fibronectin expressed by stromal cells with its ligand, VLA4 on the surface of PCs as well as PC surface proteins such as CD44 have been also shown to be critical in PC longevity in other studies (131, 132). Nonetheless, *in vitro* experiments do not reflect the complexity of *in vivo* systems; therefore, interpretations should be made cautiously. Later, murine studies provided new evidence which further confirmed some of the previous findings. For example, APRIL was highlighted as a significant survival factor for long-lived PCs following the observation that bone marrow PCs were significantly reduced in APRIL or BCMA (APRIL receptor) deficient mice. Of note, it has been shown that APRIL is expressed from Gr-1^{int}CD11b^{hi}F4/80^{+} myeloid cells including monocyte/macrophages and eosinophils in BMs (133). Furthermore, another group revealed a potential mechanism of function for APRIL such that APRIL-induced expression of the prosurvival factor, Mcl-1 favors the persistence of PCs in the BM (134).

Given the importance of BM homing for PCs to establish in survival niches, it has been shown that the majority of newly formed PBs in early stages of TD immune responses die due to failure to establish within survival niches. Then, the remaining PB pool closely interacts with reticular stromal cells through CXCL12 as well as some adhesion molecules such as VCAM-1 (expressed by stromal cells), extracellular matrix proteins fibronectin or hyaluronic acid which leads to their retention within BM niches (135). Interestingly, studies of KLF2-deficient mice revealed the significant role of VCAM-1 and its ligand integrin α4β7 in BM homing. Here, despite the normal generation and entrance in circulation, the KLF2-deficient PCs were incapable of homing into the BM. Of note, KLF2 is a known transcription factor regulating T cell egress from lymphoid organs whereas in B cells, it has been shown that KLF2 regulates B cell homeostasis and in particular mediates BM homing of PCs (136).

3.4 The Nature of T cell Help to B cells

Production of sustained long-lived antibody secreting PCs is the key function of humoral immunity; however, the fine-tuning of immune responses is pivotal. Indeed, immunity performs at the cost of autoimmunity and stronger immune responses have greater risk of inducing autoreactivity and harming self-tissues. This concept mainly applies to the GCs where SHM not only favors the increasing of BCR affinity, but also potential pitfalls arise by generating autoreactive BCRs. Therefore, several checkpoints operate in the GC and several helper T cell subsets are known to play key roles in this aspect. Given the central role of Tfh cells in helping B cells during humoral immune responses, in this section I will describe the mechanisms of Tfh cell development and function.

3.4.1 T Cell Help for Follicular and Extrafollicular Antibody Responses

Over 50 years ago, the crucial role of T: B collaboration in the induction of antibody responses has been shown (137). Later on, studies using hapten conjugated to a protein carrier revealed that carrier specific T-cells are indispensable in providing help for maturation of hapten-specific B cells and thereafter the role of T helper cells in antibody production was established (138). A decade later, studies confirmed that full differentiation of B and Tfh cells is driven by sequential recognition of peptide-MHC complexes by T cells (139). Indeed, APC-

activated antigen-specific T cells provide CD40L signal as well as cytokines such as IL-21 and IL-4 to previously primed cognate B cells and enable them to fully activate, divide and migrate to the periphery of the follicles which consequently differentiate in either a follicular or extrafollicular manner (140). In 2012, it was indicated that CD40L signaling triggers cell cycle entry of B cells while stimulating the expression of AID and thereby SHM and CSR (141). Importantly, following cognate interactions at the T and B cell interface, T cells also migrate to follicles which then provide surviving signals for GC B cells, thus these cognate interactions further sustains the GC structure. In 2009, following the discovery that BCL6 is an essential regulator for Tfh cell differentiation and suppressor of other T cell lineages such as Th1, Th2, Th17 and Tregs, Tfh cells were recognised as a distinct subset of specialized cells to provide help to B cells (100, 142, 143). Therefore, lack of T cell-driven BCL6 expression leads to impaired B cell responses to protein antigens due to failure of GC formation (142).

In follicles, following the formation of GCs by highly proliferative B cells, antibody diversification and affinity maturation occur through rounds of SHM in the DZ and selection in the LZ. The ultimate outcome of cognate T and B cell interactions is generation of long-lived PCs and Bmem which reside in the bone marrow or secondary lymphoid organs, respectively (144). In contrast, the extrafollicular route is known to generate short-lived PCs with fewer SHM, although recently, it has been demonstrated that extrafollicular SHM leads to affinity maturation of antibody responses in mouse models of *Salmonella* infection (145).

3.4.2 Tfh Cell Differentiation

Given the flexibility of naïve T cells to differentiate into diverse subsets of effector cells that provide help and perform distinct functions during the course of an immune response, it is therefore critical to generate an appropriate type of cell that leads to proper protection. To this point, it is known that different stimulatory cytokines generate various effector cells. Of note, it is known that in T helper 1 (TH1) cells, Interleukin-12 (IL-12) induces the T-box transcription factor T-bet (a.k.a. TBX21), whereas IL-4 induces GATA-binding protein 3 (GATA3) in TH2 cells and finally retinoic acid receptor-related orphan receptor-γt (RORγt) is induced by IL-6 or IL-23 in the case of TH17 cells. However, in the current view it is believed that the ultimate

outcome of Th cell differentiation relies on the coordinated functions of multiple regulators instead of being affected by only a simple master regulator (106).

As mentioned earlier, production of antibody secreting cells from differentiated B cells in GCs is highly dependent on the help provided by Tfh cells (**Fig. 4**). Indeed, Tfh cell differentiation occurs following upregulation of multiple markers including surface receptors such as CD40L, CD28 family including inducible T cell costimulator (ICOS) and programmed cell death protein 1 (PD-1) as well as cytokine associated signaling pathways such as signal transducer activator (STAT3) or SLAM-associated protein (SAP) which are mainly controlled by BCL6 as an important regulator of these processes. Additionally, other transcription factors including interferon-regulatory factor 4 (IRF4), basic leucine zipper transcriptional factor ATF-like (BATF), and microRNAs are also known to mediate Tfh cell development. Optimal Tfh differentiation has been shown to rely on the cognate ICOS–ICOSL interactions of activated CD4 T cells with B cells that express ICOSL through BAFF–BAFFR–NIK (NF-κB-Inducing Kinase) signaling (106).

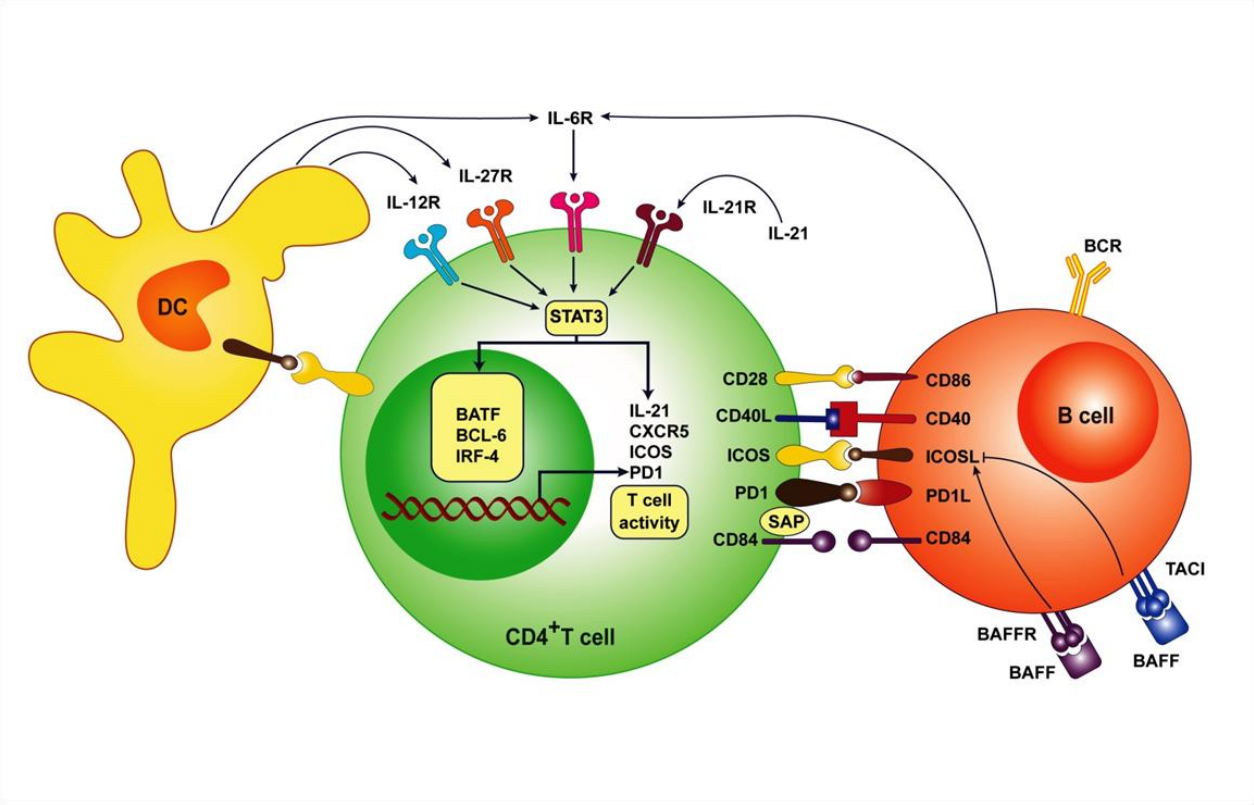

Figure 4. In T cell zone, $CD4^+$ T cells get primed by antigen presenting DCs leading to expression of CXCR5 and BCL6 to become early T_{FH} cells. Following migration to the T cell–B cell border and interactions with cognate B cells, early T_{FH} cells differentiate into germinal center T_{FH} cells. STAT3-activating cytokines are secreted by DCs (IL-6, IL-12 and IL-27), B cells (IL-6) and $CD4^+$ T cells (IL-21). These cytokines expression then induce or enhance expression of the transcription factors BCL6, MAF, BATF and IRF4, imprinting the T_{FH} cell fate by inducing the transcription of signature genes, including *CXCR5*, *ICOS*, *IL21* and *PD1*.

Despite that other effector T helper subsets have been shown to differentiate only within the T cell zones, Tfh cells are believed to require 2 different compartments for their differentiation. Inside the T cell zone, the first stage of differentiation initiates with antigen loaded DCs providing signals to antigen-specific CD4 T cells. Receiving of these cues such as TCR-mediated signals, costimulatory signals and STAT3 activating cytokines leads to polarization of pre-Tfh cells. Accordingly, it has been shown that stronger TCR signals induce differentiation of $CXCR5^+CCR7^-BCL6^+$ cells which reside inside the B cell follicles whereas weaker signals lead to $CXCR5^{lo}BCL6^-Blimp^+$ cell differentiation and further emigration of these cells into periphery giving rise to Th1, Th2 and Th17 subsets (146). However, more recently, Jenkins's group contradicted this finding and showed that TCR-peptide:MHC encounter time rather than TCR affinity is a key player that influences Tfh cell differentiation (147). Collectively, it is speculated that overall, the strength of TCR signaling positively impacts Tfh cell differentiation.

In addition, DCs also provide costimulatory signals such as CD28 and ICOS. Early studies of CD28-deficient mice revealed a crucial role of CD28 costimulation in Tfh cell differentiation following the observation of impaired TD humoral immune responses and complete loss of T-cell mediated GCs in these mice (148, 149). Lastly, T cells need STAT3 activation cytokines, in particular IL-21 and IL-6, in order to differentiate into Tfh cells (150). Indeed, DC-driven IL-6 induces IL-21 production in T cells (151). Of note, it has been shown that the synergic function of IL-21 and IL-6 is required for Tfh differentiation. Therefore, DC-dependent early stages of differentiation give rise to pre-Tfh cells. Later, upregulation of CXCR5 and downregulation of CCR7 by pre-Tfh cells enables them to migrate towards the T: B border and thereby contact their cognate B cells for further differentiation and maturation.

The second stage of differentiation is B cell-dependent in which the T-B contact leads to the second wave of BCL6 upregulation and thereby CXCR5 and PD1 expression in pre-Tfh cells. Nonetheless, due to the observation of $CXCR5^+$ pre-Tfh cells inside follicles in the absence of B cells, it has been speculated that B cells are critical for the maintenance of pre-Tfh cells rather than differentiation and entry into follicles (152). In 2010, Tangye's group indicated that the major impact of B cells on Tfh cell differentiation is to perform as classical antigen presenting cells rather than providing a specific signal (102). B cells are known to capture antigens via various routes. For example, they capture antigens from neighboring antigen-coated macrophages in a complement dependent manner within lymph nodes (153) or through calcium-dependent signaling pathways from antigen-loaded DCs (154). Collectively, B cell mediated antigen uptake triggers intracellular signaling cascades and thereby induces B cell proliferation, differentiation as well as migration towards the T-B border through which they can induce a second wave of BCL6 upregulation in their cognate pre-Tfh cells and further their full differentiation.

3.4.3 Tfh cell Characteristics

Gene expression profiling experiments in both humans and mice indicated that Tfh cells are distinct from other effector CD4 helper subsets. As such, they showed culminated expression of the transcriptional repressor B cell lymphoma 6 (BCL6), cytokine IL-21, PD-1, and ICOS genes whereas they did not express T-Bet or GATA3 and had limited expression of Th1, Th2 or Th17 cytokines (142). Importantly, in other attempts it was indicated that CD4 T cells with high levels of CXCR5 and PD-1 display gene expression patterns consistent with the behaviour of Tfh cells (155, 156). CXCR5 is a B cell zone-homing chemokine receptor that enables Tfh cells and B cells to localize inside B follicles including GCs in response to CXCL13 (157). In addition, Tfh cells downregulate the T cell zone-homing receptor CC-chemokine receptor 7 (CCR7) and IL-7 receptor-α (IL-7Rα) and these modified expression patterns facilitate the T-cell dependent B-cell responses by bringing T and B cells in close proximity. Therefore, fully differentiated Tfh cells are defined as $CD4^+CXCR5^+CCR7^-BCL6^{hi}PD1^{hi}ICOS^{hi}$ cells that express high levels of IL-21 as well as IL-4 and localize inside B follicles. In particular, Tfh cells that are strictly localized within the GCs express even higher levels of BCL6 and CXCR5 as well as ICOS and PD-1 and are referred to as mature GC Tfh cells.

