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Langerhans Cells in Autoimmunity:

New Perspectives in Function and Homeostasis

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy in

Molecular, Cellular and Integrative Physiology

by

Miguel-Angel Gutierrez, Jr

2018

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ABSTRACT OF THE DISSERTATION

Langerhans Cells in Autoimmunity: New Perspectives in Function and Homeostasis

by

Miguel-Angel Gutierrez, Jr

Doctor of Philosophy in Molecular, Cellular and Integrative Physiology

University of California, Los Angeles, 2017

Professor Ram Raj Singh, Chair

Langerhans cells (LC) are the premier antigen presenting cells on epithelial surfaces. The key to LCs' functions is their residence in peripheral tissues as well as their capacity to migrate to local draining lymphoid organs. The present dissertation's goal is to elucidate the specific roles LCs play in the context of autoimmunity, by employing a mouse model genetically prone to develop multi-organ autoimmune disease, in which LCs can be traced and selectively depleted *in vivo*.

Previous studies have elucidated LCs' ability to induce tolerance against skin-specific self-antigen and as well as to protect against the development of autoimmune skin inflammation. However, LCs' role in other, non-skin, organs where they are present, such as in cornea on eye surface and in gastric and genital mucosa, remains largely unknown. We found that LCs were altered in distribution and density in the cornea of autoimmune-prone mice. Importantly, a

selective depletion of LCs led to acceleration of corneal inflammation, along with increased T cell activation and proliferation in eye-draining lymph nodes. These data allowed us to show for the first time that LCs play a protective role against corneal autoimmune disease, potentially through inhibition of T cell activation in a tissue-specific manner.

Homeostatic migration of LCs from their tissue of residence to local draining lymph nodes is a hallmark of LC biology, however, the underlying mechanisms are largely unclear. We studied mechanism of LCs migration using models of autoimmune dermatitis, where LC migration was previously shown to be defective prior to the onset of clinical disease. We demonstrate that skin-resident dendritic epidermal T cells are reduced in the epidermis of these autoimmune-prone mice, and that a glycolipid that ameliorates skin inflammation restores DETCs and LC migration. This rescue of LC migration in autoimmune mice by skin-resident dendritic epidermal T cells is mediated, at least in part, via CD40–CD40L signaling. Such ‘local’ control of migratory behavior of tissue-resident dendritic cells can regulate systemic immune response in a tissue-specific manner, which has important implications for protection against inflammation, autoimmune diseases and graft rejection, and vaccination against cancers and infections.

The dissertation of Miguel-Angel Gutierrez, Jr is approved.

Kristina Bostrom

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University of California, Los Angeles

2018

Dedication

To my wife, Sana Ahmed, whose love and support served as north star and sextant in this grand voyage. Even in my most fantastic of dreams I couldn't imagined a more perfect partner for this journey.

To my mother Frances Franco, whose suffering and determination inspired me to study autoimmune diseases. Your strength in the face of adversity is and forever will be wind my sails.

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List of Acronyms

LC	Langerhans Cells
DC	Dendritic Cells
APC	Antigen presenting cell
MHC-II	Mayor histocompatibility complex
CD207	Cluster of differentiation 207
TRM	Tissue resident memory cell
Ag	Antigens
cDCs	Classical Dendritic cell
$Lang^+$ dDC	Langerin positive dermal dendritic cell
Flt3L	FMS-like tyrosine kinase 3 ligands
TGF-β	Transforming growth factor-β
T_{reg}	T regulatory cells
DNTB	2,4-dinitrothiocyanobenzene
DNFB	dinitrofluorobenzene
$CD8^+$ T cells	Cluster of differentiation 8 positive T cells
huLang-DTA	human langerin-diphtheria toxin A
CHS	Contact hypersensitivity
huDTR	human diphtheria toxin receptor
C. albicans	Candida albincans
MRL	Murphy Roth large
lpr	lymphoproliferative
SLE	Systemic lupus erythematosus

Dsg3	Desmoglein 3
CD103	Cluster of differentiation 103
EpCAM	Epithelial cell adhesion molecule
CXCR4	C-X-C Chemokine receptor 4
CCR7	C-C Chemokine receptor 7
CCL19	C-C Chemokine ligand 19
CCL21	C-C Chemokine ligand 21
IL-1β	Interleukin 1β
IL-18	Interleukin 18
TNF-α	Tumor necrotic factor α
MyD88	Myeloid differentiation primary response 88
Tgfbr1	Transforming growth factor receptor 1
NOD $\delta^{-/-}$	Non-obese diabetic mice T cell receptor delta knock out
eGFP	enhanced green fluorescent protein
CD62L	Cluster of differentiation 62 ligand
KS	Keratoconjunctivitis sicca
H&E	Hematoxylin and eosinophil
TCR	T cell receptor
DETC	Dendritic epidermal T cells
CD40L	cluster of differentiation ligand
iNKT	invariant natural killer T cells
αGC	α-galactosylceramide
CLN	cutaneous lymph node
ITGAE	Integrin, alfa E

CTL	Cytotoxic T lymphocytes
Th 17 cells	T helper 17 cells
IL-17	Interleukin 17
IL-10	Interleukin 10
CAR-JAML	Coxsackie adenovirus receptor-Junctional Adhesion Molecule-Like pathway

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The person I think the most for this achievement is my advisor and mentor Dr. Ram Raj Singh. I arrived at his laboratory after switching fields, with a great interest in studying autoimmunity. Dr, Singh recognized in me such interest and not only did he open the doors of his lab but nurtured such desire and passion. Through every grant proposal, abstract submission, presentation and the writing of this dissertation, Dr. Singh shared with me knowledge and wisdom form his personal and professional life story, for which I will forever be grateful. Also, I will forever be grateful for his generous counsel every time I had questions about my mother's illness. You shared your expert opinions on the case, even though she was not your patient, and for that you will have my family's gratitude and as well as my own.

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Dr. Ram R. Singh, principal investigator supervised the research included in the dissertations. He also provided guidance and critical comments after reviewing each draft of this thesis.

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Chapter 1-

Langerhans Cells: sentries at the wall

Introduction

First identified by Paul Langerhans in the 19th century, Langerhans cells (LCs) are the primary antigen presenting cells (APC) that express major histocompatibility complex (MHC) class II under non-inflammatory conditions in the epidermis and other epithelial layers [1, 2]. Due to their strategic location at the interface between organism and the environment, LCs are regarded as crucial *sentinels at the wall*s, where they have easy access to epithelial pathogens, commensal organisms, allergens, epidermal self-antigens and skin contact sensitizers [3]. In the epidermis, LCs live in close contact with keratinocytes, the main cellular component of the epidermis, as well as skin lymphocytes such as tissue resident memory T cells (TRM) and regulatory T cells (Treg).

Between the 1970s to the early 1990s, work by Ralph Steinman and others demonstrated that LCs as well as immature dendritic cells (DCs) were able to up take antigens (Ag) and efficiently process it, further solidifying LCs' role as crucial immunological sentries in the periphery [4]. For a while LCs have been regarded as the prototypic migratory tissue resident DC, and their functions in secondary lymphoid organs have been the focus of investigations. Yet, for all that is known about LC biology, their close resemblance to other migratory DC types in the skin and other organs has mystified the specific role that LCs play in epithelial tissue biology. While in the epidermis LCs reign supreme as the only APC, in the dermis migrating LCs are accompanied by two types of classical (cDCs), including cDC1 that are characterized by expression of CD103 and Langerin (Lang, CD207) and lack of CD11b. These $CD11c^{+}CD103^{+}Lang^{+}CD11b^{-}$ cells have been implicated in the pathogenesis of allergy and autoimmunity [3, 5-9] . Because of their Lang expression, these dermal-resident DCs were long confused for LCs, and a major confounding

factor in earlier studies of LCs. These dermal DCs are now referred to as $Lang^+$ dermal DCs ($Lang^+$ dDC).

LCs differ from cDC, however, on their ontogeny. LCs derive from primitive myeloid progenitor cells that originate from, at first, yolk sack, and subsequently the fetal liver [10]. While cDCs originate from circulating bone marrow precursors, depend on stimulation by FMS-like tyrosine kinase 3 ligand (Flt3L), and lack capacity for self-renewal, LCs precursors arrive at tissue of residence during embryonic development. The self-renewal nature of LCs in peripheral tissue makes them resemble tissue macrophages, such as microglia, rather than cDCs. Indeed, LCs depend on IL-34 stimulation from neighboring keratinocytes as well as signaling through transforming growth factor-β (TGF-β) [11-14]. However, the idea of categorizing LCs as a subset of resident tissue macrophages encounters the crucial functional distinction: migration to regional lymphoid organs, a feature that aligns LCs with cDCs, rather than with microglia, alveolar macrophages or hepatic Kupffer cells. Therefore, LC migration is a feature central to their nature as *sentries* at the interface between environment and the organism.

LCs in autoimmunity and tolerance

LC involvement in peripheral tolerance and protection against autoimmunity has been the focus of research by us and others. The working paradigm states that presentation of self-antigen by LCs or cDCs in a non-inflammatory environment will lead to anergy and/or apoptosis of self-reactive lymphocytes, thus preventing autoimmunity [15]. Promotion of tolerance is by no means an exclusive feature of LCs, and classical antigen targeting experiments have clearly established all DCs' capacity for induction of peripheral tolerance [16-18]. Dermal DCs have been found to also promote tolerance, while all migratory DCs share an immune suppressive gene-expression

profile [19-21]. LCs' role in tolerance, on the other hand, is a story long unfolding. *In vitro* studies showed that resting human LCs can activate and promote proliferation of skin-resident T_{reg} cells, while activated LCs selectively induce proliferation of skin-resident memory cells [22]. Furthermore, in vivo targeting of self-antigens, but not of foreign antigens, to LCs promotes proliferation of T_{reg} cells [23, 24]. There is evidence that LCs promote suppressive responses against infections such as *Leishmania major*, potentially through expansion of T_{reg} cells [25]. Additionally, mice treated with specific doses of innocuous hapten 2,4-dinitrothiocyanobenzene (DNTB) had a reduced response to subsequent exposure to strong hapten dinitrofluorobenzene (DNFB) in a mouse model for allergic contact dermatitis [26]. This mechanism of tolerization by weak hapten to a subsequent strong sensitizer was LC-dependent and involved $CD8^+$ T cell tolerance and T_{reg} cell activation. Targeted *in vivo* ablation studies in contact hypersensitivity (CHS), a mouse model of human allergic contact dermatitis, gave further evidence of LCs credentials as an immune modulator. Mouse models where LCs were constitutively (by transgenic expression of huLang-DTA) or conditionally ablated (by expression of huLang-DTR and timed injections of human diphtheria toxin A) showed enhanced CHS responses to various degrees, and cells isolated form lymph nodes of huLang-DTA mice promoted overt CHS responses after adoptive transfers [27-29]. Lastly, there was a similarly enhanced delayed-type hypersensitivity response to *C. albicans* in huLang-DTA mice as well as in huLang-DTA naïve mice after intradermal *C. albicans* injections [30, 31]. Collectively, these evidences support that LCs may be a modulator of the "base" cellular immune response to sensitizers. However, an immune response is still elicited even in the presence of LCs, and thus, an intact LC compartment is not able to override such response. This is an issue echoed by LC depletion studies in healthy mouse models conducted by us and others, where complete deletion of LCs

does not lead to development of autoimmunity [3]. This is because autoimmunity is dependent on other factors, including genetics. Thus, a LC depletion model would most likely require an autoimmune genetic background in order to test the contribution of LCs in the pathogenesis of autoimmunity.

Evidence of the potential role of DCs in autoimmunity stems from DC depletion studies in the context of the lupus-prone mouse strain MRL/MpJ-*Fas*$^{lpr/lpr}$ (MRL-*lpr*) [32, 33]. Murphy Roth Large (MRL) is a mouse strain that develops systemic lupus erythematosus (SLE)-like phenotype that resembles human disease in both pathology and progression. The MRL genetic background and the *lpr* mutation, which is a null mutation in the *fas* gene, accelerates the development of the SLE phenotype. In this study, a constitutive depletion of all CD11c^{+} in MRL-*lpr* mice leads to an annulment of organ pathology associated with lupus. This suggested that, among the greatly diverse DC compartment, some cells may be involved in driving the tissue pathology of lupus and some may not. In the context of skin lupus, a chronic autoimmune condition, suspicion of LC involvement comes from immunohistochemistry analysis of skin lesions from patients with SLE, where density and distributions of LCs was altered, in some instances increased and on others decreased [34-36]. Another analysis of skin lesions showed an evidence of migration of LC into lupus skin lesions preceding infiltration of lymphocytes [37]. More recently, the direct evidence for the role that LCs play in the maintenance of tolerance to skin-specific antigen was brought forth by members of our group [38]. First, it was observed that preclinical MRL-*lpr* mice exhibit vigorous reactivity of lymphocytes to skin antigen desmoglein 3 (Dsg3), indicating that there is a breakdown of tolerance preceding the onset of tissue pathology in a genetically autoimmune-prone background. In lymphocytes from the lymph node of mice that were topically (epicutaneously) exposed to a Dsg3 emulsion, the vigorous response

observed before was no longer there, indicating that lymphocytes have been tolerized. To test if such epicutaneous tolerance induction to skin antigen Dsg3 is mediated via Dsg3-loaded LCs migrating from skin to skin-draining lymph node, we depleted LCs in MRL-*lpr* mice that express diphtheria toxin receptor (DTR) driven by the Lang promoter (Lang-DTR). This model allows the *in vivo* specific removal of LCs without a possible compensatory mechanism arising from the life-long absence of LCs in the constitutive knockout mice. Furthermore, the ablation using i.p. diphtheria toxin injections (D Tox) in Lang-DTR mice follows specific time dynamics that render the skin devoid of LCs for about 7-10 days post-injection [7]. Importantly, this dynamics is different from the repopulation dynamics of $Lang^{+}$ dDCs, a bone marrow derived DC that reside in the dermis, and is differentiated from LCs by minimal expression of CD11b and the expression of skin homing receptor CD103 [5, 8, 39]. In the absence of LCs (D Tox injection 10 days before cutaneous application of Dsg3), the in vitro lymphocytic response to Dsg3 was as robust as that of unexposed mice, indicating that the tolerance to skin antigen is dependent on LCs migrating from skin to tolerize Dsg3 specific lymphocytes in skin-draining lymph nodes. Secondly, continuous depletion of LCs by repeated injections of D Tox every 7-10 days led to increased serum levels of autoantibodies against skin antigens but there was no effect on autoantibodies to systemic, non-skin, autoantigens, again suggesting a role of LCs in maintaining tolerance to skin antigens. Lastly, upon continuous depletion of LCs by injection of D Tox every 7-10 days, the onset of lupus skin pathology in MRL-*lpr* mice was accelerated, without changes in the systemic disease. These observations indicate that in the context of autoimmunity, LCs promote organ specific tolerance that, due to as-of-yet unknown factors, breaks down, leading to autoimmune pathology. These results offer a possible explanation for the heterogeneity of multiple-organ involvement in lupus. Furthermore, this provides a framework for the study of

LC biology and tissue specific autoimmunity in organs beyond the skin. For Instance, LCs are present in other epithelial layers such as the gut and genital mucosa as well as the ocular surface [40]. The presence of LCs and Lang^{+}dDCs in the cornea has been characterized and, much like the skin, LCs are exclusive corneal epithelium dwellers while Lang^{+}dDCs are limited to the stroma [41]. Furthermore, the cornea is a tissue affected by keratoconjunctivitis sicca or secondary Sjogren's syndrome, the autoimmune dry eye condition that develops in SLE patients as well as in SLE mouse models such as MRL [42-44]. With the lessons learned from studies on the role of LCs in skin autoimmunity, we hypothesize a role of LCs in the development of autoimmune disease in cornea and other organs that possess LCs.

LC migration

Migration from resident tissues to regional secondary lymph nodes is an important functional feature that distinguishes LCs from tissue resident macrophages. LCs migrate at a slow rate under steady state conditions and at a higher rate under activating conditions. Activation induced migration can be elicited by several stimuli such as UV light exposure and haptens and is dependent on the reduction of E-cadherin, and expression of CXCR4 and EpCAM [45-48]. Activated or mature LCs express chemokine receptors CCR7 which will allow them to follow CCL19 and CCL21 through the dermal lymphatics [49]. Triggering of LC migration under inflammatory conditions is dependent on the coordinated stimulation by IL-1β, IL-18 and TNF-α [50-54]. The nature of LC stimulation by these cytokines is rather unclear, as LC-specific insensitivity to IL-1β and IL-18, or Toll-like receptor 2 stimulation, does not seem to be impaired in commensal or hapten based in vivo migration models [55]. Instead, LC migration is

reduced after protein immunization in keratinocyte-specific MyD88 deficient mice, suggesting that such inflammatory push for LC migration may operate indirectly through keratinocytes [56].

