Investigating role of IL-4 receptor alpha (IL-4Rα) in murine models of *atopic dermatitis*

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DECLARATION

I, *Martyna Scibiorek*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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List of abbreviations

Ig	Immunoglobulin
Th	T helper
FcεRI	High-Affinity Receptor for IgE
IL	interleukin
FcγRIIb	Fc gamma receptor IIb
CD	cluster of differentiation
AD	atopic dermatitis
TEWL	transepidermal water loss
NMFs	natural moisturizing factors
TSLP	thymic stromal lymphopoietin
KRT	Keratins
IVL	involucrin
TGM	transglutaminases
LOR	loricrin
FLG	filaggrin
ECM	extracellular matrix
γδ T	gamma delta
LCs	Langerhans cells
Bregs	B regulatory cells
LN	lymph nodes
Tregs	T regulatory cells
TLRs	Toll-like receptors
KCs	keratinocytes
PAMPs	pathogen-associated molecular patterns
DMAPs	danger-associated molecular patterns
PPRs	pathogen recognition receptors
ILC	Innate lymphoid cells
CLA	cutaneous lymphocyte antigen
CXCR	C-X-C chemokine receptor
ICOS	inducible T-cell costimulatory
OX40L	TNF receptor superfamily ligand
CysL	cysteinyl leukotrienes
CysLT	cysteinyl leukotrienes receptor
CCR	C-C chemokine receptor type
Ly6G/GR-1	Lymphocyte antigen 6 complex locus G6D
GC	germinal center
DP	double positive
DN	double negative
SP	single positive
T-bet	T-box transcription factor TBX21
GATA3	GATA Binding Protein 3
RORγt	RAR-related orphan receptor gamma
Foxp3	forkhead box P3
DETC	Dendritic epidermal T cells
IGF-1	insulin growth factor -1
Skint1	selection and upkeep of intraepithelial T cells protein 1

CTLA	cytotoxic T-lymphocyte-associated protein
MHC	major histocompatibility complex class
AIRE	autoimmune regulator
MPP	multipotent progenitor
CLP	common lymphoid progenitor
HSC	hematopoietic stem cell
LMPP	and lymphoid-primed multipotent progenitor
CILP	common innate lymphoid progenitor
TSP	thymus-settling progenitor
Foxp3	follicular
MZ	marginal zone
SHM	somatic hypermutation
CSR	class switch recombination
Abs	antibodies
TGFβ	transforming growth factor β
(LCs)	Langerhans cells
MC	Mast cells
CNS	central nervous system
CCL5	Chemokine ligand 5
HFSC	hair follicle stem cells
IFN-γ	interferon -γ
LEKTI	lymphoepithelial Kazal-type trypsin inhibitor
KLLK	kallikreins
PAR2	with protease activated type 2 receptor
SNP	single nucleotide polymorphisms
CLDN-1	claudin-1
IL-4Rα	interleukin 4 receptor alpha
JAK/STAT6	Janus kinase 1/ Signal transducer and activator 6
AhR	aryl hydrocarbon receptor
AMPs	antimicrobial peptides
PIA/PNAG	polysaccharide intercellular adhesin/ poly-N-acetyl-glucosamine
ROS	reactive oxygen species
NOS	reactive nitrogen species
NET	neutrophil extracellular traps
ICAM-3	intercellular adhesion molecule-3
CLR	C-type lectin receptor
AMPs	atopic march
OVA	ovalbumin
HDM	house dust mite
PBS	Phosphate-Buffered Saline
EtOH	ethanol
PFA	paraformaldehyde
RPMI	Roswell Park Memorial Institute
BAL	Broncho-alveolar lavage
H&E	hematoxilin and eosin
PAS	Periodic acid–Schiff
ELISA	enzyme-linked immunosorbent assay
BSA	bovine serum albumin

AP	alkaline phosphatase
PNP	4-Nitrophenylphosphate

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Abstract

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease with complex environmental and genetic predisposition factors. Primary skin barrier dysfunction and aberrant T helper 2 (TH2) responses to common allergens, together with increased serum IgE antibodies, characterise the disease. B and T cells are essential in the disease manifestation, however, the exact mechanism of how these cells are involved in skin sensitization to allergens is unclear.

Clinical studies investigating the efficacy of monoclonal antibody to IgE such as omalizumab and ligelizumab do not show efficacy in AD patients. However, targeting IL-4/IL-13 signalling axis with dupilumab show efficacy in AD. We investigated the importance of interleukin 4 receptor alpha (IL-4Rα) signalling specifically on B and T cells to understand the requirement of this signalling axis in epicutaneous skin sensitisation during AD.

We investigated 3 models of AD using House dust mite (HDM), Ovalbumin (OVA) and low-calcemic analog of vitamin D (MC903) on mouse strains lacking IL-4Ra on various B and T cells. We used mb1^{cre}IL-4Rα$^{-/lox}$ (mice lacking IL-4Rα on B cells), iLcK^{Cre} IL-4Rα$^{-/lox}$ (mice lacking IL-4Rα on all T cells), LcK^{Cre} IL-4Rα$^{-/lox}$ (mice lacking IL-4Rα on CD4+ and CD8+ T cells), CD4^{Cre} IL-4Rα$^{-/lox}$ (mice lacking IL-4Rα on CD4+ Tcells), Foxp3^{Cre} IL-4Rα$^{-/lox}$ (mice lacking IL-4Rα on Foxp3+ T regulatory cells) and IL-4Rα$^{-/lox}$ littermate controls. We analysed cellular infiltrate in the skin and inguinal lymph nodes (LN) by flow cytometry, histology of the skin, serum antibodies and cytokines by ELISA.

Mice lacking IL-4Rα-responsive B cells showed a reduced serum IgE levels, but no significant differences in epidermal thickening compared to littermate control in HDM or MC903 models. Mice investigated in the T cell arm of the study showed reduced epidermal thickening in pan-T cell IL-4Rα knock-out, but not in groups lacking IL-4Rα signalling in adaptive T cells, suggesting importance of IL4/IL13 signalling axis in ydT cells during AD.
Overall, our results suggest that deletion of IL-4Rα on innate T cells regulates inflammatory response in atopic dermatitis.

Key words: atopic dermatitis, interleukin 4 receptor alpha, allergy, T cells, B cells**,** murine model

Chapter 1

Literature Review

1.1 Allergy overview

Allergic diseases are one of the biggest groups of non-communicable diseases characterized by hypersensitivity of the immune system to an inappropriate external antigen. Allergic diseases include asthma, urticaria, angioedema, allergic rhinitis, food allergy, drug hypersensitivity, and atopic dermatitis [10–12].

The development of the allergic reaction is not fully understood. The rise of allergy cases has been mostly seen in industrialized countries, with a sensitization rate to one or more allergen between 40 to 50% of school children [13]. The economic burden of allergic rhinitis and urticaria alone has been estimated to USD 105,4 billion per year in Asia alone [14]. The development of allergic diseases has been tightly connected with urbanization and industrialization therefore, to prevent the high costs a better treatment and management are required.

According to Coombs and Gell, there are four main types of hypersensitivity [15]. Type I is mediated by immunoglobulin E (IgE), where mostly T_{helper} (Th)-2 cells as well as B cells are involved. The IgE binds to its high-affinity receptor Fc epsilon RI (FcεRI) on mast cells and basophils, where it activates cells and leads to the release of cellular content [16]. Other mediators stimulating type I hypersensitization are interleukins (IL): -4, -13, and -5. Type II reaction is mitigated by IgG and IgM antibodies, which lead to activation of cytotoxic response [15]. This reaction can be further divided into type IIa and type IIb. Type IIa is characterized by complement-dependant cytotoxicity. In type IIb IgG antibodies bind to Fc gamma receptor IIb (FcγRIIb) on natural killer cells and macrophages leading to cell activation and release of cytotoxic granules such as perforins and granzymes [17]. Type III responses are characterized by the formation of immune complexes, which deposit in blood vessels leading to activation of complement pathway and organ damage [17]. This response is associated with soluble antigens such as thymoglobulin or penicillin [15]. Lastly, type IV sensitivity also called delayed reaction, involves T cell cytotoxicity as well as recruitment of other immune cells such as macrophages or eosinophils [15]. Depending on the T cell mechanism involved, the further subclassification can be used; type IVa where Th1 cells activate macrophage-induced cytotoxicity, type IVb where Th2 cells recruit eosinophils to site of inflammation, type IVc where cluster of differentiation (CD) 8+T directly mediate cytotoxicity, and finally IVd where T cells recruit neutrophils to the site of inflammation [15].

The predisposition to mount IgE response to an innocuous allergen is called atopy [18]. Often the atopic individuals will develop more than one allergic disorder throughout their life for instance early-childhood onset of atopic dermatitis is an indicator of later development of food allergy or asthma, this mechanism is known as atopic march[19].

Various environmental, immunological, and genetic factors can contribute to the development of atopy, proving an understanding of allergic disease more complex.

1.2 Atopic Dermatitis

Atopic dermatitis (AD) is a complex disorder of the immune system, which affects about 20% of children and 3% of adults[20]. The major differences between young infants and adults are the physiology of skin and as a result the pathophysiology of AD. The differences include smaller corneocytes, thinner epidermis and stratum corneum, increased transepidermal water loss (TEWL), proliferation rate of keratinocytes, surface lipid concentration as well as natural moisturizing factors (NMFs). As a result, infants have impaired epidermal barrier structure, leaving them more vulnerable to sensitization by higher absorption of various antigens[21]. The disease presentation can vary between adults, young children, and infants, which adds complexity to diagnostic practice[21].

The exact etiology of the disease has not been fully understood, however, there are few intrinsic factors, such as disruption of skin barrier integrity due to genetic predisposition, tight junction dysfunction or impairment of immune system[20].

Eczema can be considered an umbrella term for various skin disorders which present as eczematic, inflamed skin. In contact eczema, the skin becomes red and itchy or burning consequently to direct contact with skin irritant[22]. The allergic contact eczema appears red and itchy as a result of to the contact with foreign antigen[23]. Seborrheic eczema presents as mild skin inflammation most and commonly it is associated with Malassezia yeast[24]. In the clinical presentation seborrheic eczema shows as yellowish, oily, and scaly patches on scalp, face and ears[24]. Nummular eczema demonstrates as round and isolated patches of irritated skin on arms and lower legs, the patches might be crusted, scaling and extremely itchy[22,25]. Lichen simplex chronicus, otherwise known as localized neurodermatitis is induced by long-term rubbing, scratching or picking of the skin, the cause might be sensitivity or irritation which results in repeated itching and scratching cycles which resolve when irritation stops[26]. Stasis dermatitis is localized mainly on the lower legs and it is directly associated with circulatory problems[27]. Xerotic eczema is an age-associated disorder, which presents mainly in the elderly, as very dry skin on the lower legs[28]. Dyshidrotic eczema presents as irritation of the skin on palms of the hands and soles of the feet resulting in clear, very deep-seated blisters which itch and burn sensations[29]. Dyshidrotic eczema is associated with an allergic reaction to skin residing

microbiota or occupational contact allergens.

Despite the variation in presentation, the main treatment regime revolves around the maintenance of skin barrier using topical emollients as skin barrier integrity is crucial for atopy.

AD has a detrimental influence on patients quality of life as well as due to its chronicity it is a high burden on medical system[23]. The study on the association of AD and burden on the US medical system found that patients with AD despite being insured, had their AD prescriptions denied by insurers resulting in a higher financial burden of the disease[30]. This was also correlated with more often visits to the emergency department, which directly increases the cost for health care system[30]. As the AD patients report decreased quality of sleep and increased depression rate, it remains of utter importance to investigate the mechanism behind AD to alleviate patients quality of life[31,32].

1.3 Skin as a primary barrier

Skin is one of the biggest organs of the human body among lung and gut, on average weighing about 3 kg[33]. In classical physiology its measure was $2m^2$, however, a revised overview of skin including hair follicle surface area, which also contributes to basic functions of the skin, suggests at least $25m^2$ [33,34]. The main function of the skin is to serve as protection from the outer environment as well as to establish interplay with skin-residing microbiota[33]. Right after birth, skin becomes colonialized by the mother's vaginal microbiome, which has been correlated to healthy immune response further in child's life[35,36]. Therefore, its structure needs to be considered as one of the primary immune-regulatory organs. The skin consists of 3 major components: epidermis, dermis, and subcutaneous tissue. Each layer can be further divided into sublayers, where the structural cells playing various roles (Fig. 1.1).

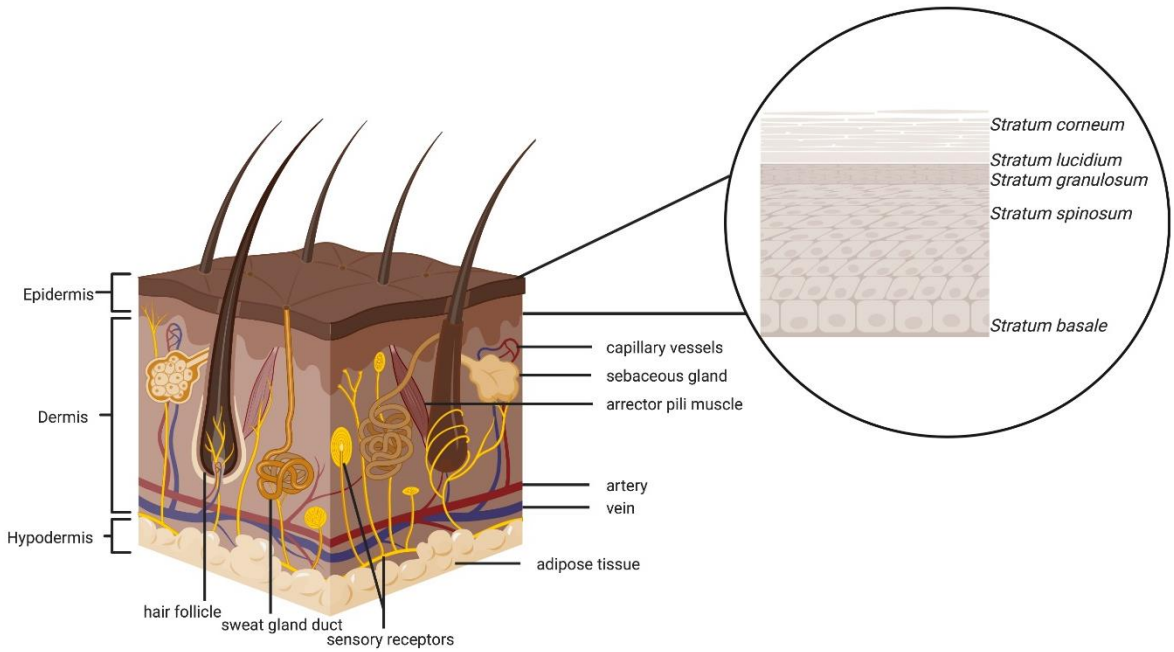


Figure 1.1 Skin structural composition. Skin can be divided into 3 major layers: hypodermis, dermis and epidermis. Hypodermis consists mostly of adipose tissue as well as various capillaries, which penetrates apically through to dermis. The dermis consists of keratinocytes, neuronal cells, muscle cells, melanocytes, sebaceous glands, stem cells including hair follicular stem cells, immune cells, arteries and veins. Epidermis consists of maturating keratinocytes migrating apically through *Stratum basale, spinosum, granulosum, lucidium,* and *corneum* using Ca^{2+} gradient[1]. Created in BioRender.com

The main structural cells in the skin are the keratinocytes, which migrate superficially from *stratum basale* towards *stratum corneum*, where they directly interact with skin microbiome and are exposed to unfavourable environmental conditions e.g. Ultra Violet rays, physical or chemical skin damage[37]. As the first line of defence, keratinocytes are capable of activation of immune cells by secretion of various alarmins such as thymic stromal lymphopoietin (TSLP), interleukin -25 (IL-25) or interleukin -33 (IL-33), which results in downstream activation of residing immune cells[38]. The disruption of keratinocytes integrity or in the microbiota can lead to leakage of microbial products or antigens into skin. In here they interact with various skin residing cell types such as Langerhans cells, intraepithelial T cells, gamma delta (γδ)T cells, or Th17, amongst others resulting in immune education, which later in life can be detrimental for the host[39–41].

1.4 Skin barrier integrity

The outermost layer of skin – epidermis consists of maturating keratinocytes which are migrating outwards from *stratum basale* through *stratum spinosum, stratum granulosum, stratum lucidium* towards *stratum corneum*, where they die off (Fig. 1.1). This differentiation is characterized by upregulation of layer-specific genes like keratins (KRT), cornified envelope precursors involucrin (IVL), transglutaminases (TGM), subsequently cornified envelope protein, loricrin (LOR) and precursor of the

keratin cross-linking protein filaggrin (FLG)[1]. Interestingly, Calcium forms a gradient across differentiating keratinocytes from 5 uM to >20 uM, therefore it is possible to study gene upregulation in primary human keratinocyte cultures by manipulation of calcium concentration[42–44].

The second most important compartment of the skin is the dermis, which is a mesenchymal component consisting of a papillary and reticular layer. The papillary dermis contains nerve endings and microvasculature vessels with a higher density of cells, but a weaker alignment of collagen fibres and higher proteoglycans than reticular layer[45–49]. Most abundant cells in dermis are fibroblasts with the primary function of synthesize and remodel the extracellular matrix (ECM). Aside from fibroblasts, CD31+ endothelial, CD45+ hematopoietic cells, neuronal cells, and sweat gland cells as well as keratinocytes can be found in dermis.

1.5 T and B cell development

T and B cells are derived from common lymphoid progenitor (CLP), where activation of RAG – mediated recombination of antigen receptors is promoted by E-proteins[2]. The transcription factors required for T cell differentiation are *Notch1, Gata3 and Tcf7*, while EBF1 and Pax5 is required for B - lineage commitment[2,50].

1.5.1 T cell development

As bone marrow cells expressing low levels of Notch1, they can stimulate the LMPP/CLP development, but it is insufficient for T cell lineage commitment[2]. The high levels of Notch1 in thymocytes promote further T cell development. The double negative (DN) cell can be further be transformed to either double positive (DP) and further to αβT cell or γδT cell by *Id2* and *Id3*[51]. As DP cells undergo further differentiation to single positive CD4+ or CD8+ T cells, their transcription is tightly regulated by *bcl11b* transcription factor. Upon this process, cells are also undergoing both positive and negative selection. The initial step is recognition of particular MHC molecule; CD8+ T cells recognize major histocompatibility complex class (MHC) I whilst CD4+ T cells recognize MHCII[52]. Cells that do not recognize MHC molecule on thymic stromal cells undergo apoptosis at this stage. Further, they interact with MHC expressed on thymic stromal cells expressing self-antigen, which is regulated by autoimmune regulator (AIRE)[53]. Upon strong recognition of self-peptide, T cells undergo apoptosis, this process is called negative selection. It is crucial to avoid auto-responsive T cells. Occasionally, the auto-responsive CD4+ T cells expressing Forkhead-family transcription factor (FoxP3), as well as CD25 and cytotoxic T-lymphocyte-associated protein (CTLA)-4 surface proteins are developed, those cells are called natural regulatory T cells (Treg)[54]. In periphery, the single positive CD4+ T cells can further be differentiated, depending on the cytokine environment into one of the Th subsets[2]. Those subsets

are characterized by the transcription factors T-box transcription factor TBX21 (T-bet), GATA Binding Protein 3 (GATA3), RAR-related orphan receptor gamma (RORγt) and Foxp3, each subset has a unique array of cytokine expression however, certain degree of differential plasticity remains (Fig. 1.2).

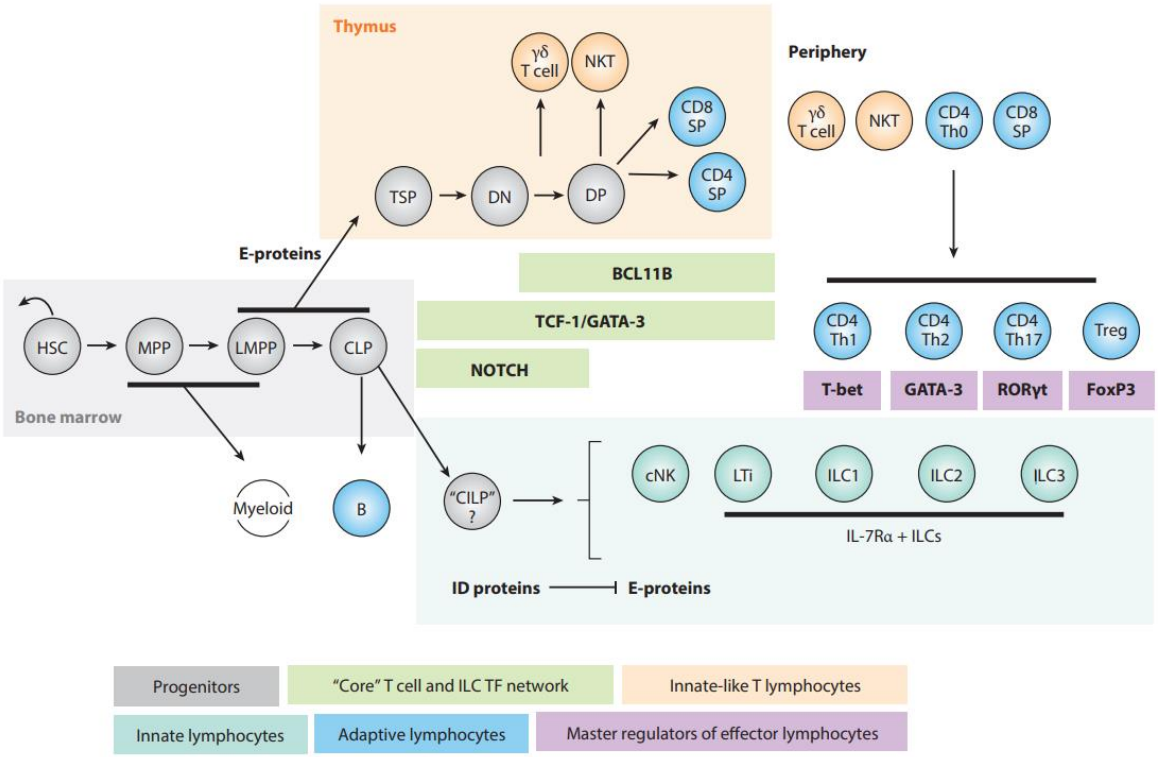


Figure 1.2 T cell development. The T cell development starts in bone marrow, where hematopoietic stem cell (HSC) differentiates to multipotent progenitor (MPP) and lymphoid-primed multipotent progenitor (LMPP). From there the myeloid cells can branch off or under control of E-proteins the common lymphoid progenitor (CLP) can be further differentiate towards B or T cells or hypothetical common innate lymphoid progenitor ("CILP"). Further T cell development occurs in thymus under control of NOTCH, TCF-1, GATA-3 and BCL11B transcription factors. Cells go from thymus-settling progenitor (TSP) to double negative (DN) and double positive (DP) stages, from which both γδT cells and NK T cells can differentiate. In periphery, the final differentiation occurs under control of T-bet, GATA-3, RORγt or Foxp3. Adapted from De Obaldia and Bhandoola 2015[2].

1.5.2 B cell development

Similarly, to T cells the wide range of B cells subsets exists including B-1a, B-1b, B-2, and Breg cells. The B-1 cells are derived from foetal liver and spleen and are self-renewable population capable of secretion of IgM and IgG3[55]. The B-2 cells are derived from bone marrow, where under control of EBF1, E2A, and Pax5 transcription factors, the cells go through pro-B and pre-B cell stages[50]. In T1/T2 transitional stage the cells can either become follicular (FO) or marginal zone (MZ) B cells, split of

cellular faith occurs in secondary lymphoid organs[56]. While MZ cells, can become short-lived plasmablast, and further plasma cell, with limited IgM repertoire. The FO cells can produce IgM and IgD can become short-lived plasma cells or enter to germinal centre (GC) stage where they can proliferate and diversify the IgV genes by somatic hypermutation (SHM) process, and their antibody repertoire undergoes class-switch recombination (CSR) providing different effector function for the antibodies (Abs)[57]. As a result, the Ab repertoire increases the affinity and specificity to the antigen.

Lastly, the Breg population of B cells, is capable of inducing immune tolerance and suppress the inflammation by secretion of IL-10, IL-35 and transforming growth factor β (TGF-β) , yet the exact aetiology of those cells is not fully elucidated[58] (Fig. 1.3).

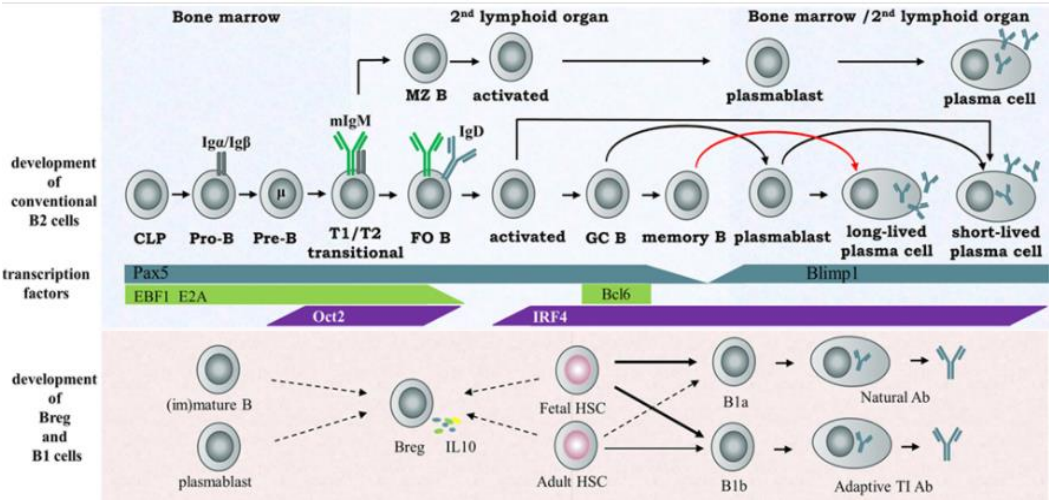


Figure 1.3 B cell development scheme. The development of conventional B2 cells starts in bone marrow, further the common lymphoid progenitor (CLP) is transformed to pro-B cell and further pre-B cells under control of Pax5 and EDF1 and E2A transcription factors. Further, the T1/T2 transitional B cell can develop to either marginal zone (MZ) B cell or follicular (FO) B cell in secondary lymphoid organ, under control of Oct2 transcription factor. Once activated, the FO B cell can enter germinal centre (GC) to undergo somatic hypermutation (SHM) and class-switch recombination (CSR) to differentiate into long-lived plasma cells producing array of specific antibodies. This differentiation happens under the control of IRF4, Blimp1 and Bcl6 transcription factors. The activated B cells can also differentiate into short-lived plasma cells under control of IRF4 alone. Once activated, the MZ cells can differentiate to plasmablast and further to plasma cells. The B-1 and Breg differentiation depicted in is not fully understood, the dashed arrows show suggested development, while solid arrows depict known routes of Breg development. Adopted from Zou et al. 2018. [3]

1.6 Skin as an immune organ

Skin residing cells both structural such as keratinocytes as well as infiltrating leukocytes like γδ T cells, and B cells are capable of interaction by secretion of various cytokines and chemokines, therefore skin can be perceived as an immune organ (Fig. 1.4). Upon epithelial damage, keratinocytes secrete alarmins such as IL-31, IL-25 and TSLP, which further activates and recruits immune cells like Langerhan cells amongst others [47,59–62]. At the same time, the skin residing immune cells provide both

supportive as well as immunosurveillance functions[4,6]. This bi-directional information exchange contributes to immune tolerance and host protection.

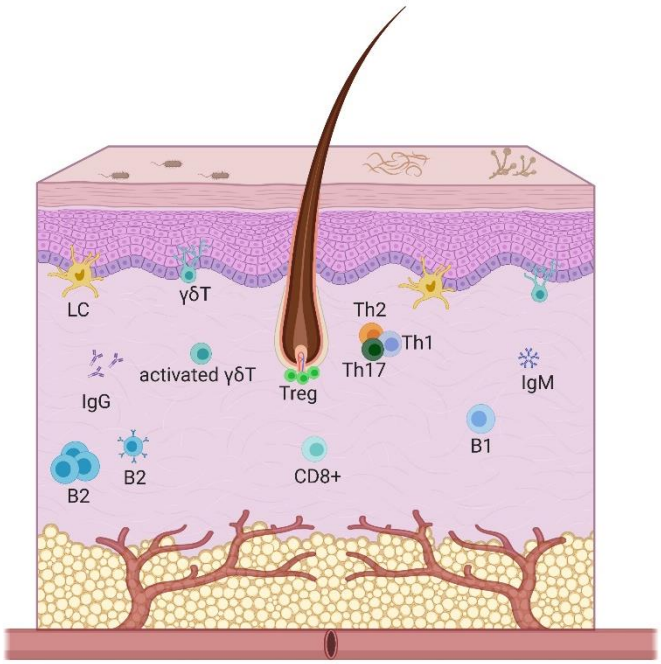


Figure 1.4 Immune system landscape of the skin. The primary cells interacting with commensal microbiota as well as invading pathogens are epidermis forming keratinocytes capable of secreting danger signals upon damage. The epidermis is resident by γδ T cells and Langerhans cells (LCs) which provide sentinel function. The dermis is populated by CD4+ T cells: Th1, Th2, Th17 as well as CD8+ T cells. CD4+ T regulatory cells (Tregs) can be found in hair follicles, where they provide supportive function for hair growth. Skin-associated B cells like B1 secreting IgM, or B regulatory cells (Bregs) as well as conventional B cells (B2) secreting IgG antibodies can also be found in the dermis. Most of those cells are capable of utilizing capillaries to further migrate to skin draining lymph nodes (LN)[4–6]. Created in BioRender.com

1.6.1 Keratinocytes

One of the major immune cells, which do not come from hematopoietic common progenitor are keratinocytes (KCs). As immune cells, they use Toll-like receptors (TLRs) system to recognize pathogen-associated molecular patterns (PAMPs) as well as danger-associated molecular patterns (DAMPs) to trigger an adequate response to external environment signals[63,64]. They are capable of secretion of a limited array of cytokines and chemokines, which can instruct surrounding cells like Langerhans cells or multiple types of T cells for a more mature response[63,65].

The multifactorial development of AD involves environmental and genetic factors for AD development. Upon damage, keratinocytes are capable of secretion alarmins such as IL-33, IL-31, IL-25, and TSLP to activate the immune response[59,66,67]. At the same time, the lack of structural integrity like tight junction, inappropriate expression of claudin-1 or filaggrin can lead to the permeability of

skin barrier, increased TEWL and increased absorption of allergens and consequently the development of eczema[65,68].

1.6.2 Langerhans cells

Langerhans cells (LCs) populate skin initially prenatally and they are capable of self-renewal to maintain the protective barrier, however, upon severe inflammation, they repopulate skin in a second wave directly from bone marrow-derived monocytes population[69]. LCs' self-renewal capacity is strictly dependant on IL-34 secreted by KCs[70]. LCs morphologically resemble dendritic cells (DCs) and macrophages, as they can use the cellular dendrites to conduct immunosurveillance of skin and further they are capable of migration to skin draining lymph nodes (sdLN) for antigen presentation[71,72]. Upon infection, LCs recognize pathogens via pathogen recognition receptors (PRRs) and migrate to sdLNs to facilitate adaptive immunity activation[69]. These leads to infiltration of the skin by Gr-1+ monocyte– derived LCs, which are short-lived and only temporary populate dermis[73]. Once the inflammation passes, the secondary wave of long-lived Langerin+ LCs substitutes monocyte-derived LCs[73].

In AD, LCs are accumulated in skin lesions and they remain in an activated state[74,75]. In patients with filaggrin null mutation, which results in higher skin permeability, the skin-residing LCs are more mature irrespectively of an ongoing flare-up[76]. At the same time, the cells express FcεRI in AD lesions, which is consistent with self-perpetuating effect of IgE in AD[77].

1.6.3 Innate lymphoid cells type 2 (ILC2)

The newly discovered ILC2 can be found in the dermal layer of skin, where they facilitate immune signal transduction and rapid cytokine production upon danger signals[78]. Similarly, to T and B cells, ILCs are derived from bone marrow, where they develop into 3 subgroups ILC1, ILC2 and ILC3[79]. It has been found that ILCs can express cutaneous lymphocyte antigen (CLA) and C-X-C chemokine receptor type 4 (CXCR-4), therefore migrate to the skin[80]. Additionally, ILC2 are capable of local Th2 activation and proliferation by inducible T-cell costimulatory (ICOS) and TNF receptor superfamily ligand(OX40L) ligand-receptor binding [81,82].

The exact mechanism of ILC2 activation remains unclear, however, it is known that the cells can be IL-33, IL-25 and TSLP responsive in different models[78,83,84]. Interestingly, in AD activation of ILC2 lead to the secretion of IL-5 and IL-13 and as a result of the infiltration of both eosinophils and mast cells[78,83]. Furthermore, the ILC2 are responsive to cysteinyl leukotrienes (CysL) secreted by mast cells[85]. Upon

leukotriene receptors ($CysLT_1$) activation, ILC2 upregulate IL/33-IL25 receptors expression, which further perpetuates inflammation in AD lesions[85].

1.6.4 Mast cells (MCs)

Mast cells are derived from myeloid lineage of hematopoietic stem cells (HSC) and are abundantly present across various tissues in the body, where they promote quick inflammatory reaction[86]. Those cells are capable of secretion of various substances such as prostaglandins, leukotrienes as well as histamine, which are released by cell degranulation in AD as well as in infection[86].

In AD, mast cells play a detrimental role when they are activated by cross-linking of IgE and FcεRI, which leads to histamine release as well as cysteinyl leukotrienes[16,86]. As MCs reside in close proximity to nociceptors in skin, they can activate the sensory terminals of the central nervous system (CNS), by inducing to pruritis[87].

1.6.5 Eosinophils

Other bone marrow-derived degranulating cells are eosinophils characterized by IL-5 receptor, C-C chemokine receptor type 3 (CCR3) and most recently by Lymphocyte antigen 6 complex locus G6D (Ly6G/GR-1) in presence of IL-5[16,88]. Their granules mostly compose of cationic proteins, leukotrienes, prostaglandins, and free radicals, which under infectious conditions are toxic to bacteria and parasites[16]. Those cells do not reside in the skin, however upon secretion of multiple chemotactic signals such as IL-5, Chemokine ligand 5 (CCL5 or RANTES) and eotaxin-1 (CCL11), the eosinophils are recruited from the circulatory system, where they provide protective, antimicrobial as well as proinflammatory functions[16].

In allergic diseases, the impaired expression of chemotactic factors by keratinocytes and ILC2 recruits CCR3 positive eosinophils to the dermis, where they degranulate and produce cytokines such as IL-5, which in an autocrine manner perpetuates the disease[79,89].

1.6.6 Dendritic epidermal T cells

Dendritic epidermal T cells (DETC) are a unique population, which originates from yolk sac and remains in the murine basal layer of epidermis[90]. The DETC have an invariant Vγ5Vδ1 T-cell receptor in mice, in other species it is referred to as γδ T cells population, which originates from thymus and is dependent on selection and upkeep of intraepithelial T cells protein 1 (Skint1) expression[90]. In humans

mainly Vδ1 T cells reside in the epidermis and they do not resemble the shape of DETC, however, they do share functions such as secretion of cytokines to facilitate wound healing[63,91,92]. In steady-state they have dendritic morphology with dendrites reaching apically to provide immune surveillance[6,90,93]. Once activated, cells resume the round shape and further secrete cytokines and chemokines to activate appropriate immune reactions[90,94]. The DETCs are capable of secretion of insulin growth factor -1 (IGF-1) to maintain their own population in an autocrine manner[95].

In spontaneous models of murine AD, the DETCs accumulates in the epidermis in an IL-2, IL-7 dependant manner, however, their exact role has not been fully elucidated[93]. As DETCs are capable of IL-17 secretion, their function is critical in murine psoriatic form of eczema induced by IL-23[96].

1.6.7 T cells

The classical αβ TCR expressing CD4+ and CD8+ T cells populates skin in the epidermal-dermal junction[97,98]. The CD4+ T cell consists of various Th subtypes such as Th2, Th1 and Th17, as well as Treg cells[97,99]. Their presence serves as both protection and structural support[98,100]. As T cells reside near antigen-presenting cells, they can easily perpetuate the immune response upon antigen presentation by secretion of cytokines and chemokines[98]. They can have both memory, and effector activation status, therefore, they respond instantaneously to the triggers[101]. The Treg population can be found in hair follicles where it stimulates hair follicle stem cells (HFSCs) to regenerate and upon mechanical skin injury, the same cells can promote localized tissue recovery[102,103].

The role of CLA+ T cells in AD has been vastly associated with the pathogenicity of eczema[104–106]. Upon activation of LCs or ILC2, cells are capable of upregulation of OX40L, which in turn binds to OX40 on T cells to form an immunological synapse for priming of the response[81,107]. Additional detrimental effect of T cells in AD is induced by the secretion of interferon -γ (IFN-γ) which perpetuates KCs apoptosis, which leads to further epidermal barrier degradation and ultimately leading to morphological changes of epidermal barrier called spongiosis[108,109]. The elevated levels of cytokines such as IL-4 and IL-2 contribute to T cell survival in eczematous lesions[97].

