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The Role of CD1a and Phospholipase A_2 in Psoriasis

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By

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Contents

Contents i

Abstract iv

Acknowledgements v

List of Figures vi

List of Tables ix

Abbreviations x

1. General Introduction 1

1.1 Immunity of the skin 1

1.1.1 The skin 1

1.1.2 Keratinocytes 2

1.1.3 Langerhans cells 3

1.1.4 Dermal dendritic cells 5

1.1.5 Plasmacytoid dendritic cells 6

1.1.6 Mast cells 8

1.1.7 T cells 11

1.1.8 Innate and adaptive immunity in skin 13

1.2 Psoriasis 18

1.2.1 Overview of psoriasis 18

1.2.2 Histological features of psoriatic lesions 20

1.2.3 Pathogenesis of psoriasis 22

1.2.4 Psoriasis-associated genes 26

1.2.5 Treatments of psoriasis 27

1.2.6 Langerhans cells and dermal dendritic cells in psoriasis 29

1.2.7 In search of antigens of psoriasis 31

1.3 CD1a and CD1 family 33

1.3.1 Presentation of non-peptide antigens 33
1.3.2 CD1 family 34
1.3.3 CD1 antigens and CD1-restricted T cells 35
1.4 Phospholipase A_2 38
1.4.1 Introduction of phospholipase A_2 38
1.4.2 Secretory PLA_2 38
1.4.3 Cytosolic PLA_2 41

2. Aims and Objectives 44

3. Materials and Methods 46

3.1 Reagents 46
3.2 Isolation of PBMC from blood 50
3.3 Isolation of $CD3^+$ T cells from donor PBMC 50
3.4 Monocyte-derived dendritic (mDC) cell culture 51
3.5 K562 cell culture 52
3.6 LAD2 cell culture 52
3.7 Human cord blood-derived mast cell (CBMC) culture 52
3.8 Isolation of $CD3^+$ T cells from skin 53
3.9 Flow cytometry 54
3.10 *Ex vivo* ELISpot assay 55
3.11 Cultured ELISpot assay 56
3.12 ELISA 58
3.13 Gene Expression Omnibus (GEO) study 59
3.14 Immunofluorescence microscopy 60
3.15 Immmunoprecipitation 61
3.16 Exosome preparation 62
3.17 Cytosolic PLA_2 biochemical activity assay 63
3.18 SDS-PAGE and Western Blot 63
3.19 Statistics 65

4. CD1a autoreactivity in Psoriasis 66

4.1 Introduction and Aims 66
4.2 Results 70
4.2.1 Enriched CD1a-autoreactive T cells in psoriasis patients 70
4.2.2 CD1a-autoreactive T cells produce IL-17A and IL-22 79
4.2.3 CD1a presents host-derived lipids to T cells 80

4.3 Discussion 89

5. Phospholipase A_2 activates CD1a-reactive T cells in Psoriasis ..97

5.1 Introduction and Aims 97

5.2 Results 102

5.2.1 PLA2G4D expression in psoriatic lesions 102

5.2.2 LAD2-derived PLA2G4D activates CD1a-restricted T cells 123

5.2.3 Cytosolic PLA_2 inhibitor reduces PLA2G4D-dependent CD1a reactivity... 129

5.2.4 Autologous mDCs activate PLA2G4D-responsive CD1a-reactive T cells . 131

5.2.5 PLA2G4D-responsive CD1a-reactive T cells produce IL-17A and IL-22 .. 133

5.2.6 Cord blood mast cells-derived PLA2G4D activates CD1a-reactive T cells 137

5.3 Discussion 139

6. Mast Cell Exosome-responsive CD1a Reactivity in Psoriasis ...145

6.1 Introduction and Aims 145

6.2 Results 154

6.2.1 Cytosolic PLA_2 activity in LAD2 exosomes 154

6.2.2 Uptake of LAD2 exosomes by K562 cells 160

6.2.3 LAD2 exosomes activate CD1a-reactive T cells 162

6.2.4 Exosome-responsive CD1a-reactive T cells produce IL-17A and IL-22.... 167

6.2.5 Autologous mDCs activate exosome-responsive CD1a-reactive T cells .. 169

6.2.6 Inhibition of endocytosis reduces exosome-responsive CD1a reactivity... 171

6.2.7 IFN-α potentiates exosome-responsive CD1a reactivity 175

6.2.8 Enriched exosome-responsive CD1a-reactive T cells in psoriatic lesions 177

6.3 Discussion 184

7. General Discussion 191

7.1 General discussion 191

7.2 Future works 206

8. References 208

Abstract

The Role of CD1a and Phospholipase A_2 in Psoriasis

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Psoriasis is a chronic inflammatory skin disease associated with a T-helper 17 (Th17) response, yet it has proved challenging to identify relevant peptide-based T cell antigens. Langerhans cells show a differential migration phenotype in psoriatic lesions and express constitutively high levels of CD1a, which presents lipid antigens to T cells. In addition, phospholipase A_2 (PLA_2) is highly expressed in psoriatic lesions and is known to generate neolipid skin antigens for recognition by CD1a-reactive T cells. Here we observed expression of a cytosolic PLA_2 (PLA2G4D) in mast cells in psoriatic lesions but not in healthy skin, but unexpectedly also found the PLA2G4D activity to be extracellular. This could be explained by IFN-α-induced mast cell release of exosomes, which transferred cytosolic PLA_2 activity to neighbouring CD1a-expressing cells. This led to the generation of neolipid antigens and subsequent recognition by lipid-specific CD1a-reactive T cells inducing production of IL-22 and IL-17. Circulating and skin-resident T cells from patients with psoriasis showed elevated PLA2G4D-responsiveness compared to healthy controls. Overall these data present an alternative model of psoriasis pathogenesis in which unconventional lipid-specific CD1a-reactive T cells contribute to psoriatic inflammation. The findings suggest that PLA_2 inhibition may have therapeutic potential for psoriasis.

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List of Figures

Figure 1.1	Structure and cellular components of skin in mice and humans	2
Figure 1.2	Clinical features of chronic psoriasis vulgaris	20
Figure 1.3	Histological features of psoriasis	21
Figure 1.4	Comparison of human and mouse CD1 and MHC molecules	35
Figure 1.5	Arachidonic acid cascade	41
Figure 4.1	CD3 expression of T cells verified by flow cytometry	71
Figure 4.2	Circulating CD1a-autoreactive T cells in healthy and psoriasis individuals using K562 as antigen-presenting cells	74
Figure 4.3	Circulating CD1a-autoreactive T cells in healthy and psoriasis individuals using mDCs as antigen-presenting cells	77
Figure 4.4	Circulating CD1a-autoreactive T cells produce IL-17A and IL-22	79
Figure 4.5	CD1a-reactivity of T cells in response to arachidonic acid	81
Figure 4.6	CD1a-reactivity of T cells in response to lipids relevant to lipoxygenase pathways	84
Figure 4.7	CD1a-reactivity of T cells in response to prostaglandins	85
Figure 4.8	CD1a-reactivity of T cells in response to lysophospholipids and fatty acids	87
Figure 5.1	Gene Expression Omnibus shows elevated *PLA2G4D* gene expression in psoriatic skin	107
Figure 5.2	PLA_2- and psoriasis-relevant gene expression results in GEO DataSet GDS2518	108
Figure 5.3	*PLA2G4B* gene expression results in GEO Series	110