The interaction of LCs and their cellular neighbors in the epidermis has important functional significance. TGF-β1 is an important factor for the development and maintenance of the epidermal cell network. Constitutive absence of TGF-β1, or the absence of components of its signaling pathway, results in the defective development of LCs that goes from a great reduction in numbers to the complete absence of LCs [12, 13, 57-59]. Furthermore, TGF-β1 signaling is required for the retention of LCs in epidermis after development. Conditional ablation of TGF-β receptor 1 gene (*Tgfbr1)* or *Tgfbr2* or other components of the TGF-β signaling pathway leads to spontaneous emigration of LCs from the epidermis to regional lymph nodes [28, 60, 61]. This is mirrored by conditional ablation of *Tgfb1* in LCs, which also results in spontaneous LC migration [28, 62]. On the other hand, a constant signaling through the TGF-β1 pathway causes a failure in homeostatic migration of LCs in steady state or after stimulation [63]. All these evidences suggest that TGF-β1 signaling, either via paracrine or autocrine modality, may be fundamental for the retention of LCs in the epidermis. This also highlights mobility as a feature of LC biology that can be modulated for retention or promotion of LC emigration. For example, exposure of skin to ultraviolet irradiation causes a reduced release of active TFG-β1 locally mediated by keratinocytes, resulting in emigration of LCs from the epidermis [63].

Changes in LC migration within the context of skin pathology have been reported by us and others. As mentioned before, analysis of human skin from lupus patients showed LCs migrating to lesion sites prior to lymphocytic infiltration [37]. Our group has reported that in preclinical MRL mice, there is a retention of LCs in the epidermis with their aberrant distribution, concomitant with a dramatic reduction in the number of LCs in the skin draining lymph nodes

[64]. Like from keratinocytes, epidermal homeostatic stimuli might originate from other sources, such as dendritic epidermal T cells (DETCs), the epidermal resident γδ T cell population [65]. DETCs are known mediators and regulators of epidermal keratinocyte homeostasis and wound healing [66]. Deficiency of γδ T cells in non-obese diabetic mice (NOD $\delta^{-/-}$) induces dermatitis [67], suggesting a role of T cells in preventing skin inflammation. Furthermore, *in vitro* studies have suggested a role of DETCs in activating LCs through cytokine signaling [68]. Lastly, DETCs have been shown to play both pro- and anti-inflammatory roles in CHS [69-72]. Thus, it is possible to suggest that DETCs may play a role in the modulation of LC homeostasis too. Furthermore, it would also be conceivable that changes to the distribution and availability of homeostatic signals in the epidermal environment would change LC functions, such as migration.

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Chapter 2-

A protective role of Langerhans cells in the pathogenesis of autoimmune corneal inflammation

Abstract

Ocular surface inflammation commonly occurs in systemic autoimmune diseases, such as lupus. Mechanisms underlying ocular surface autoimmunity are largely unclear. Previous studies have suggested a role of tissue-resident dendritic cells (DC) in the pathogenesis of specific organ involvement in autoimmune diseases. Here, we evaluated the role of Langerhans cells (LC), prototypic tissue-resident DCs, in the pathogenesis of corneal autoimmunity using the MRL model including MRL-lpr (MRL-Fas$^{lpr/lpr}$) and MRL+/+ (MRL-Fas$^{+/+}$) mice. MRL-lpr mice exhibited corneal inflammation, calcium deposition, scarring, erosion, and leukocyte infiltration. Mild leukocyte infiltration was also seen in MRL+/+ mice that develop a delayed disease. Lang-eGFP (enhanced green fluorescent protein under the langerin promoter) knock-in MRL mice exhibited altered distribution and frequency of LCs in cornea compared to control mice. To directly assess the role of LCs, we depleted LCs by diphtheria toxin injection in Lang-eGFP.DTR knock-in mice. Serial diphtheria toxin injections every 7-10 days in this model leads to continuous LC depletion. LC-depleted MRL-lpr mice had significantly increased corneal lesions compared to LC-intact littermates. Compared to LC-intact mice, LC-depleted mice had increased T-cell proliferation, particularly of $CD8^+$ T-cells, in eye-draining lymph nodes, but not in spleen. Long-term LC-ablation also led to reduced CD62L on T-cells in eye-draining lymph nodes in MRL, but not in C57/Bl6 mice. Such local regulation of T-cells may play a role in immune tolerance locally in a systemic autoimmune-prone background. Thus, LCs play a protective role in corneal autoimmune disease and in modulating local T-cell activation.

Introduction

Keratoconjunctivitis sicca (KS) or secondary Sjogren's syndrome is the most common ocular manifestation of systemic lupus erythematosus (SLE) affecting about 30% of patients. It is also known as dry eye disease in part due to a reduction in aqueous tear production, leading to discomfort and reduction in visual acuity, and in severe cases, it can lead to scarring of the ocular surface [1]. This condition is manifested by inflammation in periocular regions as well as periorbital edema, and anterior and posterior scleritis [2]. The anterior scleritis involves tissues such as conjunctiva, episclera and cornea. Corneal involvement manifests with epitheliopathy, scarring, ulceration, and filamentary keratitis [3], and sometimes leading to corneal erosion, and potentially sight-threatening peripheral ulcerative keratitis [3, 4]. Such corneal involvement may include deposition of immune complexes in the basement membrane of endothelial cells of the limbal vasculature, promoting immune cell infiltration [5]. This phenomenon is of interest since cornea has long been considered an immune-privileged tissue. In this regard, it would be important to determine the role of antigen presenting cells (APCs) to understand mechanisms underlying corneal autoimmune pathology.

The cornea contains dendritic cell (DC) populations as the main APC, which are major cellular initiators and mediators of immune response [6]. Among these are Langerhans cells (LC), the prototypical APC of epithelial layers characterized by the presence of Birbeck granules and the expression of c-type lectin langerin (CD207) [7, 8]. The location and distribution of LCs in the cornea is still subject of study, but the evidence so far points to LCs to be mainly present in the peripheral layer of the corneal epithelium [6, 7, 9]. These epithelial resident LCs do not express EpCAM and express low levels of CD11b, which is in contrast to epidermis-resident LCs that are $EpCAM^{hi}CD11b^{hi}$, but common to epithelial DCs [9]. In vivo confocal studies have detected

morphological changes in the intraepithelial LCs of dry eye patients, suggesting a possible involvement of LCs in the pathogenesis [10, 11]. Additionally, recent *in vivo* evidence has shown that LCs may play a potentially protective role against dry eye associated nerve damage [12]. However, no study has addressed the LC's role in the pathogenesis of corneal inflammation in dry eye disease.

In the present study, we set out to understand the possible role that LCs might play in the pathogenesis of corneal inflammation associated with dry eye disease secondary to SLE in a genetically prone mouse model. We employed the knock-in mouse expressing human Diphtheria toxin receptor-green fluorescent protein (huDTR-GFP) gene cassette under the Langerin promoter [13] which allows for the selective depletion of Langerin expressing cells in vivo, bypassing any form of adaptation or redundancy that may develop in the absence of LCs in constitutively langerin knockout mice. This knock-in mutation was introgressed onto the lupus-prone Murphy Roth Large (MRL) mouse background. We observed a complete depletion of LCs from the corneal epithelium of huDTR-GFP MRL-*lpr* mice up to 7 days after injection with human Diphtheria Toxin A (huDTA). Furthermore, when preclinical MRL mice where repeatedly depleted of LCs over several weeks, there was an acceleration in the development of corneal pathology as compared with non-depleted MRL-*lpr* mice. This acceleration in chronically depleted mice was concomitant with a reduction of *in vivo* naïve T cell phenotype and heightened *in vitro* reactivity of $CD8^+$ T cells. Overall, these data suggest a possible protective role of LCs against autoimmune disease in cornea.

Results

Spontaneous development of corneal disease in SLE mouse model

To better understand the dynamics of cornea pathology in autoimmune mouse model, we compared corneal histology in lupus-prone MRL and healthy control mice and detected leukocytes (CD45-expressing cells) in the cornea. MRL mice spontaneously develop a multi-system autoimmune disease that greatly resembles SLE in humans [14]. Furthermore, MRL model has been widely used in the study of dry eye disease associated with the autoimmune destruction of lachrymal glands [15]. In the current study, we employed MRL mice homozygous for the lymphoproliferation mutation Fas$^{lpr/lpr}$ (MRL-*lpr*) as well as wildtype counterpart MRL*fas*$^{+/+}$ (MRL$^{+/+}$). MRL-*lpr* mice present with lymphadenopathy and lupus-like nephritis, dermatitis and circulating autoantibodies at an earlier age (4-6 months of age) when compared to MRL$^{+/+}$ mice that develop lupus-like disease at 8-10-months of age. H&E, Masson's trichrome and von Kossa stains showed corneal inflammation, fibrosis, calcium deposition, scarring and erosion in MRL-*lpr* mice. Immunohistochemistry showed accumulation of CD45^{+} cells in the basal aspect of the corneal epithelium of MRL-*lpr* and MRL$^{+/+}$ mice as compared to healthy control mice, where the presence of CD45^{+} is more evenly distributed (Figure 1). MRL-*lpr* mice in early stages of disease (between 12-14 weeks of age) had an increased infiltration with CD45^{+} cells in the basal aspect of the corneal epithelium, concomitant to marked stromal edema (thickening). Leukocyte infiltration and corneal changes, including scarring and erosion, are worse at later ages (20-24 weeks). These results indicate that the progression of corneal disease parallels lupus disease in other tissues [14].

Changes in LC distribution in the corneal epithelium of MRL+/+ and MRL-*lpr* mice as compared to healthy control cornea

Recent characterization of DC populations in healthy cornea showed that LCs are the sole langerin expressing cells in the epithelium, while the stroma contains mainly non-LC langerin expressing DCs [9]. Furthermore, these populations are mainly distributed along the paracentral and peripheral regions of the cornea, while the central region of the cornea is relatively devoid of these cell populations. To evaluate the distribution of langerin-expressing cells in autoimmune-prone mice, we harvested corneas from MRL-*lpr* mice, separated epithelium from stroma, and stained each layer using fluorescently labeled antibodies for langerin (CD207), CD11c, and MHCII. Figure 2A shows a representative whole mount fluorescent photomicrograph, where epithelial layer is shown in the top and stroma in the bottom. As expected, most MHCII+ cells in the corneal epithelium were LCs ($CD207^+$ $CD11c^+$ MHC II^+), whereas stroma had mostly other DCs ($CD11c^+$ $CD207^-$ MHC II^+) and macrophages ($CD11c^-$ $CD207^-$ MHC II^+). The stroma did have some DCs that expressed lower levels of Langerin, previously described as $Lang^+$ DCs that are located in the stroma at much reduced numbers and with low expression levels of langerin as compared to epithelium [9].

To determine if LCs and other APCs undergo changes during the development of autoimmune corneal disease, we used mice that express GFP under the langerin promoter in autoimmune-prone (MRL-lpr and MRL+/+) and healthy (C57/Bl6) backgrounds. Representative whole mount photomicrographs of the corneas are shown, with epithelium in the top and stroma in the bottom row (Figure 2B), with Langerin-GFP in green and MHC-II stained cells in red. A relatively more

robust presence of LCs in the epithelium of MRL-*lpr* (left top) as compared to MRL+/+ mice (center top) can be observed. Furthermore, the stroma of MRL-*lpr* mice showed a different distribution of $Lang^+$ DCs as compared to MRL+/+ and B6 mice. These consistent changes in the distribution and density of LCs in the corneal epithelium of MRL-*lpr* mice that develop disease at an early age compared to MRL+/+ mice that develop a delayed disease and healthy B6 mice suggests changes in the population dynamics of LCs as disease progresses, as also reported in the skin [16]. To quantitate differences in the proportions of these cells in relation to autoimmune disease, we stained cells isolated from corneal epithelium and stroma for MHC II and langerin (GFP) expression. As shown in Figure 2C, langerin-expressing as well as non-langerin $MHCII^+$ cells in corneal epithelium and stroma were more in MRL-*lpr* mice than in MRL+/+ mice. These observations suggest qualitative and quantitative changes in LCs and other DCs in animals prone to develop spontaneous autoimmune corneal disease.

Injection of human diphtheria toxin A ablates Langerin expressing cells in cornea

To study the in vivo role that LCs may play in cornea pathology, we employed an inducible and selective ablation system for Langerin expressing cells. For this purpose, we employed MRL-*lpr* mice that express human diphtheria toxin receptor (huDTR) linked to green fluorescent protein (GFP) under the control of langerin promoter [17]. This knock-in mouse strain was generated through crossing the knocked in mutation from the stock B6 strain on to the MRL-*lpr* background for more than 10 generations [18]. In this mouse strain, a single i.p. injection of human diphtheria toxin A depletes all langerin-expressing cells in the skin, including both LC and langerin-expressing dermal DCs ($Lang^+$ dDCs) [8]. Furthermore, the dermis begins to be

repopulated by day 3 post injection with Lang$^+$ dDCs, while the epidermal LCs begin to repopulate after day 14 post injection.

To evaluate the dynamics of LC depletion and repopulation in cornea, we injected female Lang-huDTR-GFP MRL-*lpr* mice with 100µg of huDTA at 6 weeks of age, and harvested corneas 7 days after injection, separated epithelium from stroma and incubated each layer with conjugated anti-MHC II antibodies (Figure 3). The GFP$^+$ population in the epithelium of DT treated mice were still absent at the time of analysis, mirroring what would be expected in skin. In the stroma, we detected a few GFP$^+$ cells, which suggest that the dynamics of Lang$^+$ dDCs in the stroma may resemble that of the dermis, as previously described [8]. This means that there is a window of about 10 days when LCs are absent while Lang$^+$ dDCs are present. This is of importance for the understanding of LC function in vivo since it minimizes the impact that Lang$^+$ dDCs absence may have on any possible phenotype.

Repeated ablation of LCs accelerates the development of corneal pathology in an autoimmune mouse model

To evaluate the effects of LC ablation in the development of SLE associated corneal pathology, we performed a series of LC ablation studies in MRL-*lpr* mice. Animals received 6 weekly i.p. injections of huDTA starting at 6 weeks of age, which is before the development of clinical disease [19]. 7 days after administering the last injection, eyeballs where harvested, fixed and prepared for histology. Figure 4A shows representative photomicrographs of H&E stained tissues of huDTA treated (right) or control mice (left), focusing on the central region of cornea.

The corneal tissue from huDTA treated mice reveals a drastic change in appearance among all three layers (epithelium, stroma and endothelium), as compared to control mice. Such changes include, but are not limited to, the thickening of corneal stroma, possibly due to edema, the thinning and erosion of epithelium, as well as the increased cellularity in the anterior portion of the stroma (or basal region of the epithelium). Masson's trichrome stained sections of cornea further highlighted the significantly increased thickening of stroma in LC-depleted as compared to LC-intact animals (Figure 4B, middle panels, and 4C, left panel).

Calcification in cornea is commonly associated with inflammation in spontaneous autoimmune mouse models such as MRL mice [20]. Calcium depositions, which resemble band keratitis, are characterized by granular layers forming in the anterior aspect of the corneal stroma (in the Bowman's layer in humans) and it typically underlines areas of thinning epithelium. These calcareous formations are considered early signs of corneal inflammation, which precedes areas of neovascularization in latter stages of disease [20]. To evaluate that LC depletion also accelerates calcium deposition, we stained cornea sections with von Kossa stain (Figure 4B, bottom row). PBS-treated control MRL-*lpr* mice that have milder corneal disease (Figure 4A, and 4B, upper and middle panels) showed formations of calcium stained tissue, suggesting that calcium formations represent early lesions during the progression of autoimmune corneal disease. In comparison with controls, huDTA treated mice showed longer and more pronounced bands of granular dark stain as compared to controls. These bands were significantly longer in depleted mice as compared to control mice (Figure 4C, right panel). These observations indicate an acceleration of local inflammation in LC-depleted animals. Previous studies from our laboratory has shown that LC depletion does not affect systemic disease in organs, such as

kidneys, in MRL-*lpr* mice [18]. Overall, acceleration of corneal disease in the absence of LCs indicates a protective role of LCs against autoimmune disease in cornea.

