The physiological role of polycomb group protein Ezh2 in dendritic cells

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The Physiological Role of Polycomb Group Protein Ezh2 in Dendritic Cells

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Abbreviations

BM	Basement membrane
BMDC	Bone marrow-derived dendritic cell
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
cDC	Classical dendritic cell
CHS	Contact hypersensitivity
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR	CXC chemokine receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DN	Double negative
DNFB	Dinitrofluorobenzene
DNTB	Dinitrothiocyanobenzene
DP	Double positive
dSEARCH	Dendrite surveillance extension and retraction cycling habitude
EED	Embryonic ectoderm development
EpCAM	Cancer-associated epithelial cell adhesion molecule
ESAM	Endothelial cell-selective adhesion molecule
EZH1	Enhancer of zeste homolog 1
EZH2	Enhancer of zeste homolog 2
FAK	Focal adhesion kinase
FCS	Fetal calf serum
Flt3L	FMS-like tyrosine kinase 3 ligand
fMLP	N-formylmethionine-leucyl-phenylalanine
H2AK119ub1	Monoubiquitylated histone 2A lysine 119

H3K27me3	Trimethylated histone 3 lysine 27
HBSS	Hanks' balanced salt solution
ICAM	Intercellular adhesion molecule
IFN γ	Interferon gamma
IL	Interleukin
IMQ	Imiquimod
iSALT	Inducible skin-associated lymphoid tissue
LC	Langerhans cell
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MDP	Macrophage dendritic cell progenitors
MHC	Major histocompatibility complex
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
mTEC	Medullary thymic epithelial cell
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PcG	Polycomb group protein
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PRC	Polycomb repressive complex
PRR	Pattern recognition receptor
PTM	Post-translational modification
RLR	Retinoic acid-inducible gene-I-like receptor
RT-qPCR	Quantitative real-time polymerase chain reaction
SP	Single positive
SUZ12	Suppressor of zeste 12
TCR	T cell receptor
TGF β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF α	Tumour necrosis factor alpha

Treg	Regulatory T cell
UT	Untreated
VCAM	Vascular cell adhesion protein
VLA-1	Very late antigen-1
YFP	Yellow fluorescent protein

Abstract

The contribution of epigenetics in the regulation of leukocyte development and functions has gained increasing attention over the past years. Polycomb group protein Enhancer of Zeste homolog 2 (Ezh2) is a histone methyltransferase which regulates gene expression through the modification of chromatin structure via the addition of H3K27me3 marks. In this study, we aim to characterize the role of Ezh2 using Langerhans cells (LCs) and thymic dendritic cells (DCs) as model systems, and further elucidate the molecular mechanism and *in vivo* relevance underlying such regulation.

Surprisingly, the well-established epigenetic repressor Ezh2 was found to control LC integrin-dependent migration via an epigenetic-independent mechanism involving the direct methylation of an extra-nuclear substrate Talin1. Deficiency in Ezh2 impairs the disassembly of adhesion structures which enhances the adhesive properties of LCs, leading to their reduced transmigration across the skin basement membrane. Using a mouse model for allergic contact dermatitis, we demonstrated that reduced migration of Ezh2-deficient LCs to the skin-draining lymph nodes resulted in the development of an exaggerated contact hypersensitivity (CHS) response despite tolerization with low dosage of an innocuous hapten 2, 4- dinitrothiocyanobenzene. Such breakdown in skin tolerance arises from the compromised activation of regulatory T cells, which consequently promotes an enhanced CD8⁺ T cell response in the sensitization phase of CHS.

In addition, DC-intrinsic expression of Ezh2 was found to regulate early T cell developmental program particularly in adult mice. Deficiency of Ezh2 in thymic DCs favours the $\gamma\delta$ T cell lineage and leads to a profound developmental block characterized by a decrease in DP thymocytes, indicating their roles in directing lineage choices and DN thymocyte development. These developmental defects are accompanied by a change in the cytokine milieu of the thymus, suggesting the importance of DC-intrinsic Ezh2 in sustaining a proper thymic cytokine environment for normal T cell development.

Collectively, our data highlighted the importance of Ezh2 in LCs for the regulation of their integrin-dependent migration, and also in thymic DCs for the maintenance of adult-stage early T cell development.

(1) Introduction

1.1 Dendritic cells

Dendritic cells were first identified by Steinman and Cohn in the 1970s as a rare population of cells residing in the spleen with a stellate and elongated morphology (Steinman & Cohn, 1973). However, their role as sentinels of the immune system was only recognized after culmination of decades of efforts. Even though DCs can now be broadly defined as a family of professional antigen-presenting cells capable of priming naïve T cell response, it is becoming increasingly clear that they are actually a heterogeneous mix of populations with unique ontogeny, distinct localization and specialized function. At present, DCs can be classified into 4 major subtypes, namely the classical DC (cDC), Langerhans cell (LC), plasmacytoid DC (pDC), and monocyte-derived DC.

1.1.1 Classical Dendritic Cells

The DC family is composed predominantly of the classical DCs. cDCs are a group of specialized antigen-processing and –presenting cells responsible for the surveillance of tissues against invading pathogens, while at the same time, capable of maintaining tolerance towards self-antigens. To achieve these, they are sparsely but strategically positioned in most lymphoid and non-lymphoid tissues including the thymus, spleen, lymph node and skin.

1.1.1.1 Immunogenic function of cDCs

cDCs have been proposed to exist in 2 distinct functional and phenotypic states, depending on their maturation status (Albert et al., 1998; Platt et al., 2010). Immature cDCs are highly phagocytic cells with a low surface expression of major histocompatibility complex (MHC) class I and II proteins. During this stage, the cDCs continuously sample the tissue environment for microbial products and necrotic cells through the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) via their expression of diverse pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene-I-like receptors (RLRs) and NOD-like receptors (NLRs). Upon antigen capture, these cells quickly lose their phagocytic capacity and undergo rapid terminal differentiation to form mature DCs

which accumulate the peptide-MHC II complexes on their surfaces (signal 1). During the maturation process, DCs also undergo transcriptional reprogramming to upregulate their surface expression of various costimulatory molecules like CD40, CD80 and CD86 (signal 2), and increase their secretion of proinflammatory cytokines including $\text{TNF}\alpha$, IL-6, IL-12 and IL-23 (signal 3). By providing these 3 signals, DCs are able to trigger the proliferation of antigen-specific T cells and subsequently polarize them towards the correct effector phenotype (Curtsinger, Lins, & Mescher, 2003). Hence, these contribute to their transformation from an immune sentinel into an exceptional T cell stimulator.

Figure 1. Mature DCs provide 3 signals for T cell activation and polarization. Signal 1 involves the presentation of a peptide-MHC II complex on the DCs to the T-cell receptor. Signal 2 is the delivery of costimulatory molecules such as CD80 and CD86 on DCs to CD28 expressed on the T cells. Lastly, signal 3 consists of the DC-secreted cytokines, which are responsible for the polarization of T cells towards a Th1 or Th2 phenotype. Taken from Kapsenberg, 2003.

1.1.1.2 Tolerogenic function of cDCs

Apart from their crucial role in T cell priming, the ability of cDCs in establishing immunological tolerance towards innocuous self and non-self antigens is becoming evident in recent years. During the maintenance of central tolerance, thymic DCs strategically located in the medulla and cortico-medullary perivascular space mediate the clonal deletion of self-reactive thymocytes and their clonal diversion to form regulatory T cells (Tregs) through the presentation of self-antigens acquired from

either the medullary thymic epithelial cells (mTEC) or the bloodstream respectively (Coquet et al., 2013; Proietto et al., 2008). However, since it is impossible to fully eliminate all self-reactive T cells in the thymus, peripheral tolerance exists as an additional mechanism to inhibit T cell responses against antigens previously not encountered in the thymus, or those which arise during the later stages of life, in particular the food and developmental antigens. In this case, tolerogenic DCs in the periphery can promote the generation of Tregs (Sela, Olds, Park, Schlesinger, & Steinman, 2011), bring about the anergy of T cells through negative signalling exerted by the expression of PD-1 or CTLA-4 (Probst, McCoy, Okazaki, Honjo, & van den Broek, 2005), or induce the deletion of self-reactive T cells via Fas- and Bim-mediated apoptosis (Steinbrink, Graulich, Kubsch, Knop, & Enk, 2002). Previously, it was believed that such tolerance induction is a consequence of antigen presentation by immature DCs with a low surface expression of MHC molecules to the T cells (signal 1) in the absence of costimulation (signal 2). However, accumulating evidence suggests that this paradigm is not totally true. DCs expressing high levels of MHC II and costimulatory molecules have been reported to harbour tolerogenic activity as well. These DCs, which frequently produce anti-inflammatory cytokines such as IL-10 and TGF β rather than pro-inflammatory cytokines, are being labelled as 'semi-mature' DCs. In fact, these semi-mature DCs seem to bear greater tolerogenic potential than immature DCs due to their superior lymph node homing ability (Verginis, Li, & Carayanniotis, 2005). Thus, the therapeutic potential of semi-mature DCs as treatment options for autoimmune diseases has been greatly exploited in the recent years.

1.1.1.3 Developmental origins and homeostasis of cDCs

cDCs are cells of hematopoietic lineage derived largely from the common myeloid progenitors (CMPs), and to a smaller extent from the common lymphoid progenitors (CLPs), in the presence of the DC poietin Fms-related tyrosine kinase 3 ligand (Flt3L). In the bone marrow, these early progenitors differentiate into the macrophage dendritic cell progenitors (MDPs) before committing to the dendritic cell lineage to eventually give rise to the cDC-restricted precursors (pre-cDCs) (Waskow et al., 2008). These pre-cDCs which have not yet expressed surface MHC II then circulate through the blood and seed the periphery, where they subsequently differentiate locally to establish a network of dendritic cells in both the lymphoid and

non-lymphoid organs. Contrary to previous dogma which describes the cDC as non-dividing and 'end-stage', BrdU labelling studies revealed that these steady state cDCs were capable of undergoing limited number of cell division in the periphery (Ginhoux et al., 2009). However, since they do not self-renew, they have to be continuously replenished by blood-borne precursors approximately every 3 to 6 days (K. Liu et al., 2007). As such, the maintenance of the steady state cDC homeostasis depends on the interplay among their replenishment from the bone marrow-derived precursors, their local proliferation, and ultimately, their cell death.

1.1.1.4 cDC subsets

Despite the traditional viewpoint that all DCs are highly adept at antigen processing and presentation, it is becoming increasingly clear that extensive functional heterogeneity exists within the cDCs. Currently, cDCs can be further divided into two main categories, namely the CD8⁺ (equivalent to CD103⁺ in non-lymphoid tissues) and the CD11b⁺ cDC subsets. These distinct cDC subsets are segregated not only on the basis of their phenotypic markers, but also their functionality and ontogeny (Table 1). The precise roles and anatomical locations of these heterogeneous DC subsets in various lymphoid and non-lymphoid organs will be further discussed below.

1.1.1.4.1 CD8⁺ and CD103⁺ cDCs

CD8⁺ and CD103⁺ cDCs, which populate the lymphoid and non-lymphoid organs respectively, are the most well-defined cDC subsets identified to date. The functional equivalence and shared origin of these 2 cDC subsets regardless of their tissue residency have been established based on their common gene signature identified through the profiling of their transcriptome network (Miller et al., 2012). In the spleen and lymph nodes, approximately 20 to 40% of the DCs belong to the CD8⁺ DC subset, while on the other hand, almost the entire thymic DC compartment is comprised of the CD8⁺ DCs (Vremec, Pooley, Hochrein, Wu, & Shortman, 2000). In the periphery, CD103⁺ DCs are distributed among organs such as the lung, skin and intestinal lamina propria. These CD8⁺ and CD103⁺ cDCs are highly specialized in the cross presentation of exogenous antigens to cytotoxic CD8⁺ T cells, thereby playing an important role in the generation of an effective immune response against

DC subset	Phenotypic markers													Location	Function	Ref
	CD11c	MHC II	CD8	CD11b	CD103	Langerin	EpCAM	B220	CD24	Clec9a	CD205	CD209	F4/80			
CD8⁺ cDC	+++	++	+	-	+/-	+/-	-	-	++	++	++	-	-	Spleen, lymph nodes, thymus	Cross presentation	(Gallegos & Bevan, 2004)
CD11b⁺ cDC	+++	++	-	+	-	-	-	-	+	-	+	+	+	Spleen, lymph nodes	MHC II presentation	(Dudziak et al., 2007)
CD103⁺ CD11b⁻ cDC	++	++	-	-	++	+	-	-	++	++	++	-	-	Connective tissues, peyer's patches, dermis, lungs	Cross presentation	(Bedoui et al., 2009)
CD103⁻ CD11b⁺ cDC	++	++	-	+	-	-	-	-	+/-	-	ND	+/-	+	Dermis, lamina propria, lungs	MHC II presentation, tolerance	(Guilliams et al., 2010)
LC	++	++	-	+	-	++	++	-	++	-	++	-	+	Epidermis	Promote Th17 differentiation, tolerance	(Gomez de Agüero et al., 2012; Igyarto et al., 2011)
pDC	+	+	+/-	-	-	-	-	+	ND	+	-	++	-	Blood, lymphoid tissue, thymus	Type I IFN production	(Jego et al., 2003)

ND, not determined

Table 1. Phenotype and functions of murine DC subsets. Adapted from Merad, 2013.

intracellular pathogens (Bedoui et al., 2009; del Rio, Rodriguez-Barbosa, Kremmer, & Forster, 2007). On the other hand, thymic CD8⁺ DCs utilize their efficient cross-presentation capacity for the maintenance of central tolerance to negatively select against self-reactive CD8⁺ T cells during the developmental process (Gallegos & Bevan, 2004).

1.1.1.4.2 CD11b⁺ cDCs

Unlike the CD8⁺ and CD103⁺ DC subsets which consist of a unified population sharing a core gene signature, CD11b⁺ DC subset represents a highly heterogeneous population according to their transcriptome analysis (Miller et al., 2012). Given that their current phenotypic classification is insufficient, attempts have been made to further distinguish the CD11b⁺ DCs using markers such as Endothelial cell-selective adhesion molecule (ESAM) and CD4, albeit being unsuccessful in yielding homogeneous subsets. These CD11b⁺ DCs are found predominantly in lymphoid organs except for the thymus (which consists of less than 30% CD11b⁺ DCs), as well as various non-lymphoid organs like the muscle and lamina propria. Although the function of CD11b⁺ DCs remains less well defined, some evidence suggest that they are highly specialized in CD4⁺ T cell priming due to their exceptional MHC II presentation machinery (Dudziak et al., 2007).

1.1.2 Langerhans Cells

The Langerhans cell (LC) was named after Paul Langerhans, who first reported the presence of a non-pigmentary, dendritically shaped population residing in the epidermis. Although these cells were once perceived to be receptors of the nervous system residing in the epidermis, this “nerve theory” was gradually disproved a century later owing to accumulating evidence on the hematopoietic origin of LCs. Today, LCs are recognized as a subset of skin-resident DCs which strategically populates the epidermis, thereby forming the first line of immunological defense against the external environment. Despite being classified under the DC family, the gene signature and origin of LCs were found to be more closely related to that of macrophages than the cDCs (Hoeffel et al., 2012; Miller et al., 2012). Thus, this has led to emerging debate over the classification of LCs, and whether the existing classification system for monocytes, DCs and macrophages should be revisited.

1.1.2.1 The Langerhans cell paradigm

Langerhans cell paradigm, coined by Wilson and Villadangos, serves to describe the prototypic function of LCs as sentinels of the immune system (Wilson & Villadangos, 2004). In their immature state, sessile LCs survey the epidermis through their characteristic dendrite surveillance extension and retraction cycling habitude (dSEARCH) (Gaiser et al., 2012). These immature LCs are equipped with high endocytotic capacity for efficient uptake of antigens from the periphery, and upon encountering such danger signals, their mobilization to the skin-draining lymph nodes is augmented via a series of coordinated events. Initially, E-cadherin downregulation facilitates the disengagement of epidermal LCs from their surrounding keratinocytes (T. Jakob, Brown, & Udey, 1999), thus enabling them to crawl in between the keratinocyte gaps via extension of their pseudopodia (Stoitzner, Pfaller, Stossel, & Romani, 2002). Later, LCs transit across the basement membrane through enzymatic digestion of the dense collagen fibrils by matrix metalloproteinases (MMPs), a type IV collagenase secreted by the LCs (Kobayashi, 1997; Ratzinger et al., 2002). This results in the creation of a migratory path with focal absence of lamina densa, thus allowing LCs to traffic towards the underlying dermis. These activated LCs subsequently upregulate their surface CCR7 expression for a directional migration along the CCL21 chemotactic gradient,

towards the lymphatic vessels (Ohl et al., 2004). *En route* to the skin-draining lymph nodes, these LCs undergo phenotypic maturation, enhancing their antigen presenting capacity and their expression of costimulatory molecules in preparation of triggering a T cell-specific response. As such, LCs function to initiate the adaptive immunity through their acquisition and presentation of cutaneous antigens.

Figure 2. Key events during LC-mediated immune response. Pathogen invasion or hapten application leads to the capture of antigens by LCs, the professional antigen-presenting cells residing in the epidermis. This induces the release of pro-inflammatory cytokines by LCs and their adjacent keratinocytes, thus promoting the maturation and migration of LCs to the T cell zones of the skin-draining lymph nodes. Once in the lymph nodes, activated LCs can either present the captured antigens directly to naïve T cells, or transfer the antigens to the lymph node-resident CD8⁺ DCs for indirect presentation to the T cells. These trigger an antigen-specific T-cell proliferation and differentiation, subsequently leading to the development of an adaptive immune response. Taken from “Complexity of the immune system: Changing soldiers to battle,” Brian Cheng, 2012, symposium.com.

1.1.2.2 Development origins of LCs

Despite their functional similarity, LCs were found to originate from a unique precursor distinct from that of cDCs. The steady state LC pool arises from two different progenitors of distinct origins which seed the epidermis independently during embryonic development. The first wave of progenitor recruitment involves the yolk sac-derived macrophages, which infiltrate the epidermis early in embryogenesis and eventually account for approximately 10% of the adult LC population. This is followed shortly by a second wave during late embryogenesis, in which the fetal liver monocyte precursor eventually contributes to majority of the LC population during adult stage (Hoeffel et al., 2012). Immediately after birth, these 2 types of progenitors differentiate locally into MHC II⁺ CD11c⁺ LCs before undergoing a proliferative burst to expand their numbers by more than 10-fold. The result is an establishment of a LC network which remains stable throughout life in steady state.

1.1.2.3 LC homeostasis during steady state and inflammatory state

Unlike cDCs which have limited proliferative capacity and need to be constantly replenished by circulatory bone marrow-derived precursors, LCs can be maintained locally over extended period of time, independent of any input from the bone marrow. Studies have demonstrated that host LCs persisted indefinitely in the skin of chimeric mouse after congenic bone marrow transplantation despite the rapid repopulation of donor DCs in other peripheral organs such as the spleen and kidney (Merad et al., 2002). In the absence of inflammation, LCs are maintained stably with an estimated half-life of between 53 to 78 days (Vishwanath et al., 2006), contrary to the short half-life of cDCs which is around 5 to 7 days (K. Liu et al., 2007). Such self-renewal of LCs are found to be mediated by a small subset of slow proliferating resident LCs which re-enters the cell cycle upon signaling from their microenvironment to maintain the homeostatic balance of LCs during steady state (L. Chorro et al., 2009).

However, under inflammatory conditions, two distinct pathways are utilized to replenish the LCs, depending on the degree of damage inflicted on them. In the event of a high dosage UVB light-induced skin inflammation, a severe depletion of LCs from the skin necessitates the recruitment of blood-borne precursors for their repopulation (Chorro & Geissmann, 2010). This process is mediated at large by CCR2 on the precursors, which respond to the CCL2 and CCL7 gradient at the

epidermal-dermal junction for their efficient recruitment (Merad et al., 2002). On the other hand, when local inflammation without the radical depletion of LCs is evoked, *in situ* proliferation of LCs is sufficient to repopulate the epidermis. During such inflammatory conditions, the number of cycling LCs in the epidermis is greatly enhanced to ensure that their massive proliferation compensates for the LCs loss (L. Chorro et al., 2009). Thus, the homeostatic maintenance of LCs is highly dependent on the amount and the type of stress exerted on them.

1.1.2.4 Functional specialization of LCs

Although LCs have been long regarded as prototypic DCs, they appear to possess unique capacity in their T cell priming ability. The immunogenic properties of LCs were first questioned when they were found to be incapable of priming a CD8⁺ T cell response in the skin-draining lymph nodes following infection with herpes simplex virus. Moreover, reports showing the functional redundancy of LCs relative to dermal DCs during contact hypersensitivity (CHS) response further highlighted their dispensability in the priming of T cell response against skin-acquired antigens (Noordegraaf, Flacher, Stoitzner, & Clausen, 2010). However, during allogeneic bone marrow transplantation, the host LCs are responsible for the activation of T cells to trigger a graft-versus-host disease (Merad et al., 2004). In addition, LCs are sufficient to generate Th17, but not cytotoxic T cell responses, in the event of infection with *Candida albicans* (Igyarto et al., 2011). Hence, the immunogenicity of LCs is highly dependent on the context of the immune responses, and they are clearly functionally distinct from other cDC subsets residing in the skin. On the other hand, mounting evidence suggest that LCs are involved in the induction of tolerance towards skin antigens (Gomez de Agüero et al., 2012; Shklovskaya et al., 2011). They are able to transport and present antigens from the periphery to the skin-draining lymph nodes, thereby promoting the deletion of CD8⁺ T cells and the activation of regulatory T cells for protection against various autoimmune diseases. However, the precise mechanism underlying the interplay between tolerogenicity and immunogenicity of LCs remains poorly understood.