Interestingly, other immunological disorder related strictly to absence of CD25+ Treg cells - Immune dysregulation, polyendocrinopathy and enteropathy X-linked syndrome (IPEX) – is also characterized by atopy-like eczema, eosinophilia and hyper IgE, suggesting that those cells can play a vital role in AD[110,111].

1.6.8 Skin associated B cells

The recent discovery of skin-associate B cells provides additional support to the complexity of skin as a tertiary immune organ[112,113]. Skin-homing innate B1 cells are capable of IL-10 secretion and expression of CD1, which can suppress the inflammation in skin by interacting with cells such as γδ T cells and natural IgM, which modulates the skin microbiota[5,114]. The conventional B cells are capable of class switch recombination and IgG antibodies production[112–114]. Interestingly, the skin-residing B cells can be drivers for germinal centre (GC)-like structures formation and neogenesis of lymphoid structures, called tertiary lymphoid organs, by lymphotoxin secretion[115]. Additionally, due to the proximity of skin-residing T cells, the quick antigen presentation can lead to efficient antibody response against pathogens[114,115]. Similarly to T cells, B1 cells secreting IL-10 and IL-35 are called B regulatory (Breg) cells[116,117]. Those cells are capable of suppression of inflammation as well as regulation of homeostasis.

In skin autoimmune disorders, the tertiary lymphoid structures initiated by skin-associated B can contribute to formation of autoantibodies, which further perpetuate inflammation [114,118]. The systemic B cell role has been vastly investigated in allergic diseases, where B cells can serve as antigen presenting cells as well as anti-allergen antibody producing cells[119,120].

1.7 Mechanism of AD

Atopic dermatitis is multifaced disease, with both acute and chronic presentation, as a result the immune response as well as treatment of the disease is not well established. AD is detrimental both to patients and medical system therefore precise and affordable therapies are needed. Despite the vast body of knowledge, the exact understanding of the mechanism has not been fully elucidated up to date.

It is not clear whether increased permeability of epidermis results from exogenous factors such as dysbiosis and predominance of *S. aureus* colonialization, increased proteases activity like house dust, mite exposure, or chemically induced damage like detergents[37,121–125]. The other contributing factor can be intrinsic filaggrin mutations, loss of tight junction functionality, impaired ceramide formation with an abnormal lamellar composition[123,126–128]. However, upon damage or increased permeability of stratum corneum, the skin residing microbiota or allergen can penetrate the non-lesional skin[20]. The KCs will send danger signals such as IL-31, TSLP and IL-25 to further prime immune response leading to activation of ILC2 and other skin residing cells[38,129–131]. As microbial endotoxins and proteases penetrate epidermis, they trigger LCs and γδT cells[72,90]. Those cells can both prime the adaptive

immunity locally or migrate to skin draining lymph nodes (LN), where they trigger naïve B cells and T cells maturation[6,62]. The local effector T cells such as Th1, Th2, Th17, and Th22 are capable of secretion array of cytokines, depending on the priming trigger, like IL-4, -13, -17 or INF-γ, which further perpetuates inflammation[65,97,137–139,101,105,129,132–136].

As acute lesion starts to form, the activated B cells secrete antibodies: IgG1 and IgE, which prime mast cells and basophils resulting in histamine release[16,140]. This further activates sensory neurons and consequently pruritis[141]. Activated Th2 cells populate skin and secrete mediators and chemotaxins such as IL-5 or eotaxin to attract eosinophils to the site of inflammation[16,142,143].

In chronic lesions, the epidermal thickness is increased due to ongoing inflammatory process and scaling[20,144]. As microbiome penetrates the skin, the Th1/Th17 immunity is primed, which further drives the inflammatory process[65,145](Fig. 1.5).

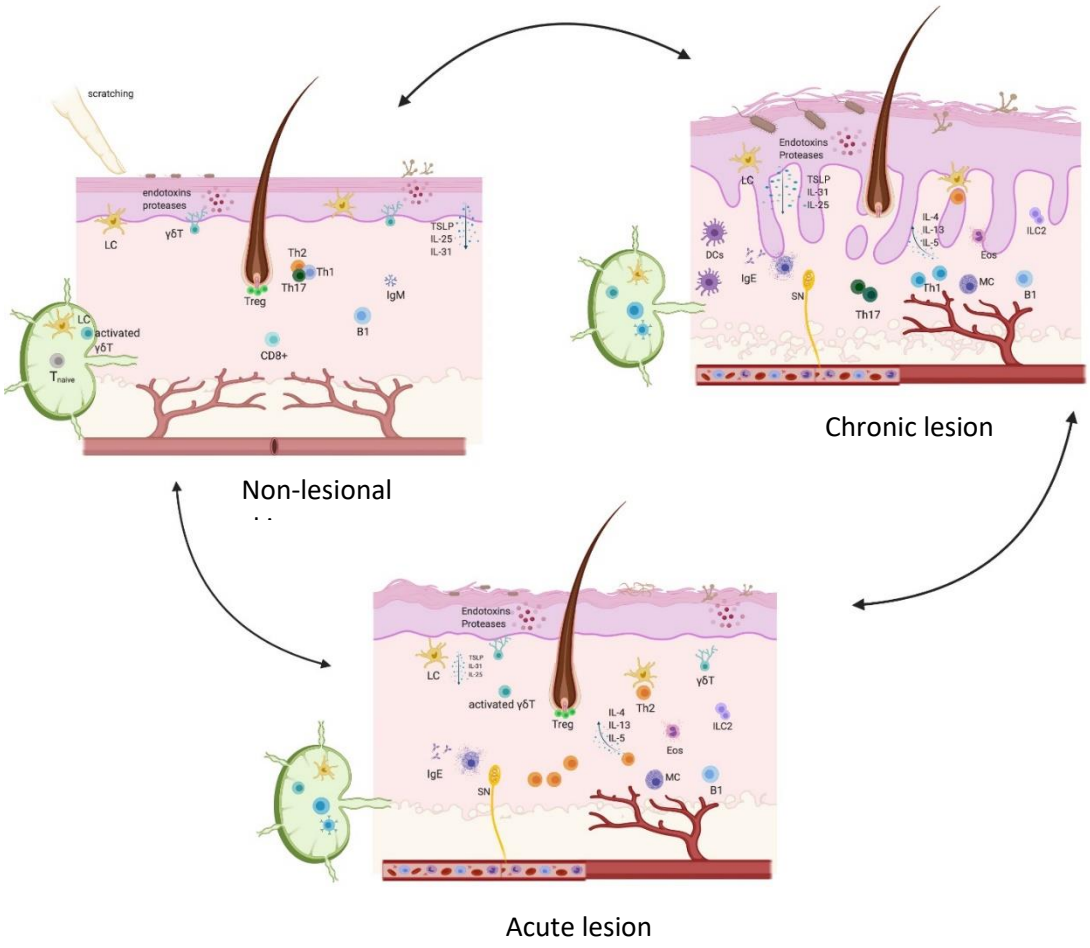


Figure 1.1 Key mechanisms in AD. The non-lesional skin in patients with AD can be altered by genetic factors such as FLG null mutation or attenuated lipid composition, alternatively, mechanical scratching can increase the barrier permeability. This can lead to trespassing of microbial peptides from skin colonizing microbiota. These peptides trigger the immune response of skin residing innate sells such as Langerhans cells (LCs) or γδ T cells. Those cells migrate to lymph nodes, where

they prime B and T cell activation. Alternatively, the immune response can be triggered by keratinocytes secreting alarmins IL-33, -31 and TSLP, which further activate the immune response. In acute lesions, the skin draining lymph nodes actively produce activated B and T cells. The B cells produce IgE, which activates mast cells (MC) and eosinophils (Eos) leading to cell degranulation. MCs activate of sensory neurons promoting itch signal, while Eos perpetuate further skin inflammation. The Th2 and ILC2 cells secrete type 2 cytokines, IL-5, -4 and -13 promoting further inflammation. In chronic lesions, further epidermal damage leads to spongiosis and microbial penetration of the skin, promoting change in immune response towards Th1/Th17-driven mixed response[7]. *Created in BioRender.com*

1.8 Genetic factors in AD progression

As the exact mechanism of AD development is not fully understood, the strong implication of gene-environment interaction has been found to be a prerequisite for disease development. Considering the mechanistic of AD, various genetic biomarkers have been identified such as mutations in FLG, SPINK5 and CLDN-1 genes which allows higher barrier permeability. TLR 2 polymorphisms providing detection of harmless antigen and IL-4Ra point mutations leading to hyperactivation of inflammatory pathways. Often, the genetic association in AD, leads to higher severity of the disease, for instance approximately 50% of moderate to severe AD is associated with FLG-null mutation[128].

FLG is a structural protein, which undergoes breakdown into shorter peptides and free amino acids forming NMF, which ultimate role is to prevent the formation of gaps between corneocytes to improve the integrity of SC[146]. In recent years, the mutation in FLG gene has been vastly associated with predisposition to atopy and asthma[18,39,131,147–149]. As a result of loss-of-function mutation, the structural integrity of skin is compromised and allows the commensal microflora to become pathogenic[37,131]. The bacterial toxin skin penetration can lead to T cell aggregation and activation in the dermis, similarly, the fungal antigen can lead to host sensitization and asthmatic symptoms upon challenge. Additionally, the disruption of filaggrin metabolism leads to increase of the skin pH[125]. As naturally, skin pH is acidic, which blocks activity of serine proteases, the less acidic pH will allow proteolytic activity of the enzymes, thus degradation of corneodesmosomes and further barrier permeability[125,146].

The loss of function of lymphoepithelial Kazal-type trypsin inhibitor (LEKTI) encoded by *SPINK5* gene which blocks serine peptidase enzymatic activity (kallikreins), has been associated with Netherton Syndrome – a severe AD-like hereditary genetic disorder[128,150–152]. As a result, the SC is severely thinned, due to the degradation of corneodesmosomes by increased activity of kallikreins (KLK) [151]. The murine genetic knockout model, provided insight to KLKs activity, which together with protease activated type 2 receptor (PAR2) stimulates TSLP secretion through NF-κB pathway[124].

Defects in tight-junction formation and stability, by single nucleotide polymorphisms (SNPs) in claudin-1 (*CLDN-1)* gene results in increased skin permeability to external allergens and microbacterial toxins[153].

Aside from loss of barrier function in skin, the mutations associated with immune response can also lead to AD. Single nucleotide polymorphism in TLR encoding genes such as TLR2 polymorphisms have been found in AD patients and can result in eczema driven by *S. aureus* colonialization[128,154]. As TLR signalling leads to downstream immune activation, multiple cytokines were disrupted in patients with those mutations, including IL-6 and IL-12[155].

Interestingly, when analysing type 2 immune response which is predominantly associated with allergic diseases, few distinct polymorphisms were found in genes encoding receptors of type 2 cytokines: interleukin 4 receptor alpha (IL-4Rα), IL-5Rα and IL-13Rα[143,156,157]. Most SNPs have been identified in the IL-13Rα gene, while in IL-4Rα more prevalent was chain polymorphism Gln551Arg[157,158]. In IL-5Rα, the SNP and haplotype T-C-T have been associated with circulating eosinophils in AD patients[143].

1.9 IL-4Rα signal transduction in AD

IL-4Rα is a pleiotropic receptor present on multiple cells including, but not limited to, keratinocytes, subpopulations of T cells and subpopulations of B cells[159–164]. Chain α can form a heterodimeric receptor with IL-13Rα1 to detect both IL-4 and IL-13. At the same time, IL-4Rα can bind to the γ chain and detect solely IL-4, while IL-13 can be detected by IL-13Rα2[8]. Downstream of the receptor the signal transduction leads to tyrosine phosphorylation of Janus kinase 1/ Signal transducer and activator 6 (JAK/STAT6) pathway, resulting in translocation of STAT6 transcription factor into the nucleus, where it activates IL-13 and IL-4 transcription (Fig. 1.6)[165,166].

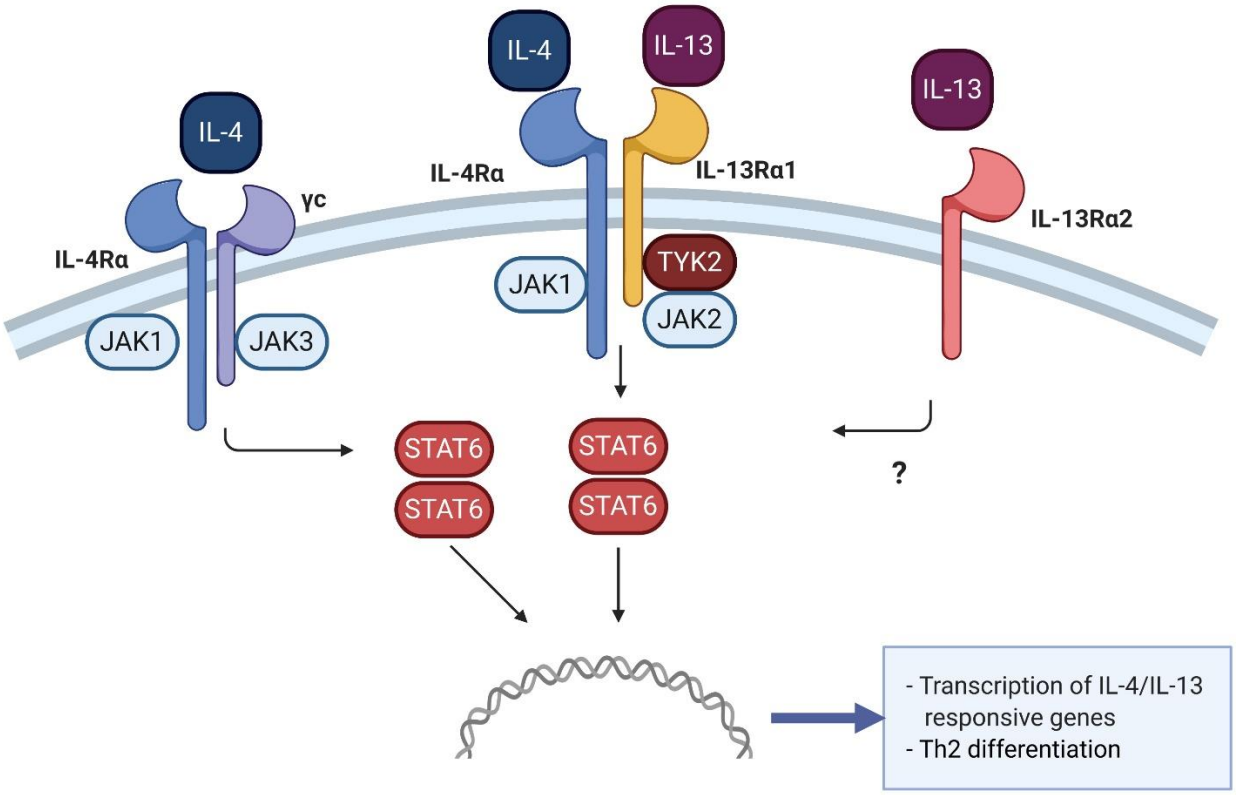


Figure 1.2 IL4Rα signal transduction. Both IL-4 and IL-13 cytokines can conduct through heterodimeric receptor consisting of IL-4rα and IL-13Rα1. IL-4 can be detected by IL-4rα together with γc subunit. Both receptors signalling cascades further transduced signal via JAK/STAT pathway. The IL-13 alone can be detected by IL-13rα2; however, the downstream signalling cascade has not been fully understood up to date[8]. *Created in BioRender.com*

Interestingly, up to date it has been believed that IL-13Rα2 is a decoy receptor, however, recent research in both allergic asthma and AD showed that the receptor is important in IL-13 signalling and can contribute to the disease[167–172].

As the efficacy of newly approved Dupilumab treatment against AD has been greatly proven, the remaining is the question of the avoidance of side effects of systemic administration of the drug. In order to avoid the systemic side effects, more understanding on the exact cell types involved in pathogenesis of AD is needed.

1.10 Skin-residing bacteria

The bacteria residing on the skin can be divided in two categories: commensal and infectious. Although the commensal bacteria compete over the niche of residence and can protect host from development of large infectious population, in some cases the host might be a carrier of infectious bacteria without

getting infected[173]. Both gram-positive and gram-negative bacteria reside in the skin, including *Staphylococcus*, *Micrococcus*, *Corynebacterium* and *Propionibacterium* genuses – gram-positive; Acinetobacter – gram-negative and their exact location vary depending on the preference of the physical and chemical conditions such as humidity level or pH as well as the availability of nutrients e.g. sweat or sebum[174]. There is a fine-tuned balance between bacterial invasion/tropism versus commensals and these relations is established early in life. For example, competitive colonialization is *Staphylococcus epidermis*, which binds to keratinocyte aryl hydrocarbon receptor (AhR) and activates highly specific antimicrobial peptides (AMPs) production, preventing the development of *Staphylococcus aureus* biofilm[175,176]. Other competitive interaction occurs between *Staphylococcus lugdunensis* and *S. aureus*, the first one produces thiazolidine-containing cyclic peptide (lugdunin), which exhibits long term therapeutic potential against *S. aureus* colonialization. As a result, it protects the host from potential infection with opportunistic pathogens[177]. These AMPs mechanisms of action have been shown to decrease colonialization of atopic dermatitis patients with *S. aureus*, who are prone to increased *S. aureus* skin invasion when reintroduced to human subjects[175].

Bacterial species that can thrive in the harsh skin environment have developed various sophisticated mechanisms to be able to complete. These include tropisms, where bacteria can extract nutrients from environmental or have developed host evasion strategies through growth[174,178,179]. Bacterial have an ability to extract nutrients from host's resources, for example *P. acnes* is capable of secretion proteases to liberate the amino acid arginine from skin proteins, as well as, lipases in order to degrade triglyceride lipids from sebum, hence it thrives in the anoxic sebaceous glands[180,181]. Another important factor in bacterial tropism is the resistance to and evasion of host defence mechanisms. One such example is the formation of polysaccharide intercellular adhesin/ poly-N-acetyl-glucosamine (PIA/PNAG) rich biofilm, which acts as a mechanical barrier to block neutrophil killing, immunoglobulin opsonization and complement deposition, this mechanism is used mainly by *S. epidermis*[182–184].

The control of *S. aureus* infection is initiated by microbial opsonization with complement factors or antibodies such as C3b and IgG leading to phagocytosis and killing of bacteria by releasing reactive oxygen species (ROS) and/or reactive nitrogen species (NO)[185]. However, some bacterial species, like *S. aureus*, can abuse host immune system and use it to its advantage e.g. to proliferate and inhibits stress regulon via lactic acid fermentation[186]. Another important host-derived mechanism of protection and bacterial regulation is neutrophil extracellular traps (NETs) secretion upon Toll-like receptor-2 (TLR-2)/MyD88 stimulation[187,188]. NETs mechanism secrets antimicrobial peptides, cathepsins, histones and proteases to limit pathogen spread. Aside from NETs secretion, neutrophils can also degranulate toxic components such as LL37, Mel4 and defensins, which are directly killing pathogen by induction of bacteriolysis and/or autolysis[189,190].

The host-bacterial commensal relationship is established early in neonatal life, due to the influx of highly activated CD44+ Tregs into neonatal skin[191]. This tolerance cannot be restored in adult mice, therefore, the initial exposure to commensal bacteria is critical for proper immune education. Tregs accumulate in the hair follicles, where one of their functions is interaction with bacteria as well as Langerhans cells[62]. Even though, the causative effect of interaction between host and microbiome in vaginal versus C-section delivered infants and its effect on non-communicable diseases development is still under debate, it is important to point out that various non-harmful bacteria compete for the local niche, which can result in potential further implication on positive effect for restoration of maternal microbiota post-C-section delivery. Example of such

The infection with topic bacteria can be secondary to host injuries such as wound formation or autoimmune skin inflammation like atopic dermatitis or systemic lupus erythematosus (SLE) and they can lead to abscess formation or even sepsis. The progression of the disease from skin rash towards sepsis is not fully understood, however, several host-derived factors can contribute to bacterial sepsis.

As bacterial infections are predominantly type-1 immune regulated it is important to take a closer look at the infection site during destabilized immune response in AD or other autoimmune diseases. *S. aureus* infection has been often reported in AD patients, where impaired skin barrier favours colonialization and penetration of the barrier[192]. The cytokine microenvironment stimulated degradation of tight junction formed by zonula occludins 1 and claudin-1 in lesioned epidermis of AD patients[68]. Together, IL-4 and IL-13 it have been shown to contribute to increase α-toxin-induced cytotoxicity of keratinocytes upon *S. aureus* skin colonialization in AD lesions[193]. IL-31 is another cytokine that is highly upregulated in AD patients and is favouring bacterial adhesion, which suppresses filaggrin and AMPs expression[59,131]. At the same time, IL-17 can lead to filaggrin monomer degradation in murine model of AD[68].

Taken together, atopic dermatitis immune dysregulation self-perpetuates further bacterial invasion of skin and worsening of patient condition due to secondary bacteraemia.

1.11 Fungal infections

Due to the strong physical integrity of skin, the development of fungal infection requires either skin damage induced by trauma or impairment of host immune protection[194]. At the same time, the fungal cell wall consists of highly immunoreactive polysaccharide and lipid moieties, which collectively belong to pathogen-associated molecular patterns (PAMPs). Example of such PAMPs are glycoproteins that incorporate N- and O-linked mannans (*C. albicans*), which activates mannose receptor and TLR4 [195]. Aside from TLR recognition, fungi can be detected by C-type lectin receptor (CLR) family in which you will find mannose receptor, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing

non-integrin (DC-SIGN), Dectin-1, Dectin-2 and the collectins [196] . Cellular activation by PAMPs leads to secretion of AMPs by keratinocytes which provides protection as well as regulates the colonialization of the healthy host.

One of the examples of skin commensal fungi are Malassezia species. The growth of Malassezia species is regulated by detection of fungal hydrophobic glycerol-glycolipid via Mincle and hydrophilic, mannobiose-rich glycoprotein by Dectin-2 [197]. The early detection of fungal antigens by innate immune cells leads to downstream naïve cell maturation. Due to complexity of fungal growth, the adaptive immune response needs to be flexible; for example, in case of *C. albicans* detection, depending on the form of the fungus yeast vs Pseudo-hyphae , host immune response will be either mature towards Th17- or Th1- driven[198].

1.12 Atopic March

As symptoms of AD predate episodes of asthma or allergic rhinitis, the immunological process behind this transition is called atopic march (AM)[199]. The exact mechanism of AM has not been fully understood, however, is supported by both human longitude studies as well as murine models[199–202]. Multiple endotypes of AM have been determined up to date including, early onset of AD, polysensitization, persistence of AD symptoms, atopy in parents, increased severity of AD as well as FLG mutation[202]. Similarly, to AD, the mechanism of AM involves higher permeability of the skin epidermal barrier, which leads to penetration of antigen into deeper layers of skin, leading to immune activation by Th2 cells and antibody production by B cells. As alarmins, cytokines, antibodies, and cells migrate to circulation, they are capable of inducing an inflammatory reaction in other sides such as lungs (asthma), nasopharyngeal (allergic rhinitis), or intestines (food allergy) (Fig.1.7)[9].

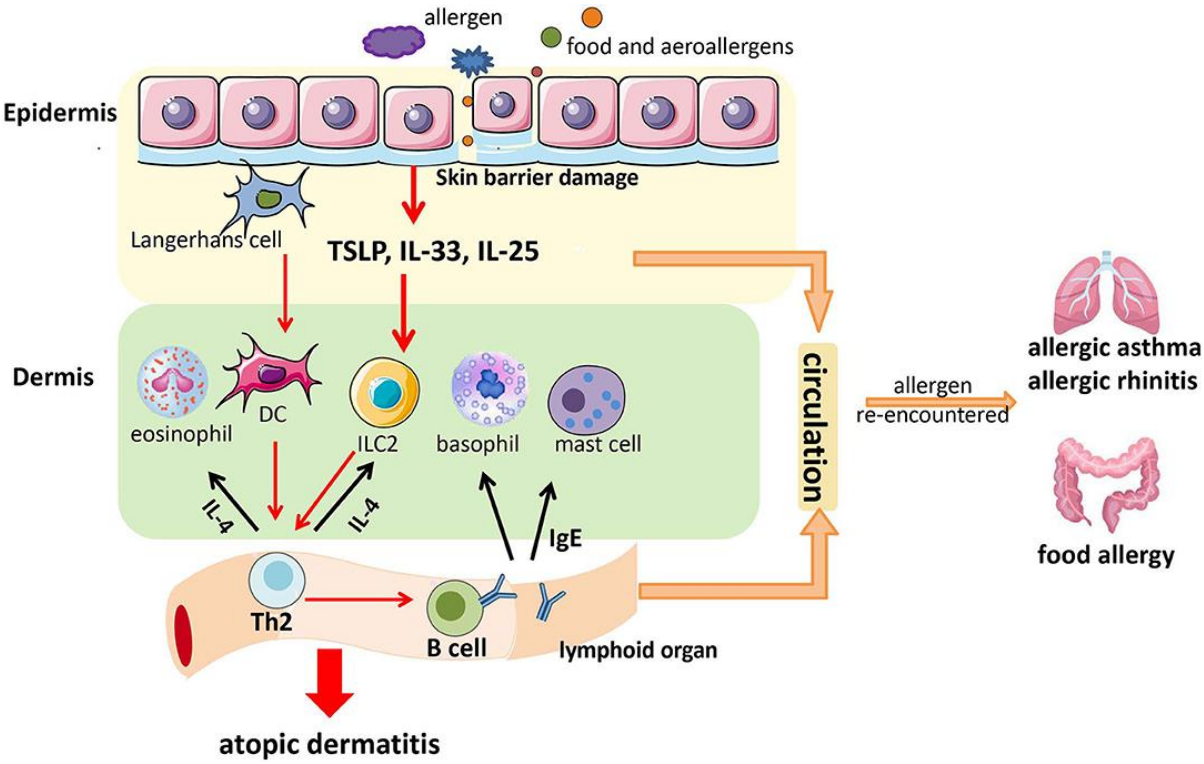


Figure 1.3 The suggested mechanism for atopic march. As the skin loses its integrity, the allergens cross the barrier and trigger the immune response, by thymic stromal lymphopoietin (TSLP), IL-33 and IL-25, which triggers type 2 immune cells to the site of inflammation. The response in skin draining lymph nodes leads to the development of the anti-allergen IgE, which can enter circulation together with TSLP, IL-33 and IL-25. Upon re-exposure to allergen in the lungs or digestive tract, the antibody and cytokines will lead to a secondary allergic reaction like allergic asthma, allergic rhinitis, and food allergy. This process is known as atopic march. Adapted from Yang et al. 2020[9].

1.13 Recommended treatment regime for AD

As AD cannot be cured, the main treatment is the maintenance of suppressed immune response and the application of topical emollients to improve the epidermal skin barrier that results in patients' quality of life.

The recommended medications include topical corticosteroids, calcineurin inhibitors, and biologicals such as anti-IgE (Omalizumab) and (anti-IL-4Rα) Dupilumab. The summary of topical treatments can be found in Table 1[203]:

Table 1.1 Treatment regime for atopic dermatitis

Treatment	Target action	Side effects	Remarks	Reference
Bleach bath	Diminish population of skin residing microbiome	Skin irritation	no long-term efficacy and safety studies	[204–206]
Emollients and moisturisers	Maintenance of skin lipid barrier, and increase in water absorption	None	Applicable to mild AD or as supportive therapy for severe AD	[207–209]
Topical Corticosteroids (Class I to VII)	Suppression of inflammatory cells	Hypothalamic-pituitary-adrenal (HPA) axis suppression, Skin atrophy, allergic contact dermatitis	Class I to VII vary in strength, applicable to acute to severe AD	[210–212]
Topical calcineurin inhibitors	Inhibition of transcription of proinflammatory genes	Pruritis, skin infection, erythema, flu-like symptoms	Low risk of lymphoproliferative disease	[11,213–215]
Janus Kinase Inhibitors	Inhibition of downstream signalling of proinflammatory cytokines	Mild infections such as nasopharyngitis	Phase II clinical trial	[216–218]
Phosphodiesterase-4 Inhibitors	Inhibits production of proinflammatory cytokines	Mild side effects including pruritis, dermatitis and site pain	Phase II clinical trial, currently in phase III	[219–223]
Benvitimod	Decreases proinflammatory cytokine production and migration of T cells, and blood mononuclear cells	Folliculitis, contact dermatitis, and headache	Phase II clinical trial	[203,224,225]

As cutaneous therapies are not always efficient, in severe cases of AD the systemic treatments are recommended, the summary of systemic therapies can be found in table 2[226,227]:

Table 1.2 Overview of systemic corticosteroids and biologicals in therapy for atopic dermatitis

Treatment	**Target action**	**Side effects**	**Remarks**	**References**
Cyclosporine	Blocks activation of Th1/Th2 cells and DCs	Nausea, headache, renal failure, hypertension	Prolonged immunosuppression is not recommended	[104,228,229]
Azathioprine	Inhibits DNA synthesis	Lymphocytopenia, gastrointestinal side effects, impairment of liver function	Used off-label for AD	[230–232]
Methotrexate	Folic acid antagonist	Impaired liver function, nausea, toxicity, pancytopenia	Used off-label for AD	[230,233,234]
Rituximab	Monoclonal anti-CD20 B cell surface marker	Well tolerated		[235,236]
Omalizumab	Monoclonal anti-IgE antibody	Well tolerated	2 randomized control trials, conflicting data on efficacy	[235,237–240]
Dupilumab	Monoclonal Anti-IL-4Rα antibody	Conjunctivitis	Highly efficient, recently approved for AD treatment	[219,227,241–247]

The recently approved treatment with Dupilumab showed the most promising results for long term management of AD with the least of side effects[219,241,245–247]. Dupilumab is a fully human monoclonal antibody directed against the alpha subunit of interleukin 4 receptor (IL-4Rα), which blocks signal transduction of IL-4 and partially IL-13.

1.14 Animal Models of AD

The human studies of AD are greatly limited due to late diagnosis and ethical concerns, therefore animal-based research models play a vital role for understanding of underlying mechanisms of AD.

As AD is multifactorial disorder, various models focus on different aspects of the disease.

1.14.1 Spontaneous models of murine AD

The animal models of AD can resemble some but not all symptoms of human disease. There are two mice strains which can develop AD in spontaneous way: Flaky Tail (ft/ft) mouse, which has frameshift mutation in FLG and MA genes and Nc/Nga mouse, of which exact genetic mechanism is not fully determined[248–254]. Both murine strains can develop acute and chronic lesions, pruritic manifestations as well as an increase in the Th2 response. The major differences can be found in total IgE production, it is increased in Nc/Nga mouse but not in Flaky Tail, this mouse is also responsive to glucocorticosteroids treatment, while the other is not. Interesting, the Flaky Tails strain is characterized by increased Th17 response and disrupted epidermal barrier, while Nc/Nga strain does not resemble these characteristics.

1.14.2 Allergen-induced murine models of AD

Due to the strong correlation between epicutaneous allergen exposure and development of human AD, the murine models that resemble the disease have been determined[255]. In animal models it remains vital the disruption of epidermal layer before the application of antigens, either by repetitive tape stripping or the use of genetically modified mice with permeable skin barrier[252,255]. Similarly to asthma models, both ovalbumin (OVA) and house dust mite (HDM) murine models are capable of inducing allergic response after epidermal damage or when mice capable of spontaneous AD such as Nc/Nga mice are treated with an allergen[256,257].

Both allergens are inducing inflammatory changes on the skin in acute and chronic phase of the disease. However, only HDM model induces pruritis in mice. In Flaky Tail mice, only OVA is able to induce increased Th2 response and elevated IgE. In Nc/Nga mice, HDM can lead to elevated Th2 cytokine and IgE production.

1.14.3 Skin irritant-induced murine model of AD

Vitamin D analogue calcipotriol (MC903) is a vastly used, reproducible model of murine AD[67,142]. MC903 does not require epithelial damage to induce inflammatory response driven by TSLP and ILC2[258]. Upon application of MC903, an increase in epithelial thickening has been reported as well as pruritic bursts in mice[84,141]. MC903 is both sex and genetic-background independent, however, in the BALB/c mice the inflammation is driven by IL-33 and IL-25 contrary to C57BL/6 background mice[258].

1.15 Thesis Aims, Hypothesis and Objectives

As the current understanding of mechanisms underlying the AD are not fully elucidated, the newly approved treatment regime using dupilumab provides a guiding path towards more precise treatment. In order to dissect the importance of IL-4/IL-13 axis of immune response in AD and better understanding of dupilumab-driven mechanism of disease alleviation, we sort to investigate the cell-specific abrogation of IL-4Rα signalling.

AIM 1: Investigation of the role of T cell-specific genetic knockout of IL-4Rα using murine models of AD.

As murine models provide to be useful in understanding the AD progression, we dissect the receptor signalling in different T cell specific murine strains: iLcK^{Cre} IL-4Rα$^{-/lox}$, which does not have the receptor on all T cell populations including iNKT, γδT cells, CD4+ T cells, and CD8+ T cells; LcK^{Cre} IL-4Rα$^{-/lox}$, without receptor signalling on CD4+ T cells, and CD8+ T cells; CD4^{Cre} IL-4Rα$^{-/lox}$, which has impaired receptor signalling on CD4+ T cells only; lastly Foxp3^{Cre} IL-4Rα$^{-/lox}$, which does not have receptor signalling on T reg cells only.

For this project we used the allergic murine model of AD using OVA and HDM as well as calcipotriol model of AD. The major objectives include:

1. The determine an atopic dermatitis model by analysis of skin thickness in both allergic and non-allergic models
2. Characterize the cell populations in skin draining lymph nodes after induction of allergic and non-allergic atopic dermatitis.
3. Characterize serum antibody composition produced by the B cells after induction of allergic and non-allergic atopic dermatitis.

AIM 2: Investigation of role of B cell-specific genetic knockout of IL-4Rα using murine models of AD.

As one of the main hallmarks of AD is elevated antibody response, it remains vital to analyse the consequence of reduced signalling IL-4Rα on B cells in murine model of AD. We hypothesized that abrogation of receptor signalling will hinder the AD antibody response and as a result dampen the AD symptoms. Secondly, knowing that atopic march is a secondary condition following allergic skin sensitization we hypothesized that mice lacking IL-4Rα on B cells will be protected from asthma symptoms. The specific objectives include:

1. Characterize the cell populations in skin draining lymph nodes in MB1^{Cre} IL-4Rα$^{-/lox}$ murine strain lacking IL-4Rα on B-2 cells after induction of allergic and non-allergic atopic dermatitis.

2. Characterize serum antibody composition produced by the B cells lacking IL-4Rα after induction of allergic and non-allergic atopic dermatitis.
3. Characterize level of protection from asthma induced by epicutaneous sensitization.

Chapter 2

Materials and methods

2.1 Animals

The genetically modified mice were generated on BALB/c background with genetic predisposition to allergic diseases, using Cre-Lox system as described previously (Hoving et al. 2012)[161] .

Table 2.3 List of mice strains used for the research

Mouse Strain	Cell Knock-out	Literature
IL-4Rα$^{-/-}$	Global IL-4Rα knock-out	201,259
MB-1Cre IL-4Rα$^{-/-}$	B-cell specific IL-4Rα knock-out	161,162,260
uMT	Pan-B cell knock-out	162,261,262
LcKCre IL-4Rα$^{-/-}$	CD4+, CD8+ specific IL-4Rα knock-out	200,259,263,264
iLcKCre IL-4Rα$^{-/-}$	CD4	259,263
CD4Cre IL-4Rα$^{-/-}$	CD4+ specific IL-4Rα knock-out	Darby et al data unpublished
Foxp3Cre IL-4Rα$^{-/-}$	Foxp3+ specific IL-4Rα knock-out	159,265

Mice were bred at the University of Cape Town Animal Facility, Cape Town under protocol number 017/004. Only adult (6-8 weeks old) females were used for the study (Fig. 2.1). Mice were housed in specific pathogen-free conditions in individually ventilated cages in a controlled day-night cycle and given food and water *ad libidum.* The genetic phenotype was confirmed by polymerase chain reaction (PCR) for presence of Cre recombinase and the gene knockout, by Miss Munadia Ansari.