GSE13355
Figure 5.4 PLA_2- and psoriasis-relevant gene expression results in GEO DataSet GDS3539 111
Figure 5.5 *PLA2G4B* gene expression results in GEO Data Series GSE30999 115
Figure 5.6 Mast cells are a source of PLA2G4D in psoriatic lesions 117
Figure 5.7 PLA2G4D protein expression in LAD2 mast cell-like line and cord blood-derived mast cells 119
Figure 5.8 Column purification of PLA2G4D protein from LAD2 cell lysate 121
Figure 5.9 LAD2-derived PLA2G4D protein activates CD1a-restricted T cells in blood of psoriasis patients 126
Figure 5.10 Dose-dependent PLA2G4D-responsive CD1a reactivity of T cells from healthy and psoriasis individuals 128
Figure 5.11 Reduction of PLA2G4D-responsive CD1a reactivity by a $cPLA_2$ inhibitor 130
Figure 5.12 PLA2G4D-responsive CD1a reactivity using autologous mDCs as antigen-presenting cells 132
Figure 5.13 IL-17A and IL-22 production by PLA2G4D-responsive CD1a-reactive T cells 136
Figure 5.14 PLA2G4D derived from cord blood-derived mast cells activates CD1a-reactive T cells 138
Figure 6.1 LAD2-derived exosomes activate T cells from psoriasis individuals 157
Figure 6.2 Cytosolic PLA_2 activity in LAD2 supernatant and exosomes 158
Figure 6.3 PLA2G4D protein expression in LAD2 exosomes 159
Figure 6.4 Uptake of LAD2 exosomes by K562 cells 161
Figure 6.5 Exosome-responsive CD1a reactivity of T cells from psoriasis patients 163
Figure 6.6 Dose-dependent exosome-responsive CD1a reactivity 166

	of T cells from psoriasis patients	
Figure 6.7	IL-17A and IL-22 production by exosome-responsive CD1a-reactive T cells	168
Figure 6.8	Exosome-responsive CD1a reactivity using autologous mDCs as presenting cells	170
Figure 6.9	Clathrin-mediated endocytosis is critical for exosome-responsive CD1a reactivity of T cells	173
Figure 6.10	IFN-α potentiates exosome-responsive CD1a reactivity	176
Figure 6.11	Skin-resident CD1a-autoreactive and exosome-responsive CD1a-reactive T cells in psoriatic lesions	182
Figure 7	A proposed model for CD1a-mediated pathogenesis of psoriasis	203

List of Tables

Table 1	Gene Expression Omnibus DataSets and Series relevant to psoriasis studies in humans	103
Table 2	Five studies reporting the presence of PLA_2 lipids in extracellular vesicles from immune cells and fluids	150
Table 3	Four studies reporting the presence of mRNA and protein of cytosolic PLA_2 in extracellular vesicles	152

Abbreviations

12-HETE	12-*L*-hydroxy-5Z,8Z,10E,13Z-eicosatetraenoic acid
13-HpODE	13-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid
5-HpETE	5-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
15-HpETE	15-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid
AD	Atopic dermatitis
ALOX5	Arachidonate 5-lipoxygenase
ALOX12	Arachidonate 12-lipoxygenase
ALOX15	Arachidonate 15-lipoxygenase
ATK	Arachidonoyl trifluromethyl ketone
β2m	β-2 microglobulin
BCA	Bicinchoninic acid assay
BDCA	Blood dendritic cell antigen
BSA	Bovine serum albumin
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDSN	Corneodesmosin
CLA	Cutaneous leukocyte antigen
CBMC	Cord blood-derived mast cell
CML	Chronic myelogenous leukaemia
CNV	Copy number variation
$cPLA_2$	Cytosolic phospholipase A_2
CTLA	Cytotoxic T-lymphocyte-associated protein
CXCL	CXC chemokine ligand
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DTNB	5,5’dithiobis(2-nitrobenzoic acid)

EDC	Epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EpCAM	Epithelial-cell adhesion molecule
EPCR	Endothelial cell protein C receptor
FA	Fatty acid
FasL	Fas ligand
FITC	
FLG	Filaggrin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HDM	House dust mite
HFE	Hemochromatosis protein
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILT7	Immunoglobulin-like transcript 7
iNKT	Invariant natural killer T cell
iNOS	Inducible nitric oxide synthase
IVL	Involucrin
K562-CD1a	CD1a-transfected K562 cell
K562-EV	Empty vector-transfected K562 cell
LCE	Late cornified envelope
LFA	Lymphocyte function-associated antigen
LOR	Loricrin

LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LT	Leukotriene
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
mDC	Monocyte-derived dendritic cell
MHC	Major histocompatibility complex
miRNA	Micro RNA
mRNA	Messenger RNA
MVB	Multivesicular body
NK	Natural killer cell
NKT	Natural killer T cell
NSAID	Non-steroidal anti-inflammatory drug
PA	Phosphatidic acid
PAF-AH	Platelet-activating factor acetylhydrolase
PASI	Psoriasis Activity and Severity Index
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PG	Prostaglandin
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLA2R1	Phospholipase A_2 receptor 1
PLAA	Phospholipase A_2 activating protein
PMA	Phorbol myristate acetate
PS	Phosphatidylserine
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RORγt	Retinoic acid receptor-related orphan receptor-γt

SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SPH	Sphingosine
$sPLA_2$	Secretory phospholipase A_2
SPRR	Small proline-rich protein
STAT3	Signal transducer and activator of transcription 3
Tc	Cytotoxic T
Tc17	Cytotoxic T 17
Tc22	Cytotoxic T 22
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
Temra	Effector memory RA T cell
TGF	Transforming growth factor
Th	T-helper
Th1	T-helper 1
Th2	T-helper 2
Th17	T-helper 17
Th22	T-helper 22
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Treg	Regulatory T cell
Trm	Resident memory T cell
tRNA	Transfer RNA
Tscm	Stem memory T cell
TSLP	Thymic stromal lymphopoietin

UVA	Ultraviolet A
UVB	Ultraviolet B
VEGF	Vascular endothelial growth factor

1. General Introduction

1.1 Immunity of the skin

1.1.1 The skin

Belonging to the integumentary system, the skin is comprised of three layers: epidermis, dermis, and hypodermis. Epidermis is the outermost region of the skin made up of several layers, including (from innermost to outermost) stratum basale (basal layer), stratum spinosum, stratum granulosum (granular layer), stratum lucidum, and stratum corneum (cornified layer) (the latter four layers form the suprabasal region). Keratinocytes at the basal layer proliferate and then move outwards, and differentiate as they approach the upper layers, and finally become anucleated corneocytes when they reach stratum corneum. Apart from keratinocytes, a population of dendritic antigen-presenting cells, namely Langerhans cells, is also resident in the epidermis. Other cells in the epidermis include melanocytes, and sensory receptors, and some resident immune cells, as discussed later. Underneath the epidermis is the dermis, which is composed of collagenous connective tissue, blood vessels, sweat glands, sebaceous glands, hair follicles, nerves and other cells including immune cells. The third layer, hypodermis, contains fat-rich

adipose tissue. Apart from host-derived cells of the skin, a number of commensal microbes are resident on the outermost layer of the skin. These microbes include *Actinobacteria*, *Firmicutes*, and *Proteobacteria* [1] [2].

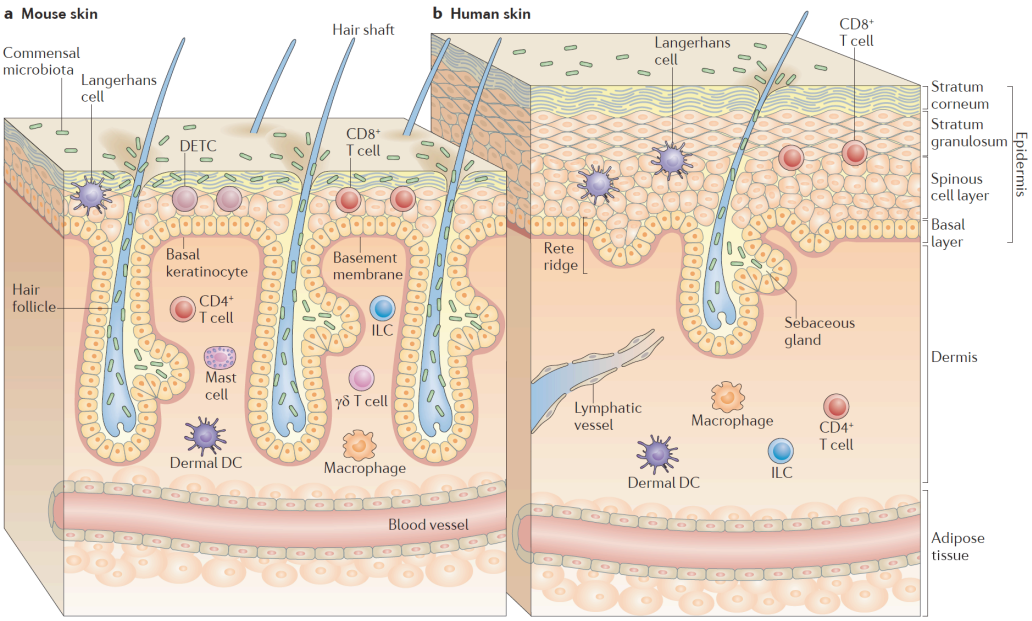


Figure 1.1 Structure and cellular components of skin in mice and humans [3]

1.1.2 Keratinocytes

The skin possesses various biological mechanisms to deal with foreign pathogens should they penetrate the physical barrier of the skin. Keratinocytes play a major role for rapid innate immune responses. Upon pathogen challenge, the keratinocytes at the granular layer can produce

antimicrobial peptides including cathelicidin, β-defensins, lipocalin-2, psoriasin, calgranulin A, and calgranulin 2 [4, 5] and cytokines such as interleukin (IL)-1, IL-10, IL-20 and tumour necrosis factors (TNFs). Acidic breakdown products of filaggrin, which is derived from keratinocytes, can also inhibit microbial growth [6, 7]. Endogenous RNA released from necrotic cells can activate keratinocytes to produce cytokines via Toll-like receptor 3 (TLR3) pathway [8, 9]. These mechanisms are important for innate immune defence against bacterial infection and chemicals from the environment. However, the cutaneous immunity does not only rely on keratinocytes, but also some immune cells as listed below.