3.5 Non-conventional Tfh-like cells

As mentioned earlier, in TD immune responses, B cells receive help from Tfh cells which further leads to activation and proliferation of these B cells. The outcome of these early T-B interactions and B cell differentiation favors one of the three alternate fates including extrafollicular PCs, GC B cells or early memory B cells. Recently, new evidence emerged that proposed some Tfh-like cells can provide help to B cells and thus participate in antibody production in a non-conventional manner. In this section I will describe only two groups of Tfh-like cells that are more related to my study.

3.5.1 Extrafollicular T helper cells

Several groups demonstrated that receiving help from T cells in the T: B interface channels a subset of B cells to upregulate CD138 and initiate an extrafollicular focus. These extrafollicular helper T cells have been shown to be CD4 T cells that induce the production of short-lived PBs in extrafollicular foci. Given that changes in chemokine receptors play a key role in dictating the B cell fate, it has been shown that migration toward $CD11c^+$ dendritic cells at bridging channels promotes PC differentiation through enhanced delivery of survival signals. Importantly, extrafollicular helper T cells lose CXCR5 expression while they are known to highly express CXCR4 and thereby CXCR4-CXCL12 axis enables them to reach the extrafollicular sites (107).

3.5.2 Follicular Treg cells

Although $Foxp3^+$ Treg cells were first found inside the GCs in early studies of Lim's group in 2004 (158), it was more recently that these cells became the topic of interest in the field. In 2011, three independent studies reported that 10-15 % of "Tfh cells" have hybrid features of Tfh and Treg cells, which have been referred to as follicular Treg cells (Tfr) (159-161). These Tfr cells express Tfh markers such as CXCR5, PD-1, Bcl6 and ICOS while lacking expression of CD40L, IL-4 and IL-21 and have been shown to arise from thymus-derived Treg cells (160). Given that PD-1 expression levels are higher in Tfr compared to Tfh cells and PD-1-deficiency leads to reduced Tfr cell frequency (162), PD-1 appeared to be crucial in Tfr development. However, in another study, ICOS has been proposed to mediate Tfr cell homeostasis in a mouse

model of diabetes (163). Therefore, further investigation is required to fully identify the mediators of Tfr cell development.

Another key issue regarding Tfr cells and their function is whether they use similar inhibitory mechanisms as Tregs in order to negatively regulate Tfh cell-mediated B cell help. Given the high expression level of IL-10 in Tfr cells much like Treg cells (160), and the fact that IL-10 has been shown to inhibit Tfh cell-mediated B cell help, there might be similar inhibitory mechanisms used by Treg and Tfr cells. However, it remains to be seen if Tfr cells have unique mechanisms that are optimized to control germinal center reactions.

Initial adoptive transfer experiments using CXCR5- or Bcl6-deficient Treg cells have shown that lack of Tfr cells enhances GC reaction and thereby plasma cell generation and antibody production suggesting that the role of Tfr cells is to downregulate the overall germinal center reaction (159, 161). However, these approaches are prone to misinterpretation due to the potential dysregulation of T cell functions associated with the genetic modification. Indeed, recent studies using mice in which the *Bcl6* gene is selectively deleted only in Foxp3^{+} cells provided a different view. They clearly showed that the impact of Tfr-deficiency on the overall antibody titers upon immunization or infections is rather minor (164, 165). More importantly, Tfr-deficient mice developed multi-organ spontaneous autoimmunity at 7 months of age presumably due to the accumulation of tissue-specific autoantibodies (165). Therefore, it appears that the main role of Tfr cells is to prevent generation of autoantibodies in the germinal center.

4. Rationales and Objectives: Examining the Function of Mst1 in Humoral Immunity

The ser/thr kinase Mst1 is a core component of the Hippo pathway that was originally discovered as a regulator of organ size in *Drosophila* (166). In the classical view, it has been known that upstream signals lead to a cascade of protein phosphorylation/activation that culminates in the cytoplasmic sequestration of oncogenic transcription factor YAP suppressing cell proliferation. However, more recent studies provided new evidence of multiple non-canonical mechanisms of function for Mst1 within mammalian cells in a highly context-dependent manner. In this regard, several groups revealed that Mst1/2 mediate critical functions

in mammalian adaptive immunity through non-canonical pathways. In 2012, MST1 drew a lot of attention following reports of human patients harboring autosomal recessive null-mutations in *MST1* gene that suffered from primary immunodeficiency (65, 167-170). Thus, many groups dedicated their work to investigate the role of MST1 in the immune system using both mouse models of Mst1-deficiency and human patient samples.

Accordingly, the roles of Mst1 in T cell generation and function have been investigated in mouse models (69, 171-173). All studies concur that naïve T cells are susceptible to apoptosis, consistent with the behavior of MST1-deficient human T cells (65, 167). Furthermore, thymocyte selection and Treg generation and function have been shown to be defective in Mst1-deficient mice in accordance with the autoimmune symptoms found in both mice and human patients (68, 69, 71, 174). However, some of the observations in mouse studies were paradoxical with the consequence of Mst1-deficiency in host defense. For example, Mst1 KO mice displayed greatly reduced neutrophil extravasation into the inflamed tissues upon bacterial infection (175) and ROS-mediated bacteriocidal activities of phagocytes (176) which could explain the recurrent bacterial infections in human patients. In other settings, Mst1-deficiency augmented IRF3-dependent type I interferon production and protection against viral infections (177) and further, it has been shown that DC-specific Mst1 KO mice mounted greater Th17 responses and better controlled lethal doses of *Candida albicans* infection (178). Hence, these results were in contrast with recurrent viral and fungal infections observed in human patients.

More questions were raised in MST1-deficient patients knowing that despite the reduced number of T and B cells, serum IgG and IgA titers were moderately higher. Furthermore, in some but not all patients, IgE titers were highly elevated with a potential association with mild atopic dermatitis (169). However, the basis of this hyper-IgE phenotype in Mst1-deficient patients remained unclear. Overall, it remained to be seen if these apparently normal or higher levels of class-switched antibodies are providing sustainable protection against pathogens.

4.1 Hypothesis

Our central hypothesis was that Mst1 plays a role in regulation of humoral immunity, thus MST1-deficiency leads to the production of antibodies with poor quality that fail to provide

efficient protection via humoral immune defense mechanism(s) that are critical for a wide range of pathogens.

4.2 Objective 1:

As mentioned earlier, human patients with MST1-deficiency have been reported with moderately elevated levels of class-switched IgG while struggling with recurrent bacterial, viral, and fungal infections. Using Mst1-deficient mouse models, I will investigate the IgG1 antibody responses in Mst1-deficient mouse models and will provide the cellular and molecular basis if they are defective.

4.3 Objective 2:

Another phenotype in patients with MST1-deficiency is hyper IgE serum levels. Therefore, I will study the IgE antibody responses in Mst1-deficient mouse models to see whether this phenotype is consistent and, if so, I will identify the underlying mechanisms causing this phenotype.

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Chapter II

Hippo pathway kinase Mst1 is required for long-lived humoral immunity

Hippo pathway kinase Mst1 is required for long-lived humoral immunity

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Running title: Role of Mst1 in long-term humoral immunity

1. Abstract

The protein kinase Mst1 is a key component of the evolutionarily conserved Hippo pathway that regulates cell survival, proliferation, differentiation, and migration. In humans, MST1-deficiency causes primary immunodeficiency. Patients with *MST1*-null mutations show progressive loss of naïve T cells but, paradoxically, mildly elevated antibody titers. Nonetheless, the role of MST1 in humoral immunity remains poorly understood. Here we found that early T-dependent IgG1 responses in young adult Mst1-deficient mice were largely intact with a minor defect in affinity maturation. However, Mst1-deficient mice progressively lost antigen-specific IgG1 titers concomitant with selective reduction of antigen-specific bone marrow plasma cells. The radio-resistant stromal tissues of Mst1-deficient mice had a normal capacity to sustain antibody titers indicating that the defects arise from hematopoietic compartments. Counterintuitively, both under steady state and upon immunization, Mst1-deficient mice

produced more abundant plasma cells displaying apparently unaltered functions and plasma cell gene signatures. We provide evidence that dysregulated germinal center reactions in the absence of Mst1 allow accelerated transition of germinal center B cells into the plasma cell lineage. Thus, it appears that overproduction of plasma cells secreting lower affinity antibodies and an increased turnover of bone marrow plasma cells underlie the premature loss of antigen-specific plasma cells in Mst1-deficient mice. Together, our findings suggest that vaccination of MST1-deficient human patients, even at the early stage of life, may fail to establish long-lived high affinity humoral immunity and that prophylactic antibody replacement therapy can be beneficial to patients.

2. Introduction

Mst1 is a Ser/Thr kinase that was originally discovered as a regulator of organ size in *Drosophila* known as Hippo (1). In the canonical Hippo signaling pathways, upstream sensors of cell-cell contact and hormonal signals lead to a cascade of protein phosphorylation that ultimately suppresses the key oncogenic transcription factor YAP thereby slowing down cell proliferation. Consistent with a high level of evolutionary conservation, a combined deficiency of Mst1 and its homolog Mst2 in hepatocytes leads to increase of hepatic cell size, hepatomegaly, and hepatocellular carcinoma (2, 3). However, the roles of Mst1 in mammalian cells are highly context-dependent such that Mst1-deficiency in different tissues results in a wide spectrum of defects such as survival, proliferation, differentiation, migration, and invasion.

Importantly, humans harboring autosomal recessive null-mutations in the *MST1* gene suffer from primary immunodeficiency featured by recurrent infections by viruses, bacteria, and fungi at early ages (1-10 years) (4-8). Sometimes, immunodeficiency is accompanied by autoimmune symptoms (4-7). MST1-deficient patients manifest T cell and B cell lymphopenia due to increased apoptosis of naïve T cells (4, 5). Despite the reduced number of T and B cells, serum IgG, IgA, and IgE titers are moderately higher in MST1-deficient patients. However, it remains to be seen if these apparently normal levels of class-switched antibodies are providing sustainable protection against pathogens.

The roles of Mst1 in T cell generation and function have been investigated in mouse models (9-12). All studies concur that naïve T cells are susceptible to apoptosis, consistent with the behavior of MST1-deficient human T cells (4, 5). Also, Mst1 knockout (KO) mice have

impaired thymocyte selection and Treg generation and function through multiple mechanisms explaining the autoimmune symptoms found in mice and human patients (10, 13-15). However, mouse studies have generated conflicting results in terms of the consequence of Mst1-deficiency in host defense. On the one hand, Mst1 KO mice displayed greatly reduced neutrophil extravasation into the inflamed tissues upon bacterial infection (16) and ROS-mediated bactericidal activities of phagocytes (17) consistent with recurrent bacterial infections in human patients. On the other hand, Mst1-deficiency augmented IRF3-dependent type I interferon production and protection against viral infections (18). Further, DC-specific Mst1 KO mice mounted greater Th17 responses and better controlled lethal doses of *Candida albicans* infection (19). These results contrast recurrent viral and fungal infections observed in human patients. One possible explanation reconciling these contradictory findings is that Mst1-deficiency causes defects in other immune defense mechanism(s) that are critical for a wide range of pathogens.

Here we used Mst1-deficient mice to gain insight into the role of Mst1 in regulation of humoral immunity. We found that the early antibody response is minimally affected by Mst1-deficiency as long as young adult mice were immunized. However, Mst1-deficient mice could not sustain antibody titers against the immunizing antigens due to a reduced number of antigen-specific plasma cells in the bone marrow. Our data suggest that dysregulated germinal center reactions in the absence of Mst1 overproduce low-affinity plasma cells and this may increase plasma cell turnover in the bone marrow compromising the longevity of antigen-specific humoral immunity. Thus, Mst1 plays critical roles in long-lived humoral immunity by controlling plasma cell output from germinal centers.

3. Materials and Methods

Mice

Mst1 germline KO (11), Mst1 conditional KO mice (3), and BLIMP1-GFP reporter mice (20) have been previously described. For tissue-specific KO models, we used CD4-Cre transgenic (21) (Jax 000664), MB1-Cre knockin (22), or CD11c-Cre BAC transgenic (23) mouse lines. All the mice were in C57BL/6 background (>12 generations) and housed in the IRCM Animal Facility under specific pathogen-free conditions. For steady-state serum antibody titers, 8-10 weeks old mice were used. For immunization or infection, young mice at 7-8 weeks of age were

used unless otherwise indicated. Animal experiments were performed according to animal use protocols approved by the IRCM Animal Care Committee.

Immunization, BrdU labelling and infection

For immunization, mice received a single intraperitoneal injection of 4-hydroxy-3-nitrophenylacetyl-ovalbumin (NP_{16}-OVA) (Biosearch Technologies) in alum (Thermo Fisher Scientific) at 100 μg in 200 μl per mouse. For BrdU labelling experiments, mice were i.p. injected with BrdU (BD Biosciences) and analyzed after a chase period. For *Heligmosomoides polygyrus* (Hp) infection experiments, mice were infected by gavage and bled at indicted time points. For elimination of adult worms, two doses of pyrantel pamoate (100 mg/kg, gavage) were given 2 weeks post infection.

Antibodies

For flow cytometry and immunofluorescence, all primary antibodies and streptavidin conjugates used were purchased from Thermo Fisher Scientific unless otherwise specified: anti-mouse B220, Bcl6 (BD Biosciences), CD3, CD4, CD11b, CD11c, CD44, CD95 (Fas), CD138, CD62L (BioLegend), CD162 (PSGL-1, BD Biosciences), CXCR5 biotin, IgD, F4/80, GL7 (BD Biosciences), Ly-6G/Ly-6C, NK1.1, PD-1, streptavidin, and Ter119. To prevent non-specific binding, anti-CD16/CD32 was used prior to surface staining. For intracellular staining of BrdU, BrdU Flow kit (BD Biosciences) was used, following manufacturer's instructions. To exclude dead cells, 7-AAD (BD Biosciences) or fixable viability dye was used for surface or intracellular staining, respectively.

Flow cytometry

Single cell suspensions were prepared in FACS buffer (1% BSA/PBS) by gentle mechanical digestion through a 70μm filter. After red blood cell lysis and blocking, cells were stained with primary antibodies, followed by streptavidin conjugates. For intracellular staining, cells were fixed and permeabilized with Intracellular Fixation & Permeabilization Buffer set (Thermo Fisher Scientific), according to manufacturer's instructions. Stained cells were analyzed using

BD LSR Fortessa and Flowjo software. Plasma cells were identified according to the gating strategy described in Supplementary Fig. 1.