LC ablation results in an increased proliferation of $CD8^+$ T-cells in the eye-draining lymph nodes

In steady state, skin-resident LCs sample the epidermal environment, take up tissue antigens, and migrate to the local draining lymph nodes, where skin antigens are presented to T-cells in order to induce tolerance and prevent self-reactivity [6, 18, 21-23]. However, corneal resident LC's behavior is not so clear, though they have been regarded as analogous to langerin expressing cells found in the epidermis. There is evidence of antigen trafficking to the eye-draining lymph nodes by eye-resident DCs in experiments where antigen delivered to the cornea as plasmids, which lack secretion signals, were traced to the submandibular lymph nodes in mice [24]. In other experiments where ovalbumin injection in the posterior chamber of eyes was followed by clonal expansion of ovalbumin-specific OT-1 T cells in the submandibular lymph nodes [25]. All these evidences support the notion that eye resident professional antigen presenting cells (APC) are involved in the trafficking of corneal antigens to regional draining lymph nodes. Therefore, to test if the effects of LC depletion on corneal tissue are a consequence of loss of LC's tolerogenic function, we analyzed the effects of LC depletion on T cell activation in submandibular lymph nodes.

Lymphocytes from submandibular lymph nodes of MRL+/+ mice were cultured with or without T-cell activating antibodies CD3/CD28 [6]. After 3 days of stimulation, lymphocytes were stained with Ki67-FITC antibody to measure proliferation by flow cytometry. Proliferation of

$CD8^+$ T-cells was significantly higher in the absence of LC (huDTA treated mice) than in PBS treated controls (Figure 5B). The *in vitro* response to T cell stimulation in $CD4^+$ cells was also slightly higher in LC-depleted mice than in LC-intact mice, but the differences were not statistically significant (Figure 5A). To verify that the impact of LC ablation was a localized phenomenon, we stimulated lymphocytes from spleens of chronically LC depleted MRL+/+ mice (Figure 5C and D). In contrast to $CD8^+$ cells from eye-draining lymph nodes, there was no detectable difference between LC-depleted and LC-intact mice in proliferation of $CD8^+$ or $CD4^+$ lymphocytes.

Overall, the increased T cell reactivity observed in LC-depleted mice suggests a role of LCs in limiting T cell activation and inducing local T cell tolerance. This role of cornea-resident LCs is consistent with the role of skin-resident LCs in inducing T cell tolerance in a local tissue-specific manner in autoimmune conditions [18].

LC ablation results in accelerated loss of naïve T cell phenotype in eye-draining lymph node lymphocytes.

To further understand the impact of LC ablation on T cell activation in autoimmune conditions, we analyzed the activation state of lymphocytes in submandibular lymph nodes of MRL+/+ mice continuously depleted of LCs. Lymphocytes from freshly dissected submandibular lymph nodes from 9-month old MRL+/+ mice were analyzed for expression of CD62L, an L-selectin that is expressed in naïve lymphocytes but is shed from the cell membrane upon T cell activation. We observed a significant reduction in the expression of CD62L, as denoted by the increase in $CD62L^{-/low}$ T cells, both $CD8^+$ and $CD8^-$, in MRL+/+ mice treated with DT as compared to PBS injected control mice (Figure 6A, upper panels). The mean fluorescent intensity of CD62L and

the percentage of CD62L positive cells were significantly lower in LC-depleted mice than in LC-intact MRL+/+ mice (Figure 6B). The loss of CD62L expression on T-cells indicates T cell activation and acquisition of effector / effector memory phenotype. Reduced CD62L expression prevents effector T cells from trafficking to lymph nodes, which shifts T cell trafficking from secondary lymphoid organs to tertiary tissues. Interestingly, $CD8^+$ as well as $CD8^-$ T cells from age/sex-matched B6 mice showed no such change in CD62L expression upon LC depletion (Figure 6A, lower panels). These observations suggest a role of LCs in suppressing ongoing autoimmune T cell activation. Furthermore, this immune suppressive effect of LCs is not apparent under normal steady state condition.

Discussion

In this study, we assessed the *in vivo* role that LCs may play in autoimmune corneal pathology, and to do so, we employed the spontaneous lupus keratitis mouse models, MRL+/+ and MRL-*lpr*. These mouse models provide the advantage that they develop tissue pathology spontaneously as it occurs in humans [14]. We demonstrate that the hallmark features associated with lupus keratitis, including epitheliopathy, stromal edema, and leukocyte infiltration, appear in MRL+/+ and in MRL-*lpr* mice. We further observed an alteration in the distribution and density of LCs in the cornea of MRL-*lpr* mice in early stages of disease as compared to corneas from healthy mouse controls. The frequency of langerin-expressing as well as of other MHC class II expressing cells increased with disease in these mice. Lastly, by using a targeted ablation system for langerin expressing cells, we showed for the first time that chronic depletion of LCs in the epithelium leads to an accelerated onset of corneal pathology in MRL-*lpr* mice, marked by increased stromal edema, epithelial erosion and heightened calcium deposition, as compared to

LC-intact mice. The chronic LC depletion brought about the increased T cell proliferation, particularly of $CD8^+$ T cells, and a loss of naïve T cell phenotype in the eye-draining lymph nodes. Collectively, the evidence presented here suggests that LCs play a protective role against the development of autoimmune corneal disease in lupus-prone mice. Our study provides a model for studying mechanisms, particularly the protective role of LCs, underlying dry eye disease that develops in patients with SLE.

The focus of the study of LC's function has shifted relatively recently. LC's role as a major antigen presenter in peripheral tissues frames them as major regulators/ mediators of local immune and tolerogenic functions. For instance, LCs have been shown to suppress contact hypersensitivity via CD4 cognate interactions as well as through the production of immunosuppressive cytokine IL-10 [26]. LCs have also been shown to promote tolerance and prevent autoimmunity through expansion of skin antigen specific regulatory T cells [27]. Such antigen specific tolerance was shown to be LC dependent in vivo too [18]. Additionally, recent *in vivo* studies support LC's role as eye tissue protectors, by showing increased nerve damage in LC depleted mice after induction of dry eye disease [12]. Our findings seem to fit this narrative, in the sense that in the absence of LCs, there was an increased corneal disease. Similar findings have been reported in the skin [18]. This highlights the notion that the functions of LCs in the cornea may be analogous to those in skin, and that LCs suppress inflammation to maintain the integrity of the corneal tissue.

Further support for this explanation will require testing the induction of tolerance to corneal specific antigens that could be used to inoculate the surface of autoimmune dry eye mice. To our knowledge, serological studies in autoimmune dry eye disease or secondary Sjogren's syndrome patients haven't yielded any cornea antigens that lack cross reactivity with other tissues. An

adequate method of antigen delivery would be required too. Skin inoculation can be easily achieved by several methods that do not compromise the integrity of tissue [18, 27]. However, the ocular surface is protected by tears that are in constant, mechanical movement, rendering any topical application of antigen difficult to control [6, 22, 24]. Furthermore, the corneal antigen will need to be delivered to the corneal epithelium without causing any local damage.

The present study raises another important issue that must be addressed. We observed an altered distribution and density of LCs in corneal epithelium and increased frequency of LCs and other APCs in the cornea of autoimmune-prone mice. This phenomenon was also observed in the inducible dry eye model, where there was a 1.5 fold increase in the number of LCs in corneal epithelium in comparison to control mice [28]. This is consistent with what was observed in Sjogren's syndrome dry eyes versus control patients [29]. There were significant changes in the distribution of LCs within the cornea too. This indicates changes in LCs density and distribution in relation to disease onset or manifestation. Such changes raise the possibilities that there may be changes in the emigrational dynamics of LCs within and out of the cornea. As mentioned above, LCs migrate from the cornea to eye-draining lymph nodes at a faster rate than they do in the skin. This could be explained by the almost null expression of EpCAM [6, 22] and a low expression of CD11b [9], which suggests a rapid turnover of corneal LCs in a steady state. Thus, an increased presence of LCs in the cornea in autoimmune mice could suggest a reduction in their mobility and/or emigration towards eye-draining lymph nodes, which precedes the onset of disease. This reduction in the migration of LCs out of resident tissue and towards local lymphatics is a phenomenon observed before in the skin, where LCs accumulated in the skin and were markedly reduced in the skin-draining lymph nodes prior to onset of lupus dermatitis [16]. Our initial attempts to investigate in vivo migration of cornea-resident LCs and other DCs didn't

succeed, as fluorochromes applied to corneal surface led to marked local inflammation and sloughing of cornea.

In conclusion, our data indicate a protective role of corneal LCs in the development of corneal autoimmune disease and in preventing local T cell activation under autoimmune conditions. Our data also raise a potential role of altered LC trafficking from the cornea to eye-draining lymph nodes in the pathogenesis of autoimmune corneal disease. Thus, the modulation of LC migration from the cornea to local draining lymph nodes may be a potential therapeutic avenue in autoimmune eye inflammation.

Chapter 2 Figures

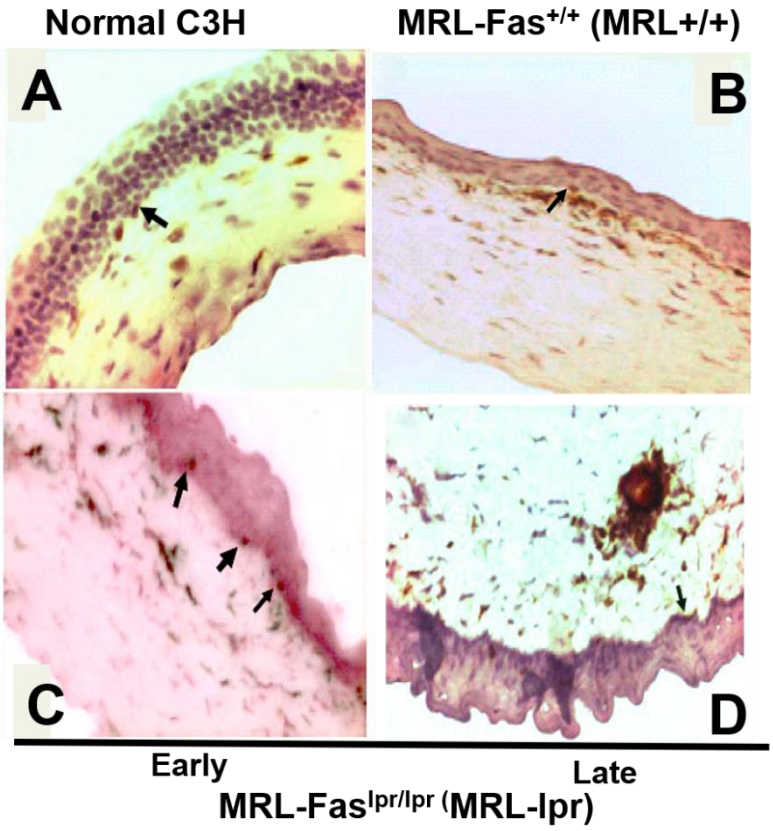

Figure 1 MRL+/+ and MRL-*lpr* develop corneal pathology characterized by immune infiltrates, stromal aedema, and epitheliopathy

Mouse eyeballs were dissected out and embedded in OCT for cryosection . 6µm sections were stained to detect CD45 for leukocyte infiltrates by immunohistochemistry (n = 4-6 female mice per group). Representative photomicrographs show cornea from healthy control 4-6-month-old C3H mice (A), MRL+/+ mice at a preclinical stage (4-6-month-old) (B), and MRL-*lpr* mice at early (8-10-week-old) and later (4-6-month-old) stages of disease (C and D, respectively). Immune infiltrates are located in basal aspect of the corenal epithelium (black arrows) as well as in the stroma.

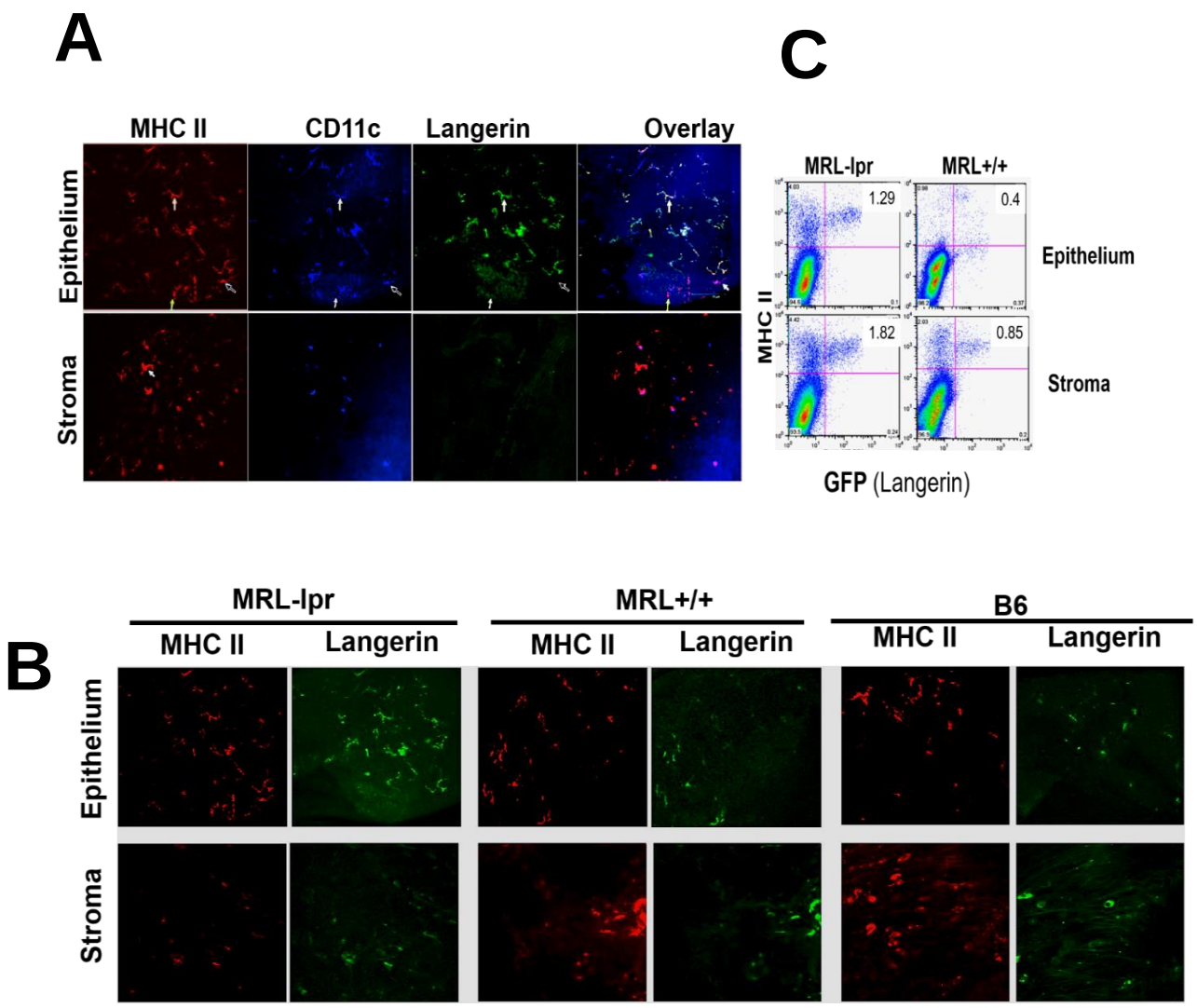

Figure 2 Changes in LC density and distribution in the epithelium of MRL-*lpr* mice prior to onset of tissue pathology. A) Corneas were dissected and digested to separate epithelium from stroma. The separated corneal epithelium and stromal sheets were stained with PE-conjugated anti-MHC II (red), anti-CD11c–APC (blue), and anti-CD207–FITC (green), and analyzed for stained cells by confocal microscopy. Representative whole mount photomicrographs of corneal epithelium and stroma from 8-week-old female MRL-*lpr* mice are shown. Note that most MHCII^{+} cells in the corneal

epithelium are LCs ($CD207^+$ $CD11c^+$ MHC II^+), whereas stroma has mostly other DCs ($CD11c^+$ $CD207^-$ MHC II^+) & macrophages ($CD11c^-$ $CD207^-$ MHC II^+). B) Representative whole mount photomicrographs of corneas from Lang.eGFP-knock-in mice show MHC II and langerin (GFP) expression in corneal epithelium and stroma. Results represent 6 each of 8-week-old MRL-*lpr* mice, and 3-month-old MRL+/+ and B6 mice. C) Corneas were harvested from 10 each of 8-week-old Lang.eGFP-knock-in MRL-*lpr* and MRL+/+ mice, and epithelium and stroma were separated. Cells isolated from corneal epithelium and stroma were separately pooled, and pooled cells analyzed for MHC II and langerin (eGFP) expression by flow cytometry. Numbers represent % of gated live $FSC^{hi}SSC^{hi}$ cells. Gating scheme: $FSC^{hi}SSC^{hi}$ CD11+→MHCII vs GFP