1.2 Dendritic cell migration

DCs play a crucial role in bridging the innate and adaptive immunity during both physiological and pathological conditions. As a prerequisite for the elicitation of an effective immune response, they have to be positioned precisely, in a timely manner, at specific lymphoid and non-lymphoid organs. To achieve this, the cytoskeleton of DCs is highly flexible and versatile to support their rapid and drastic reorganization in response to various stimuli. As such, they are able to generate rapid gliding movements with a migratory velocity of up to 10µm/min (Gunzer et al., 2000), almost 100-fold faster than other mesenchymal and epithelial cell types.

1.2.1 Amoeboid migration of DCs

DCs exhibit an amoeboid migratory pattern reminiscent of the amoeba *Dictyostelium discoideum* (Barry & Bretscher, 2010). During such movement, the polarized DCs first extend their plasma membrane at the leading edge in response to chemoattractants through the polymerization of filamentous actin to form a growing pseudopod. Following attachment of the pseudopod to the underlying substrate, actomyosin contraction at the cell centre transduces an internal force to propel the rigid cell nucleus forward. The trailing edge of the DC then detaches itself from the substrate, facilitating advancement of the cell (Friedl, Borgmann, & Brocker, 2001). Such frequent shape changes experienced by the DCs during amoeboid migration enables them to squeeze through physically constrained regions of the extracellular matrix, circumventing the need for proteolytic degradation and therefore preserves the structural integrity of the tissue environment (Wolf, Muller, Borgmann, Brocker, & Friedl, 2003). In order to fulfil their immune functions, DCs have to transverse through different tissues composed of distinct extracellular matrices in the body. Depending on the topology of the tissue structures encountered, they can adopt either an adhesion-dependent or adhesion-independent form of amoeboid movement.

1.2.1.1 Adhesion-dependent amoeboid migration of DCs

When transmigrating across two-dimensional tissue surfaces like the vessel walls and basement membranes, DCs have to rely largely on integrin-mediated adhesion for their effective migration. In this case, the integrin repertoire expressed on their surfaces dictates their affinity for the matrix present in the tissue environment. For

instance, circulating DCs in the blood make use of integrins LFA-1 and VLA-4 for their binding to endothelial ligands ICAM-1 and VCAM respectively, hence allowing for their firm arrest onto the endothelial barrier to overcome the shear stress from the flowing blood, thereby facilitating their trafficking into target tissues during both homeostatic and inflammatory conditions such as experimental autoimmune encephalomyelitis (Jain, Coisne, Enzmann, Rottapel, & Engelhardt, 2010). On the other hand, LCs exiting the epidermis have to transverse through a basement membrane network enriched in laminin, type IV collagen and proteoglycans (Fleischmajer et al., 1998) before arriving at the dermal afferent lymphatics for further migration to the skin-draining lymph nodes. Here, $\alpha 6$ integrin-mediated adhesions have been reported to be critical for their attachment and subsequent transmigration across the basement membrane (Price, Cumberbatch, Kimber, & Ager, 1997).

1.2.1.2 Adhesion-independent amoeboid migration of DCs

Intriguingly, DC migration in a confined three-dimensional environment was found to be driven primarily by actomyosin contractions rather than integrin-based adhesions (T. Lammermann et al., 2008). This was demonstrated by integrin- and talin1-deficient DCs, which surprisingly were able to migrate from the dermis to the skin-draining lymph nodes, a route which does not require crossing of any tissue barrier, in a manner indistinguishable from their wild type counterparts. In this case, protrusion of actin filaments is sufficient for the DCs to initiate movement in regions of low matrix density while myosin II-mediated contraction is only essential when they have to squeeze through dense regions of the tissue interstitium. Indeed, disruption of the actin cytoskeletal network and inhibition of the myosin contractile activity through latrunculin B and blebbistatin treatment respectively resulted in the migration arrest of DCs in a three-dimensional environment (T. Lammermann et al., 2008). Such adhesion-independent migration strategy serves to endow DCs with the capacity to navigate through different organs rapidly without being restricted to predefined routes due to the limited integrin repertoire expressed on their cell surfaces.

1.2.2 Dynamic switch between different modes of migration

Rather than being confined to a particular migratory speed, DCs are capable of alternating among a fast migratory, slow migratory and an arrest phase. Such dynamic and plastic nature of their locomotion, which depend critically on the shift between adhesion-dependent and –independent migration, is tailored to support their diverse functions. The dermal DCs are highly motile cells under steady state conditions, actively crawling through the dermal interstitium in an amoeboid fashion to survey the skin microenvironment for any invading pathogens. However, upon infection with the protozoan parasite *Leishmania major*, they undergo rapid migratory arrest at the infected site to maximise their antigen uptake capacity (Ng et al., 2008). On the other hand, antigen-presenting DCs ceased migration upon contact with the antigen-specific T cells in the lymph nodes, forming a stable immunological synapse mediated by LFA-1-ICAM-1 interaction to facilitate full T cell activation (Shakhar et al., 2005). As such, striking a balance between cell adhesion and migration in DCs at each of their distinct functional phases plays a key role in modulating a protective immune response.

1.3 Post-translational modifications

Post-translational modifications (PTMs) are the covalent processing of amino acids in proteins to alter their biochemical properties. To date, more than 400 different kinds of modifications have been identified, with the more common ones including acetylation, phosphorylation, methylation and ubiquitylation (Lothrop, Torres, & Fuchs, 2013). These modifications, which can either act alone or in combination, help to massively expand the genetic code by controlling the activities, regulating the structures and increasing the functional diversity of proteins. Even though these combinatorial PTMs lay the basis for information processing in organisms, mapping of these modifications and the identification of their functional implications remain challenging.

1.3.1 Post-translational regulation on leukocyte migration

The cell migration machinery comprises of a myriad of temporally and spatially segregated signalling and adhesion molecules which need to be orchestrated in a rapid and precise manner to support an efficient immune response. To achieve this, PTM has been employed as an ideal tool to confer dynamic and reversible regulation on the migrating leukocytes. The major PTMs which have been reported for various cytoskeletal and adhesion-related molecules are summarized in table 2 and their importance with regards to leukocyte migration is further highlighted below.

Molecule	PTM	Site	References
Actin	Acetylation	Met-1, Asp/Glu-2, Asp-3, Lys-326, Lys-328: Strengthen actomyosin interaction Lys-61: Stabilization of F-actin	(Abe, Saeki, Yasunaga, & Wakabayashi, 2000; Viswanathan, Blice-Baum, Schmidt, Foster, & Cammarato, 2015) (S. C. Kim et al., 2006)
	ADP-ribosylation	Thr-148: Promotes actin polymerization Arg-177: Promotes actin depolymerization Arg-206: Reduces actin polymerization	(Aktories, Lang, Schwan, & Mannherz, 2011) (Aktories et al., 2011) (Terashima, Yamamori, &

			Shimoyama, 1995)
	Arginylation	Asp-3: Promotes actin polymerization	(Karakozova et al., 2006)
	Glutathionylation	Cys-374: Reduces actin polymerization	(Dalle-Donne, Giustarini, Rossi, Colombo, & Milzani, 2003)
	Methylation	His-73: Stabilization of F-actin Lys-84me1: Disrupts actomyosin interaction	(Nyman et al., 2002) (M. M. Li et al., 2013)
	Oxidation	Met-44, Met-176, Met-190, Met 269, Cys-374: Promotes actin depolymerization	(Dalle-Donne et al., 2002)
	Phosphorylation	Tyr-53: Reduces actin polymerization Thr-201, Thr-202, Thr-203: Promotes actin polymerization	(X. Liu, Shu, Hong, Levine, & Korn, 2006) (Furuhashi, Hatano, Ando, Nishizawa, & Inagaki, 1992)
	Sumoylation	Lys-68, Lys-284: Retention of nuclear actin	(Hofmann et al., 2009)
Arp2/3 complex	Phosphorylation	Thr-237, Thr-238: Promotes actin polymerization	(LeClaire, Baumgartner, Iwasa, Mullins, & Barber, 2008)
Cortactin	Acetylation	Lys-87, Lys-161, Lys-189, Lys-198, Lys-235, Lys-272, Lys-309, Lys-319: Inhibits association with F-actin	(X. Zhang et al., 2007)
	Phosphorylation	Tyr-421, Tyr-466: Regulates Nck 1-dependent actin polymerization	(Oser et al., 2010)
Tubulin	Detyrosination	C-terminal: Stabilization of microtubules	(Kreitzer, Liao, & Gundersen, 1999)
	Acetylation	Lys-40: Stabilization of microtubules Lys-252: Inhibits tubulin incorporation into microtubules	(Al-Bassam & Corbett, 2012) (Chu et al., 2011)
	Phosphorylation	Ser-172: Inhibits tubulin incorporation into microtubules	(Fourest-Lieuvin et al., 2006) (Vogel et al., 2001)

		Tyr-445: Promotes assembly of astral microtubules	
Myosin II	Phosphorylation	Thr-18, Ser-19: Increase association with actin filaments Ser-1917, Ser-1943: Promotes disassembly of myosin filaments	(Vicente-Manzanares, Ma, Adelstein, & Horwitz, 2009) (Vicente-Manzanares et al., 2009)
Talin	Methylation	Lys-2454me3: Promotes disassembly of adhesion structures (Gunawan et al., 2015)	
	Proteolysis	Gln-433 Gln-434: Promotes disassembly of adhesion structures	(Franco et al., 2004)
	Phosphorylation	Ser-425: Stabilizes adhesion structures	(Huang et al., 2009)
	Arginylation	Ala-1903: Promotes formation of cadherin-dependent cell-cell adhesion	(F. Zhang, Saha, & Kashina, 2012)
Paxillin	Phosphorylation	Tyr-31, Tyr-118: Promotes docking of SH2 domain proteins Ser-85: Promotes formation of focal adhesions Ser-126, Ser-130: Promotes cell spreading Ser-188, Ser-190: Regulates paxillin degradation Thr-403: Promotes recruitment of paxillin to adhesion structures Thr-538: Promotes actin depolymerization	(Petit et al., 2000) (Kwak et al., 2012) (X. Cai, Li, Vrana, & Schaller, 2006) (Abou Zeid, Valles, & Boyer, 2006) (Brown, Perrotta, & Turner, 1998) (Romanova et al., 2010)
	Proteolysis	Ser-96 Ala-97: Inhibits disassembly of adhesion structures	(Cortesio, Boateng, Piazza, Bennin, & Huttenlocher, 2011)
Vinculin	Phosphorylation	Tyr-822: Promotes binding to β -catenin for cell stiffening	(Bays et al., 2014)
	ADP-ribosylation	Arg-433: Promotes disassembly of adhesion structures	(Simon & Barbieri, 2014)

Kindlin-2	Phosphorylation	Tyr-193: Promotes interaction with Migfilin	(Z. Liu et al., 2015)
Integrin	Phosphorylation	Thr-758: Promotes integrin activation	(Takala et al., 2008)
	Glycosylation	Asn-327, Asn-491, Asn-579, Asn-617, Asn-695: Modulates integrin binding to other molecules	(Kariya & Gu, 2011)

Table 2. Post-translational modification sites identified on cytoskeletal and adhesion molecules.

1.3.1.1 Regulation of actomyosin contraction

During adhesion-independent migration, actomyosin-mediated contraction of the cell body occurs to generate a forward locomotion. Here, reversible phosphorylation of the motor protein myosin II plays an essential role in controlling force generation during migration. Unphosphorylated myosin is inactive and exists in a compact, assembly-incompetent conformation through head-head and head-tail interactions (Jung, Komatsu, Ikebe, & Craig, 2008). Upon phosphorylation of Thr-18 and Ser-19 on the regulatory light chain by myosin light chain kinase (MLCK), myosin unfolds to adopt an assembly-competent conformation, thereby increasing its association with actin filaments and also its ATPase activity for the initiation of contraction. Several phosphorylation sites have also been identified on the C-terminal region of the myosin heavy chain, including Ser-1917 and Ser-1943. Unlike those found on the regulatory light chain, phosphorylation sites on the heavy chain are associated with the disassembly of myosin filaments. Hence, by making use of phosphorylation switches, myosin is able to modulate its actin-binding and contractile activities to control cell migration.

1.3.1.2 Regulation of cell adhesion and migration

Although fast-migratory cell types like leukocytes do not usually form visible adhesion structures to mediate their rapid gliding movements, larger adhesion structures may be induced by stimuli to cause the arrest of their migration (Huttenlocher & Horwitz, 2011). Since the dynamic assembly and disassembly of adhesion structures is pivotal to the balance between migration and adhesion, many of these adhesion-related molecules are heavily modified by PTMs. Paxillin is one

such focal adhesion-associated adaptor molecule that serves to recruit various cytoskeletal proteins for the transmission of downstream signalling. Phosphorylation of two sites in paxillin (Tyr-31 and Tyr-118), mediated by Src, function to facilitate docking of downstream SH2-containing signalling molecules that subsequently recruit focal adhesion kinase (FAK) to promote the turnover of adhesion structures. Therefore, cells are able to control their adhesion dynamics by regulating the relative proportion of unphosphorylated and phosphorylated paxillin (Zaidel-Bar, Milo, Kam, & Geiger, 2007). In addition, paxillin is also susceptible to calpain-mediated proteolysis, which generates an inhibitory paxillin fragment that impairs the turnover of adhesion structures and hence impedes cellular migration (Cortesio et al., 2011).

Integrins are transmembrane receptors that help to link the extracellular matrix with the actin cytoskeleton. Regulation of these proteins during cell migration and adhesion occurs either through direct PTM or through the PTM of various adaptor proteins such as paxillin, talin, and vinculin. Calpain-mediated cleavage of the integrin cytoplasmic domain has been shown to regulate its bidirectional signalling properties, thereby impacting processes such as cell spreading (Pfaff, Du, & Ginsberg, 1999; Xi, Bodnar, Li, Lam, & Du, 2003). This proteolysis, however, can be antagonized by the phosphorylation of the integrin cytoplasmic domain at Tyr-747 and Tyr-759. Furthermore, the interaction of the integrin cytoplasmic domain with either filamin or talin, which share overlapping binding sites, but possess opposing functions, is modulated through phosphorylation of Thr-758 by protein kinase C (PKC) (Takala et al., 2008). This modification favours the binding of talin over filamin, thereby promoting the activation of integrins and their downstream signalling pathways. Such binding of talin to the integrin cytoplasmic tail can, in turn, be antagonized by calpain-mediated proteolysis of talin (Franco et al., 2004), resulting in the formation of separate head and rod domains. The head domain of talin, which remains integrin-bound, can be ubiquitinated by the E3 ligase, Smurf1, and subsequently degraded, leading to the disassembly of adhesion structures. However, phosphorylation of the head domain by Cdk5 can inhibit the binding of Smurf1 and stabilize focal adhesions (Huang et al., 2009). Collectively, these data demonstrate how adhesion dynamics and cell migration can be intricately regulated by an extensive array of PTMs.

1.3.2 Ezh2-mediated talin1 methylation

Enhancer of Zeste homolog 2 (Ezh2) is an evolutionarily conserved SET domain-containing methyltransferase which forms the catalytic component of the Polycomb Repressive Complex (PRC) 2. Together with the core cofactors suppressor of Zeste 12 (SUZ12) and embryonic ectoderm development (EED), and also the non-essential cofactors RBBP4/7, JARID2 and PCL proteins, Ezh2 is able to specifically catalyse the mono-, di- and tri-methylation of histone 3 Lys 27 (H3K27), thereby maintaining the repressive state of genes through modification of the chromatin structure (G. Li et al., 2010). Given its well-defined role as a transcriptional silencer, Ezh2 has been implicated in the suppression of many genes, including those involved in host cell defence like IL-4 and IL-13 (Koyanagi et al., 2005). In addition, through the addition of methylation marks on H3K27, Ezh2 is able to modify the chromatin structure to facilitate rearrangement of the immunoglobulin heavy chain gene during V(D)J recombination, thereby controlling the development of early B cells (Su et al., 2003). Apart from histone substrates, Ezh2 has also been reported to methylate non-histone targets in the nucleus. For instance, direct methylation of the transcription factor GATA4 by Ezh2 results in their reduced recruitment to the chromatin and hence inhibits their transcriptional activities through impairment of their acetylation by p300 (He et al., 2012).

Interestingly, despite their well-characterized nuclear role as a histone methyltransferase, mammalian Ezh2 was first reported as an interacting partner of Vav1 (Hobert, Jallal, & Ullrich, 1996), the master regulator of cytoskeletal reorganization, hence suggesting the possibility of their non-nuclear localization. Indeed, Ezh2 was later reported to exist as a methyltransferase complex together with Vav in the cellular cytosolic compartment, whereby it regulates ligand-induced actin polymerization in T cells as well as dorsal ruffle formation in fibroblasts (Su et al., 2005). Recently, further insight regarding the cytoplasmic function of Ezh2 has been gained through the identification of their novel extra-nuclear substrate talin1 (figure 3A) (Gunawan et al., 2015). Ezh2 was found to directly tri-methylate talin1 at Lys2454 (K2454) (figure 3B), a residue located within their actin-binding domain (figure 3C), thereby disrupting their binding to F-actin (figure 3D and E). Since talin1 is a critical component of the focal adhesion complex, it raises the question of

whether such Ezh2-mediated talin1 methylation could impact cellular adhesion and migration processes.

Figure 3. Ezh2 mediates tri-methylation of talin1. (A) *In vitro* methyltransferase assay was performed with radioactive methyl donor [³H]S-adenosyl methionine and active (rPRC2a) or inactive (rPRC2i) recombinant Ezh2 complex containing Ezh2, Suz12, EED and PHF1 on talin1 immunoprecipitated from mouse DC cell line DC2.4. Fluorograph indicates methylation of talin1 by active PRC2 complex. (B) Mass spectrometry analysis revealed the Ezh2-mediated talin1 tri-methylation site to be at Lys15 (42.05-Da shift). (C) Electrostatic profiling of the talin1 carboxy-terminal actin-binding domain and the position of Lys2454 relative to other residues identified to be important for actin binding. (D) Binding of wild type talin1 or talin1 mutant (K2454Q or K2454F) peptides to various concentrations of F-actin is determined by actin co-sedimentation studies. (E) Dissociation rate of F-actin from wild type talin1 or talin1 mutant (K2454Q or K2454F) peptides at 4, 20 and 40 μM was determined by surface plasmon resonance studies. Taken from Gunawan et al., 2015.

1.4 Aim

The involvement of epigenetics in the regulation of leukocyte development and functions has been extensively studied over the past years. In this study, we aim to characterize the functional role of Ezh2 in DCs, and further elucidate the molecular mechanism and *in vivo* relevance underlying such regulation. Surprisingly, the well-established epigenetic repressor Ezh2 was found to control LC integrin-dependent migration via an epigenetic-independent mechanism involving the direct methylation of an extra-nuclear substrate talin1. In addition, the presence of Ezh2 in thymic DCs was found to be critical for the regulation of early T cell development in adult thymus, although the underlying mechanism remains elusive.

(2) Materials and Methods

2.1 Materials

2.1.1 Mice

CD11c-cre;Ezh2^{fl/fl}, CD11c-cre;CD11c-YFP;Ezh2^{fl/fl} and Mx-cre;Ezh2^{fl/fl} mice were bred and maintained under specific pathogen-free conditions at the animal facility of School of Biological Sciences, Nanyang Technological University. Mice were identified by toe clipping, and tail DNA was acquired for genotyping for the presence of loxP-floxed Ezh2 allele and other transgenes by PCR. Primer sequences are listed in table 3 below. Deletion of Ezh2 in Mx-cre;Ezh2^{fl/fl} mice was induced by 3 consecutive i.p. injections of 300µg Poly(I)poly(C) (Amersham Biosciences) at two-days interval. Mice were sacrificed at least 4 weeks after last injection. All mouse protocols were performed with approval of the NTU Institutional Animal Care and Use Committee (IACUC). Mice used were between 6 to 12 weeks of age, unless otherwise stated.