1.1.3 Langerhans cells

Langerhans cells, named after Paul Langerhans, a German physician and anatomist who mistakenly recognized them as part of the nervous system [10], are mononuclear phagocytic antigen-presenting cells derived from the bone marrow [11], and they are the predominant haematopoietic cells found in the epidermis in humans [12]. Birbeck granules, named after their discoverer Michael Birbeck, are tennis-racket-shaped intracellular granules uniquely found in Langerhans cells [13, 14]. A Langerhans cells-specific type II C-type lectin receptor, namely langerin or CD207, binds to glycosylated ligands and has been thought to be associated with cell adhesion, endocytosis, and dendritic cell activation

and antigen handling [12]. Langerin is trafficked from cell surface to Birbeck granules upon binding of anti-langerin monoclonal antibody, suggesting that formation of Birbeck granules is a result of the capture of antigens by langerin [15]. The expression of epithelial-cell adhesion molecule (EpCAM) allows the anchoring of Langerhans cells to neighbouring keratinocytes [16]. Also, they express E-cadherin which allows them to interact with $CD103^+$ dendritic cells and T cells through integrin $\alpha^E\beta_7$ [17]. They also express CD11b, CD11c, and high level of class II major histocompatibility complex (MHC) [12]. In particular, human, but not mouse, Langerhans cells express CD1a, an MHC-like antigen-presenting molecules belonging to the CD1 family [18]. A brief review of CD1a and CD1 family will be discussed in the next section. Interestingly, co-expression of CD1a and langerin was found in Birbeck granules [19, 20], however their relationship has not yet been well elucidated.

In the quiescent skin, Langerhans cells form a network of immune cells among the keratinocytes, and repopulate locally and are independent of circulatory progenitor cells [21] with a half-life from about 53 to 84 days [22]. Repopulation of these cells after depletion by ultraviolet radiation, which only damaged epidermis but not dermis, suggests that they can be regenerated from progenitor cells from hair follicles [23]. Transforming grow factor β1 (TGF-β1), secreted by keratinocytes, as well as Langerhans cells themselves in an autocrine manner, is crucial to the

differentiation and survival of these cells [16]. In the steady state, they carry antigens and migrate to draining lymph nodes through dermal lymphatic vessels and reach the T cell area of the skin-draining lymph nodes for presentation of antigens to T cells [12, 24-27].

During inflammation, the migration rate of Langerhans cells increases [28]. Migratory Langerhans cells up-regulate MHC II molecules and CD40, which is a co-stimulatory molecule [29-31]. Concurrently, the expression of CC-chemokine receptor 7 (CCR7) decreases, while EpCAM expression remains [32, 33]. Alterations of normal function, mobilization for example, of Langerhans cells have been related to several diseases, such as Langerhans cell histiocytosis and psoriasis [20, 34-36].

1.1.4 Dermal dendritic cells

Dermal myeloid dendritic cells (DCs) are derived from dermal-resident or circulating haematopoietic stem cells (HSCs), monocytes, and other dendritic cell precursor cells [33, 37-39]. In the steady state, the recruitment of dermal dendritic cells from the circulation requires CCR2, E-selectin, and P-selectin [12]. Dermal DCs are generally identified as $CD11c^+$ myeloid cells found in the dermis [40]. The staining of a panel of blood dendritic cell antigens (BDCA) demonstrated different subsets of dermal DCs [41]. In healthy skin, the major subset was BDCA-1 (CD1c)-

positive, while the minor was BDCA-3 (CD141)-positive, and both subsets were identified as originating in part of circulating DCs [42]. In particular, BDCA-3$^+$ DCs were able to cross-present endogenous antigens to conventional CD4$^+$ T cells by MHC II molecules [43, 44]. BDCA-1$^+$ dendritic cells were immature dendritic cells in steady state, but could be matured by cytokines including interleukin (IL)-1β (IL-1β), IL-6, prostaglandin E_2 (PGE_2), and tumour necrosis factor (TNF) [45]. Apart from BDCA, different subsets of DCs can also be identified according to other markers such as CD1a and CD14. CD1a$^+$ DCs also express BDCA-1 [46]. However, CD1a is not a specific marker for dermal myeloid DCs, as CD1a$^+$ migratory Langerhans cells can also be found in the dermis. Dermal DCs also contain langerin$^+$ and langerin$^-$ subsets, suggesting that they might have different functions [12]. In particular, DCs in psoriatic skin are able to produce TNF, inducible nitric oxide synthase (iNOS), IL-20, and IL-23 [47]. On the other hand, the BDCA-3$^+$ DCs are able to produce IL-10 and hence are thought to contribute to suppression of skin inflammation under some circumstances [48].

1.1.5 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDCs) are CD4$^+$CD45RA$^+$IL-3Rα$^+$ILT3$^+$ILT1$^+$CD11c$^-$ immune cells especially important against viral infections in the skin due to their ability to produce large amounts of type I

IFNs (IFN-α and IFN-β) to confer antiviral immunity [49-51]. They are mainly found in the peripheral blood, bone marrow, thymus, and secondary lymphoid organs [52, 53]. Only the pDCs in bone marrow and peripheral blood express BDCA-2 and BDCA-4 [54]. Although they were found in very low amounts in quiescent skin, they are rapidly recruited to the skin, via a CCR6-dependent mechanism, during the inflammation of the skin and especially in the skin of psoriasis [55, 56].

pDCs can sense the presence of viral pathogens through Toll-like receptors (TLR). TLR9 on the surface of the pDCs can bind to bacterial DNA, as well as self DNA that are rich in CpG [57], while TL7 can bind to RNA derived from viruses [58-60]. The TLR-ligand binding activates a MyD88-dependent signalling pathway and leads to the secretion of type I interferons by pDCs [61]. Type I interferons can act on a number of immune cells. For instance, type I interferons can activate natural killer (NK) cell-mediated cytotoxicity [62]. They can also promote differentiation, maturation and immunostumulatory functions of myeloid DCs [63]. In addition, these interferons can induce B cells to differentiate to plasma cells for the production of antibodies [64, 65]. Moreover, pDCs can present antigens to T cells, although inefficiently due to low expression of MHC molecules and inefficient endocytosis of antigens [49, 66, 67]. However, the production of type I interferons can inhibit the production of IL-12 from myeloid DCs [68]. Considering the diverse effects of pDCs on different

immune cells, pDCs serve as an important link between the innate and adaptive immune responses. Recent studies have suggested that pDCs are important for the initiation of psoriasis. For example, the complex of antimicrobial peptide LL-37 and self DNA can bind to TLR9 of pDCs and lead to the production of IFN-α by pDCs in the skin of psoriasis [56, 69, 70].

The pDC responses can be regulated through various pathways. For example, the type I interferon production by pDCs via TLR7 or TLR9 pathways can be inhibited by immunoglobulin-like transcript 7 (ILT7) [71]. The production of IFN-α by pDCs can also be inhibited by cross-linking of NKp44, as well as IgG binding to FcγRIIA [72-74]. All the pDC regulations mentioned above are related to ITAM signalling pathway, which eventually leads to the inhibition of production of type I interferons [70].

1.1.6 Mast cells

Mast cells are bone marrow progenitor derived cells found rarely in the blood but mainly in the tissues, such as skin and mucosal sites, where they mature under the influence of local growth factors including stem cell factor (SCF). A distinguishable histological feature of mast cells is their large lysosome-like secretory granules in the cytoplasm, which were initially thought to be responsible for nourishing neighbouring cells. Hence

mast cells were named as such by their discoverer Paul Ehrlich due to the meaning of “mast” in German as “fattening” [75]. Mast cells and mast cell granules play a major role in IgE-mediated allergic reactions. Although IgE antibodies have a very short lifetime in circulation (around 2 days), they can bind to FcεRI, the high affinity Fc receptor for IgE highly expressed by mast cells. Upon antigen challenge and cross-linking of IgE antibodies on mast cell surfaces, mast cells are then activated and release the granular content to extracellular environment via degranulation, and hence initiate a series of physiological events [76-80].