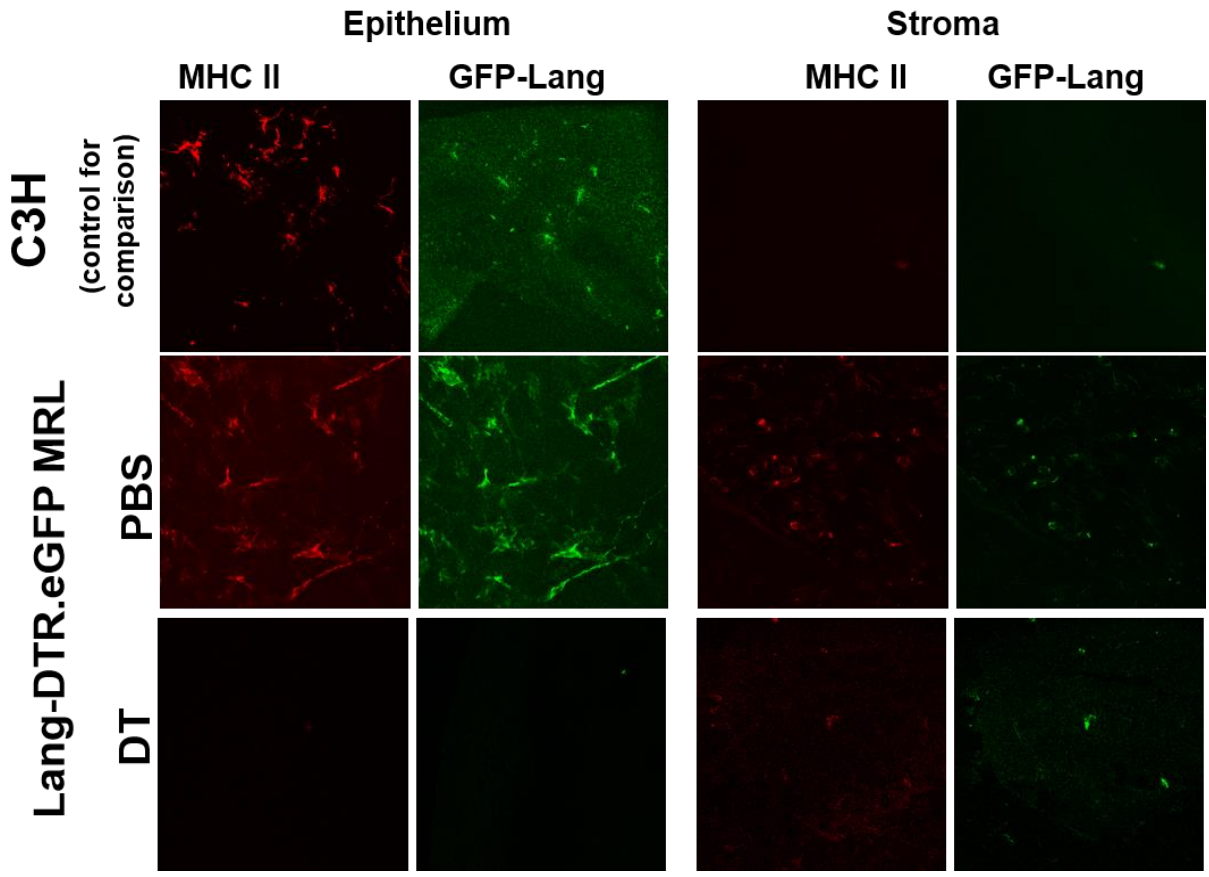

Figure 3 Corneal LCs were depleted by DT injection in knock-in mice

Lang-DTR.eGFP knock-in female MRL-*lpr* mice were injected with diphtheria toxin A (DT) (100μg) or PBS (control) and analyzed for LC depletion 7 days post-injection. Corneas were dissected and digested to separate epithelium from stroma. Each layer was further stained with PE-labeled anti-MHC II antibody (red).

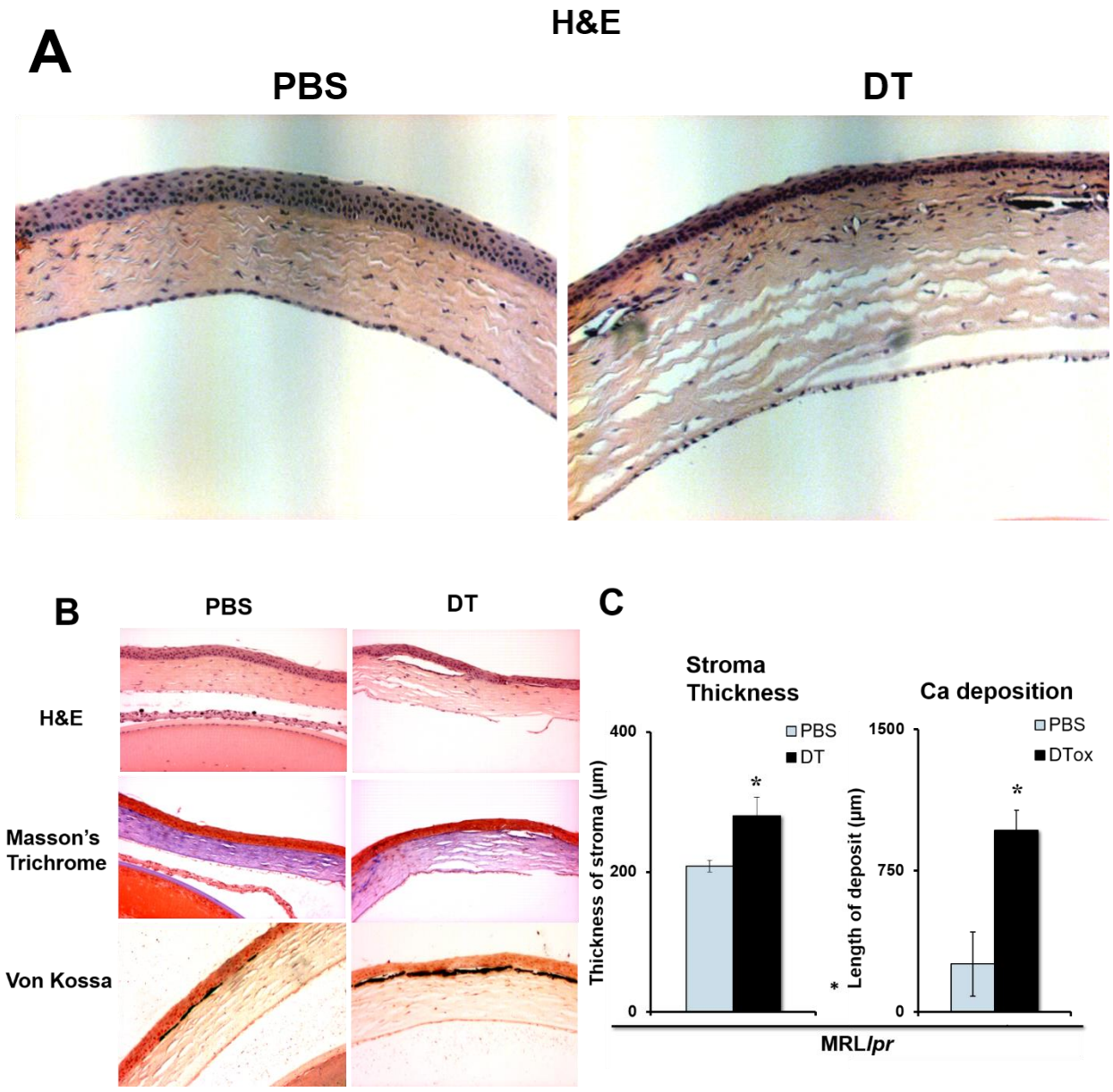

Figure 4 Cornea tissue pathology is increased in LC-depleted MRL-*lpr* mice.

A Representative H&E stained paraphing section of eye ball from LC depleted (DT) and controls (PBS) 14-week-old mice. Six-wk-old mice received 6 i.p. injections of DT or

PBS once a week for 6 weeks. 2 of 4 LC-ablated and 0 of 4 control mice showed corneal infiltration with thickened stroma.

B Representative sections of H&E, Massons's trichrome, and Von Kossa stained corneas. Nine-week-old MRL-lpr mice received 4 i.p. injections of DT or PBS every 10 days. Eye balls were harvested for histology at 20 weeks of age. 9 of 11 mice LC-depleted and 2 of 11 control mice exhibited chronic inflammatory changes in the cornea

C Quantification of cornea pathology defined as central stroma thickening and calcium deposition. This chart represents compilation of 3 separate experiments, with 3 mice per group per experiment ($p < 0.01$, Fisher's exact test).

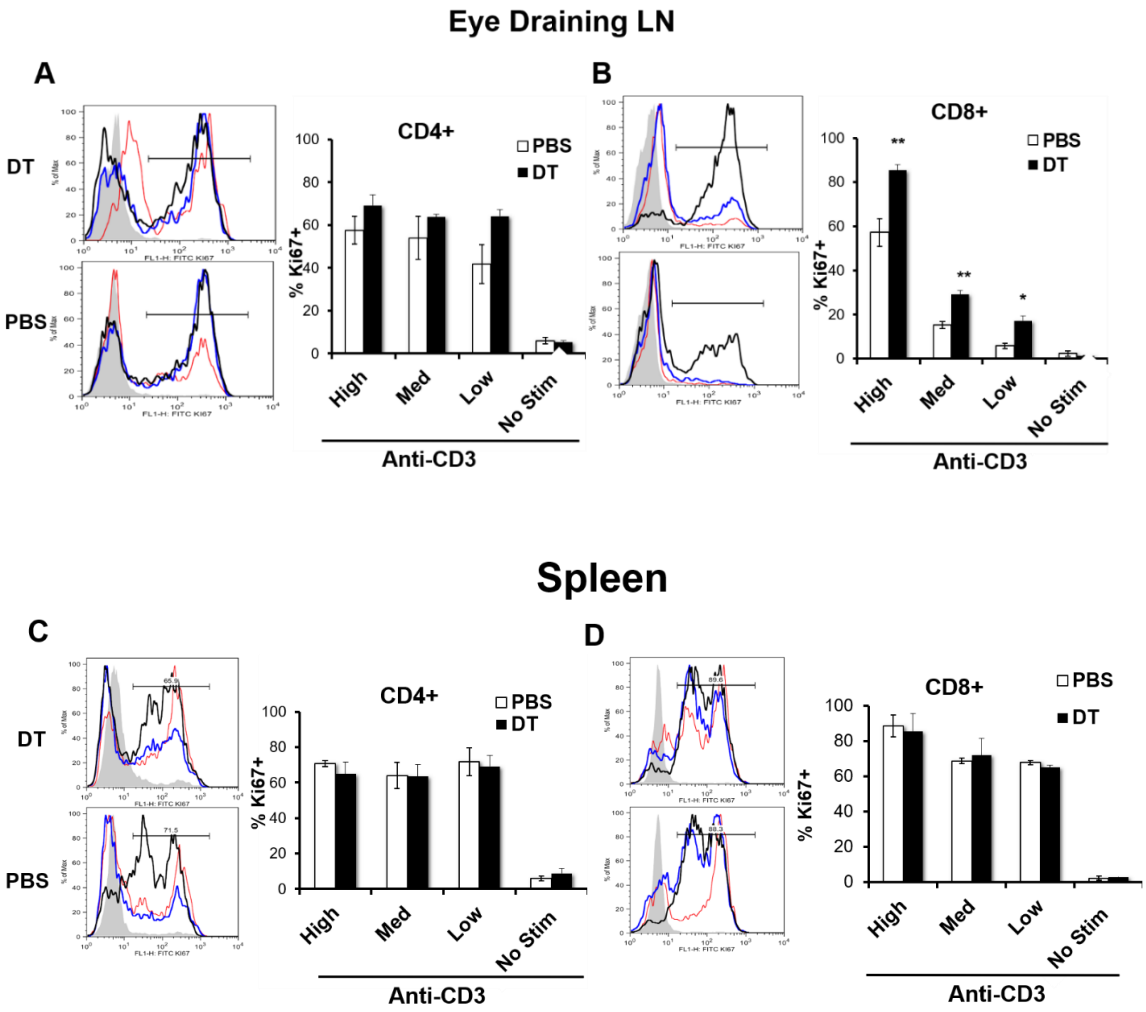

Figure 5 Higher proliferation activity of CD8⁺ T-cells in the absence of LC.

Lang-DTR.eGFP knock-in female MRL+/+ mice were treated weekly with 100µg huDTA or PBS for 6 weeks. Eye-draining lymph nodes (submandibular lymph nodes) (A-B) and spleens (C-D) were harvested from these animals, and their single cells isolated and plated to isolate non-adherent cells. Isolated cells were cultured for 3 days without or with anti-CD3/CD28 (at three different concentrations of anti-CD3: high=1µg/mL ----, medium=0.5µg/L -----, and low=0.25µg/mL ----). Shaded area, no stimulation. Proliferation was measured by Ki67 detection. n=4, *p<0.05; **p<0.001.

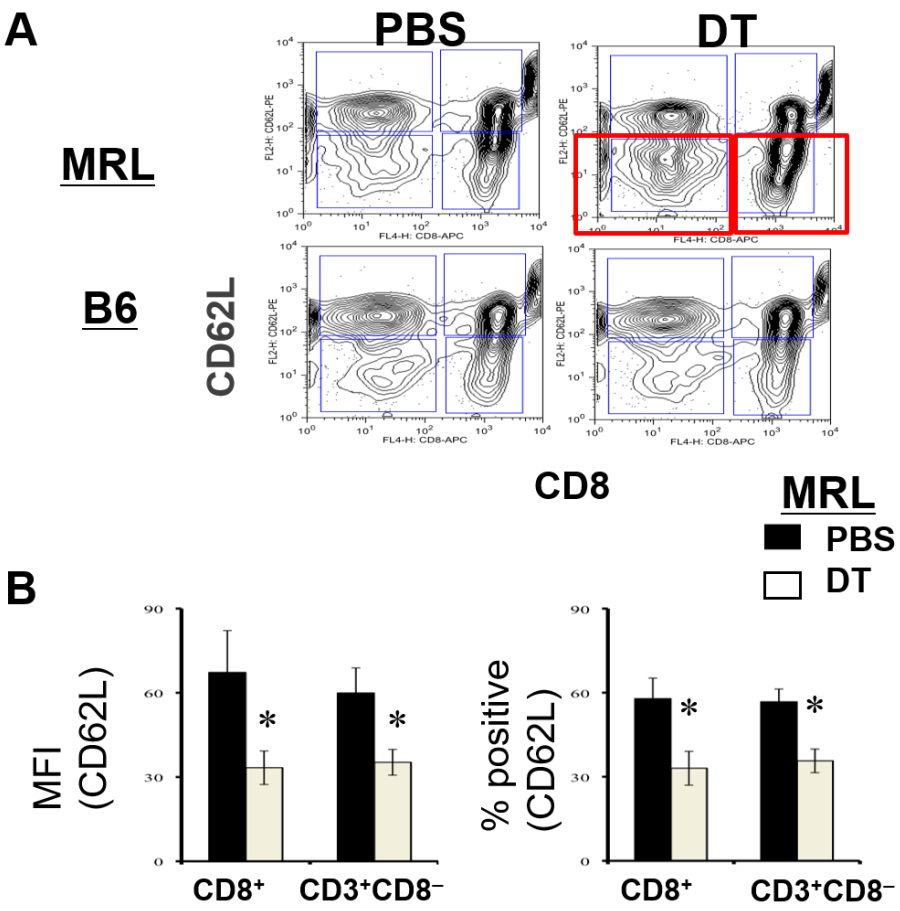

Figure 6 Reduced naïve T cells in LC-depleted animals in the autoimmune background.