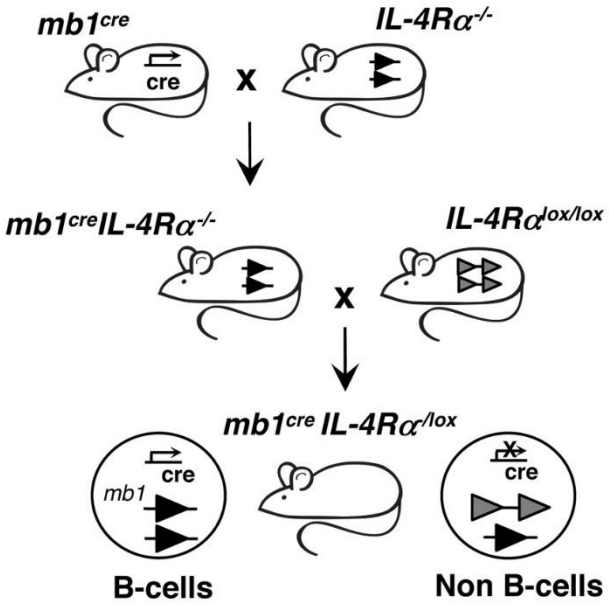


Figure 2.1 Example of generation of gene-specific knockout mice. Adapted from J. Hoving et al. 2011

2.1 Model of atopic dermatitis

2.1.1 House dust mite induced atopic dermatitis model

Mice of 6-8 weeks were anesthetized with ketamine (Fresenius, South Africa) and xylazine (Bayer, South Africa) mix and shaved on ventral side 1x1 cm with single-blade disposable razor (BIC, South Africa). Mice were treated with 10 or 100 ug of House Dust Mite (*D. Pteronyssinus*) extract (HDM, Depr 1 1142.12 mcg/vial, Endotoxin 7925 EU/vial, Stellargen, Germany) or 200 ug ovalbumin (OVA, grade V; Sigma-Aldrich, South Africa) diluted in 200 µL of 1 x Phosphate-Buffered Saline (PBS, Thermofisher, South Africa) or vehicle control – 200 µL of 1x PBS using sterile band aid (Elastoplast, Baiersdorf, South Africa). Patch was removed 24 hours post treatment. Treatments were repeated on day 0, 14 and 28 and mice were killed on day 35 (Fig. 2.2).

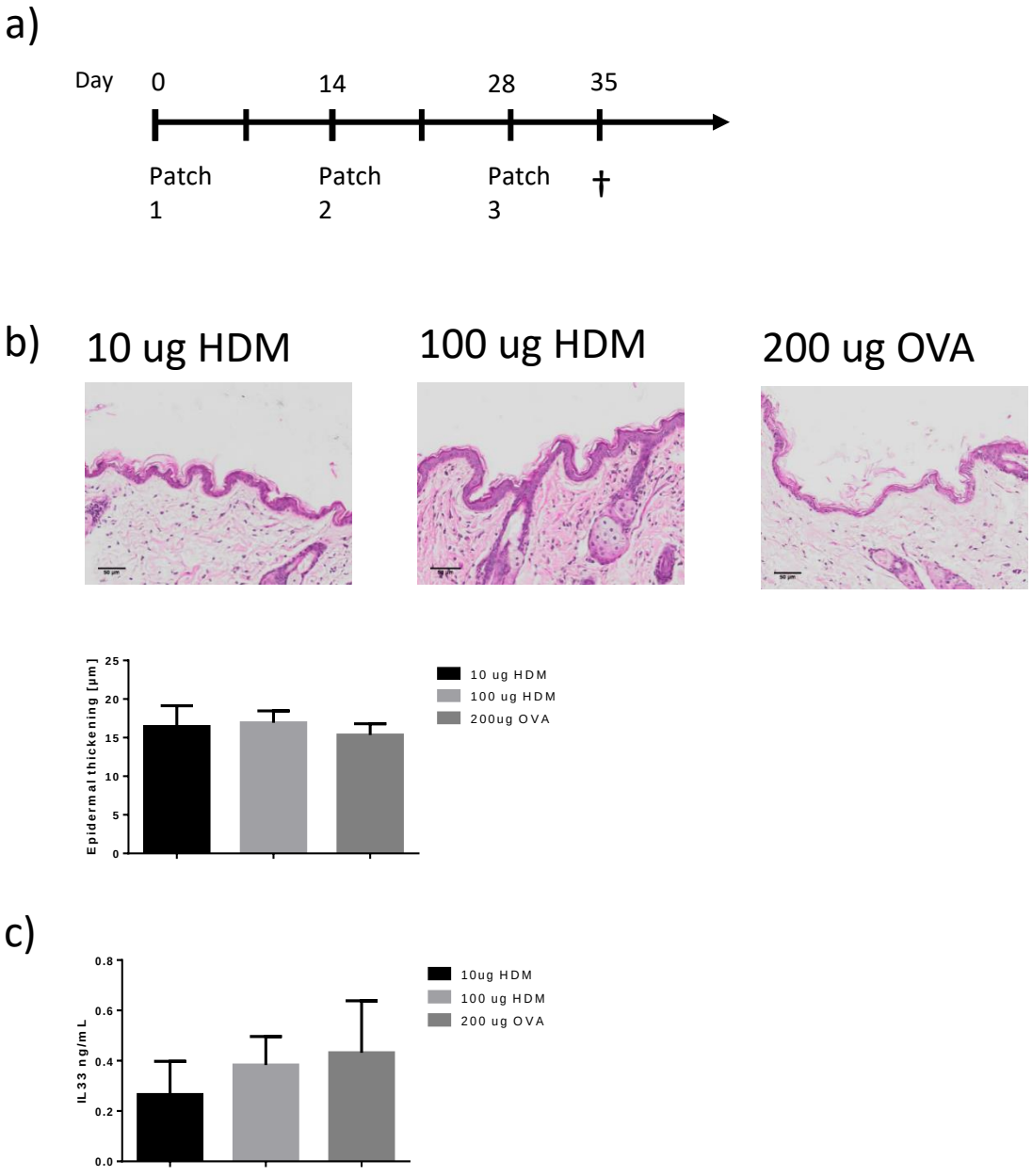


Figure 2.2 Titration of HDM and OVA for epicutaneous sensitization. a) Model for allergen skin sensitization b) comparison of histology images of skin biopsies of ventral side from HDM or OVA treated mice stained with H&E, scale bar 50um, quantification of epidermal thickening using QuPath software, c) quantification of serum IL33 using ELISA. Representative of one experiment is shown with mean ± SD. n=5 mice per group. one-way ANOVA

2.2.2 MC903 induced atopic dermatitis model

Adult mice were shaved using single-blade disposable razor (BIC, South Africa) on ventral side 1x1 cm, 3 days prior start of the experiment. Mice were treated with 100 µL of various doses (45 µM 90 µM) of calcineurin inhibitor, MC903 (Tocris Bioscience, United Kingdom) in 100% ethanol - vehicle (EtOH, Thermofisher, South Africa) or with vehicle (100% EtOH) for 10 consecutive days. The weight loss was closely monitored. Mice were killed on day 11 and organs were collected for further analysis (Fig. 2.3).

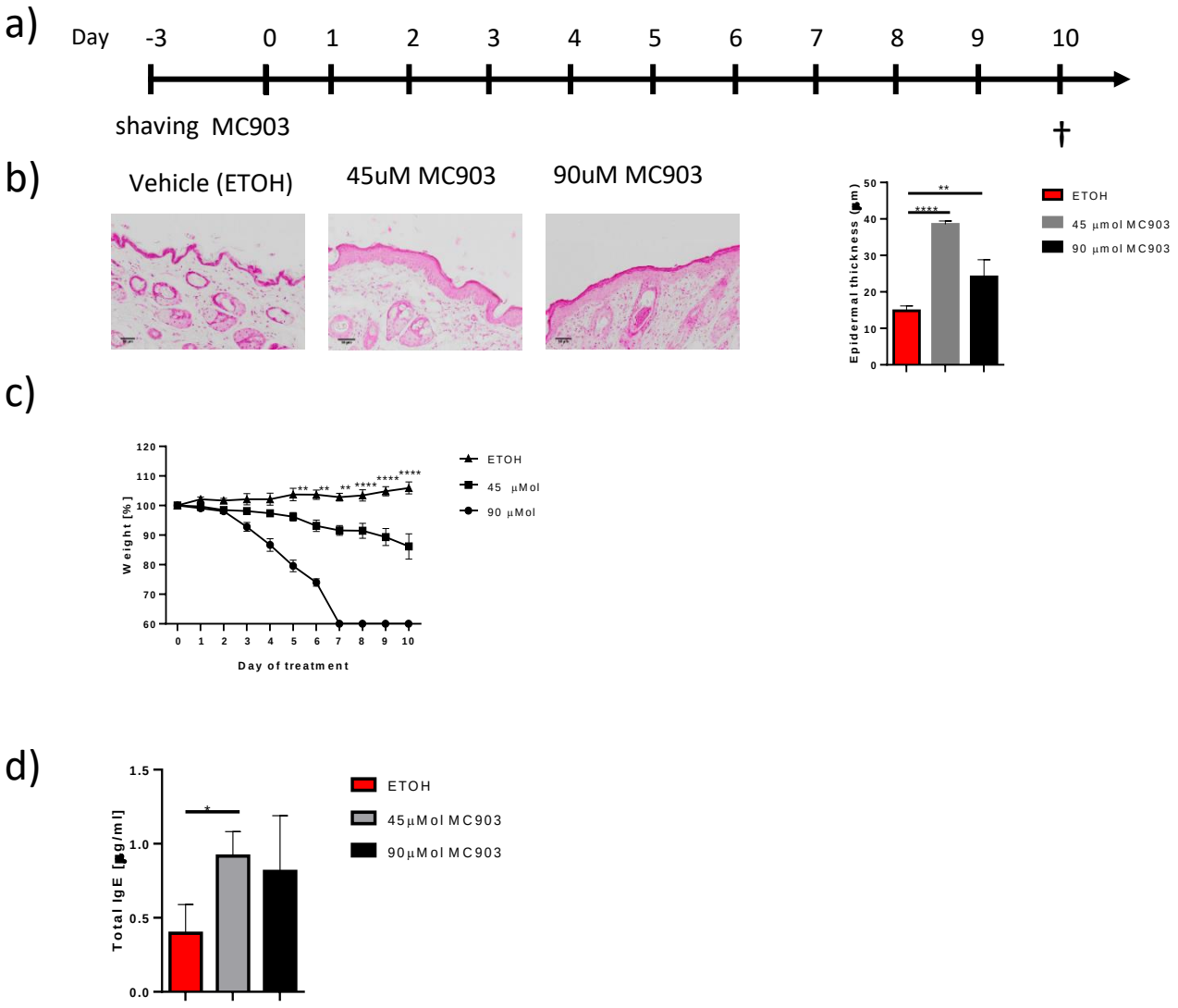


Figure 2.3 The titration of MC903 epicutaneous treatment. a) model of MC903 epicutaneous treatment b) comparison of histology images of skin biopsies of ventral side from MC903 or vehicle treated mice stained with H&E, scale bar 50um, quantification of epidermal thickening using QuPath software, c) weight loss tracing where statistical analysis was performed defining differences to 45uM dose to control group as significant, d) quantification of serum total IgE using ELISA. Representative of one experiment is shown with mean ± SD. n=5 mice per group, Student t-test and one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

N.B., We found MC903 to be biologically unstable, therefore titration had to be performed two times.

2.3 House dust mite model of atopic march

Adult mice were sensitized as previously described (2.1.1) with 10 µg HDM (Stellargen, Germany) or PBS (Thermofisher, South Africa) solution 3 times with 2 weeks intervals. Further mice were challenged intranasally with 10 µg of HDM (Stellargen, Germany) in 50 µL PBS (Thermofisher, South Africa) under ketamine (Fresenius, South Africa) and xylazine (Bayer, South Africa) anaesthesia for 3 consecutive days. The airway hyperresponsiveness was measured. (Fig. 2.4)

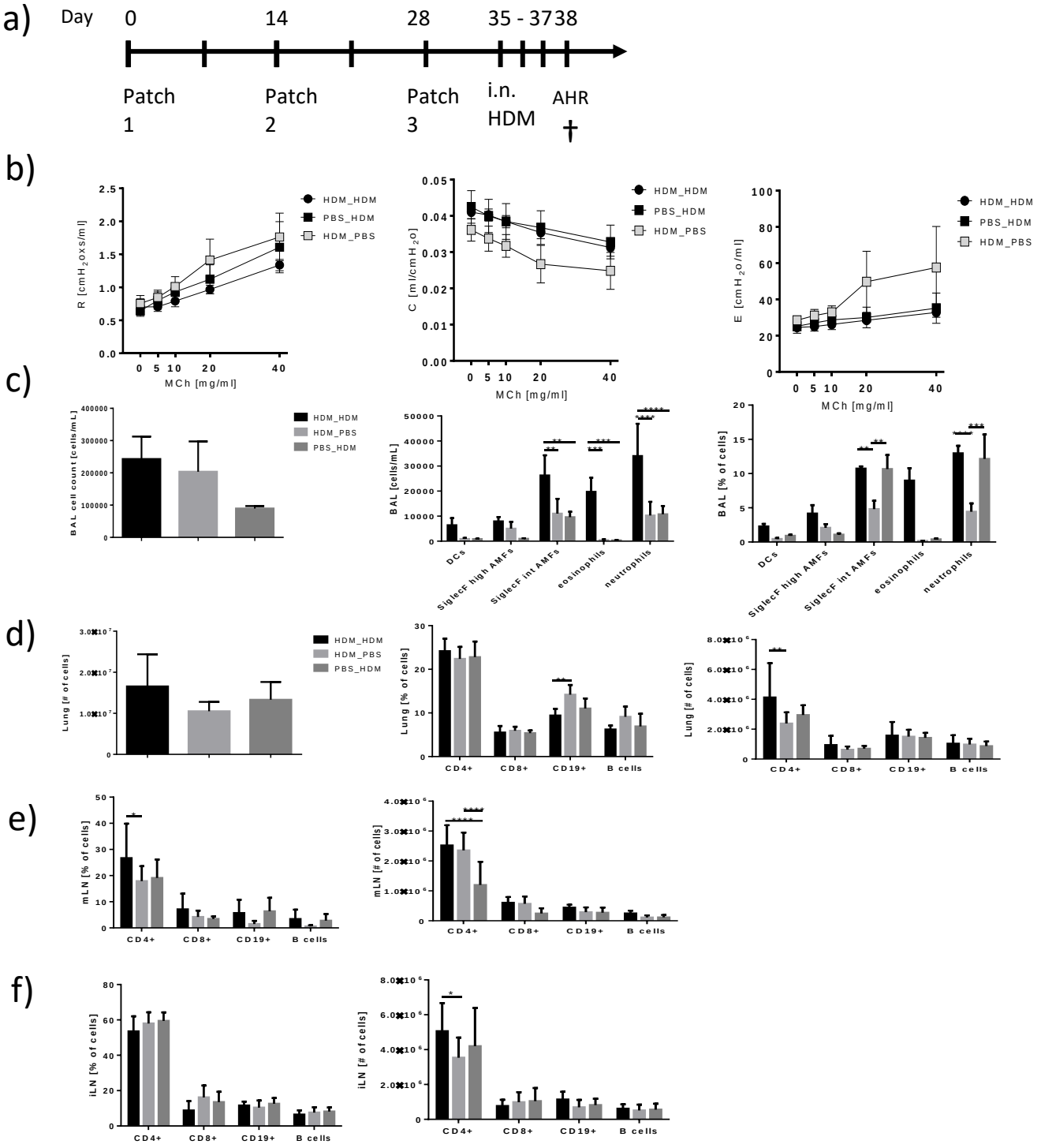


Figure 2.4 Atopic march model quantification. a) model of atopic march b) airway hyperresistance quantification in upon increasing dose of metacholine challenge c) quantification of BAL d)and lung cellular infiltrates in FACS comparison e) quantification e) mediastinal and f) skin draining lymph node B and T cells infiltrates using FACS. Representative of one experiment is shown with mean ± SD. n=5 mice per group, one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

2.3.1 Airway Hyperresponsiveness

Airway Hyperresponsiveness was measured using SCIREQ flexiVent Instrument (SCIREQ, Canada) as described previously[266]. Mice were paralyzed under ketamine (Fresenius, South Africa) and xylazine (Bayer, South Africa) anaesthesia. The tracheotomy procedure was performed, and mouse trachea was cannulated with 18G metal cannula and mechanically ventilated with frequency of 2.5 Hz. The increasing dose of acetyl-β-methylcholine chloride (Sigma-Aldrich, South Africa) in sterile 1x PBS (Thermofisher, South Africa): 0, 5, 10, 20, 40 mg/mL was administered to mice by nebulization. The consecutive measurement of respiratory resistance (R), elastance (E) and compliance (C) were performed. The mice were killed by lethal dose of halothane (Piramal Healthcare Limited, India).

2.4 Sample Collection

2.4.1 Collection and processing of skin tissue

Mice were euthanized using by halothane (Piramal Healthcare Limited, India) inhalation and shaved on the ventral side. Patch size of the skin (19 mm x 10 mm) was cut out and placed in 4% formalin for histology analysis or in 1xPBS for Flowcytometry.

2.4.2 Skin processing

The shaved skin from mouse was collected as described previously. The processing protocol was adopted from Jensen et al. with modifications[267]. The skin was sterilized by emersion for 1 min. before further processing in the following order: 10% BETADINE Antiseptic Solution (10% povidone-iodine), 70% ethanol (Thermofisher, South Africa), 1x sterile PBS (Thermofisher, South Africa). The tissue was soaked in 32U/mL trypsin (Gibco, USA) with epidermal side up for 3 hrs at 37 ^{0}C. Consecutively, the epidermis was scrapped off using sterile surgical blade. The dermis was further transferred to sterile Hank's balanced salt solution (HBSS, MERCK, USA) for subsequent secondary digestion. The epidermis was sieved through 70 µm cell strainer into 50 mL Falcon tubes with Roswell Park Memorial Institute -1640 (RPMI-1640) medium (Thermofisher, South Africa). The epidermis solution was further spun at 800x g for 10 min at room temperature. The supernatant was discarded and cell pellet was re-suspended for cell count and staining using 1mL RPMI-1640 medium (Thermofisher, South Africa). The dermis was minced using sterile scissors and re-suspended in 5 mL of HBSS solution supplemented with 3 mM Calcium chloride (Sigma, USA) containing 150 U/mL collagenase type IV from *Clostridium histolyticum* (Sigma, USA) in 15 mL Falcon tubes. The secondary digestion was done in incubator at 220 rpmi at 37 ^{0}C for 1 hr. The single-cell suspension was sieved via 70 µm cell strainer into 50 mL Falcon tubes and re-suspended with 15 mL RPMI-1640 medium (Thermofisher, South Africa). The cell solution was spun at 800x g for 10 min at room temperature. The supernatant was discarded and cell

pellet was re-suspended with 1 mL RPMI-1640 medium (Thermofisher, South Africa) for further cell count and processing.

2.4.3 Lung collection and processing

Superior lung lobe was preserved in 4% paraformaldehyde (PFA) and stored at room temperature for histological staining performed at the department of surgery of Groote Schuur Hospital, Cape Town. Inferior lung lobe was snap frozen in liquid nitrogen for further processing and stored and -80^{0}C. The left lung lobe was collected and transferred directly into ice-cold digest buffer (Supplementary, Table 1) which was stored on ice for processing. The lungs were incubated for 1 hour at 37 ˚C in digest buffer (Supplementary, Table 1) and further crushed through 70 µm cell strainer (Falcon, USA) into single-cell suspension. Cells were spun at 1500 rpm at 4 ^{0}C. The pellet was re-suspended in 5 mL osmotic lysis buffer (Supplementary, Table 1) for 5 min at room temperature. The incubation was stopped using 3 mL of RPMI-1640 Medium (Thermofisher, South Africa). Cells were spun at 1500 rpm at 4 ^{0}C. The pellet was re-suspended in 1 mL of RPMI-1640 Medium (Thermofisher, South Africa). Cells were counted in order to determine the cell number per lung. Cells were stained according to the protocol shown in table 2.

2.4.4 Broncho alveolar lavage collection and processing

Broncho-alveolar lavage (BAL) was collected by flushing lungs with 1 mL of sterile 1x PBS. Samples were collected and spun down for 5 min., 1500 rpm at 4 ^{0}C, the supernatant was stored at -80 ^{0}C for further processing and the pellet of the cells was re-suspended with 200 µL osmotic lysis buffer (Supplementary Table1) and incubated for 60 sec at room temperature. The incubation was stopped by adding 700 µL of 1x PBS. Samples were spun down for 5 min., 1500 rpm at 4 ^{0}C and re-suspended in 500 µL of 1xPBS). Cells were counted in order to determine the cell number per 1 mL of BAL fluid and stored at 4^{0}C for further analysis.

2.4.5 Lymph nodes re-stimulation

Inguinal/mediastinal lymph nodes (LN) were collected into 1 mL of non-supplemented RPMI-1640 Medium (Thermofisher, South Africa). The lymph node was crashed through 70 µm cell strainer (Falcon, USA) into single-cell suspension. Cells were spun at 1500 rpm at 4 ^{0}C. The pellet was re-suspended in 1 mL of RPMI-1640 Medium (Thermofisher, South Africa) supplemented with 10% heat-inactivated foetal bovine serum (Biochrom, Germany) and 10 UI penicillin/streptomycin (LONZA). Cells were counted and seeded 2x10^6 cells per well in 96-well U-bottom shaped plate (Thermo Scientific, USA). Cells were re-stimulated using ionomycin (250ng/mL, Sigma-Aldrich, USA), phorbal myristate acetate (PMA; 50ng/mL, Sigma- Aldrich, USA) and monensin (200mM, Sigma-Aldrich, USA) for 5 hours

at 37 ^{0}C in 5% CO_2. Cells were then spun at 1500 rpmi at 4 ^{0}C for 5 minutes and media was collected. Cells were further stained for flow cytometry.

2.4.6 Flow Cytometry staining

Cells were seeded in 96-well V-bottom plates (Thermo Scientific, USA) and spun at 1500 rpmi at 4 ^{0}C for 5 minutes. The supernatant was discarded. Surface staining containing surface cell markers in FACS buffer (1% BSA, 2% inactivated rat serum, 1% Fc block). The samples were incubated at 4 ^{0}C for 45 min in dark. For intracellular staining, we used the BD Pharmingen Transcription Buffer Set (BD Biosciences) as per manufacturer's instructions to detect Foxp3 expression or cytokine staining. In short, the samples were washed with 200 µL of permeabilization-wash buffer (BD Biosciences) and spun at 1500 rpmi at 4 ^{0}C for 5 minutes. The samples then were incubated with 100 uL of fix permeabilization buffer (BD Biosciences) at 4 ^{0}C for 1 hour in dark. The plate was washed again with 100 µL of permeabilization-wash buffer (BD Biosciences) and spun at 1500 rpmi at 4 ^{0}C for 5 minutes and intracellular staining was added according to table 2. The staining was prepared in permeabilization-wash buffer (BD Biosciences) and samples were incubated in dark at 4 ^{0}C for 45 min. Samples were acquired on LSR Fortessa (BD immunocytometry system, San Jose, CA, USA) and data were analysed using Flowjo software (Treestar, Ashland, OR, USA).

Single cell suspensions from lymph nodes were stained for markers with the following antibodies (Table 2):

Table 2.4 Flow Cytometry Antibodies list

Antibody	Clone	Fluorochrome	Producer	Dilution
CD3	500A2	Alexa 700	BD Science	1:320
CD8	53,6-7	V500, APC	BD Science	1:320
Foxp3	MF23	APC	BD Science	1:100
CD44	IM7	FITC	BD Science	1:320
CD62L	MEL-14	V450	BD Science	1:320
CD4	RM4-5	PerCp, V421	BD Science	1:320
CD19	1D3	APC-Cy7	BD Science	1:320
IL-4Rα	mIL4R-M1	PE	BD Science	1:160
γδTCR	GL3	biotin	BD Science	1:160
IFN-γ	XMG1.2	Alexa Fluor 700	BD Science	1:100
B220	RA3-6B2	V500	BD Science	1:320
TNFα	MP6-XT22	APC-Cy7	BD Science	1:100
IL-17A	eBio17B7	PerCp	BD Science	1:100
IL-13	eBio13A	PE-Cy7	BD Science	1:100
IL-5	TRFK5	PE	BD Science	1:100
CD21/35	7G6	APC	BD Science	1:80
CD24	M1/69	FITC	BD Science	1:320
CD23	B3B4	PE-Cy7	BD Science	1:320
GL7	GL7	PE	BD Science	1:320
FAS	MFL3	biotin		1:200
CD138	281-2	APC	BD Science	1:320
IgM	R4-22	FITC	BD Science	1:80
IgE	RME-1	PE	BD Science	1:80
IgG	A85-1	V450	BD Science	1:80
IL-4	11B11	FITC	BD Science	1:100
Streptavidin	none	PE-CF594/Texas Red	BD Science	1:1000

For the gating strategy for data analysis please refer to Appendix 1.

2.4.7 Histology

Formalin fixed (4%) skin and lung biopsies from mice were stained with hematoxilin and eosin (H&E), for lung biopsies additional Periodic acid–Schiff (PAS) staining was used. All processing and procedures were performed in Department of Surgery, Groote Schuur Hospital by Lizette Fick. Image acquisition was done using VS120 Virtual Slide Microscope (Olympus Global, USA) x20 magnification and quantification of epidermal thickening was performed using QuPath.Ink open software. Lung PAS histology was processed using ImageJ (NIH) software and analysed using NIS element microscope imaging software.

2.4.8 Antibody responses

Antibody responses were measure using enzyme-linked immunosorbent assay (ELISA) method. Briefly after each experiment the blood was collected post-mortem via cardiac puncture into separator tubes (BD Bioscience, San Diego, USA). Tubes were then spun for 5 min at room temperature at 6000 rpm according to manufacturer's instruction. Separated serum was then stored at -80 ^{0}C. The sandwich ELISA was performed for detection of total IgE, IgG1, IgG2a, IgG2b.

2.4.8.1 Total IgE ELISA

Wells were coated with IgE clone 84.1C in 1xPBS buffer and incubated overnight at 4°C. Wells were washed, blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich, USA) solution for 3 hrs at 37°C and sample added, followed by the addition of recombinant IgE (BD Biosciences, USA) and incubated overnight at 4°C. Further, plates were washed and Rat anti-mouse IgE biotinylated alkaline phosphatase (AP) antibody was added and incubated for 3 hrs at 37 °C. After incubation with AP-labelled antibody, plates were washed and 4-Nitrophenylphosphate (PNP) added (1 mg/mL dissolved in substrate buffer), (Sigma-Aldrich, USA). Optical density was measured at 405 nm with 492 nm as a reference wavelength. Micro-titre plate readings were carried out with a VersaMax™ ELISA plate reader and data were analysed using 62 SoftMax Pro 6.

2.4.8.2 HDM Specific antibody response

Wells were coated with HDM with 5 µg/mL of HDM in 50 µL PBS and incubated overnight at 4°C. The rest of the procedure was performed as for the total IgE ELISA (1.4.6.1). In short, wells were washed, blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich, USA) solution for 3 hrs at 37°C and sample added and incubated overnight at 4°C. Further, plates were washed and followed by the addition of recombinant isotype-specific AP conjugated secondary antibody (BD Biosciences, USA) and incubated for 3 hrs at 37 °C. After incubation with AP-labelled antibody, plates were washed and 4-Nitrophenylphosphate (PNP) added (1 mg/mL dissolved in substrate buffer), (Sigma-Aldrich, USA). Optical density was measured at 405 nm with 492 nm as a reference wavelength. Micro-titre plate

readings were carried out with a VersaMax™ ELISA plate reader and data were analysed using 62 SoftMax Pro 6.

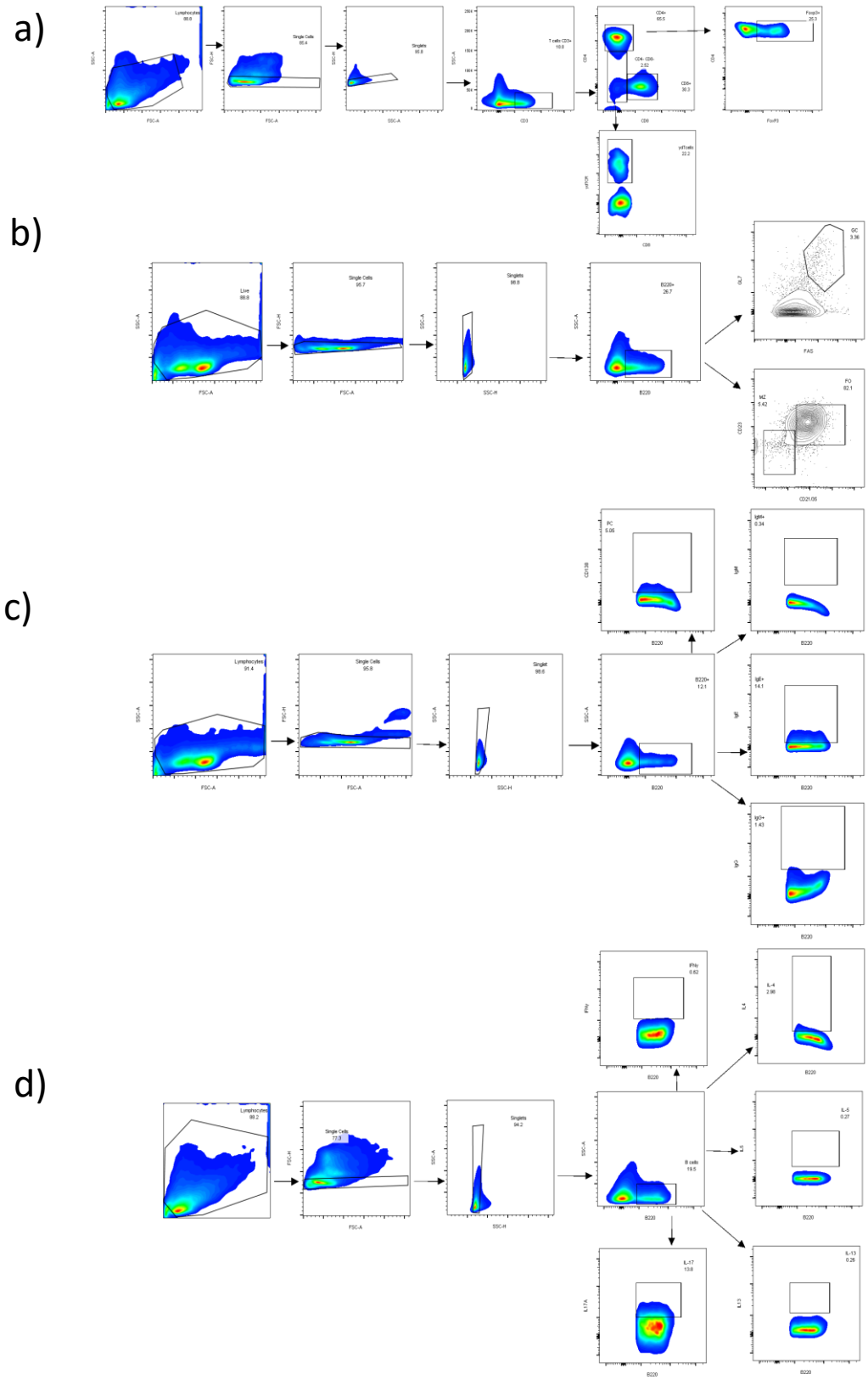
2.4.8.3 Cytokines responses

Supernatants collected from skin draining lymph nodes were used to determine cytokine production using ELISA as described above. Plates were coated using: anti-IL-4 (clone 11 B11), anti-IL-5 (BD, USA), anti-IL-13, IL-33 (R&D systems, USA), anti-IL-17 (Biolegend, USA) or anti-IFNγ (Clone AN18.KL6,) and plates were incubated overnight at 4°C. Serum or supernatant samples were added the next day as well as the recombinant protein for cytokines, followed by overnight incubation at 4°C. On the final day, the biotinylated detection antibody was added, and plates were incubated for 3 hrs at 37°C. Plates were washed and HRP-labelled Streptavidin (KPL, USA) was added and incubated for 1 hour at 37°C. Thereafter, plates were incubated with TMB peroxidase substrate mix (KPL, USA) until colour developed. The reaction was stopped using Sulfuric Acid. Micro-titre plate readings were carried out with a VersaMax™ ELISA plate reader and data were analysed

2.5 Statistics

All data were analysed using Graph-Pad Prism 6.0. Data were calculated as mean ± SD. Statistical significance was calculated with one or two-way ANOVA with post-hoc test Bonferroni or t-student test with Kruskal–Wallis test was used, where indicated. A *p value of less than 0.05 as considered significant, with **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Appendix 1



Appendix 1 Figure 1 Gating strategies for FACS analysis. a) T cell panel, b) and c) B cell panel analysis d) example of cytokine panel analysis

Supplementary

Table 1 *The buffer recipes used in the project*

Buffer	Recipe
10x Phosphate-buffered saline	1.37 M NaCl
	0.03 M KCl
	0.01 M Na_2HPO_4
	0.02 M KH_2PO_4
	1 L H_20
Digestion Buffer	0.002 g DNase I
	0.02 g Collagenase Type IV
	150 mL complete RPMI
Osmotic lysis buffer	155 mM NH_4Cl
	12 mM $NaHCO_3$
	0.1 mM EDTA
	1 L H_20
FACS buffer	0.5% Bovine Serum Albumin
	1 L 1x PBS
ELISA blocking buffer	20 g Bovine Serum Albumin
	1 L 1x PBS
ELISA dilution buffer	10 g Bovine Serum Albumin
	1 L 1x PBS

Chapter 3

The role of interleukin receptor alpha on B2 cells in atopic dermatitis

3.1 Introduction

Atopic dermatitis (AD) is non-communicable disease, which severely impairs the patients' quality of life[246,268,269]. The clinical presentation varies, depending on the severity of the disease, the most common presentation is pruritic visible flexural rashes combined with various morphological abnormalities. AD is occasionally linked to other allergic co-morbidities such as asthma, rhinoconjunctivitis and food allergy particularly in children [144]. The immunological presentation of allergic atopic dermatitis is mainly characterized by increase in serum immunoglobulin E (IgE) and allergen-specific IgE by skin prick test[20,203]. There is also an increase of T helper 2 (Th2) cytokines such as interleukin -4 (IL-4), interleukin-5 (IL-5), interleukin -13 (IL-13) and interleukin -31 (IL-31) [144,247,270]. Such presentation and diagnosis of the disease indicates a strong involvement of humoral response in development of the disease. The recent treatment proposed for atopic dermatitis patients involves: anti-IgE monoclonal antibody (omalizumab), anti-IL-4 receptor alpha (IL-4Rα) (dupilumab) as well as IgE-targeted immunoadsorption (AI) combined with topical glucocorticosteroids application[237,238,246,271]. As various strategies focus on the skin morphological presentation of AD patients, the exact mechanism of immune modulation has not been fully elucidated. Both anti-IgE treatments and anti-IL-4Rα therapies have shown reduction in allergen-specific IgE and total IgE at the end of treatment[220,238,271]. The exact mechanisms of how these therapies influence B cells ability to secrete IgE in AD is unclear and requires further investigation.

IL-4Rα is a pleiotropic receptor, which in heterodimeric form with IL-13Rα1 can transduce signals from both IL-4 and IL-13[8,168,272,273]. At the same time IL-4 signalling can be also executed by IL-4Rα combined with gamma (γ) chain subunit, while IL-13 can be detected solely by IL-13Rα2[167,168,274]. In rodents, IL-13Rα2 occurs in soluble form, while in humans the receptor has transmembrane domain lacking cytoplasmic signalling motifs, therefore it is considered to be a decoy receptor for IL-13 signalling[275,276]. As B cells can be both IL-4-responsive due to presence of the receptor on the cell surface, as well as they can secrete the cytokine, the deletion of the receptor can have both autocrine and paracrine consequences[260]. The effect of IL-4/IL-13 binding on IL-4Rα is required for B cell maturation, formation of germinal centre (GCs), B-T cell interaction and adequate antibody class switched IgE production[277,278]. Interestingly, in the skin gamma-delta (γδ)T cells have been shown to be essential in producing IL-4 production and influencing B cell class switching to IgE, which is essential in skin

tumours surveillance activation status along with autoantibody production as well as loss of B cell - mediated tolerance[279,280].

Previously in our group we found that deletion of IL-4Rα on B cells plays a pivotal role in establishing the Th2 immune response in infectious models of *Leishmania major*, *Schistosoma mansoni* and *Nippostrongylus brasiliensis* infections[162,260,262]. Upon deletion of IL-4Rα on B cells, the production of cytokines such as IL-4, IL-13 was downregulated, while interferon gamma (IFN-γ) was upregulated in those mice. Additionally, we found that deletion of IL-4Rα on B cells mediates class switching leading to impaired IgE antibody production in model of oxazolone induced colitis[161].

Our group found that inhibition of IL-4Rα signalling does not prevent airway hyperresponsiveness (AHR) in *Anisakis* -induced model of asthma due to IFN-γ signalling[201]. Despite absence of the receptor, B cells were still capable of IgG2a production. This effect was only present when mice were subcutaneously infected with the parasite, not by intranasal sensitization. This study emphasized the importance of the route of antigen administration as well as provides insight into IL-4Rα signalling on B cells.

Atopic dermatitis belongs to so called atopic triad: atopic dermatitis, asthma and rhinoconjunctivitis[281]. The secondary allergic reaction such as asthma or rhinoconjunctivitis preceded by atopic dermatitis is known as atopic march (AM)[18,199,202,282]. We and others studied atopic march in context of Th2 response in various models of AM[126,160,248]. Both Langerhans cells and skin residing γδ T cells (DETC) are capable of detecting antigen or other danger signal, they can secrete cytokines to recruit other lymphocytes as well as migrate to skin draining lymph nodes (LN) where they can interact with other cells[6,71]. Therefore, the systemic sensitization to epicutaneously induced allergen can lead to systemic reaction[61]. Of interest remains influence of B cell specific IL-4Rα removal, which abrogates the co-stimulation between B and T follicular cells in germinal center[283]. As previously shown, in the general absence of the receptor, cells are still capable of IgG2a production as well as in asthma model, IFN-γ-mediate AHR[160,201].