Enzyme-linked immunosorbent assay (ELISA)

For analysis of serum antibody titers, MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with either goat-anti mouse Ig (Southern Biotech) for naïve mice or with NP_4 or NP_{30}-BSA (Biosearch Technologies) for immunized mice and ELISA assays were performed as previously described (24). For analysis of Hp-specific antibodies, plates were coated with Hp excreted and secreted proteins (HES) at 4°C overnight. After incubation with serum samples, antibodies were detected using rat anti-mouse IgG1-Biotin (SB77E) and Streptavidin-HRP (Southern Biotech). Plates were read at 405 or 450 nm (AP-pNPP or HRP-TMB, respectively) using a Molecular Devices microplate reader and SoftMax Pro 4.7 software.

Bone marrow transplantation experiments

Mst1 HET or KO recipient mice were lethally irradiated with a single dose of 900 cGy and immediately received bone marrow cells from 7-8-week-old Mst1 KO or HET mice (2×10^6 cells per mouse, tail vein injection). Bone marrow reconstitution was confirmed by flow cytometric analysis of blood samples six weeks later. The bone marrow transplanted mice were immunized with NP_{16}-OVA/alum 7 weeks post-BMT and sera were taken at day 12 and day 50 post-immunization to monitor the level of anti-NP IgG1.

Enzyme-linked immunospot (ELISPOT) assay

MultiScreen 96-well plates (Millipore) were coated with NP_{30}-BSA, NP_4-BSA or goat anti-mouse IgH+L in PBS and incubated overnight at 4°C. Plates were washed with PBS and blocked with RPMI 1640 media containing 1 % fetal bovine serum, penicillin, streptomycin, and glutamine for at least 2 hours at 37° C. Cell suspensions were plated and incubated overnight at 37°C. Plates were washed and detected with anti–IgG1-horseradish peroxidase conjugate (Invitrogen) and developed using AEC substrate set according to manufacturer's instructions (BD Bioscience).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from FACS-sorted plasma cells harvested from spleens using RNeasy Mini Kit (Qiagen). Subsequently, cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen), according to manufacturer's instructions. PowerUp SYBR Green Master Mix (Life technologies) and 400 nM of forward and reverse primers were used for qPCR reactions. The names of genes and their primer sequences are listed in Supplemental Table 1. All reactions were performed on the ViiA7 Real-time PCR System (Life Technologies) as follows: 2 min at 50°C, 2 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles. The amount of a given transcript was normalized against β-actin, and the relative abundance was determined against the less abundant sample (set to a unit) among Mst1 HET and Mst1 KO samples.

In vitro migration assay

Plasma cell migration assays was performed using a 24-well transwell plate (5.0 μm pore size, Costar). Splenic B cells were isolated using EasySep B cell negative enrichment kit (STEMCELL) and added to the upper chamber. Media containing varying concentrations of CXCL12 (SDF-1α, Peprotech) was added to the lower chamber. After 4 hours of incubation at 37°C, cells from the upper and lower chamber were collected and normalized by adding Sphero beads (Spherotech) and enumerated by flow cytometry.

Histology

For hematoxylin and eosin (H&E) staining, serial paraffin-embedded sections (5μm) were used. For immunofluorescence staining, spleen samples frozen in OCT were sectioned (8μm) and fixed in cold acetone for 10 min. Samples were washed with PBS 3 times, blocked with 3% BSA/PBS (1 hour at room temperature), and then stained overnight at 4 °C with directly conjugated antibodies against CD4, IgD, GL7, or CD138 (all in 1:100 dilution). After washing in PBS, slides were mounted in Aqua-Mount (Lerner Laboratories) and fluorescence images were taken using DMRB microscope (Leica) equipped with a digital camera.

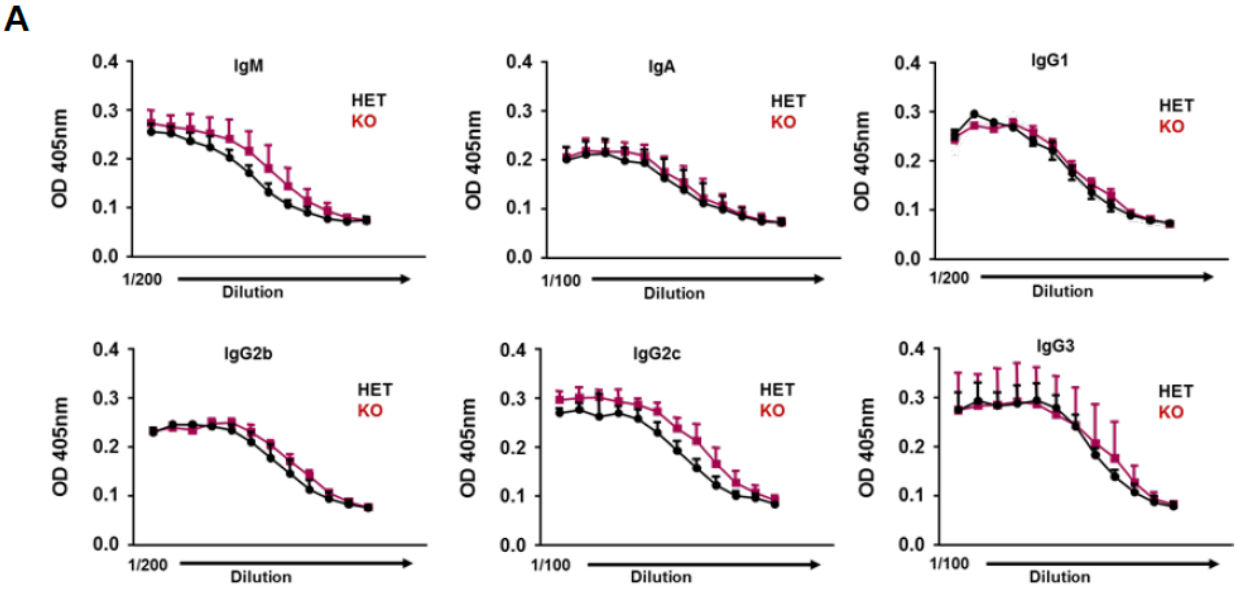
Statistical analysis

All data are presented as mean and standard error of the mean (SEM). Student's *t*-test (Graphpad Prism 7) was used to analyze statistical significance: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$.

4. Results

4.1 Mst1 KO mice display immune features characteristic of MST1-deficient human patients

Despite T and B cell lymphopenia, human patients possessing MST1-null mutations have moderately increased levels of class-switched antibody (Ab) titers, whereas heterozygous (HET) parents and siblings do not show these symptoms (4-8). To test if Mst1-deficent mice can serve as an animal model to understand human MST1-deficiency, we first surveyed the main parameters of humoral immunity in Mst1 germline knockout (KO) mice housed in our SPF colonies without immunization. We did not detect alterations in the serum titers of total IgM, IgA, and IgG isotype antibodies in 8-week-old Mst1 KO mice (Figure 1A). Importantly, the serum IgG1 level increased in aged Mst1 KO mice consistent with findings made in human patients (Figure 1B). Heterozygous mice showed similar antibody titers as those of wild-type mice (data not depicted). Therefore, Mst1 germline KO mice closely reflect humoral immune features seen in human patients.



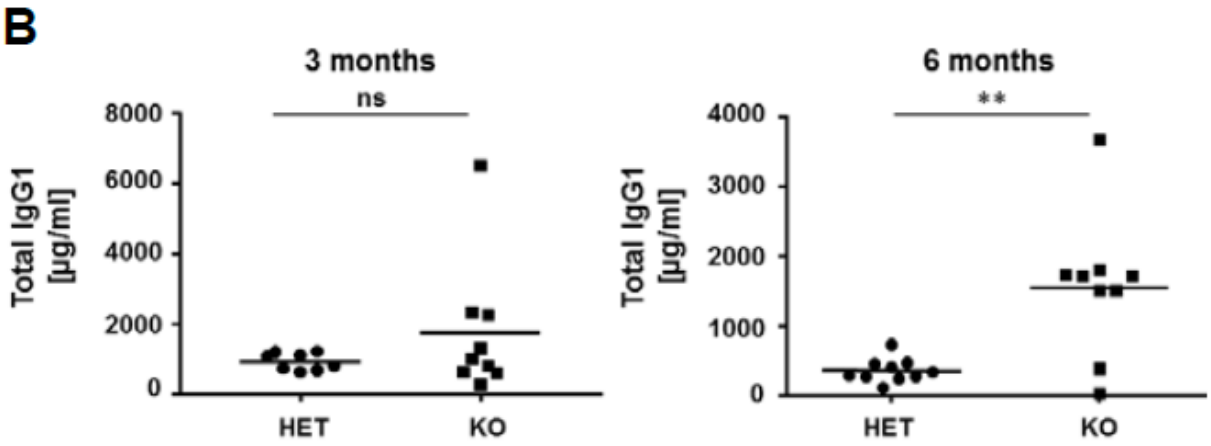
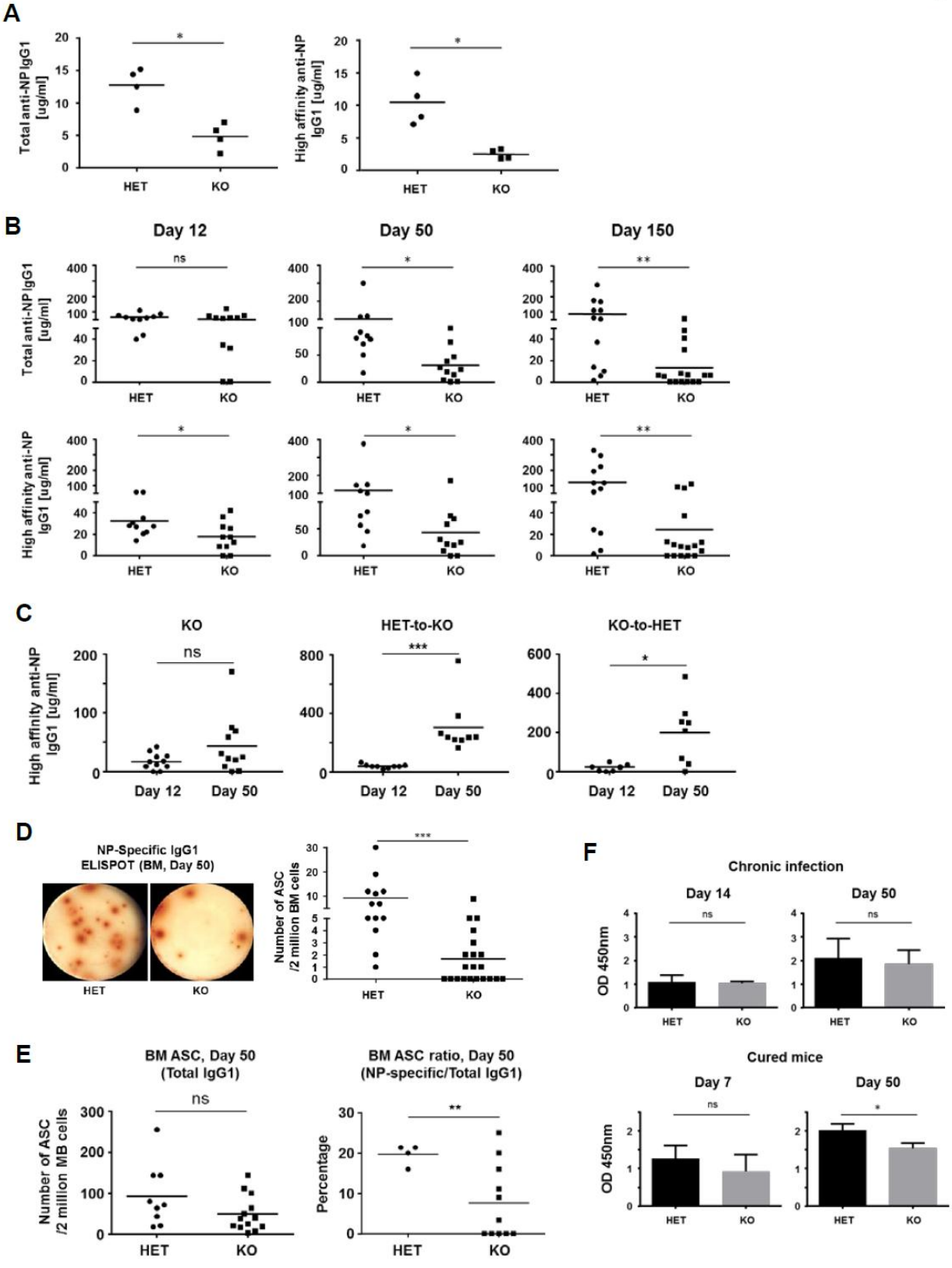


Figure 1. Mst1 KO mice display humoral immune features characteristic of MST1-deficient human patients. (A) Comparison of steady-state serum immunoglobulin levels by ELISA in Mst1 HET vs. KO mice at 8 weeks of age. Data presented as average values of optical density (OD405nm) with SEM using sera diluted by 2-fold serial dilution (n = 8 HET and 10 KO). (B) Moderate increase of serum IgG1 in KO mice upon aging. Data represent antibody concentrations measured by ELISA using standard curves (n=10 HET and 9 KO for IgG1).