2.1.2 Primers

Transgene	Primer seq
Ezh2 floxed	F: 5'-TTA TTC ATA GAG CCA CCT GG
	Left F: 5'- ACG AAA CAG CTC CAG ATT CAG GG
	R: 5'-CTG CTC TGA ATG GCA ACT CC
Mx-Cre	F: 5'-CAT GTG TCT TGG TGG GCT GAG
	R: 5'-CGC ATA ACC AGT GAA ACA GCA T
CD11c-Cre	F: 5'-TTC TGT GGT GAC TTG GCA GC
	R: 5'-ATC GAC CGG TAA TGC AGG
CD11c-YFP	F: 5'-TGC TGG TTG TTG TGC TGT CTC ATC
	R: 5'-GGG GGT GTT CTG CTG GTA GTG GTC
Vβ1	5'-AAA TGA GAC GGT GCC CAG TCG TT
Vβ2	5'-TCC TGG GGA CAA AGA GGT CAA ATC
Vβ4	5'-AGC TAT CAA AAA CTT ATG GAC AAT CA
Vβ8	5'-TAT ATG TAC TGG TAT CGG CAG GAC A
Vβ10	5'-CAA ACT CTG GGC CAC GAT AC
Vβ14	5'-GGC AAG CAA GCT GGT GTG T

Vβ16	5'-GAT TTT AGG ACA GCA GAT GGA GTT TC
DJβ1	5'-CCT GAC TTC CAC CCG AGG TT
YZ-95	5'-AGT TTG AGA AGG AGG CTG CT
YZ-96	5'-CAA CAA TCA GGA CAG CAC AGT C
Ezh1	F: 5'- ACT GAG TCT TAC AGC GAC AAC C R: 5'-CTA ACT CTC GCT GTC AGA CC

Table 3. PCR Primers

2.1.3 Antibodies

Antibody	Clone	Dilution	Application	Company
CCR7-PE	4B12	1:200	FACS	BD Pharmingen
CXCR4-PE	L276F12	1:200	FACS	Biolegend
CD3ε-biotin	145-2C11	1:200	FACS	Biolegend
CD3ε-PE	145-2C11	1:200	FACS	Biolegend
CD4-PerCP/Cy5.5	GK1.5	1:200	FACS	Biolegend
CD8-Brilliant Violet 605	53-6.7	1:200	FACS	Biolegend
CD11b-biotin	M1/70	1:200	FACS	Biolegend
C11b-Brilliant Violet 605	M1/70	1:200	FACS	Biolegend
CD11c-PE	N418	1:200	FACS	Biolegend
CD11c-PerCP/Cy5.5	N418	1:100	FACS	Biolegend
CD11c-FITC	N418	1:100	IF	Biolegend
CD103-biotin	2.00E+07	1:200	FACS	Biolegend
CD103-APC	2.00E+07	1:200	FACS	eBioscience
CD326-PECy7	G8.8	1:200	FACS	eBioscience
FoxP3-FITC	FJK-16s	1:100	FACS	eBioscience
BrdU-FITC	3D4	1:100	FACS	BD

				Pharmingen
Annexin V-FITC		1:100	FACS	Biolegend
CD278-PE	7E.17G9	1:200	FACS	Biolegend
CD44-FITC	IM7	1:200	FACS	Biolegend
CD69-FITC	H1.2F3	1:200	FACS	Biolegend
CD62L-PE	MEL-14	1:200	FACS	Biolegend
CD19-biotin	6D5	1:200	FACS	Biolegend
B220-biotin	RA3-6B2	1:200	FACS	Biolegend
CD45-APC/Cy7	30-F11	1:200	FACS	Biolegend
CD49f-FITC	GoH3	1:200	FACS	eBioscience
CD207-PE	eBioL31	1:200	IF	eBioscience
I-A/I-E-Pacific blue	M5/114.15.2	1:100	FACS	Biolegend
I-A/I-E-biotin	M5/114.15.2	1:200	FACS, IF	Biolegend
CD31-APC	MEC 13.3	1:100	IF	BD Pharmingen
Gr-1-biotin	RB6-8C5	1:200	FACS	Biolegend
Ly6G-PE	1A8	1:100	FACS, IF	Biolegend
TCR γ/δ-biotin	GL3	1:200	FACS	Biolegend
TCR γ/δ-FITC	GL3	1:200	FACS	Biolegend
TER-119-biotin	TER-119	1:200	FACS	Biolegend
Streptavidin-Alexa 647		1:200	FACS	Invitrogen
Streptavidin-PE/Cy7		1:400	FACS	eBioscience
IgG2a-APC	RTK2758	1:200	FACS	Biolegend
IgG2a-FITC	RTK2758	1:200	FACS	Biolegend
IgG2a-PE	RTK2758	1:200	FACS	Biolegend

IgG1-PE/Cy7	RTK2071	1:200	FACS	Biolegend
Anti-E-cadherin	DECMA-1	1:100	FACS	Sigma-Aldrich
Anti-H3K27me3	mAbcam6002	1:100	FACS	abcam
Anti-Ezh2	D2C9	1:100	FACS	Cell signaling
Anti-TIMP2	3A4	1:100	FACS	abcam
Anti-TIMP3	Polyclonal	1:100	FACS	abcam
Anti-laminin	Polyclonal	1:200	IF	abcam
Anti-Talin	8d4	1:200	IF	Sigma-Aldrich
Anti-Paxillin	5H11	1:200	IF	Millipore
Anti-cytokeratin5	Polyclonal	1:100	IF	abcam

Table 4. Antibodies list

2.2 Cell Biology techniques

2.2.1 Isolation and preparation of organs

Skin draining lymph nodes, spleen and thymus were harvested, cut into small pieces, and digested in RPMI medium 1640 (Invitrogen) with 10% FCS (PAA) containing Liberase TM Research Grade (Roche) and DNase I (Roche) for 30 minutes at 37°C. Tissue fragments were then homogenized with a syringe and the cells were passed through a 70µm nylon mesh. Erythrocytes in the splenic cell suspension were lysed by incubating with 140mM NH₄Cl and 17mM Tris-Cl for 3 minutes at room temperature.

2.2.2 Preparation of single cell suspension from skin

Ears were split into the dorsal and ventral halves and floated on HBSS containing Dispase (Invitrogen) for 45 minutes at 37°C to allow for the separation of epidermis from the dermis. Epidermal sheet and dermis were subsequently cut into small pieces and digested in RPMI medium 1640 (Invitrogen) with 10% FCS (PAA) containing Liberase TM Research Grade (Roche) and DNase I (Roche) for 1.5 hours at 37°C. Cell suspension was then passed through a 19-gauge needle and filtered through a 70µm nylon mesh to obtain a homogeneous single cell suspension.

2.2.3 Isolation and culture of BMDCs

Bone marrow cells were flushed from the mice femurs and tibias and erythrocytes were lysed as described above. The cells were subsequently cultured in complete RPMI medium 1640 (Invitrogen) with 10% FCS (PAA) in the presence of 20ng/ml GM-CSF and 10ng/ml IL4 (Prospec Tany). On day 2 of culture, nonadherent cells were gently washed off and fresh complete medium with 10ng/ml GM-CSF was added. Cells were cultured for 6 days and fresh medium was added as necessary. To induce maturation of DCs, immature cells were stimulated with 100ng/ml of LPS (Sigma-Aldrich) overnight. DCs were harvested by gentle pipetting.

2.2.4 Isolation of neutrophils

Bone marrow cells were isolated and erythrocytes were lysed as described above. Neutrophils are purified from bone marrow cells using the neutrophil isolation kit (Miltenyi Biotec), according to the manufacturer's protocol.

2.2.5 Retroviral transduction of BMDCs

BMDCs were transduced with retrovirus based on the mouse stem cell virus (MSCV)-internal ribosomal entry site (IRES)-GFP (MIG) vector encoding wild type talin, talin K2454F and talin K2454Q mutants. Cells were transduced on day 3 and 4 of culture by spin infection with the virus supernatant in the presence of 8µg/ml polybrene. The virus supernatant was removed after 4 hours and fresh complete RPMI medium with 10ng/ml GM-CSF was added.

2.2.6 Skin explant assay

Split ear halves were cultured in 1.5ml of complete medium in 24-well tissue culture plate for 18 hours at 37°C. In some experiments, epidermal sheet was separated from the dermis prior to culture for 4 hours.

2.2.7 Flow cytometry

Flow cytometric studies of single cell suspension were performed on LSRII or LSRFortessa (BD Biosciences), and subsequently analyzed with FlowJo software (Tree Star). All surface staining were performed for 10 minutes at 4°C in staining buffer containing PBS, BSA and sodium azide. Intracellular staining was performed for 1 hour at 25°C, following fixation and permeabilization of the cells with Cytofix/Cytoperm kit (BD) or Foxp3 staining buffer set (eBioscience), according to the manufacturer's protocol. The fluorochrome-conjugated mAbs used for staining are listed in table 4. Fixable viability dye (ebioscience) was used for dead cells exclusion.

2.3 Imaging techniques

2.3.1 Preparation of epidermal sheet for immunofluorescence

Epidermal sheet was fixed in acetone for 20 minutes at -20°C. Isolated sheets were stained with biotin I-A/I-E antibody (clone M5/114.15.2) and Streptavidin Alexa Fluor 647 conjugate (Life Technologies) for 30 minutes at 4°C before mounting in mounting medium (Vector Laboratories).

2.3.2 Whole mount ear staining

Split ear halves were fixed with acetone for 20 minutes at -20°C or with 2% paraformaldehyde containing 30% sucrose overnight at 4°C. Ear halves were stained overnight at 4°C with anti-CD31 (clone MEC 13.3; BD Biosciences) and anti-Ly6G (1A8; Biolegend). Ear halves were mounted with mounting medium (Ibidi) containing DAPI (Sigma-Aldrich).

2.3.3 Skin sectioning

Footpad skin were fixed with 2% paraformaldehyde containing 30% sucrose overnight at 4°C. Fixed skin samples were embedded in OCT compound and sliced into 20µm-thick sections and stained with anti-laminin (abcam) and Alexa Fluor 647-X-AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) before mounting with mounting medium (Ibidi) containing DAPI(Sigma-Aldrich).

2.3.4 Thymus sectioning

Frozen thymuses embedded in OCT were sliced into 20µm-thick sections and stained with anti-cytokeratin5 (abcam), Alexa Fluor 647-X-AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) and anti-CD11c (Biolegend) or hematoxylin and eosin. The area of cortex and medulla were measured with ImageJ software.

2.3.5 Cell motility assay

LCs, mature DCs, or neutrophils were allowed to adhere to 8-well culture slides (BD Falcon) coated with 10µg/ml of Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma-Aldrich), 10µg/ml of fibronectin (Sigma-Aldrich), 10µg/ml of VCAM-1 (Sino Biological), 12.5µg/ml of ICAM-1 (Sino Biological)

or Poly-L-lysine (Sigma-Aldrich). Nonadherent cells were removed by gentle washing, and adherent cells were supplemented with complete medium with or without 100ng/ml of CCL19 (DENOVO) or 10^{-5} M of fMLP (Sigma-Aldrich). Culture slides were maintained at 37°C in 5% CO₂ on heated microscope stage. Images of live cells were recorded at a time-lapse interval of 5 mins for LCs and BMDCs, or 30 secs for neutrophils. Individual cell trajectories and distance travelled were analyzed on ImageJ with manual tracking plugin and chemotaxis-migration tool (Ibidi). Tracking was restricted to cells which remain visible within the observation field for more than half the total duration of experiment.

2.3.6 Focal adhesions imaging

LCs, mature DCs, or neutrophils were allowed to adhere to 8-well culture slides (BD Falcon) coated with 10µg/ml of Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma-Aldrich), 10µg/ml of fibronectin (Sigma-Aldrich), 10µg/ml of VCAM-1 (Sino Biological) or Poly-L-lysine (Sigma-Aldrich). Cells were incubated at 37°C for 2 hours before removal of the nonadherent cells by gentle washing with PBS. Adherent cells were fixed with 2% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.5% Triton X-100 for 5 minutes at 4°C. Fixed cells were blocked with 10% normal donkey serum (Sigma-Aldrich) in PBS containing BSA, sodium azide and 0.5% saponin for 1 hour before staining with monoclonal anti-Talin (clone 8d4, Sigma-Aldrich) or anti-Paxillin (clone 5h11, Millipore) overnight at 4°C and then with Rhodamine Red- or fluorescein isothiocyanate-X-AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 hour at room temperature. F-actin was stained with fluorescein isothiocyanate- or rhodamine red-conjugated phalloidin (Invitrogen). LCs were identified by staining with biotin I-A/I-E antibody and Streptavidin Alexa Fluor 647 conjugate at 4°C for 30 minutes respectively prior to mounting.

2.3.7 Image acquisition and analysis

Images were visualized with Zeiss LSM 510 Meta or LSM 710 Confocal Microscope at 10x, 20x or 63x magnification. Z-stacks were acquired for the epidermal sheets and whole mount ears and projected at maximal intensity. Thymus H&E sections were visualized with Olympus SZX7 Stereo microscope. All image analyses were performed on LSM Image Browser, Zen 2009 or ImageJ software.

2.4 Molecular Biology techniques

2.4.1 RNA isolation, cDNA synthesis and real-time quantitative PCR

Total mRNA was extracted from cells using Trizol (Thermofisher) or QIAshredder (Qiagen) and purified with RNeasy mini kit (Qiagen). cDNA was synthesized using SuperScript III first-strand synthesis system with random hexamers (Invitrogen). RT-qPCR was performed using KAPA SYBR Fast ABI Prism 2x qPCR master mix (KAPA Biosystems, Inc.) with the primers indicated in primer list. Data were normalized to expression levels of housekeeping gene GAPDH.

2.4.2 Analysis of V(D)J recombination products

Total DNA was extracted from thymus and VDJ β joints were analyzed using a touchdown PCR strategy: 5 min at 94°C, followed by 5 cycles of 30s at 94°C, 30s at annealing temperature, and 1 min at 72°C, and a 10-min extension at 72°C. Annealing temperature was held at 68, 65, and 62°C for 5 cycles each and at 58°C for 15 cycles. Amplicons were electrophoresed on 1.3% agarose gel and visualized by ethidium bromide staining.

2.4.3 Cytokine proteome array

Thymic cell suspension was lysed in Nonidet P-40 buffer (50mM Tris-HCl, pH8.0, 500mM NaCl, 1mM EDTA, 1% Nonidet P-40, 10% glycerol) and sonicated (Bioruptor). Protease inhibitor cocktail (Sigma-Aldrich), sodium fluoride (Sigma-Aldrich) and activated sodium-orthovanadate (Sigma-Aldrich) were added to buffer before use. Cytokine levels in the cell lysate were determined using the Mouse Cytokine Antibody Array Panel A kit (R&D Systems), according to the manufacturer's protocol.

2.5 *In vivo* models

2.5.1 Oxazolone painting

25 μ l of 0.5% oxazolone (Sigma-Aldrich) dissolved in acetone:olive oil (4:1) was painted on both the dorsal and ventral sides of the mouse ears. Mice were sacrificed 24 or 48 hours after sensitization and their ears or skin draining lymph nodes were harvested respectively.

2.5.2 *In vivo* BrdU labeling

Mice were injected intraperitoneally with 1mg of BrdU (Sigma-Aldrich) in PBS, and given fresh drinking water supplemented with 0.8mg/ml of BrdU daily for an additional 7 days. Single cell suspension was prepared as described above and intracellular staining against BrdU was performed using the FITC BrdU Flow kit (BD Pharmingen) according to the manufacturer's protocol.

2.5.3 Croton oil induced ear inflammation

20 μ l of 1% croton oil (Sigma-Aldrich) in acetone were applied to the dorsal side of mouse ears. Mice were sacrificed 3 hours after sensitization, and their ears were harvested for immunofluorescence or flow cytometry analysis.

2.5.4 Induction of tolerance to contact hypersensitivity

Mice were tolerized by painting 100 μ l of 0.1% or 1% DNTB (Lancaster Synthesis) in vehicle consisting of acetone and olive oil (AOO) (4:1, v/v) on shaved abdominal skin. 7 days later, they were sensitized with 25 μ l of 0.5% DNFB (Sigma-Aldrich) in AOO on shaved back skin. 5 days later, organs were either harvested for analysis, or the mice were subsequently challenged with 4 μ l of 0.1% DNFB in AOO on both dorsal and ventral sides of the ear. Ear swelling was measured using a micrometer (Mitutoyo).

2.5.5 Induction of IMQ-induced psoriasis

Mice received a daily topical dose of 10 mg of IMQ cream (5%) (Aldara; 3M Pharmaceuticals) on the dorsal side of both ears for 4 consecutive days. Ear swelling was measured daily using a micrometer (Mitutoyo). Skin-draining lymph nodes were harvested on day 5 for analysis.

2.5.6 Bone marrow chimeras

Bone marrow cells isolated from femurs and tibias of mice were injected intravenously into lethally irradiated recipients (2x5.5 Gy, 4 hours apart). Mice were maintained on antibiotic-supplemented water (Neomycin sulfate; Sigma-Aldrich) for 2 weeks. Mice were used for experiments 8 weeks after transplantation.

2.6 Statistical analyses

Data were analyzed by two-tail Student's t test or 2-way ANOVA. Differences were considered significant when the p value is less than 0.05.

(3) Results

3.1 Ezh2 regulates integrin-dependent migration of LCs

3.1.1 Ezh2 regulates migration of LCs from epidermis to skin-draining lymph node

To investigate the role of Ezh2 in LCs, the epidermal LC population was analyzed during steady state in 6- to 8-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. The deletion of *Ezh2* gene from skin DCs of CD11c-cre;Ezh2^{fl/fl} mice is efficient as demonstrated previously (Gunawan et al., 2015).

As shown in figure 4A, immunofluorescence staining of epidermal sheets from CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice revealed no morphological difference between their LCs, in which a characteristic dendritic shape was observed in both strains. However, a small but significant increase in the density of LCs was observed in the CD11c-cre;Ezh2^{fl/fl} mice (figure 4A). Flow cytometric analysis of the epidermal cell suspensions (figure 4B) further supported this consistent increase in percentage of MHC II⁺ CD11c⁺ EpCAM⁺ CD103⁻ LCs in the CD11c-cre;Ezh2^{fl/fl} mice.

The increased LCs in the epidermis during steady state suggests that Ezh2 might be involved in the regulation of their migration. To address this possibility, dermal cell suspensions were analyzed by flow cytometry (figure 4B). Although the percentage of MHC II⁺ CD11c⁺ total DCs were not reduced in the dermis of the CD11c-cre;Ezh2^{fl/fl} mice, the EpCAM⁺ CD103⁻ LCs were found to be significantly lowered in these mice. To further support this observation, the percentage and absolute number of migratory LCs in the skin-draining lymph node were also reduced in the CD11c-cre;Ezh2^{fl/fl} mice during steady state (figure 4C). Hence, these data suggest that accumulation of LCs in the epidermis of the CD11c-cre;Ezh2^{fl/fl} mice arises from their deficiency in migration, thereby contributing to the reduced migratory LC numbers observed in the dermis and skin-draining lymph nodes.

LCs are considered to be relatively sessile during steady state, with their trafficking to the skin-draining lymph nodes being greatly enhanced following activation (Nishibu et al., 2006). Since the results described above indicate the dampened migration of LCs in the CD11c-cre;Ezh2^{fl/fl} mice during steady state, the relevance of this phenotype during *in vitro* and *in vivo* activation was studied. The mouse skin

explant culture model, which has been widely demonstrated to promote the migration and activation of LCs (Larsen et al., 1990; Stoitzner et al., 2003), was used to induce *in vitro* activation of LCs. Epidermal sheets isolated from the mice ear skin explants after 18 hours of culture were subsequently analyzed by immunofluorescence staining. Indeed, during this period of culture, LCs from CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice underwent spontaneous activation and phenotypic maturation, as exhibited by their increase in cell size and staining intensity for MHC II (figure 4A). In the Ezh2^{fl/fl} skin explant, approximately 30% of the LCs were found to have migrated away from the epidermis after culture. In contrast, migration of LCs away from the CD11c-cre;Ezh2^{fl/fl} skin explant was significantly reduced, with majority of the LCs remaining in the epidermis despite being activated after culture.

A similar trend was also observed following *in vivo* activation of LCs through the painting of contact sensitizer oxazolone on the mice ears. Even though LCs from both CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice were activated and display an increase in cell size and MHC II expression, migration of LCs away from the epidermis was found to be significantly reduced in the CD11c-cre;Ezh2^{fl/fl} mice (figure 4A). Moreover, a significant increase in migratory LCs was detected in the skin-draining lymph nodes of the Ezh2^{fl/fl} mice following contact sensitization with oxazolone, while no significant increase in the migratory LC numbers was observed in the lymph nodes of the CD11c-cre;Ezh2^{fl/fl} mouse (figure 4C). On the other hand, migration of dermal DCs to the skin-draining lymph nodes during steady state and upon oxazolone application were unaffected by their deficiency in Ezh2 (figure 4C), suggesting that the migration defect was limited to only the LCs.

Since these data demonstrate that Ezh2-deficient LCs were less able to reach the dermis and skin-draining lymph nodes, they imply that Ezh2 is required for the trafficking of LCs away from the epidermis during both steady state and under inflammatory conditions.

Figure 4. Characterization of LCs in 6- to 8-week-old *Ezh2^{fl/fl}* and *CD11c-cre;Ezh2^{fl/fl}* mice. (A) Immunofluorescence analysis of epidermal sheets isolated from freshly excised mice ears at steady state, from skin explants which have been cultured for 18 hours, and from mice ears 18 hours after application of 0.5%

oxazolone. LCs were identified by immunostaining with anti-MHC II antibody. Images shown are representative of 3 independent experiments. Scale bars, 50 μ m. Bar graph indicates LC numbers/mm² (mean $\pm$ s.d.). Data is based on 3 independent experiments. 3 mice per group were analyzed, and a total of 6 fields were counted for each group. * p <0.05, *** p <0.001 (two-tailed Student's t -test). (B) Flow cytometric analyses of LCs in epidermis and dermis during steady state. Contour plots for epidermis were pregated on singlet, live cells. Contour plots for dermis were pregated on singlet, live, CD45⁺ cells. CD11c⁺ and MHC II⁺ DCs in the epidermis and dermis were further analysed for CD103⁻ and EpCAM⁺ cells to define LCs population. Percentages of gated cells were indicated. Data shown is representative of 3 independent experiments. Bar graph indicates LC percentages in epidermis and dermis (mean $\pm$ s.d.). Data is based on 3 independent experiments. 7 mice per group were analyzed. * p <0.05, ** p <0.01 (two-tailed Student's t -test). (C) 0.5% oxazolone was painted on mice ears, and skin-draining lymph node was harvested after 48 hours for flow cytometric analyses of LCs. Dot plots were pregated on singlet, live cells. CD11c⁺ and MHC II^{hi} migratory DCs were further analysed for CD103⁻ and EpCAM⁺ cells to define LCs population, and EpCAM^{+/lo} cells to define dermal DCs population. Percentages of gated cells were indicated. Data shown is representative of 3 independent experiments. Bar graphs indicate total lymph node cell numbers, and absolute numbers of LCs and dermal DCs reaching the lymph nodes of Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice during steady state (ss) and after oxazolone painting (oxa) (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 6 mice per group were analyzed. * p <0.05, ** p <0.01, *** p <0.001 (two-tailed Student's t -test).