We therefore investigated the AD changes in murine models of AD in mice lacking fully functional B cells - $MB1^{Cre}IL\text{-}4R\alpha^{-/lox}$. We found that despite the repetitive exposure to allergen, we were unable to induce skin changes in mice, however, the humoral response was altered resulting in increased total IgE in wild-type (WT) mice – one of the hallmarks of AD in patients. Mice lacking IL-4Rα specifically on B cells were unable to produce total IgE. Further we showed that B cell specific IL-4Rα abrogation does not protect from non-allergic atopy in MC903 model of AD. We further confirmed that removal of the

receptor influenced the B cell maturation, germinal centre formation and IgE antibody production. Additionally, we investigated if those mice are protected from atopic march – one of the major consecutive allergic disorder following atopic dermatitis. We found that despite absence of total IgE, mice lacking IL-4Rα on B cells were still capable of producing IgG2a and they were more prone to increased mucus production upon intranasal challenge. This study gives insight on reduction of IgE production upon dupilumab treatment.

3.2 Results

3.2.1 Presence of IL-4Rα on B cells does not influence epidermal thickness in mice.

We have previously shown that IL-4Rα in CD4 T cells is redundant in *Anisakis* induced AD[160]. Furthermore, we found an IL-4Rα dependent and independent role in AD using OVA model [272]. Because of the important role of B cells and IgE particularly as a marker for sensitisation to allergens in skin prick test, we wondered whether IL-4Rα signalling would be essential in AD. In order to verify the influence of B cells and B cell activation via IL-4Rα signalling on epidermal thickening at baseline, we euthanized naïve mice from $MB1^{Cre}$IL-4R$\alpha^{-/lox}$, uMT and IL-4R$\alpha^{-/lox}$ (WT) strains and collected shaved skin biopsies. Skin samples were stained with H&E and epidermal thickening was measured using QuPath software (Fig.3.1a). The lack of B cell activation via IL-4Rα did not influence the keratinization of skin in naïve mice (Fig. 3.1b). To understand whether B cells have a function during epicutaneous inflammation, we sensitised mice with increasing doses of House dust mite (HDM) and a single high dose of ovalbumin (OVA) (Fig. 3.1c). We found no differences in inflammation and epidermal thickness between HDM 10μg, 100μg and OVA 200μg (Fig. 3.1d). We also observed a modest serum IL-33 levels when comparing HDM 10μg, 100μg and OVA 200μg (Fig. 3.1e). We then chose to use low dose HDM of 10μg for subsequent experiments.

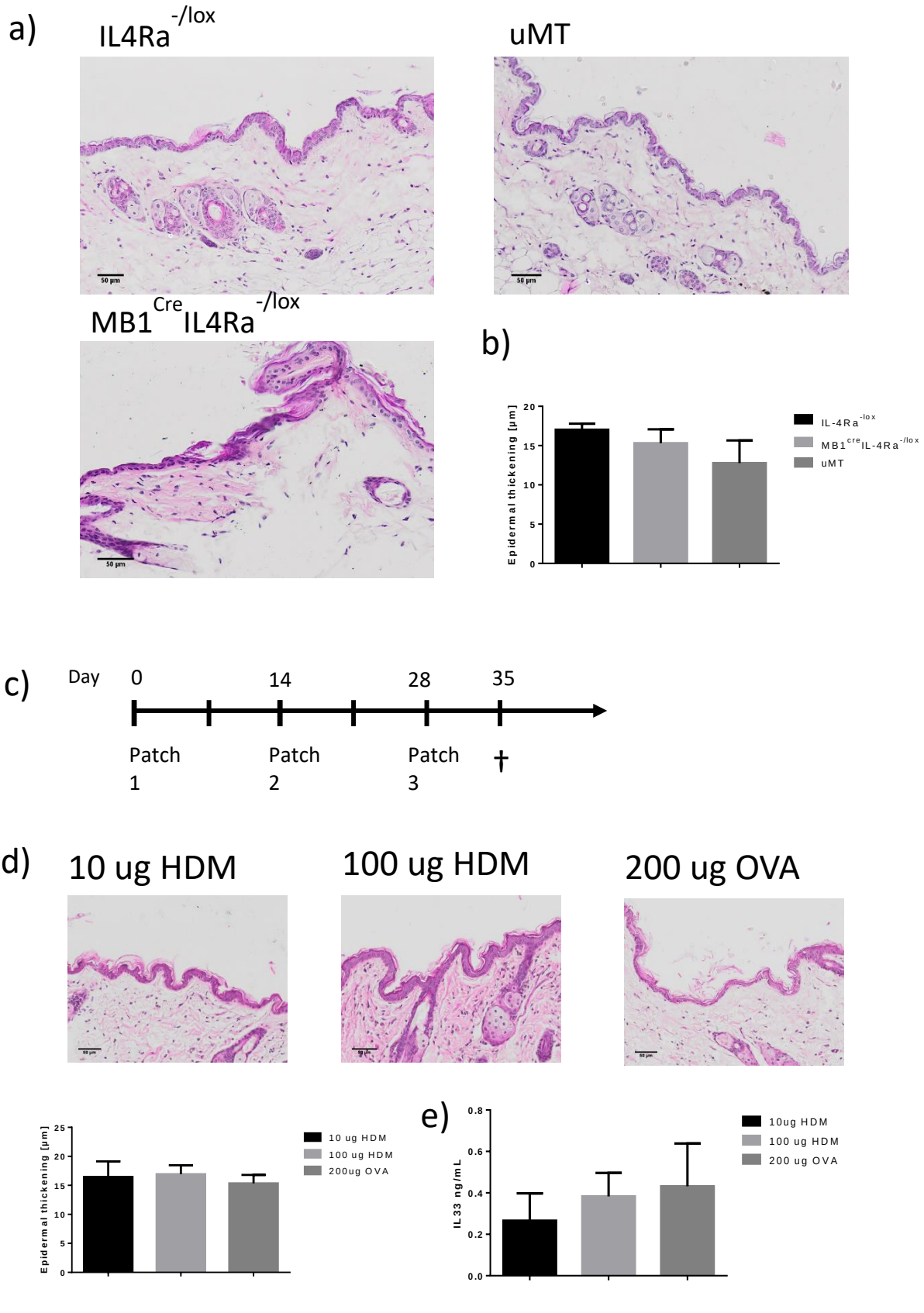


Figure 3.1 Naive mice of global IL4Ra knockout, MB1CreIL4Ra-/lox and uMT do not differ in epidermal thickening. a) Comparison of skin biopsies of ventral side, collected from naive mice after post mortem shaving, histology was stained with H&E, scale bar 50um, b) quantification of epidermal thickening using QuPath software. Representative of one experiment is shown with mean ± SD. One-way ANOVA statistical analysis was performed n=5 mice per group

3.2.2 IL-4Rα plays minimal role in inflammation during HDM epicutaneous sensitization in Atopic Dermatitis but is important for IgE production.

Dupilumab is an Food and Drug Administration (FDA) approved monoclonal antibody that targets IL-4Rα signalling and has shown promise in phase II/III clinical trials in AD patients with improvement in severity and IgE [284–286]. We investigated the B cell activation during allergic sensitization in mouse model of HDM. We epicutaneously sensitized mice using 10 ug of HDM on pre-shaved skin over period of 5 weeks, with 2 weeks intervals (day 0, 14, 28), patches were placed for 24hrs (as shown Fig. 3.1c). Mice were killed on day 35, skin biopsies, blood and inguinal lymph nodes (iLN) were collected for further analysis as described in Chapter 2 Materials and Methods.

Mice sensitized with HDM did not show significant increase in epidermal thickening between IL-4Rα$^{-/lox}$ and PBS control, however both MB1^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ did not change at all after HDM treatment in contrast to IL-4Rα$^{-/lox}$ (Fig 3.2a, b). The results suggest lack of adequate sensitization to HDM allergen.

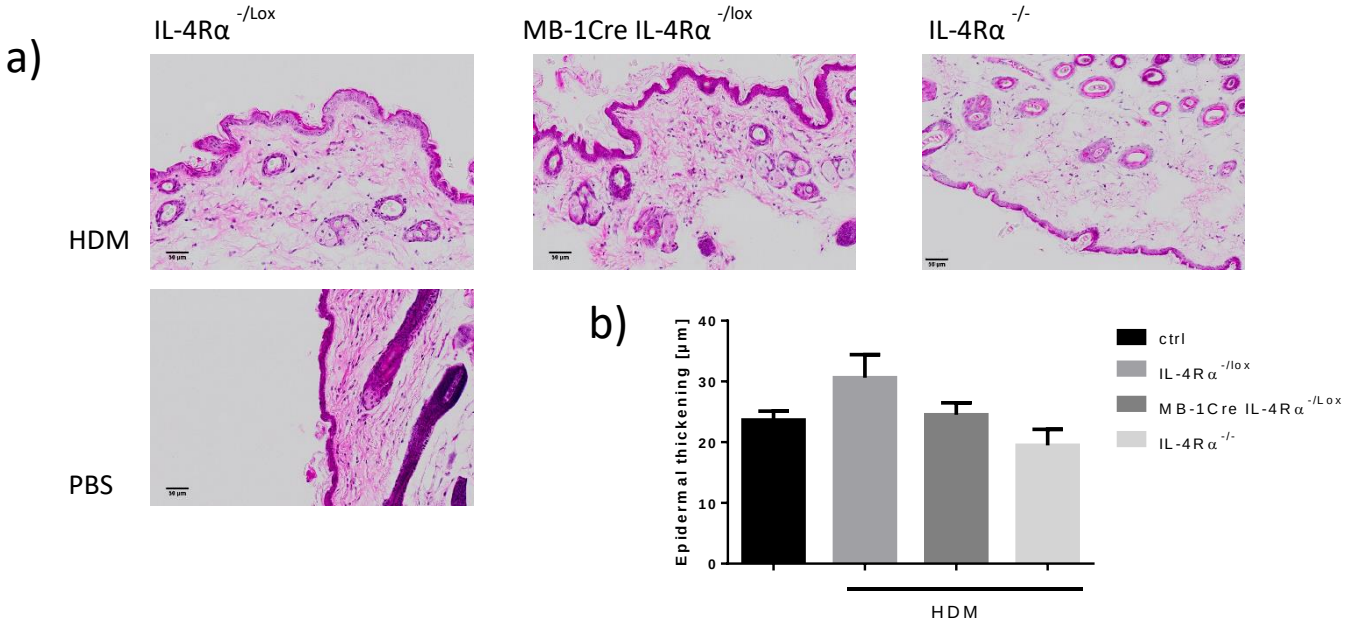


Figure 3.2 Treatment with 10ug HDM does not induce epidermal thickening in mice. a) Comparison of skin biopsies of ventral side, from mice treated with 10ug HDM histology was stained with H&E scale bar 50um, b) and quantification of epidermal thickening using QuPath software. Representative of two experiment is shown with mean ± SD. One-way ANOVA statistical analysis was performed, n=5 mice per group

One of the hallmarks of AD is increased level of total IgE in patients [287]. To analyse the level of IgE in mice, we collected blood *post-mortem*, by cardiac puncture and separated serum. We further analysed levels of total IgE and cytokines (IL-33 and IL-13) by ELISA. Treatment with HDM increased

level of total IgE in IL-4Rα$^{-/lox}$ mice, but not in mice lacking IL-4Rα (Fig. 3.3a). Additionally, we did not detect any HDM specific antibodies (data not shown). Interestingly there was no change in IL-33 and IL-13 cytokine levels between PBS and HDM treated littermate controls, however, the IL-33 and IL-13 levels were undetectable in MB1^{Cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα global knockout mice (Fig. 3.3b).

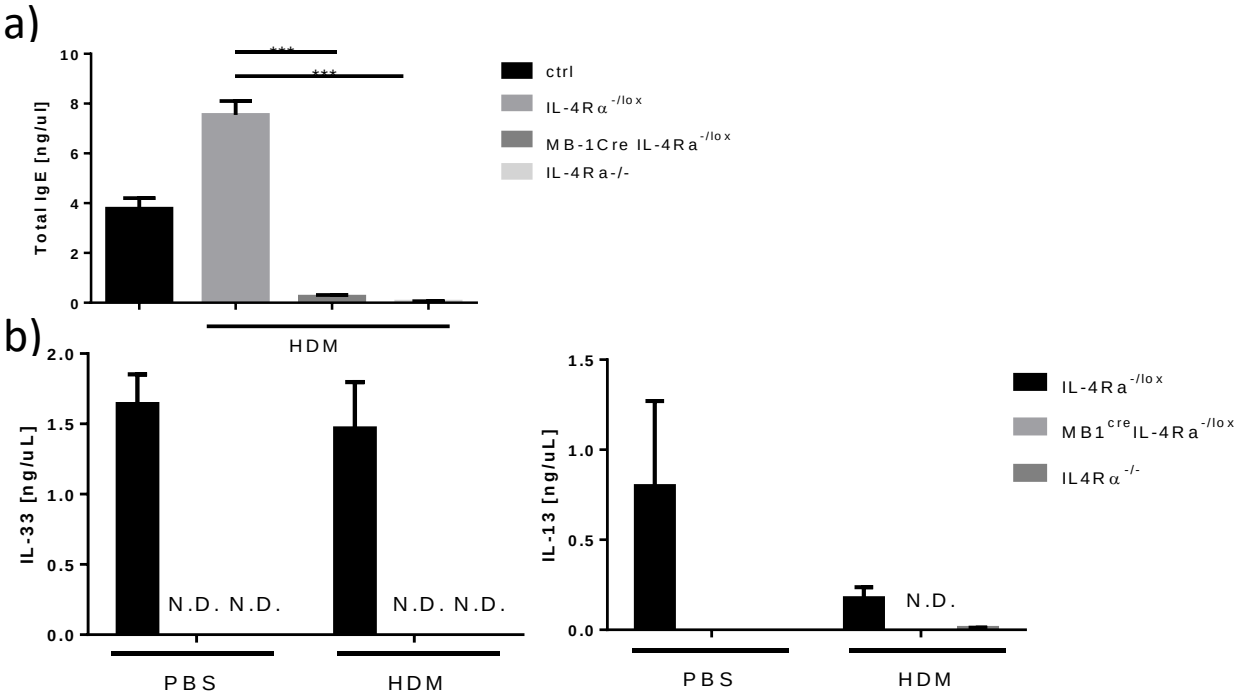


Figure 3.3 MB1^{Cre} IL4Rα $^{-/lox}$ and IL4Rα $^{-/-}$ mice are not producing IgE upon exposure to HDM. a) Quantification of total IgE and b) serum cytokine using ELISA. Representative of two experiment is shown with mean ± SD. One-way ANOVA statistical analysis was performed *P<0,01, **P<0,001, ***P<0,0001 n=5 mice per group

Collectively, our results show that IL-4Rα is necessary for induction of IgE antibodies and type 2 cytokines as well as alarmins.

3.2.3 Cellular infiltration is not attenuated upon HDM sensitization

Repetitive exposure to exogenous antigen such as HDM leads to formation of type 2 immunity against the allergen in various models of asthma [288–290]. Similarly, to asthmatic models we predicted that upon repeated epicutaneous exposure to HDM, type 2 immunity predominantly rich in CD4+ cells as well as IgE producing CD19+ MHCII+ B cells would be formed in inguinal LN[291] . On day 35 we collected inguinal LN (iLN) and analysed cellular composition using FACS, gating for various T and B cells according to gating strategy presented in Appendix 1 in Chapter 2 Materials and Methods. (Fig. 3.4).

Upon treatment with HDM, the total number of cells was increased in IL-4Rα$^{-/lox}$ littermate control mice, but not in MB1^{Cre}IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/-}$ groups, when compared to PBS control (Fig. 3.4a). We

then analysed the frequencies and cell numbers of CD19+ MHCII+ B cells. We observed no significant increase in IL-4Rα$^{-/lox}$ group after HDM sensitization when compared to PBS control. Additionally, the numbers of B cells in MB1^{Cre}IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/-}$ groups remained unchanged when compared to PBS group (Fig. 3.4b). We also verified the IL-4Rα knock-down, using mean fluorescence intensity of IL-4Rα on those cells and found that MB1^{Cre}IL-4Rα$^{-/lox}$ group has partial knock-down of the receptor expression, while IL-4Rα global knockout group shows complete receptor knockout when compared to both littermate treated with HDM and PBS (Fig. 3.4b). We then analysed the various T cell subsets in the iLN (Fig. 3.4c). The frequency of CD4+ T cell population or cell numbers were not changed after treatment with HDM in any of the groups when compared to PBS control. Similarly, The CD8+ T cell composition was not changed after allergen exposure in IL-4Rα$^{-/lox}$, MB1^{Cre}IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/-}$ when compared to PBS control. We found increased γδ+ T cell numbers upon sensitization in IL-4Rα$^{-/lox}$ mice when compared to PBS control; this increase was not visible in mice lacking IL-4Rα on B cells (Fig. 3.4c).

Interestingly, both CD4+ and CD19+ MHCII+ cells showed reduction in IL-4Rα in MB1^{Cre}IL-4Rα$^{-/lox}$ mice suggesting need for adequate B cell IL-4Rα signalling for proper co-stimulation in T cells (Fig. 3.4b and c).

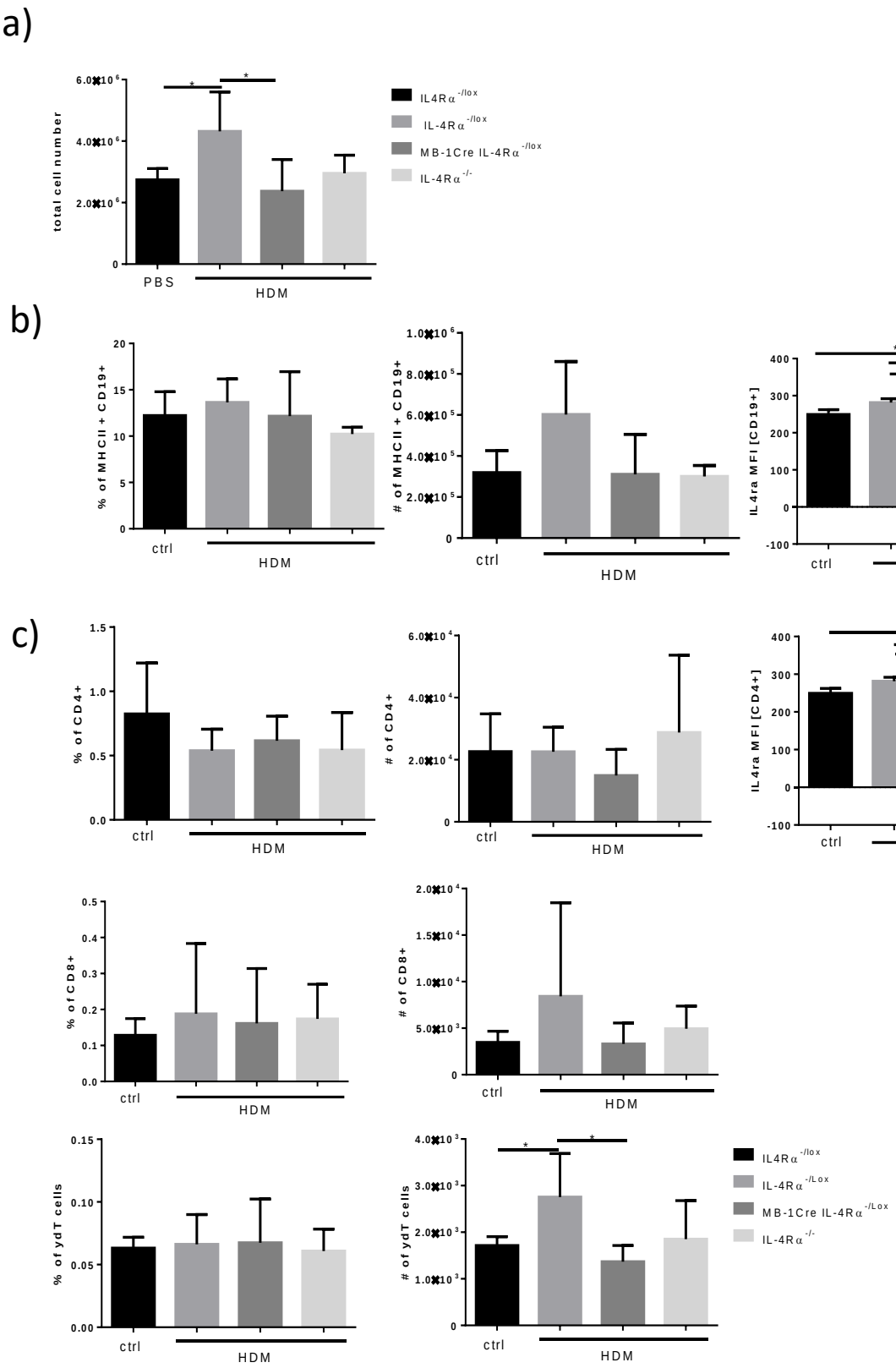


Figure 3.4 Cellular composition of the dLNs is not changed by HDM sensitization. a) Total cell number, b) analysis of B cell and c) T cell populations using FACS. Representative of two experiment is shown with mean ± SD. One-way ANOVA statistical analysis was performed *P<0,01, **P<0,001, ***P<0,0001 n=5 mice per group

Overall, the treatment with HDM was insufficient to induce all hallmarks of atopic dermatitis in murine model. Importantly, IgE was completely abrogated in mice lacking IL-4Rα on B cells epicutaneously sensitized to HDM.

3.2.4 IL-4Rα on B cells remains redundant in MC903-induced model of skin inflammation

We found HDM to not induce a strong skin inflammation and this may be due to lack of proteases which break down skin barrier [253]. To explore possibility that HDM model might be insufficient for skin inflammation, we performed a short model of non-antigen induced AD using low-calcaemic analogue of vitamin D (MC903)[142]. The murine model of acute skin irritation with MC903 characterizes as formation of itchy skin plaques on site of treatment, increase in skin cell infiltrates, elevation of inflammatory markers such as total IgE, TSLP as well as reduction of body weight [67,141,142].

Mice were treated with 45 uM of MC903 or with ethanol (vehicle control) over period of 10 days during which weight loss was monitored and were killed on day 11 as described in Chapter 2 Materials and Methods (Fig. 3.5a). We collected shaved skin biopsies from treatment site, inguinal lymph nodes and blood for further analysis.

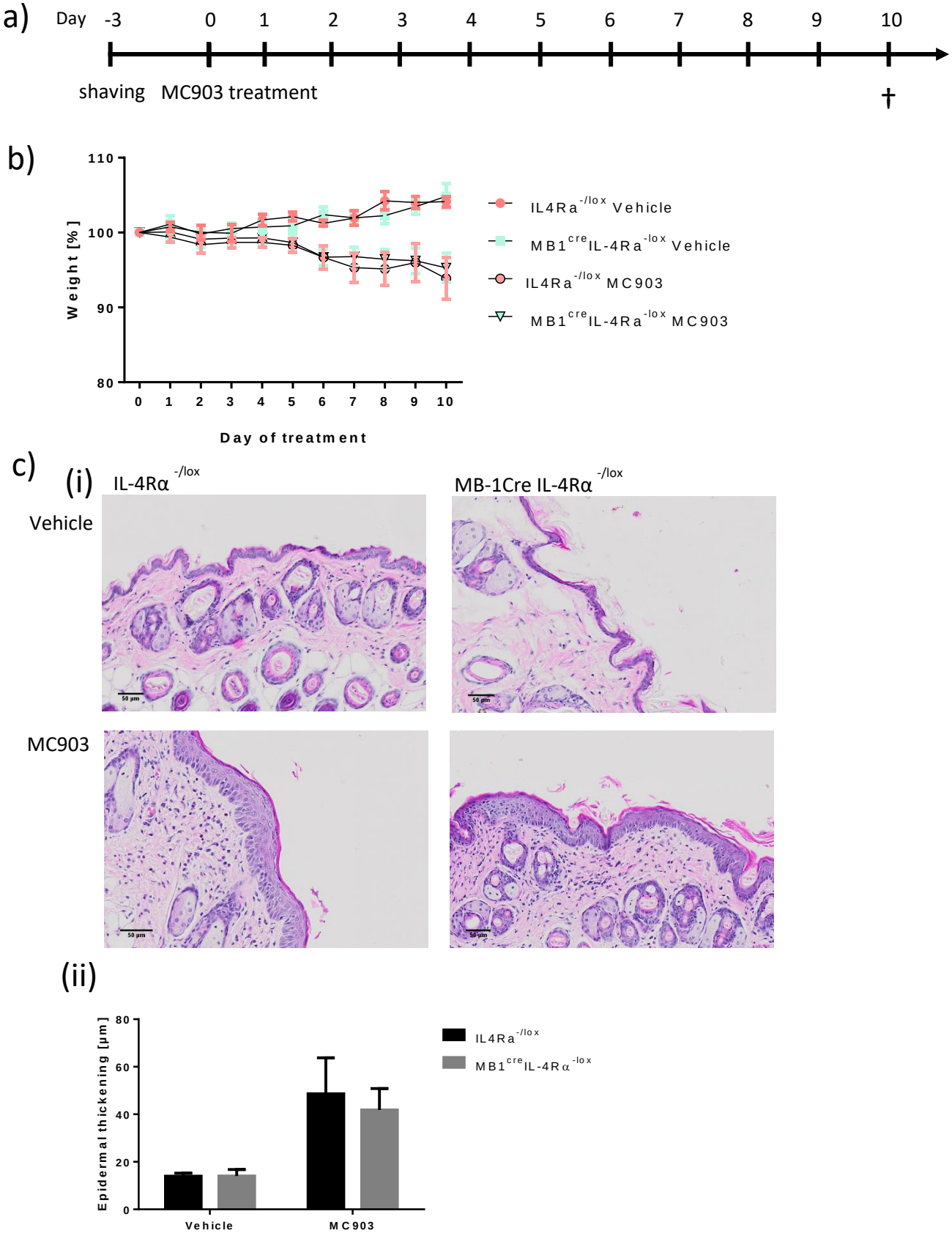


Figure 3.5 Treatment with 45uM MC903 induces similar inflammation in both MB1^{Cre} IL4Rα $^{-/lox}$ mice and B cell competent mice. **a**) scheme of the treatment, b) weigh loss tracing, c) (i) comparison of skin biopsies of ventral side, from mice treated with 45 uM MC903 or vehicle histology was stained with H&E (ii) quantification of epidermal thickening using QuPath software. Multiple group comparison; graph showing one experiment is shown with mean ± SD. Two-way ANOVA with Turkey post-test analysis was performed n=3 - 7 mice per group,

During the experiment we observed induction of severe skin inflammation upon MC903 treatment as well as mild weight loss (Fig. 3.5b). We did not see a significant difference between weight loss MB1^{Cre}IL-4Rα$^{-/lox}$ mice and their littermate controls during MC903 treatment (Fig. 3.5b). The analysis of epidermal thickening did not show difference between MB1^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ group after MC903 treatment (Fig. 3.5c, i). Treatment with vehicle did not influence the keratinization of epidermis (Fig. 3.5c, ii).

3.2.5 IL-4Rα is important for CD4+ T cell accumulation in secondary lymphoid organs

In mice the exposure to MC903 led to weight loss and epidermal hyperplasia. The epidermal thickening stimulates immune response in human AD lesion[67]. Thus, we analysed the inguinal lymph nodes immune cell infiltrates. We found no differences in frequencies and numbers of B cells between MB1^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ group after MC903 treatment (Fig. 3.6a). We confirmed the phenotype of MB1^{Cre}IL-4Rα$^{-/lox}$ mice by analysing the expression of IL-4Rα. The receptor expression was reduced in MB1^{Cre}IL-4Rα$^{-/lox}$ mice after MC903 treatment, when compared to littermate control (Fig. 3.6a). We also found that the main cytokine produced by B cells was IL-17A in IL-4Rα$^{-/lox}$ group but not in MB1^{Cre}IL-4Rα$^{-/lox}$ after MC903 treatment (Fig. 3.6a). To better understand the interaction between B cells and various T cell populations we analysed the T cell composition and their cytokine production in the iLN (Fig. 3.6b). The CD4+ and CD8+ T cell populations showed trend in reduction in numbers but not in frequencies in MB1^{Cre}IL-4Rα$^{-/lox}$ group treated with both vehicle and MC903 when compared to IL-4Rα$^{-/lox}$ group. Additionally, their cytokine production profile was not altered by absence of IL-4Rα (Fig. 3.6b). The expression of IL-4Rα on CD4+ T cells was not changed in MB1^{Cre}IL-4Rα$^{-/lox}$ when compared to littermate control, showing specificity of Cre recombination (Fig. 3.6b). The γδ+ T cell populations was significantly reduced cell numbers but not in frequencies in IL-4Rα-deficient B cells when compared to littermate control mice treated with skin vehicle only (Fig. 3.6b). Moreover, γδ+ T population produces IL-17A after MC903 treatment in littermate control group but not in MB1^{Cre}IL-4Rα$^{-/lox}$ group (Fig. 3.6b).

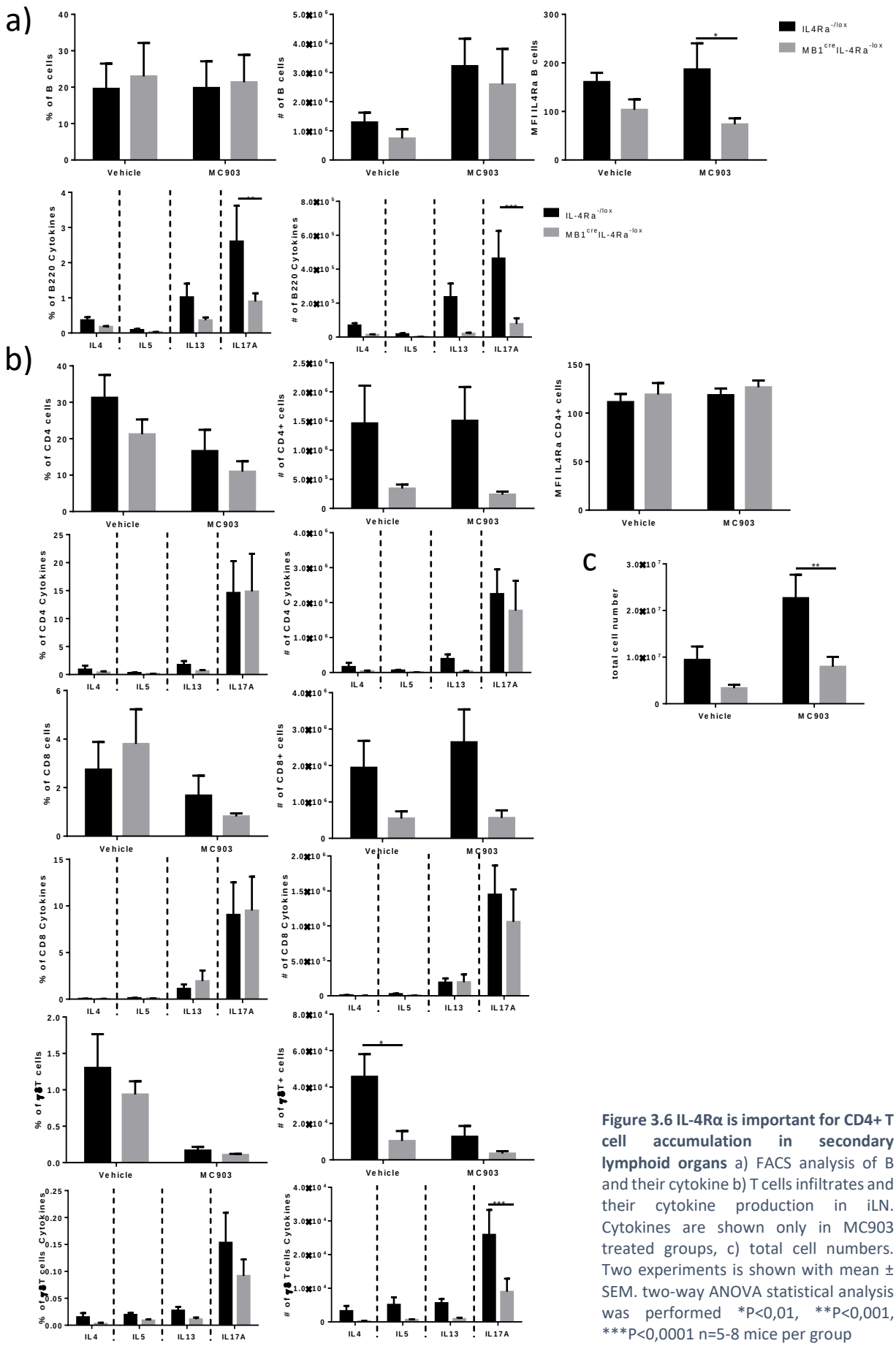


Figure 3.6 IL-4Rα is important for CD4+ T cell accumulation in secondary lymphoid organs a) FACS analysis of B and their cytokine b) T cells infiltrates and their cytokine production in iLN. Cytokines are shown only in MC903 treated groups, c) total cell numbers. Two experiments is shown with mean ± SEM. two-way ANOVA statistical analysis was performed *P<0,01, **P<0,001, ***P<0,0001 n=5-8 mice per group

Collectively this part of the investigation suggests that upon skin inflammation, B cell population is expanded in skin draining lymph nodes. The reduction of CD4+ T cells in MB1^{Cre}IL-4Rα$^{-/lox}$ suggests importance of the receptor in B -T cells interaction possibly in follicles, however further investigation is necessary. Additionally, the inflammation is mainly IL-17A driven.

3.2.6 Presence of IL-4Rα signalling on B cells is necessary for germinal centre formation

To investigate the B cell and follicular T cell interaction we further analysed various B cell subpopulations in iLNs: follicular (FO), marginal zone (MZ), germinal centre (GC) and plasma (PC) cells. Follicular B cells numbers, but not frequencies were significantly reduced upon the MC903 treatment when comparing IL-4R$^{-/lox}$ and MB1^{cre}IL-4Rα$^{-/lox}$ (Fig 3.7a). MB1^{Cre}IL-4Rα$^{-/lox}$ mice showed reduction in marginal zone cell count but not in cell frequencies when compared to their littermate control group (Fig. 3.7b). Of interest, the germinal centre B cells count, and frequencies were reduced in MB1^{Cre}IL-4Rα$^{-/lox}$ mice when compared to IL-4Rα$^{-/lox}$ (Fig. 3.7c). Since B-T cell interaction occurs in germinal centre and it is IL-4 dependant, it could explain the lower numbers of CD4+ T cells (Fig. 3.6b) in MB1^{Cre}IL-4Rα$^{-/lox}$ mice [292].