4.2 Lack of sustainable IgG1 responses in Mst1 KO mice

To gain insight into the impact of Mst1-deficiency on sustainable Ab responses, we immunized mice with NP-OVA/alum and monitored NP-specific IgG1 titers over time. Mst1 KO mice at 12 weeks of age showed much weaker antibody responses compared to Mst1 HET mice, presumably due to severe T cell lymphopenia (11) (Figure 2A). In order to investigate the potential defect in duration of a humoral response after its initial establishment, we immunized mice at 7-8 weeks age. At this age, we found that early antibody titers at day 12 post-immunization were equivalent between the genotypes, but that the KO sera contained anti-NP Abs of slightly lower affinity (Figure 2B, day 12). Importantly, the total NP-specific IgG1 antibody titer (measured against NP_{30}-BSA) was not sustained in Mst1 KO mice. Whereas the average titers in HET mice remained constant (~100 µg/ml), those of Mst1 KO mice declined quite substantially down to 25% and 10% of the control HET groups at day 50 and day 150, respectively (Figure 2B). However, high-affinity antibodies (measured against NP_4-BSA) in Mst1 KO mice were not decreased at day 50 compared to those at day 12 indicating that antibodies with lower affinities are selectively lost (Figure 2C, KO). To determine if non-

hematopoietic compartments in Mst1 KO mice are able to maintain long-term antibody titers, we performed bone marrow transplantation experiments. For this, lethally irradiated Mst1 KO host mice were reconstituted with Mst1 HET bone marrow cells, immunized with NP_{16}-OVA/alum, and high affinity antibody titers were monitored at d12 and d50 post-immunization. Interestingly, high affinity anti-NP antibody titers substantially increased over time (Figure 2C, HET-to-KO). Similar results were obtained when KO bone marrow cells were reconstituted in HET recipients (Figure 2C, KO-to-HET). We speculate that the increase of antigen-specific antibody titers over time in the bone-marrow transplanted recipients is likely due to the lack of pre-existing bone marrow plasma cells in the irradiated hosts that providing ample niches for newly generated PCs upon immunization. Regardless, it is clear that Mst1 deficiency in radio-resistant stromal compartments cannot explain the progressive loss of NP-specific antibodies in Mst1 KO mice. Since long-term humoral immunity relies on bone marrow PCs continuously producing antibodies, we enumerated antibody-secreting cells (ASCs) in the Mst1 KO bone marrow by ELISPOT assays. Indeed, the decrease of serum antibody titer in Mst1 KO mice correlated with a lower number of antibody secreting cells (ASCs) in the bone marrow at day 50 (Figure 2D). However, the numbers of ASCs producing steady-state IgG1 were normal in Mst1 KO mice indicating that the antigen-specific ASC populations were selectively lost (Figure 2E). Based on this dysregulated antibody response, we examined antibody responses following oral infection with *Heligmosomoides polygyrus*, an intestinal helminth that elicits a robust antibody response (Figure 2F). We did not see a significant deficit in antigen-specific IgG1 levels over time during chronic infection (Figure 2F, Chronic infection). However, when worms were eliminated by an anti-parasite drug 2 weeks post infection, the IgG1 Ab titer dropped, consistent with data obtained from the protein immunization model (Figure 2F, Cured). Taken together, these data indicate that normal levels of antigen-specific IgG1 antibodies are generated with a minor defect in affinity maturation in young adult Mst1 KO mice, but the antibody titers cannot be maintained over time due to a selective loss of antigen-specific bone marrow PCs.


A
Total anti-NP IgG1 [ug/ml]
High affinity anti-NP IgG1 [ug/ml]
HET
KO
B
Day 12
Day 50
Day 150
ns
C
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HET-to-KO
KO-to-HET
Day 12
Day 50
D
NP-Specific IgG1
ELISPOT (BM, Day 50)
Number of ASC /2 million BM cells
E
BM ASC, Day 50 (Total IgG1)
BM ASC ratio, Day 50 (NP-specific/Total IgG1)
Number of ASC /2 million MB cells
Percentage
F
Chronic infection
Day 14
Day 50
OD 450nm
Cured mice
Day 7

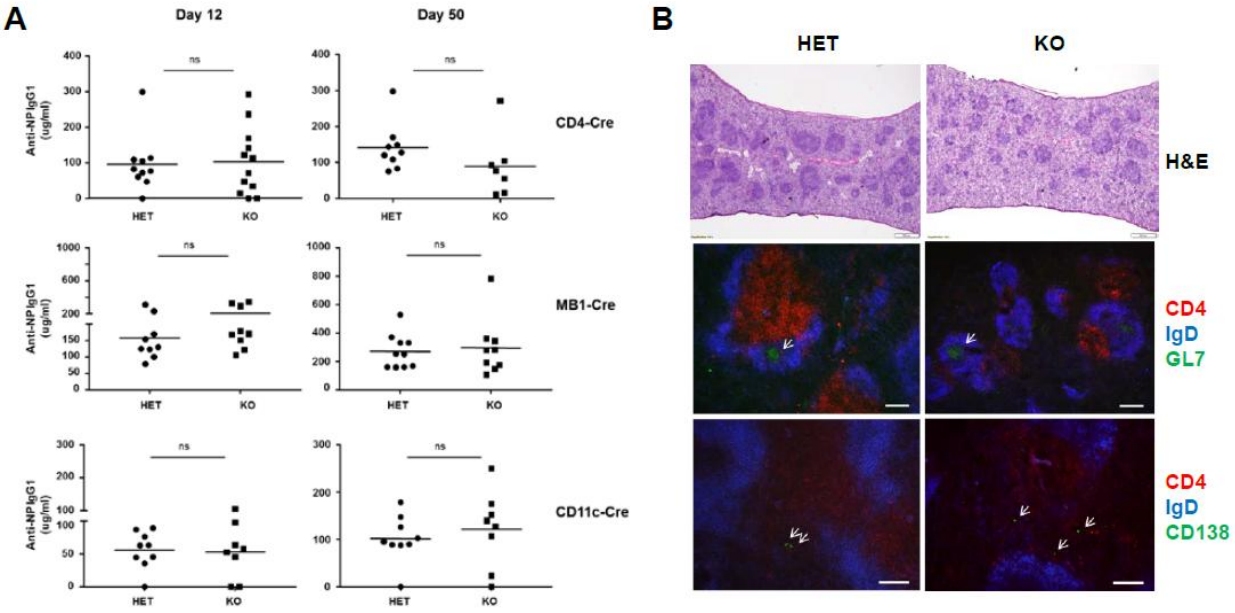
Figure 2. Lack of sustainable IgG1 responses in Mst1 KO mice. (A) Twelve-week-old mice were immunized by NP-OVA/alum and anti-NP IgG1 titers were measured at day 12 by ELISA. n=4 each HET and KO. (B) Seven- to eight-week-old mice were immunized by NP_{16}-OVA/alum and anti-NP_{30} (Total) and anti-NP_4 (High affinity) IgG1 titers were measured by ELISA at day 12, 50, and 150 post-immunization. Data pooled from two independent experiments using more than ten mice per genotype. (C) High-affinity anti-NP antibody titers were analyzed at day 12 and day 50 post-immunization by ELISA using sera from Mst1 KO recipient reconstituted with Mst1 HET bone marrow (HET-to-KO, n=9) or vice versa (KO-to-HET, n=8) in comparison with Mst1 germline KO mice (KO, n=11). (D) Bone marrow cells from day 50 post-immunization were used for ELISPOT assays to detect anti-NP IgG1 antibody secreting cells. A representative ELISPOT result (Left) with a summary of ASCs per 2 million bone marrow cells (Right, n=13 HET and 21 KO). (E) A summary of ELISPOT data depicting the total IgG1 secreting cells in BM at day 50 post-immunization (Left, n=9 HET and 13 KO). Also shown is a representative data set describing the ratio of NP-specific ASCs over the total IgG1 ASCs (Right, n=4 HET and 11 KO). (F) IgG1 titers against Hp antigens were measured by ELISA at day 14 and 50 post-infection (Chronic infection) or day 7 post-infection before curing (Cured mice, Day 7) and day 50 after curing (Cured mice, Day 50). Data are representative of two independent experiments with similar results. Mean ± SEM values of OD 450 nm at dilution 1:1458 (Chronic infection, Day 50) or 1:9 (the others). n= 5 HET and 5 KO.

4.3 Dysregulated GC reaction and increased plasma cell generation in Mst1 KO mice

Since our NP-OVA/alum immunization relies on priming of T cells by dendritic cells followed by intimate T-B interactions, we sought to determine the main immune subset(s) that requires Mst1 to sustain IgG1 responses. To this end, we compared anti-NP IgG1 responses in mice lacking Mst1 specifically in T cells, B cells, or dendritic cells using CD4-Cre, MB1-Cre, and CD11c-Cre system, respectively (Cre^+; $Mst1^{f/+}$ vs. Cre^+; $Mst1^{f/-}$; Figure 3A). It became clear that B- or DC-specific deletions of *Mst1* gene do not cause any defect in sustainable IgG1 responses. Although T cell-specific Mst1 KO mice showed a trend of reduced NP-specific Ab titers at day 50 post-immunization (~35% reduction at day 50) it did not reach statistical significance (p=0.195). Thus, it appears that there is no single dominant immune cell subset that explains Mst1 germline KO phenotypes.

The reduced affinity of NP-specific IgG1 in immunized Mst1 KO mice suggests that the germinal center reaction could be defective. Consistent with previous observation that Mst1 KO mice have severe defects in marginal zone B cell development (9), we found that the white pulp area is much reduced in Mst1 KO spleen (Figure 3B, H&E). Despite this, there was no obvious

defect in the number and the size of germinal centers in Mst1 KO mice (Figure 3B, Middle). In addition, $CD138^{+}$ plasma cells were detected in the absence of Mst1 (Figure 3B, Bottom). However, quantitative flow cytometric analysis of GC B cells revealed reduced GC B cell populations at day 9 post-immunization (Figure 3C). In addition, the GC B cells generated in the absence of Mst1 showed much lower levels of Bcl6 at day 9 post-immunization (Figure 3D). On the other hand, the Tfh cell population showed a trend of increased frequency with lower Bcl6 expression in Mst1 KO mice (Figure 3E and 3F). It appears that the early phase of GC reaction could be normal or even hyperactive since there were trends of elevated GC B (Figure 3G, day 6) and Tfh cell (Figure 3H, day 9) populations. Since GC B cells and Tfh cells interact each other to maintain high levels of Bcl6 during active GC reaction (25, 26), these data may indicate that T-B collaboration and GC B cell differentiation into plasma cells are prematurely terminated in the absence of Mst1. Alternatively, it is also possible that hyperactive GC reactions accelerate the transition of GC B cells into the plasma cell lineage reducing GC B cell numbers and Bcl6 expression levels earlier than normal.



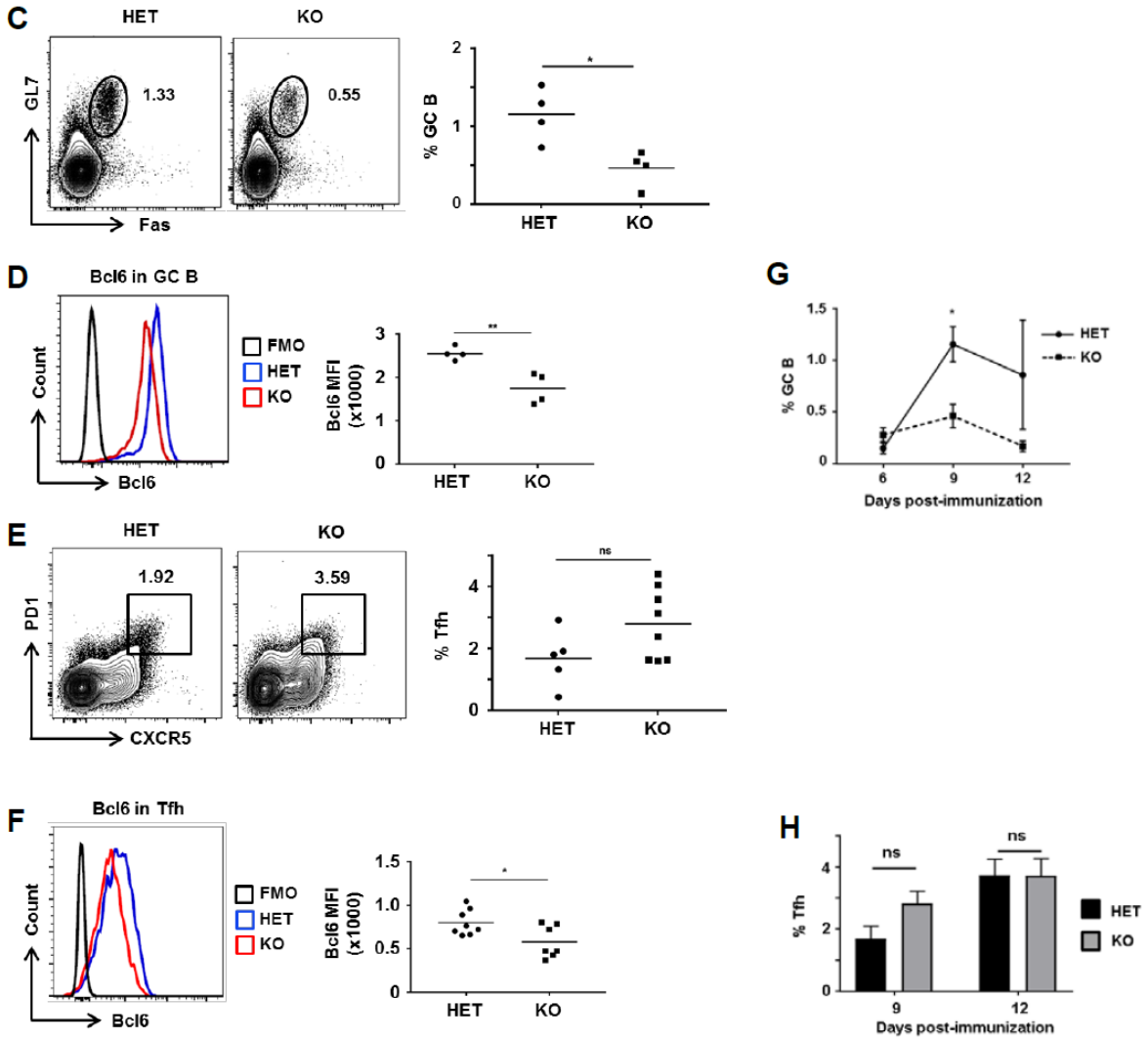
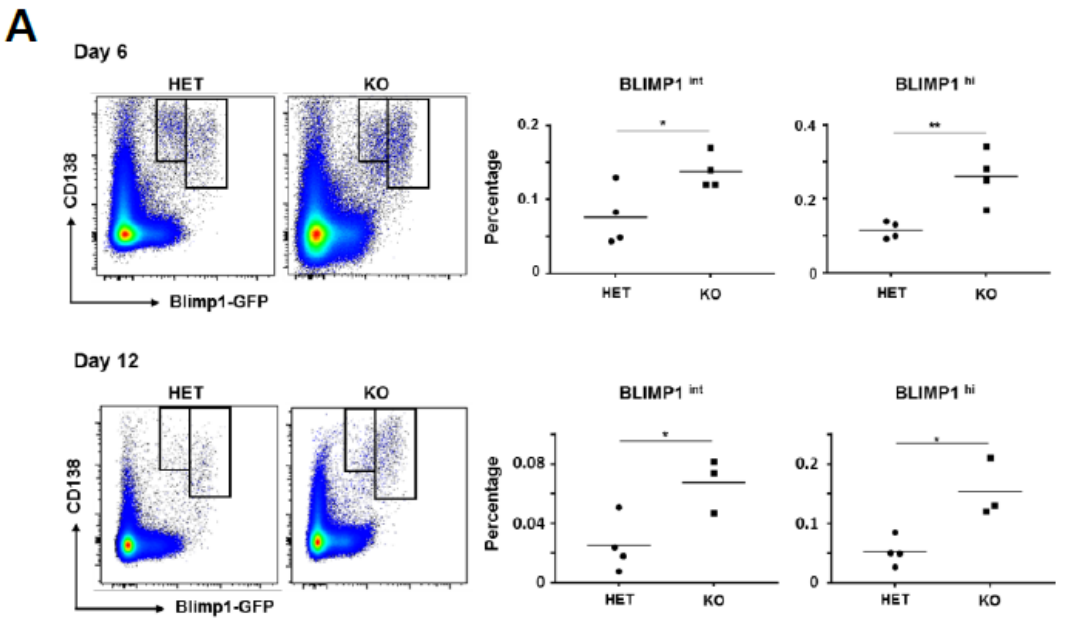
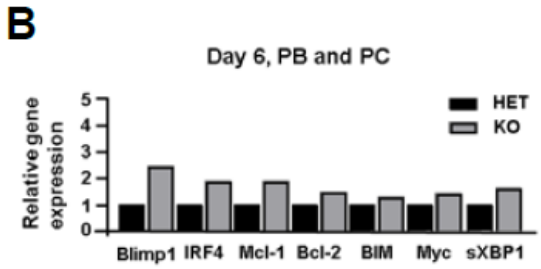