Mast cell granules contain a wide range of preformed mediators, including biogenic amines (e.g. histamine, serotonin, dopamine), lysosomal enzymes (e.g. cathepsin, β-hexosaminidase), cytokines (TNF, IL-4, IL-5, IL-6, IL-15, VEGF, TGFβ, SCF), mast cell-specific enzymes (e.g. tryptase, chymase), non-mast-cell-specific enzymes (e.g. renin, caspase 3, granzyme B), membrane-associated proteins (e.g. CD63, MHC II), and other proteins such as LL37 [80]. The biogenesis of secretory granules is initiated at the trans-Golgi network, where granular proteins are synthesized and sorted into the pro-granules (i.e. immature granules). After the pro-granules leave the Golgi, more substances are transported and sorted into the granules until the granules mature. During degranulation, adjacent granules fuse together to form a degranulation channel, followed by the fusion of granular membrane and plasma

membrane, resulting in the release of granular content. Apart from IgE cross-linking, mast cells can also be activated by IgG via FcγRI, complement proteins C3a and C5a via C3Ra and C5aR, neuropeptides, and TLR pathway [76, 80].

In addition to releasing preformed mediators, activated mast cells also newly synthesize lipid mediators including prostaglandins (PG) and leukotrienes (LT), cytokines and chemokines. In particular, upon activation by SCF via c-KIT (a receptor for SCF) or FcεRI, mast cells rapidly synthesise *de novo* eicosanoid mediators, such as PGD_2 via the COX pathway, and LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4 via the 5-lipoxygenase pathway. These eicosanoids, together with histamine, tryptase, and proteases, contribute to the pathophysiology of allergic inflammation [76, 81]. Mast cells can also be activated by secretory phospholipase A_2 ($sPLA_2$) to produce PGs [82], as well as by PLA_2 derived from bee venom to produce IL-4 [83]. Cytokines such as IL-1, IL-3, IL-33, and thymic stromal lymphopoietin (TSLP) can also activate mast cells [84-87]. Mast cells are also associated with other diseases such as rheumatoid arthritis, bullous pemphigoid, and encephalomyelitis owing to the wide range of mediators they can produce. Under certain conditions, mast cells produce IL-6, IL-21, IL-23, and TNF, which drive Th17 differentiation [88].

1.1.7 T cells

T cells play a central role in the cell-mediated immunity in the human body. While there are a large amount of T cells in the circulation, the skin contains almost twice as many in comparison [89]. They express CD3 and T cell receptors (TCRs), of which about 95% of them are αβ and about 5% of them are γδ. Both β and δ chains contain variable (V), diversity (D), joining (J), constant (C) regions, while α and γ chains only contain V, J and C regions, lacking D region. TCRs are associated with CD3 molecule to form the TCR-CD3 complex, and the majority are thought to recognise peptide antigens presented by MHC molecules.

Conventionally, T cells are categorised into different types according to their expression of surface markers and cellular functions. The functions of $CD4^+$ T helper (Th) include assisting B cell maturation to become plasma cell and memory B cells, as well as activating cytotoxic T (Tc) cells and macrophages. Upon activation by MHC II-peptide antigen presentation by antigen-presenting cells, conventional Th cells can rapidly proliferate and differentiate into different subsets of Th cells, including Th1, Th2, Th17, and Th22 subsets. Th1 cells produce cytokines such as IFN-γ, IL-12, and TNF-α, while Th2 cells produce IL-4, IL-5, and IL-13. The Th1 differentiation of naive T cells is mutually exclusive to the Th2 differentiation. For instance, IL-4 promotes the Th2 differentiation and inhibits Th1 differentiation, while IL-12 and IFN-γ do the opposite. Th17

cells are able to produce IL-17, and the differentiation of Th17 cells is driven by the Th17-polarising cytokine IL-23 [90, 91]. Th22 cells are IL-22-producing T cells, which differentiation is driven by TGF-β and IL-6 [92]. IL-22 released by these T cells can promote the production of chemokines CXCL9, CXCL10 and CXCL11, and cytokines IL-7, IL-15, and IL-32 by keratinocytes [93]. In particular, IL-22 can also stimulate the proliferation, yet inhibit the differentiation, of keratinocytes, and hence Th22 responses are thought to play a major role in the pathogenesis of psoriasis [94].

On the other hand, Tc cells are $CD8^+$ T cells mainly for cell-mediated cytotoxicity, and the majority can be activated by MHC I-peptide antigen presentation by antigen-presenting cells. Memory T cells are a subset T cells that possess long-term immunological “memory”, allowing them to mount rapid immunological responses upon secondary antigen challenges. They are subdivided into stem memory T (Tscm) cells ($CD45RA^+$, $CCR7^+$), central memory T (Tcm) cells ($CD45RA^-$, $CCR7^+$), effector memory T (Tem) cells ($CD45RA^-$, $CCR7^-$), effector memory RA T (Temra) cells ($CD45RA^+$, $CCR7^-$), and resident memory T (Trm) cells ($CD69^+$). In particular, Trm can enter the skin during effector phase of immune responses in response to chemokine CXCL9 and CXCL10, and a substantial amount of Trm cells remain after the clearance of antigens [95]. Regulatory T (Treg) cells are important for the homeostasis of immune responses. They are responsible for suppressing immunity in

order to restore the steady state, as well as supressing autoreactive T cells. Skin-resident Foxp3$^+$ Treg cells can be induced by IL-10 produced by resident BDCA3$^+$ DCs for the suppression of inflammation in the skin [48].

1.1.8 Innate and adaptive immunity in skin

In the quiescent state of the skin, Langerhans cells in the epidermis are in part responsible for the immunosurveillance; they constantly sense and capture antigens from the external environment before the antigens can penetrate to deeper skin. Keratinocytes produce antimicrobial peptides constitutively to provide an innate immunity, and can enhance expression on activation. Different subsets of DCs are located in the dermis. While some subsets can produce a range of pro-inflammatory cytokines, others produce regulatory cytokines such as IL-10. pDCs are usually absent in the quiescent skin as they are only recruited to the skin upon chemokine attraction. Skin-resident macrophages, like Langerhans cells, can also constantly survey the skin for the detection of antigens. Mast cells in the dermis contain numerous preformed pro-inflammatory molecules in their granules, which are ready to be released to elicit a rapid response. Innate lymphoid cells (ILCs) are also found in the skin, and with ILC3 being the major population of ILCs in the normal skin [3]. Mast cell functions can be suppressed by IL-13 produced by ILC2 in the dermis [96].

Also, a large population of non-circulating $CD45RO^+$ $CCR4^+$ effector memory T cells (tissue resident T cells) that express cutaneous lymphocyte antigen (CLA), which allows T cells to bind to E-selectin expressed on cutaneous blood vessels [97]. In addition, keratinocytes constitutively release CCL27, which attracts skin-homing $CCR10^+$ T cells to the quiescent skin for immune surveillance [98, 99].

When the physical barrier of the skin is breached due to injury, the deeper skin may be exposed to microbes, such as bacteria, fungi, and viruses. Keratinocytes, mast cells and pDCs, are important immune cells conferring the innate immunity in the skin. Increased amounts of β-defensins and cathelicidins, the two major antimicrobial peptides in the skin, are produced by keratinocytes, as well as macrophages, mast cells, and infiltrating neutrophils [100]. These antimicrobial peptides target the cell wall or cell membrane of the microbes and promote their lysis [101-103]. Mast cell degranulation can be triggered by the presence of exotoxins, such as δ-toxin from *Staphylococcus aureus* [100, 104]. Activated keratinocytes also produce different cytokines, such as TNF and IFN-γ triggered by infections. On the other hand, pDCs can recognise the complex of foreign DNA and cathelicidin LL-37 (LL-37/DNA) via TLR9 and produce type I interferons, which reduce the ability of virus to infect host cells [105-107]. Altogether these immune cells provide rapid innate immune responses in skin inflammation.