3.1.2 Age-dependent accumulation of Ezh2-deficient LCs in the epidermis

The increased recruitment of LC progenitors to the epidermis during development is an unlikely cause of their increased numbers in CD11c-cre;Ezh2^{fl/fl} mice, since these progenitors do not express CD11c when they seed the epidermis. Differentiation of these progenitors into CD11c-expressing cells, and hence the excision of Ezh2 locus, occur only after the recruitment phase is completed (Laurent Chorro et al., 2009). To control for any initial difference in the rate of proliferative burst by the CD11c⁺ precursors, the LC numbers were analyzed in 2-week-old mice, shortly after the occurrence of proliferative burst in the epidermis. No significant difference in LC numbers was observed between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice at this age (figure 5A and B), suggesting that the rate of LC expansion shortly after birth is indistinguishable between these two strains of mice. During the adult stage, epidermal LC numbers remained relatively constant in the 6- to 16-week-old Ezh2^{fl/fl} mice. However, they were found to increase over time in the CD11c-cre;Ezh2^{fl/fl} mice. In particular, a 1.3-fold difference in LC numbers was observed between the 6- to 8-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice, and this difference escalated to approximately 2-fold 10 weeks later (figure 5B). Taken together, these results suggest that early development and expansion of LCs from their precursors are unaffected by Ezh2 deficiency. However, in the CD11c-cre;Ezh2^{fl/fl} mice, it may be possible that the intact renewal of epidermal LCs in spite of their reduced steady state migration consequently led to their subtle but significant accumulation over time.

Figure 5. Steady state accumulation of *Ezh2*-deficient LCs over time. (A) Epidermal sheets isolated from mice of different age groups (as indicated) were stained with anti-MHC II antibody. Images shown are representative of 2 (2 weeks, 16 weeks) or 3 (6-8 weeks) independent experiments. 3 to 4 mice per group were analyzed. Scale bars, 50 μ m. (B) MHC II⁺ LCs in the immunofluorescence assay were counted to determine their density in the $Ezh2^{fl/fl}$ and CD11c-cre; $Ezh2^{fl/fl}$ mice (mean $\pm$ s.d.). Data is based on 2 (2 weeks, 16 weeks) or 3 (6-8 weeks) independent experiments. 3 to 4 mice per group were analyzed, and at least 6 fields were counted for each group. *** p <0.001 (two-tailed Student's t -test).

3.1.3 Unaltered proliferative capacity of Ezh2-deficient LCs

To address the possibility that accumulation of epidermal LCs in the CD11c-cre;Ezh2^{fl/fl} mice is due to their enhanced proliferative capacity, an *in vivo* BrdU-labeling assay was performed. However, no difference in the rate of BrdU incorporation was found between the wild type and Ezh2-deficient LCs (figure 6A and B). This excludes an altered self-renewal rate to be responsible for the increased epidermal LC numbers observed in the CD11c-cre;Ezh2^{fl/fl} mice during steady state.

Figure 6. LC proliferation is not altered in Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice. (A) 6- to 8-week-old mice were injected intraperitoneally with BrdU and subsequently administered with BrdU-supplemented drinking water for 7 days. MHC II⁺ CD11c⁺ EpCAM⁺ CD103⁻ live epidermal LCs from Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice were analyzed for their incorporation of BrdU by flow cytometry. Percentages of BrdU-positive LCs are indicated. (B) Bar graph indicates the percentage (mean $\pm$ s.d.) of BrdU-positive LCs in Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice. Data is based on 1 experiment. 3 mice per group were analyzed.

3.1.4 Ezh2 deficiency does not affect LC viability

Epidermal LCs have been reported to be long-lived cells, with their half-life estimated to be between 53 to 78 days based on intravital confocal imaging studies (Vishwanath et al., 2006). If Ezh2 is involved in regulating the survival of LCs, the accumulation of LCs in the CD11c-cre;Ezh2^{fl/fl} mice over time (figure 5) could have resulted from their prolonged lifespan. To test this hypothesis, the percentages of apoptotic and dead LCs were analyzed in CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. According to the annexin V and viability dye staining, no difference in survival of wild type and Ezh2-deficient LCs was observed (figure 7A). Since LCs are long-lived and a relatively small amount of apoptotic cells was captured by annexin V staining, FLICA caspase assay, which measures the amount of active caspases, was performed to detect early apoptotic events in LCs. Similarly, no significant difference in the incidence of apoptotic events was observed (figure 7B). These data collectively suggest that Ezh2 is not implicated in the regulation of LC survival, and thus prolonged lifespan is an unlikely contributing factor for the increase in Ezh2-deficient LCs in the epidermis of adult mice.

Figure 7. LC viability is not altered in Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice. (A) The frequency of apoptotic LCs in 6- to 8-week-old Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice was analyzed by Annexin V staining. Dead LCs are identified by their positive

staining for the viability dye. Bar graph indicates the percentages of apoptotic (lower right quadrant) and dead (upper quadrants) LCs (mean $\pm$ s.d.). Data is based on 3 independent experiments. 4 mice per group were analyzed. (B) Apoptotic LCs are determined by labeling with FLICA. Bar graph indicates percentage of apoptotic FLICA-positive LCs (mean $\pm$ s.d.). Data is based on 3 independent experiments. 5 mice per group were analyzed.

3.1.5 Ezh2 is dispensable for the downregulation of E-cadherin in LCs

To unravel the mechanism underlying the migration defect in Ezh2-deficient LCs, various pathways involved in the regulation of LC migration were investigated. During steady state, LCs express high levels of E-cadherin, which mediate their homophilic adhesion to neighbouring keratinocytes and therefore their retention in the epidermis. Upon stimulation with haptens or allergens, downregulation of E-cadherin on LCs' surfaces enables them to dissociate from the surrounding keratinocytes, as such facilitating their migration (Thilo Jakob & Udey, 1998). To evaluate whether E-cadherin-mediated adhesion to keratinocytes contributes to the retention of Ezh2-deficient LCs in the epidermis, their expression levels were compared in steady state LCs and activated LCs isolated from skin explants which have been cultured for 18 hours. Flow cytometric analysis in figure 8 revealed that LCs from both CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice were capable of downregulating their surface E-cadherin upon maturation. This suggests that the impaired migration of LCs in CD11c-cre;Ezh2^{fl/fl} mice was not due to their inability to modulate E-cadherin level, which would have led to their tight adhesion with the surrounding keratinocytes.

Figure 8. Ezh2 is dispensable for the downregulation of surface E-cadherin on LCs following activation. E-cadherin expression on epidermal LCs isolated from freshly excised mice ears was compared with the expression on epidermal LCs isolated from skin explants which have been cultured for 18 hours by flow cytometry. Histograms were pregated on singlet, live, CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ cells. Solid line represents LCs at steady state, and dotted line represents LCs which have been activated by culture. Filled histogram represents isotype control. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed.

Intriguingly, Ezh2 has been implicated in tumorigenesis, in which its overexpression in prostate and breast cancer promotes H3K27me3-mediated silencing of the tumour suppressor gene E-cadherin, thus leading to enhanced aggressiveness and metastasis of cancer cells (Cao et al., 2008). While the dispensability of Ezh2 in the downregulation of E-cadherin in LCs was in contrary to what have been reported in the context of cancer cells, it could possibly reflect the compensatory effects exerted by Ezh1, another member of the polycomb group of histone methyltransferase which shares overlapping target genes with Ezh2, but resides solely in the nucleus (Margueron et al., 2008). Indeed, LCs from both CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice express comparable levels of H3K27me3 (figure 9A). In addition, an increased amount of Ezh1, as reflected by their elevated transcript level, was detected in Ezh2-deficient bone marrow-derived dendritic cells (BMDCs) (figure 9B). These indicate that Ezh1 may possibly compensate for the loss of Ezh2 and thereby maintain the overall level of H3K27me3 in the Ezh2-deficient LCs. Such redundancy with Ezh1 has also been previously reported in resting Ezh2-deficient T cells, which harbor an equivalent H3K27me3 content and gene expression pattern as their wild type counterparts. Hence, the suppression of E-cadherin in Ezh2-deficient LCs upon activation is likely to be compensated by the presence of Ezh1.

Figure 9. Ezh2-deficient LCs display comparable levels of H3K27me3. (A) Total H3K27me3 levels in epidermal LCs isolated from freshly excised mice ears were analyzed by intracellular staining and flow cytometry. Histograms were pregated on singlet, live, CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ cells. Solid line represents wild type LCs, and dotted line represents Ezh2-deficient LCs. Filled histogram represents isotype control. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed. (B) Ezh1 expression in wild type and Ezh2-deficient mature BMDCs. Total RNA extracted was used for cDNA synthesis before conducting RT-qPCR. Gene expression was normalized against GAPDH. Data

shown is representative of 3 independent experiments. 3 mice per group were analyzed.

3.1.6 Ezh2 deficiency does not affect TIMPs expression

Migratory LCs are known to secrete matrix metalloproteinases (MMPs) 2 and 9 to facilitate the localized proteolysis of basement membrane during their migration to the skin-draining lymph nodes (Kobayashi, 1997; Ratzinger et al., 2002). The activities of MMPs are regulated by the tissue inhibitors of metalloproteinases (TIMPs), which keep them in an inactive form through direct binding to their catalytic domain. Ezh2 overexpression has been implicated in the suppression of TIMP2 and 3 in various forms of cancer (Shin & Kim, 2012), thus leading to the increased activity of MMP9 and an enhanced invasiveness of cancer cells. To understand whether Ezh2 deficiency in LCs contributes to the elevated expression of TIMPs and as such an impaired migration, the intracellular expression of TIMP2 and 3 were analyzed by flow cytometry. Contrary to the findings in cancer cells, comparable levels of TIMP2 and 3 were found in both wild type and Ezh2-deficient LCs, indicating that their expression are not under the control of Ezh2 (figure 10). Likewise to the case of E-cadherin, the suppression of TIMPs by Ezh2 could be specific to the context of cancer cells, or could possibly reflect the compensatory effects exerted by Ezh1. Hence, Ezh2 does not appear to be essential for the regulation of MMP activities via TIMPs in LCs.

Figure 10. Ezh2 deficiency does not affect expression of TIMPs. TIMP2 and TIMP3 expression in epidermal LCs isolated from freshly excised mice ears were analyzed by intracellular staining and flow cytometry. Histograms were pregated on singlet, live, CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ cells. Solid line represents wild type LCs, and dotted line represents Ezh2-deficient LCs. Filled histogram represents isotype control. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed.

3.1.7 Unaltered expression of chemokine receptors on Ezh2-deficient LCs

Migration of LCs to the skin-draining lymph nodes follows a two-step paradigm involving two temporally dissociated chemokine receptors CXCR4 and CCR7 (Villablanca & Mora, 2008). To determine the contribution of these receptors to the migration defect of Ezh2-deficient LCs, their surface expressions were analyzed by flow cytometry. Comparable levels of CXCR4 were found to be expressed on both wild type and Ezh2-deficient LCs, suggesting that their capacity to respond to CXCL12 was not compromised by the absence of Ezh2 (figure 11A). On the other hand, no expression of CCR7 was detected on the epidermal LCs at this time point (figure 11B). This is consistent with reports that migration of LCs from the epidermis to dermis is CCR7-independent, and their upregulation occurs only at a later stage to promote their entry into the dermal lymphatic vessels. Moreover, comparable levels of CCR7 were detected on wild type and Ezh2-deficient migratory DCs in the skin-draining lymph nodes (figure 11B), suggesting that deletion of Ezh2 does not affect CCR7 expression. Thus, the impaired migration of Ezh2-deficient LCs is not due to a differential expression of these chemokine receptors.

Figure 11. Ezh2 is dispensable for the expression of chemokine receptors. (A) Expression of chemokine receptor CXCR4 on epidermal LCs isolated from freshly excised mice ears was analyzed by flow cytometry. Histograms were pregated on singlet, live, CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ cells. Solid line represents wild type LCs, and dotted line represents Ezh2-deficient LCs. Filled histogram represents isotype control. Data shown is representative of 4 independent experiments. 4 mice per group were analyzed. (B) Expression of chemokine receptor CCR7 on epidermal LCs isolated from freshly excised mice ears and migratory DCs in the skin-draining

lymph nodes were analyzed by flow cytometry. Histograms for LCs were pregated on singlet, live, CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ cells. Histograms for migratory DCs were pregated on singlet, live, CD11c⁺ MHC II^{hi} cells. Solid line represents wild type LCs, and dotted line represents Ezh2-deficient LCs. Filled histogram represents isotype control. Data shown is representative of 7 (LCs) and 3 (migratory DCs) independent experiments. 7 (LCs) and 3 (migratory DCs) mice per group were analyzed.

3.1.8 Inability of Ezh2-deficient LCs to pass through basement membrane

Upon dissociation from the keratinocytes, LCs have to encounter the basement membrane, a dense network composed of mainly type IV collagen and laminin, before reaching the dermis. To understand whether the basement membrane poses a barrier to the migration of LCs in the CD11c-cre;Ezh2^{fl/fl} mice, epidermal sheets isolated from mice ears by dispase digestion were cultured on medium for 4 hours, allowing LCs to be activated *in vitro*. Dispase can disrupt the integrity of basement membrane through specific cleavage of type IV collagen in the lamina densa, while at the same time keeping the epidermis intact (Stenn, Link, Moellmann, Madri, & Kuklinska, 1989). In figure 12A, immunofluorescence analysis of the dispase-treated epidermal sheet explants revealed that approximately 50% of the Ezh2-deficient LCs were able to leave the epidermis upon activation in the absence of an intact basement membrane, contrary to what was observed previously when the basement membrane was not disrupted (figure 4A). Furthermore, similar percentages of live LCs were retrieved from the cultured medium, hence excluding the possibility of increased cell death being the underlying cause of the decline in LC numbers in the CD11c-cre;Ezh2^{fl/fl} epidermal sheet explants. As such, these results suggest that Ezh2 is important for LCs to overcome the basement membrane barrier during their migration.

To assess the *in vivo* relevance of this finding, mice skin was examined by sectioning to visualize the longitudinal distribution of LCs in the epidermis. In this case, footpad skin, instead of ear skin, was used for a better resolution of the epidermal layer and basement membrane. Similar to the ear skin, an accumulation of epidermal LCs was observed in the footpad skin of CD11c-cre;Ezh2^{fl/fl} mice during steady state (figure 12C). Transverse sections of the footpad skin revealed that majority of the steady state LCs in the Ezh2^{fl/fl} mice adopted a round morphology and were distributed among the keratinocytes, approximately 8µm away from the underlying basement membrane. Upon induction of contact sensitization by oxazolone, these LCs became activated and move closer to the basement membrane. Occasionally, transiting LCs were observed to extend their dendrites and cell body through the basement membrane (figure 12D and E). However, Ezh2-deficient LCs were frequently found to be lining up along the basement membrane layer during steady state, and this association with the basement membrane

increased following their activation by oxazolone. Moreover, they tend to attach themselves flatly onto the basement membrane layer, in contrast to the rounded morphology of the wild type LCs (figure 12D and E). These suggest that Ezh2-deficient LCs were able to detach themselves from the surrounding keratinocytes, but were unable to pass through the underlying basement membrane, possibly due to their enhanced binding to the extracellular matrix components, thereby causing them to spread out flatly. Altogether, these data show that Ezh2 helps to regulate the transmigration of LCs across the basement membrane.

Figure 12. Ezh2 regulates the transmigration of LCs across basement membrane. (A) Epidermis was separated from the dermis and basement membrane by dispase digestion. Epidermal sheets were cultured on medium supplemented with GM-CSF for 4 hours and stained with anti-MHC II antibody. Images shown are representative of 2 independent experiments. Scale bars, 50 μ m. MHC II⁺ LCs in the immunofluorescence assay were counted to determine their density in the Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice (mean $\pm$ s.d.). Data is based on 2 independent experiments. 3 mice per group were analyzed, and a total of 6 fields were counted for each group. ***p<0.001 (two-tailed Student's *t*-test). (B) LCs were retrieved from the medium 4 hours following epidermal sheet culture. Percentages of live CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ LCs were determined by flow cytometry (mean $\pm$ s.d.). Data is based on 2 independent experiments. 3 mice per group were analyzed. (C) Immunofluorescence analysis of epidermal sheets isolated from footpad skin at steady state. Images shown are representative of 2 independent experiments. Scale bars, 50 μ m. MHC II⁺ LCs in the immunofluorescence assay were counted to determine their density in the Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice (mean $\pm$ s.d.). Data is based on 2 independent experiments. 3 mice per group were analyzed, and a total of 11 fields were counted for each group. ***p<0.001 (two-tailed Student's *t*-test). (D) 0.5% oxazolone was painted on the footpad skin before harvesting them 18 hours later. Steady state and oxazolone-painted footpad skin sections were stained with anti-laminin (red) and DAPI (blue). Endogenous CD11c-YFP⁺ LCs were depicted in green. Images shown are representative of 3 independent experiments. 3 mice per group were analyzed. Scale bars, 100 μ m. Outlined boxes in (D) were enlarged in (E). (E) Scale bars, 10 μ m. (F) Bar graph indicates distance of epidermal LCs (green) away from the basement membrane (red) during steady state and after oxazolone application (mean $\pm$ s.e.m. of n>200 cells from 3 mice), as depicted in (D). ***p<0.001 (two-tailed Student's *t*-test).

3.1.9 Ezh2-deficient LCs show comparable integrin $\alpha 6$ expression as wild type LCs

Integrin $\alpha 6$ has been shown to play crucial roles during the early stages of LC migration, mediating their adhesion to laminin, one of the major components in the underlying basement membrane (Price et al., 1997). Since the results above revealed that LCs in CD11c-cre;Ezh2^{fl/fl} mice were unable to pass through the basement membrane, it raises the possibility that they might be trapped in the epidermal compartment due to dysregulation of integrin $\alpha 6$ expression. To study this, the surface expression of integrin $\alpha 6$ on LCs was analyzed by flow cytometry. As shown in figure 13, comparable level of integrin $\alpha 6$ was observed in wild type and Ezh2-deficient LCs, during both steady state and upon activation by *in vitro* culture. Hence, the inability of Ezh2-deficient LCs to cross the basement membrane is unlikely to be caused by a dysregulated integrin expression.

Figure 13. Ezh2 deficiency does not affect expression of integrin $\alpha 6$. Integrin $\alpha 6$ expression on epidermal LCs isolated from freshly excised mice ears was compared with the expression on epidermal LCs isolated from skin explants which have been cultured for 18 hours by flow cytometry. Histograms were pregated on singlet, live, CD11c⁺ MHC II⁺ EpCAM⁺ CD103⁻ cells. Solid line represents LCs at steady state, and dotted line represents LCs which have been activated by culture. Filled histogram represents isotype control. Data is based on 5 independent experiments. 5 mice per group were analyzed.

3.1.10 Ezh2 regulates adhesion characteristics of LCs

Migration of LCs across the basement membrane is an integrin-dependent process which requires the dynamic formation and turnover of adhesion structures. To understand the migration defect of Ezh2-deficient LCs, their integrin-dependent movements were studied *in vitro* on a two-dimensional laminin-coated slide, a system which resembles their *in vivo* migration on the basement membrane. Time-lapse imaging revealed that wild type LCs were highly motile on laminin, while Ezh2-deficient LCs appeared to be tightly adhered onto the laminin surface and were unable to move much (figure 14A).

This impaired integrin-dependent migration of Ezh2-deficient LCs further prompts the investigation of their adhesion characteristics. To this end, LCs were allowed to attach onto a laminin-coated surface and the adhesion structures formed were visualized by immunostaining with anti-talin antibody. Dendritic cells which adopt a quick migratory phenotype to fulfill their sentinel function do not usually form visible adhesion structures in order to facilitate their movement in the extracellular environment (T. Lammermann et al., 2008). Indeed, as shown in figure 14B, majority of the Ezh2^{fl/fl} LCs did not form visible focal adhesion when attached onto a laminin-coated surface. In contrast, enlarged focal adhesions were frequently observed in the Ezh2-deficient LCs upon attachment to laminin. Moreover, consistent with what was observed previously in the skin sections (figure 12E), these LCs tend to spread excessively, giving rise to an enlarged surface area which is almost 2-fold higher as compared to the wild type LCs (figure 14D). These results suggest that Ezh2 is required to promote the turnover of focal adhesion in LCs, and hence regulate their integrin-dependent migration process.