Nine-month-old female Lang-DTR.eGFP knock-in MRL$^{+/+}$ or B6 mice were treated with 6 i.p. injections of DT or PBS every 10 days to achieve a long-term depletion of LCs. Eye-draining cervical lymph nodes from these animals were analyzed for CD62L expression on T cells by flow cytometry. A) Representative contour plots show CD62L expression on gated CD3^{+} cells. Note the reduced proportions of both CD8^{+} and CD8$^{–}$ T cells that express CD62L in LC-depleted as compared to LC-intact MRL mice. Such

change in CD62L expressing cells upon LC depletion was not seen in B6 mice. B) Quantification of CD62L expression as MFI or as % $CD62L^+$ cells. * $p<0.05$ (n=4 per group).

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Chapter 3-

Skin γδ T Cells Promote Langerhans Cell Migration

Abstract

Acquired or self-antigens in tissues are taken to lymphoid organs to elicit protective immunity against infection and cancer or to acquire ability to avoid self-reactivity. This important immune function is accomplished by dendritic cell (DC) populations that primarily reside in tissues. Mechanisms that regulate the migration of tissue-resident DC from tissues to lymph nodes are unclear. We demonstrate that skin-resident specialized γδ T cells called dendritic epidermal T cells (DETC) promote the migration of skin-resident DC from skin to cutaneous lymph nodes via CD40–CD40L interaction. Autoimmune-prone mice that exhibit reduced skin-DC migration have reduced DETCs, whereas a glycolipid that ameliorates skin inflammation restores DETCs and skin-DC migration. Such 'local' control of migratory behavior of tissue-resident DC can regulate systemic immune response in a tissue-specific manner, which has important implications for protection against inflammation, autoimmune diseases and graft rejection, and vaccination against cancers and infections.

Introduction

The immune system deploys many mechanisms that allow the host to mount immune responses against pathogens while avoiding self-reactivity [30]. Though many of these regulatory mechanisms systemically affect the host immune repertoire, some control mechanisms act at the local tissue-level. Tissue-resident dendritic cells (DC) are believed to transport antigens from tissues such as skin, gut and lungs to local draining lymph nodes, where they instruct T cells to mount immunity or tolerance against specific tissue based antigens [31]. Mechanisms that regulate the migration of tissue-resident DCs from tissues to lymph nodes are unclear.

Skin contains a major DC population, Langerhans cells (LC) [32, 33] that are believed to maintain tolerance to skin antigens by constant low migration to skin draining lymph nodes (SDLN) [31, 34]. Epithelial tissues of all mammalian species also contain a resident population of γδ T cells [35, 36]. All resident lymphocytes in the murine epidermis and ~20% of T cells in the human epidermis express a canonical γδ TCR [37-39]. In the mouse, epidermal γδ T cells express a canonical TCR Vγ3Vδ1 (alternate nomenclature Vγ5Vδ1) and display a dendritic type of morphology, hence these cells are called dendritic epidermal T-cells (DETC). A previous study showed that LC lines from the epidermis of BALB/c mice activated DETC lines from AKR mice. Such activated DETC lines secrete cytokines that then induce the proliferation of epidermal LC lines [40]. These observations suggest a possible interaction between LC and DETC. The in vivo functional significance of these interactions remains largely unclear. Here we demonstrate the role of DETC in regulating the migration of their DC neighbors under steady state and autoimmune conditions.

Materials and Methods

Mice

Lupus-prone MRL/MpJ-Fas$^{lpr/lpr}$ (MRL-lpr) and MRL/MpJ-Fas$^{+/+}$ (MRL+/+) mice, their MHC (H-2^{k})-matched control strains (B10.BR and C3H/HeOuJ), C57Bl/6 (B6), TCR $\delta^{-/-}$ ($\delta^{-/-}$) (B6.129P2-*Tcrd*^{tm1Mom}/J), CD40L$^{-/-}$ B6, and BALB/c mice were purchased (Jackson Laboratory, Bar Harbor, Maine) and/or bred locally. MRL-lpr mice develop a systemic autoimmune disease including lupus dermatitis at ~4-mo of age, whereas MRL+/+ mice develop disease at ~8-10-mo of age [41]. Lang-eGFP knock-in MRL-lpr mice were generated by introgression of the knock-in mutation from the stock B6 onto the MRL-lpr background for 8 generations. Lang-eGFP B6 stock mice that express enhanced green fluorescent protein (eGFP) driven by langerin (lang) promoter were kindly provided by Dr. B. Malissen [13]. Animal experiments were performed following the approved institutional guidelines.

Antibodies and reagents

Following antibodies were used in flow cytometry and in situ staining: mouse Langerin (CD207) (205C1, 929F3) (ABCys, France); mouse CD207 (eBioRMUL.2), NK1.1 (PK136) and CD11c-Biotin (N418) (eBioscience); IE^{k} (14-4-4S) (Biolegend); and CD1d (1B1), CD3 (145-2C11), IE/IA (2G9), CD8α (53-6.7), CD4 (GK1.5), TCRβ (H57-597), TCRγδ (GL3), TCRVγ3 (536), avidin-pCP, CD16/CD32 (2.4G2) (all from BD PharMingen). Purified antibodies were conjugated to Alexafluor 488, 568, or 647 using a mAb labeling kit (Molecular Probes), following the manufacturer's instructions. Mouse IgG2a conjugated to Alexafluor-488, rat IgG2a-PE, avidin-

pCP, and Armenian hamster-APC were used as isotype controls. PBS57 loaded CD1d–tetramers conjugated with Alexa-488, PE or Alexa-647 were provided by the NIH Tetramer core facility. αGC was dissolved in vehicle (0.025% polysorbate-20 in PBS). IL-1β and TNF-α (Calbiochem, USA) and CCL19 (R&D Systems, USA) were used in skin cultures.

In situ staining

Ear skin explants were harvested, and dorsal halves separated with the aid of forceps. Epidermal and dermal sheets were recovered by floating the dorsal halves on 4% NH_4SCN in PBS for 40 min. Epidermal and dermal sheets were washed in PBS, fixed in ice-cold acetone for 4 min, and blocked in 1% BSA, 5μg/ml anti-CD16/CD32 (2.4G2) in PBS before staining with conjugated antibodies. Intensity of cellular markers was determined from maximum projections of Z-stacks of 8 sections spanning ~8-10μm to cover all visible cells in focus using a confocal microscope. For counting of epidermal cells, Z-stacks of 3-8 sections (Δd~2μm/section) at 20-40x magnification were recovered from different areas of epidermis. Numbers of LC and DETC were determined post-acquisition by counting the fluorescent cells within a defined area using a grid. An average number of cells were obtained by counting 4-10 series (depending on cellular distribution) for each epidermal sheet covering 1-2mm^2. Acquisition and counting of cells was performed in a blinded manner. In situ studies shown in were conducted in B6, BALB/c, B10.BR and C3H mice with similar results.

Epidermal cell preparation

Epidermal and dermal sheets were recovered by floating dorsal ear halves from 10-15 mice per group on 2.5% trypsin solution for 40min at 37°C. After the separation, epidermal cell suspensions

were incubated with 0.1 % DNAse I for 5min at 37°C, washed, and cultured overnight in 5% CO_2 at 37°C in complete RPMI-1640 to allow re-expression of surface markers.

For large scale isolation of LC and DETC, fur hair was removed using nair (CVS pharmacy) and whole flank skin was recovered, cut in 0.5 cm^2 pieces and digested in 2.5% trypsin and 0.1% DNAse I (Invitrogen) solution at 37°C. After 40min digestion epidermal sheets were gently separated from dermis. Epidermal cell suspensions were obtained by vigorous shaking and filtered through Sera sepra filter columns (Evergreen Scientific). Live cells were collected by gradient centrifugation on lympholyte-M. LC and T-cells ($Thy1^+$) were purified by FACS sorting.

Short-term DETC lines were generated using isolated epidermal cells, as described [40]. The DETC were maintained at 10^6 per ml in complete RPMI-1640 supplemented with 10% FCS and 10U IL-2, and repeatedly stimulated with 2μg/ml con A (Sigma-Aldrich) every 14 days.

In vitro LC migration assay

LCs were isolated by floating ear skin explants on complete RPMI [42]. After resting in fresh media overnight, LCs were transferred into the top chamber of a transwell chamber. Complete medium containing CXCL12 (10ng/ml) was placed into the bottom chamber and cells were allowed to migrate for 4 h [34]. Cells in the top and bottom chambers were collected and analyzed by FACS along with a fixed number of CaliBrite (BD PharMingen) beads to normalize for cell recovery. Fraction migrated was calculated as LC ($MHCII^{hi}$) in the bottom chamber divided by total LC. Few (<0.5%) LC migrated to lower chamber in the absence of CXCL12.

To determine the effect of DETC on LC migration, DETC were isolated and cultured for at least three weeks to reach a purity of >80% $CD3^+V\gamma3^+$ DETC [43]. Equal numbers of DETC and LC were added to the top chamber and the migration experiment conducted.

Flow cytometry

$5x10^6$ (for DC) and $1–2x10^6$ (for T-cells) Fc-blocked cells were stained with indicated antibodies at 0.5μg/ml in 1% BSA in PBS for 30min on ice. Samples were acquired and analyzed using FACSCalibur. 10,000-20,000 events gated on lymphocytes were collected for T-cells. For DC, 10,000 $CD11c^+$ events gated on FSC^{hi}/SSC^{hi} cells were collected.

Skin explant cultures

Ear skin explants were floated on complete RPMI-1640 supplemented with IL-1β (10ng/ml), TNF-α (10ng/ml) and CCL19 (100ng/ml) for 4 days, with exchange of culture media on day 2. Epidermal and dermal sheets were separated, acetone-fixed, blocked and stained to analyze on a confocal microscope [44], as described above.

In vivo skin DC migration assay

The dorsal side of the ear of animals was painted with 1% FITC in DBP-acetone, as described [44]. At the indicated time-points, animals were euthanized and their CLN harvested. The CLN cells were stained with indicated antibodies to detect and analyze $FITC^+$ cells by flow cytometry.

In vitro interaction between LC and DETC

To determine if LC and DETC interact with each other and form strong cell-cell adhesion, DETC and LC isolated from the epidermis were cultured alone or together for 3 h. Cells were then collected with vigorous pipetting and stained for CD3 and MHCII. Samples were vortexed at full speed prior to FACS analysis. To visualize if LC and DETC form strong cell-cell adhesion, LC and DETC were co-cultured for 3 h, and then stained with FITC anti-Thy1 and PE anti-MHCII. Cells were vortexed, agitated with vigorous pipetting, and wet-mounted on a slide for fluorescence microscopy.

In vitro DETC activation

DETC (~10^5) rested 14 days since the last con A stimulation were re-stimulated with 10^4 CD1d-transfected or untransfected RMA-S cells or RPMI alone, with or without αGC (100ng/ml) or ConA, in 96-well flat bottom plates. All wells were supplemented with 10% FCS and 10U IL-2.

Detection of CD40L on DETC

To detect CD40L on cell surface of in vitro activated T-cells[45], an anti-CD40L Ab was added to cultures at time 0 which avoids missing CD40L expression due to internalization and degradation.

Statistics

Statistical comparisons between groups were made using two-tailed Student's *t*-test and ANOVA for comparison of two groups, and one-way ANOVA with Tukey as post hoc when more than two groups were compared.

Results

γδ DETCs promote LC migration

Since DETCs reside in close contact with LCs in the epidermis (**Fig. 1A**), we reasoned that DETCs might regulate the migration of LCs. To address this, we isolated LCs ($MHCII^{hi}$) and DETCs ($CD3^+V\gamma3^+$) from the mouse epidermis and cultured them alone or together in a trans well culture chamber (**Fig. 1B**). Results show that LCs migrated more efficiently in the presence of DETCs than when cultured alone. To determine if DETCs affect LC migration *in situ*, we performed an *ex vivo* migration assay [44] where skin explants are cultured with cytokines to induce LCs to emigrate from the epidermis and migrate through dermal lymphatics into culture chamber. Fewer LCs emigrated from the epidermis of $\delta^{-/-}$ mice that have no γδ DETCs compared to wild-type (WT) B6 mice (**Fig. 1C**). While WT LCs migrated through the dermis forming characteristic

dermal cords, such organized LC migration was absent in $\delta^{-/-}$ dermis (**Fig. 1D**). To determine if DETCs play a role in LC migration in vivo in the steady state, we enumerated langerin$^+$ skin DCs in CLN. Whereas the proportion and number of blood-derived myeloid DC (mDC) were similar between the two groups, the proportion and total number of langerin$^+$ skin DC were reduced in $\delta^{-/-}$ mice (**Fig. 1E, 1F**) compared to control mice. Thus, DETCs seem to stimulate LCs migration *in vitro*, emigration from epidermis and migration through dermal lymphatics *in situ*, and their steady state immigration to CLN *in vivo*. These data indicate a role of DETCs in LC migration.

Role of γδ DETCs in LC migration in mice prone to develop autoimmune skin inflammation

To evaluate the functional consequences of DETC-induced LC migration, we used genetically autoimmune-prone MRL-lpr and MRL+/+ mice that develop skin inflammation that resembles lupus dermatitis in humans [46, 47]. These mice exhibit impaired migration of LCs from skin to CLN prior to the onset of skin inflammation [44]. We reasoned that the LC migration defect in MRL mice might be related to impairment in DETC. Indeed, analysis of epidermis for CD3$^+$ cells by confocal microscopy showed that epidermal T cells are reduced in MRL-lpr and MRL+/+ mice compared to control C3H and B10.BR mice (**Fig. 2A, 2B**). We found reduced numbers of DETCs, but increased numbers of LCs, in the epidermis of Lang-EGFP knock-in MRL-lpr mice compared to Lang-EGFP knock-in B6 mice. To further determine any relation between the disease state and DETC numbers, we enumerated DETCs in MRL and control mice at different age groups. Results show that DETC numbers decrease with age in MRL-lpr mice, with a profound reduction around 10-wk of age (**Fig. 2A**) when these mice begin to exhibit serological features of autoimmunity. The reduction of DETCs is more drastic in MRL-lpr mice than in MRL+/+ mice that develop a delayed disease accompanied by reduced LC migration later in life [44]. We further show that like

in normal mice, all T cells in the epidermis of MRL mice express γδ TCR (**Fig. 2C**). Thus, like the LC migration defect [44], reduction in γδ DETCs in MRL mice starts prior to the onset of dermatitis and appears to correlate with the severity of dermatitis.

Treatment with glycolipid α-galactosylceramide (αGC) ameliorates lupus dermatitis in MRL-lpr mice [47]. To determine if αGC treatment affects DETCs in vivo, we enumerated DETCs after an i.p. or topical administration of αGC (**Fig. 2D, 2E**). Results show that DETCs were restored in αGC-treated MRL-lpr mice. Thus, αGC treatment that ameliorates lupus dermatitis [47] expands DETC population.

We then reasoned that since αGC treatment expands DETCs (**Fig. 2D, 2E**) that are required for normal LC migration (**Fig. 1**), treatment with αGC might restore LC migration in MRL-lpr mice. Indeed, αGC-treated MRL-lpr mice had increased proportions and total numbers of langerin$^+$ DCs, but not of mDC, in their CLN compared to control mice (**Fig. 3A, 3B**). A more dramatic and direct effect of αGC on LC migration was seen in an *ex vivo* LC emigration assay which showed that LC emigration from epidermis in cultured skin explants was normalized after αGC injection (**Fig. 3C**). Increased numbers of LCs were also detected in the dermis of cultured skin explants from αGC-treated MRL-lpr mice, with restored migration pattern and dermal cord formation (**Fig. 3D**). This was further confirmed in an *in vivo* activation-induced immigration assay where skin is painted with FITC and newly immigrant (FITC$^+$) LCs are enumerated in CLN by flow cytometry [44]. We found increased percentages (**Fig. 3E**) and total numbers (**Fig. 3F**) of FITC$^+$MHCII^{hi}CD11c$^+$CD86$^+$ cells in CLN of αGC-treated MRL-lpr mice. Thus, αGC treatment improves the capacity of LCs to migrate, resulting in their reduced numbers in the epidermis, increased dermal cord formation, and increased numbers in the CLNs of MRL-lpr mice.