Adaptive immune responses occur in the later phase of skin inflammation, and provide long-term immune protection against foreign pathogens. Some components in the innate response can facilitate the adaptive response by recruiting different types of immune cells to the site of inflammation. For example, IFN-γ can induce the release of CXCL10 and CXCL11 by keratinocytes, leading to the recruitment of Th1 cells [108]. TNF can induce the production of CCL20 by keratinocytes to attract $CD11c^+$ dendritic cells and $CCR6^+$ IL-17-producing $CD4^+$ and $CD8^+$ T cells [109]. TNF can also induce the production of CXCL1, CXCL2, and CXCL8 by keratinocytes to recruit neutrophils to the site of inflammation [45]. On the other hand, type I interferons secreted by pDCs can stimulate the maturation of Th17 cells [100]. Activated Langerhans cells can induce IL-22-producing $CD4^+$ T cells, of which the IL-22 can induce the increase in proliferation of keratinocytes [110]. Accelerated turnover of keratinocytes can, therefore, help eliminate the pathogens by physical removal. The production of IL-12 and TNF by the activated DCs in the dermis can promote the differentiation of Th1 cells for Th1 response in the skin, while the production of IL-23 by the DCs can promote Th17 response. IL-17 produced by the Th17 cells can induce the production of antimicrobial peptides by keratinocytes [111].

Although numerous mechanisms are activated during inflammation, the reaction is constantly under tight regulation in order to restore the steady state of the skin for homeostasis. For instance, skin-resident $CD141^+$ dendritic cells can produce IL-10, which activate Treg cells to suppress inflammation [48]. Also, IL-2 produced by activated mast cells in the dermis can promote regulatory functions by the Treg cells. Treg cells express $Foxp3^+$, and different subsets of $Foxp3^+$ Treg cells can suppress different types of immune responses. For instance, $Tbet^+Foxp3^+$ Treg cells can regulate Th1 response, while $GATA3^+IRF4^+$Foxp3+ Treg cells can suppress Th2 response [112]. In particular, Treg cells residing in the skin express CD45RO, therefore they are referred as effector-memory regulatory T cells, and might play a potential role in inflammatory diseases including psoriasis [113, 114].

Given the complex interplay among the immune cells in the skin, dysregulation of any participant in the cutaneous immunity can give rise to an ill-controlled state of inflammation. Psoriasis is though to be a lymphoid cell-mediated chronic inflammatory skin disorder, and, as discussed in the next section, is mainly driven by complex feedback loops contributed by T cells, innate lymphoid cells, antigen-presenting cells, keratinocytes, neutrophils, and other cells. Over the decades, various studies have attempted to explain the pathogenesis of the disease, in particular there has been an intense search for the antigen sources. This thesis proposes

an alternative mechanism and reveals sources of antigens for T cell responses in psoriasis, based on our experiments and analyses. However, before a detailed discussion can begin, we have to first understand the clinical features, the current explanations of pathogenesis, and the remaining questions about psoriasis with relevance to treatment.

1.2 Psoriasis

1.2.1 Overview of psoriasis

Psoriasis is a chronic inflammatory skin disorder affecting about 1.5% of the population in the United Kingdom, European and North American population, and about 2-4% of the worldwide population [115-119]. While both sexes have approximately equal prevalence of the disease, severe forms are more frequent in men than women [120]. In addition, sun exposure, ethnicity, and latitude also attribute to the prevalence [121].

Psoriasis vulgaris, being the most common type accounting for about 90% of cases, can be identified as red plaques with well-defined borders and shiny silvery or white and dry scales. Plaques of psoriasis can vary in sizes and locations on the body, and usually affect areas of elbows, knees, scalp, and lumbosacral regions [122]. Other types of psoriasis include guttate, inverse, erythrodermic, and pustular psoriasis [45, 123]. Symptoms include pain, pruritus, burning sensations, and bleeding [122, 124-126]. The Psoriasis Activity and Severity Index (PASI) is used to rank the severity of psoriasis, by measuring the area and intensity of erythema, induration, and desquamation of plaques.

Microbes in the skin were thought to be one of the triggers of psoriasis. Similar to the healthy skin, the skin microbiota of psoriasis skin consists of the three main phyla, *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. However, there are major differences in the frequency of these microbes in the psoriasis compared to healthy skin, according to several microbiome studies [1, 127]. In general, higher frequency of *Proteobacteria* and low frequency of *Actinobacteria* were found in psoriasis skin. At the genus level, *Staphylococcus* (belonging to *Firmicutes*) and *Propionibacteria* (belonging to *Actinobacteria*) were present in lower frequencies in psoriasis skin. The relative frequency of *Streptococcus* (belonging to *Firmicutes*) to *Propionibacteria* was found higher in psoriasis [1, 127].

Psoriasis can also be induced by non-specific triggers such as scratching, piercings, and tattoos; or sunburn, chemicals, or psoriasis-exacerbating drugs including beta-blockers, lithium, anti-malarials, and non-steroidal anti-inflammatory drugs (NSAIDs) [128]. The association of beta-blockers and lithium with psoriasis is thought to be relevant to the decrease of intracellular levels of calcium, leading to increased cellular proliferation and decreased differentiation [129]. Inhibition of transglutaminase by anti-malarials are thought to influence cellular proliferation [129]. Inhibition of cyclooxygenase pathway by NSAIDs can

lead to accumulation of leukotrienes, which are thought to aggravate psoriasis [129]. HIV infection may also be a trigger of psoriasis [129]. Comorbidity of psoriasis includes psoriatic arthritis, metabolic syndrome, cardiovascular diseases, inflammatory bowel disease and psychological conditions, such as anxiety and depression [130].

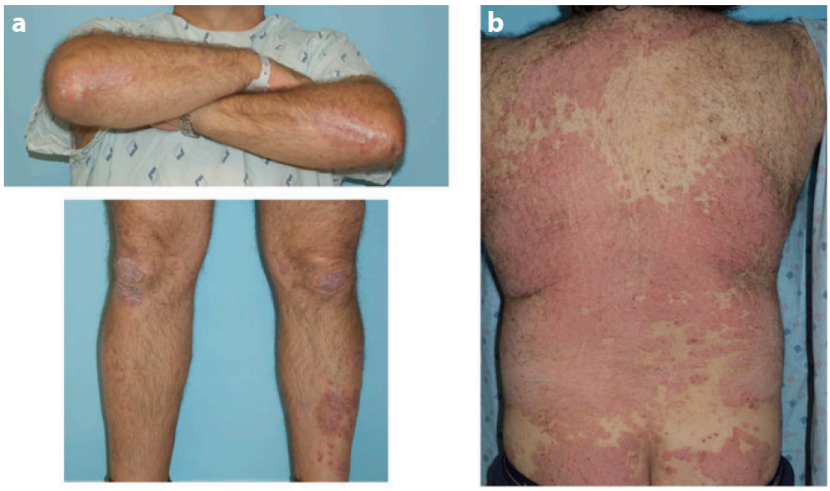


Figure 1.2 Clinical features of chronic psoriasis vulgaris [45]

1.2.2 Histological features of psoriatic lesions

In contrast with normal healthy skin and non-lesional skin of psoriasis, psoriatic lesions have several significant clinical histological features. One major feature is acanthosis, which is the thickening of epidermis, due to the accelerated proliferation of keratinocytes in response to, for example, IL-22. Keratinocytes in psoriatic lesions present a few

features, including hyperkeratosis, which is the abnormal differentiation of keratinocytes, resulting in thickened stratum corneum and the loss of normal granular layer; and parakeratosis, the retention of nuclei in the upper layers and stratum corneum [122, 131]. Apart from abnormalities of keratinocytes, there are dilated and contorted blood vessels in the psoriatic dermis thought to be due to elevated expression of vascular endothelial growth factor (VEGF) in psoriatic lesions [132]; and elongation of rete ridges and the presence of micropustules of Kogoj and microabscesses of Munro [122, 133]. Increased mast cell density in the dermis was reported in the lesional skin of psoriasis compared to normal skin [134]. Increased T cell density in both epidermis and dermis of psoriatic lesions, compared to healthy skin, were also reported, and a higher CD8:CD4 ratio was found in the psoriatic epidermis compared to the psoriatic dermis [135].

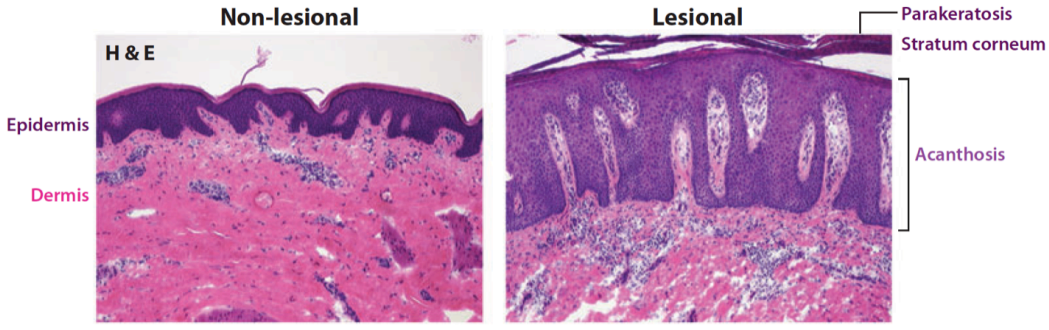


Figure 1.3 Histological features of psoriasis [45]

1.2.3 Pathogenesis of psoriasis

As a complex inflammatory disorder, psoriasis is a consequence of crosstalk between innate and adaptive immunity, and various cell types are involved in the pathogenesis of the disease. In psoriatic lesions, infiltration of $CD4^+$ and $CD8^+$ T cells, neutrophils, natural killer (NK) cells, natural killer T (NKT) cells, mast cells, macrophages and innate lymphoid cells are usually found [130, 136-143]. $CD45RO^+$ memory T cells are also present in psoriatic lesions [144].