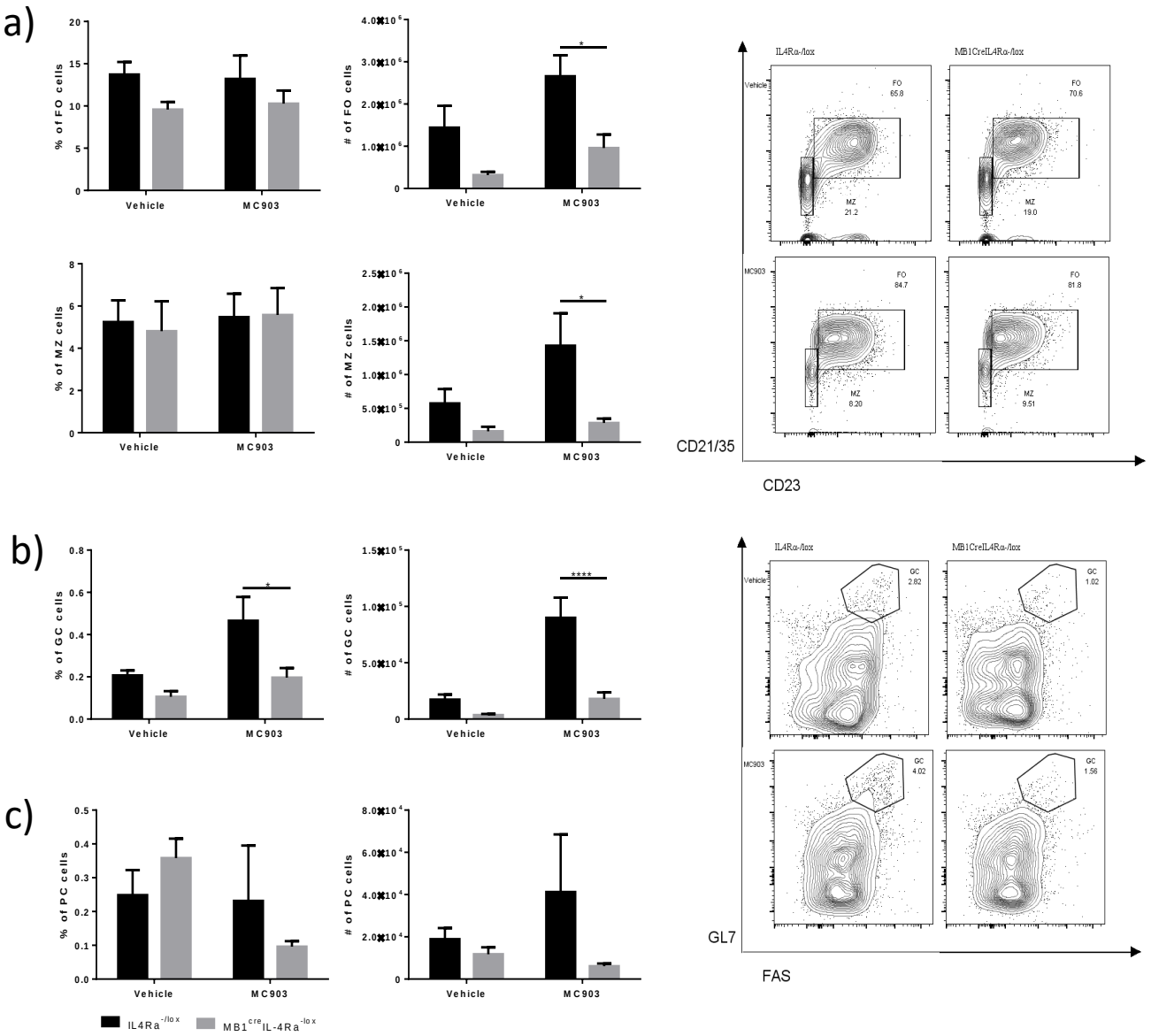


Figure 3.6 Treatment with MC903 leads to increase in germinal centre B cells in IL4Ra$^{-/lox}$ mice, but not in the MB1^{Cre}IL4Ra$^{-/lox}$ mice. FACS analysis of B cell subpopulations: a) follicular cells (FO) and marginal zone (MZ), b) germinal centre (GC) and c) plasma cells (PC). Two experiments are shown with mean ± SEM. two-way ANOVA statistical analysis was performed *P<0,01, **P<0,001, ***P<0,0001 n=5-8 mice per group

As germinal centre interactions are crucial for high affinity antibody maturation and class switching, we sought to investigate the different antibody producing B cells in iLNs[113,135,293]. The analysis of antibody producing B cells showed reduction of IgG+ B cell numbers, but not frequencies in MB1^{Cre}IL-4Rα$^{-/lox}$ group after treatment with MC903, when compared to littermate control (Fig. 3.8a). Of interest, the IgE+ B cells were significantly reduced in MB1^{Cre}IL-4Rα$^{-/lox}$ mice in both frequencies and number upon MC903 treatment (Fig. 3.8b). We found no significant changes between the groups in IgM+ B cells (Fig. 3.8c)

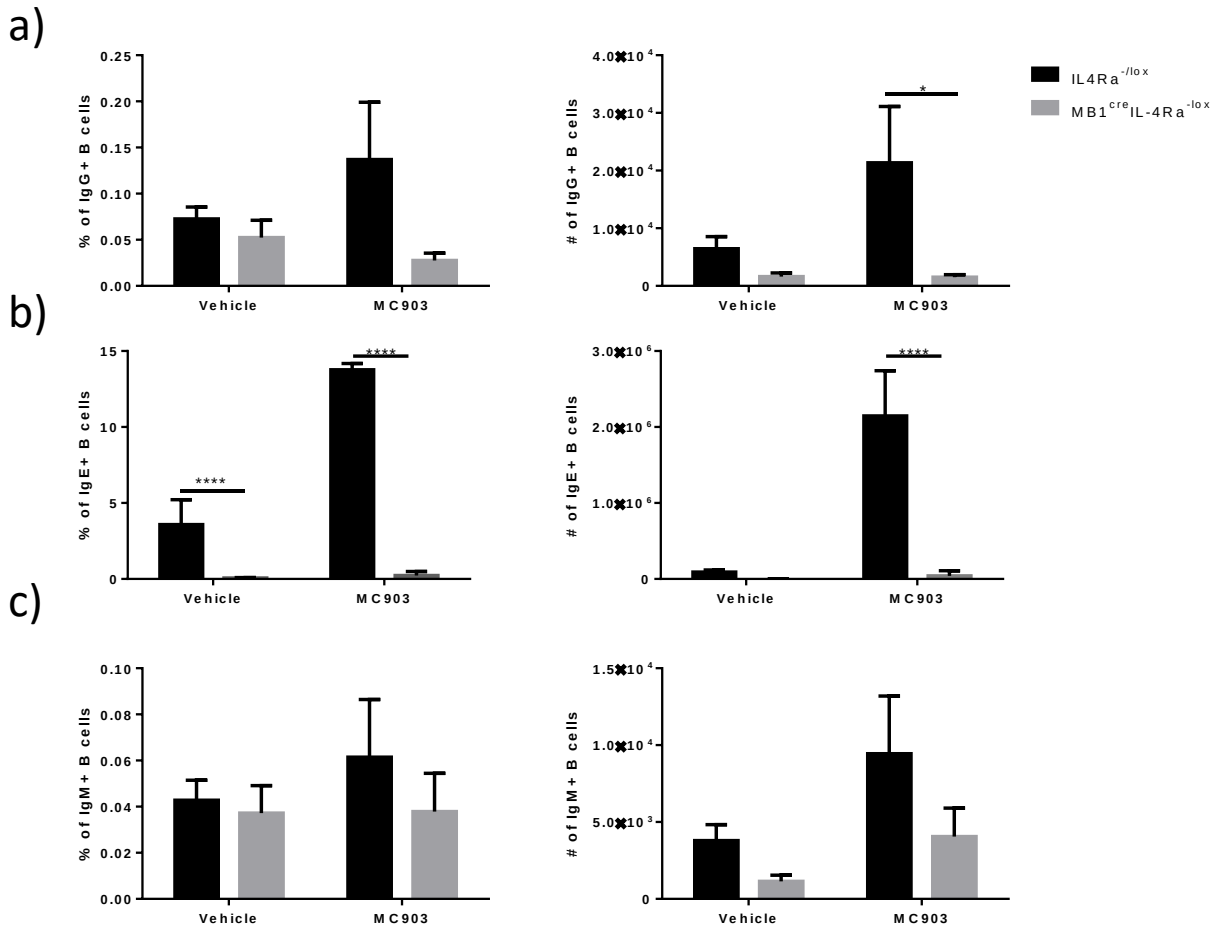


Figure 3.8 Treatment with MC903 leads to increase in IgE producing B cells in IL4Ra$^{-/lox}$ mice, but not in the MB1^{Cre}IL4Ra$^{-/lox}$ mice. FACS analysis of antibody production: a) IgG+ b) IgE+ c) IgM+ B cells. Two experiments are shown with mean ± SEM. two-way ANOVA statistical analysis was performed *P<0,01, **P<0,001, ***P<0,0001 n=5-8 mice per group

Taken together, the absence of IL-4Rα contributes to impaired functionality of B cells, which results in failed specific antibody production. However, mice lacking IL-4Rα on B cells did not show protection from the MC903-induced weight loss, therefore suggesting redundancy of specific antibody production this model. At the same time, the numbers of CD4+T cells was reduced, which indicates a role of IL-4Rα signalling in T-B interaction in secondary lymphoid organs.

3.2.7 Epicutaneous sensitization with HDM can lead to mild lung inflammation upon HDM challenge

The skin sensitization with an allergen can lead to further food or airway allergies, which can further lead to anaphylactic shock or asthma [61,160]. This immunological mechanism is known as atopic march (AM) [282]. Despite various research on the molecular mechanism driving AM, the exact order of biological events has not been established. Previously in our lab we showed that repetitive skin exposure to food allergen such as OVA and Anisakis can lead to consecutive anaphylactic shock and/or asthma [160,200,294].

In our AD study, we found IL-4Rα signalling on B cells to play minimal role in skin inflammation, although IgE levels were dependent on IL-4Rα signalling on B cells (Figure 3.2a and 3.3a). To determine the importance of repetitive skin exposure to allergen we investigated progression of AD to AM by challenging mice intranasally with 10ug HDM for 3 days after (Fig. 3.9a). On the last day we measure AHR, collected BAL, lungs, blood, mediastinal LN for further analysis.

We analysed cellular composition of BAL and lungs cells using FACS in $MB1^{Cre}$IL-$4R\alpha^{-/lox}$ and littermate control mice sensitised through skin and challenged with HDM intranasally. We first analysed the cellular composition of the BAL and lungs (Fig. 3.9). We found that IL-$4R\alpha^{-/lox}$ mice epicutaneously sensitized to HDM and challenged intranasally have increased cell numbers when compared with $MB1^{Cre}$IL-$4R\alpha^{-/lox}$ group in BAL (Fig. 3.9b). The frequencies and numbers of dendritic cell infiltrates showed trend in elevation in IL-$4R\alpha^{-/lox}$ mice, but not in $MB1^{Cre}$IL-$4R\alpha^{-/lox}$ after HDM sensitization (Fig. 3.9b). Additionally, the eosinophils frequencies and numbers were increased in IL-$4R\alpha^{-/lox}$, but not in $MB1^{Cre}$IL-$4R\alpha^{-/lox}$ strain after HDM exposure (Fig. 3.9b). We further analysed lung cell composition; we found no change in cell numbers after HDM sensitization (Fig. 3.9c). Similarly, to BAL DC in lung the frequencies of DCs were increased, however the numbers of DC infiltrates did not reflect the same trend (Fig. 3.9c). As in BAL, the eosinophilic infiltrates were significantly elevated both in frequencies and in cell counts in IL-$4R\alpha^{-/lox}$ when compared to $MB1^{Cre}$IL-$4R\alpha^{-/lox}$ strain after HDM exposure (Fig. 3.9c). Lastly, we verified the expression level of IL-4Rα on B cells using FACS (Fig. 3.9d), where $MB1^{Cre}$IL-$4R\alpha^{-/lox}$ mice showed reduction in IL-4Rα expression on B cells in mediastinal lymph nodes after HDM exposure. This reduction was not observed in their littermate control, nor on CD4+ T cells, showing specific deletion in B cells (Fig. 3.9d).

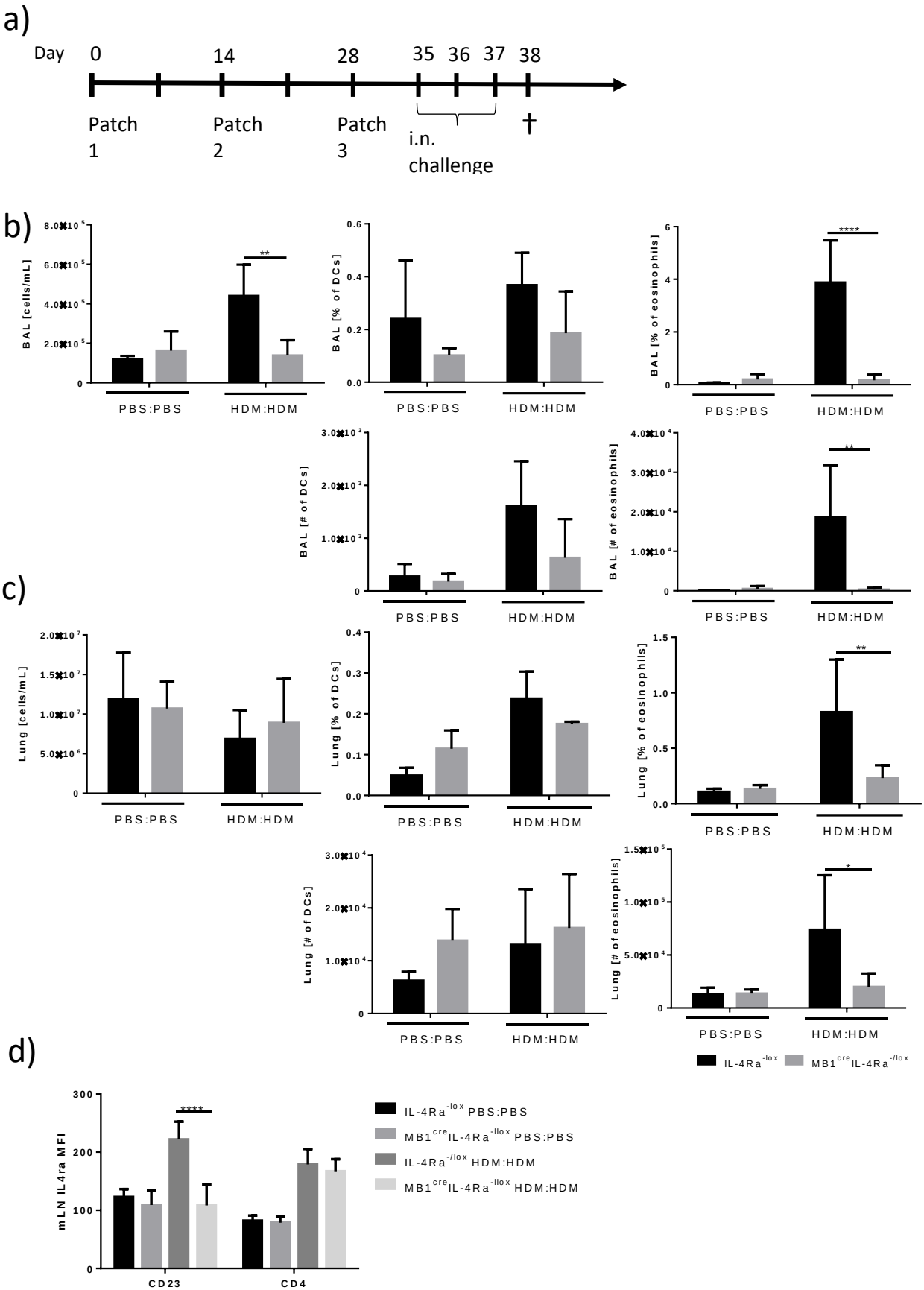


Figure 3.9 Epicutaneous sensitization with HDM increase in BAL and lung eosinophils infiltration. a) Schematic of HDM treatment, b) quantification of BAL and c) lung infiltration. d) Mean fluorescence intensity of IL4Ra on B and T cells. Representative of one experiment is shown with mean ± SD. One-way ANOVA statistical analysis was performed *P<0,01, **P<0,001, ***P<0,0001 n=5 mice per group

Collectively, we found that repetitive epicutaneous exposure to antigen can lead to systemic reaction such as increase in eosinophilic cellular infiltrates in BAL and lung, when challenged intranasally with the same allergen. At the same time, the mice lacking IL-4Rα on B cells are protected from lung eosinophilia upon allergic challenge.

3.2.8 IL-4Rα signalling on B cells is not essential for airway hyperresponsiveness or airway inflammation but is required for Th2-associated antibody production after epicutaneous sensitization with HDM

The allergic reaction to HDM is driven by anti-HDM antibody production, therefore we measured serum levels of anti-HDM specific antibodies after HDM epicutaneous sensitisation and HDM i.n. challenge. We found littermate control mice to have increased Th2 associated antibody response, shown by increased HDM-specific IgE and IgG1, while $MB1^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice did not show the same trend (Fig. 3.10a). Conversely, the mice lacking IL-4Rα on B cells presented with increased IgG2a HDM-specific antibody, which suggest possible increase in Th1 associated antibody production in those mice (Fig. 3.10a). Both littermate controls and $MB1^{Cre}IL\text{-}4R\alpha^{-/lox}$ treated with HDM and PBS showed elevated IgM levels. This phenomenon could be explained by mild inflammation induced by shaving as well as activity of B1 cells in those mice[61,112].

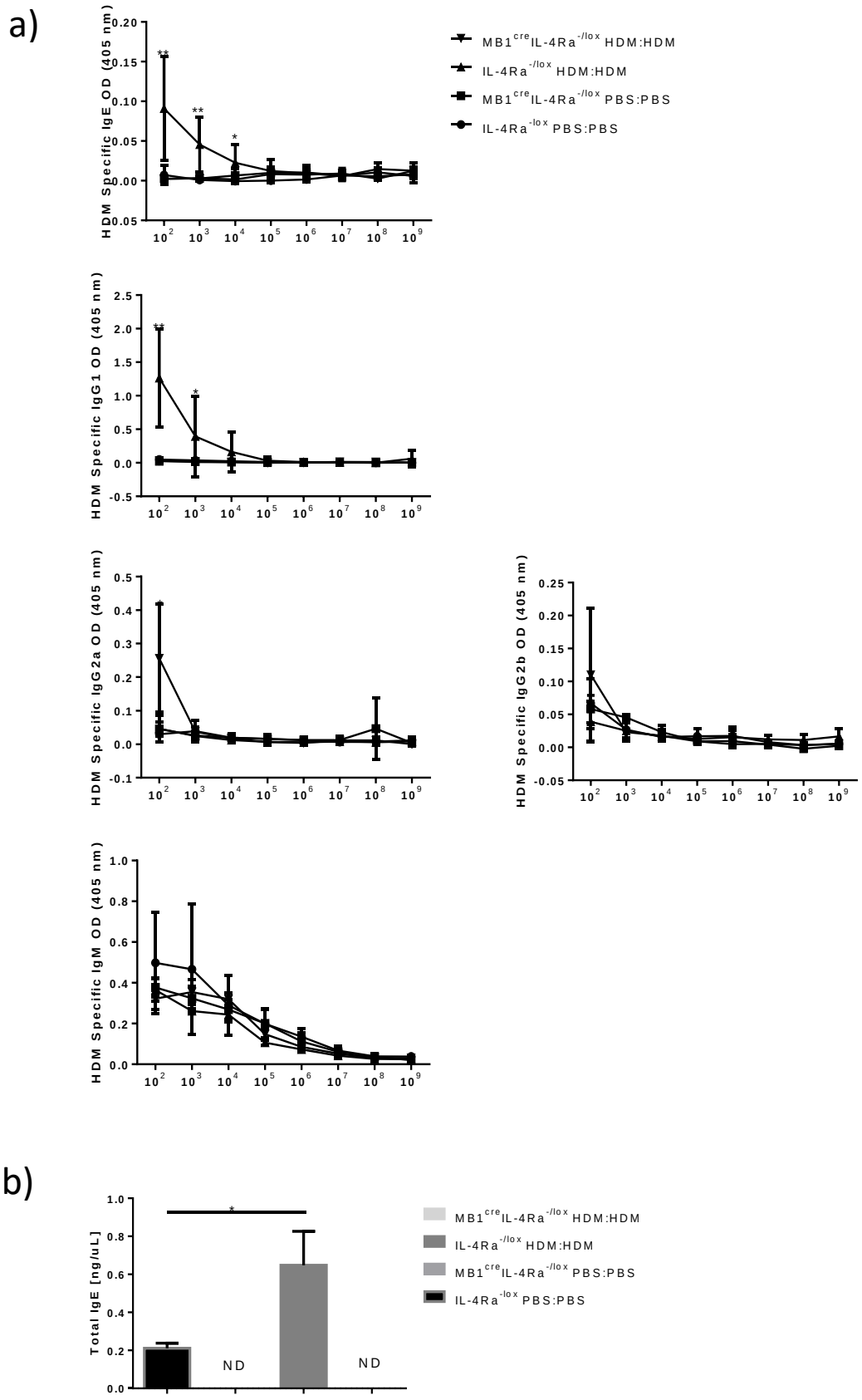


Figure 3.10 MB1CreIL4Ra-/lox mice are protected from Th2 inflammation after epicutaneous sensitization with HDM a) HDM specific antibody production and b) total IgE quantified using ELISA. Representative of one experiment is shown with mean ± SD. Student t-test statistical analysis was performed *P<0,01, **P<0,001 n=5 mice per group

Lastly, we analysed HDM-induced airway hyperresponsiveness, lung inflammation and mucus production (Fig. 3.11). None of The AHR parameters (elastance, compliance and resistance) were elevated upon dose-dependent methacholine challenge in IL-4Rα$^{-/lox}$ and MB1^{Cre}IL-4Rα$^{-/lox}$ strain after HDM exposure (Fig. 3.11a). We further stained lung histology with periodic acid shift (PAS) to visualize the mucus production in lung airways, as described in chapter Materials and Methods (Fig. 3.11b). The staining was quantified using NIS-Elements software (Fig. 3.11c). Interestingly, the data showed increase in mucus production in MB1^{Cre}IL-4Rα$^{-/lox}$ group treated with HDM, when compared to its littermate control (Fig. 3.11c). We stained lung histology with H&E staining and found lung inflammation to be increased in HDM treated mice when compared to PBS challenged mice (Fig. 3.11d).

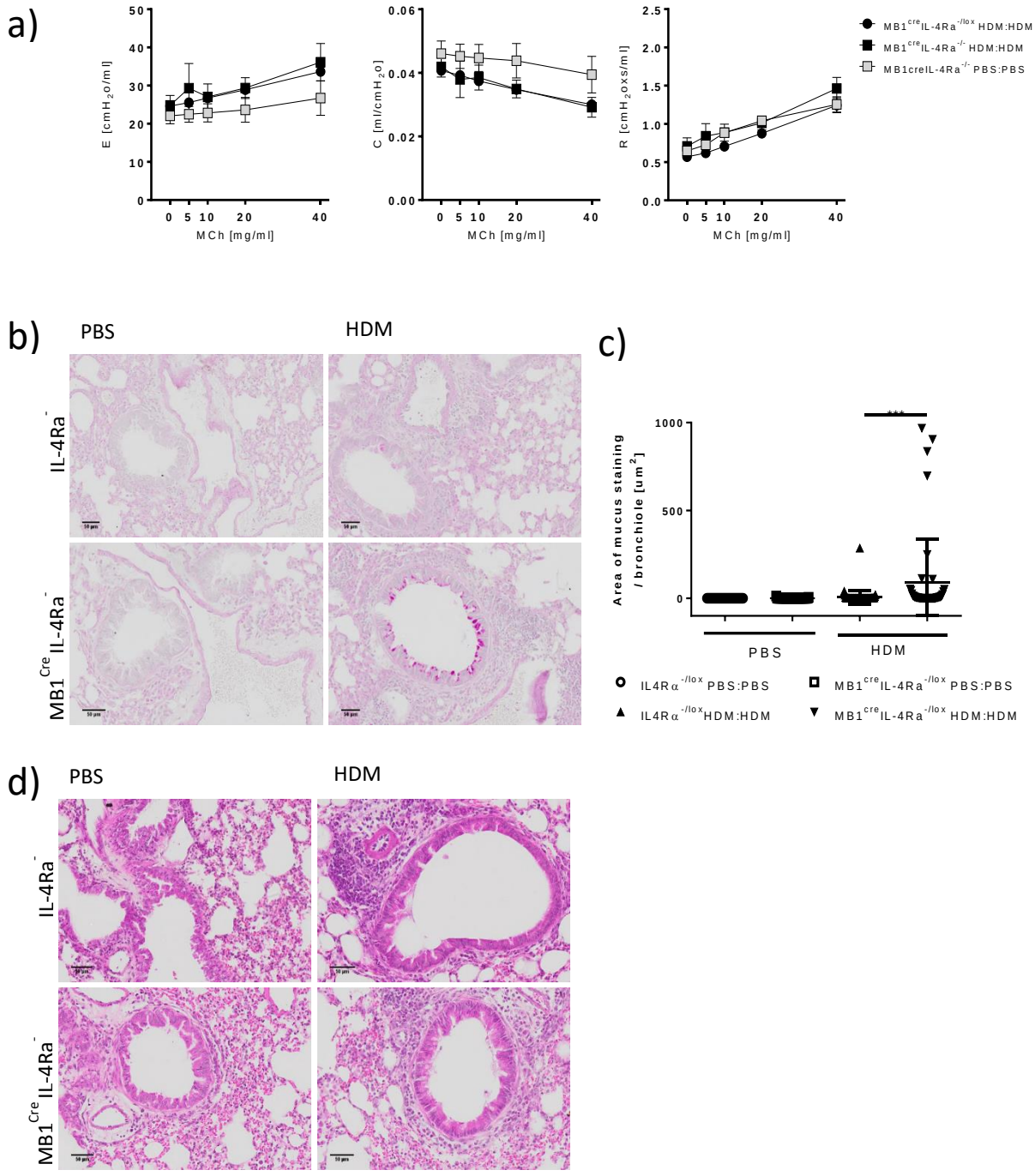


Figure 3.11 Epicutaneous sensitization with 10ug HDM and subsequent i.n. challenge did not induce airway hyperresponsiveness or airway inflammation. a) AHR analysis of elastance, compliance and resistance, b) representative of lung histology stained periodic acid shift (PAS) staining, scale bar 50 um c) quantification of PAS staining using NIS-Elements software, d) representative lung cell infiltrates using H&E staining, scale bar 50um. Representative of one experiment is shown with mean ± SD. One-way ANOVA statistical analysis was performed, *p < .05, **p < .01, ***p < .001, ****p<.0001 n=5 mice pre group

Collectively these findings suggest that presence of IL-4Rα on B cells is necessary for the induction of some asthmatic features, particularly eosinophilic inflammation and type 2 antibody class switching

in allergic model of atopic march. Our protocol did not result in increase of classic asthmatic parameters such as AHR.

3.3 Discussion

Atopic dermatitis is non-infectious skin disease affecting between 15-20% of children and up to 3% of adults world-wide resulting in high costs of extended medical care[20,144]. Often, The disease progresses into other forms of co-morbidities such as asthma or rhinoconjunctivitis[20,23,144]. Most recently approved treatment using dupilumab shows promising results for patients resistant to other forms of treatment such as corticosteroids, however systemic administration of the drug induces side effects such as conjuctivits[219,220,227,242,245]. Dupilumab is a monoclonal antibody targeting IL-4Rα, a signalling receptor for IL-4 and IL-13 type 2 cytokines. As the receptor is present on many hematopoietic and non-hematopoietic cells, the effect of the drug is broad, therefore more targeted therapies are needed. Previously, we showed that global abrogation of IL-4Rα in murine Anisakis asthma model can reduce total IgE production, but it does not diminish AHR[201]. We also found that reaction to the parasite is dependent on the route of administration of allergen[201]. In this study, we investigated B cell specific signalling on the IL-4Rα in HDM allergen and skin irritant models of AD. We found that allergen sensitization using HDM does not induce AD lesions similar to disease presentation in patients, however it can lead to systemic sensitization to allergen. Moreover, mice lacking IL-4Rα on B cells cannot produce high affinity total IgE antibody. Additionally, we found that MC903 induces skin inflammation resembling clinical presentation of AD, and absence of IL-4Rα on B cells does not prevent from disease progression despite impaired antibody production. We also found that B- T cell interaction in germinal centres is impaired in those mice, which leads to impaired class switch recombination and lack of antigen-specific antibody production.

The skin associated B cells have been described in context of keratinization in various diseases including scleroderma and wound healing[4,295–298]. Therefore, initially we analysed the epithelialization of murine skin in steady state in mice lacking B cells (uMT) and mice lacking IL-4Rα on B cells $MB1^{Cre}$IL-$4R\alpha^{-/lox}$. We found that skin keratinization is not dependent on IL-4Rα-responsive B cells, however uMT mice skin was seemingly thinner (observation), despite the lack of significance in the quantification of skin samples. Therefore, we suggest that solely presence of skin-associated innate B cells can be vital for structural rigidity of skin, however it requires further investigation in tight junction formation between skin forming cells such as keratinocytes.

In this study we looked at the role of IL-4Rα specifically on B cells in murine models of AD. For this purpose, we used mouse models of allergic AD – the HDM model and inflammatory model – MC903-induced AD. We found that allergic model using HDM is insufficient to induce strong inflammatory changes on murine skin. This is potentially result of a fault in the study design, where we did not induce

epithelial damage such as tape stripping prior the treatment. In mice with disrupted epithelial barrier, such as Nc/Nga mice, the HDM treatment results in similar presentation of AD as in human pathogenesis, with increased hyperparakeratosis, hyperplasia, elevated IgE and TH2 response [61,251,299]. Other possible fault could be difference in Derp1/Derp2 proportion and activity in our HDM solution, which can result is inadequate inflammatory response[300,301]. Other studies have used skin irritant or bacterial exotoxin SEB from S. aureus in conjunction with HDM strain Dermatophagoides farina to observe other features of AD[302]. Although epidermal thickening did not reach statistical significance, our future studies should use similar approaches to delineate the vital role of IL-4Rα signalling on B cells.

We showed that absence of IL-4Rα leads to impairment of B cell functionality resulting in lack of IgE production in both cell-specific MB1^{Cre}IL-4Rα$^{-/lox}$ group and global IL-4Rα$^{-/-}$ group. This result supports our previous findings in infectious and non-infectious models, where signalling of IL-4Rα was impaired leading to change in B cell antibody producing capacity[162,201,260].

Previously, our group showed that epicutaneous sensitization to *Anisakis* extract is capable of inducing systemic effect such as anaphylaxis in mice and that this effect is mainly IL-13 -dependent[160]. For this reason, we investigated whether epicutaneous sensitization with HDM, will induce airway hyperresponsiveness similar to asthma models. Despite lack of severe skin inflammation induced by HDM, the epicutaneous exposure to allergen resulted in increased lung and BAL eosinophilic inflammation after intranasal challenge with HDM in IL-4Rα$^{-/lox}$ mice, but not in MB1^{Cre}IL-4Rα$^{-/lox}$ group. At The same time, mice lacking IL-4Rα on B cells, were not protected from increased mucus production, which could be a consequence of switch in the immune response from Th2-driven to Th1, by attenuation of antibody production. Previously in our laboratory we found that in the global absence of IL-4Rα mice are not protected from asthma responses such as AHR in allergic sensitization to *Anisakis* and this mechanism is IFNγ-dependant [201]. Induction of this mechanism is relying on the route of sensitization. On contrary to this finding, we did not find increase of IFNγ in this study. It remains important that IFNγ was induced by intraperitoneal *Anisakis* treatment, not by epicutaneous sensitization. Similarly, to the study by Kirstein et al. we showed increase in IgG2a, but not IgG1 in absence of IL-4Rα, which can explain the switch from Th2 to Th1 response [201]. The study by McKnight et al. showed that in limited dose of allergen, the AHR can be IgE/FcεRIα independant[303]. In our study we did not find increase of AHR parameters in both IL-4Rα$^{-/lox}$ and MB1^{Cre}IL-4Rα$^{-/lox}$ mice, but we did find other symptoms of airway inflammation such as increase in mucus secretion in MB1^{Cre}IL-4Rα$^{-/lox}$ mice which was not present in littermate control group, however the IL-4Rα$^{-/lox}$ mice showed eosinophilic infiltration, which was absent in MB1^{Cre}IL-4Rα$^{-/lox}$ group. As neutralization of IgE with omalizumab can reduce lung eosinophilia in patients with asthma, it could be speculated that

abrogation of IL-4Rα leads to impairment of antibody secretion and therefore lack of lung infiltrates in MB1^{Cre}IL-4Rα$^{-/lox}$ mice[133].

In the acute AD model of skin inflammation MB1^{Cre}IL-4Rα$^{-/lox}$ mice were not protected from MC903-induced weight loss or hyperkeratinisation of skin epidermis when compared to littermate control group. This suggest that the absence of the signalling of the receptor on B cells does not play a significant role in this model of AD. Additionally, we found that lack of adequate germinal centre formation is dependent on IL-4Rα presence and it is required for production of high affinity antibodies such as IgE. As IL-4 non-responsive mice did not differ in the weight loss or epidermal thickening from their littermate controls we concluded that IgE does not play a detrimental role in MC903 murine model of AD. This conclusion is in contrast with the current treatment regime for AD patients, where use of omalizumab and IgE-targeted immunoadsorption is recommended course of action [238,271]. Similarly to our results, treatment with anti-IL-4Rα biological (dupilumab), which targets whole Th2 axis of immune response, shows better efficacy than solely antibody-targeting omalizumab [220].

In this study we found that both B220+ B cells and γδT cells are main source of IL-17. Since IL-17 can promote keratinocyte proliferation in tumour oncogenesis, we suspect it to be a reason for epidermal hyperplasia in our model of AD [304]. Additional reason behind the increase in IL-17 upon MC903 treatment is inflammatory disruption of epidermal lining, which leads to open permeability for toxins from commensal bacteria and fungi, which would not be possible in untouched epidermis. *Staphylococcus aureus* is one of the most prevalent skin colonizing bacteria, which produces alpha-toxin[37]. In patients with AD and psoriasis, disruption of epidermal barrier leads to Th17 priming of naïve T cells by *S. aureus* produced alpha toxin[193,305].

As mice lacking IL-4Rα showed impaired B cell functionality, the germinal centre B cells numbers after MC903 treatment were reduced in comparison with its littermate control. The MB1^{Cre}IL-4Rα$^{-/lox}$ mice presented decreased numbers of FO, MZ and GC B cells in comparison with their littermate controls after MC903 treatment. Of interest the GC B cells were reduced in both frequencies and numbers, providing strong evidence of importance of IL-4Rα signalling in B cell functionality in secondary organs. Additionally, low numbers of GC B cells support the lack of IgE which is produced by B cells after class-switch recombination. Interestingly, when exposed to HDM in atopic march study, we observed increased IgG2a, which is produced after CSR as well, therefore it is worth noticing that partial potential of B cell CSR remained in mice lacking IL-4Rα, which suggests that B cell CSR might not be fully dependant on IL-4Rα signalling. This result was also reported previously in our laboratory by Kirstein et al. where group showed that IL-4Rα$^{-/-}$ mice were capable of IgG2a production upon allergic sensitization[201]. As both IgG and IgE antibodies are developed in T-cell dependant (TD) phase of B cell

activation, the presence of IgG2a over IgE suggests that IgE production is IL-4Rα signalling dependent, while IgG2a is not[56,306]. Of interest, various subclasses of IgG antibodies have different effector function e.g. IgG2a has high activator to inhibitory binding ratio to FcγRIV with downstream signalling of immunoreceptor tyrosine-based activation motif (ITAM) resulting in activation of immune cell, while IgG1 has low affinity to this receptor[307]. This mechanism could potentially explain the increase in mucus production by goblet cells in $MB1^{Cre}$IL-4$Rα^{-/lox}$ mice, but it requires further investigation.

3.4 Conclusion

Collectively, our results suggest that abrogation of IL-4Rα on B cells in model of atopy protects host from detrimental IgE production, however it does not translate to full protection from atopic march due to dichotomy of B cells and their unsuppressed capability of producing type 1 antibodies, which does not depend on IL-4 signalling. In acute model of atopic dermatitis, we found that IL-4Rα on B cells does not prevent from disease progression, despite of partial abrogation of B cell functionality.

Chapter 4

The role of interleukin receptor alpha on T cells in atopic dermatitis

4.1 Introduction

Atopic dermatitis (AD) affects approximately 20% of children and up to 3 % of adults world-wide[270]. It is a reoccurring chronic skin disorder with flare-ups characterized by eczematous lesions of dry skin and pruritis[23]. AD leads to decrease in patients' health-related quality of life (HRQoL) and costly to health-care system[23,243]. AD patients report higher incidence of depression, anxiety and suicide as well as pain, fatigue and insomnia [31,32,308,309]. The immune responses in AD are dependent on the stage of disease progression. Initially, the keratinocytes secrete interleukin (IL)-33, -31 and thymic stromal lymphopoietin (TSLP), which activates the skin residing cells such as Langerhan cells (LCs) and innate lymphocyte type 2 (ILC2s), which drive predominantly type 2 immune response by activation of T_{helpe}(TH)2 cells[20,38]. In chronic phase of the disease, upon epithelial damage and microbial skin penetration type 1 immune response is additionally activated giving a mixed profile of the disease[20,68,310]. This is characterized by infiltration of TH1 and TH17 cells in site of inflammation.

The current treatment regime focuses on use of topical glucocorticosteroids and calcineurin inhibitors as well as systemic immunosuppressants in severe cases [11]. For some patient's additional photo therapy as well as antimicrobial therapies and anti-pruritic agents are advised[11,246]. The recent studies on AD show that both intrinsic (non-allergic) and extrinsic (allergic) factors can play a role in pathogenesis of disease progression[268]. As the endotypes vary in immunoglobulin E (IgE) levels, the type 2 cytokines namely interleukin 4 and 13 (IL-4 and IL-13) remain elevated in both types of the disease[269,311]. Dupilumab, an interleukin 4 receptor alpha (IL-4Rα) antagonist was recently approved by FDA as treatment for AD after it showed positive effects in patients with moderate to severe AD, with reduced blood type 2 signature, and reversed epidermal abnormalities [285]. This clinical trials provide a strong evidence for IL-4/IL-13 as a key target in controlling debilitating AD disease [243].

Both cytokines are detected by pleotropic receptor, IL-4Rα subunit [273,312,313]. IL-4Rα forms a heterodimer with IL-13Rα1 to transduce signals for both IL-4 and IL-13 cytokines, the IL-4 can also be detected by its own receptor formed by IL-4Rα and common gamma chain (cγ) subunits, whilst IL-13 can be detected by IL-13Rα2[167,168,172,273,314,315]. In models of allergic asthma and AD, IL-4Rα independent mechanisms have been observed, where IL-13-responsiveness is observed via IL-13Rα2 [167,168]. Canonically, the signal transduced by signal transducer and activator of transcription (STAT) 6

phosphorylation and further increase of GATA3 expression in TH2 cells[316]. This further leads to cell proliferation and perpetuates type 2 inflammation[317]. Interestingly, the presence of the receptor is redundant on the T cells in early stages of TH2 priming, where cells are still capable of IL-4 production, despite being unresponsive to IL-4/IL-13, in contrast it is crucial for later T-B cell interaction and germinal centre formation[135].