Figure 14. Ezh2 regulates adhesion characteristics of LCs. (A) CD11c-YFP⁺ LCs (green and outlined) were plated out and allowed to adhere onto laminin-coated slides. The migration of attached LCs in the presence of CCL19 was tracked over a period of 80 minutes by time-lapse imaging. Individual cell trajectories were tracked with the “Manual tracking tool” and compiled using the “Chemotaxis Plug-in” tool in ImageJ. Bar graph indicates total distance moved by the LCs (mean $\pm$ s.e.m. of $n > 40$ cells from 4 mice per group in 4 independent experiments). ** $p < 0.01$ (two-tailed Student’s *t*-test). (B) LCs were allowed to attach onto laminin-coated slides prior to immunostaining with DAPI (blue) and anti-Talin antibody (green). Representative images of the focal adhesions formed in wild type and Ezh2-deficient LCs from 4 independent experiments were shown. 4 mice per group were analyzed. Scale bars, 10 μ m. (C) Analysis of adhesion structures was performed on cells from (B) according to the following criteria: Nascent adhesions $\leq 0.2 \mu\text{m}^2$, $0.2 \mu\text{m}^2 < \text{focal complexes} \leq 0.8 \mu\text{m}^2$, $0.8 \mu\text{m}^2 < \text{focal adhesions} \leq 19 \mu\text{m}^2$, fibrillar adhesions $> 19 \mu\text{m}^2$. Bar graph indicates mean $\pm$ s.e.m. Data is based on 4 independent experiments. 4 mice per group were analyzed. *** $p < 0.001$ (two-tailed Student’s *t*-test). (D) Surface area of the LCs from (B) were measured using ImageJ software and represented by a bar graph (mean $\pm$ s.d.). Data is based on 4 independent experiments. 4 mice per group were analyzed. *** $p < 0.001$ (two-tailed Student’s *t*-test).

3.1.11 Ezh2 regulates adhesion characteristics of BMDCs

To gain mechanistic insights underlying Ezh2 regulation of adhesion structures turnover, bone marrow-derived dendritic cells (BMDCs) were employed as a tool owing to their easier manipulation *in vitro* and higher yield in comparison to LCs. Ezh2-deficient BMDCs were generated from bone marrow cells of poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} mice, and they were able to differentiate into mature DCs phenotypically indistinguishable from the wild type BMDCs (figure 15A).

Similar to LCs, these Ezh2-deficient BMDCs frequently form numerous and enlarged adhesion structures when plated onto their endothelial ligand VCAM-1, unlike the wild type BMDCs which do not usually form any visible adhesion structures (figure 15B and C). In addition, they spread out extensively on VCAM-1 in the absence of Ezh2. These phenotypes resemble those of Ezh2-deficient LCs when they were plated out on laminin-coated surfaces, hence suggesting that Ezh2 also regulates the turnover of adhesion structures in BMDCs.

Figure 15. Ezh2 regulates adhesion characteristics of BMDCs. (A) Generation of BMDCs from poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice in the presence of GM-CSF and IL-4. Percentages of MHC II^{hi} CD11c⁺ cells obtained on day 6 of

culture, with and without overnight LPS stimulation, were indicated. Data shown is representative of 6 independent experiments. 6 mice per group were analyzed. (B) Mature BMDCs were allowed to attach onto VCAM-1-coated slides prior to immunostaining with DAPI (blue) and anti-Talin antibody (green). Representative images of the focal adhesions formed in wild type and Ezh2-deficient BMDCs from 3 independent experiments were shown. 3 mice per group were analyzed. Scale bars, 10 μ m. (C) Analysis of adhesion structures was performed according to the criteria mentioned in figure 14C. Bar graph indicates mean $\pm$ s.e.m. Data is based on 3 independent experiments. 3 mice per group were analyzed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed Student's *t*-test). (D) Surface area of the BMDCs from (B) were measured using ImageJ software and represented by a bar graph (mean $\pm$ s.d.). Data is based on 3 independent experiments. 3 mice per group were analyzed. *** $p < 0.001$ (two-tailed Student's *t*-test).

3.1.12 Methylation of Talin1 at Lys2454 promotes the disassembly of adhesion structures

Talin1 is a component of the focal adhesion complex which couples the cytoplasmic domain of integrins to the actin cytoskeleton. During integrin-dependent migration, these adhesion structures need to be assembled and disassembled dynamically to promote a polarized movement along the substratum (Tim Lammermann et al., 2008). To achieve such dynamic regulation on protein stability, adhesion structure components including talin1 are frequently subjected to post-translational modifications. Indeed, in our earlier studies, talin1 was identified to be methylated by Ezh2 at K2454 (Gunawan et al., 2015).

To assess the relevance of talin1 K2454 methylation on the abnormal adhesion characteristics observed in Ezh2-deficient BMDCs, BMDCs were transduced with wild type talin1 or talin1 mutants in which K2454 is replaced with either an unmethylatable glutamine (K2454Q) or a methyl-mimicking phenylalanine (K2454F). Expression of unmethylatable talin1 (K2454Q) converted the Ezh2^{fl/fl} BMDCs into Ezh2-deficient DC-like cells, where they spread out excessively on VCAM-1 surfaces and frequently form enlarged adhesion structures connected to actin stress fibres (figure 16A and B). Moreover, live-cell imaging revealed that these cells were highly immotile as compared to Ezh2^{fl/fl} BMDCs expressing the wild type talin1 (figure 16C). On the other hand, expression of methyl-mimicking talin1 (K2454F) was able to rescue the excessive spreading phenotype of Ezh2-deficient BMDCs, and also diminish the number and size of their adhesion structures (figure 16A and B). These cells regained their migratory capacity on VCAM-1, unlike Ezh2-deficient BMDCs expressing wild type talin1 which remain tightly attached to the integrin ligand (figure 16C). These data collectively show that Ezh2-mediated methylation of talin1 at K2454 controls cell migration through regulating the disassembly of adhesion structures.

Figure 16. Ezh2-mediated Talin1 methylation at Lys2454 regulates adhesion dynamics. Mature BMDCs generated from poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice were transduced with different GFP-tagged talin1 variants (K2454, K2454F, K2454Q). Untransduced or transduced BMDCs were allowed to adhere onto 10 or 20 μ g/ml VCAM-1-coated slides prior to immunostaining with anti-paxillin

(red), phalloidin (green) and DAPI (blue) (A, B) or live cell imaging (C). (A) Representative images of the untransduced and transduced wild type and Ezh2-deficient BMDCs from 3 independent experiments were shown. 3 mice per group were analyzed. Scale bars, 10 μ m. (B) Surface area of the BMDCs from (A) were measured using ImageJ software and represented by a bar graph (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 3 mice per group were analyzed (at least 30 cells per group). **p<0.01, ***p<0.001 (Black asterisks, two-tailed Student's *t*-test. Red asterisks, one-way ANOVA). (C) Migration of BMDCs in the presence of CCL19 was tracked over a period of 2 hours by time-lapse imaging. Bar graph indicates total distance moved by the BMDCs (mean $\pm$ s.e.m.). Data is based on at least 2 independent experiments. 3 mice per group were analyzed (at least 100 cells per group). **p<0.01, ***p<0.001 (two-tailed Student's *t*-test).

3.1.13 Ezh2 does not regulate integrin-independent migration

Unlike integrin-dependent migration which requires the turnover of adhesion structures, integrin-independent migration is driven primarily by actomyosin contractions (T. Lammermann et al., 2008). To understand whether Ezh2 also regulates such myosin-dependent migration pattern, BMDCs were plated out on poly-L-lysine-coated surface which promotes a charge-based adhesion rather than adhesion structures formation. However, time-lapse imaging revealed that both wild type and Ezh2-deficient BMDCs exhibited little movement on this surface even in the presence of chemokine CCL19 (figure 17A). In this case, the appearance of wild type and Ezh2-deficient BMDCs were similar, with their cell size remaining small and no actin stress fibres being assembled (figure 17B). This suggests that their immotility was not due to a defect in adhesion structure disassembly, but rather an enhanced attraction to opposite charges on the coated surface.

Furthermore, when fibronectin used for surface coating was titrated to reduce the amount of integrin ligands available for adhesion structure formation, the migratory defect of Ezh2-deficient BMDCs was gradually rescued back to wild type level (figure 17C). Consistent with this, the adhesion structures of Ezh2-deficient BMDCs formed at such low fibronectin concentration were small and comparable to that of the wild type cells. As such, these cells were able to overcome the weaker adhesive forces with their actomyosin contractions, thereby allowing them to migrate like their wild type counterparts. Here, the importance of myosin contraction was demonstrated by the addition of Y27632 (an inhibitor of ROCK, which is the downstream effector of Rho) or blebbistatin (an inhibitor of myosin II), which inhibited the migration of both wild type and Ezh2-deficient BMDCs at low fibronectin concentration (figure 17C).

In addition, the integrin-independent migration of dermal DCs to the skin-draining lymph nodes (T. Lammermann et al., 2008) during steady state and upon oxazolone (figure 4C) or FITC painting (Gunawan et al., 2015) was unaffected by their deficiency in Ezh2. These data collectively show that Ezh2 is dispensable for actomyosin contraction, and hence does not regulate integrin-independent migration.

Figure 17. Ezh2 is dispensable for integrin-independent migration of BMDCs. (A) BMDCs were plated out and allowed to adhere onto poly-L-lysine-coated slides. The migration of BMDCs in the presence of CCL19 was tracked over a period of 1 hour by time-lapse imaging. Individual cell trajectories were tracked with the “Manual

tracking tool” and compiled using the “Chemotaxis Plug-in” tool in ImageJ. Data shown is representative of 2 independent experiments. 2 mice per group were analyzed. (B) BMDCs were plated on poly-L-lysine coated slides and immunostained with phalloidin (green), anti-talin1 (red) and DAPI (blue). Data shown is representative of 2 independent experiments. 2 mice per group were analyzed. Scale bars, 10 μ m. (C) BMDCs were plated on 1.3 or 5 μ g/ml of fibronectin, with or without treatment with ROCK inhibitor Y27632 (Y27) or myosin II inhibitor blebbistatin (ble). *Left*, time-lapse images were taken every 5 min for 2 hours. Bar graph indicates total distance moved by the BMDCs (mean $\pm$ s.e.m.). *** $p < 0.001$ (two-tailed Student’s *t*-test). *Right*, surface area of the BMDCs were measured using ImageJ software and represented by a bar graph (mean $\pm$ s.e.m.). *** $p < 0.001$ (two-tailed Student’s *t*-test). *Bottom*, BMDCs were immunostained with anti-paxillin (red), phalloidin (green) and DAPI (blue). Representative images of the BMDCs from 3 independent experiments were shown. 3 mice per group were analyzed. Scale bars, 10 μ m.

3.1.14 Ezh2 regulates integrin-dependent migration, but not integrin-independent migration, in other leukocytes

To understand the relevance of Ezh2-regulated integrin-dependent migration in other leukocytes apart from DCs, irritant contact dermatitis was induced by croton oil in poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} and control Ezh2^{fl/fl} mice, and the neutrophilic response to the irritant was subsequently evaluated. At 3 hours post-induction, similar percentages of wild type and Ezh2-deficient neutrophils were recruited to the site of inflammation (figure 18B). Whole mount ear staining revealed that majority of the wild type neutrophils were already located in the interstitial tissue at this time point. In contrast, a significant proportion of Ezh2-deficient neutrophils remains trapped within the blood vessels and were unable to move into the interstitial tissue (figure 18A). Consistent with this, when taken *in vitro*, these Ezh2-deficient neutrophils displayed a compromised migration on ICAM-1-coated surface despite stimulation with the chemoattractant fMLP (N-formyl-methionine-leucine-phenylalanine) (figure 18C). These show that Ezh2 is also involved in the regulation of integrin-dependent migration of neutrophils.

Figure 18. Ezh2 regulates integrin-dependent migration of neutrophils. (A) Croton oil was applied topically for 3 hours to induce ear inflammation in the poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Whole mount ear was stained with anti-Ly6G to identify neutrophils (red) and anti-PECAM to visualize the blood vessels (green). Scale bars, 50μm. Bar graph indicates percentage of diapedesis, as determined from the images (left) (mean ± s.d.). Data shown is based

on 2 independent experiments. 2 mice per group were analyzed. *** $p < 0.001$ (two-tailed Student's t -test). (B) The recruitment of neutrophils to the site of inflammation was determined by flow cytometry. Percentages of Ly6G⁺ CD11b⁺ neutrophils in the ears are indicated. Data shown is representative of 4 independent experiments. 4 mice per group were analyzed. (C) Neutrophils were isolated and purified from the bone marrow cells of poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice using the murine neutrophil isolation kit. Migration of neutrophils on ICAM-1-coated slides in the presence or absence of fMLP was tracked over a period of 30 minutes through time-lapse imaging. Bar graph indicates total distance moved by the neutrophils (mean $\pm$ s.e.m. of $n=100$ cells from 2 mice per group in 2 independent experiments). *** $p < 0.001$ (two-tailed Student's t -test).

Unlike transmigration of neutrophils across the vascular endothelium which depends on the dynamic turnover of adhesion structures, the circulation of neutrophils *in vivo* is largely integrin-independent. Flow cytometric analysis of various lymphoid and non-lymphoid organs during steady state shows that the distribution of neutrophils in poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} mice was comparable to that in the control mice, hence suggesting that integrin-independent migration of neutrophils is also unaffected by the deficiency of Ezh2 (figure 19A and B). Moreover, likewise to the BMDCs, the Ezh2-deficient neutrophils acquired a similar actin and talin staining as the wild type neutrophils when plated on poly-L-lysine-coated surface, indicating that their integrin-independent adhesion was not affected by Ezh2 deficiency (figure 19C). Furthermore, upon stimulation of the wild type and Ezh2-deficient neutrophils with the chemoattractant fMLP in solution, which activates them independently of the integrin signaling pathway, no difference in actin polymerization was observed (figure 19D), unlike in Ezh2-deficient BMDCs which frequently form more F-actin fibres than control BMDCs when attached to VCAM-1 surfaces (figure 16A). Hence, these data collectively show that Ezh2 regulates integrin-dependent but not integrin-independent migration and adhesion processes in various leukocytes.

Figure 19. *Ezh2* deficiency does not affect integrin-independent migration of neutrophils. (A) Steady state distribution of neutrophils in bone marrow, spleen, blood and ear was analyzed by flow cytometry. Dot plots are pregated on singlet, live, CD45⁺ cells. Percentages of Ly6G⁺ CD11b⁺ neutrophils were indicated. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed. (B)

Bar graph indicates the absolute numbers (left) or percentages (right) of neutrophils from (A) (mean $\pm$ s.d.). Data is based on 3 independent experiments. 3 mice per group were analyzed. (C) Neutrophils isolated from the bone marrow cells of poly(I)poly(C)-treated Mx1-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice were plated on poly-L-lysine coated slides and immunostained with phalloidin (green), anti-talin1 (red) and DAPI (blue). Data shown is representative of 2 independent experiments. 2 mice per group were analyzed. Scale bars, 10 μ m. (D) Purified neutrophils as from (C) were stimulated with fMLP for the indicated time points. F-actin content was determined by phalloidin staining and flow cytometry analysis. Data shown is representative of 4 independent experiments. 4 mice per group were analyzed.

3.1.15 Comparable CHS response in CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice

Allergic contact dermatitis is one of the most prevalent occupational diseases which impairs the patients' quality of life due to their chronic and recurrent nature. To date, the role of LCs in mediating contact hypersensitivity (CHS), a mouse model for allergic contact dermatitis, remains controversial. The traditional viewpoint that LCs play a crucial role in the initiation of CHS has been challenged by recent development of various LC ablation mouse models which yield conflicting results (Noordegraaf et al., 2010). Taking advantage of the impaired migration of Ezh2-deficient LCs (integrin-dependent) but intact migratory capacity of Ezh2-deficient dermal DCs (integrin-independent) to the skin-draining lymph nodes, CHS response was assessed in 8-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice to determine the involvement of LCs during such cutaneous inflammation. Here, the mice were topically painted with a contact sensitizer DNFB on the back skin and subsequently challenged 5 days later with a second dose of DNFB on the ear. Consistent with current findings, no difference in the magnitude of ear swelling was observed between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (figure 20), indicating that LCs are functionally redundant for the initiation of CHS. Furthermore, the ability of Ezh2-deficient dermal DCs to trigger a comparable CHS response as the control mice suggests that Ezh2-mediated signaling is dispensable in the DCs during the development of CHS.

Figure 20. Contact hypersensitivity response is unaltered between CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Mice were sensitized with 0.5% DNFB on the shaved back skin before ear challenge with 0.1% DNFB 5 days later. The ear swelling response was determined daily over a period of 5 days after challenge as an

indicator of ear inflammation (mean $\pm$ s.e.m.). Data is based on 2 independent experiments. 8 mice per group were analyzed.

3.1.16 Ezh2 in LCs is essential for tolerance induction during contact hypersensitivity

In contrast to their redundancy during CHS, LCs have been shown to play a crucial role in mediating immune tolerance to innocuous hapten and thus protect against the development of CHS through the activation of Foxp3⁺ regulatory T cells (Tregs) (Gomez de Aguero et al., 2012). Since Ezh2-deficient LCs were less able to reach the skin-draining lymph nodes (figure 4), their ability to mount a protection against CHS may be compromised. To address this, 8-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice were first tolerized with a weak hapten DNTB prior to the induction of CHS with the strong hapten DNFB (figure 21A). Both DNTB and DNFB are able to generate similar dinitrophenyl (DNP) motifs, which permit their recognition by the same antigen-specific T cells. In this case, tolerization with 0.1% DNTB prior to the induction of CHS with DNFB suppressed the development of ear swelling in wild type mice (figure 21B). However, a robust CHS response peaking at day 2 to 3 was observed in the CD11c-cre;Ezh2^{fl/fl} mice despite pretreatment with DNTB, implying a breakdown of cutaneous tolerance in these mice.

To control for any difference in CHS response arising from the deletion of Ezh2 in other skin DC subsets apart from LCs in the CD11c-cre;Ezh2^{fl/fl} mice, a series of adoptive transfer experiments were performed to selectively delete Ezh2 in either the LCs or dermal DCs. Using the same tolerization and sensitization regimen, an enhanced ear swelling was observed in the BM chimeric mice where Ezh2 deletion is restricted to only the epidermal LCs. On the other hand, ear inflammation was significantly attenuated in BM chimeras harbouring wild type LCs and Ezh2-deficient dermal DCs (figure 21C). However, the overall ear swelling response observed in the chimeric mice was higher as compared to the non-chimeric mice, probably due to other non-specific inflammatory effects triggered by the irradiation process. As such, these data collectively demonstrate the importance of Ezh2 in LCs, but not dermal DCs, during tolerance induction.

3.1.17 Ezh2 regulates LC migration in response to DNTB

To determine whether the breakdown of cutaneous tolerance in CD11c-cre;Ezh2^{fl/fl} mice was due to the impaired migration of Ezh2-deficient LCs to the skin-draining lymph nodes, epidermal sheets were analyzed 18 hours after painting with 0.1% DNTB. Indeed, wild type LCs but not Ezh2-deficient LCs were able to leave the epidermis following exposure to the weak hapten (figure 22A and B). Consistent with this, an increase in arrival of LCs was observed in the skin-draining lymph nodes of Ezh2^{fl/fl} mice, while no significant increase was found in the CD11c-cre;Ezh2^{fl/fl} mice after DNTB application (figure 22C). Hence, these data show the importance of Ezh2-regulated LC migration during the tolerogenic phase of CHS.

Figure 22. Impaired migration of Ezh2-deficient LCs in response to 0.1% DNTB. (A) Immunofluorescence analysis of epidermal sheets isolated from freshly excised mice ears at steady state and from mice ears 18 hours after application with 0.1% DNTB. LCs were identified by immunostaining with anti-MHC II antibody. Images shown are representative of 2 independent experiments. 3 mice per group were

analyzed. Scale bars, 50 μ m. (B) MHC II⁺ LCs in the immunofluorescence assay (A) were counted to determine their density in the Ezh2^{fl/fl} and CD11c-cre;Ezh2^{fl/fl} mice (mean $\pm$ s.d.). Data is based on 2 independent experiments. 3 mice per group were analyzed, and a total of 6 fields were counted. ***p<0.001 (two-tailed Student's *t*-test). (C) 0.1% DNTB was painted on shaved abdominal skin, and skin-draining lymph node was harvested after 48 hours. Bar graph indicates the absolute numbers of singlet, live, MHC II^{hi} CD11c⁺ Langerin⁺ CD103⁻ LCs in lymph nodes as determined by flow cytometry analysis (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 6 mice per group were analyzed.

3.1.18 Ezh2 expression in DCs regulates the activation of regulatory T cells

To determine whether the impaired migration of Ezh2-deficient LCs to the skin-draining lymph nodes contributes to the reduced priming of Tregs, the Treg activation status as indicated by their surface ICOS expression was assessed. As expected, activated ICOS⁺ Foxp3⁺ Tregs were increased in the Ezh2^{fl/fl} mice upon DNTB tolerization, thus conferring protection against CHS upon subsequent sensitization and challenge with DNFB (figure 23A). However, the number of activated ICOS⁺ Foxp3⁺ Tregs did not increase significantly in the CD11c-cre;Ezh2^{fl/fl} mice even after tolerization, suggesting that migration of LCs to the skin-draining lymph nodes is required for the activation of Tregs.

In accordance with the reduced Treg activation, the number of cytotoxic CD8⁺ T cells, the main effector cell type mediating CHS, as well as $\gamma\delta$ T cells, were significantly higher in the skin-draining lymph nodes of DNTB-tolerized CD11c-cre;Ezh2^{fl/fl} mice following sensitization with DNFB (figure 23B). Moreover, significantly higher number of these CD8⁺ T cells adopted a CD44⁺ CD62L^{lo} effector memory and CD44⁺ CD62L⁺ central memory phenotype (figure 23C). The absolute number of IFN γ -producing CD8⁺ T cells was also significantly higher in the tolerized CD11c-cre;Ezh2^{fl/fl} mice after DNFB sensitization (figure 23D). However, no difference in the number of CD4⁺ T cells was found between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice, possibly due to their functional irrelevancy in the initiation of CHS. Together, these results suggest that the enhanced ear swelling observed in CD11c-cre;Ezh2^{fl/fl} mice could be attributed to reduced Treg activation due to impaired migration of LCs to the skin-draining lymph nodes, which consequently promotes an increased priming of cytotoxic CD8⁺ and $\gamma\delta$ T cells following DNFB sensitization.