Figure 3. Dysregulated GC reaction in Mst1 KO mice. (A) T-, B-, or DC-specific Mst1 HET and KO mice were immunized with NP-OVA/alum and anti-NP antibody titers were monitored at day 12 and day 50. (B) Histological analyses of spleens. Shown are representative pictures of 2-4 spleens depicting splenic architecture after immunization (H&E, day 12), $GL7^+$ GCs in the context of IgD^+ B cell follicles and $CD4^+$ T cell zone (Middle, day 9) or $CD138^+$ PCs (Bottom, day 12). Scale bars represent 50 μm for H&E and 100 μm for immunofluorescence images. (C-F) Flow cytometric analysis of GC B cells and Tfh cells at day 9 post-immunization. Shown are a representative of GC B cell staining patterns (C, Left) and a summary of GC B cell percentages (C, Right); a representative Bcl6 intracellular staining of GC B cells with a summary of MFI (D); a representative of Tfh staining and a summary (E); and a representative Bcl6 intracellular staining of Tfh cells with a summary of MFI (F). (G-H) Flow cytometric analysis of GC B cell populations at day 6, 9, and 12 (G) and Tfh populations at day 9 and 12 post-immunization.

To examine the kinetics of plasma cell generation in Mst1 KO mice, we crossed BLIMP1-GFP reporter mice with Mst1-deficient animals. It has been shown that the level of BLIMP1-GFP protein well reflects the maturation status of plasma cells (20). Importantly, we found substantially augmented production of $CD138^{+}$ $BLIMP1^{+}$ plasmablasts (BLIMP1 intermediate) and plasma cells (BLIMP1 high) in the spleen of immunized Mst1 KO mice throughout GC reaction (Figure 4A). The equally elevated immature and mature PC populations indicate that there is no major block in the PC maturation step. To understand the molecular features of the resulting PCs generated in Mst1 KO mice, we sorted $B220^{lo}$ $CD138^{hi}$ cells at day 6 post-immunization and performed RT-qPCR analysis to assess expression of genes involved in plasma cell maturation and function (27) (Figure 4B). In support of the enhanced production of PCs in the absence of Mst1, PCs generated in Mst1 KO mice had normal to high levels of mRNA encoding BLIMP1, IRF-4, Mcl-1, Bcl-2, and XBP-1, hallmark gene signatures of plasma cell differentiation. Lastly, we observed that even under steady state, Mst1 KO mice had more $BLIMP1^{int}$ and $BLIMP1^{hi}$ $CD138^{+}$ cell populations in the spleen compared with Mst1 HET mice (Figure 4C). Interestingly, there was no difference in the bone marrow $BLIMP1^{+}$ PC populations between genotypes suggesting that limited bone marrow niches constrain the BM-PC pool size (28, 29). Collectively, these results indicate that there is ongoing overproduction of PCs in young Mst1-deficient mice probably through dysregulated GC reactions.


A
Day 6
HET
KO
CD138
Blimp1-GFP
BLIMP1 int
BLIMP1 hi
Percentage
HET
KO
Day 12


B
Day 6, PB and PC
Relative gene expression
HET
KO
Blimp1
IRF4
Mcl-1
Bcl-2
BIM
Myc
sXBP1

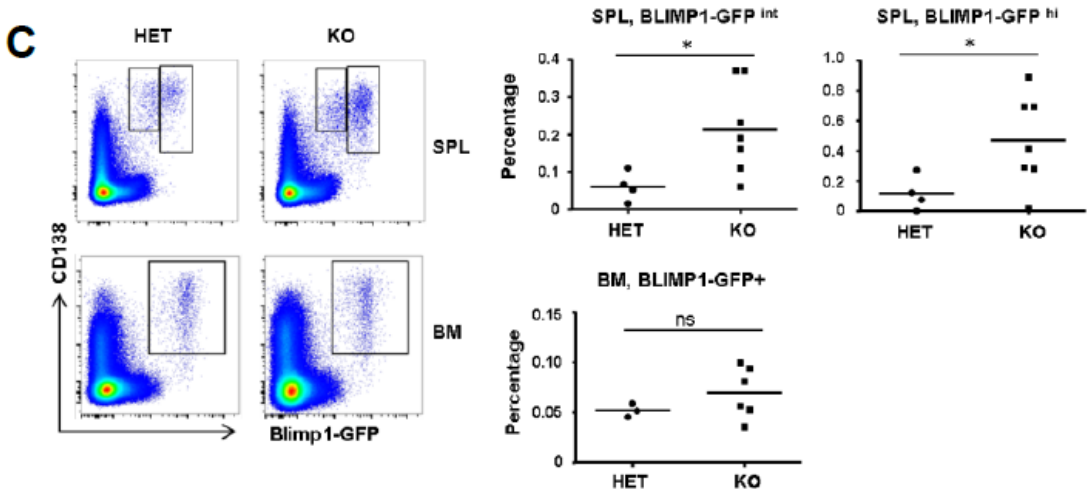
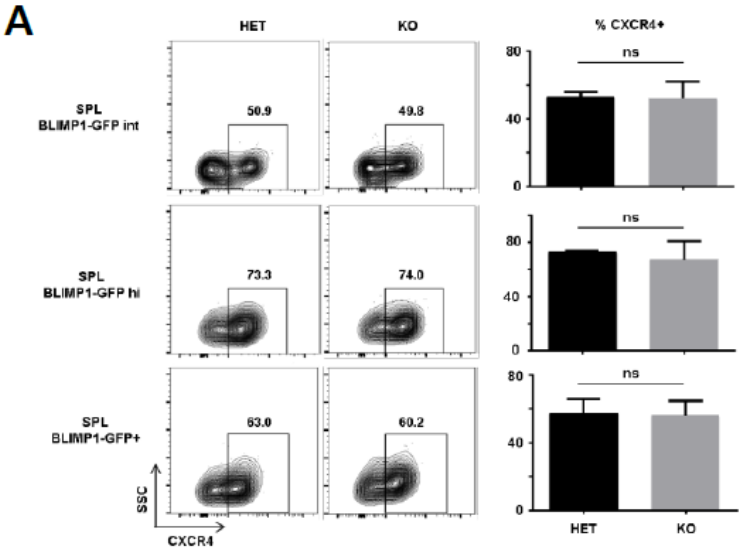
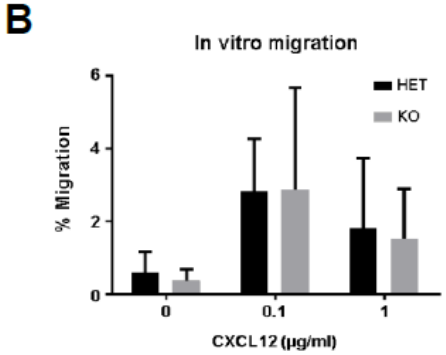

C
HET
KO
SPL
BM
CD138
Blimp1-GFP
SPL, BLIMP1-GFP int
SPL, BLIMP1-GFP hi
BM, BLIMP1-GFP+
Percentage
ns

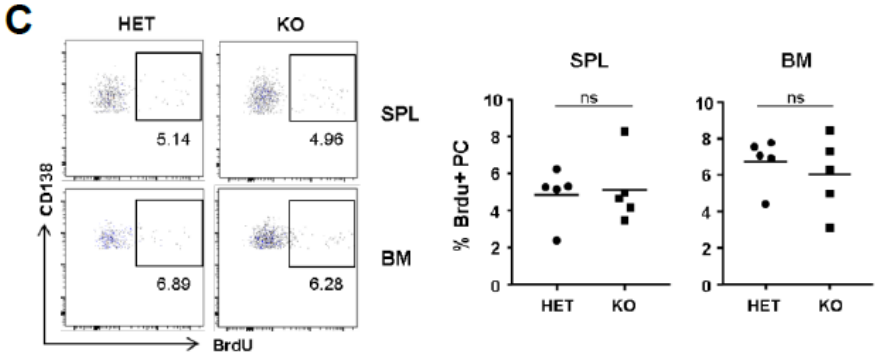
Figure 4. Augmented plasma cell generation in Mst1 KO mice. (A) Flow cytometric analysis of plasmablasts ($CD138^+$ $BLIMP1^{int}$) and plasma cells ($CD138^+$ $BLIMP1^{hi}$) at day 6 and 12 post-immunization. Shown are representative plots (Left) and percentages of PBs and PCs (Right). (B) Expression levels of genes involved in PCs differentiation are analyzed by RT-qPCR. $CD138^+$ $B220^{lo}$ cells at day 6 post-immunization were isolated by cell sorting and used for RT-qPCR analysis. A presentative of three independent experiments with similar results is presented. (C) Steady-state splenic and bone marrow PCs were analyzed by flow cytometry using Mst1 HET and KO mice expressing BLIMP1-GFP reporter at 8 weeks of age.

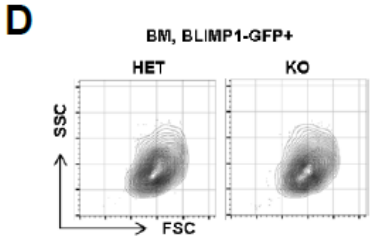
4.4 Splenic $BLIMP1^+$ PCs in Mst1 KO mice have normal migratory and secretory capacities

The heightened level of $BLIMP1^+$ PCs in the spleen may represent a transient accumulation of PCs due to higher production or an intrinsic problem of PC migratory properties. To distinguish these possibilities, we examined expression levels of CXCR4, a key chemokine receptor for PC migration to the bone marrow. As shown in Figure 5A, all the $BLIMP1^+$ PC subsets showed normal levels of CXCR4 expression. Also, Mst1 KO PC displayed normal migration capacity in transwell assays (Figure 5B). Further, PCs pulse-labeled by BrdU at day 11 post-immunization showed normal migratory patterns when assessed seven days later (Figure 5C). Lastly, we asked whether PCs generated in the absence of Mst1 have normal capacities to secrete antibodies. It has been shown that elevated levels of forward scatter and side scatter in flow cytometric profiles driven by BLIMP1 and XBP1 are good indicators of antibody secreting mature PCs (30, 31). Consistent with the intact ability to express BLIMP1 and XBP1, Mst1 KO PCs had similar levels of forward scatter and side scatter compared to those of Mst1 HET (Figure 5D). This is also well reflected in the normal number of total IgG1-secreting BM ASCs in Mst1 KO mice at steady state (Figure 5E). Taken together, these results indicate that overproduced PCs in Mst1 KO mice can migrate into BM niches and secrete antibodies.


A
HET
KO
% CXCR4+
SPL
BLIMP1-GFP int
50.9
49.8
ns
SPL
BLIMP1-GFP hi
73.3
74.0
ns
SPL
BLIMP1-GFP+
63.0
60.2
ns
SSC
CXCR4
HET
KO


B
In vitro migration
HET
KO
% Migration
CXCL12 (µg/ml)


C
HET
KO
SPL
BM
5.14
4.96
6.89
6.28
CD138
BrdU
% Brdu+ PC
ns
HET
KO


D
BM, BLIMP1-GFP+
HET
KO
SSC
FSC

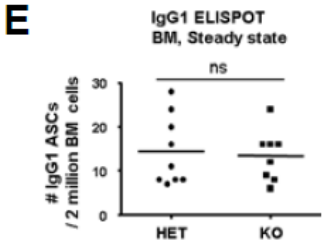

E
IgG1 ELISPOT
BM, Steady state
ns
IgG1 ASCs
/ 2 million BM cells
HET
KO

Figure 5. Plasma cells in Mst1 KO mice have normal migratory and secretory capacities. (A) CXCR4 expression levels were analyzed by flow cytometry gated on $CD138^+$ BLIMP1-GFP^+ splenic and bone marrow cells at day 12 post-immunization. (B) Migratory capacities of BLIMP1-GFP^+ splenic PCs were tested by transwell assay using CXCL12 as ligand. (C) Mice were injected with BrdU at day 11 post-immunization and the distribution of $CD138^+$ $BrdU^+$ cells in the spleen and bone marrow were assessed seven days later by flow cytometry. (D) A representative FSC/SSC profile of steady-state BM BLIMP1+ PCs. (E) ELISPOT analysis of BM ASCs secreting IgG1 at 8 weeks of age without immunization.

5. Discussion

In this study, we found that Mst1 KO mice cannot mount robust T cell-dependent antibody responses upon aging consistent with the progressive T cell lymphopenia that has been shown in Mst1-deficient mice and humans. Young adult Mst1 KO mice can produce normal antibody titers albeit with reduced affinity. However, the antigen-specific antibody titers diminish rapidly over time with concomitant selective loss of antigen-specific bone marrow plasma cells. We found that this defect is associated with dysregulated GC reactions and continuous oversupply of splenic PCs that have normal migratory, survival, and secretory features. Our data indicate that Mst1 plays important role in long-term humoral immunity by controlling PC production.