The early phase of psoriasis can be triggered by the skin microbiota. Changes in the skin microbiota of the psoriasis skin, compared to the healthy skin, can lead to an imbalanced symbiotic relationship between the host and the foreign microbes, and hence eliciting the innate immune responses of the host. Upon physical injury and hence a breach in the skin barrier, antimicrobial peptides, including β-defensins and cathelicidins, are produced by keratinocytes for the clearance of pathogens. In particular, LL-37, a cathelicidin produced by keratinocytes, can bind to nucleic acids released from necrotic cells due to physical injury or infections. The complexes of LL-37 and nucleic acids can activate a number of cells in the following ways: LL-37/DNA complexes can bind to TLR9 on the surface of pDCs, resulting in IFN-α production [56, 69]. LL-37/RNA can activate pDCs and myeloid DCs through TLR7 and TLR8

pathways respectively [145]. In addition, imiquimod can activate pDCs via TLR7 pathway [146]. IFN-α produced by pDCs can then activate myeloid DCs to release IL-12 and IL-23 [45]. While IL-12 stimulates the differentiation of naïve $CD4^+$ T cells to Th1 cells that produce IFN-γ and TNF-α, IL-23 promotes Th17 differentiation and hence leading to the production of IL-17A, IL-17F, and IL-17A/F [147]. Also, TNF-α and IFN-α can activate Th17 cells and group 3 innate lymphoid cells (ILC3s) to produce IL-17 and IL-22, both of which stimulate the proliferation of keratinocytes, as well as the expression of antimicrobial peptides and chemokines such as CCL20, CXCL8 (IL-8), and CXCL1 to attract neutrophils and lymphocytes [148-150]. To summarise, the early phase of psoriasis is initiated by the innate immune responses against foreign pathogens, and the cytokines produced during this phase can bring about the adaptive immune responses in the chronic phase of psoriasis.

Adaptive immune response of psoriasis was initially regarded as being dominated by Th1 responses due to high expression of Th1 cytokines, including IFN-γ, IL-1, and IL-12 in psoriatic lesions [151]. This was consistent with the relatively lower expression of Th2 cytokines such as IL-4 [152, 153]. However the discovery of increased number of an IL-17-secreting Th17 subset and the elevated level of Th17-polarizing cytokine IL-23 in psoriatic lesions suggested the central role of Th17 response in psoriasis, hence leading to a paradigm shift from Th1 to Th17

responses in the contribution to the pathogenesis of the disease [94, 154-156]. Th17 differentiation is stimulated by IL-23, and regulated by the transcription factors signal transducer and activator of transcription 3 (STAT3) and retinoic acid receptor-related orphan receptor-γt (RORγt). IL-17 cytokine family consists of six members: IL-17A (CTLA-8), IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. Different IL-17 members are responsible for various physiological functions. For example, IL-17A and IL-17F are involved in the initiation of inflammation and autoimmunity, while IL-17E is thought to promote Th2 responses, yet IL-17B, IL-17C and IL-17D functions are less defined [157]. Among different isoforms of IL-17, IL-17A, IL-17C, and IL-17F, but not IL-17B and IL-17D, were found in higher levels in lesional skin of psoriasis compared to non-lesional skin [158]. Apart from IL-17, Th17 cells can also produce IL-21, IL-22, and GM-CSF [159].

In addition to Th17 cells, Th22 cells are also though to be involved in the pathogenesis of psoriasis, as elevated IL-22 level was found in psoriatic lesions, compared to normal skin, as well as in circulation [149, 160, 161]. Increased number of circulating Th17 and Th22 cells in psoriasis patients was also reported [94]. Although Th17 cells can also produce some Th22 cytokines such as IL-22, Th22 cells are characterised by the secretion of IL-22 and TNF-α, but not IFN-γ, IL-4, or IL-17 [93]. Langerhans cells can effectively induce the differentiation of Th22 cells

from naïve $CD4^+$ T cells, as well as the expansion of memory Th22/Tc22 cells [110]. Th22 differentiation is mainly driven by cytokines TNF-α and IL-6 [162].

The chronic phase of psoriasis is sustained in different ways. TNF-α, produced by keratinocytes, macrophages, lymphocytes, and endothelial cells, plays a major role in the chronic phase of psoriasis by inducing secondary mediators and adhesion molecules [122, 163], and the activity of TNF-α is substantially increased in psoriatic lesions [164]. Infiltration of leukocytes, including neutrophils, DCs, and T cells, occurs in response to chemokines secreted by proliferating and activating keratinocytes. Activated neutrophils can release TNF-α, IL-1, and IL-6. The recruitment of T cells, particularly Th17 and Th22 cells, leads to the increased production of the hallmark cytokines IFN-γ, TNF-α, IL-17 and IL-22, and hence contributing to the amplification of Th17 and Th22 responses in the psoriatic lesions. Furthermore, TNF-α and IFN-α can strengthen the action of IL-22 on keratinocytes by stimulating their expression of IL-22 receptor [165, 166]. Apart from $CD4^+$ T cells, increased number of $CD8^+$ T cells was also found in the epidermis of psoriatic lesions, including IFN-γ-producing Tc1 cells, IL-17-producing Tc17 cells, and IL-22-producing Tc22 cells according to recent studies [147, 156]. Moreover, other immune cells such as mast cells, ILC3s, and neutrophils can also release IL-17 [140, 167].

Altogether, a positive feedback loop occurs between keratinocytes and activated immune cells. Cytokine responses from immune cells can lead to the induction of keratinocyte proliferation and production of chemokines and other cytokines, which lead to recruitment and activation of immune cells. Further evidence supporting a key role for immune cell derived cytokines comes from targeted therapeutic interventions as discussed below. To summarize, the pathogenesis of psoriasis is driven by, TNF-α pathway, IL-23/IL-17 pathway, and IL-22 pathway.

1.2.4 Psoriasis-associated genes

While physical injury to the skin, psychological stress, and drugs can trigger the onset of psoriasis, the initiation of the disease can be predisposed due to genetic factors. The inflammatory nature of psoriasis has led to the search for genetic causes to identify the risk factors, especially those related to innate and adaptive immunity.

Genome-wide association studies (GWAS) have been performed to seek potential genetic component and search for any susceptible loci in the human genome. A number of linkage analyses have identified at least nine loci related to psoriasis, termed *PSORS1* to *PSORS9*. To date more than 20 psoriasis susceptibility loci being have been identified by GWAS

and candidate gene approaches. *PSORS1*, harbouring HLA-Cw6 and corneodesmosin (*CDSN*), is the strongest and most susceptible locus for psoriasis [168-170]. Increased expression of early differentiation markers including CDSN, involucrin (*IVL*), small proline-rich proteins (*SPRR*s), crystatin A, and transglutaminase 1, as well as decreased expression of late differentiation markers including loricrin (*LOR*) and filaggrin (*FLG*) were found in psoriatic skin [171, 172]. The *PSORS4* locus is located in the epidermal differentiation complex (EDC) on chromosome 1q21, which includes *LOR*, *IVL*, *FLG*, *SPRR*s, and the late cornified envelope (LCE) genes. LCE genes consist of a cluster of 18 members, divided into 6 groups, namely *LCE1* to *LCE6*. An association of copy number variation (CNV) in the LCR common deletion in the *LCE3B* and *LCE3C* genes (LCE3C_LCE3B-del) was found in different ethnic backgrounds [173-177]. Other than psoriasis, LCE3C_LCE3B-del has also been found in psoriatic arthritis, rheumatoid arthritis, systemic lupus erythematosus, and allergic contact dermatitis, implying that the deletion might be a common risk factor for various inflammatory diseases [178-182].

1.2.5 Treatments of psoriasis

Conventional treatments of psoriasis include topical therapy such as glucocorticoids, vitamin D derivatives, or a combination, and phototherapy including narrow-band UVB, and photochemotherapy using

psoralen plus UVA. Systemic drugs are also used for the treatment of psoriasis, such as methotrexate, ciclosporin, acitretin, and fumaric acid esters [122].