αGC treatment differentially affects the migration of the two subsets of langerin-expressing skin DC subsets, namely LCs and Lang$^+$ dDCs

In addition to LCs, there are other migratory DC populations in the skin, one of which also expresses Langerin (CD207) and skin homing integrin, α E (ITGAE) or CD103, referred to as Langerin$^+$ dermal DC (Lang$^+$ dDC) [8, 33]. [48, 49]. [50]. Recent studies using viral, bacterial, or allergic immune response models have shown that skin-resident DC subsets may promote distinct and opposing antigen-specific responses [48, 51-54]. For example, in a Candida albicans skin infection model, LCs directly presented antigen to induce antigen-specific Th17 cells whereas Lang$^+$ dDCs induced antigen specific CTL and Th1 cells. Lang$^+$ dDCs also inhibited the ability of LCs and classical DCs to promote Th17 cell responses [48]. In other studies, migratory LCs have been shown to promote the maintenance of peripheral tolerance through deletion of self-reactive T cells, induction of regulatory T cell (Tregs), and promotion of IL-10 secretion [55-57] . In contrast, Langerin-expressing dermal CD103$^+$ DCs are specialized for cross-presentation of viral Ags, as well as uptake and delivery of Staphylococcus epidermidis-derived Ags from skin to skin-draining lymph nodes for efficient induction of Ag-specific IL-17–producing CD8$^+$ T cells[51-54]. Although many studies have suggested the functional specialization of different tissue-resident DC populations in infectious and allergic responses, the roles and regulation of different skin DC subsets in systemic autoimmune responses, such as in lupus, are unclear.

To determine if αGC treatment differentially affects the migration of different skin DC subsets, we analyzed the migration patterns of LCs and Lang$^+$ dDCs in steady state using Lang-GFP knock-in MRL+/+ and MRL-*lpr* mice 7 days after a αGC i.p. injection (**Fig. 4**). Compared to vehicle-injected control animals, the proportions of migratory Lang$^+$ cells (defined as Lang-GFP$^+$

CD8α^{-}) significantly increased in the cLNs of αGC-treated MRL+/+ and MRL*lpr* strains which exhibit LC migration defect (**Fig. 4A, B)**. Then, to distinguish LCs from Lang^{+} dDCs, Lang^{+} CD8α^{-} cells were analyzed further for CD11b and CD103 expression (**Fig. 4C**). αGC treatment led to a significant increase in LCs (CD11b$^{+/lo}$CD103^{-}), but a significant reduction in Lang^{+} dDCs (CD11b^{-}CD103^{+}) in both MRL+/+ and MRL*lpr* mice (**Fig. 4D**). Thus, the ratio of LCs to Lang^{+} dDCs was markedly increased in αGC-treated mice as compared to vehicle injected control animals (**Fig. 4F**). Lastly, MRL*lpr* mice develop lymphoproliferative disorder with increased lymph node cellularity, which was markedly reduced after αGC treatment whereas αGC treatment had no significant effect on lymph node cellularity in MRL+/+ and B6 mice that don't exhibit lymph node hypercellularity (**Fig. 4E**), which suggest that a recovery of LC migration, with a concomitant reduction in Lang^{+} dDCs, may result in a reduction in proliferative responses in the cLNs. Overall, LC migration rescue may have a more significant impact on the development of tissue autoimmunity, which may explain why absence of LCs in lymph nodes precedes skin pathology.

Role of CD40L in mediating DETC-induced LC migration

Since DETCs and LCs appear to be in direct contact *in vivo* (**Fig. 1A**) and the two cell types interact with each other *in vitro* and form pairs in cultures (**Fig. 5A**), we envisioned a role of cell-cell contact in mediating DETC effect on LC migration. A previous study suggested a role of CD40-CD40L interactions in LC migration [58]. However, the cell type in the epidermis that provides CD40 ligation is not known. Since αGC treatment that corrects LC migration defect (**Fig. 3**) also recovers the population of DETCs in the epidermis of MRL-lpr mice (**Fig. 2**), we first tested if the interaction between DETCs and LCs changed in any way the expression of CD40L. We found increased *Cd40l* mRNA levels in freshly isolated DETCs upon co-culture with LCs (**Fig. 5B**) and

increased CD40L expression in a DETC line cultured with CD1d transfected RMA-S cells with αGC (**Fig. 5C**). To directly test the role of CD40L expressed on DETC in modulating LC migration, we performed an *in vitro* LC migration assay where DETCs from the WT or CD40L-deficient mice were cultured with LCs isolated from the epidermis of syngeneic WT mice (**Fig. 5D**). Results show that CD40L deficiency on DETC abolished their ability to promote LC migration. Similar results were obtained using Ab blockade of CD40L (**Fig. 5E**). These data identify a new mechanism whereby DETC interact with their DC neighbors to facilitate their migration to CLN.

Discussion

Our results identify a new mechanism of regulating tissue-resident DC migration from tissues to tissue-draining lymph nodes. We demonstrate that DETCs and LCs interact with each other via CD40–CD40L to promote LC migration (**Fig. 5**). Genetic deficiency of each of these partners disrupts skin homeostasis and elicits skin inflammation. For example, $\delta^{-/-}$ non-obese diabetic mice develop skin inflammation [59], and the conditional ablation of LCs [18], or germline deletion of CD40L [60] accelerates lupus dermatitis. Furthermore, genetically autoimmune dermatitis-prone MRL mice exhibit reduced DETC numbers (**Fig. 2A, 2B**), and defective LC migration [44] (**Fig. 3C–F**). Conversely, αGC treatment expands DETCs (**Fig. 2D, 2E**), increases CD40L on DETCs (**Fig. 5D**), augments LC migration (**Fig. 3**) but not $Lang^{+}$dDCs migration (**Fig. 4C, D**), and prevents lupus dermatitis [47]. Taken together, these findings indicate a role of DETC–LC interactions in preventing autoimmune skin inflammation.

DETCs play a role in the homeostasis of keratinocytes and promote wound healing in mice [38, 61-63]. Similar to murine DETCs, freshly isolated human epidermal Vδ1 T-cells are activated in acute human wounds and secrete IGF-1 to promote wound healing [39]. DETCs have also been shown to play roles, both pro- and anti-inflammatory, during the development of contact hypersensitivity [64-67]. In this article, we provide several lines of evidence demonstrating that DETCs can regulate the homeostasis of another skin-resident cell, LC, by activating them and promoting their migration from skin to CLNs (**Fig. 1**). In a previous study, DETC and LC cell lines activated each other *in vitro* via cytokines [40]. Consistently, fewer LCs stained brightly for MHCII in cultured skin explants in $\delta^{-/-}$ epidermis than in the epidermis of WT mice (**Fig. 1C**), suggesting a role of DETCs in activating LCs. In turn, a co-culture with LCs resulted in increased

Cd40l expression on DETCs (**Fig. 5C**). These data indicate a cross-talk between LCs and DETCs, leading to their activation. The activation status of DETCs is important in the regulation of LC migration, as the resting DETCs have a weak effect on LC migration when compared to freshly activated DETCs (our unpublished data). Taken together, DETCs appear to interact with major cell types in the skin, thus endowing important roles in maintaining skin homeostasis.

Glycolipids that bind CD1d activate natural killer T-cells [68]. They have been shown to modulate many immune cells and exert many immune functions [47, 69, 70]. Here we report a novel effect of treatment with these lipids whereby *in vivo* treatment with αGC caused an expansion of DETCs (**Fig. 2D, 2E**) and improved LC migration (**Fig. 3**). Furthermore, the addition of CD1d-transfected cells plus αGC to a DETC line enhanced their CD40L expression (**Fig. 5D**), suggesting a direct effect of CD1d in activating DETCs. A role of CD1 in activating tissue γδ T cells has been suggested in a previous study showing that human Vδ1 γδ T cells recognize CD1c, a CD1 isoform present in humans, on the surface of immature DCs, secrete cytokines, and selectively stimulate human monocyte-derived DCs to undergo maturation *in vitro* [71]. Furthermore, mature keratinocytes do express CD1d and the addition of ceramides to in vitro cultures of keratinocyte precursors increases CD1d gene expression and its surface translocation, while inhibition of endogenous ceramide synthesis reduces CD1d in keratinocytes [72]. Thus, αGC may enhance the expression of a DETC ligand(s) on cells such as keratinocytes. Similar ligands may appear on the stressed keratinocytes that are known to activate DETC through their γδ TCR [73]. Thus, stressed keratinocytes might provide a physiological stimulus for LC migration *in vivo*.

DETCs co-exist with LCs *in vivo* (**Fig. 1A**) and appear to bind tightly to each other in co-cultures (**Fig. 5A, B**). To identify possible molecular interactions responsible for the augmentation of LC migration, we searched the Gene Expression Omnibus database for LC and DETC datasets to obtain a useful "first pass" look at possible candidates. Expression and mAb blocking studies were conducted for a set of these molecules involved in cell adhesion and DC/T cell interactions, including CD40L, CD48, integrin α4, integrin β7, LFA1, LFA2, and TCR. While previous studies have suggested a role of CD40-CD40L interactions in LC migration [58, 74], the cell type in the epidermis that provides CD40 ligation is not known. Our data clearly show that CD40L increased on DETCs upon activation and co-culture with LCs (**Fig. 5C–E**), and blockade or deficiency of CD40L abrogated the DETC-mediated increase in LC migration (**Fig. 5F, G**). In preliminary studies, blocking CD49d, CD48 and TCR Vγ3 also negatively affected, albeit less profoundly than CD40L, LC migration (our unpublished data). Junctional adhesion molecule-like (JAML) has recently been identified as a costimulatory signal for DETC [75]. JAML is expressed on DETC and is the receptor for Coxsackie adenovirus receptor (CAR). The CAR-JAML pathway does not activate conventional T cells but is a potent stimulator of γδ T cells. The GEO database entry for GDS3575 indicates that LC express moderate amounts of CAR. Our preliminary studies to activate or block this pathway have thus far not revealed a role of this pathway in regulating LC migration. Our ongoing global gene expression studies in LCs and DETCs cultured alone and together will more comprehensively map the molecular interactions responsible for DETC-induced LC migration.

In summary, we show that the germline deletion of DETCs or treatment with anti-TCRδ Ab in normal mice and spontaneous reduction of DETCs in autoimmune mice results in reduced migration of LCs in steady state as well as upon activation, whereas increase in DETCs enhances

LC migration. DETCs have been previously shown to participate in wound healing, tumor rejection, and regulation of graft-versus-host disease and inflammation [38, 59, 76]. Findings in this manuscript ascribe a new role for these tissue-resident specialized T cells, whereby they orchestrate peripheral tolerance to skin tissue by sending their tolerogenic neighbors (skin DC) to lymphoid organs. In fact, reduced DETC population and impaired LC migration correlate with the development and severity of autoimmune skin inflammation that develops spontaneously in MRL mice, whereas increased DETC numbers and LC migration correlate with improved skin inflammation. We anticipate that a similar mechanism may operate in other barrier sites such as gastrointestinal and genital tracts as well as in other internal organs. Improved understanding of crosstalk between T cells and DCs at the tissue level will have implications for protection against autoimmune diseases and graft rejection as well as for wound healing and vaccination against infections and cancers.

Figures

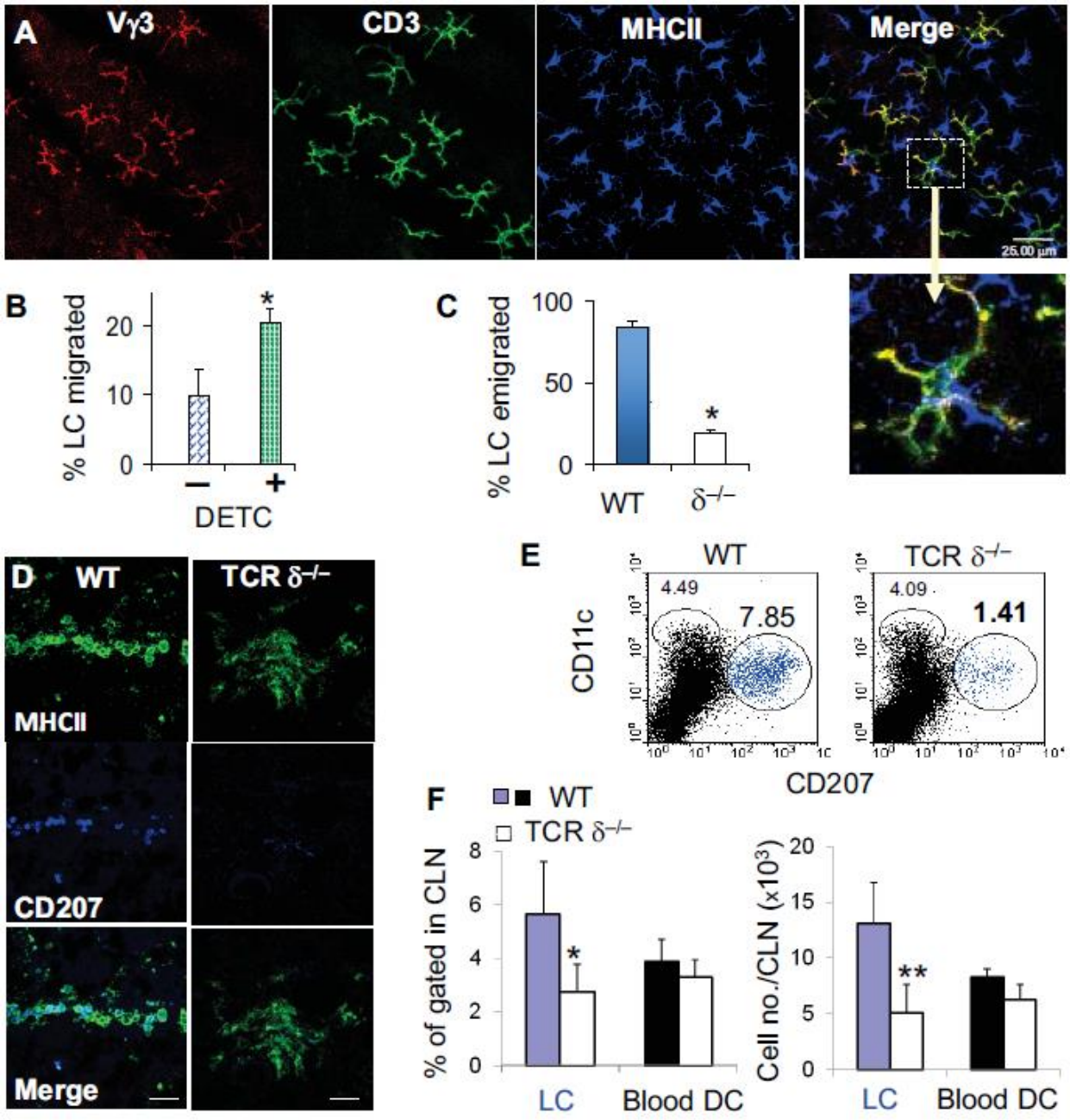

Fig. 1. **γδ DETCs promote LC migration**.

(**A**) **DETCs make a close contact with LCs *in vivo***. Epidermal sheets from mouse ear were stained for DETCs (CD3 and TCR Vγ3) and LCs (MHCII). Images are maximum projections of 8 sections spanning ~12 μm, at 63x magnification. Scale bars represent 25μm.

(**B**) **DETCs increase LC migration *in vitro***. LCs isolated from the epidermis of B6 mice were added to the upper chamber of transwells with or without DETCs. The proportion of LCs migrating to lower chamber was calculated (*$p < 0.05$). Bars, means ± SD of triplicates.

(**C, D**) **Reduced *in situ* LC migration in $\delta^{-/-}$ mice**. Dorsal ear half explants from WT and $\delta^{-/-}$ mice were cultured with cytokines (Supplemental Fig. 1). Epidermal and dermal sheets were then separated and stained for LCs. (***C***) LCs in epidermal sheets recovered before and after culture were counted. LC emigration was assessed by a reduction in epidermal LC counts after culture, and calculated as (1–[numbers of LC in cultured / numbers of LC in fresh]*100). Results are expressed as the mean ± SEM % emigrating cells (*$p < 0.01$, $n = 5$ mice per group). (**D**) Confocal images of dermis show migrating LCs as 'dermal cords' that represent LCs migrating through dermal lymphatics in the WT, but not in $\delta^{-/-}$ mice. Scale bars represent 40μm.