Multiple animal studies have shown that Mst1-deficiency augments innate and adoptive immune responses. First, Mst1 KO mice show enhanced type I interferon responses due to increased IRF3 activation and function (18). Consequently, Mst1-deficient cells and mice are more resistant to infections by herpes simplex virus and others. Second, dendritic cell-specific deletion of the *Mst1* gene enhanced IL-6 production by DCs and thereby promotes Th17 differentiation (19). Consistently, Mst1 KO mice were more resistant to a lethal dose of *Candida albicans*. Third, Mst1-deficient CD8 cytotoxic T lymphocytes displayed heightened cytotoxicity producing more IFN-γ and granzyme B with a concomitant increase of T-bet expression in anti-tumor responses suggesting stronger cytotoxic capacities against intracellular pathogens (32). In sharp contrast, humans lacking MST1 are highly susceptible to viruses (including HSV), fungi

(including *C. albicans*), and bacteria (4-8). In this context, our work suggests that a lack of protective antibodies may underlie immunodeficiency in humans as well as in mice.

There has been no in-depth study on the sustainable protective antibodies in MST1-deficient patients. However, 3 out of 5 case studies reported absence of antigen-specific antibody responses against vaccines despite moderately elevated serum IgG and IgA titers (5, 6, 8). Importantly, protein vaccines administered at early life (6 weeks) such as diphtheria and tetanus toxoids often failed to elicit protective titers in MST1-deficient patients, reminiscent to results produced in mouse models as presented here. This implies that Mst1-deficiency may cause a similar problem both in mice and humans: a lack of sustainable antigen-specific humoral immunity. Consistent with this idea, prophylactic immunoglobulin replacement therapy (performed in 5 patients out of 13 cases reported) appears to be beneficial to MST1-deficient patients (5-7). Taken together, these findings suggest that the apparently normal to moderately elevated serum antibody titers in MST1-deficient patients often fail to provide protective immunity and thus immunoglobulin replacement therapy should be considered as a standard prophylactic practice in clinic.

What causes the loss of long-lasting humoral immunity in Mst1 KO mice? Although we confirmed that Mst1-deficient radio-resistant stromal tissues cannot explain this defect, we failed to pinpoint a single immune cell population responsible for this phenotype by tissue-specific KO models. However, it appears that increased plasma cell production associated with dysregulated T-B interaction during GC reaction contributes to this phenomenon. Clearly, BLIMP1^{+} plasmablasts and plasma cells are over produced in Mst1-deficient mice. Also, they express intact levels of BLIMP1 and other PC markers, can normally migrate to bone marrow, and secrete antibodies. However, the antigen-specific PCs cannot sustain themselves after arriving at the bone marrow. Since the total steady-state BM-PC pools are normal in Mst1 KO mice, it appears that the loss of antigen-specific PC pools is caused by accelerated replacement by newly arriving PCs. However, it remains possible that there are intrinsic defects in Mst1-deficient PCs that we have not been able to detect.

In summary, our data indicate that the long-term antigen-specific humoral immunity is compromised in Mst1-deficient mice providing a potential explanation as to why MST1-deficient patients suffer from recurrent infections.

6. Acknowledgements

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7. Footnotes

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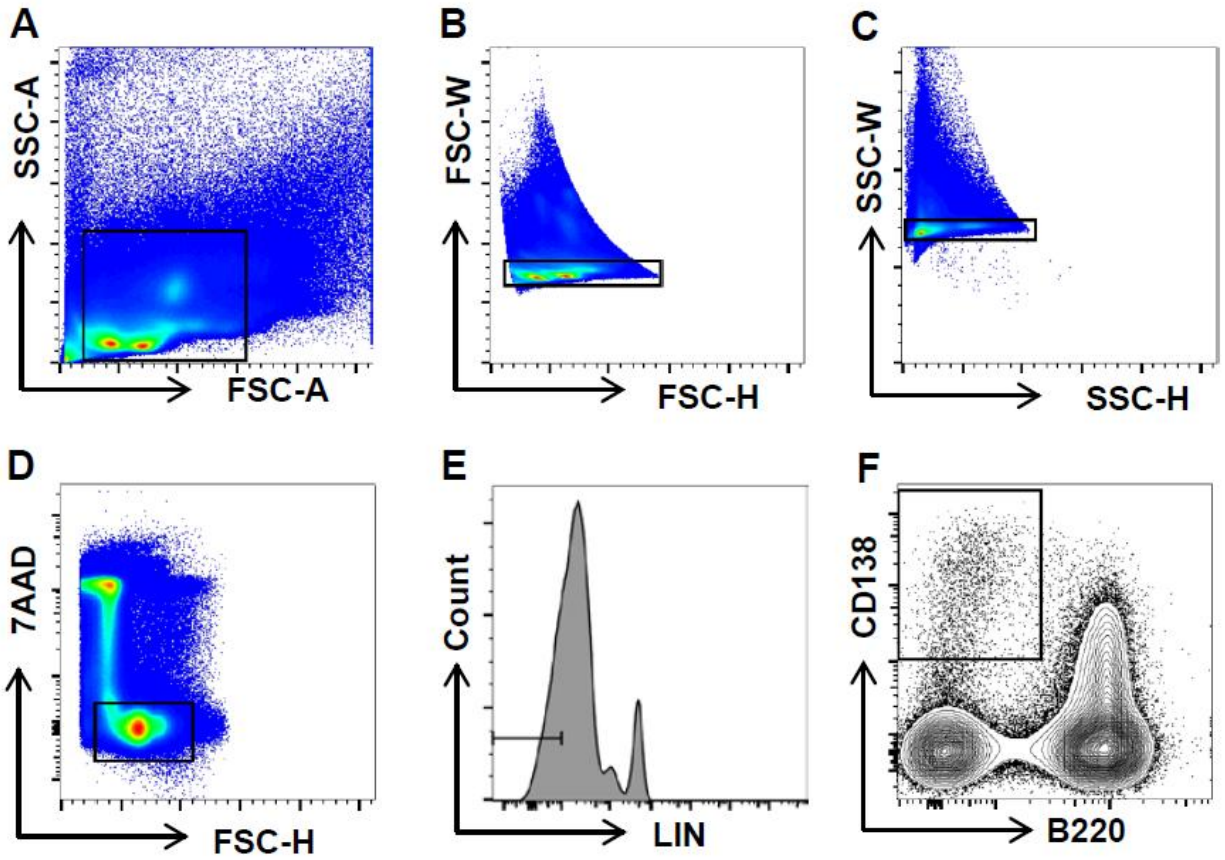
Abbreviations used in this article: Mst1, Mammalian sterile 20-like kinase 1; PB, plasmablast; PC, plasma cell; HET, heterozygous; KO, knockout; BM, bone marrow; ASC, antibody secreting cell.



Supplementary Fig. 1 Gating strategy for splenic plasma cells. Single cell suspensions were stained with antibodies against lineage markers (CD3, IgD, NK1.1, F4/80, CD11b, CD11c, Ly-6G, Ly-6C, and TER119), CD138, and B220. After gating in single cells based on FSC and SSC features (A-C), 7AAD-negative live cells were gated (D). After gating out cells expressing high levels of linage markers (E), B220- CD138+ plasma cells were identified (F). For intracellular staining, 7ADD was replaced by a fixable viability dye. Data shown represent Mst1 heterozygous mice and there were no differences between Mst1 heterozygous and knockout mice in gates A-E.

Gene	Primer Sequence (5'-3')	
	Sense	Anti-sense
BCL6	GCC CAC GTT CCC GGA GA	CGT CTG CAG CGT GTG CCT CT
BCL2	CAC CCC TGG TGG ACA ACA TC	GTT CCA CAA AGG CAT CCC AG
MCL1	AGA AAG CTT CAT CGA ACC ATT AGC	AAA CCC ATC CCA GCC TCT TTG
CMY	AAA CGA CAA GAG GCG GAC ACA	AAA GCT GCG CTT CAG CTC GTT C
IRF4	AGG TCT GCT GAA GCC TTG GC	CTT CAG GGC TCG TCG TGG TC
SXBP1	GAG TCC GCA GCA GGT G	GTG TCA GAG TCC ATG GGA
BIM	TCT TTT GAC ACA GAC AGG AGC C	ATG GAA GCC ATT GCA CTG AGA TAG
BLIMP1	GTG TGG TAT TGT CGG GAC TTT G	GGA CAC TCT TTG GGT AGA GTT C

Supplementary Table 1. List of qPCR primer sets.

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Chapter III

Dysregulation of immunoglobulin E responses in Mst1-deficient mice

Dysregulation of immunoglobulin E responses in Mst1-deficient mice

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1. Abstract

Some MST1-deficient human patients display autoimmune symptoms and highly elevated serum immunoglobulin E (IgE) titers with a potential association with mild atopic dermatitis. Although autoimmunity can be explained by defective Treg development and functions, the basis of hyper-IgE phenotype has not been addressed. Here, we found that mice with germline Mst1-deficiency accumulate high levels of serum IgE upon aging or immunization. The elevated IgE levels can be pathogenic since immunized Mst1-deficient mice suffer from anaphylactic shock when re-challenged with the immunizing antigens. Using a conditional knockout model, we demonstrated that mice lacking Mst1 selectively in $Foxp3^+$ Treg cells develop multi-organ autoimmune inflammation as expected. Further, Treg-specific Mst1-deficiency also led to pronounced hyper-IgE phenotypes. Our work clearly indicates that Mst1 plays an important role in regulating IgE production by controlling Treg cells.

2. Introduction

There are three known mechanisms that control self-reactivity caused by adaptive immune mediators: central tolerance, peripheral tolerance, and Treg-mediated dominant tolerance. Treg cells have been identified as key players to maintain self-tolerance by preventing excessive immune cell activation (1). Mutations in the Treg lineage-specific transcription factor forkhead box P3 (Foxp3) have been reported in both mouse and humans with autoimmune manifestations (2-5). Natural Treg (nTreg) cells arise in the thymus (6) whereas inducible Treg cells can be generated from conventional T cells in the periphery (7). In germinal centers, a subset of nTreg precursors further differentiate and constitute a unique subset of cells called T follicular regulatory cells (Tfr) that suppress germinal center reactions (8-10).

Mst1-deficiency has been shown to compromise Treg generation and function through multiple mechanisms (11-14). Mst1-deficient thymocytes produced reduced numbers of $Foxp3^+$ Treg cells due to decreased migratory capacity and cell-cell contact between Treg and thymic medullary epithelial cells (11). Also, peripheral Treg cells are unstable in the absence of Mst1 due to reduced acetylation of Foxp3 (13) and their suppressive function is compromised because of dysregulated immunological synapses between Treg and dendritic cells (14). Consistently, *lck-cre; Mst1*$^{f/f}$ mice have been shown to develop progressive multi-organ inflammation and autoantibody generation (11). However, the impact of defective Treg generation vs. dysregulated Treg stability and function has not been addressed in previous studies since Mst1 was deleted in all T cells at the early stages of thymic development.

Previous findings in MST1-deficient human patients revealed that immunoglobulin E (IgE) titers are highly elevated in some patients (15-18). However, the basis of this hyper-IgE phenotype and its significance has not been addressed in any previous studies. In mice, it has been shown that Mst1-deficient T cells promote plasma cell differentiation and thus leads to elevated levels of IgG, IgA and IgE (19). Also, Foxp3 deficiency in both humans and mice leads to dysregulation of humoral immune responses such as spontaneous GC reaction and elevated levels of IgE (8, 20-22). Moreover, Sakaguchi's group studied the impact of acute Treg cell depletion on humoral immunity using the DEREG system in which Treg cells expressing diphtheria toxin receptor (DTR) were deleted following exposure to DT (23). Their findings

indicated increased antigen-specific humoral responses with elevated levels of total IgE and IgG isotypes.

Using the Foxp3-Cre system, we observed that Treg-specific deletion of Mst1 induces a severe form of spontaneous autoimmune symptoms typical of *scurfy* mice (24) or Foxp3 KO mice (25, 26) - invariable skin/lung/liver inflammation at 3-4 weeks of age. Importantly, we found variably elevated serum IgE levels in Treg-specific Mst1 KO mice rendering these mice susceptible to anaphylaxis.

3. Materials and Methods

Mice

Mst1 germline KO (27) and Mst1 conditional KO mice (28) have been previously described. For Treg-specific KO model, we used Foxp3-YFP-Cre (29) (Jax016959) mouse line. All the mice were in C57BL/6 background (>12 generations) and housed in the IRCM Animal Facility under specific pathogen-free conditions. For steady-state serum antibody titers, 3-month-old mice were used. For immunization, young mice at 7-8 weeks of age were used. Animal experiments were performed according to animal use protocols approved by the IRCM Animal Care Committee.

Enzyme-linked immunosorbent assay (ELISA)

MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with capture antibody for naïve mice or with NP_4 or NP_{30}-BSA (Biosearch Technologies) for immunized mice. IgE ELISAs were performed according to manufacturer's instructions using IgE Mouse Uncoated ELISA Kit (Thermo Fisher Scientific). Plates were read at 450 nm (HRP-TMB) using a Molecular Devices microplate reader and SoftMax Pro 4.7 software.

Immunization

For immunization, mice received a single intraperitoneal injection of 4-hydroxy-3-nitrophenylacetyl-ovalbumin (NP_{16}-OVA) (Biosearch Technologies) in alum (Thermo Fisher Scientific) at 100 μg in 200 μl per mouse and received the second injection at 50 μg in 200 μl per mouse 30 days after primary immunization.

Histology

For hematoxylin and eosin (H&E), serial paraffin-embedded sections (5μm) were used for staining.

Anaphylactic Shock

The severity of anaphylaxis was judged by monitoring changes in mouse body temperature following secondary antigenic challenges of mice that had been immunized with NP_{16}-OVA as described above. Mouse body temperature was monitored using a TH-5 Thermalert Monitoring Thermometer equipped with a RET-3 rectal probe (Physitemp Instruments, New Jersey) at 10-minute intervals.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Student's *t*-test was used to analyze statistical significance: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ (Graphpad Prism 7).