Apart from the above treatment methods, biologics are also commonly used. The efficacy of these biologics has indicated the significant role of lymphoid cell-mediated response in psoriasis. Initially, targeting IL-2 receptor (IL-2R) by using a DAB389IL-2 agent (Denileukin diftitox) proved successful [183]. Later, the use of CTLA-4-Ig (Abatacept) to block B7 (CD80/CD86)-mediated costimulation was found to reduce the frequency of T cells in psoriatic lesions [184]. Large reduction in DC and T cell populations can be achieved with the use of Alefacept, an LFA-3-Ig fusion protein that blocks CD2 [185]. Efalizumab, a monoclonal antibody targeting CD11a subunit of LFA-1, inhibits the migration of T cells to peripheral tissues as well as the simulation of T cells [186]. Reduction of circulating effector memory T cells was observed in patients treated with Alefacept [187]. Targeting TNF pathway also proved successful. Biologics intervening TNF pathway include Inflixamab and Adalimumab, which are monoclonal antibody against TNF-α [188]; and Etanercept, a TNFR-Ig fusion protein [189].

Better understanding of the pathogenesis of psoriasis with time has stimulated the advancement of the treatments using biologics. During the

recent decade, biologics have been designed to target and intervene the IL-23/Th17 pathway. Notably, the use of Ustekinumab, an antagonist of IL-12/23p40 and also IL-23p19 have both shown clinical efficacy and hence supported the role of IL-17 response in psoriasis [190-192]. Other interventions included the use of Secukinumab, a monoclonal antibody against IL-17A [193, 194]; and Brodalumab, a monoclonal antibody against IL-17RA [195-197].

1.2.6 Langerhans cells and dermal dendritic cells in psoriasis

It has been widely accepted that immune cell-mediated responses are involved in the pathogenesis of psoriasis [122, 198, 199]. Expressing high levels of MHC II, Langerhans cells and dermal DCs are professional antigen-presenting cells which are resident in quiescent skin, and capture, process, and carry peptide antigens during inflammation, and migrate to skin-draining lymph nodes, and present antigens to T cells [12, 24, 28]. Being an early line of immunological defence in the skin, they play a significant role in immune surveillance and the initiation of peptide-specific T cell responses.

Over the recent decade, the association between the alteration of migration of Langerhans cells and psoriasis has been investigated. Cumberbatch *et al.* reported that although the frequency and morphology

of epidermal Langerhans cells in uninvolved skin of psoriasis patients remained normal, their mobilisation was impaired in response to stimuli which normally induce migration [200]. Such impairment of mobilization of Langerhans cells in psoriasis was also reported in elsewhere [35, 201]. Shaw *et al.* proposed that the impaired Langerhans cells migration might be due to epidermal microenvironment rather than abnormality of the cells themselves, as they demonstrated that TNF-α inhibitors, fumaric acid esters, and Ustekinumab, an IL-12/23p40 inhibitor, were able to restore the migration of Langerhans cells [202]. This was further confirmed by a recent report revealing that IL-23 pathway negatively regulated the mobilization of Langerhans cells in uninvolved skin of psoriasis patients [36]. All these reports have confirmed the involvement of Langerhans cells migration in psoriasis.

Besides the epidermal Langerhans cells, dermal DCs also play a crucial role in cutaneous immune system. Dermal DCs were found in large amounts in psoriatic lesions, immediately beneath the epidermis and surrounded by T cells [203]. Furthermore, Zaba *et al.* reported that Th1/Th17-polarizing myeloid dermal dendritic cell populations were present in psoriatic lesions, and they could induce T cells to produce IL-17 and IFN-γ [204]. Also Lowes *et al*. demonstrated the production of TNF-α and inducible nitric oxide synthase (iNOS) by the dendritic cells, and the

reduction of infiltration by these cells with the use of Efalizumab as a treatment of psoriasis [47].

1.2.7 In search of antigens of psoriasis

Given the crucial role of antigen-presenting cells, namely Langerhans cells and dermal dendritic cells, in the pathogenesis of psoriasis, investigators have sought the sources of antigens and identified a number of candidates. Bacterial antigen from the skin microbiome was thought to be one of the psoriasis-associated antigens. In the past decades, the association of antigens from Staphylococcus and Streptococcus and acute guttate psoriasis was proposed [205-212]. Apart from foreign antigens, several self-antigens were also proposed, including keratins, antimicrobial peptide LL-37, and melanocyte antigen [213-216]. However these studies have rarely been replicated by different groups and there remains the challenge of identifying the relevant targets of infiltrating T cells.

Besides peptide antigens, some indirect evidence suggested a potential role for non-peptide antigens for the pathogenesis of psoriasis. Langerhans cells and some dermal dendritic cells express CD1a, which belongs to CD1 family and allows the presentation of non-peptide antigens to T cells [217-221]. Furthermore, some CD1a-restricted T cells express skin-homing markers and were found in the dermis [162, 218, 222].

Therefore, we hypothesize that non-peptide antigens might play a role in T cell-mediated response of psoriasis via non-peptide antigen presentation by CD1a. While this topic will be discussed in greater details in Chapter 4, an overview of CD1a molecule and CD1 family will be introduced in the next section.

1.3 CD1a and CD1 family

1.3.1 Presentation of non-peptide antigens

The significant role of peptide antigens in adaptive immune response has been extensively studied over decades since the discovery of restriction of MHC complexes by Rolf Zinkernagel and Peter Doherty in the 1970s [223-228]; as well as the structure of complex between αβ T cell receptor (TCR), peptide, and MHC molecule [229]. Genes for classical MHC molecules (MHC I and II) are located within the MHC region on chromosome 6 in human. While MHC II is composed of an α and a β chain, MHC I is made up of a larger α chain which is associated with β_2-microglobulin (β_2m). Exhibiting high polymorphism, both MHC I and II can present peptides of varying lengths (although within certain range) and amino acid residues. Successful peptide binding to MHC molecules would depend on the proper length, size, shape, and electrostatic complementarity to satisfy the steric requirement, hydrogen bonding, and hydrophobicity of the antigen-binding groove of the MHC molecules [230, 231].

Apart from classical antigen-presenting molecules, there exist non-classical antigen-presenting molecules, including CD1 and MR1; and other

MHC I homologs such as the human hemochromatosis protein (HFE) and endothelial cell protein C receptor (EPCR), which is a CD1-like molecule [230]. The findings of the presentation of non-peptide antigens to restricted T cells by CD1 and MR1 molecules has opened up a new perspective on antigen presentation by non-classical antigen-presenting molecules, as well as antigen recognition and subsequent T cell responses [232, 233].

1.3.2 CD1 family

CD1 molecules were first described in the 1970s, although their functions were not well understood until most recent two decades [234]. CD1 family consists of five members, namely CD1a, CD1b, CD1c, CD1d, and CD1e. The first three molecules are grouped as Group 1 CD1 (expressed in human and mice), and CD1d represents Group 2 CD1 (expressed in human only) [235-237]. CD1e serves as a chaperone-like, rather than antigen-presenting, molecule to assist antigen processing [238-241]. CD1 genes are located on chromosome 1q22, and exhibit low level of allelic polymorphism compared to the highly polymorphic MHC I and II genes [230, 242]. CD1 molecules are homologous to MHC I but not MHC II, due to its composition of a heavy chain with three extracellular domains (α1, α2, and α3), and a non-covalently bound β_2m. Similar to MHC I, CD1 molecules possess an antigen-binding groove, although it is narrower and deeper than that of MHC I [243-245]. In addition, the inner

surface of CD1 antigen-binding groove is predominantly lined with non-polar amino acids, which facilitates the binding with of non-polar antigens through hydrophobic interactions. CD1 isoforms have different tissue distributions and intracellular trafficking patterns, suggesting their non-redundant roles in the immune system [246, 247]. While MHC I molecules contain antigen-binding pocket A to F, CD1 molecules contain only two antigen-binding pockets, namely A' and F' (Figure 1.4).