(**E, F**) **Reduced *in vivo* steady state migration of skin DCs in $\delta^{-/-}$ mice**. CLN cells from 8–12-wk-old mice were analyzed for langerin$^+$ skin DCs (blue gate, CD207$^+$CD11c$^+$) and other mDC (black gate, CD11c^{hi}CD207$^-$) among FSC^{hi}SSC^{hi} CD8α$^{lo/-}$ cells. (**E**) Numbers on a representative dotplot indicate percentage of langerin$^+$ skin DCs and other mDC of gated cells. (**F**) Proportion and total numbers of langerin$^+$ skin DCs per CLN are shown as the mean ± SD from 5 mice per group (*$p = 0.01$).

Results are representative of three (**A–D**) or two (**E, F**) independent experiments.

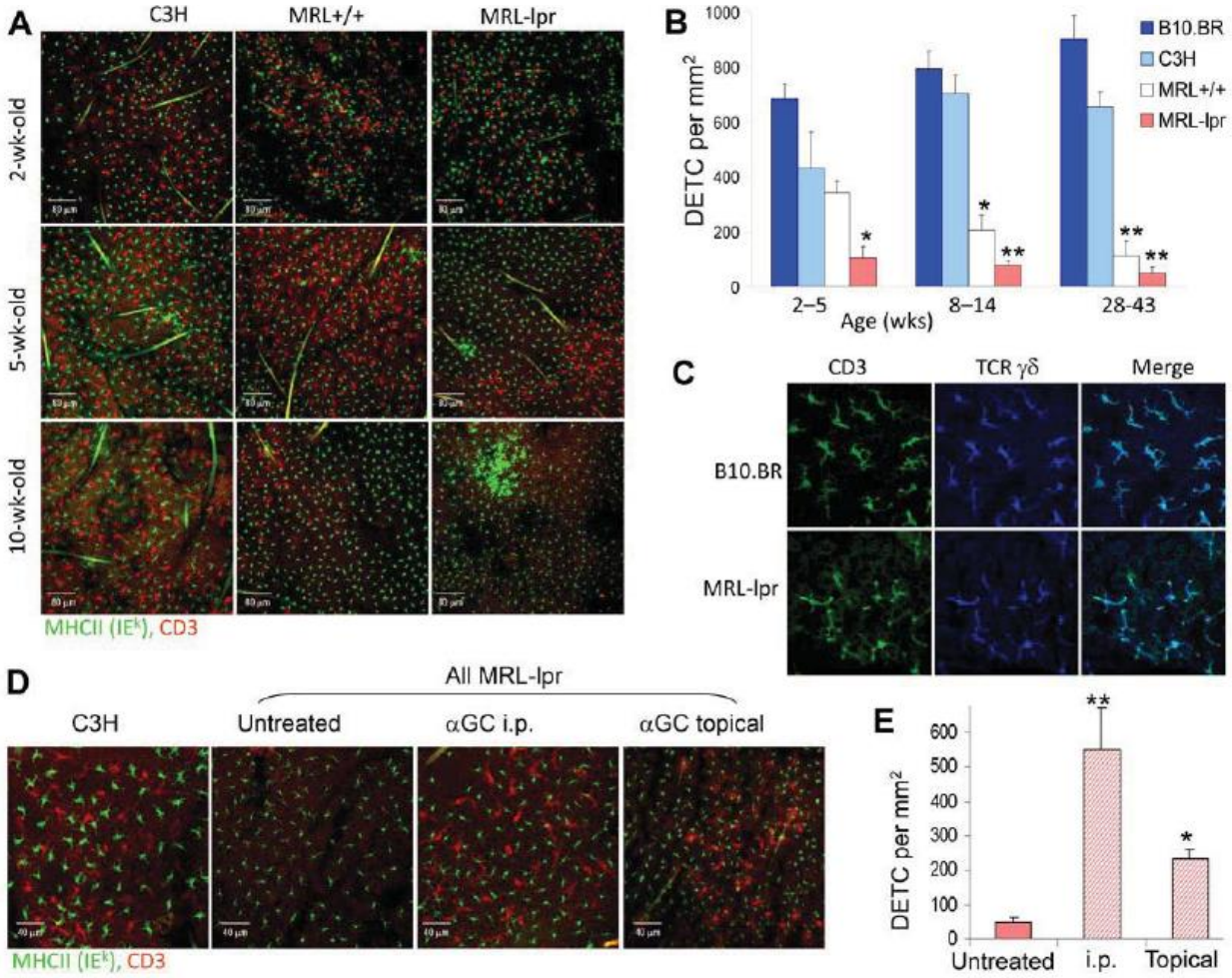

Fig. 2. **γδ DETCs in autoimmune skin inflammation-prone MRL mice.**

(A-C) DETCs are reduced in MRL mice. Epidermal sheets were stained for T cells (CD3) and LCs (MHCII). (**A**) Confocal images are shown as the maximum projections of 8 sections spanning ~8μm, 20x magnification. T-cell numbers decline with age in MRL mouse strains, which is summarized in (**B**) as the mean ± SEM DETC per mm^2 of epidermal sheet (*$p < 0.05$, **$p < 0.001$ vs. control strains; n = 3-5 mice per strain at each age group). (**C**) Representative DETCs ($CD3^+TCR\gamma\delta^+$) rich area in epidermis from 4-wk-old mice.

(**D, E**) **αGC treatment enhances DETCs in MRL-lpr mice**. αGC was administered i.p. or topically, and epidermal sheets were harvested after 3-14 days or 7 days, respectively, and stained for CD3 (red) and MHCII (green). An epidermal sheet from control C3H mice is shown for comparison. Shown are maximum projections of 5 sections spanning ~6 μm. Scale bars, 80μm.

The mean ± SEM T cell numbers in epidermal sheets are shown in **E** (*p = 0.001; **p = 0.0003, vs. untreated; *n* = 7 untreated, and 5 i.p. and 2 topical αGC treated 10–14-wk-old mice).

Data represent three to five independent experiments.

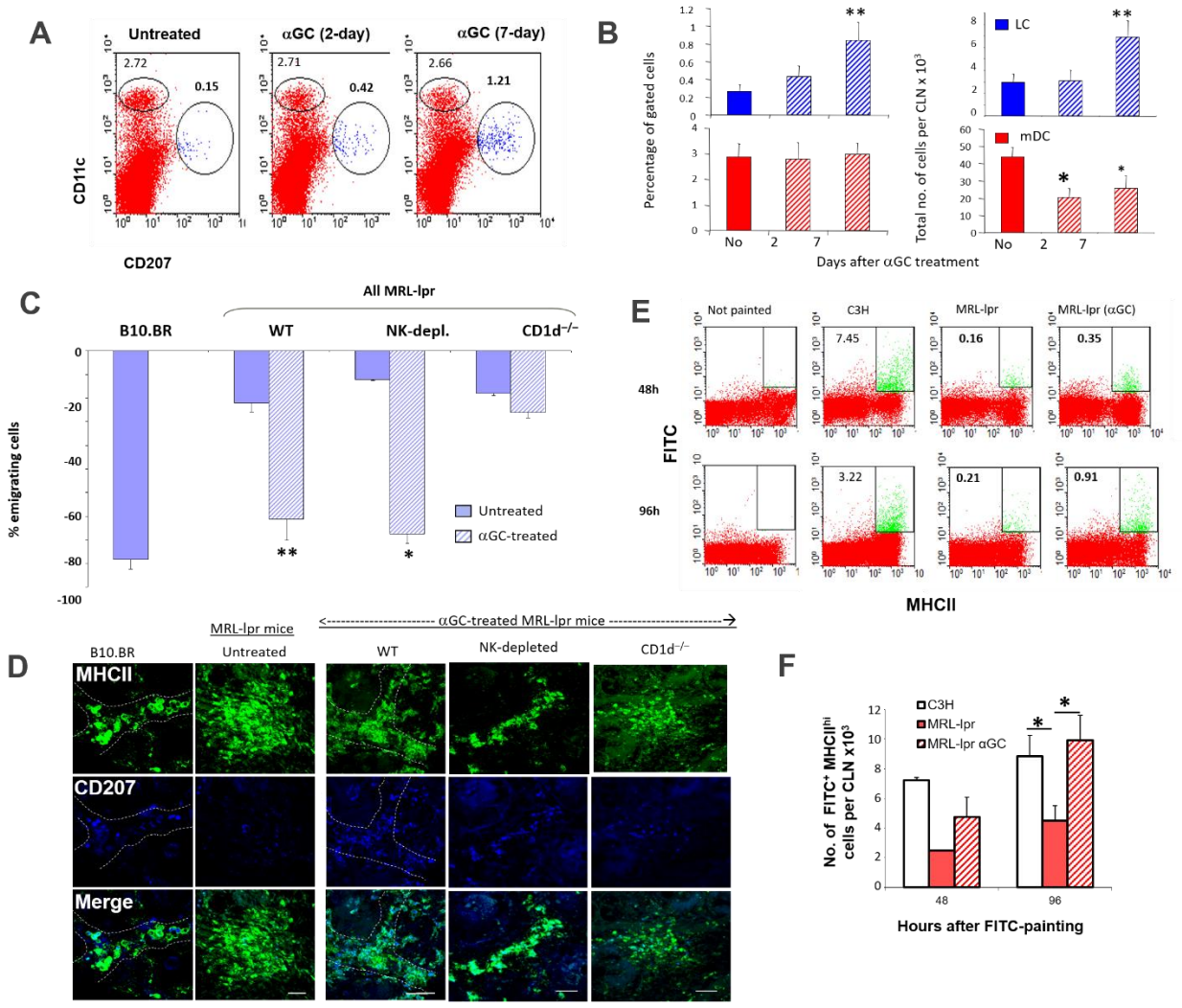

Fig. 3. αGC treatment restores LC migration in MRL-lpr mice.

(A, B) Steady state in vivo migration of langerin$^+$ skin DC. MRL-lpr mice were treated with αGC at the indicated timepoints prior to harvesting CLN, and CLN cells analyzed for langerin$^+$ skin DCs (blue, CD11c$^+$CD207$^+$) and other mDC (red gate, CD11c^{hi}CD207$^-$) on gated FSC^{hi}SSC^{hi} CD8α$^{lo/-}$ cells. (**A**) Numbers on dotplots indicate % of langerin$^+$ skin DCs and mDC of gated cells. (**B**) The mean ± SD % and numbers of langerin$^+$ skin DCs and mDC are shown (*p<0.05, ** p<0.01, vs. untreated, *n*=9-11 mice per group).

(C, D) In situ LC migration. Dorsal ear half explants from 9–12-wk-old mice were cultured with cytokines, after which epidermal and dermal sheets were separated and stained for LCs, as in Supplemental Fig. 1. (**C**) LCs were counted in epidermis before and after culture, and

LC emigration was assessed, as in **Fig. 1C**. Results are expressed as the mean ± SEM % LCs emigrating from the epidermis. LC emigration that is lower in MRL-lpr mice than in control mice is restored to normal after αGC injection (*p<0.001, *n*=4-6 mice per group). (**D**) Confocal images of post-culture dermal sheets show maximum projections of 8 sections spanning 15-20μm. Scale bars represent 40μm. Dotted lines represent formations called "dermal cords" containing LC (blue/green) and dermal DC (green) in lymphatic vessels. Such cords are not seen in untreated MRL-lpr mice but reappear in mice treated with αGC (4μg i.p.) a week ago).

(**E, F**) ***In vivo*** **activation-induced skin DC migration.** Ears of 10–13-wk-old mice were painted with FITC. After the indicated timepoints, CLN were harvested and CLN cells analyzed for immigrant DC ($FITC^+MHCII^{hi}$) that are indicated as % of gated cells on dotplots (**E**). All $FITC^+MHCII^{hi}$ cells (green) were $CD11c^+$ and expressed high levels of CD86 (not shown). Dotplot from a non-painted C3H mouse is shown as a control. One group of MRL-lpr mice were treated with αGC (4μg i.p.) 24h prior to FITC painting. (**F**) Total numbers of $FITC^+MHCII^{hi}$ cells per CLN at the indicated timepoints after painting (*p < 0.05, n = 4 mice per group).

Data are representative of four (**A–D**) or two (**E, F**) independent experiments.

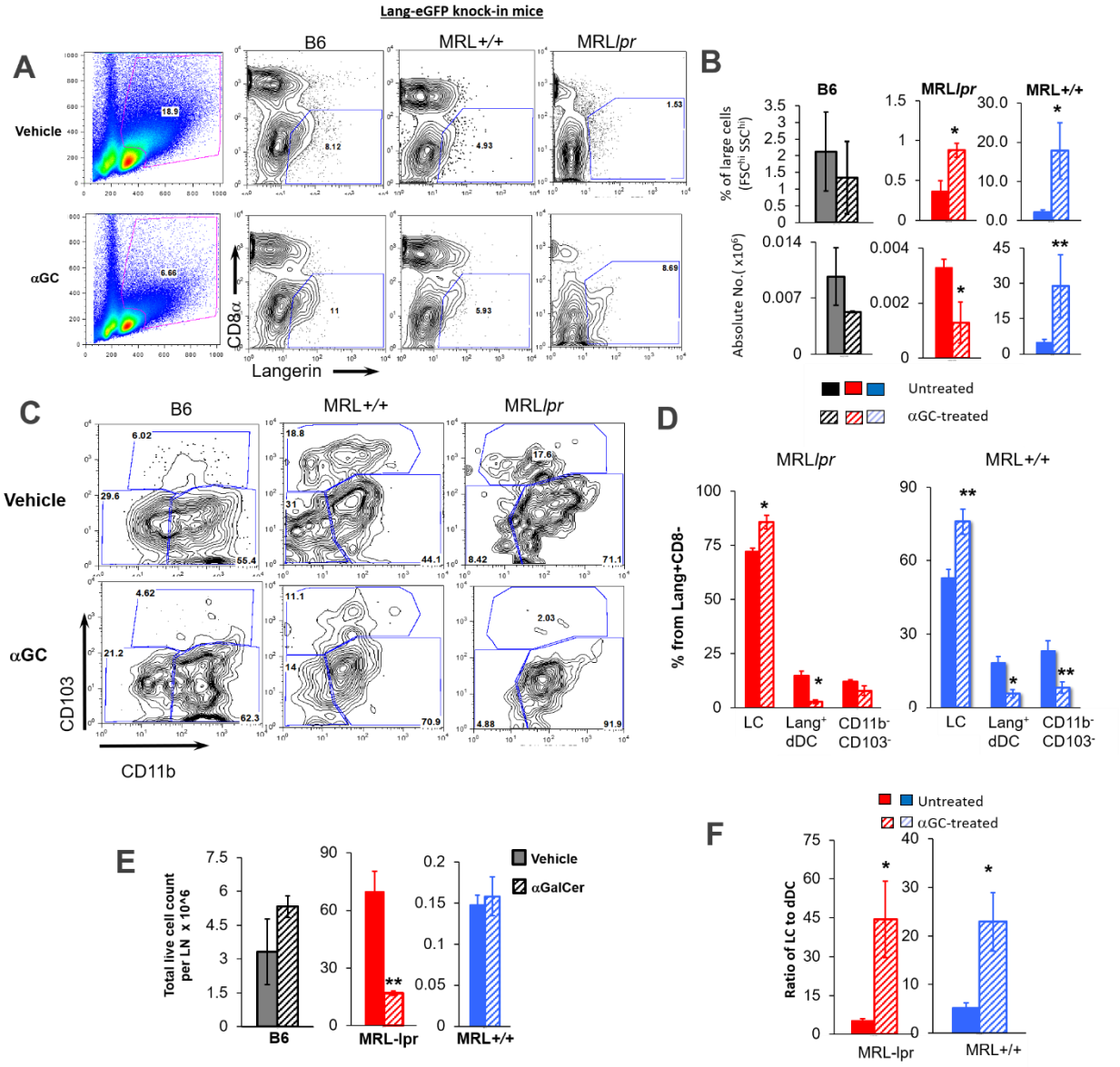

Fig. 4. αGC treatment differentially affects the migration of LCs versus Lang$^+$ dDCs.