Figure 23. Treg activation in tolerization phase of CHS is required to reduce cytotoxic T cell response in elicitation phase. (A) 8-week-old mice were tolerized on shaved abdomen skin with 0.1% DNTB and Treg activation statuses were determined by flow cytometry on day 5. Dot plots were pregated on singlet, live, CD3 ϵ ⁺ CD4⁺ Foxp3⁺ Tregs. Percentages of gated cells were indicated. Bar graph

indicates absolute numbers of ICOS⁺ Tregs in the skin-draining lymph nodes of untreated mice (UT) or mice tolerized with 0.1% DNTB (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. At least 5 mice per group were analyzed. * p <0.05, ** p <0.01 (two-tailed Student's t -test). (B, C, D) 8-week-old mice were tolerized on shaved abdomen skin with 0.1% DNTB before sensitization with 0.5% DNFB on shaved back skin 7 days later. Skin-draining lymph nodes were harvested after 5 days. (B) Bar graph indicates absolute numbers of CD8⁺, CD4⁺ and $\gamma\delta$ T cells in the skin-draining lymph nodes of untreated or tolerized and sensitized mice (mean $\pm$ s.e.m.), as determined by flow cytometry. Data is based at least 3 independent experiments. At least 6 mice per group were analyzed. * p <0.05, ** p <0.01, *** p <0.001 (two-tailed Student's t -test). (C) Bar graph indicates absolute numbers of CD44⁺ CD62L^{lo} effector memory and CD44⁺ CD62L⁺ central memory CD8⁺ T cells from (B) (mean $\pm$ s.e.m.). Data is based at least 3 independent experiments. At least 6 mice per group were analyzed. * p <0.05, ** p <0.01, *** p <0.001 (two-tailed Student's t -test). (D) Bar graph indicates absolute numbers of IFN γ -producing CD8⁺ T cells from (B) (mean $\pm$ s.e.m.). Data is based on at least 3 independent experiments. At least 6 mice per group were analyzed. * p <0.05, ** p <0.01, *** p <0.001 (two-tailed Student's t -test).

3.1.19 Increased DC clustering in skin of CD11c-cre;Ezh2^{fl/fl} mice

Since CD11c-cre;Ezh2^{fl/fl} mice experienced an increase in ear swelling despite tolerance induction, the formation of dermal DC cluster, an indicator of skin inflammation (Natsuaki et al., 2014), was examined during the elicitation phase of CHS in both CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Whole mount ear staining was performed on the DNTB-tolerized and DNFB-sensitized mice 48 hours after ear challenge with DNFB. This time point enables the visualization of DNP-specific immune responses in the skin, as opposed to non-specific DC clustering events triggered by the vehicle which would have dissipated within 48 hours (Natsuaki et al., 2014). As shown in figure 24, DCs were distributed diffusely in the dermis during steady state in both CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Upon ear challenge with DNFB, few DC clusters were formed in the dermis of Ezh2^{fl/fl} mice, suggesting the absence of an antigen-specific inflammation due to prior tolerization with DNTB. In contrast, accumulation of enlarged DC clusters was observed in the CD11c-cre;Ezh2^{fl/fl} mice, indicating the breakdown of cutaneous tolerance leading to the elicitation of CHS. These clusters resemble the 'inducible skin-associated lymphoid tissue (iSALT)' structure which is essential for the elicitation of an acquired immune response (Natsuaki et al., 2014). Collectively, these data suggest that LCs play a critical role in the tolerogenic phase of CHS to prevent the subsequent development of skin inflammation.

Figure 24. Increased DC clustering in dermis of CD11c-cre;Ezh2^{fl/fl} mice following tolerization to contact hypersensitivity. 8-week-old mice were tolerized with 0.1% DNTB on the shaved abdomen skin prior to the induction of CHS by DNFB. CD11c-YFP⁺ dermal DC clustering events were visualized in the whole mount ear

during elicitation phase of CHS, 48 hours following ear challenge with DNFB. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed. Scale bars, 50 μ m.

3.1.20 Higher DNTB dose restores tolerance induction in CD11c-cre;Ezh2^{fl/fl} mice

Interestingly, upon tolerization with increasing dosage of DNTB, the exaggerated ear swelling response observed previously in the CD11c-cre;Ezh2^{fl/fl} mice could be rescued. At 1% DNTB, no significant difference in ear swelling was found between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (figure 25A). Consistent with this, the formation of dermal DC clusters was also attenuated in the CD11c-cre;Ezh2^{fl/fl} mice which were treated with higher tolerizing dose of DNTB (figure 25B). Indeed, a significant increase in the number of activated ICOS⁺ Tregs was observed in the skin-draining lymph nodes of CD11c-cre;Ezh2^{fl/fl} mice upon tolerization with 1% DNTB. Even though their numbers remain significantly lower than that in the Ezh2^{fl/fl} mice, the higher DNTB dose was able to bring the activated Tregs in CD11c-cre;Ezh2^{fl/fl} mice to comparable numbers as what was observed in the Ezh2^{fl/fl} mice with 0.1% DNTB, which probably is sufficient to confer protection from the development of CHS (figure 25C).

This rescue effect was, however, not due to the regained migratory capacity of Ezh2-deficient LCs, since their migration away from the epidermis remains defective at 1% DNTB (figure 25D). Studies have demonstrated that application of high-dose haptens facilitate the dissemination of antigens into the dermis, thereby activating other dermal DC subsets to participate in the cutaneous immune response (Noordegraaf et al., 2010). Hence, to test the hypothesis of whether restoration of Tregs activation was due to migration of dermal DCs at higher DNTB dose, the arrival of different skin DC subsets at the skin-draining lymph nodes was analyzed 48 hours following DNTB application. Indeed, following skin painting with 1% DNTB, both wild type and Ezh2-deficient migratory Langerin⁻ dermal DCs were significantly increased in the skin-draining lymph nodes, while LCs were increased significantly only in the Ezh2^{fl/fl} mice (figure 26A and B). The number of Langerin⁺ dermal DCs in the skin-draining lymph nodes were not increased following 1% DNTB application, possibly due to their smaller proportion in the dermis relative to the Langerin⁻ DC subset, which could have impeded their access to antigens. On the other hand, recruitment of dermal DCs to the lymph nodes was not observed when 0.1% DNTB was applied. In this case, since antigen dissemination into the dermis is limited, the only mobilized skin DC subset was the epidermal LCs (figure 26A and B). Taken

together, these data suggest that tolerization can be restored in the CD11c-cre;*Ezh2*^{fl/fl} mice with higher DNTB dose, when the impaired migration of *Ezh2*-deficient LCs can be compensated by the migration of dermal DCs.

Figure 25. Higher dose of DNTB restores tolerance in *CD11c-cre;Ezh2*^{fl/fl}. (A, B) 8-week-old mice were tolerized on shaved abdomen skin with 1% DNTB before sensitization with 0.5% DNFB on shaved back skin 7 days later. After 5 days, mice were re-challenged on ears with 0.1% DNFB. (A) Ear swelling response was determined daily over a period of 5 days after challenge in the *CD11c-cre;Ezh2*^{fl/fl} and *Ezh2*^{fl/fl} mice (mean $\pm$ s.e.m.). Data is based on 2 independent experiments. At least 8 mice per group were analyzed. (B) *CD11c-YFP*⁺ dermal DC clustering events were visualized in the whole mount ear during elicitation phase of CHS, 48 hours following ear challenge with DNFB. Scale bars, 50μm. Data is based on 2 independent experiments. 2 mice per group were analyzed. (C) Bar graph indicates absolute number of ICOS⁺ Tregs in the skin-draining lymph nodes of untreated mice (UT), and of mice after tolerization with 0.1% or 1% DNTB on shaved abdominal skin (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. At least 5 mice per group were analyzed. **p*<0.05, ***p*<0.01, ****p*<0.001 (two-tailed Student's *t*-test). (D)

Bar graph indicates the density of MHC II⁺ LCs in the epidermal immunofluorescence assay from freshly excised mice ears at steady state and from mice ears 18 hours after application of 1% DNTB mice (mean $\pm$ s.d.). Data is based on 3 independent experiments. 3 mice per group were analyzed. ** $p < 0.01$, *** $p < 0.001$ (two-tailed Student's t -test).

Figure 26. Migration of skin DC subsets to the draining lymph nodes upon DNTB tolerization. (A) Flow cytometric analyses of DCs in skin-draining lymph nodes during steady state and 48 hours after tolerization with 0.1% and 1% DNTB. Dot plots were pregated on singlet, live cells. CD11c⁺ and MHC II^{hi} migratory DCs were further analysed for CD103 and Langerin expression to define LCs (Langerin⁺ CD103⁻), Langerin⁺ dermal DCs (Langerin⁺ CD103⁺) and Langerin⁻ dermal DCs (Langerin⁻). Percentages of gated cells were indicated. Data shown is representative of 4 independent experiments. At least 4 mice per group were analyzed. (B) Bar graph indicates absolute numbers of LCs, Langerin⁺ dermal DCs and Langerin⁻

dermal DCs reaching the lymph nodes in the untreated (UT) and 0.1% or 1% DNTB painted mice (mean $\pm$ s.e.m.). Data is based on 4 independent experiments. At least 4 mice per group were analyzed. * $p < 0.05$, ** $p < 0.01$ (two-tailed Student's t -test).

3.2 Ezh2 in DCs regulates thymocyte development

3.2.1 Overrepresentation of thymic DCs in CD11c-cre;Ezh2^{fl/fl} mice

Since earlier results revealed that Ezh2 controls integrin-dependent migration of DCs, it was hypothesized that their distribution across various organs, which occurs either through integrin-independent means or before CD11c-cre-mediated deletion of Ezh2 takes place, would be unaffected by their deficiency in Ezh2. Indeed, comparable percentages and numbers of resident DCs were found in the skin-draining lymph nodes and spleen of 10-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice during steady state (figure 27A). Surprisingly, higher percentages of MHC II^{hi} CD11c⁺ cDCs and MHC II^{int} CD11c⁺ pDCs were found in the thymus of 10-week-old CD11c-cre;Ezh2^{fl/fl} mice (figure 27B). Similar to LCs, such accumulation of thymic DCs correlates positively with age of the mice. In 4-week-old mice, no difference in the percentage of DCs was observed between the thymus of CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Upon reaching 10 weeks of age, an age-dependent expansion of DC percentage occurred in the CD11c-cre;Ezh2^{fl/fl} mice, which was further increased at 16 weeks of age. Intriguingly, this increase in percentage was not accompanied by an increase in their absolute numbers (figure 27B). The total DC numbers remain comparable between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice throughout the 16 weeks where they were analyzed. As such, this suggests that the overrepresentation of cDCs and pDCs in the CD11c-cre;Ezh2^{fl/fl} mice upon hitting 10 weeks of age was not due to their expansion, but rather, was resulting from the age-related decline of other cell populations in the thymus.

Figure 27. Increased proportion of Ezh2-deficient thymic DCs with age. (A) Flow cytometric analyses of resident and migratory DCs in skin-draining lymph node and spleen of 10-week-old mice. Percentages of MHC II^{int} CD11c^{hi} resident DCs and MHC II^{hi} CD11c^{int} migratory DCs in the skin-draining lymph node and MHC II⁺ CD11c⁺ DCs in the spleen are indicated. Data shown is representative of 3 independent experiments. 3 to 4 mice per group were analyzed. Bar graph indicates the absolute numbers of resident lymph node, migratory lymph node and splenic DCs (mean ± s.e.m.). Data is based on 3 independent experiments. 3 to 4 mice per group were analyzed. (B) Flow cytometric analyses of DCs in thymus at various age as indicated. Dot plots are pregated on singlet, live, CD3ε⁻ cells. Percentages of MHC II^{hi} CD11c⁺ cDCs and MHC II^{int} CD11c⁺ pDCs were indicated. Data shown is representative of 3 independent experiments. 4 mice per group were analyzed. Bar

graph indicates the absolute numbers of thymic cDCs and pDCs (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 4 mice per group were analyzed.

3.2.2 Ezh2 expression in thymic DCs prevents premature thymic involution

To ascertain the possibility of other declining thymic cell populations in the CD11c-cre;Ezh2^{fl/fl} mice, the total thymic cell numbers were examined. Indeed, an accelerated thymic involution was observed in the CD11c-cre;Ezh2^{fl/fl} mice (figure 28A and B). According to the histological analysis, this reduction in thymus size was due to a proportional shrinkage of both cortex and medulla, as revealed by the maintenance of their cortico-medullary ratio (figure 28B).

Figure 28. Accelerated thymic atrophy in CD11c-cre;Ezh2^{fl/fl} mice. (A) Bar graph indicates total cell numbers in the thymi of CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice at indicated age (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 3 mice per group were analyzed. * $p < 0.05$, *** $p < 0.001$ (two-tailed Student's *t*-test). (B) *Top*, gross morphology of 10-week-old thymi of CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice as shown by H&E staining. *Bottom*, 10x magnification of thymus sections. Scale bars, 100 μ m. Images shown are representative of 3 independent experiments. 3 mice per group were analyzed. *Right*, area of cortex and medulla were determined from the thymus sections by ImageJ. Bar graph indicates the thymic cortico-medullary ratio in the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 3 mice per group were analyzed.

Further analysis of these CD11c-cre;Ezh2^{fl/fl} mice revealed an altered development of thymocytes, which became evident upon reaching 10 weeks of age. The percentage of their CD4⁺CD8⁺ double-positive (DP) thymocytes was significantly reduced, while the percentages of the mature CD8⁺ and CD4⁺ single-positive (SP) and $\gamma\delta$ T cells were markedly increased. Although the double-negative (DN) thymocytes from the 10-week-old CD11c-cre;Ezh2^{fl/fl} mice displayed no significant change in their percentage, further discrimination of the DN subsets based on their surface expression of CD44 and CD25 revealed a partial arrest of the thymocytes at DN1 (CD44⁺ CD25⁻) stage (figure 29A). These differences in thymocyte percentages were also reflected in terms of their absolute numbers, with the exception of CD4⁺ SP T cells which appear to be similar to the wild type mice (figure 29B). Such altered development of thymocytes, however, does not exist in 4-week-old young mice. Moreover, DCs percentages and numbers were also similar between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice at this age (figure 27B). This suggests that the dependency on DCs for thymopoiesis is only required during a later stage of life. Hence, the altered development of wild type T cells in the CD11c-cre;Ezh2^{fl/fl} mice indicates that DCs play a critical role in regulating early T cell development in adult thymus.

Figure 29. Altered T cell development in CD11c-cre;Ezh2^{fl/fl} mice. (A) Flow cytometric analyses of thymocytes in 4- (top), 10- (middle), 16-week-old mice (bottom). *Left*, dot plots were pregated on singlet, live, lin⁻ cells. *Middle*, γδTCR⁻ cells from left panel were further analysed for their CD4 and CD8 expression. *Right*, CD4⁻ CD8⁻ cells from middle panel were further analysed for their CD25 and CD44

expression. Percentages of gated cells are indicated. Data shown is representative of 3 independent experiments. 3 to 4 mice per group were analyzed. (B) Bar graph indicates the absolute numbers of DN ($CD4^- CD8^-$), DP ($CD4^+ CD8^+$), $CD4^+$ SP, $CD8^+$ SP and $\gamma\delta$ T cells in thymus of 10- to 12-week-old mice (mean $\pm$ s.e.m.). Data is based on 4 independent experiments. 7 mice per group were analyzed. * $p < 0.05$ (two-tailed Student's t -test).

3.2.3 Specific deletion of Ezh2 in thymic DCs

Since only the thymocyte numbers, but not DC numbers, were found to be reduced in the CD11c-cre; Ezh2^{fl/fl} mice, it remains the possibility that the accelerated thymic atrophy observed was brought about by the deletion of Ezh2 in other CD11c-expressing cell types, such as macrophages, NK cells and a subset of T cells (Abram, Roberge, Hu, & Lowell, 2014). As such, to assess the specificity and efficiency of CD11c-cre-mediated gene deletion in our CD11c-cre; Ezh2^{fl/fl} mice strain, the expression of Ezh2 in various thymic cell populations was examined by intracellular staining and flow cytometry. Apart from CD11c⁺ MHC II⁺ DCs where Ezh2 level was markedly reduced, all other analysed cell populations including F4/80⁺ macrophages, Dx5⁺ NK cells, CD3ε⁺ T cells and EpCAM⁺ thymic epithelial cells (TECs) in the CD11c-cre;Ezh2^{fl/fl} mice were found to harbour comparable level of Ezh2 as their counterparts in the control Ezh2^{fl/fl} mice (figure 30). Hence, this suggests that the premature thymic involution of CD11c-cre; Ezh2^{fl/fl} mice is unlikely to be a consequence of Ezh2 deletion in other thymic cell populations, and further supports the importance of DCs in the regulation of early T cell development.

Figure 30. Ezh2 expression in thymic cell populations of CD11c-cre;Ezh2^{fl/fl} mice. Total Ezh2 level in different thymic cell populations of 10-week-old mice was analyzed by intracellular staining and flow cytometry. Histograms were pregated on singlet, live, CD11c⁺ MHC II⁺ cells (DCs), F4/80⁺ cells (macrophages), Dx5⁺ cells (NK cells), CD3ε⁺ cells (T cells) or EpCAM⁺ cells (TECs). Filled histogram represents isotype control. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed.

3.2.4 Increased peripheral memory-like T cells in CD11c-cre;Ezh2^{fl/fl} mice

Since the differential thymocyte development and accelerated thymic atrophy of the CD11c-cre;Ezh2^{fl/fl} mice translate to an altered thymic output, the peripheral T cell compartment was analyzed in the secondary lymphoid organs, in particular the spleen and lymph nodes, of 10-week-old mice. A significant increase in $\gamma\delta$ T cells was observed in the spleen of CD11c-cre;Ezh2^{fl/fl} mice, which correlates with their increased thymic production (figure 31B). Although no difference in the numbers and percentages of peripheral CD4⁺ and CD8⁺ T cells were observed between the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice at this age (figure 31A and B), the relative ratio of their naïve to memory T cells was changed significantly. In both the spleen and lymph nodes of CD11c-cre;Ezh2^{fl/fl} mice, a higher proportion of CD4⁺ and CD8⁺ T cells were found to express an activated, memory-like phenotype (CD44⁺ CD62L^{lo} effector memory and CD44⁺ CD62L⁺ central memory), unlike in the Ezh2^{fl/fl} mice where they exist predominantly as naïve T cells (CD44^{lo} CD62L⁺) (figure 31C and D). However, such increased expression of memory surface markers on T cells were not evident in 8-week-old CD11c-cre;Ezh2^{fl/fl} mice (figure 23C). Hence, the expansion of memory-like T cell compartment in CD11c-cre;Ezh2^{fl/fl} mice is likely to be a compensatory response for the maintenance of peripheral T cell homeostasis in face of a declining thymic output due to premature thymic involution.

Figure 31. Increased peripheral T cells with memory surface markers in CD11c-cre;Ezh2^{fl/fl} mice. (A) Flow cytometric analyses of T cells in lymph nodes and spleen of 10-week-old mice. *Left*, dot plots were pregated on singlet, live, CD3 ϵ ⁺ cells. Percentages of CD4⁺ and CD8⁺ T cells are indicated. *Right*, dot plots were pregated on singlet, live cells. Percentages of $\gamma\delta$ T cells are indicated. Data shown is representative of 3 independent experiments. 4 mice per group were analyzed. (B) Bar graphs indicate the total cell numbers in lymph nodes and spleen, and absolute

numbers of CD4⁺, CD8⁺ and $\gamma\delta$ T cells in lymph nodes and spleen (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 4 mice per group were analyzed. (C) Flow cytometric analyses of naïve (CD44^{lo} CD62L⁺), effector memory (CD44⁺ CD62L^{lo}) and central memory (CD44⁺ CD62L⁺) T cells in lymph nodes and spleen. Dot plots were pregated on singlet, live, CD3 ϵ ⁺, CD4⁺/CD8⁺ cells. Data shown is representative of 3 independent experiments. 4 mice per group were analyzed. (D) Bar graphs indicate the absolute numbers of naïve (nai), effector memory (eff) and central memory (mem) T cells in lymph nodes and spleen (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 4 mice per group were analyzed. *p<0.05 (two-tailed Student's *t*-test).

3.2.5 Localization of thymic DCs unaffected by Ezh2 deficiency

The thymic microenvironment is enriched in extracellular matrices such as fibronectin and laminin which help regulate thymocyte development and migration (Villa-Verde et al., 1994). Since Ezh2-deficient DCs exhibit an enhanced adhesion to integrin ligands, it raises the possibility that their localization in the thymus may be altered, thus affecting early T cell development which occurs predominantly in the cortex. However, according to the histological analysis, no obvious difference in thymic DC localization was observed between the 10-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Majority of the CD11c⁺ DCs were accumulated in the medullary compartment, with few DCs being detected in the cortex (figure 32). These suggest that localization of thymic DCs was not affected by Ezh2 deficiency, and hence unlikely to be the cause of the altered T cell development observed in the CD11c-cre;Ezh2^{fl/fl} mice.

Figure 32. Distribution of thymic DCs is unaffected by Ezh2 deficiency. Frozen thymus sections from 10-week-old mice were fixed and stained with DAPI (blue), anti-cytokeratin 5 (red) to delineate the medulla, and anti-CD11c (green) to identify the DCs. Images shown are representative of 3 independent experiments. 3 mice per group were analyzed. Scale bars, 200 μ m.