The caveats of systemic IL-4Rα blockade with dupilumab results in various adverse effects such as conjunctivitis, nasopharyngitis and exacerbation of viral skin infections, due to the presence of the receptor in many immune and non-immune cells [246].

On T cells, IL-4Rα is present mainly on CD4+ T cells, however it can also be found of γδ+ T cells, CD8+ T cells or NKT [263]. Various T cell populations are capable of migration between secondary lymphoid organ and site of exposure to allergen, thus exacerbating immune response [105]. For those reasons, it is important to understand how cell-specific attenuation of IL-4Rα functionality will result in atopic dermatitis. Previously our group showed that global absence of IL-4Rα can protect mice from Anisakis-induced food allergy [200]. At the same time, we showed that upon epicutaneous exposure to the extract, mice showed reduced symptoms of atopic dermatitis such as epidermal hyperplasia as well as total IgE level in global IL-4Rα knockout, but not in CD4 T cell deficient mice (LCK^{Cre} IL-4Rα $^{-/lox}$) [160]. More recently, our group developed a new mouse strain, where IL-4Rα expression is disrupted specifically for CD4+ cells upstream the CD4+/CD8+ stage of T cell development (Darby et al. data unpublished). Whether deletion of the IL-4Rα at various stages of T cell development (i.e. LCK^{Cre} IL-4Rα $^{-/lox}$ vs $CD4^{Cre}$ IL-4Rα $^{-/lox}$ strains) is crucial in AD protective effects observed in global IL-4Rα is currently unknown.

Additional important subpopulation of T cells in skin is CD4+ Foxp3+ T regulatory (Treg) group, which can be found in hair follicles[102,103,318]. Their role has been attributed to maintenance of barrier-tissue integrity and also facilitation of hair growth in inflammatory conditions [102,103]. The absence of Tregs leads to Th17-driven skin inflammation, increased trans-epidermal water loss (TEWL), downregulation of integrins such as filaggrin (FLG)[102]. Treg population has been vastly described in context of allergic asthma, but their role in atopic dermatitis has not been fully understood [127,139,159,319]. In patients with AD, the circulatory memory Tregs cells are higher than in non-atopic individuals [139]. We recently showed that, absence of IL-4Rα on Tregs results in increased type 2 innate lymphoid cells, mainly driven by uncontrolled IL-33 secretion in HDM-induced allergic asthma [159].

Lastly, the skin residing gamma-delta (γδ) T cell population has been shown to contribute to maintenance of skin homeostasis and repair [72,94,320,321]. Similarly to dendritic cells (DCs), DETCs main function is immunosurveillance in skin, where upon transcription of CC chemokine receptor 4 (CCR4)

and CCR10 are able to migrate from skin draining lymph nodes to skin [6,72]. γδ T cell migrate between skin tissue and skin draining lymph nodes using cell-intrinsic signals such as Sphingosine-1-phosphate receptor 2 (S1PR1) and CCR6 [322,323]. Interestingly, absence of IL-4Rα all T cells using iLCK^{Cre} IL-4Rα $^{-/lox}$ knockout system resulted in increase of γδT cell population development (Darby et al. data unpublished). As γδT cells are capable of immunosurveillance, migration between skin and draining lymph nodes, are capable of IL-4, IL-13 and IL-17 production, we wondered whether their responsiveness to IL-4Rα would be crucial in AD [6,93,94,323–325].

We investigated possible role of IL-4Rα on T cells in pan-T cell IL-4Rα knockout (iLCK^{Cre} IL-4Rα $^{-/lox}$), CD4+, CD8+ - restricted IL-4Rα knockout (LCK^{Cre} IL-4Rα $^{-/lox}$), CD4+ - restricted (CD4^{Cre} IL-4Rα $^{-/lox}$) and T regulatory specific IL-4Rα knockout (Foxp3^{Cre}IL-4Rα$^{-/lox}$) in allergen-induced (house dust mite or OVA) or non-allergen induced (calcipotriol) murine models of atopic dermatitis. We showed that IL-4Rα signalling on all T cells had little impact allergen induced allergen induced AD. Interestingly, we found that, absence of IL-4Rα signalling on γδ+ T cells abolishes epidermal skin thickening following calciprol-induced acute skin inflammation, with ablation of IgE production.

4.2 Results

4.2.1 Insertion of Cre – lox system attenuates epidermal thickening in mice

Our previous studies on mouse model of iLCK^{Cre}, LCK^{Cre}, CD4^{Cre} mice did not provide information on epidermal thickening between knock-out mice and their littermate controls [326266,327328]. For this purpose, we killed naïve female mice, shaved *post-mortem* and collected skin samples for histology analysis, H&E stained samples were analysed using QuPath software (Fig. 4.1a, b). We observed significant differences in keratinization of epidermis between various types of Cre – lox system at steady state; we therefore compared each Cre – lox mice with its littermate control.

Previous studies on mechanical injury mimicking scratch in AD patients, showed that tape stripping can lead to increase in mast cells population [61,280,329]. We further wanted to know if process of multiple shaving and application of Phosphate buffered saline (PBS) covered patch can increase epidermal

thickening. For this purpose, we compared the naïve, non-treated mice to PBS control (Fig. 4.1c). The mechanical injury induced by multiple shaving (on day 0, 14, 28) and consecutive PBS patch application leads to increase in epidermal thickening in comparison with naïve non-treated mice.

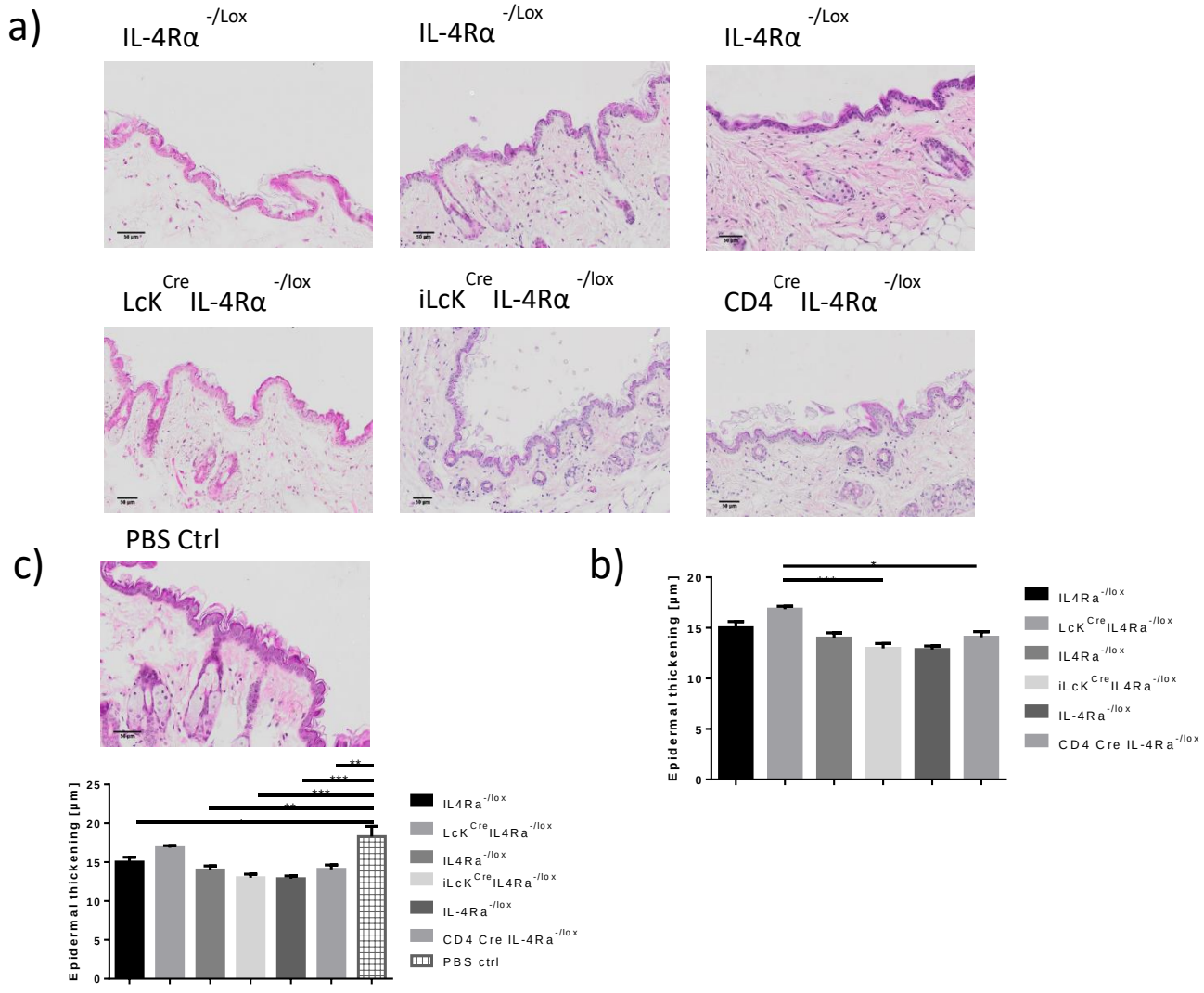


Figure 4.1 Naive mice of T cell IL-4Rα knockout differ in epidermal thickening. a) Comparison of histology images of skin biopsies of ventral side from naive mice stained with H&E, scale bar 50um, b) quantification of epidermal thickening using QuPath software, c) quantification of epidermal thickening in 3-times PBS treated mice compared to naive, untreated mice. Representative of one experiment is shown with mean ± SEM. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

4.2.2 Treatment with 10ug HDM does not induce epidermal thickening

To understand whether IL-4Rα would be essential in a type 2 AD skin inflammatory model, we shaved mice and sensitised them 3 times at 2 week intervals with HDM 10μg, 100μg and also ovalbumin (200μg), known to induced AD skin inflammation (Fig 4.2a). We found no significant changes in epidermal thickness (Fig 4.2b) and levels of IL-33 in serum (Fig 4.2c) between HDM 10μg, 100μg and OVA 200 μg.

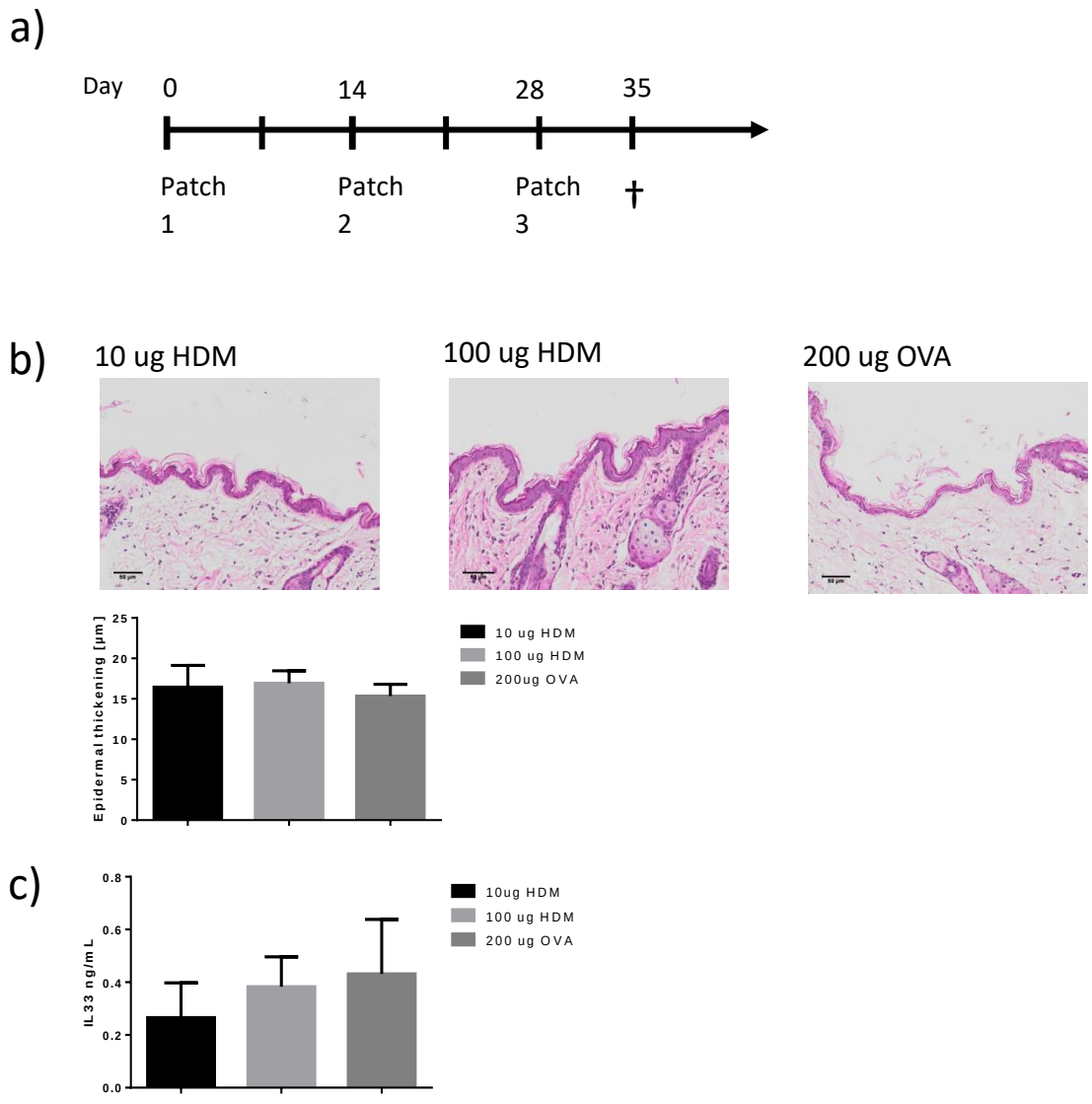


Figure 4.2 Titration of HDM and OVA for epicutaneous sensitization. a) Model for allergen skin sensitization b) comparison of histology images of skin biopsies of ventral side from HDM or OVA treated mice stained with H&E, scale bar 50um, quantification of epidermal thickening using QuPath software, c) quantification of serum IL33 using ELISA. Representative of one experiment is shown with mean ± SD. n=5 mice per group. one-way ANOVA

We then chose to treat mice with 10ug HDM in 200 uL PBS on day 0, 14, 28 for all subsequent experiments. We killed mice on day 35, collected shaved skin samples, blood and inguinal lymph nodes for further analysis. Skin was stained with H&E stain and epidermal thickening was quantified using QuPath software. We compared iLCK^{Cre} IL-4Rα $^{-/lox}$, LCK^{Cre} IL-4Rα $^{-/lox}$, CD4^{Cre} IL-4Rα $^{-/lox}$ mice to their littermate controls IL-4Rα $^{-/lox}$ and to PBS control (Fig 4.3). We did not observe any epidermal thickening changes between iLCK^{Cre} IL-4Rα $^{-/lox}$, LCK^{Cre} IL-4Rα $^{-/lox}$, CD4^{Cre} IL-4Rα $^{-/lox}$ mice compared to littermate control treated with HDM. We observe a non-significant increase in epidermal thickening between HDM exposed mice compared to PBS control mice, however this was significant when compared to naïve untreated controls.

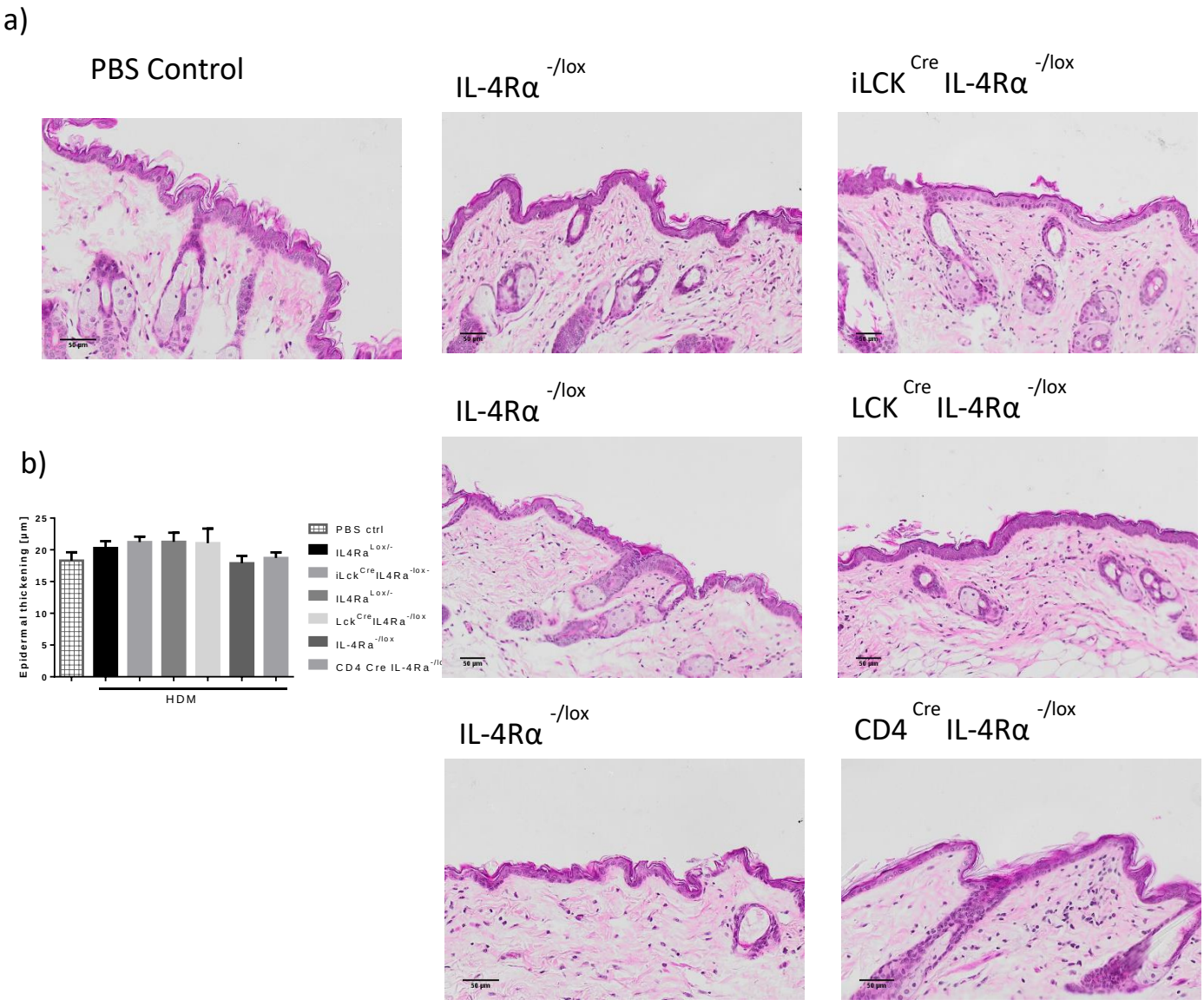


Figure 4.3 The epicutaneous sensitization with 10ug HDM does not induce epidermal thickening. a) Representative images of histology images of skin biopsies of ventral side from HDM or PBS treated mice, stained with H&E, scale bar 50um b) quantification of epidermal thickening using QuPath software. Representative of one experiment is shown with mean ± SEM. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, n=3,4,5 mice per group. one-way ANOVA

4.2.3 Epicutaneous sensitization with 10ug HDM leads to increase in total IgE, and γδT cell proliferation in pan T cell-IL-4Rα-deficient mice.

As increase of blood IgE levels is a hallmark of AD in sensitised patients, we analysed IgE and cytokine levels in serum by ELISA [284]. Total IgE concentration was increased in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice and their littermate controls, when compared to PBS treated group (Fig. 4.4a). LCK^{Cre} IL-4Rα $^{-/lox}$ mice showed increase in total IgE in comparison with its littermate control and with PBS control (Fig. 4.4a). None of the mice strains showed increase in serum IL-17 (Fig. 4.4b). We measured level of serum IL-33 as well, but it was not detectable in our system. Overall, treatment with HDM does only partially reflects patient AD symptoms.

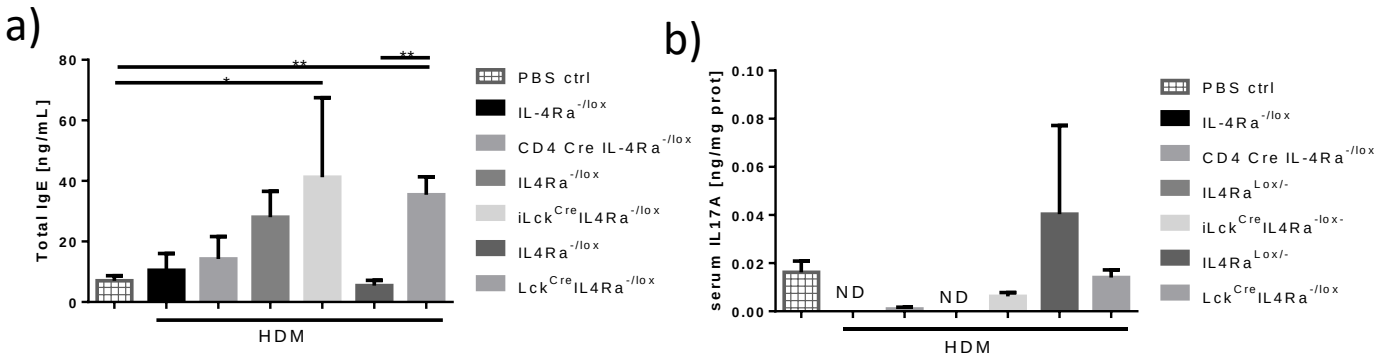


Figure 4.4 The epicutaneous sensitization 10ug HDM induces total IgE production in iLcK^{Cre}IL-4Rα$^{-/lox}$ mice and their littermate controls as well as in iLcK^{Cre}IL-4Rα$^{-/lox}$ mice but not in its littermate control. a) Quantification of total IgE and b) serum IL17A using ELISA Representative of one experiment is shown with mean ± SEM. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, n=3,4,5 mice per group. Student t-test *P<0,01, **P<0,001,

Teraki et al. showed that in patient with AD, the predominant subset of T cells is IL-13/IL-22-coproducing T cells [137]. We therefore analysed inguinal cell infiltrates using FACS, we gated for CD4+ T cells, CD8+ T cells, γδT cells and CD19+ B cells (Fig. 4.5). We found that treatment with HDM does not significantly change the frequencies and numbers of CD4+ and CD8+ T cells between Cre -lox and their littermate controls (Fig. 4.5a, b). Using mean fluorescence intensity of IL-4Rα, we confirmed the IL-4Rα knock down in CD4+ cells in iLCK^{Cre} IL-4Rα $^{-/lox}$, LCK^{Cre} IL-4Rα $^{-/lox}$ and CD4^{Cre} IL-4Rα $^{-/lox}$ mice in comparison with their littermate controls (Fig. 4.5a). Similarly, we analysed expression of IL-4Rα on CD8+ cells, which was reduced in all knockout strains in comparison with their littermate control groups (Fig. 4.5b). Given that γδ+ T cells play a controversial role in AD, we analysed the γδ+ T induced by HDM exposure (Mraz, Geisler, and Bonefeld 2020; Sulcova et al. 2015; Cairo et al. 2005) (Fig. 4.5c). Treatment of iLCK^{Cre} IL-4Rα $^{-/lox}$ mice, led to expansion of frequencies and numbers of γδ+ T cells when compared to its littermate control (Fig. 4.5c). Similar observations of reduced IL-4Rα were seen in CD4^{Cre} IL-4Rα $^{-/lox}$, but not LCK^{Cre} IL-4Rα $^{-/lox}$ strain (Fig. 4.5c). This suggest a possible role for IL-4Rα on γδ+ T cells in model of AD. We then analysed CD19+ B cells population, in all mice strains we did not

see any significant difference between Cre -lox and its respective littermate controls (Fig. 4.5d). The analysis of IL-4Rα expression on CD19+ cells showed that insertion of Cre-lox system did not influence receptor expression on B cells (Fig. 4.5d).

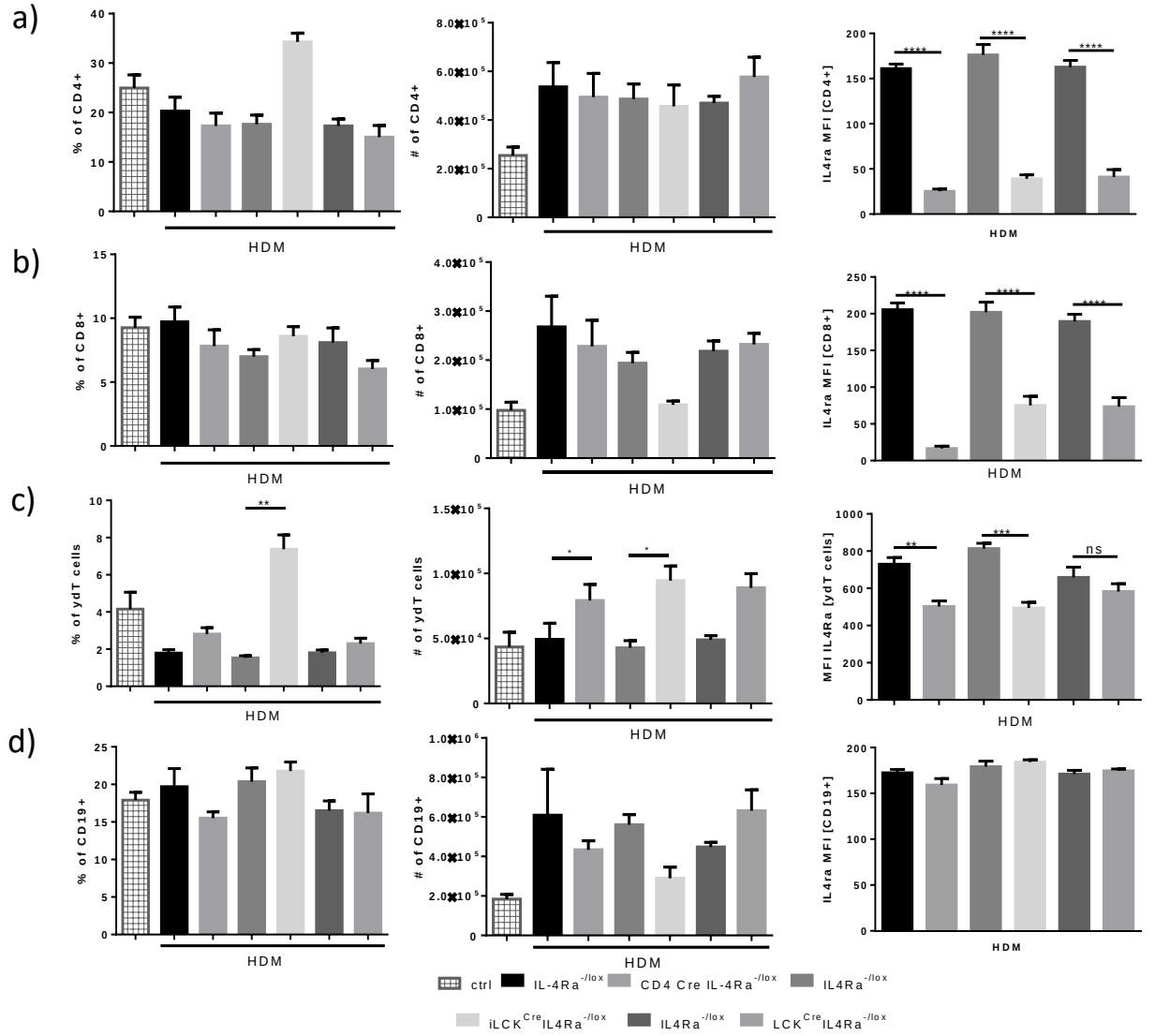


Figure 4.5 The epicutaneous sensitization 10ug HDM does not induce increased adoptive immunity response in mice, however iLcK mice show increased γδT cell proliferation. Quantification of CD4+ a) and b) CD8+ cell proliferation, c) γδT cell proliferation, d) CD19+ B cell proliferation using FACS. Representative of one experiment is shown with mean ± SEM. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, with one-way ANOVA *P<0,01, **P<0,001 n=4 and 3 mice per group.

Overall, these finding suggest that absence of IL-4Rα on pan-T cells results in increased IgE production and γδ+ T cells expansion upon HDM treatment.

4.2.4 IL-4Rα-responsiveness in all T cells is detrimental in MC903-induced AD inflammation

5 MC903 is analogue of Vitamin D3, which in mice induces AD-like lesions, when applied topically [331]. Initially we tested increasing doses of MC903 (45 uM and 90 uM) dissolved

in 100% ethanol and we recorded weight loss and skin changes on WT (Balb/c) mice (Fig. 4.6a and b). We shaved mice 3 days prior treatment, we treated mice for 10 consecutive days on shaved skin (Fig. 4.6a). On last day of the experiment (day 11) we collected skin and blood for further analysis. We found 45uM to be sufficient in inducing AD like

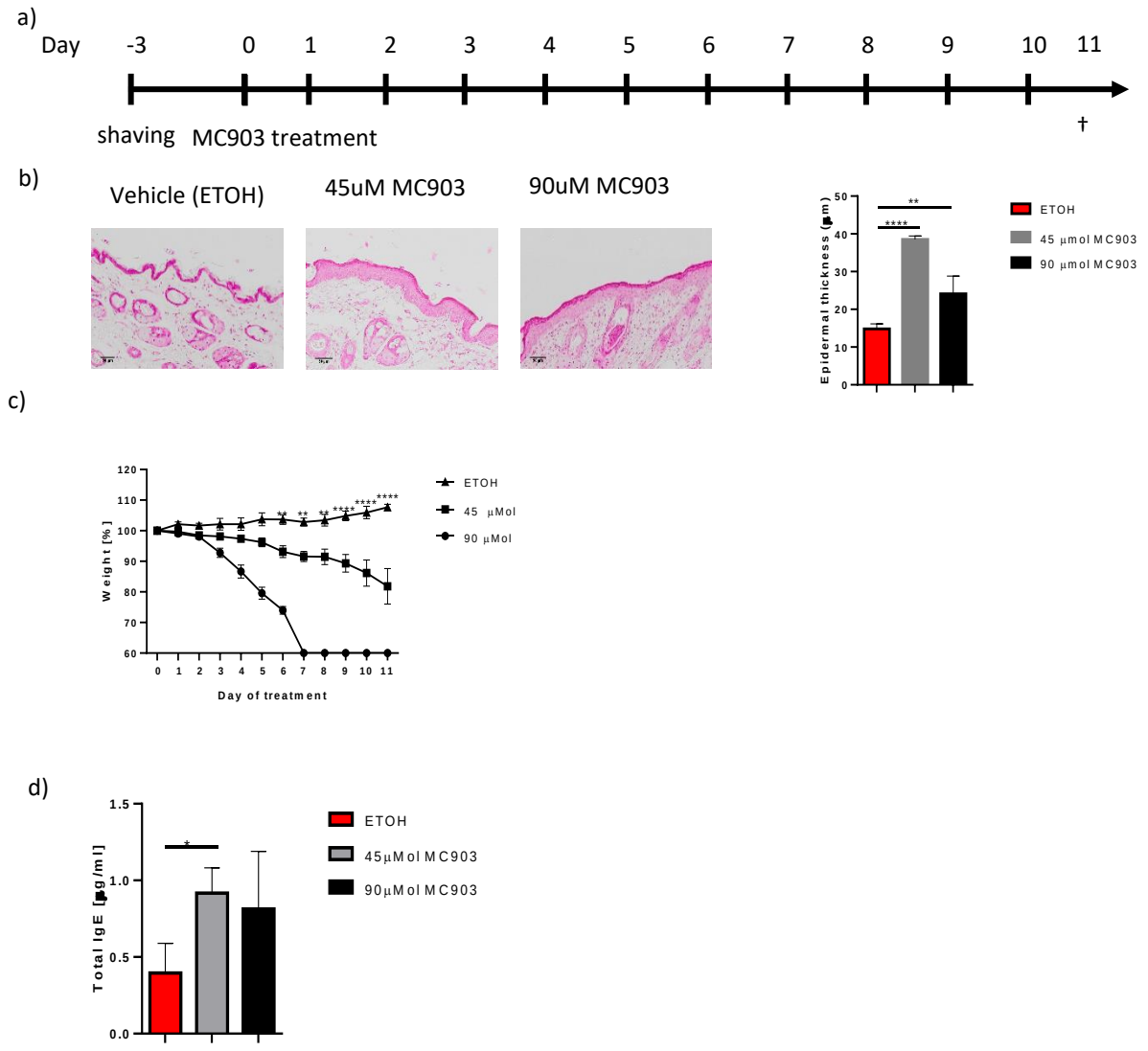


Figure 4.6 The titration of MC903 epicutaneous treatment. a) model of MC903 epicutaneous treatment of Balb/c mice b) comparison of histology images of skin biopsies of ventral side from MC903 or vehicle treated mice stained with H&E, scale bar 50um, quantification of epidermal thickening using QuPath software, c) weight loss tracing where statistical analysis was performed defining differences to 45uM dose to control group as significant, d) quantification of serum total IgE using ELISA. Representative of one experiment is shown with mean ± SD. n=5 mice per group, Student t-test and one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

symptoms which included skin redness, rash, and scab as well as weight loss of 10-20% when compared to ethanol treated mice (Fig. 4.6c).

We then used 45 uM MC903 in 100% ethanol and applied it for 10 consecutive days on shaved skin. On last day of treatment, we killed mice and collected shaved skin, blood and

inguinal lymph nodes for further analysis (Fig. 4.7). Upon treatment we recorded weight loss, on day 7 WT mice treated with MC903 started losing weight in comparison with iLCK^{Cre} IL-4Rα $^{-/lox}$ mice and in ethanol (EtOH) treated groups (Fig. 4.7a). The knockout mice were protected from MC903-related weight loss throughout the experiment (Fig. 4.7a). LCK^{Cre} IL-4Rα $^{-/lox}$ mice and their littermate control did not show any significant changes in

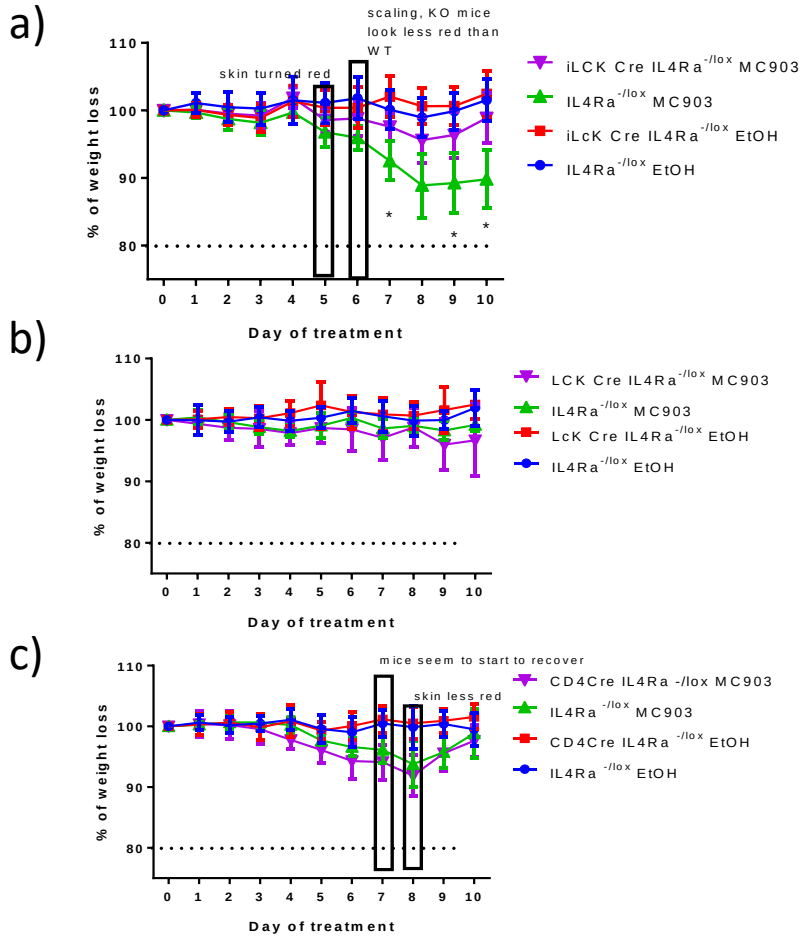


Figure 4.7 iLcK KO mice are protected from MC903 – induced weight loss a)-c) weight loss tracing over 10 day period of MC903 treatment in iLcK KO, LcK KO and CD4 KO strains compared to its littermate controls. Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, with Mann-Whitney analysis was performed (*P<0,05), n=5 mice per group.

weight during treatment (Fig. 4.7b). CD4^{Cre}IL-4Rα$^{-/lox}$ and its littermate control IL-4Rα$^{-/lox}$ showed similar weight loss upon MC903 application, importantly their weight loss seemed to be transient and on the last day of experiment, mice had similar weight as in the beginning of treatment (Fig. 4.7c).