(A-B) CLNs from Lang-eGFP C57Bl/6 (B6), MRL+/+ and MRL-*lpr* mice that express GFP under Langerin promoter (Lang-GFP) were harvested 7 days after receiving an i.p. injection of αGC. Isolated cells were counted (**E**) and analyzed by flow cytometry for LCs that were defined as GFP$^+$ (langerin$^+$) CD8α$^-$ cells on gated FSC^{hi}SSC^{hi} cells. Numbers on FACS plots represent the proportions of FSC^{hi}SSC^{hi} cells. Representative contour plots from vehicle injected control mice (upper panels) and αGC-treated mice (lower panels) are shown. Results are summarized in **B** as

the proportions and absolute numbers of langerin-expressing skin DCs ($CD8\alpha^-$ $langerin^+$). (**C**-**D**) $CD8\alpha^-GFP^+$ cells were then analyzed for LCs as $CD11b^+CD103^-$ cells, and $langerin^+$ dermal DCs ($Lang^+$ dDC) as $CD11b^{lo/-}CD103^+$ cells, and $CD11b^{lo/-}CD103^-$ cells. Representative contour plots from vehicle injected control mice (upper panels) and αGC-treated mice (lower panels) are shown. Results are summarized in **D** as proportions of LCs and $Lang^+$ dDCs. (**E**) Total live cell count per lymph node in vehicle injected control mice and αGC-treated mice. (**F**) Ratio of LC to $Lang^+$ dDC in the CLN 7 days after a αGC injection.

Results represent five separate experiments, each using 3-5 animals per group of 10-week-old MRL-*lpr*, 20-22-week-old MRL+/+ and 20-week-old B6 mice. * $p<0.05$, ** $p<0.01$; n = 3-5 mice per group; mean ± SE.

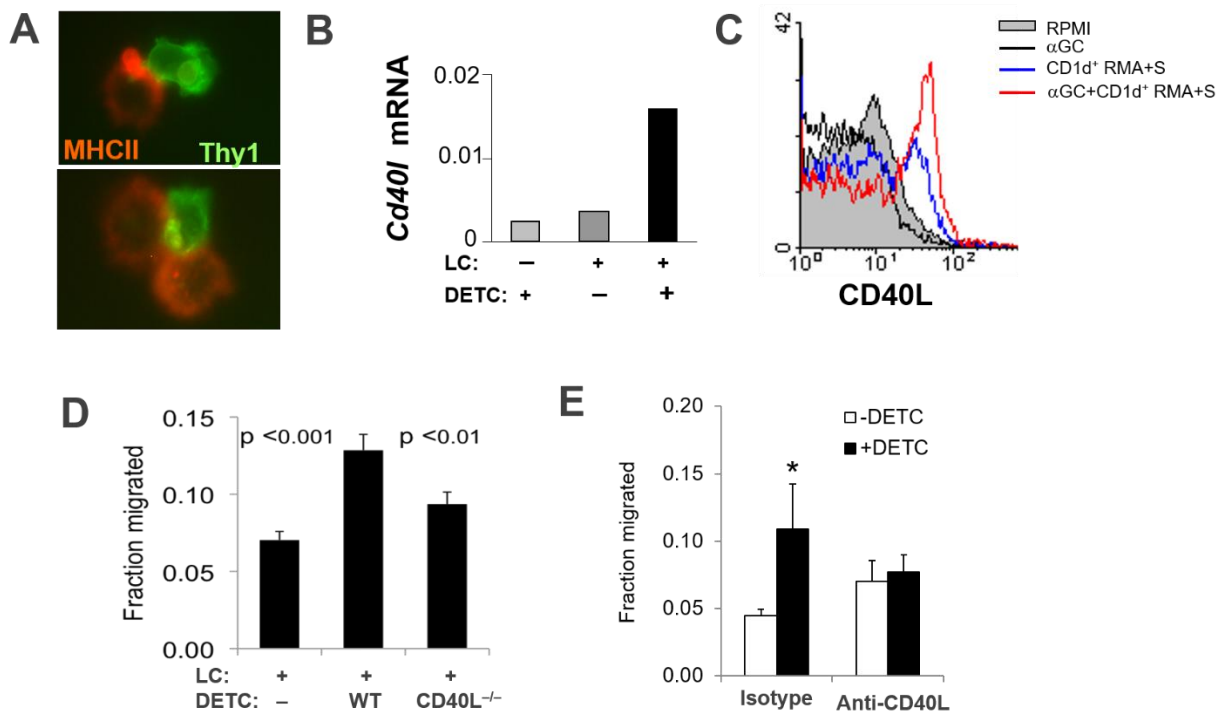

Fig. 5 Role of CD40L on DETCs in mediating LC migration.

(**A**) **DETCs and LCs directly interact with each other**. DETCs and LCs isolated from the epidermis were cultured alone or together for 3 h. Cells were then collected with vigorous pipetting and stained as indicated. Cells were vortexed, agitated with vigorous pipetting, wet-mounted on a slide for fluorescence microscopy (**A**). A population of large cells stained for both MHCII and CD3 (**A**) and ~5% of cells showed pairing between red (LC) and green (DETC) cells.

(**B,C**) **Induction of CD40L expression on DETC**. (**B**) Freshly isolated DETCs and LCs from the epidermis of B6 mice were cultured alone or together. RNA was extracted and analyzed for *Cd40l* mRNA expression using real-time quantitative PCR. Results are expressed as *Cd40l* mRNA levels relative to β-actin expression. Short-term DETC cell lines were generated. After resting these cells for 14 days since the last con A stimulation, 10^5 DETCs were cultured in complete medium with 10% FCS+10U IL-2 and 100ng/ml αGC and/or 10^4 CD1d-transfected RMA-S cells (**C**). CD40L expression was detected by flow cytometry.

(**D, E**) **Deficiency or Ab blockade of CD40L abolishes DETC-induced enhancement of LC migration**. LCs from WT B6 mice were added to transwell culture chambers alone or

with DETCs from WT or CD40L$^{-/-}$ B6 mice (**D**). In (**E**), LCs were added to transwells alone or with DETCs in the presence of a blocking anti-CD40L or isotype matched control Ab. *In vitro* migration of LCs was assessed, as described in **Fig. 1B**. Bars, means ± SD of triplicates.

Data represent three (**A, C**) or two (**B, D, E**) independent experiments.

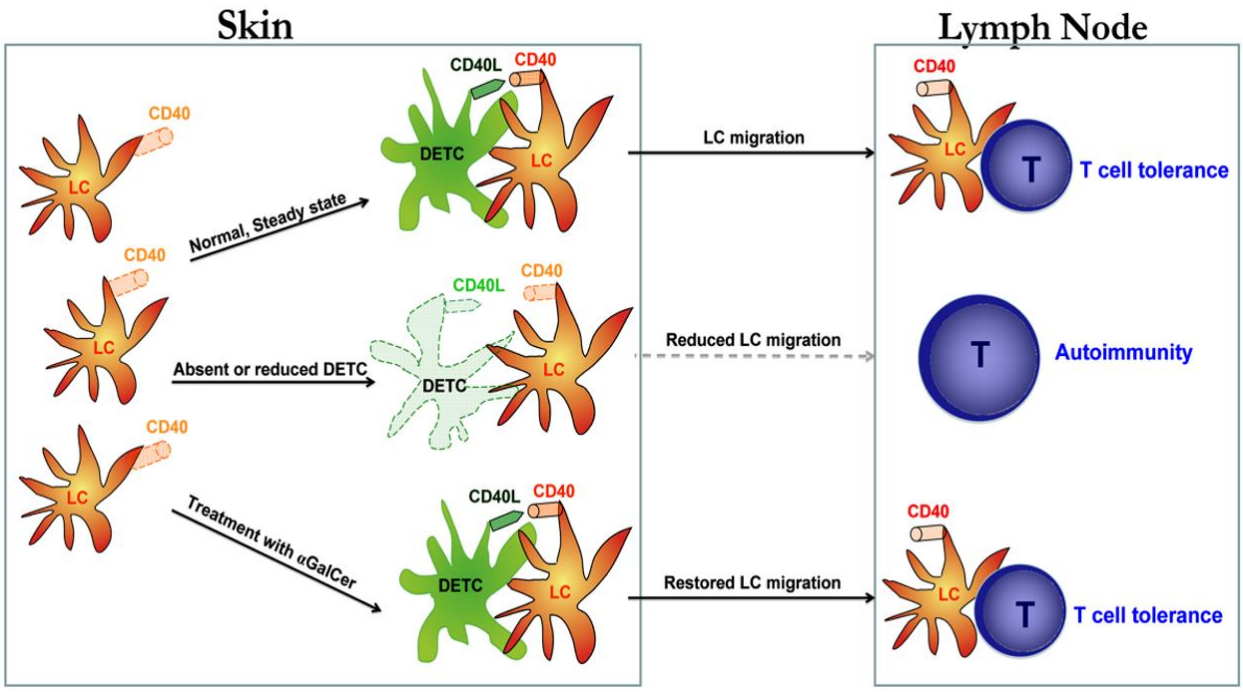

Fig. 6. Graphical representation of summary of our data and its implications for the induction of autoimmunity.

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Chapter 4-

Summary

The purpose of this dissertation was to expand on the current understanding of Langerhans cell (LC) biology, by addressing LC's in vivo functions from different perspectives. We found that LCs protect against ocular surface inflammation, which resembles LC's role in skin autoimmunity. Additionally, we discovered a new mechanism of regulation of LC migration from skin to skin-draining lymph nodes, which may be central to the understanding of tolerance breakdown and autoimmunity in a tissue-specific manner. These important additions to the growing body of knowledge on LC biology provide new perspectives on the role of this unique cell type in tissue homeostasis.

One of the tenets of the evolving LC paradigm is that LCs promote immune tolerance in a tissue-specific manner [77, 78]. This has been extensively studied in the context of skin contact hypersensitivity [21, 26, 56], but only recently has it been addressed in the context of systemic autoimmunity. A germline knockout of *Cd11c*, which leads to depletion of all dendritic cells, led to reduced systemic organ damage without affecting T and B cell activation in MRL-*lpr* mice [79]. In contrast, repeated LC depletion in adult MRL-*lpr* mice led to accelerated onset of more severe autoimmune skin disease along with increased autoantibodies against skin antigens, without any effect on kidney disease or autoantibodies against systemic autoantigens [80]. LC depleted MRL-*lpr* mice had less IL-10 producing regulatory T cells than their LC-intact littermates. Furthermore, epicutaneous delivery of a skin specific antigen, desmoglein 3 (Dsg3), led to antigen-specific tolerance along with an expansion of $CD4^+CD25^+FoxP3^+$ regulatory T cells in skin-draining lymph nodes. Such induction of tolerance and expansion of regulatory T cells did not occur in MRL-lpr mice depleted of LCs [80]. These observations suggest that LCs protect against autoimmune skin disease via promoting tolerance to skin antigens. This was further supported by other reports showing that LCs prevented autoimmunity through

keratinocyte antigen specific expansion of regulatory T cells [81]. While these studies firmly established LC's protective role in skin autoimmunity, it was unclear if LCs have the capacity to protect against autoimmune disease in other, non-skin, tissues where they may reside. LCs are present in almost all epithelial layers, such as gut and genital tracts, as well as on the surface of eyes [77]. We focused on the eyes, because the eye surface is affected by autoimmune dry eye disease known as keratoconjunctivitis sicca (KS) or secondary Sjogren's syndrome, which is the most common manifestation of systemic lupus erythematosus (SLE) in the cornea [1]. We set out to answer this question by employing an *in vivo* mouse model for LC depletion in an autoimmune genetic background [80]. We showed that MRL+/+ and MRL-*lpr* mice develop the hallmark corneal pathology associated with lupus keratitis, such as epitheliopathy, stromal edema, and leukocyte infiltration. Using Lang-eGFP knockin mice, we showed an altered distribution of LCs in the cornea in early stages of lupus keratitis, which suggested that the dynamic mobility of LCs in the cornea previously described [6] may be altered prior to the onset of autoimmune corneal inflammation. Lastly, by continuously depleting LCs in vivo, we showed a significant exacerbation of corneal pathology, marked by increases in stromal thickening (edema), epithelial erosion, and calcium deposition. Continuous LC depletion also caused an increased $CD8^+$ T cell proliferation of lymphocytes from eye-draining lymph nodes, but not those from spleen. LC-depleted mice also exhibited a loss of naïve T cell phenotype in eye-draining lymph nodes. Thus, LC depletion may lead to an expansion of activated effector cells in local tissue-draining lymph node, which can promote autoimmune disease in the respective tissue. Overall, this study makes a strong case that cornea resident LCs, similar to their skin counterparts, protect the local tissue from autoimmune pathology. It remains to be determined whether this protection is based on tolerance to cornea-specific antigens or through another

mechanism. Nevertheless, we, for the first time, provide evidence for a protective role of LCs in vivo in cornea.

Migration is a fundamental trait of LCs that differentiates them from tissue-resident macrophages and portrays them to resemble classical DCs [77]. Steady-state migration from skin to lymph nodes is fundamental for their tolerogenic role, and disruption in LC trafficking has been associated with the onset of autoimmunity [44, 82]. Our results uncovered a new mechanism of LC migration regulation that can potentially explain events that lead to the breakdown of immune tolerance in skin. We demonstrated that dendritic epidermal T cells (DETCs), one of the skin's resident T cells, promoted LC migration *in vitro* in normal mice. In genetically autoimmune dermatitis-prone MRL mice, DETCs were markedly reduced in the epidermis prior to the onset of clinical skin disease, which temporally coincided with the accumulation of LCs in the epidermis that is a consequence of defective migration of LCs from skin to skin-draining lymph nodes [44]. Furthermore, the numbers of DETCs inversely correlated with the numbers of LCs in the epidermis of MRL-*lpr* mice, suggesting a possible relationship between reduced DETCs and impaired LC migration. In resonance with this notion, treatment of MRL mice with the CD1d binding glycolipid α-galactosylceramide (αGC), which has been reported to ameliorate lupus dermatitis [47], rescued LC migration while also restoring the presence of DETCs in epidermis. Finally, a complete absence of DETCs (in γδ T cell-deficient in normal B6 background) led to reduced migration of LCs from the skin to skin-draining lymph nodes. These results indicate a role of DETCs in promoting LC migration.

How might DETCs interact with LCs to promote LC migration? We found that co-culture with LCs increased *Cd40l* gene expression in DETCs, and a cell line transfected with CD1d and loaded with αGC enhanced the expression of CD40L on DETCs after co-culture. These

observations along with previous studies suggesting a role of CD40-CD40L interactions in increasing LC migration [83-85] led us to ask if DETC may promote LC migration via CD40-CD40L interaction. Indeed, germline deficiency or antibody blockade of CD40L in DETCs abrogated DETC-induced increase in LC migration in vitro. Taken together, these results indicate the role of crosstalk between LCs and DETCs via CD40-CD40L in modulating LC migration.

αGC activates natural killer T cells and has modulatory effects on several immune cells and functions, which include a protective effect in MRL mice [46, 47, 68-70]. However, improvement in LC migration was independent of αGC-induced activation of natural killer T cells. Instead, in vivo effect of αGC treatment was CD1d-dependent, as LC migration and DETC presence was not rescued by αGC in CD1d-deficient MRL mice. In the epidermis, in addition to LCs, CD1d can be expressed by keratinocytes in a ceramide-dependent way [72], and keratinocytes has been shown to be important mediators of activation-induced LC migration [86].

In addition to LCs, skin contains a subpopulation of Langerin-expressing migratory DCs in the dermis, called Langerin$^+$ dermal DCs (Lang$^+$ dDCs) [32, 52]. Treatment with αGC increased the migration of LCs but not of Lang$^+$ dDCs, resulting in a dramatic shift in the ratio of LCs to Lang$^+$ dDCs in skin-draining lymph nodes. The latter population has been shown to exhibit increased migration from skin to skin-draining lymph nodes upon activation of tissue-resident autoreactive effector T cells; such increased migration of Lang$^+$ dDCs has been implicated in the development of autoimmune disease [50]. Thus, a relative decrease in Lang$^+$ dDCs in skin-draining lymph nodes after αGC treatment might underlie αGC's and LC's protective effects in lupus dermatitis.

Taken together, our data support a mechanism of local control of LC migration that is dependent on interactions between DETCs and LCs via CD40-CD40L. This regulatory axis seems to breakdown in autoimmune-prone conditions, leading to development of autoimmune disease in local tissues. Similar tissue-specific mechanisms may exist in other organs. Thus, our observations may have broad implications for the pathogenesis and immune intervention for autoimmune diseases as well as for graft rejections, wound healing, cancer, and vaccination.

References 4

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