3.2.6 Ezh2 deficiency in DCs does not affect V(D)J recombination

Rearrangement of the TCR V β locus is initiated during the transition from DN2 to DN3 stage of thymocyte development, and their successful rearrangement is a prerequisite for thymocytes to progress beyond DN3 (J. Kim et al., 2014). To investigate whether the impaired development of thymocytes in CD11c-cre;Ezh2^{fl/fl} mice at DN stage was attributed to defects in the formation of a functional TCR β , their intracellular expression in the DN thymocytes was determined by flow cytometry. As shown in figure 33A, approximately 90% of the DN thymocytes in Ezh2^{fl/fl} mice expressed the TCR β chain. However, this population was reduced in the CD11c-cre;Ezh2^{fl/fl} mice, suggesting a decline in the DN thymocytes harboring a functional TCR β chain.

To identify whether this reduced expression of TCR β in the DN thymocytes from the CD11c-cre;Ezh2^{fl/fl} mice was due to inefficient V(D)J recombination, the genomic DNA from total thymus was analyzed by PCR to examine the relative frequencies of the various recombination events. No major difference in the repertoire of the rearranged TCR V β gene was found between the total thymocytes from CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (figure 33B), implying that the decreased expression of TCR β at DN stage was not caused by an impairment of either the distal or proximal recombination, but rather reflects an overall decline in the frequencies of rearrangement.

Figure 33. V(D)J recombination is unaffected in *CD11c-cre;Ezh2^{fl/fl}* mice. (A) Flow cytometric analyses of intracellular TCRβ expression in DN thymocytes isolated from 10-week-old mice. Dot plots are pregated on singlet, live, lin⁻ CD4⁻ CD8⁻ cells. Percentages of TCRβ⁺ DN thymocytes are indicated. Data shown is representative of 4 independent experiments. 4 mice per group were analyzed. Bar graph indicates the percentages of TCRβ⁺ DN thymocytes (mean $\pm$ s.d.). Data is based on 4 independent experiments. 4 mice per group were analyzed. ***p*<0.01 (two-tailed Student's *t*-test). (B) Primers recognizing the various Vβ genes (as indicated) and the DJβ1 gene were used to amplify the rearranged TCR Vβ gene in the genomic DNA isolated from total thymus using touchdown PCR. DNA isolated from the thymus of *RAG^{-/-}* mice serves as a negative control. PCR amplification of EF gene serves as a loading control. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed.

3.2.7 Ezh2 deficiency in DCs does not affect thymocyte selection

During the developmental process, DP thymocytes undergo a classical affinity model of selection to positively select for thymocytes expressing a TCR with low affinity for self-peptide MHC complex and subsequently negatively select against those expressing a high affinity for self-peptide MHC complex. Such selection processes which serve to shape the TCR repertoire are mediated by three major groups of thymic antigen-presenting cells, namely the cortical thymic epithelial cells (cTECs), medullary TECs (mTECs) and the thymic DCs. Given that a significant reduction in DP population was observed in the thymus of CD11c-cre;Ezh2^{fl/fl} mice, it raises the possibility that Ezh2 in DCs is required for the selection process.

To address this, the expression of CD69, a marker for successful positive selection (D. Wang et al., 2012), was examined in the thymocytes. Comparable percentages of pre-positive selection CD69^{lo} DP thymocytes and CD69^{hi} DP thymocytes which are engaging in positive selection were observed in the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice. Moreover, among the CD4⁺ and CD8⁺ SP cells, the percentages of post-positive selection CD69^{hi} cells and mature CD69^{lo} SP cells ready for thymic egress were similar between these two strains of mice (figure 34A and B). Thus, these data suggest that positive selection is not affected in the CD11c-cre;Ezh2^{fl/fl} mice.

To further investigate if the negative selection process is affected by Ezh2-deficient DCs, the expression of Nur77, a transcription factor critical for the regulation of TCR-mediated apoptosis of DP thymocytes during negative selection (Moran et al., 2011), was evaluated. Comparable percentages of Nur77-expressing DP thymocytes were found in both the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (figure 34C), suggesting that the strength of TCR signaling during negative selection is not different between them. In addition, no difference in the apoptotic and death rates of DP thymocytes, as determined by annexin V and viability dye staining, during resting state or upon calcium ionophore and phorbol ester stimulation (which mimics TCR activation) was observed in the CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (figure 34D and E). As such, these data suggest that the decline in DP population was not due to an altered selection process, which would have led to differences in the rate of activation-induced apoptosis or death by neglect.

Figure 34. Ezh2 deficiency in thymic DCs does not affect selection process. (A) Expression of CD69 in DP, CD4⁺ SP and CD8⁺ SP thymocytes from 10-week-old mice was determined by flow cytometry. Dot plots were pregated on singlet, live, lin⁻ CD4⁺CD8⁺ (DP)/ CD4⁺CD8⁻ (CD4⁺ SP)/ CD4⁻CD8⁺ (CD8⁺ SP) cells. Percentages of

gated cells were indicated. Data shown is representative of 3 independent experiments. 4 mice per group were analyzed. (B) Bar graphs indicate percentages of CD69^{lo} and CD69^{hi} DP, CD4⁺ SP and CD8⁺ SP cells (mean $\pm$ s.d.). Data shown is based on 3 independent experiments. 4 mice per group were analyzed. (C) Expression of Nur77 in DP thymocytes from 10-week-old mice was determined by flow cytometry. Dot plots were pregated on singlet, live, lin⁻ CD4⁺ CD8⁺ cells. Percentages of gated cells were indicated. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed. Bar graph indicates percentages of Nur77⁺ DP thymocytes (mean $\pm$ s.d.). Data shown is based on 3 independent experiments. 3 mice per group were analyzed. (D) The frequencies of apoptotic DP thymocytes from 10-week-old mice during resting state and upon calcium ionophore and phorbol ester stimulation for various time points as indicated were analyzed by Annexin V staining. Dead DP thymocytes were identified by their positive staining for the viability dye. (E) Bar graph indicates the percentages of apoptotic (lower right quadrant) and dead (upper quadrants) DP thymocytes (mean $\pm$ s.d.). Data shown is based on 3 independent experiments. 3 mice per group were analyzed.

3.2.8 Differential expression of cytokines in the thymus of 10-week-old CD11c-cre;Ezh2^{fl/fl} mice

The thymus consists of a spontaneously expressed pool of cytokines which serve to coordinate the interactions between different thymic resident cell types as well as play a crucial role in the maintenance of constitutive processes like thymocyte development. This spectrum of cytokines is produced mainly by the stromal connective tissue cells, the TECs, DCs, as well as the thymocytes. Since cytokines are critical for shaping thymocyte development, the altered development of T cells in the adult CD11c-cre;Ezh2^{fl/fl} mice could possibly arise because of a dysregulated cytokine environment. According to the cytokine proteome array performed on the total thymic cell extracts, similar levels of cytokines were expressed in 4-week-old CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice (figure 35), which is consistent with their normal thymocyte development at this age. However, the cytokine environment in 10-week-old CD11c-cre;Ezh2^{fl/fl} mice was vastly different as compared to that of the Ezh2^{fl/fl} mice (figure 35). Comparative analysis indicates that a large number of cytokines were upregulated in the thymus of CD11c-cre;Ezh2^{fl/fl} mice. Although this difference in cytokine expression could be attributed to a change in relative proportion of thymocytes, various cytokines which are known to be produced specifically by the stromal connective tissue cells, the TECs and DCs were also observed to be elevated in the CD11c-cre;Ezh2^{fl/fl} mice. Since Ezh2 has been implicated in the suppression of cytokines in lymphocytes, it is likely that they could also control the expression of various cytokines in the thymic DCs. These cytokines could possibly influence thymocyte development directly or indirectly via the stroma cells and TECs, thereby contributing to the observed phenotype in the CD11c-cre;Ezh2^{fl/fl} mice.

Indeed, we observed that the global level of H3K27me3 was reduced in the Ezh2-deficient thymic DCs (figure 36), suggesting that the overall gene expression pattern may be altered in these cells. As such, it is likely that the dysregulated cytokine environment, and hence the altered thymocyte development, in the thymus of CD11c-cre; Ezh2^{fl/fl} mice arise due to a de-repression of various genes in the thymic DCs in the absence of Ezh2.

Figure 35. Cytokine expression in thymi of 4- and 10-week-old CD11c-cre;Ezh2^{fl/fl} mice. The expression of different cytokines in thymic cell lysates were determined using the cytokine proteome array. Pixel intensity of each cytokine in the immunoblot was determined using ImageJ, and expressed as a fold change of the

CD11c-cre;Ezh2^{fl/fl} mice relative to the Ezh2^{fl/fl} mice (mean $\pm$ s.e.m.). Data is based on 3 independent experiments. 3 mice per group were analyzed. Cell types responsible for the production of the cytokines are indicated. *p<0.05, **p<0.01 (one-tailed Student's *t*-test).

Figure 36. Ezh2-deficient thymic DCs display reduced levels of H3K27me3. (A) Total H3K27me3 levels in thymic DCs from 10-week-old mice were analyzed by intracellular staining and flow cytometry. Histograms were pregated on singlet, live, CD3 ϵ ⁻, CD11c⁺, MHC II⁺ cells. Solid line represents wild type DCs, and dotted line represents Ezh2-deficient DCs. Filled histogram represents isotype control. Data shown is representative of 3 independent experiments. 3 mice per group were analyzed.

3.2.9 Increased susceptibility of CD11c-cre;Ezh2^{fl/fl} mice to psoriasis

Psoriasis is a chronic and inflammatory immune-mediated skin disorder which, although rarely life-threatening, has devastating psycho-social impact on an individual's life. With the discovery of the imiquimod (IMQ)-induced psoriasis model, great advances have been made in the understanding of the etiology of this disease. In particular, the IL17-producing $\gamma\delta$ T cells were found to be the primary driver of psoriatic inflammation (Y. Cai et al., 2011). Intriguingly, a massive expansion of $\gamma\delta$ T cells was observed in the thymus, and to a lesser extent in the peripheral lymphoid organs, of CD11c-cre;Ezh2^{fl/fl} mice. To examine whether these $\gamma\delta$ T cells were functional and contribute to the development of psoriasis, CD11c-cre;Ezh2^{fl/fl} and Ezh2^{fl/fl} mice were treated topically with a low daily dosage of 10mg IMQ cream on the dorsal side of each ear for 4 consecutive days. Measurement of ear thickness revealed that Ezh2^{fl/fl} mice experienced minimal skin inflammation, possibly due to the suboptimal amount of IMQ administered as opposed to the higher dose frequently used to induce psoriatic inflammation (figure 37A). To further support the absence of inflammation in the Ezh2^{fl/fl} mice, the skin-draining lymph nodes were analyzed on day 5. In line with the minimal ear swelling, no significant increase in CD4⁺ and CD8⁺ T cells were observed, although $\gamma\delta$ T cells were increased by approximately 3-fold (figure 37B). However, even at low dose, IMQ was able to trigger significant ear swelling in the CD11c-cre;Ezh2^{fl/fl} mice. Moreover, their skin-draining lymph nodes were found to be enlarged, and the $\gamma\delta$, CD4⁺ and CD8⁺ T cell numbers were significantly higher as compared to the wild type controls. Collectively, these data demonstrate that an exaggerated psoriatic response was observed in the CD11c-cre;Ezh2^{fl/fl} mice, although at this stage it remains elusive whether such difference was a direct result of the Ezh2-deficiency in DCs or could be attributed to the expansion of $\gamma\delta$ T cells, or a combinatorial effect of both.

Figure 37. Increased susceptibility of CD11c-cre;Ezh2^{fl/fl} mice to IMQ-induced psoriasis. (A) 10-week-old mice were treated daily with 10mg IMQ cream on dorsal side of each ear. Ear swelling response was determined daily over a period of 4 days (mean $\pm$ s.e.m.). Data is based on 2 independent experiments. 6 mice per group were analyzed. (B, C) Skin-draining lymph nodes were harvested on day 4. (B) Bar graph indicates absolute numbers of cells in the skin-draining lymph nodes. Data is based on 3 independent experiments. 4 mice per group were analyzed. * $p < 0.05$, ** $p < 0.01$ (two-tailed Student's *t*-test). (C) Flow cytometric analyses of T cells in skin-draining lymph nodes of untreated and IMQ-treated mice. *Left*, dot plots were

pregated on singlet, live cells. *Right*, dot plots were pregated on singlet, live, TCR β^+ , TCR $\gamma\delta^-$ cells. Data shown is representative of 3 independent experiments. 4 mice per group were analyzed. Bar graphs indicate absolute numbers of CD8 $^+$, CD4 $^+$ and $\gamma\delta$ T cells in the skin-draining lymph nodes of untreated or IMQ-treated mice (mean $\pm$ s.e.m.), as determined by flow cytometry. Data is based on 3 independent experiments. 4 mice per group were analyzed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed Student's *t*-test).

(4) Discussion

4.1 Role of Ezh2 in LCs

Ezh2 is a well-established histone methyltransferase which plays a pivotal role in mediating gene silencing through the regulation of chromatin compaction. Apart from histones, only a limited number of non-histone nuclear substrates have been identified to be the direct methylation targets of Ezh2 (Dasgupta, Dermawan, Willard, & Stark, 2015; He et al., 2012; J. M. Lee et al., 2012). Intriguingly, the cytosolic localization and function of Ezh2 was first brought to attention by Su et al. whom describe their importance in regulating ligand-induced actin polymerization and dorsal ruffling in T cells and fibroblasts respectively (Su et al., 2005). Although the exact mechanism remains unknown, it suggests the existence of potential Ezh2 methylation targets in the cytosolic compartment of cells. Indeed, this study demonstrates that Ezh2 participates in the cytoplasmic tri-methylation of an integrin adaptor protein talin1, thereby controlling adhesion dynamics and consequently the integrin-dependent migration of LCs as well as other leukocytes.

4.1.1 Ezh2 regulates integrin-dependent migration of cells

Unlike other DC subsets residing in the periphery which rely predominantly on an integrin-independent actomyosin contraction-mediated movement to reach the lymph nodes, LCs have to first adopt an integrin-independent migration, squeezing through the tight keratinocyte layer in the epidermis, before switching to an integrin-dependent migratory mode in order to adhere to the underlying basement membrane and subsequently transit across them to reach the dermis. In the absence of Ezh2, these LCs experienced defects in the turnover of adhesion structures which consequently promote cell spreading and an arrest in their migration (figure 14). Accordingly, histological analysis of the skin revealed that Ezh2-deficient LCs were frequently trapped on the basement membrane layer, where they spread out extensively, indicating their enhanced adhesiveness to the integrin ligand, laminin, present in the basement membrane due to perturbations in the turnover of adhesion structures (figure 12D and E). Interestingly, this phenomenon was also observed in the case of neutrophils extravasating across the blood vessels during irritant-induced dermatitis (figure 18A), and also for the CNS-infiltrating DCs during experimental autoimmune encephalomyelitis (Gunawan et al., 2015). Since the repertoire of

integrins differ among leukocytes (for example integrin $\alpha 6\beta 1/4$ in LCs, $\alpha 4\beta 1$ and $\alpha v\beta 8$ in DCs, $\alpha M\beta 2$ in neutrophils) (Jain et al., 2010; Price et al., 1997; C. Wang et al., 2002), such regulation exerted by Ezh2 on integrin-dependent migration via talin1 methylation is unlikely to be restricted to specific integrin molecules.

Ezh2 has been implicated in multiple aggressive and metastatic forms of cancers, and previous studies attribute this to their transcriptional silencing properties and the associated loss of tumour suppressors (Zingg et al., 2015). Our study suggests that methylation of talin1 by Ezh2 could be a conserved mechanism regulating adhesion dynamics not only in leukocytes, but also across various cell types (Venkatesan and Wong., unpublished data). In this case, the overexpression or gain of function mutation of Ezh2 may promote the turnover of adhesion structures, thereby enhancing the migratory capacity and hence metastasis of cancer cells. Such changes in adhesive properties may further contribute to the aggressiveness of cancer cells in addition to the transcriptional silencing of tumour suppressor genes.

4.1.2 Ezh2-mediated talin1 methylation regulates turnover of adhesion structures

Talin1 K2454 has been previously identified to be a methylation target of Ezh2 (Gunawan et al., 2015). To decipher if methylation at this site is associated with the observed abnormal adhesion characteristics of Ezh2-deficient BMDCs, wild type or Ezh2-deficient BMDCs were transduced with wild type talin1 or talin1 mutants where K2454 is replaced with either an un-methylatable glutamine (Q) or a methyl-mimicking phenylalanine (F). Indeed, expression of talin1 mutant K2454F was able to rescue the excessive spreading phenotype and enlarged adhesion structures of Ezh2-deficient BMDCs, while expression of talin1 mutant K2454Q was able to convert the wild type BMDCs into Ezh2-deficient DC-like cells (figure 16). In this case, these wild type BMDCs formed enlarged adhesion structures which were connected to actin stress fibres, suggesting that methylation of K2454 is required to disrupt the binding of F-actin to talin1, thereby promoting the disassembly of adhesion structures due to insufficient tension for adhesion maturation. This is consistent with earlier structural analysis of talin1 which indicates that K2454 is located within their actin-binding domain (figure 3C), raising the possibility that addition of bulky methyl groups could physically interfere with F-actin binding.

Moreover, *in vitro* actin co-sedimentation studies revealed that un-methylatable talin1 mutant K2454Q exhibit a higher binding affinity to F-actin than the wild type talin1 or methyl-mimicking talin1 mutant K2454F (figure 3D). The dissociation of talin1 mutant K2454Q from F-actin was also reduced as compared to that for wild type talin1 and talin1 mutant K2454F, as determined by surface plasmon resonance studies (figure 3E). Collectively, these *in vitro* and *in vivo* data provide compelling evidence that methylation of talin1 at K2454 by Ezh2 interferes with the binding of F-actin, therefore promoting the turnover of adhesion structures and as such regulates the integrin-dependent migration of DCs.

4.1.3 Possible existence of other PTMs on talin1 Lys2454

Glutamine (Q), which was used to mimic an un-methylatable residue in this study, is also frequently utilised as an acetyl-mimicking residue (Rothbart et al., 2012). Since wild-type BMDCs expressing the talin1 mutant K2454Q behave similarly as the Ezh2-deficient BMDCs (figure 16), it could be possible that talin1 is also subjected to acetylation on the same lysine residue *in vivo*. In this case, acetylation might enhance the binding affinity of F-actin to talin1, thereby promoting the formation of stable adhesion structures. As such, acetylation and methylation could act as molecular switches to modulate the assembly and disassembly of adhesion structures for the control of adhesion-dependent migration of cells. Such use of a PTM switch for the dynamic and reversible control on cell migration has been frequently reported. However, the existence of acetylation on talin1 K2454 remains to be determined.

4.1.4 Ezh2 deficiency does not affect integrin-independent migration

In the absence of Ezh2, the focal adhesions are unable to disassemble and the surface integrins assume a constitutively active state, resulting in an abnormally adhesive nature of the cells. Even though integrin-independent migration of leukocytes in the interstitial tissue rely on actomyosin contractions instead of integrin-mediated binding (T. Lammermann et al., 2008), it was initially hypothesized that the constitutively activated integrins present on the Ezh2-deficient leukocytes would nonetheless cause their enhanced binding to the integrin ligands encountered in the interstitial tissue, thereby impairing their integrin-independent migration as well. Surprisingly, this was not the case. The migration of dermal DCs to the skin-draining

lymph nodes during steady state and upon oxazolone (figure 4C), DNTB (figure 26) or FITC painting (Gunawan et al., 2015) were comparable between the CD11c-cre;Ezh2^{f/f} and Ezh2^{f/f} mice, suggesting that Ezh2-deficient leukocytes do not experience defect in their integrin-independent migration. This is likely due to the limited integrin repertoire expressed on the leukocyte surfaces, which restricted the diversity of integrin ligands which can be recognized by them. The interstitial tissue is composed mainly of collagens, which are known to bind to integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$ (Knight et al., 2000). However, these integrin receptors are not known to be expressed on the leukocytes. As such, although the integrins adopt a constitutively activated state on the Ezh2-deficient leukocytes, the absence of collagen-binding integrins results in their intact integrin-independent migration in the interstitial tissue.

4.1.5 Age-dependent accumulation of Ezh2-deficient epidermal LCs

In the CD11c-cre;Ezh2^{f/f} mice, an accumulation of steady state LCs was observed in the epidermis, which became more evident with age (figure 5). Since Ezh2 does not control the steady state proliferation nor death rate of LCs (figure 6 and 7), it is likely that such accumulation occurs over time due to the impaired steady state migration of the Ezh2-deficient LCs. This, however, raises the question of how the homeostatic control of LC numbers is achieved in the epidermis. Despite the importance of maintaining constant DC numbers in the peripheral lymphoid and non-lymphoid organs to ensure a balance between protective and pathogenic immune response, very little is known with regards to how this occurs. A study by Hochweller et al. has demonstrated that this is an active process intricately regulated by the size of the DC compartment. In the event of DC loss, a feedback mechanism will be triggered to replenish the DCs through the recruitment and differentiation of pre-cDCs (Hochweller et al., 2009). However, the Ezh2-deficient LCs continue to replenish themselves despite their steady state numbers remaining constant due to their impaired migration. From the histological analysis, the Ezh2-deficient LCs were frequently seen to have dislodged themselves from their steady state position, away from the surrounding keratinocytes, and line themselves up along the basement membrane layer which they were unable to cross (figure 12E). It could be likely that since they have already moved away from their original position, an empty epidermal compartment was created, which eventually signal the existing LCs or their

precursors to proliferate or differentiate to fill up the available space in the epidermis. Hence, the surrounding keratinocytes might be implicated in the feedback loop to sense and control LC numbers. Although it remains the possibility that Ezh2 is involved in the regulation of such feedback mechanism which maintains the homeostatic LC numbers, analysis of DCs in other organs such as the spleen revealed that their steady state numbers were unaffected by Ezh2 deficiency (figure 27A). Thus it is presumed that Ezh2 is unlikely to govern the homeostasis of DC/ LC numbers.