On the final day of treatment, we collected skin samples from site of treatment, samples were fixed in paraffin and further stained with H&E (Fig. 4.8a). The epidermal thickening was quantified using QuPath software (Fig. 4.8b). Similarly, to the weight loss, iLCK^{Cre} IL-

4Rα $^{-/lox}$ mice showed reduced keratinization and scaling of skin upon MC903-induced AD when compared to its littermate control (Fig. 4.8b). Other two strains investigated showed similar keratinization and scaling of skin upon MC903-induced AD when compared with their littermate controls (Fig. 4.8b). The ethanol treatment did not attenuate the epidermal thickness (Supplementary Figure 1).

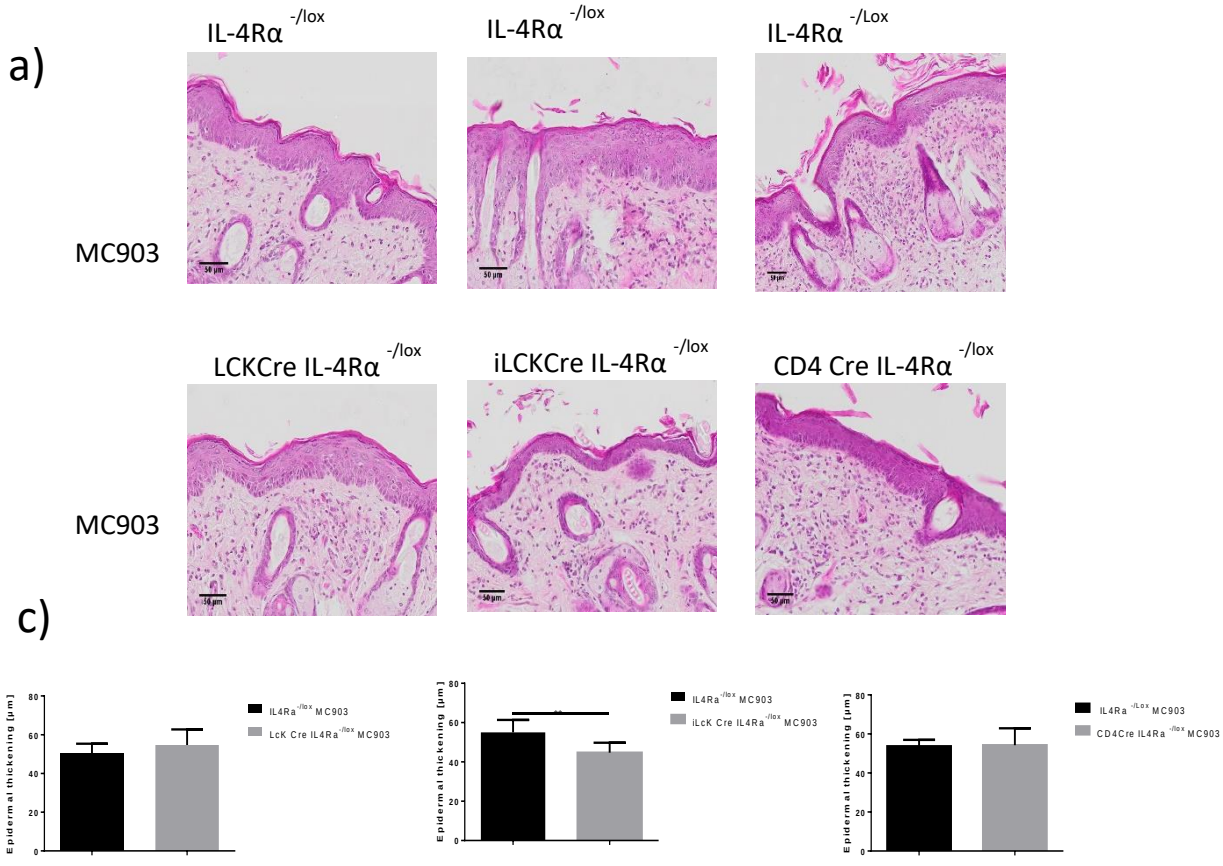


Figure 4.8 The epicutaneous treatment 45uM MC903 induces strong inflammatory response, which is augmented in iLcK KO mice a) representative images of skin biopsies from ventral side, histopathology stained with H&E showing epidermal thickening, scale bar 50um ; b) quantification of epidermal thickening using QuPath software, Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, with Mann-Whitney analysis was performed (**P<0,01), n=5 mice per group.

4.2.5 MC903 treatment does not induce activated B cell phenotype in iLcK KO mice

To assess whether changes observed in body weight and epidermal skin thickening translated in cellular infiltrate, we analysed cellular composition of B and T cells, their activation and cytokine production by FACS. Cells from the lymph nodes were either stimulated using ionomycin, PMA and monensin for 5 hours, at 37 ^{0}C and surface stained for B cells, CD4, CD8 and γδ, CD44, CD62L and CD3 cell markers or were further stained for intracellular cytokine production of IFNγ, IL-4, -5, 13 and -17A.

Upon the treatment with MC903 the cell numbers of skin draining lymph nodes was increased in littermate control mice but not in iLCK^{Cre} IL-4Rα $^{-/lox}$ strain (Fig. 4.9a). B cell population was reduced in

iLCK^{Cre} IL-4Rα $^{-/lox}$ mice (Fig. 4.9b) after MC903 treatment. Interestingly, treatment with MC903 led to reduction of IL-4Rα on those cell (Fig. 4.9b). B220+, CD44+, CD62L- B cell population was increased in littermate control mice but not in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice, similarly to B220+, CD44-, CD62L- B cells (Fig. 4.9c). We observed a significant increase in numbers of B220+ B cells secreting IL-5 and IL-13 in littermate controls mice when compared to iLCK^{Cre} IL-4Rα $^{-/lox}$ mice (Fig. 4.9d).

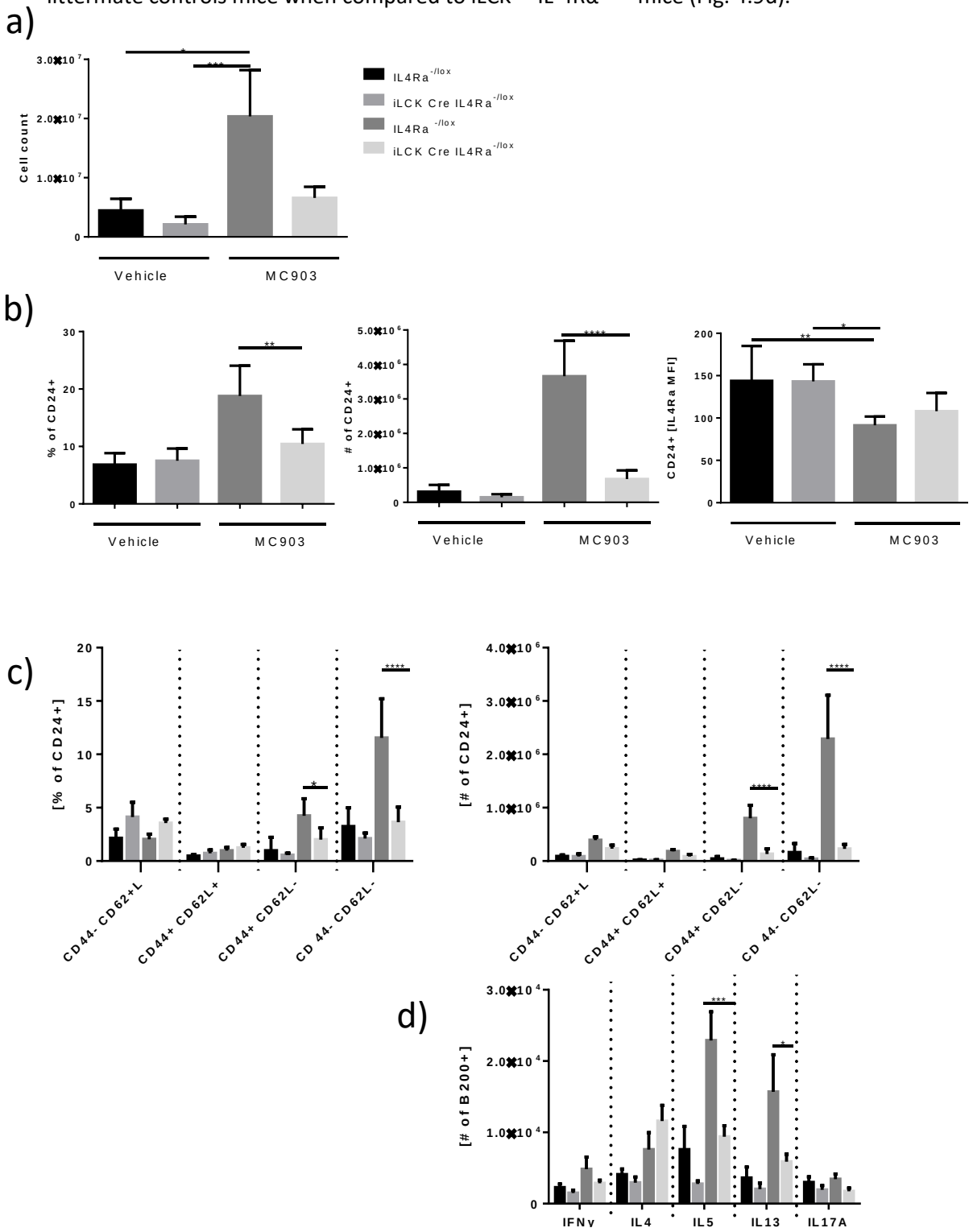


Figure 4.9 MC903 treatment does not induce activated B cell phenotype in iLcK KO mice. a) Total cell number from iLNs b) quantification of B cell infiltrates c) quantification of activated CD24+ B cells in iLN and d) B cell cytokine production. Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

4.2.6 IL-4Rα signalling in Pan-CD4+ T cells is essential for MC903 induced TH2 inflammation

We measured frequencies and total number of CD4+ T cells in iLN in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice and littermate control mice treated with ethanol or MC903 (Figure 4.10). CD4+ T cell population was not expanded in frequencies of the population, but it was significantly increased in cell numbers upon MC903 treatment in littermate control group but not in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice (Fig. 4.10a). Such difference can be possibly explained by difference in cell count, which modulates the proportion of cells. The mean fluorescence intensity of IL-4Rα was reduced in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice, confirming the phenotype of iLCK^{Cre} IL-4Rα $^{-/lox}$ mice (Fig. 4.10a). We further analysed activation status of CD4+ T cells, increase in effector (CD44+, CD62L-) CD4+ T cells was observed in numbers, but not in percentages cells in WT mice treated with MC903, but not in iLCK^{Cre} IL-4Rα $^{-/lox}$ group (Fig. 4.10b). Similarly, IL-5+ and IL-13+ CD4+ T cells were increased in MC903-treated WT mice, but not in iLCK^{Cre} IL-4Rα $^{-/lox}$ group, in cell numbers (Fig. 4.10c).

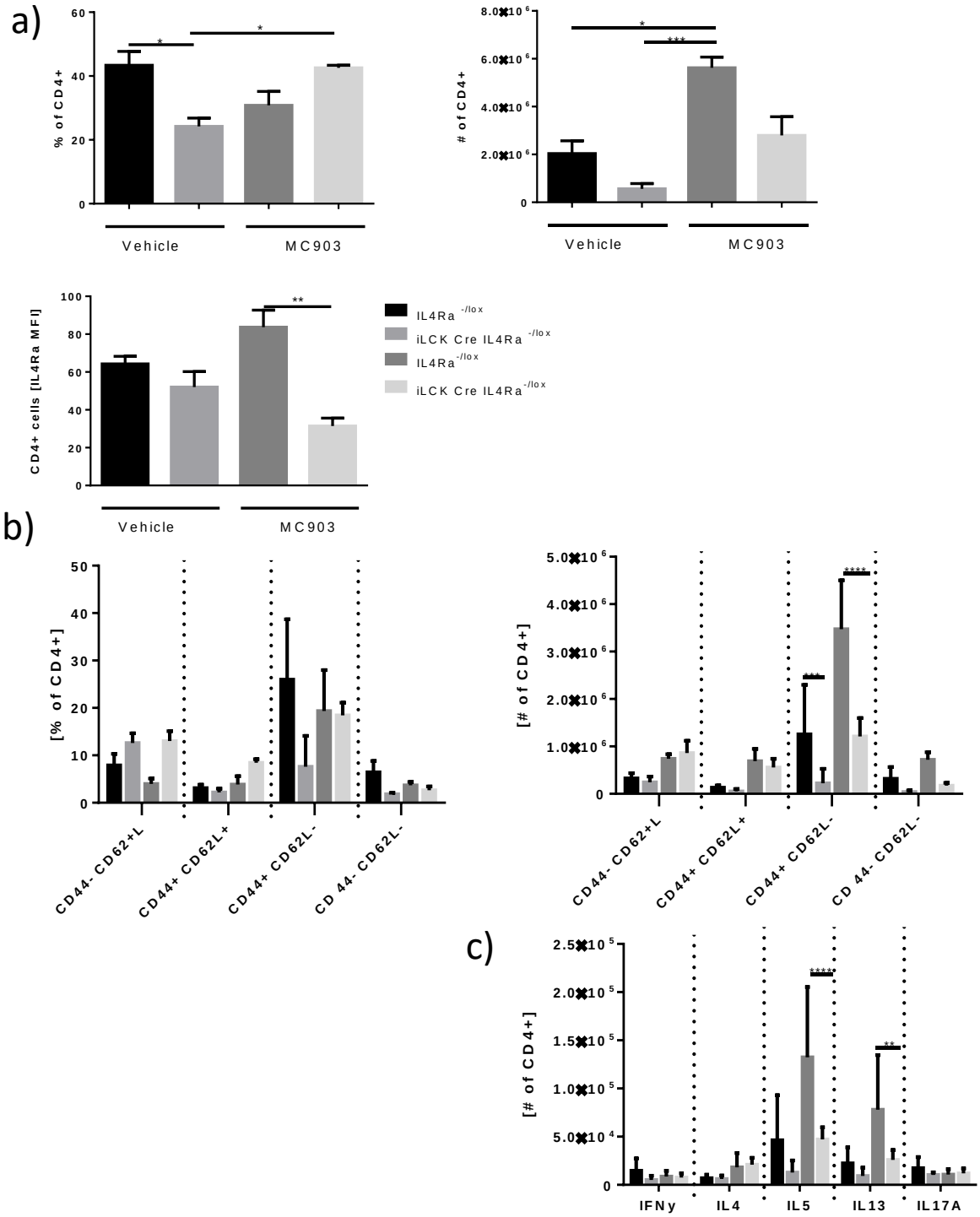


Figure 4.10 Treatment with MC903 does not induce increase of CD4+ activated T cells in iLcK KO mice a) quantification of CD4+ T cell infiltrates in iLN b) quantification of CD4+ activated T cells c) and their cytokine production in iLN. Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

4.2.7 Treatment with MC903 does not induce increase of CD8+ activated T cells in iLcK KO mice

We further, measured frequencies and total cell number of CD8+ T cells in both mouse strains (Fig. 4.11). We found that treatment with MC903 led to increase in CD8+ cell number, but not the

frequency in WT mice. However, we did not observe similar effect in iLCK^{Cre} IL-4Rα $^{-/lox}$ group (Fig. 4.11a). The mean fluorescence intensity reflected the iLCK^{Cre} IL-4Rα $^{-/lox}$ phenotype of reduced IL-4Rα on CD8+ cells (Fig. 4.11a). We then analysed the activation status of those cells and found no increase in CD44+ CD62L+ central memory CD8+ iLCK^{Cre} IL-4Rα $^{-/lox}$ mice after MC903 exposure in comparison with their littermate control (Fig. 4.11b). We observed differences in numbers of IL-5 producing CD8+ T cells, but not other cytokines in littermate control group when compared to iLCK^{Cre} IL-4Rα $^{-/lox}$ mice (Fig. 4.11c).

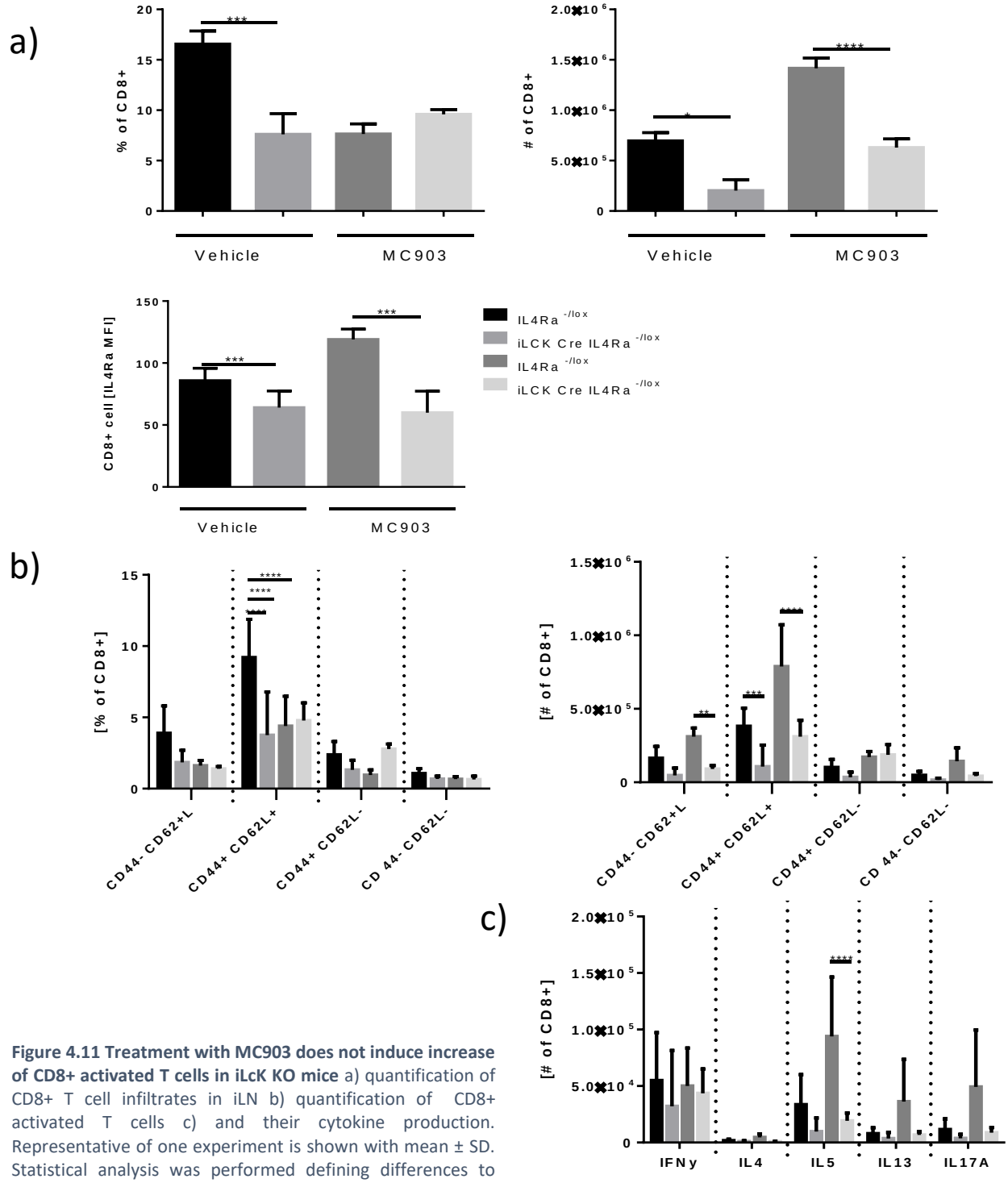


Figure 4.11 Treatment with MC903 does not induce increase of CD8+ activated T cells in iLcK KO mice a) quantification of CD8+ T cell infiltrates in iLN b) quantification of CD8+ activated T cells c) and their cytokine production. Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

4.2.8 γδT cell in Pan-IL-4Rα deficient CD4+ T cells display a naïve phenotype

Similarly, to CD4+ and CD8+ T cells, we measured frequencies and total cell number of CD3-, CD4-, CD8-, δγ+ T cells (Fig. 4.12). We observed higher numbers and frequencies of γδ T cells in MC903-treated and vehicle treated iLCK^{Cre} IL-4Rα $^{-/lox}$ mice (Fig. 4.12a). Interestingly, we found no reduction of IL-4Rα on δγ+ T cells after treatment with vehicle in iLCK^{Cre} IL-4Rα $^{-/lox}$ group, but treatment with MC903 reduced receptor expression in both littermate control and iLcK KO mice (Fig. 4.12a). We further analysed the activation status of δγ+ T cells and we observed that iLCK^{Cre} IL-4Rα $^{-/lox}$ group showed increase in CD44-, CD62L+ naïve γδ T cells frequencies and cell numbers in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice before and after MC903 treatment (Fig. 4.12b). Additionally, the CD44+ CD62+L γδ T population was increased in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice in frequencies and in cell numbers after treatment (Fig. 4.12 b). As γδ T cells are known to be the main IL-17 source in the skin, we analysed production of cytokines by γδ T cells. We saw no differences in any of the cytokines measured, except for an increased in IL-17 in iLCK^{Cre} IL-4Rα $^{-/lox}$ mice MC903 treated mice, which was significant when compared to littermate control (Fig. 4.12c).

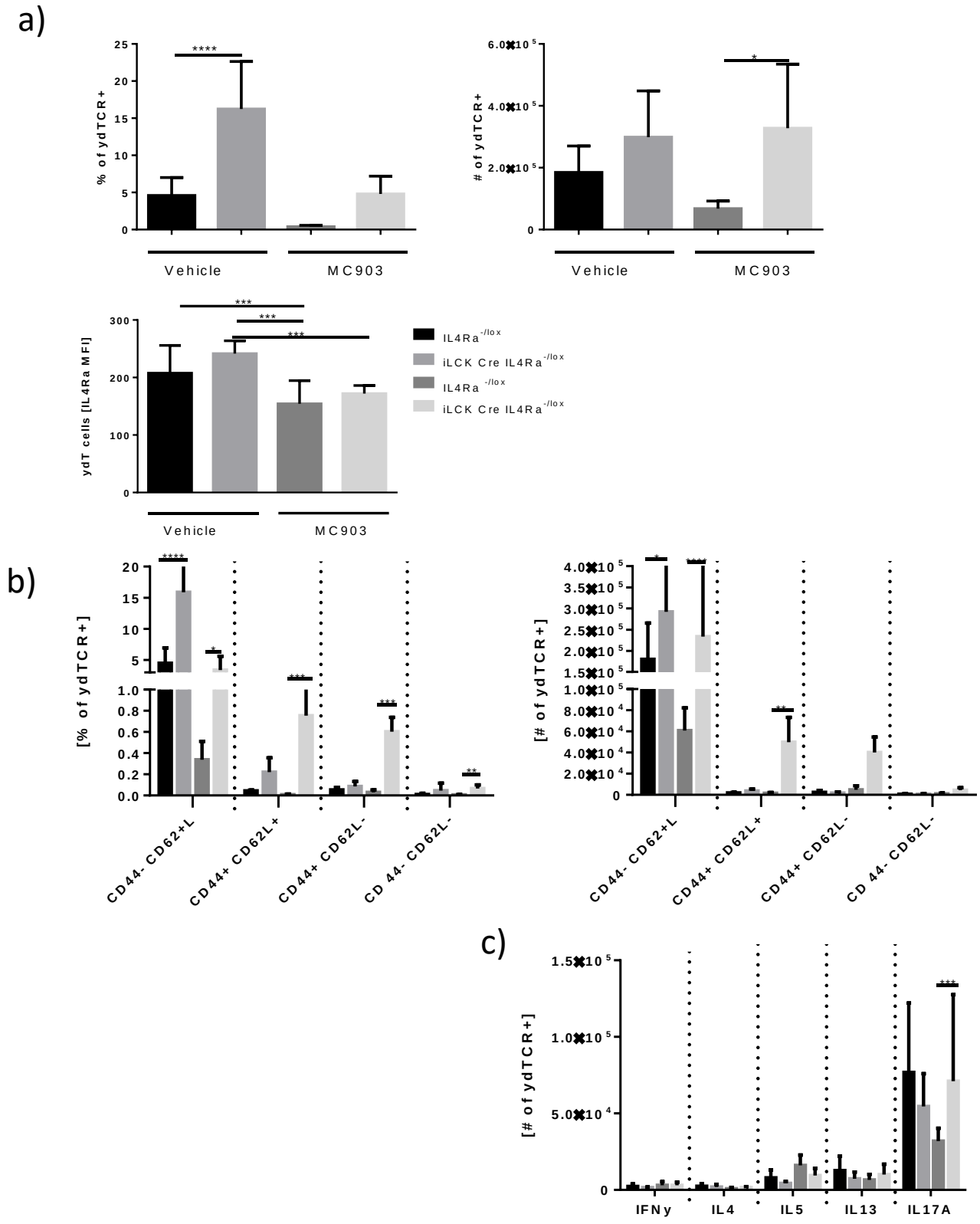


Figure 4.12 iLcK KO mice show high levels of CD44- CD62L+ γδT cell population in dLN before and after MC903 treatment a) quantification of γδ+ T cell infiltrates iLN b) quantification of activated γδ+ T cells and c) their cytokine production. Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

4.2.9 iLcK KO mice show reduced inflammatory response to MC903 treatment

We further wanted to analyse type of inflammatory response in iLN and serum. For this purpose, the supernatant from ionomycin: PMA: monensin re-stimulated iLN cell suspension as well as blood serum was collected and analysed using ELISA. The cytokines from re-stimulated LN samples were not significantly changed between IL-4Rα $^{-/lox}$ and iLCK^{Cre}IL-4Rα $^{-/lox}$ mice, nor between vehicle or MC903 (Fig. 4.13a). Mice lacking IL-4Rα on T cells were unable to produce IgE (Fig. 4.13b), however, total IgE level was not changed in WT mice upon MC903 treatment. Alarmin IL-33 was not detected in serum of MC903 mice (Fig. 4.13c). Serum IL-17A levels were lower in iLCK^{Cre}IL-4Rα $^{-/lox}$ mice than in IL-4Rα $^{-/lox}$ littermate control mice, however the difference was not significant (Fig. 4.13c). Similar trend was observed in samples of re-stimulated LN for IL17 cytokine.

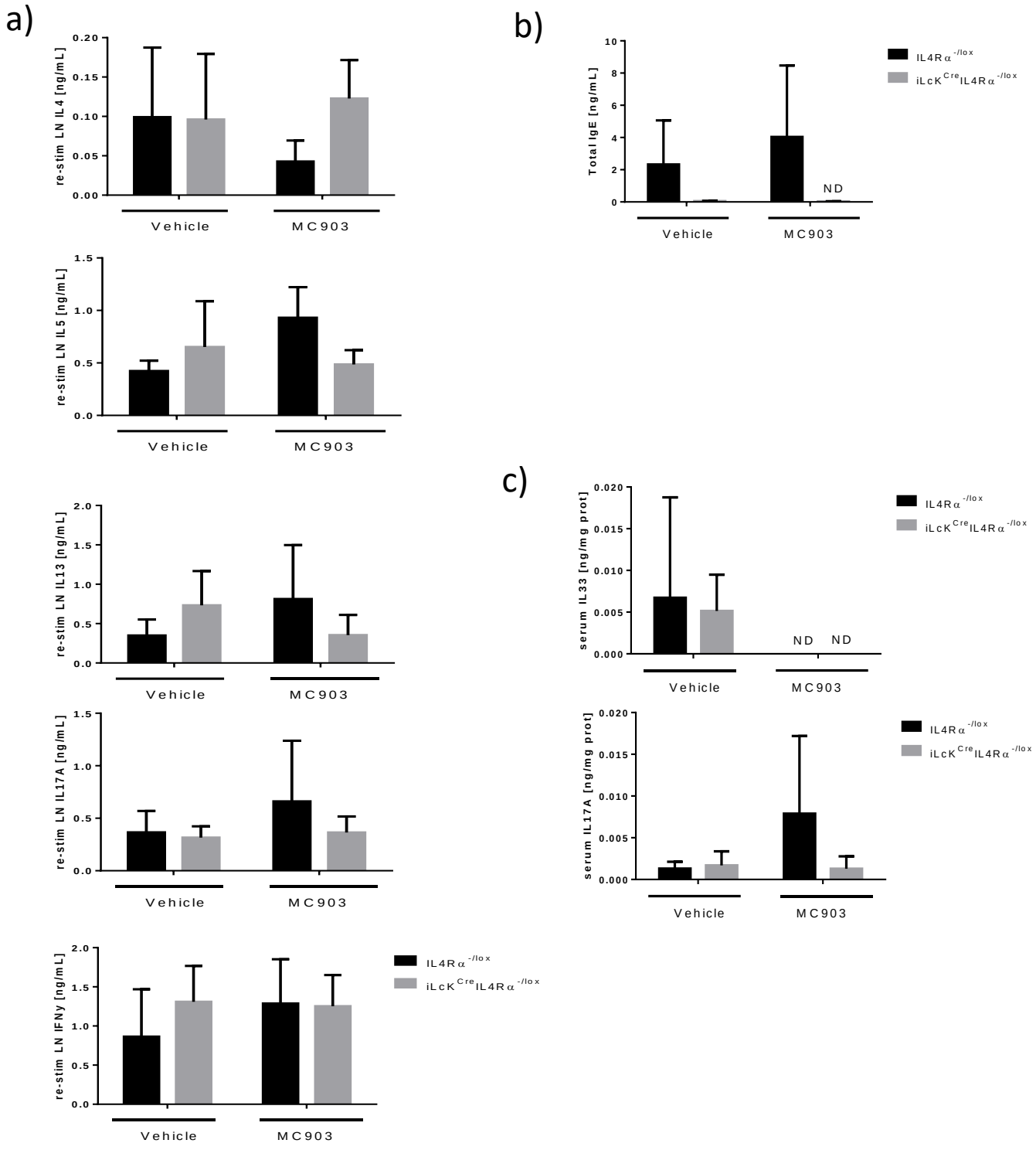


Figure 4.13 iLcK KO mice show reduced inflammatory response to MC903 treatment a) re-stimulated iLN did not show differences between iLCK^{Cre}IL-4Rα $^{-/lox}$ mice and their littermate control, however iLCK^{Cre}IL-4Rα$^{-/lox}$ mice did not produce total IgE b). The serum levels of IL33 and IL17A were similar between iLCK^{Cre}IL-4Rα$^{-/lox}$ and littermate control. Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, n=5 mice per group. one-way ANOVA

Overall, absence of IL-4Rα in pan-CD4 T cells results in protection from MC903-induced inflammation by reduction of activated B cells secreting IgE, CD4+ and CD8+ T cells at the same time the increase in population of γδ T cells.

4.2.10 Epicutaneous treatment with 10ug HDM does not induce hallmarks of AD in Foxp3^{Cre} IL-4Rα$^{-/lox}$ mice or their littermate controls

Previously in our laboratory we showed that absence of IL-4Rα on Foxp3+ T regulatory cells (Tregs) results in break of tolerance and increase of IL-33 alarmin secretion [159]. We further investigated if repetitive skin exposure to HDM would lead to break in tolerance in the skin, as T regs are shown to be critical in skin homeostasis and lack of GATA3 in Foxp3 T regs leads to skin autoimmunity [332]. We treated mice as previously described, with 10 ug HDM solution on pre-shaved ventral skin on day 0, 14, 28 and killed on day 35. We collected skin, blood by cardiac puncture and inguinal (skin draining) lymph nodes for further analysis.

The H&E stained skin keratinization was quantified using QuPath software (Fig. 4.14a, b). We did not see a significant change between PBS and HDM treated mice, nor between Foxp3^{Cre} IL-4Rα$^{-/lox}$ and their littermate controls. As one of the hallmarks of AD mice is increased level of total IgE in serum, we quantified total IgE levels using ELISA (Fig. 4.14 c). The levels of antibody were increased upon HDM treatment for both Foxp3^{Cre} IL-4Rα$^{-/lox}$ and WT mice, but the difference between strains was not significant.

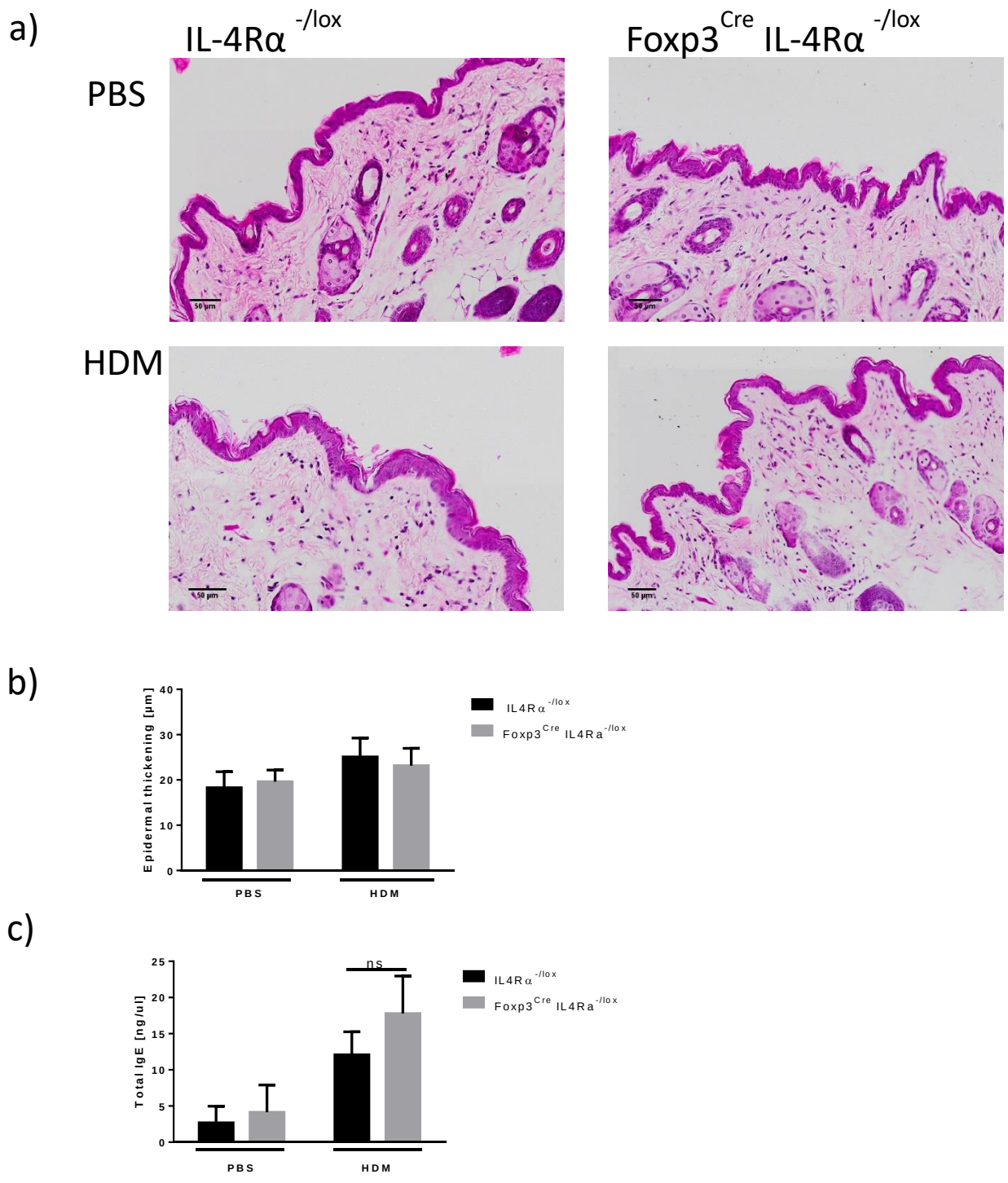


Figure 4.14 Epicutaneous treatment with 10 ug HDM does not induce signs of AD in Foxp3Cre IL-4Rα -/lox mice, but it does lead to increased total IgE production in both KO and littermate control mice a) representative images of skin biopsies from ventral side, histology stained with H&E, scale bar 50 um; b) quantification of histopathology using QuPath software, c) quantification of total IgE. Representative of two experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, n=5 mice per group. one-way ANOVA, representative of 2 experiments.

4.2.11 Epicutaneous exposure to HDM does not change immune profile of skin dLN

The collected inguinal LN were further analysed by FACS as described above. We did not see change between PBS and HDM treated mice, upon T cell analysis (CD4+, Foxp3+, CD8+ and γδ+ T cells), but we did confirm the phenotype of knockdown of IL-4Rα in Foxp3^{Cre} IL-4Rα$^{-/lox}$ mice (Fig. 4.15a).

Similarly, to T cells, we did not see difference in CD19+ B cell composition between PBS and HDM treated mice (Fig. 4.15b).