4. Results

4.1 Augmented IgE production in Mst1 KO mice

Despite T and B cell lymphopenia, human patients possessing MST1-null mutations have moderately increased levels of class-switched antibody (Ab) titers (15, 16, 30-32) and some of the patients have elevated serum IgE titers (15, 16, 31). Consistent with human findings, Mst1 KO mice showed small but significant elevation of serum IgE at 3 months of age (Figure 1A). In order to gain insight into the impact of Mst1-deficiency on Ab responses, we immunized mice with NP-OVA/alum and monitored NP-specific IgE titers over time. A portion of immunized mice had highly elevated NP-specific IgE titers and the elevated IgE levels were sustained over time (Figure 1B). Since our immunization system relies on priming of T cells by dendritic cells followed by intimate T-B interactions, we sought to determine the main immune subset(s) that requires Mst1 to produce IgE responses. To this end, we compared anti-NP IgE responses in mice lacking Mst1 specifically T cells using CD4-Cre system (Cre^{+}; $Mst1^{f/+}$ vs. Cre^{+}; $Mst1^{f/-}$). Consistent with Mst1 germline KO mice, T cell-specific Mst1 KO mice showed increased NP-

specific Ab titers upon aging (Figure 1C). Collectively, these data indicate that Mst1-deficient mice produce more IgE at steady-state and upon immunization. In addition, this defect is, at least in part, due to T cell-intrinsic Mst1-deficiency.

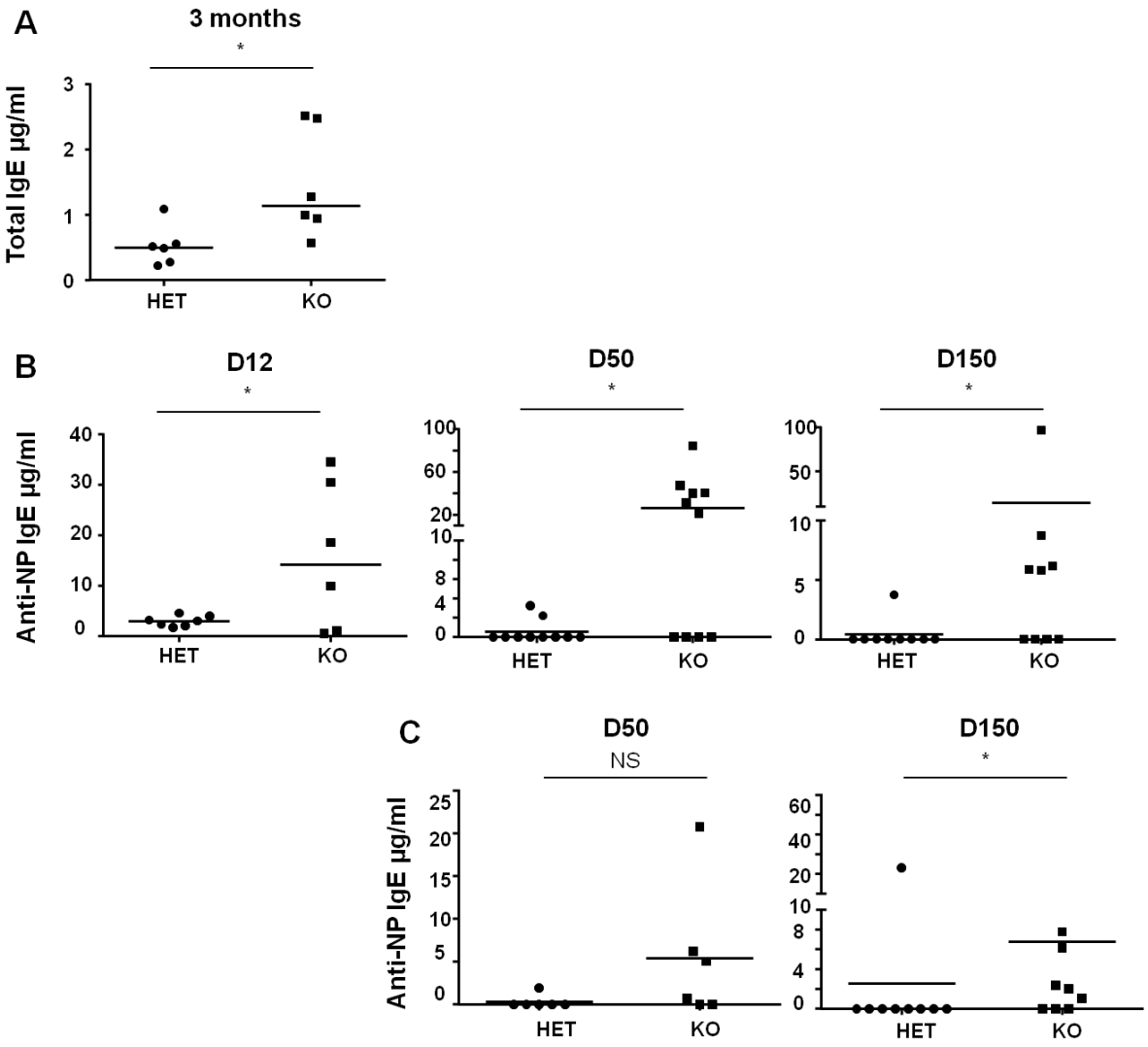

Figure 1. A) Moderate increase of serum IgE in aged Mst1 KO mice. B) Anti-NP IgE titers were measured by ELISA at day 12, 50, and 150 post-immunization. Data pooled from two independent experiments using more than six mice per genotype. C) Anti-NP IgE titers were measured in CD4-Cre MST1 mice by ELISA at 50, and 150 post-immunization.

4.2 Anaphylactic shock in Mst1 KO mice

Systemic anaphylaxis in mice has been featured by symptoms such as hypothermia, rapid and reversible hypotension, decreased mobility and scratching (33). Currently, there are two known pathways of systemic anaphylaxis in mouse models. In the classical model, anaphylaxis has been shown to be mediated by IgE, FcεRI, mast cells, histamine and platelet-activating factor (PAF). The alternative pathways are mediated by IgG, FcγRIII, macrophages, and PAF. However, to induce a similar hypothermic response as seen in the classical pathway, 100-fold more antigen is required in the alternative pathway (34). Since both unimmunized and immunized Mst1-deficient mice developed long-lasting hyper IgE responses (Figure 1), we tested if Mst1 KO mice develop anaphylactic shock through the elevated levels of IgE. For this, the mice that had been immunized with NP_{16}-OVA/alum 30 days before were challenged with the same antigen. Given that the severity of anaphylaxis in mice can be measured through changes of core body temperature (33), we measured body temperature using a digital thermometer equipped with a rectal probe 5 minutes after challenge, in 10-minute intervals until body temperature was recovered within the normal range. The baseline body temperature was measured before the mice were sensitized and was considered as time point zero. Body temperature dropped more quickly in Mst1 KO mice compared to heterozygous control group and KO mice also showed physical signs such as skin scratching and breathing distress (Figure 2A). In order to confirm that the anaphylactic shock was only driven by antibodies against NP or ovalbumin, the components of immunizing antigen NP_{16}-OVA, we injected mice with NP_{30}-BSA, or NP_{16}-OVA in the absence of alum. Although injection of NP_{30}-BSA also induced mild anaphylaxis, NP_{16}-OVA induced strongest anaphylactic reaction (Figure 2B, C). These data suggest that the elevated serum IgE specific for the immunizing antigens in Mst1 KO mice can be pathogenic.

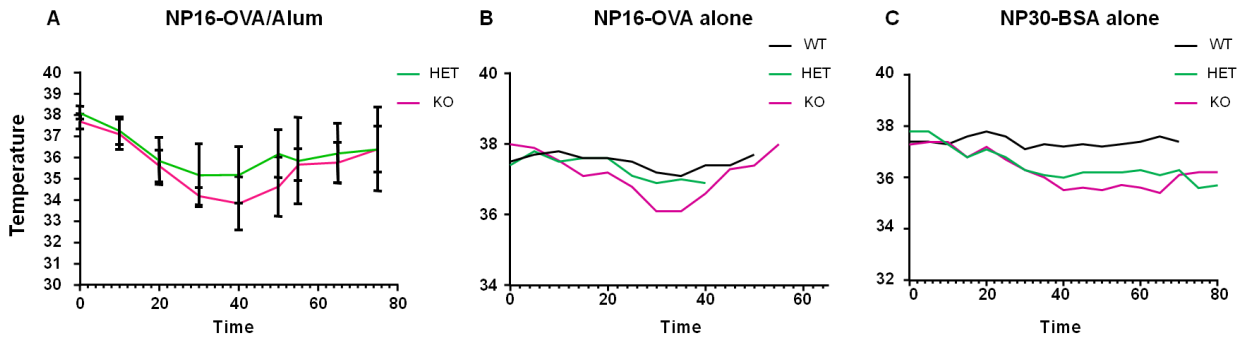

Figure 2. Anaphylactic shock in Mst KO mice. (A) Core body temperature. Data shown is representative of 5 Mst1 KO and 5 Mst1 HET mice. Mice were challenged with NP_{16}-OVA/alum 30 days post-immunization and the body temperature was measured before and after injection with 10-minute intervals until recovery. (B) Mice were injected with NP_{16}-OVA without alum or (C) NP_{30}-BSA without alum and the body temperature was measured as above.

4.3 Pronounced hyper IgE phenotype in Treg-specific Mst1 KO mice

Although mild, Mst1 germline KO mice or mice lacking Mst1 specifically in T cells (*lck-cre*$^{+}$*;Mst1*$^{f/f}$) displayed multi-organ autoimmune inflammation at ~1 year of age (11). This is presumably due to a combination of impaired thymic selection and compromised number and suppressive function of Treg cells (12-14). However, the impact of Treg-specific Mst1-deficiency has not been examined. In another study, defective Treg maintenance has been shown to increase serum IgE titers (35). To address the Treg-specific role of Mst1 in prevention of autoimmunity and IgE production, we generated a mouse line that specifically lacks Mst1 only in Treg cells using the Foxp3-Cre strain (29). Remarkably, Treg-specific Mst1-deficiency caused much more aggressive autoimmune pathology compared with Mst1 KO or *lck-cre*$^{+}$*;Mst1*$^{f/f}$ mice: all the *Foxp3-Cre*$^{+}$*; Mst1*$^{f/-}$ developed skin inflammation in the tail, eyelid, and ears (Figure 3A) and lymphocytic infiltration in the lung (Figure 3B) and liver (data not depicted) at 3-4 months of age. The accelerated autoimmune symptoms in Treg-specific Mst1 KO compared with Mst1 KO or *lck-cre*$^{+}$*;Mst1*$^{f/f}$ mice are probably due to an intact pathogenic effector T cell compartment with a selective defect in the Treg population as opposed to a global defect in all T cell compartment caused by deletion of *Mst1* gene in the germline or early stage of T cell development. Importantly, we also detected highly elevated serum IgE titers with normal levels of IgG1 in unimmunized mice at 3 months of age (Figure 3C). The elevated IgE titer was much more pronounced in Treg-specific KO: ~10-fold increase in Treg-specific KO mice vs. ~2-fold

increase in germline KO mice suggesting that Treg cells normally downregulate IgE production in an Mst1-dependent manner.

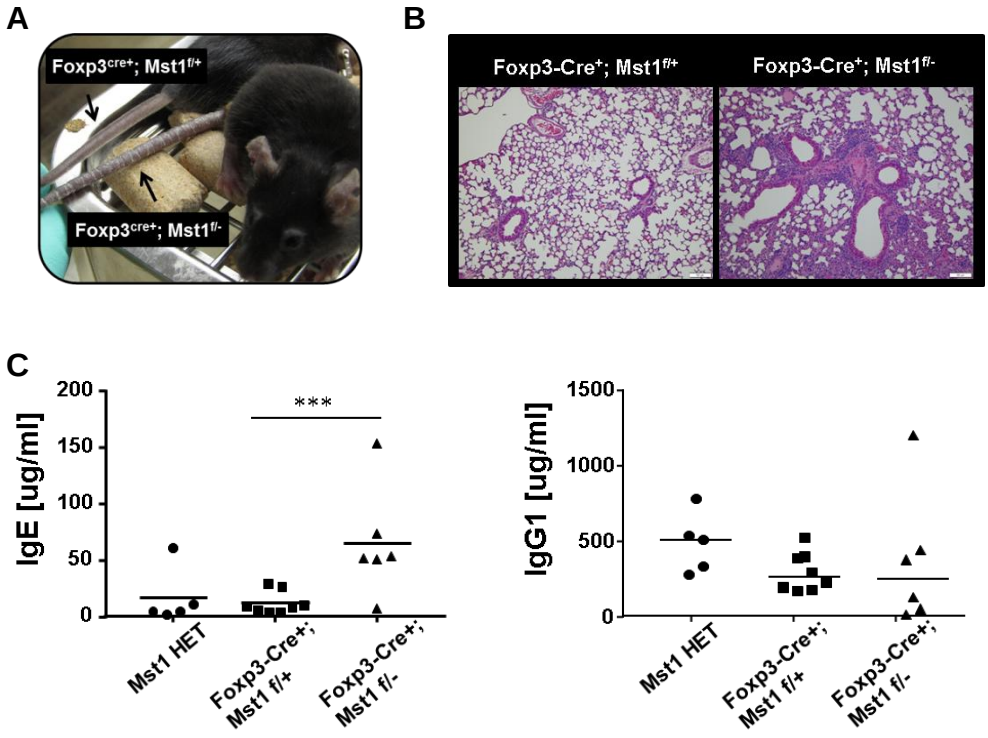

Figure 3. Pronounced hyper IgE phenotype in Treg-specific Mst1 KO mice. (A) Mice with Treg-specific deletion of Mst1 develop inflammation in the skin (A) and lung (B) at 3 months of age. Data shown are representative of eight *Foxp3-Cre*$^{+}$*;Mst1*$^{f/+}$ and six *Foxp3-Cre*$^{+}$*;Mst1*$^{f/-}$ mice. (C) Highly elevated serum IgE levels in *Foxp3-Cre*$^{+}$*;Mst1*$^{f/-}$. Sera from HET (n=5), *Foxp3-Cre*$^{+}$*;Mst1*$^{f/+}$ (n=8), and *Foxp3-Cre*$^{+}$*;Mst1*$^{f/-}$ (n=6) mice at 3 months of age were analyzed by ELISA (1:100 dilution) to detect IgE and IgG1.

5. Discussion

Here we found that Mst1 KO mice mounted and maintained higher levels of IgE antibodies, a response phenocopied by selective deletion of Mst1 in Treg cells. Given that hyperimmunoglobulin E syndrome is caused by DOCK-8 deficiency along with immunodeficiency (36), the basis of hyper IgE symptoms in MST1-deficiency is puzzling (15, 16, 31). Consistently, our data showed that Mst1 KO mice had small but significant elevation of serum IgE at 3 months of age. Similar to steady state, the antigen specific IgE levels were also elevated upon immunization and were sustained over time. However, our data showed that only 30-40% of mice had hyper IgE consistent with human patients. Collectively, this may indicate the "stochastic" nature of IgE response. In addition, our results of the CD4-cre system showed that T cell-specific Mst1 KO mice had a trend of increased NP-specific IgE titers compared to the control group. Consistent with our data in MST1 germline KO mice, the elevated IgE levels were sustained over time and were measurable around D150 post immunization. Therefore, loss of Mst1 expression in T cells appears to dysregulate IgE production. However, considering that T cell-specific KO mice had ~3-fold less IgE titers compared to germline KO mice, it is likely that non-T cell compartments contribute to this phenotype. Clearly, future work is required to address whether B cells or dendritic cells are also important in this aspect.