4.1.6 LCs induce tolerance against contact hypersensitivity

To date, the role of LCs in inducing tolerance or immunogenicity remains controversial. Even though they were once believed to play an essential role in mediating CHS response, recent observations suggested their dispensability during disease pathogenesis (Fukunaga, Khaskhely, Sreevidya, Byrne, & Ullrich, 2008). Capitalizing on the impaired migration of Ezh2-deficient LCs but intact migration of Ezh2-deficient dermal DCs to the skin-draining lymph nodes, the magnitude of CHS response was assessed to investigate the importance of LCs during sensitization phase of CHS. Indeed, in line with recent studies, the CHS response was indistinguishable between the CD11c-cre;Ezh2^{ff} and Ezh2^{ff} mice, suggesting the functional redundancy of LCs (figure 20). However, LCs are required for conferring protection against CHS upon tolerization with low dose of DNTB (figure 21). In this case, they are required to carry the antigens from the skin to the draining lymph nodes, where they activate the Tregs for suppression of the cytotoxic T cell response upon subsequent sensitization with DNFB (figure 23). Interestingly, at higher tolerizing dose of DNTB, tolerance was restored in the CD11c-cre;Ezh2^{ff} mice (figure 25A), suggesting the presence of additional mechanism which circumvents the migration defect of Ezh2-deficient LCs. A previous study by Noordegraaf et al. addresses the possibility of enhanced antigen dissemination into the dermis at higher hapten dose, thereby affecting the cutaneous DC subsets involved in the immune response (Noordegraaf et al., 2010). Indeed, an increase in arrival of Langerin⁺ dermal DCs was observed in the skin-draining lymph nodes only upon application of 1% DNTB but not 0.1% DNTB, and these DCs were able to restore the activation of Tregs in CD11c-cre;Ezh2^{ff} mice (figure 25C and 26). While Langerin⁺ dermal DCs were able to move to the lymph nodes at higher DNTB dose, the

Langerin⁺ dermal DCs were not observed to increase in numbers at the same dosage. This could be due to their relatively low cell numbers in comparison to the Langerin⁻ dermal DCs, which reduced their chances of antigen uptake in the dermis. Alternatively, this might reflect the functional specialization of different DC subsets in the skin. Since DCs are known to be heterogeneous, the tolerizing function could be characteristic of LCs and Langerin⁻ dermal DCs, thereby contributing to their involvement in Tregs activation. As such, it may be a feasible strategy to target these 2 cutaneous DC subsets for the development of tolerogenic treatments against various allergic diseases in future.

4.2 Role of Ezh2 in thymic DCs

Ezh2 is known to play an essential role in establishing the epigenetic program of T cells, thereby regulating various processes ranging from the maintenance of regulatory T cell identity (DuPage et al., 2015) to the control of CD4⁺ T cell differentiation (Tumes et al., 2013). Interestingly, apart from the well-understood T cell-intrinsic requirement for Ezh2, this study identified the contribution of Ezh2 in thymic DCs on the development of T cells. The specific deletion of Ezh2 in DCs promotes accelerated thymic atrophy and influences the $\alpha\beta/\gamma\delta$ lineage decision. Although the exact mechanism by which Ezh2 in DCs regulates T cell development remains elusive, current observations suggest that this potentially could be attributed to a dysregulated production of cytokines by the Ezh2-deficient DCs, thereby modulating the thymic microenvironment for thymopoiesis.

4.2.1 DCs regulate early thymocyte development

The current dogma suggests that thymic DCs are critically involved in the presentation of self antigens in the medulla to facilitate intrathymic negative selection of thymocytes, thereby playing a pivotal role in mediating central tolerance for the elimination of self-reactive T cells. However, the impaired thymocyte development observed in the CD11c-cre;Ezh2^{fl/fl} mice was not due to defects in the selection process, as reflected by their comparable expression of CD69 and Nur77 (figure 34). Notably, in the CD11c-cre;Ezh2^{fl/fl} mice, T cell development was affected as early as DN1 stage (figure 29A), which precedes the onset of thymocyte selection. Moreover the commitment of thymocytes to the $\gamma\delta$ lineage was also increased (figure 29), thus indicating the involvement of thymic DCs beyond negative selection during T cell development.

Since Ezh2-deficient DCs were previously shown to exhibit an enhanced adhesion to integrin ligands, their distribution in the thymus, which is enriched with various extracellular matrices, was hypothesized to be affected. However, likewise to the wild type DCs, Ezh2-deficient thymic DCs remain localized to the medullary compartment of the thymus (figure 32), suggesting that the defect in T cell development was not a consequence of an aberrant DC distribution. Since the thymus-resident and –homing DCs were derived either intrathymically from common T cell/ DC precursors or extrathymically from precursors which upregulate CD11c

only after their arrival in the thymus (J. Li, Park, Foss, & Goldschneider, 2009), it is likely that their recruitment to the medullary compartment precedes the deletion of *Ezh2*, thereby resulting in the intact distribution of *Ezh2*-deficient DCs in the thymus despite their abnormally adhesive characteristics.

Given the distinct medullary localization and minor population of DCs in the thymus, it is surprising that their deficiency in *Ezh2* could have a profound effect on early T cell development, which takes place predominantly in the cortex. This could suggest an indirect influence exerted by the DCs on the thymocytes, probably orchestrated through a complex interplay of the stroma environment involving the cortical and medullary TECs. However, it remains to be answered how exactly *Ezh2* in DCs regulates the early stage of thymocyte development, and why this defect only arises during adult stage but not in the younger mice.

4.2.2 Altered cytokine environment in the CD11c-cre;*Ezh2*^{ff} mice

T cell development is a non-cell autonomous process involving cytokine inputs from the thymic microenvironment for their intricate regulation and lineage commitment. As a consequence, aberrant expression of cytokines is often associated with altered T cell development and various immune deficiencies, which are summarized below in table 5. According to the cytokine proteome array, the cytokine environment in the thymus of 10-week-old CD11c-cre;*Ezh2*^{ff} mice was considerably different from that of the *Ezh2*^{ff} mice (figure 35), suggesting the correlation between such differential expression of cytokines and the observed T cell developmental defects. Indeed, IL-6 and IL-10, which were significantly increased in the thymus of CD11c-cre;*Ezh2*^{ff} mice (figure 35), have been previously reported to play a role in promoting thymic involution (Sempowski et al., 2000) and $\gamma\delta$ T cell development (Fine, Macosko, Grace, & Narula, 1994) respectively. Hence, the differential development of thymocytes in CD11c-cre;*Ezh2*^{ff} mice is likely to be a collective result of the dysregulated cytokine environment in the thymus.

TECs, which form the main component of the thymic stroma, represent the principal source of cytokines in thymus. Interestingly, the deletion of *Ezh2*, which is restricted to the CD11c-expressing DCs comprising less than 1% of the total thymus population, is able to trigger widespread effects in the thymic environment. *Ezh2* is an epigenetic repressor frequently implicated in the regulation of cytokine gene

expression through H3K27me3-mediated silencing (Koyanagi et al., 2005). Since a global reduction in H3K27me3 level was observed in the Ezh2-deficient thymic DCs (figure 36), it is speculated that Ezh2 controls the expression of specific cytokines in the thymic DCs, which in turn act on other thymic-resident cell types to influence their production of cytokines, ultimately leading to an amplification of the effects brought about by the minor DC population. This ability of thymic cytokines to mutually regulate each other's production has been previously reviewed by Yarilin and Belyakov (figure 38) (Yarilin & Belyakov, 2004). To further support this hypothesis, Ezh2-deficient BMDCs were shown to transiently overexpress IL-23, IL-12p40, IL-13 and IL-2 upon stimulation with LPS (Yeong, unpublished data). These cytokines were also observed to be upregulated in the thymus of the CD11c-cre;Ezh2^{ff} mice, although not statistically significant. Unlike in the periphery where cytokine secretion usually depends on cell activation by various stimuli, cytokines are constitutively produced in the thymus due to the nature of the processes they are regulating (Yarilin & Belyakov, 2004). Hence, it may be possible that the transient overexpression of cytokines seen in the BMDCs *in vitro* can be constitutively observed in the Ezh2-deficient thymic DCs. However, most of the differentially expressed DC-specific cytokines detected in the thymus of CD11c-cre;Ezh2^{ff} mice were not statistically significant, probably due to the minor population of DCs in the thymus. To better understand and verify these differences between the control and Ezh2-deficient thymic DCs, it will be useful to perform RNA-sequencing analysis on these cells in future.

Cytokine	Overexpression (+) / Knockout (-)	Phenotype	Source	References
BLC	+	-Proliferation of autologous thymic CD4 ⁺ T cells	DCs	(Sato et al., 2004)
C5a	-	-Reduced apoptosis of thymocytes during sepsis		(Guo et al., 2000)
CCL2	+	-Increased pDCs -Impaired T cell development	TECs, Endothelial cells	(Cedile et al., 2014)
CCL4	-	-Accumulation of thymic Tregs		(Kroetz & Deepe, 2011)

CXCL12	+	-Allows Notch-dependent differentiation of DN3 thymocytes in absence of stromal cells		(Janas et al., 2010)
IL-1	+	-Reduced thymocytes apoptosis		(McConkey, Hartzell, Chow, Orrenius, & Jondal, 1990)
IL-10	+	-Increased $\gamma\delta$ T cells in murine fetal thymus		(Fine et al., 1994)
IL-12p40	-	-Impairs negative selection	APCs/ stroma cells	(Ludviksson, Ehrhardt, & Strober, 1999)
IL-12p70	-	-Accelerated thymic involution -DN1 arrest	DCs	(L. Li et al., 2004)
IL-2	+	-Tregs development	DCs	(Weist, Kurd, Boussier, Chan, & Robey, 2015)
IL-22	-	-Impair thymic recovery	ILCs	(Dudakov et al., 2012)
IL-23	-	-Reduced thymocytes apoptosis -Perturbed negative selection	DCs	(H. Li et al., 2014)
IL-4	+	-Increased memory-like CD8 ⁺ T cells	iNKT	(Y. J. Lee, Holzapfel, Zhu, Jameson, & Hogquist, 2013)
IL-6	+	-Induced thymic atrophy	TECs	(Lynch et al., 2009)
IL-7	-	-Reduced $\alpha\beta$ and $\gamma\delta$ T cells -Increased proportion SP	TECs	(Shitara et al., 2013)
	+	-Coordinate proliferation,		(Boudil et

		differentiation and <i>Tcra</i> recombination during β - selection	al., 2015)
TNFα	+	-Promotes release of thymocytes	(Perez et al., 2012)

Table 5. Cytokines important for T cell development in the thymus.

Figure 38. Cytokine network in the thymus. Schematic diagram depicting the inter-relationship governing cytokine production in the thymus. Cytokines with known functions during T cell development are indicated. Cytokines whose expression were found to be significantly increased in the thymus of CD11c-cre;Ezh2^{f/f} mice according to the cytokine proteome array are labelled in red. Adapted from Yarilin and Belyakov, 2004.

4.2.3 Accelerated immunosenscence in CD11c-cre;Ezh2^{f/f} mice

In addition to the accelerated thymic involution in the CD11c-cre;Ezh2^{f/f} mice, an age-dependent accumulation of effector memory and central memory T cells were observed in their secondary lymphoid organs like the spleen and lymph nodes (figure 31). Such decline in thymic output and the consequential accumulation of peripheral memory-like T cells strikingly mirror the immune system during senescence, hence suggesting that the CD11c-cre;Ezh2^{f/f} mice are undergoing an accelerated immune ageing process. It is likely that such homeostatic expansion of memory T cells through clonal expansion serves as a compensatory mechanism to maintain the T

cell numbers in the periphery. However, in aged mice, these memory T cells have a reduced diversity and proliferative capacity as compared to the naïve T cells, thus compromising the quality of their immune response and increasing the susceptibility of the mice to infections such as influenza (Yager et al., 2008). As such, it will be worth exploring whether the expanding memory T cell population in the CD11c-cre;Ezh2^{ff} mice are less efficient, and if this translates to a declining immunity through the use of an influenza virus infection model.

4.2.4 Increased susceptibility of CD11c-cre;Ezh2^{ff} mice to psoriasis

Psoriasis is an inflammatory autoimmune disease characterized by the formation of psoriasiform plaques. Much success has been yielded in the development of anti-psoriatic therapies through targeting of the IL-23 and IL-17 pathway (Y. Cai et al., 2011; Kopp et al., 2015), thus demonstrating the importance of DCs and $\gamma\delta$ T cells in disease pathogenesis. Using a low dose IMQ-induced psoriatic model, an exaggerated ear swelling response accompanied by the expansion of various T cell subsets in the skin-draining lymph nodes were observed in the CD11c-cre;Ezh2^{ff} mice, whereas the low dose IMQ treatment had almost negligible effect on the Ezh2^{ff} mice (figure 37). This finding indicates the increased susceptibility of the CD11c-cre;Ezh2^{ff} mice towards psoriatic inflammation. Since $\gamma\delta$ T cells, the key mediator of psoriasis, were increased during steady state in the CD11c-cre;Ezh2^{ff} mice, it is tempting to speculate that expansion of this population is responsible for the predisposition of these mice to psoriasis. However, it remains to be determined whether these $\gamma\delta$ T cells found in the CD11c-cre;Ezh2^{ff} mice are functional, and if they are able to infiltrate the dermis and secrete IL-17 which plays a central role in disease pathogenesis. In addition to $\gamma\delta$ T cells, DC-derived IL-23 has also been clearly implicated in the initiation of psoriasis (Wohn et al., 2013). In fact, IL-23 is responsible for triggering the IL-17/ IL-22 cytokine milieu by the $\gamma\delta$ T cells, thereby initiating the inflammatory cascade which leads to full-blown psoriasis (figure 39). As aforementioned, Ezh2-deficient BMDCs were observed to transiently overexpress IL-23 upon LPS stimulation *in vitro*, suggesting the possibility that dermal DCs *in vivo* could likewise experience an over-production of IL-23 upon IMQ treatment, consequently evoking the downstream expansion of IL-17- and IL-22-producing $\gamma\delta$ T cells. Hence, such overexpression of IL-23 by the Ezh2-deficient DCs, together with

the accumulation of $\gamma\delta$ T cells in the periphery of CD11c-cre;Ezh2^{ff} mice, might act synergistically to promote the onset of psoriasis even upon treatment with low dosage of IMQ which do not usually cause any overt inflammation in the control Ezh2^{ff} mice. However, the exact mechanism underlying the exaggerated psoriatic inflammation in the CD11c-cre;Ezh2^{ff} mice remains to be fully elucidated.

Figure 39. Inflammatory cascade during psoriasis. During psoriatic inflammation, activated skin DCs release IL-23, which mediates the activation of $\gamma\delta$ T cells to induce their production of IL-17, IL-22 and other pro-inflammatory cytokines. These cytokines trigger various downstream responses including the recruitment of neutrophils and the further recruitment of $\gamma\delta$ T cells, as such causing the inflammation to escalate into a full blown psoriasis. Adapted from Becher and Pantelyushin, 2012.

(5) Conclusion

In conclusion, this study has demonstrated that Ezh2 is able to regulate integrin-dependent migration of leukocytes through direct methylation of a novel cytosolic substrate, talin1, at K2454. Methylation disrupts the binding of F-actin to talin1, thereby promoting the turnover of adhesion structures which is critically required for the integrin-dependent migration of leukocytes as they pass through a two-dimensional tissue barrier. Using LCs as a model system, Ezh2 deficiency was shown to impair their transmigration across the basement membrane during both steady state and upon activation. Such migration defect arises due to the enhanced adhesion of LCs onto laminin, an integrin ligand present in the basement membrane, in the absence of Ezh2-mediated talin1 methylation and hence adhesion structure turnover. As a result of their impaired migration, mice harbouring the Ezh2-deficient LCs were unable to be protected from the development of contact hypersensitivity response despite tolerance induction through the application of a low dose innocuous hapten DNTB, whereas tolerance can be induced in mice with wild type LCs and Ezh2-deficient dermal DCs.

In addition to the governing of adhesion structure dynamics, DC-intrinsic Ezh2 was also found to influence early T cell development in the adult thymus. In the presence of Ezh2-deficient thymic DCs, the $\gamma\delta$ and CD8⁺ T cell lineage were favoured, and the DN thymocytes experience a developmental block at DN1 which consequently triggered the premature involution of thymus, and an accelerated immunosenescence in the peripheral T cell compartment. Although the underlying mechanism has yet to be elucidated, the CD11c-cre;Ezh2^{f/f} mice are shown to be predisposed to psoriatic inflammation, presumably through the accumulation of peripheral $\gamma\delta$ T cells attributed to the Ezh2-deficient thymic DCs.

Collectively, these observations suggest that Ezh2 deficiency in DCs enhances the susceptibility of mice to various allergic and autoimmune diseases via novel mechanisms. Despite being frequently associated with pro-inflammatory gene silencing, Ezh2 was found to suppress such inflammatory states in mice through the regulation of leukocyte migration and T cell development. In view of these observations, it may be valuable to harness the immunosuppressive functions of Ezh2 in DCs for the future development of treatments for allergies and autoimmunity.

(6) References

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(7) Appendix

Experimental design

Figure	Panel	No of independent expt	No of mice	
4	A	3	3	6 fields per group
	B	3	7	
	C	3	6	
5	A	2 (2 weeks, 16 weeks) 3 (6-8 weeks)	3-4	
	B	2 (2 weeks, 16 weeks) 3 (6-8 weeks)	3-4	6 fields per group
6	A	1	3	
	B	1	3	
7	A	3	4	
	B	3	5	
8		3	3	
9	A	3	3	
	B	3	3	
10		3	3	
11	A	4	4	
	B	7 (LCs), 3 (migratory DCs)	7 (LCs), 3 (migratory DCs)	
12	A	2	3	6 fields per group
	B	2	3	
	C	2	3	
	D	3	3	
	E	3	3	
	F	3	3	>200 cells from 3 mice
13		5	5	

14	A	4	4	>40 cells from 4 mice
	B	4	4	
	C	4	4	
	D	4	4	
15	A	6	6	
	B	3	3	
	C	3	3	
	D	3	3	
16	A	3	3	
	B	3	3	>30 cells from 3 mice
	C	2	3	>100 cells from 3 mice
17	A	2	2	
	B	2	2	
	C	3	3	
18	A	2	2	
	B	4	4	
	C	2	2	100 cells from 2 mice
19	A	3	3	
	B	3	3	
	C	2	2	
	D	4	4	
20		2	8	
21	B	3	9	
	C	1	4	
22	A	2	3	
	B	2	3	6 fields per group
	C	3	6	
23	A	>3	>5	

	B	>3	>6	
	C	>3	>6	
	D	>3	>6	
24		3	3	
25	A	2	>8	
	B	2	2	
	C	3	>5	
	D	3	3	
27	A	3	3-4	
	B	3	4	
28	A	3	3	
	B	3	3	
29	A	3	3-4	
	B	4	7	
30		3	3	
31	A	3	4	
	B	3	4	
	C	3	4	
	D	3	4	
32		3	3	
33	A	4	4	
	B	3	3	
34	A	3	4	
	B	3	4	
	C	3	3	
	D	3	3	
	E	3	3	
35		3	3	
36		3	3	
37	A	2	6	
	B	3	4	
	C	3	4	

(8) Author's publications

Gunawan, M.*, N. Venkatesan*, **J. T. Loh***, J. F. Wong, H. Berger, W. H. Neo, L. Y. Li, M. K. La Win, Y. H. Yau, T. Guo, P. C. See, S. Yamazaki, K. C. Chin, A. R. Gingras, S. G. Shochat, L. G. Ng, S. K. Sze, F. Ginhoux and I. H. Su (2015). "The methyltransferase Ezh2 controls cell adhesion and migration through direct methylation of the extranuclear regulatory protein talin." Nat Immunol **16**(5): 505-516. (*equal contribution)

Loh, J. T., & Su, I. H. (2016). "Post-translational modification-regulated leukocyte adhesion and migration." *Oncotarget*. doi: 10.18632/oncotarget.8135

Loh, J.T., Gunawan, M., & Su, I.H. "Ear inflammation and whole-mount ear staining." (Manuscript under revision)

J. T. Loh, B. Janela, Gunawan, M., F. Ginhoux, I. H. Su. "Ezh2 controls Langerhans cell-mediated tolerance induction in allergic contact dermatitis." (Manuscript under preparation)

(9) Posters, awards, invited talks

Posters

3rd Advanced Singaporean Immunology PhD Student Retreat

6th International Singapore Symposium for Immunology

44th Australasian Society for Immunology Annual Scientific Meeting

6th Congress of the Federation of Immunological Societies of Asia Oceania

Invited talks

International Workshop on Langerhans Cells 2013

7th Asia-Pacific Organization for Cell Biology

4th Singaporean Immunology PhD Student Retreat

8th International Singapore Symposium of Immunology

Awards

Huidfonds Travel Award (International Workshop on Langerhans Cells 2013)