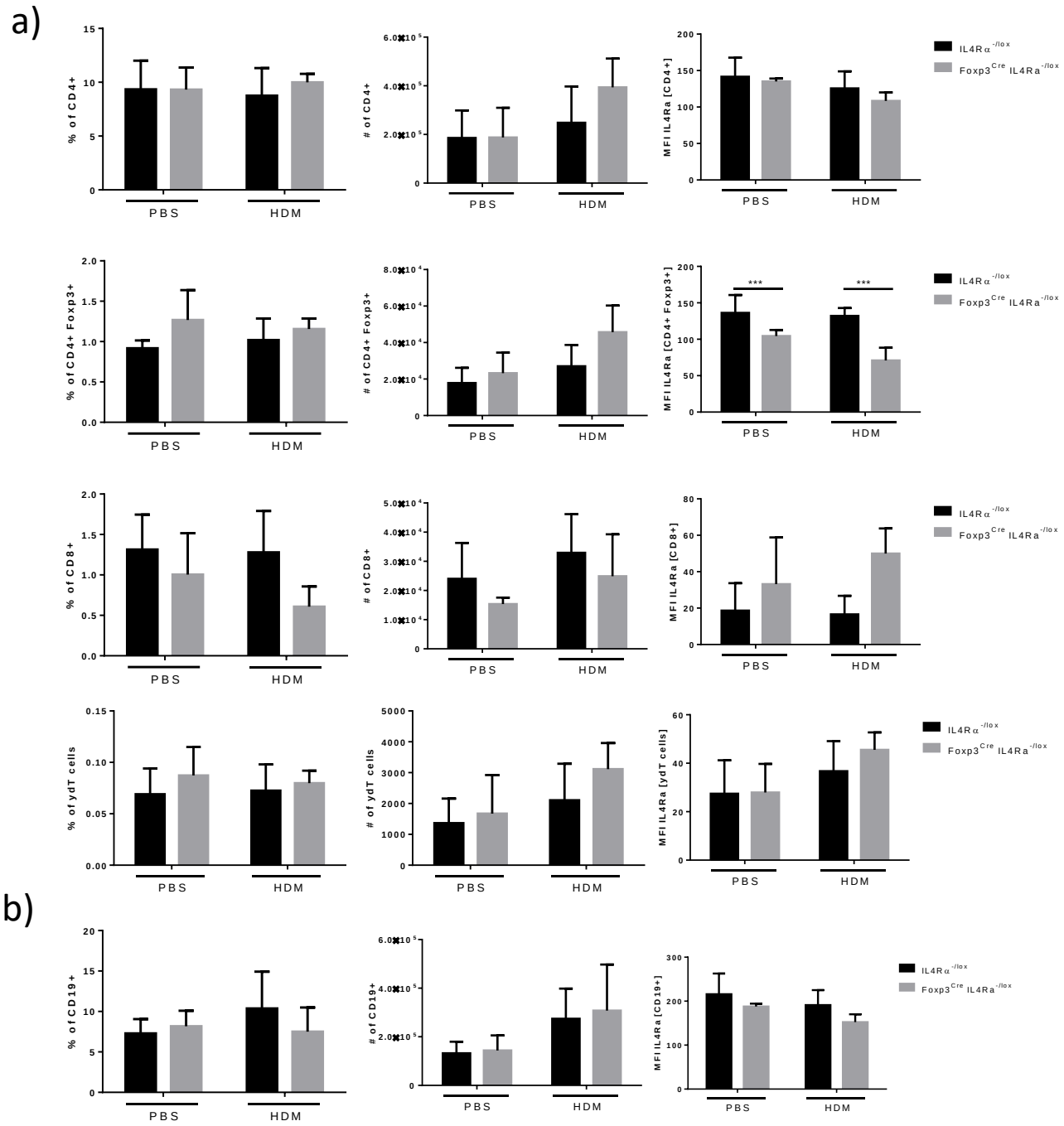


Figure 4.15 HDM does change immune profile in skin draining LN in both Foxp3^{Cre} IL-4Rα$^{-/lox}$ mice and their littermate control, a) quantification of T cells profile and b) B cell profile using FACS Representative of two experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01, **P<0,001, ***P<0,0001

4.2.12 Epicutaneous treatment with OVA does not induce AD nor change immune cell composition

Hoving et al. demonstrated that epicutaneous exposure to chicken derived ovalbumin (OVA) induces AD like symptoms in mice [272]. We replicated the model by exposing mice to repetitive treatment with 200 ug OVA on day 0, 14, 28 and killed mice on day 35. We collected skin samples, blood and inguinal lymph nodes for further analysis.

The H&E stained histology samples were quantified using QuPath software (Fig. 4.16 a, b). In our analysis we did not see differences between PBS and OVA treated mice in histology. We further analysed total IgE serum levels using ELISA (Fig. 4.16c). Mice treated with OVA did not show increase in total IgE, despite suggestions from previous studies. Importantly, we observed similar non-

significant trends in IgE production Foxp3-IL-4Rα treated with OVA as those same mice treated with HDM.

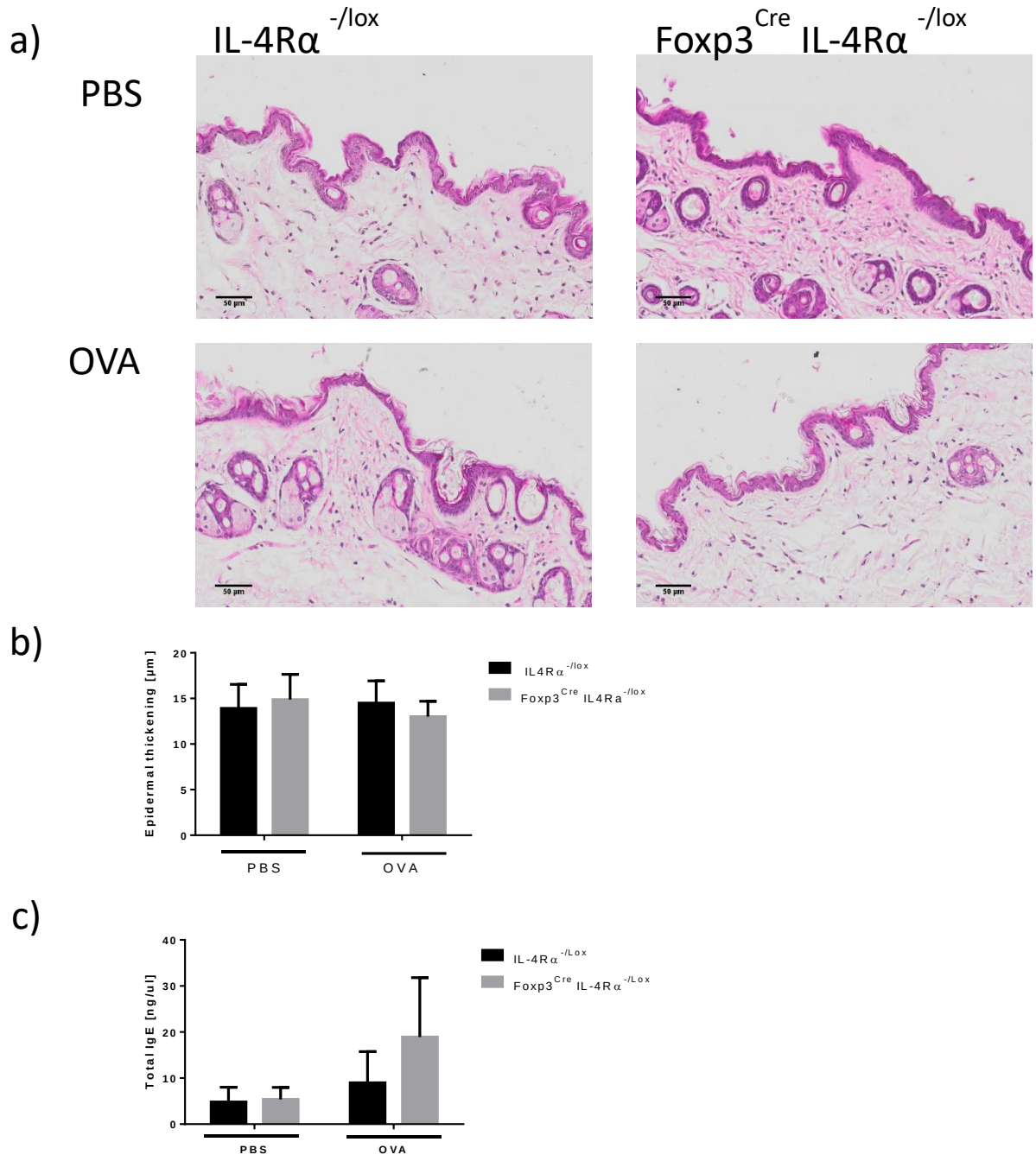


Figure 4.16 Epicutaneous treatment with 200 ug OVA does not induce signs of AD in Foxp3^{Cre}IL-4Rα$^{-/lox}$ mice. a) Representative images of skin biopsies from ventral side, histology stained with H&E, scale bar 50um, and was b) quantified using QuPath software. c) Quantification of total serum IgE using ELISA Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα$^{-/lox}$ BALB/c control mice as significant, n=5 mice per group. one-way ANOVA.

Finally, we analysed cellular composition of collected LN using FACS. We did not see differences in CD4+, Foxp3+ CD4+ or CD8+ T cells (Fig. 4.17a). Additionally, the analysis of mean fluorescence intensity of IL-4Rα on Foxp3+, CD4+ Treg cells showed that upon treatment of OVA receptor is upregulated and knock-down of the receptor is not efficient (p-0.0993). This result is similar to

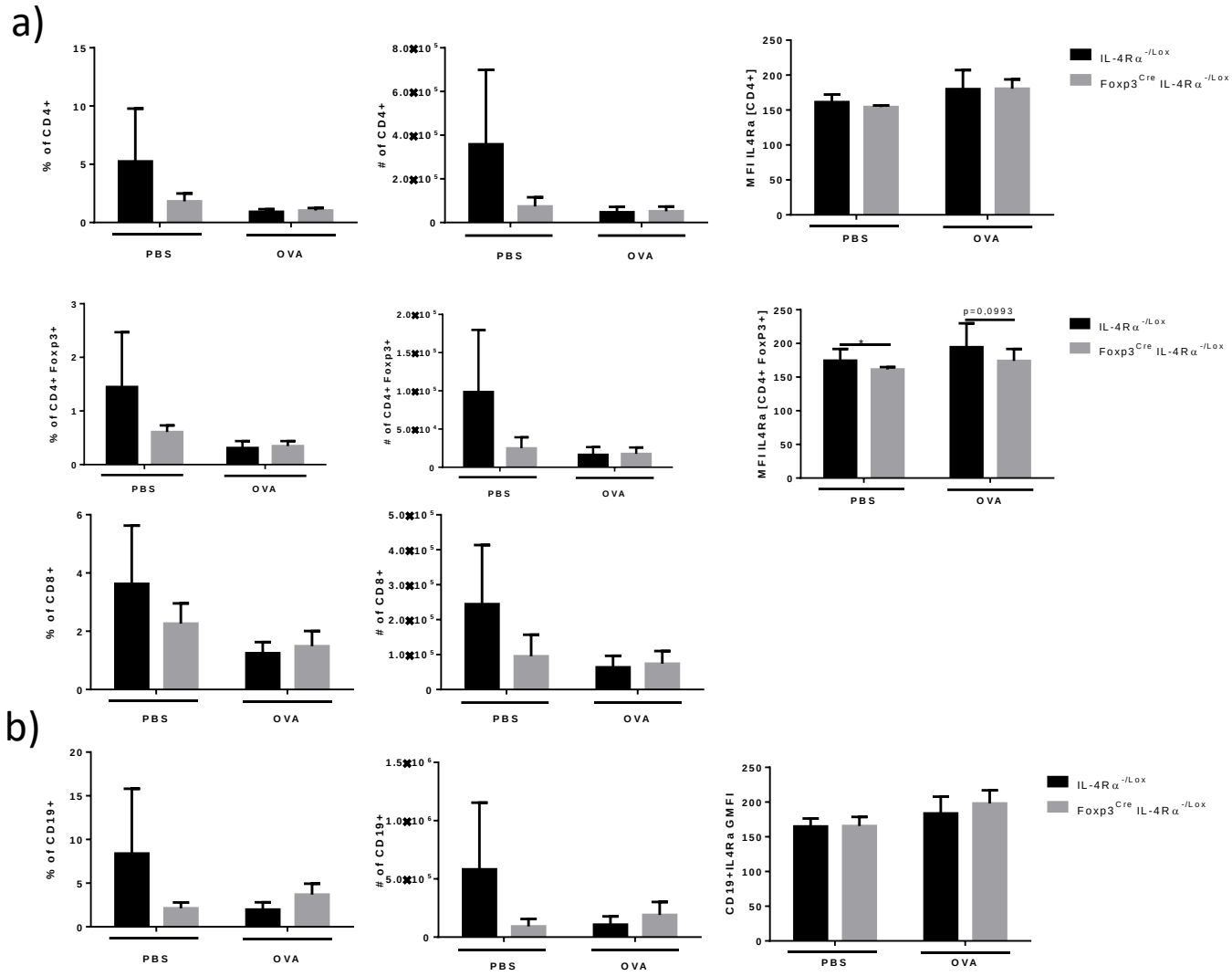


Figure 4.17 No change in cell composition was observed upon OVA induced-AD model in Foxp3^{Cre}IL-4Rα$^{-/lox}$ mice. a) quantification of CD4+, CD4+ Foxp3+, CD8+ T cells b) quantification of CD19+ B cells in skin draining LN Representative of one experiment is shown with mean ± SD. Statistical analysis was performed defining differences to littermate IL-4Rα–/lox BALB/c control mice as significant, n=5 mice per group. one-way ANOVA *P<0,01

previously shown partial knock-down in female mice of Foxp3^{Cre} IL-4Rα$^{-/lox}$ strain [265]. Similarly, to T cell analysis, we did not find differences in CD19+ B cells after OVA treatment (Fig. 4.17b).

In our study we found that HDM and OVA allergen-induced atopic dermatitis murine model does not induce strong inflammatory response in murine skin, at the same time it does not lead to increase of total IgE in serum, which is associated with AD. Additionally, we found that inflammatory model of murine AD using calcipotriol leads to increased epidermal thickening, one of the hallmarks of AD. Treatment with MC903 did not influence serum level of total IgE. Mice lacking IL-4Rα on all T cells

showed protective phenotype with reduced epidermal hyperplasia, activated B cell, CD4+ and CD8+ cell numbers in skin draining lymph nodes.

4.3 Discussion

The importance of TH2 axis of immune response has been vastly studied in context of atopic dermatitis[168,220,289,310,331,333–335]. The most recent recommended treatment is systemic administration of monoclonal antibody targeting IL-4Rα – dupilumab in conjunction with topical glucocorticosteroids[11,23,220,243]. Such regime is highly effective with alleviation of AD symptoms like reduction of the clinical score, reduction of IL-22 or eotaxin, as well as total IgE however, clinical studies show increase in IL-4 and IL-13 upon the treatment[241,244]. The most often reported side effect is conjunctivitis and blood eosinophilia. These studies suggest that despite high efficacy, the treatment could still be improved.

Previously our group showed that reduction of IL-4Rα expression on LCK^{Cre} IL-4Rα $^{-/lox}$ mouse strain contributes to reduction of IL-5 and IL-4 but not IL-13 in Anisakis-induced model of AD[160]. We further showed that in absence of IL-13 but not IL-4, we can reduce the effect of Anisakis application like epidermal hyperplasia and cellular infiltration. Recently, published paper by Bitton et al. describes mechanism of protection from oxazolone-induced AD murine model by inhibition of IL-13 detection via IL-13Rα1 [168].

In this study, we dissected role of cell specific IL-4Rα signalling on various T cell subsets. We found that insertion of Cre-loxP system influences epithelialization of skin, which has not been shown up to date. Additionally, we report that introduction of epithelial trauma such as repetitive shaving and administration of non-toxic chemical like PBS can lead to increase in epidermal thickening. Our data support the previously shown scratch-induced cutaneous hyperplasia[61,280].

We further investigated the inflammatory responses in murine skin using HDM-induced AD model previously used in our group (P. Pillay 2016 Master's thesis). In our investigation, we did not see a significant increase of hyperplasia in comparison with PBS control group. Similarly, not in all groups HDM was able to induce increase in total IgE production, which is a crucial parameter in models of allergic diseases. Furthermore, in our investigation we tried to replicate previously published model of allergic AD using OVA, however as in HDM study we did not find significant difference between OVA and control groups[272]. As the initial investigation shows that PBS alone can contribute to increase of epidermal thickening, we could speculate that the allergen application induced insufficient inflammation to show differences between the groups. The allergen sensitization of BALB/c mice has been shown to be difficult[252]. The study comparing BALB/c mice with flaky-tail mice show that maintenance of mechanical skin barrier protects from HDM-induced skin inflammation[252]. Similarly,

in OVA-induced atopy studies it has been shown that tape stripping has to be performed multiple times prior to sensitization to observe an effect [299,336]. The other plausible fault in the study design is the variance between the HDM/OVA extracts[300,301,337,338]. Not only the proportion of Derp1/Derp2 can be different between various batches of HDM, but also the variance in the microorganism diet in manufacturing facility can influence the microorganism fitness and as a result can influence the experimental design.

Due to this, we performed additional titration experiment of variable HDM dose (10 ug and 100 ug) titration in comparison with the 200 ug OVA. Unfortunately, despite increased dose of HDM antigen and repeated OVA treatment we were unable to show similar epidermal thickening change. The variability in the response to allergen-induced challenge in our study and study done by Hoving et al. might be due to variance in the degree of skin disruption or difference between batches of allergen. The comparison of murine models for AD, shows that allergen-induced models such as HDM and OVA are dependent on damage to primary layer of keratinocytes [339]. Authors suggest that allergen-induced skin exposure is a preferable method for atopic march studies rather than studying inflammation of atopic dermatitis.

As cells responsible for immunosurveillance such as DETC and T cells can migrate between skin and skin draining lymph nodes to trigger the adequate immune response, we further investigated the composition of inguinal lymph nodes after allergen exposure[60,72,164,325,335,340]. We found no increase in CD4+, CD8+ T cells after HDM and after OVA sensitization. Similarly, the population of B cells was not altered in neither of AD models. Interestingly, we found increase in γδ+ T cell population in pan-T cell IL-4Rα knockout (iLCK^{Cre} IL-4Rα $^{-/lox}$) after HDM treatment despite induction of strong inflammation. Nakajima et al. showed that those cells are the main IL-17A producers in murine AD and that they drive TH2 polarization, which could explain their role in the LN, however it does not explain their increased frequency in iLCK^{Cre} IL-4Rα $^{-/lox}$ only group[341,342].

The IL-4Rα dependent development of γδ+ T cells has been closely studied by Darby et al (data unpublished). The authors showed that alteration of IL-4Rα in early stages of thymic development lead to increase in γδ+ T cell population in inflammatory conditions such as parasitic infection. This supports our finding, that upon mild inflammation induced by HDM treatment, the γδ+ population will be increased in LNs.

Previously in our laboratory, we showed that absence of IL-4Rα in iLCK and LCK results mainly from reduction of IL-4Rα expression on CD4+ T cells [263]. The study showed that IL-4Rα expression was reduced in CD4+ T cells in iLCK and LCK strain, but γδ+ T cells IL-4Rα expression was reduced only in iLCK strain. Additionally, in steady state IL-4Rα expression on CD4+ and γδ+ T cells is reduced in iLCK^{Cre}

IL-4Rα $^{-/lox}$, but in CD4^{Cre} IL-4Rα $^{-/lox}$ the reduction is limited to CD4+ T cell population (Darby et al. data unpublished)

In our study on HDM-induced atopic dermatitis we found that, IL-4Rα was absent from γδ+ T cells in iLCK^{Cre} IL-4Rα $^{-/lox}$ and CD4^{Cre} IL-4Rα $^{-/lox}$, but not in LCK^{Cre} IL-4Rα $^{-/lox}$. Furthermore, in our study we found that upon treatment with MC903, iLcK mice treated with only vehicle show no difference in receptor expression on γδ+ T cells in comparison with littermate control. Consecutive treatment with MC903 reduced IL-4Rα expression in both groups equally. Such variance in the receptor expression can be explained by possible problematic knockout system[343,344]. The transgenic insertion of Cr-LoxP system iLcK^{Cre}, LcK^{Cre} and CD4^{Cre} knockout, can result in germline recombination and transient expression of Cre recombinase [343,344]. Further, this can potentially lead to unspecific knockout of the gene during cell development. The authors suggested few controls to be considered including reporter system to be put in place for the gene knockout or to use inducible gene knockout.

Despite the above-mentioned technical issues in the project, we found that MC903 treatment, being preferable method for AD investigation, had profound effect on skin immunobiology. We found that upon treatment on 5th day mice start losing weight as well as we could observe skin morphology changes such as redness. On day 6th skin began to scale and mice showed visible itchiness (data not quantified). Such observations were also done by Oetjen et al. while investigating role of IL-4Rα on sensory neurons [141]. The group showed that the acute itch is induced by IL-31, while in chronic itch, the inhibition of IL-4Rα can result in significant reduction of sensory neurons responsiveness to histamine secreted by mast cells in the MC903 model. In our investigation we found that pan-T cells IL-4Rα knockout ameliorated the skin inflammation. Since the iLCK^{Cre} IL-4Rα$^{-/lox}$ mice showed reduction of IL-4Rα expression on CD4+ and CD8+ T cells but not on γδ+ T cells (before the treatment with MC903) we could speculate that those cells are responsible for mast cell recruitment and further histamine secretion, which downstream leads to pruritis. However, mast cells being the innate immunity population are first one to react upon inflammatory activation independent of the antigen presentation leading to subsequent naïve T cell activation [140]. This goes in hand with the fact that mast cells do express IL-17R and IL17 was elevated in our study after MC903 treatment in both serum and re-stimulated lymph nodes supernatant of wild-type but not in the iLCK^{Cre} IL-4Rα $^{-/lox}$ [345]. IL-17 is known Th17 cytokine, however recent studies showed that IL17 can be secreted by innate subpopulation of T cells – γδ T cells[65,94,134]. At the same time, we were able to find both single positive CD62L+ cells as well as CD44+ CD62L+ double positive γδ T cells both after and prior treatment. The CD62L has been suggested to be driving the process of γδ T cells homing to dLN[6]. Together with Darby et al. study, it could explain why CD62L+ and CD44+ CD62L+ γδ T cells are more prevalent in iLCK mice but not in their littermate control group. In the study of HSV-1 corneal infections, authors showed that CCR6+,

CD44+CD62L+ γδ T cells were secreting IL17 and were showing protective phenotype against the virus [346]. In our study, we found that γδ T cells are predominant source of IL17 as well.

Additionally, treatment with MC903 reduced the IL-4Rα expression on B cells and γδ T cells in IL-4Rα$^{-/lox}$ mice, suggesting a possible receptor downregulation to reduce the treatment-induced inflammation damage [138]. Despite downregulation of the receptor in WT mice, the IL-4Rα remained upregulated in CD8+ and CD4+ cells after skin application of MC903, therefore the mice were unable to control the inflammation and as a result, they showed more severe symptoms of AD than the iLCK^{Cre} IL-4Rα $^{-/lox}$ group. Additionally, the B cells in WT were mainly CD44+ CD62L-, therefore capable of cytokine and chemokine secretion. The downregulation of the receptor on those cells can be reflected in low IgE production after MC903 treatment. Further investigation into MC903-induced downregulation and loss-of-function of those cells could be analysed using Ki67 cell marker or by assessing receptor-phosphorylation status by Western Blot.

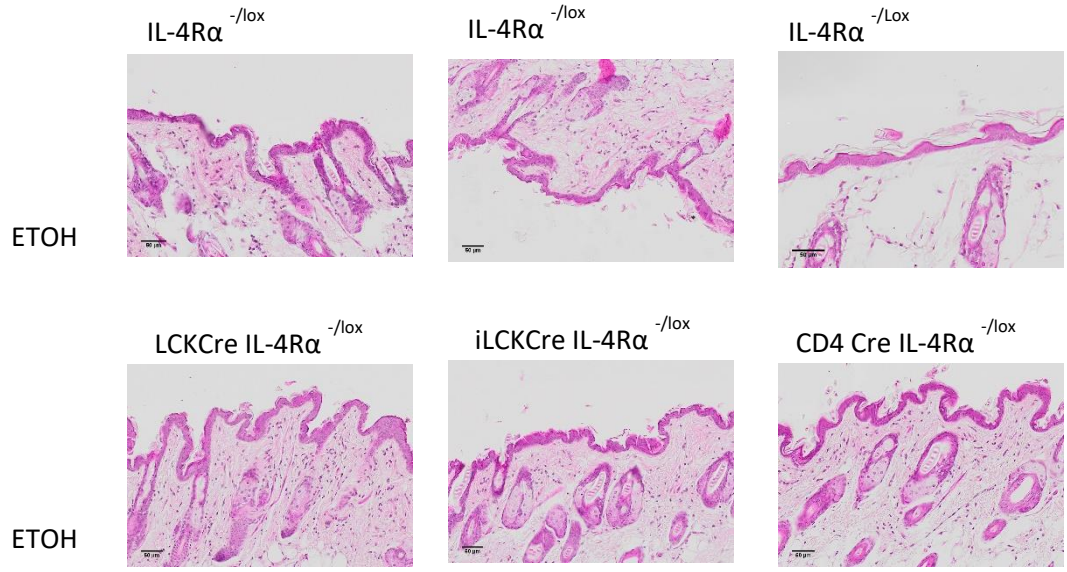
4.4 Conclusions

In conclusions we found that allergen-induced mouse models such as HDM and OVA require high dose of allergen and additional microscopic skin damage such as tape stripping to show effective induction of skin hyperplasia.

Additionally, we showed that cre-loxP system, might induce inconsistent genetic manipulation, which should be better monitored using reporter system.

iLCK^{Cre} IL-4Rα $^{-/lox}$ group showed protective phenotype in MC903-induced murine model of atopic dermatitis, by reduced epidermal hyperplasia and reduced pro-inflammatory activated CD4+ and CD8+ T cell population. At the same time, the central memory CD44+ CD62L+ γδT cells population being main IL-17 producer, are increased in those mice. Interestingly, the type 2 cytokines associated with AD were reduced in those mice, which could potentially explain the reduction in epidermal hyperplasia, however, the exact mechanism of this protection remains unclear.

Supplementary 1



Supplementary Figure 1. Comparison of ventral skin biopsies after ethanol treatment for 10 consecutive days.

Chapter 5

Final discussion, conclusions and future recommendations

Atopic dermatitis is a chronic condition of the skin, affecting up to 20% of children and 3% of adults[20]. It results in decrease of patients' quality of life as well as it has been shown to provide the ground for further development of secondary comorbidities such as allergic rhinitis and asthma[14,18,270]. As AD is a chronic condition, it provides a high burden to healthcare system, therefore better understanding of the mechanisms underlying development of AD is vital[270].

The recent body of knowledge of AD development indicates two distinct arms of disease aetiology: genetic predisposition and environmental factors[20]. One of the major genetic components is mutation in filaggrin gene, which leads to increased permeability of skin barrier and as a result sensitization to environmental antigen. Due to increased activation of immune response, the skin inflammation perpetuates further epidermal barrier damage. The main alarmins determined in AD are IL-31, TSLP, IL-25 and IL-33 which can activate skin residing ILC2, LCs and γδT cells for further type 2 immunity priming[38,83,142,310,347]. The residing cells can both secrete cytokines like IL-5, IL-4 and IL-13 to attract eosinophils and mast cells to the site of inflammation, as well as they can migrate to skin draining LN to further promote adaptive immunity by B and T cell maturation, it remains equally important to understand the mechanism by which this process can be hindered[71–73,77,109,143,168,171,272]. Previously our lab and others have shown that IL-4Rα can play a pivotal role in mechanisms of murine AD and anaphylaxis[168,264]. Interestingly, the antagonist of IL-4Rα – dupilumab, showed very high efficacy for treatment of severe and non-responsive AD supporting the murine model findings[219,220,245]. The long term effect of use of dupilumab has not been investigated as well as the systemic administration results in conjunctivitis the need for further improvement such as target cell therapy remains vital[219,227,241,242,246].

We hypothesized that cell-specific abrogation of IL-4Rα signalling will result in reduction of AD symptoms in murine model of AD, which in turn can elucidate the specific cell type necessary to target in treatment of the disease. To do this, we used various mouse strains, $MB1^{Cre}$ IL-$4R\alpha^{-/lox}$ where receptor expression was diminished on B2 cells only (Chapter 3) and in T cell (Chapter 4), we targeted specific cell populations by exclusion approach using $iLcK^{Cre}$ IL-$4R\alpha^{-/lox}$ which did not have receptor on all T cells, LcK^{Cre} IL-$4R\alpha^{-/lox}$ where receptor was absent on CD4+ and CD8+ T cells and $CD4^{Cre}$ IL-$4R\alpha^{-/lox}$ where receptor knockout was limited to CD4+ T cells. This approach allowed us to target very specific cell populations, which were not fully investigated in the past studies.

We found that genetic manipulation by insertion of Cre-lox system, can result in structural attenuation of skin thickness, which provides important point for any murine studies. Additionally, we found that lack of induction of epithelial damage such as tape-stripping can lead to mild allergic sensitization, however, it is insufficient for induction of the classical AD-related skin alteration such as increased epithelial hyperplasia and spongiosis. Additionally, other studies on AD have shown various combinations of HDM strain *Dermatophagoides farina* to be efficient in induction of AD-like lesions, when boosted with skin irritant such as MC903, or capsaicin or with bacterial toxin like staphylococcal enterotoxin b from *S. aureus*[256,302,348]. In those studies, the use of skin irritant to induce neurological response was primary goal, while immunological response was the secondary objective. At the same time, the HDM-induced sensitization cannot be neglected, because it provides a good model for studies of atopic march. We also showed that PBS alone can induce inflammatory response, similar results have been shown by Leyva-Castillo et al.[61]. The group showed that upon tape stripping alone, the inflammatory changes can be already produced in skin.

The absence of IL-4Rα in iLcK^{Cre} IL-4Rα$^{-/lox}$ resulted in increase of γδ T cell population in skin draining lymph nodes. In the model of MC903-induced AD, absence of IL-4Rα on γδ T cells contributed to protective phenotype such as reduced keratinization of epidermis and reduction of B cells as well as CD4+ and CD8+ T cells. These data demonstrate the important role of IL-4Rα signalling on γδT cells this effect has not been seen in other mouse strains. Importantly, the deletion of IL-4Rα of γδT cells, was not efficient in some of the experiments. As previously described by Song and Palmiter certain level of difficulty can be expected from Cre-loxP system and therefore suitable controls such as reporter system should be added to the experimental setup[344]. The role of IL-4 detection by those cells has not been vastly investigated in past in the context of an allergic response, but in cancer biology, γδ T cells found in circulation have been found to suppress anti-tumour immune response in presence of IL-4[349,350]. However, in skin the intraepithelial γδ T cells are capable of inducing unique autoreactive IgE production in presence of IL-4, which protects host from tumour formation[329]. These differences provide distinction between different subclasses of γδ T cells and their role in immune response. Importantly, the murine and human γδ T cells display morphological differences such as formation of dendrites. The murine the γδ T cells can form dendrites by which they conduct immunosurveillance and upon detection of antigen the dendrites are retracted while human cells do not develop dendrites[90,96,134,341]. Additionally, we found that those cells are main producers of IL-17, which the main cytokine was found in serum of mice. This cytokine is associated predominantly with psoriasis, not with atopic dermatitis, however due to epidermal damage it is likely that skin-residing microbial peptides penetrated the epidermis and skewed the cytokine production[65,92,96,248,305,320,341]. In contrast, Constantinides M., Link V., Tamoutounour S. et al. proposed a protective properties of mucosal

associated invariant T cells (MAIT) produced IL-17 in tissue repair[351]. Authors found that MAIT cells reside close to epidermal-dermal barrier and presented tissue remodelling signature. In our study we did not look at the MAIT cell composition, however it would be of value to compare the frequencies of MAIT and γδT cells as well as analyse their functionality in AD.

As previously mentioned, the skin sensitization to HDM resulted in induction of mild asthma-like lung inflammation, with increase in eosinophil infiltration in both BAL and lung samples as well as increase in antigen-specific and total IgE. This characteristic of asthma did not contribute to further development of airway hyperresponsiveness or increased mucus production in wild-type mice. On contrary, the $MB1^{Cre}$ IL-$4R\alpha^{-/lox}$ mouse strain showed increase of mucus production, despite lack of eosinophilia. Those mice showed residual antibody production with increase of IgG2a despite absence of IL-4Rα on B2 cells. This proved that abrogation of receptor signalling on B2 cells, does not protect from allergic sensitization. In this study, we did not dissect specific B cell subpopulations such as B1, B2 and Breg cells in the analysis of the receptor expression, however we used CD19 marker while analysing IL-4Rα expression, which should provide for pan-B cell population. Interestingly, in the past our group showed that mice lacking IL-4Rα on all cells are still capable of development of asthma in murine model of the disease and that the mechanism is driven by IFNγ in absence of the receptor[201]. In our study we did not find the mechanism of mucus induction.

Limitations and setbacks of the study

The model of HDM-induced AD provided to be difficult in inducing of skin changes associated with AD such as epidermal thickening and spongiosis[20]. Similarly, to HDM model, the OVA-induced AD murine model did not provide pathomorphological changes between PBS and OVA treated mice groups. The main flaw of the study design might be lack of use of epidermal tape stripping technique, prior to application of HDM and OVA, as damage to epidermal barrier seems to be the imperative for induction of epidermal spongiosis in Nc/Nga and Flaky Tail mice when used HDM protocols[250–252,257]. Importantly, those mice do depict pathomorphological changes spontaneously, therefore use of allergen is not a prerequisite for strains to develop AD. In both HDM and OVA models of AD it is plausible that the batch variability, proportion Derp1/Derp2 or level of endotoxin could play detrimental role. We further investigated whether exposure to allergen, despite lack of pathomorphological changes, induces secondary allergic comorbidities in model of HDM – induced murine AD. We found that mice exposed to allergen via epicutaneous sensitization can develop mild asthma symptoms (Figure 2.4 Atopic march model quantification). This finding proves, that epicutaneous sensitization despite the induction of morphological alterations, leads to sensitization against the allergen, which has been previously shown in models of atopic march[9,18].

Secondary to allergic sensitization we optimized the use of vitamin D3 analogue – MC903 for induction of AD. The previous studies showed that dose as low as 2 nmol can lead to inflammatory response[141]. In this study, we found that MC903 is not only highly hydrophilic, but also biologically unstable after reconstitution. Once opened, the substance was stored according to manufacturer's instruction at -20 ^{0}C in powder form, however despite recommended storage, the MC903 lost the ability to induce epidermal inflammation in low dose. Therefore, we do recommend reconstitution of substance and storage in liquid form as well as upon longer storage, reassessment of bioreactivity of the substance.

Additional aspect which was not accounted for was the receptor knockout inconsistency in iLcK^{Cre} IL-4Rα$^{-/lox}$, LcK^{Cre} IL-4Rα$^{-/lox}$ and CD4^{Cre} IL-4Rα$^{-/lox}$ murine strains. For this reason, we propose use of additional control group such as reporter strain for presence of IL-4Rα$^{-/lox}$.

Moreover, we did attempt FACS analysis on skin tissue. We found strong autofluorescence in dermis and epidermis as well as high loss of cells. It is possible that the protocol used, which was adapted from Jensen et al., was not suitable for FACS staining, but only for keratinocyte culture[267]. Other protocol published by Litchi et al., suggest shorter incubation time with trypsin or extended incubation but at low temperature (4 ^{0}C), such alteration of our protocol could prevent the cell loss. For this reason, further optimization is required for optimal preparation of dermis and epidermis for FACS.

As we did find differences in γδT cell composition in skin draining lymph nodes, we attempted quantification of those cells in skin biopsies using immunofluorescence staining. For this reason we stained the paraffin embedded tissues with anti-γδTCR antibody (BioLegend, USA). Unfortunately, despite the manufacturer's recommendations we were unable to optimize the protocol for the staining. The primary reason for this could be high background staining as the antibody was grown in mice. The secondary reason could be that the antibody is not suitable for paraffin embedded tissues, despite the manufacturer's recommendation. Formaldehyde preservation of tissue crosslinks epitopes, which can result in lack of antibody binding to the specific receptor of interest, for this reason it is recommended to cryopreserve the tissue[352].

Future recommendations

Translation of murine studies into human relevance remains difficult and adequate control experiments need to be performed.[353] Additionally, the future study in the field should take into consideration the flaws in Cre-lox system and necessity for gene-reporter murine strain.

In our study we focused predominantly on mouse models of AD. Our findings should be further investigated in human subjects in samples of both skin biopsies as well as blood.

The complexity and late detection of AD with secondary comorbidities in human provides impossible to underpin the early stages of immune response in the disease, therefore use of animal models can give insight into progression of the disease in cases such as atopic march. In the B cell arm of the study we found increase of mucus production despite absence of lung eosinophilia. These findings should be further investigated to find underlying immunological mechanism.

Considering the promising result of T cell arm of the study, we should further investigate the presence and subtypes of γδ T cells in patients with and without AD as well as before and after treatment with dupilumab. As treatment show high efficacy, it remains important to understand the exact effect it has on each cell type including γδ T cells, which could provide grounds for personalized and cell-specific AD treatment.

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