Given that immunodeficiency was accompanied with autoimmune manifestations in some MST1-deficient patients, its basis remains incompletely understood. It has been proposed that Mst1-mediated regulation of Treg development and function works through modulation of Foxo1/Foxo3 protein stability and this may explain the autoimmunity in MST1-deficient patients (13, 37). Consistently, it has been shown that Foxo protein levels were reduced in MST1-deficient human peripheral blood mononuclear cell samples (PBMCs) (16, 17) and peripheral T cells in mouse studies of Lim's group (38). Another study has shown that epigenetic methylation of *MST1* gene in Tregs leads to autoimmune pancreatitis. Thus, optimal MST1 expression in T cells appeared to be critical in preventing B cell-mediated autoimmunity (39). Importantly, we found variably elevated serum IgE levels in Treg-specific Mst1 KO mice. This might be partially due to elevated IL-4 production by Mst1-deficient T cells as shown previously (11). In addition, it has been shown that Mst1-deficiency favors Th2 cell production creating an IL-4-rich environment and Mst1 has been proposed to be critical in preventing spontaneous B cell

activation (19). However, this view is in sharp contrast with previous findings that Mst1-deficiency prevents induction of experimental autoimmune disease EAE (experimental autoimmune encephalitis) (40). Therefore, further work is required to better understand the molecular basis of autoimmunity in Mst1-deficiency. Further, it will be interesting to see if Treg (or Tfr) cells regulate the IgE class-switching process itself and possibly maintenance of IgE producing PCs.

In summary, we found that variably elevated serum IgE levels in Mst1 KO mice can lead to anaphylaxis confirming its pathological potential. These elevated IgE responses are at least in part driven by T cells as shown in CD4-Cre system. Most importantly, Mst1-dependent Treg regulatory mechanisms play critical roles not only in controlling cellular autoimmunity but also downregulating IgE responses. Clearly, further work is required to shed more light on the molecular basis of Treg-dependent mechanisms that regulate IgE responses.

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Chapter IV

General discussion

1. Summary

My thesis projects began with the following questions in mind: 1) MST1-deficient patients have moderately elevated serum IgG levels yet suffer from recurrent infections with viruses, bacteria, and fungi. Are there any hidden defects in the quality of the antibodies? 2) Some patients also display hyper IgE symptoms. Could this put the patients at risk of allergic reactions such as anaphylaxis? We decided to address these questions using mouse lines lacking Mst1 ubiquitously or in a tissue-specific manner.

Mst1 germline KO mice recapitulated features of MST1-deficient human patients, augmented steady-state IgG1 and IgE levels. However, IgG1 responses in Mst1 KO mice showed three major defects when they were immunized with T-dependent protein antigens. First, aged Mst1 KO mice (beyond 3 months of age) could not mount strong antibody responses presumably due to progressive loss of naïve T cells, a major suspected cause of immunodeficiency in humans and mice. Second, young Mst1 KO mice (before 2 months of age) were able to produce antigen-specific IgG1 to a normal level in terms of quantity but had substantially reduced amounts of high-affinity antibodies. Third, the low-quality antigen-specific IgG1 antibodies did not last long due to a reduced number of long-lived bone marrow plasma cells. Consistent with our data, many human patients did not show strong antibody responses against protein-based vaccines. Overall, our work indicates that MST1-deficiency can compromise the quality of humoral immunity at multiple levels and provide a rationale for prophylactic IgG replacement therapy.

In contrast to IgG1 responses, Mst1 KO mice mounted IgE response to much higher levels upon immunization and maintained the hyper IgE contents for a prolonged period of time (~5 months post-immunization). Importantly, the elevated antigen-specific IgE titers well correlated with the increased susceptibility of immunized Mst1 KO mice to anaphylaxis. Thus, the augmented amount of antigen-specific IgE in Mst1 KO mice had ability to cause severe allergic reactions suggesting that human patients could be also at risk.

Besides the clinical relevance, our work provided novel insights into the delicate regulatory mechanisms governed by Mst1 in humoral immunity (Figure 1). First, Mst1 appears to play a key role in controlling optimal plasma cell output during GC reaction. The premature loss of antigen-specific IgG1 antibodies in Mst1 KO mice does not seem to be due to major

defects in the quality of plasma cells produced upon immunization; it is rather ongoing overproduction of plasma cells, which presumably causes faster turnover of bone marrow plasma cells. This overproduction of plasma cells coincides with a reduced number of GC B cells at the peak of GC reaction possibly reflecting accelerated transition of GC B cells into plasma cell lineage. Second, Mst1 plays an important role in controlling IgE responses through Treg cells. Although it has been shown that Treg-specific Mst1 KO leads to autoimmune symptoms due to impaired Treg generation and function, its link to hyper IgE has not been described. It remains to be determined if the long-lasting antigen-specific IgE antibody titers rely on bone marrow plasma cells and, if so, how they persist in the face of elevated turnover. Future work should focus on cellular and molecular events during germinal center reaction that explains the overall framework presented here.

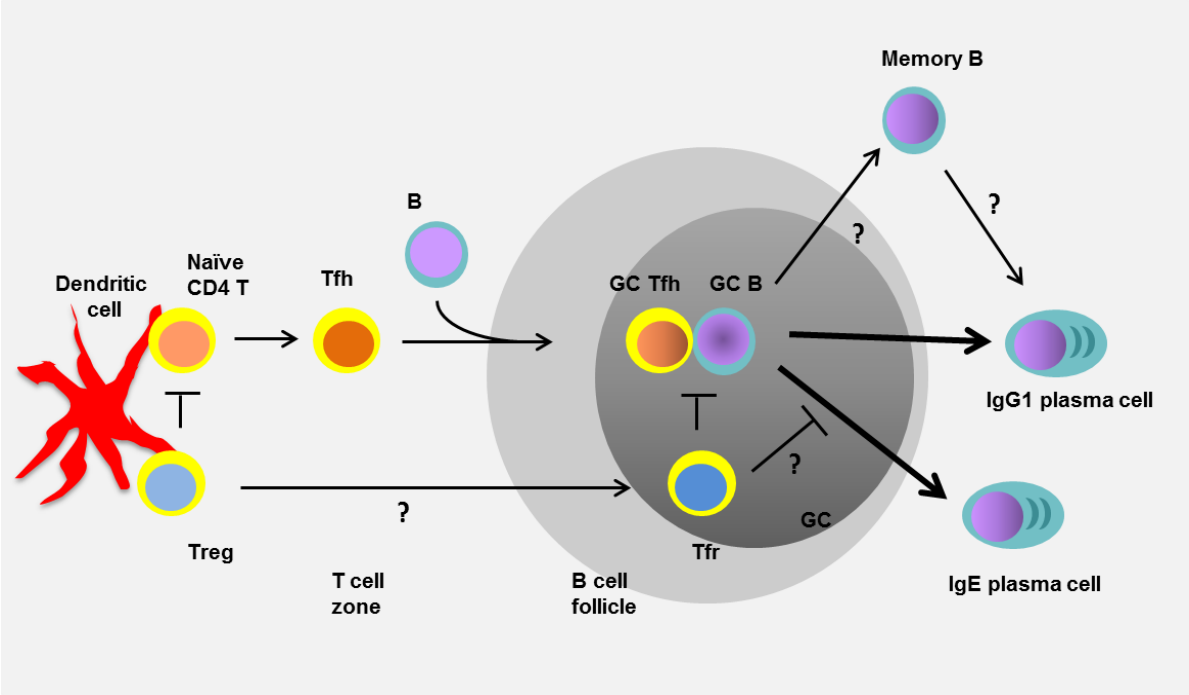

Figure 1. A working model for altered humoral immunity in Mst1-deficient mice. Dysregulated germinal center reaction, most likely accelerated transition of GC B cells into plasma cell lineage, increases plasma cell output. This may lead to faster turnover in the bone marrow plasma cell pool and early loss of plasma cells of a given antigen specificity. It remains to be seen whether Mst1-deficiency affects generation of memory B cells. Mst1-expressing Treg/Tfr cells are required to downregulate IgE production although detailed mechanisms remain to be clarified.

2. Future directions

First of all, more detailed mechanistic studies are required to understand how Mst1 regulates Tfh cells. Previous studies both in human and mice revealed that FOXO protein levels were reduced in Mst1-deficiency (1, 2). Importantly, it has been shown that a transient inactivation of FOXO1 is critical for the initiation of the Tfh program, yet restoration of FOXO1 level is critical for the generation of GC Tfh cells through upregulation of Bcl6 expression (3). One of the key downstream targets of FOXO1 relevant to a T-dependent antibody response is KLF2 (4, 5). During the course of Tfh response, KLF2 protein is downregulated in the early phase (by day 2 post-immunization) but increases by day 5, and then decreases again at days 7-14. This dynamic regulation of KLF2 level is critical to coordinate T cell trafficking and gene expression patterns for the initiation and maintenance of GCs (4). Thus, abrogation of *Mst1* gene may disrupt the dynamic changes of FOXO1 and KLF2 protein levels in Tfh cells and thereby proper control of GC reaction. Kinetic analysis of FOXO1 (through intracellular staining) and KLF2 protein levels using KLF2-GFP reporter mice (4) may help define Mst1 downstream signaling mechanisms.

Mst1-mediated clustering of high affinity LFA-1 on the T cell surface plays important role in T cell migration and interaction with antigen presenting cells (6). Also LFA-1 is known to be important for Bcl6 expression in Tfh cells (7). Thus, Mst1-deficient Tfh cells may have compromised capacity to make stable conjugates with GC B cells and lose Bcl6 expression due to the lack of high affinity LFA-1 on the cell surface. This possibility can be tested by ICAM1 binding assay (7).

It is puzzling to see lower number of GC B cells at the peak of GC reaction yet increased plasma cell generation. This can be reconciled if Tfh cells deliver more potent help signals (e.g., CD40 ligand, IL-21 or IL-4) while they interact with cognate GC B cells at the early phase of GC reaction promoting plasma cell differentiation. My preliminary data suggest that there is no difference in IL-21 expression levels in Mst1 KO Tfh cells. However, CD40L and/or IL-4 production levels by Tfh cells need to be assessed.

As mentioned earlier, following the establishment of GC in the B cell follicle, GC B cells initiate to cycle between DZ and LZ where they undergo SHM and affinity selection, respectively (8). The current model argues that GC B cells with high affinity BCRs get selected in response to signals from Tfh cells in the LZ, ultimately giving rise to high affinity plasma cells and memory B cells after rounds of proliferation, diversification and selection (8, 9). Recently, two studies have shown that FOXO1 regulates the formation and maintenance of the GC DZ. Thus, loss of FOXO1 in GC B cells disrupted the DZ and further SHM and affinity selection. CXCR4 has been shown to be directly involved in this regulation as a downstream target of FOXO1 in GC B cells. Further studies however, indicated that FOXO1 upregulates the transcription factor BATF thus regulates proliferation of GC B cells. Hence, FOXO1 has been argued to not only control GC polarization but also partially regulate the GC proliferation (10). Therefore, loss of Mst1 may further down regulate FOXO1 protein expression or function in GC B cells which could cause dysregulation of GC dynamics leading to defective affinity selection as our data showed. Hence, further studies are required to address these possibilities. It is interesting to note that KLF2 has also been reported to be expressed in B cell lineage cells. Studies of KLF2-deficient mice suggested that KLF2 plays a role in B cell subset differentiation and it has also been shown that KLF2 is critical in dictating FO B cell identity (11, 12). Furthermore, *in vitro* studies of KLF2-deficient B cells indicated defective proliferation and survival following BCR stimulation which could be corrected by simultaneous stimulation of CD40. Importantly, T cell dependent *in vivo* studies of KLF2-deficiency in B cells indicated reduced antigen-specific Ig responses and lower plasma cell numbers (12). In addition, it has been proposed that KLF2 positively regulates plasma cell migration and homing to bone marrow survival niches through regulation of S1P gene expression (13). Therefore, investigating this signaling pathway may be promising to understand the role of KLF2 in B cells downstream of Mst1 and how it impacts humoral responses.

After interaction with Tfh cells, GC B cells may differentiate into plasma cells or memory B cells. It remains poorly understood what parameters determine the fate of GC B cells. Given that the lack of Mst1 promotes plasma cells differentiation, it would be interesting to see if memory B cell differentiation is altered as well. Considering the progressive accumulation of serum antibodies in the face of T cell lymphopenia in Mst1 KO mice, it is conceivable that the memory B cell pools generated in early phase of life could continuously provide antibodies when

restimulated in a T-independent manner. Contrary to this prediction, one paper has reported reduced levels of circulating memory B cells in MST1-deficient patients (14).

Strikingly, immunization of Mst1-deficient mice elicits elevated levels of antigen-specific IgE from the beginning (compared to the normal IgG1 titers) and the IgE titers persist up to 3 months, when IgG1 antibodies drop to ~10% of those seen in Mst1 heterozygous control mice. Considering the ongoing overproduction of the total Blimp1^{+} plasma cells and apparent faster turnover of IgG1-producing plasma cells in the absence of Mst1, there should be mechanisms making IgE-producing plasma cells survive better in bone marrow environment. Given that Treg-specific Mst1 KO greatly elevates steady-state serum IgE titers upon aging, it appears that Treg/Tfr cells normally tightly control IgE class switch and/or plasma cell longevity in an Mst1-dependent manner. In-depth analysis of IgE responses using young adult mice lacking Mst1 specifically in Treg compartment may reveal unique characteristics of IgE class switch processes and maintenance of IgE-producing plasma cells.

4. Concluding remarks

Germline and tissue-specific Mst1-deficient mouse lines proved to be excellent animal models to study immune mechanisms underlying the primary immunodeficiency in MST1-deficient patients. Although my initial work mainly focused on clinically relevant questions, one could learn more about the biology of humoral immunity in the future, particularly GC B cell fate decision (memory B cells vs. plasma cells) and the role of Treg/Tfr cells in IgE responses.

5. References

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