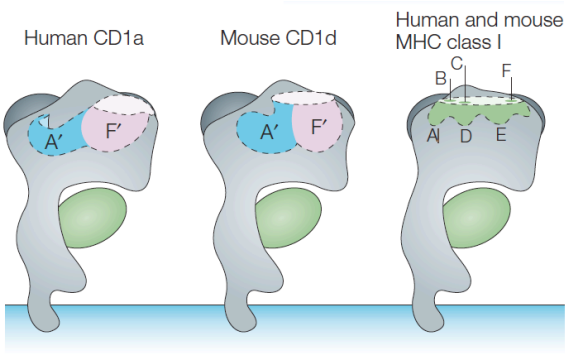


Figure 1.4 Comparison of human and mouse CD1 and MHC molecules [242]

1.3.3 CD1 antigens and CD1-restricted T cells

Since the identification of the C_{80} mycolic acid for lipid antigen presentation to $\alpha\beta^+$ T cells by CD1b-expressing antigen-presenting cells [232], many non-peptide foreign antigens for CD1-antigen presentation have been described, including lipoarabinomannan [248], phosphatidylinositol mannoside [249], glucose monomycolates [250],

diacylated sulfoglycolipids [251], and mannosyl phosphodolichols [252]. Self-antigens have also been identified such as phosphoglycerolipids [253-255], sphingolipids [256, 257], amphipatic small molecules [258], and skin oils [217].

Crystal structures of CD1-lipid complexes confirmed that the aliphatic hydrocarbon chains of non-peptide antigens were anchored deeply in the antigen-binding groove, while the polar head groups (if there are any), such as carbohydrate moieties, protruded from the groove and are exposed to TCR during CD1-TCR interactions [250, 252, 256, 259]. While the above model of CD1-antigen-TCR interactions has been commonly accepted, Birkinshaw *et al.* recently demonstrated that certain ligands of CD1a, namely permissive ligands, could inhibit CD1a-TCR interaction [260], and this will be discussed in detail in later paragraphs.

CD1-expressing antigen-presenting cells present non-peptide antigens to CD1-restricted T cells. For instance, invariant NKT (iNKT) cells recognize lipids presented by $CD1d^{+}$ antigen-presenting cells. iNKT cells possess invariant TCRα and TCRβ chains, which are TRAV10 TRAJ18 TRBV25 in human (TRAV11 TRAJ18 TRBV13-22, TRBV1 in mice) [261, 262]. These CD1d-restricted iNKT cells represent up to about 2% of peripheral blood T cells [263]. By contrast, CD1a-restricted T cells have diverse αβ TCR as opposed to an invariant TCR [218], and some studies

have suggested that some CD1a-restricted T cells can be $\gamma\delta^+$ [264]. CD1a-restricted T cells can be $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ [218, 265].

CD1a and CD1a-restricted/CD1a-autoreactive T cells have been thought to play potential roles in immune response in the skin, due to the constitutive expression of CD1a in Langerhans cells in the skin. CD1a is also expressed in thymocytes, skin-resident dendritic cells, and dendritic cells at other locations such as mouth, oesophagus, and genital tract [246, 266-271]. The importance of CD1a reactivity in the skin has been investigated recently. For example, phospholipase A_2 (PLA_2) derived from bee and wasp venom can activate CD1a-reactive T cells from the skin of healthy and venom allergic individuals [272, 273]. Apart from bee and wasp venom, house dust mite-derived PLA_2 can also lead to the CD1a reactivity, which can be inhibited by filaggrin [274].

1.4 Phospholipase A_2

1.4.1 Introduction of phospholipase A_2

The phospholipase A_2 (PLA_2) family consists of more than 30 PLA_2, divided into 6 groups, which can catalyse the hydrolysis of phospholipids at the *sn*-2 position to generate lysophospholipids and free fatty acids. Of those groups, major ones are secretory PLA_2 ($sPLA_2$) and cytosolic PLA_2 ($cPLA_2$). Other groups are Ca^{2+}-independent PLA_2, lysosomal PLA_2, adipose-specific PLA_2, and platelet-activating factor acetylhydrolase (PAF-AH). They play a major role in many biochemical processes, for example, the generation of lipid mediators, such as prostaglandins and leukotrienes, via arachidonic acid metabolism by $cPLA_2$. Apart from phospholipase activity, many of the PLA_2 also possess lysophospholipase activity. Despite having similar catalytic property, their differences in molecular structure, tissue distribution, and substrate specificity suggest their non-redundant physiological roles.

1.4.2 Secretory PLA_2

$sPLA_2$ are a group of relative low-weight (14-19 kDa) secreted enzymes, consisting a total of 11 members including G1B, G2A, G2C,

G2D, G2E, G2F, G3, G5, G10, G12A, and G12B. Different $sPLA_2$ have diverse cellular tissue localizations and physiological functions. For example, G1B is commonly known as the pancreatic PLA_2, which is a digestive PLA_2 produced by the pancreas for digestion of lipid in the intestine [275]. G10 is crucial to the normal functions of gastrointestinal and neuronal systems, as well as reproduction [276], while overexpression of G10 is related to the allergic reactions in the lungs [277]. Activity of G3 is also essential for maturation of sperm cells [278]. G1B and G2A expression are found in human epidermis [279]. Expression levels of G2A and G10 are associated with airways and severity of asthma [280]. A number of $sPLA_2$ are crucial for the maturation of dendritic cells [281-283], possibly via $sPLA_2$ receptors on the cell surface [284]. Also, G3 promotes the maturation and normal functions of mast cells [285, 286].

Although $sPLA_2$ are important for various physiological functions, abnormal expression or regulation of them can lead to several pathological conditions. For instance, in a mouse model, transgenic expression of G3 can induce spontaneous inflammation in the skin [287]. $sPLA_2$ are also present in other animals such as bird, frog, and especially G1B-like PLA_2 in large amount in snake venom, as well as bacteria and fungi [288-291]. For instance, house dust mite-derived G5 $sPLA_2$ is associated with allergic pulmonary inflammation [292].

Only G1B and G10 are secreted as proenzymes as they have N-terminal propeptide that will be cleaved off for enzymatic activation. However $sPLA_2$ require high Ca^{2+} concentration (in mM level) for active enzymatic function, therefore in general they are active only when secreted to the extracellular environment, where Ca^{2+} concentration is high [291]. After being secreted from the cells, they can cleave phospholipids of the cellular membranes to generate a number of lipids, such as arachidonic acid, oleic acid, linoleic acid, which are the precursors of pro-inflammatory lipid mediators. Apart from cellular membrane phospholipids, $sPLA_2$ can also cleave phospholipids of foreign origins, such as microbial membranes, hence providing an innate immunity against foreign microbial pathogens.

In addition to the immediate action of $sPLA_2$, some recent studies demonstrated that $sPLA_2$ also contribute to anti-inflammatory response via other pathways and confer immunological protection. Miki *et al.* showed that PLA2G2D, preferentially expressed in dendritic cells and macrophages, generated a number of polyunsaturated fatty acids for the generation of anti-inflammatory lipid mediators, leading to the resolution of contact hypersensitivity [293]. On the other hand, Palm *et al.* demonstrated the PLA_2 derived from bee venom could induce an IL-33-ST2-dependent Th2 response and group 2 innate lymphoid cell (ILC2) activation [294]. This is further confirmed by the recent findings by our

group that bee venom PLA_2 could activate CD1a-reative T cells for a Th2-biased response [273].

1.4.3 Cytosolic PLA_2

$cPLA_2$ are a group of relatively high weight (around 85kDa) family and consists of 6 members from G4A to G4F. Due to a conserved Ser/Asp catalytic dyad, instead of the classical catalytic triad, they possess enzymatic properties other than PLA_2, such as PLA_1, lysophospholipase, transacylase, and lipase activities [291]. G4A is the most extensively studied isoform in the $cPLA_2$ family, while other isoforms are less well understood until recently. One major difference of $cPLA_2$ from $sPLA_2$ is that they have hitherto been thought to remain in cytosol rather than being secreted, as the name suggests. Their catalytic reaction is Ca^{2+}-dependent owing to the C2 domain. Ca^{2+} binding to the C2 domain allows the binding of $cPLA_2$ to phospholipids of cellular membranes, where the catalytic domain can cleave the membrane phospholipids to liberate free fatty acids, such as arachidonic acid, and lysophospholipids. Therefore, $cPLA_2$ activity is crucial for the downstream production of eicosanoids and pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes [295], and the arachidonic acid cascade involving the action of PLA2 is described in Figure 1.5. PLA_2 cleaves the phospholipids at the *sn-2* position to liberate arachidonic acid, a C20:4 fatty acid. Arachidonic acid is

then further converted to various downstream lipid mediators either via cyclooxygenase pathway or lipoxygenase pathway. In the cyclooxygenase pathway, arachidonic acid is first converted to PGH_2 by cyclooxygenases including COX-1 and COX-2, then further to PGD_2, PGE_2, PGF_2, and PGI_2. In the lipoxygenase pathway, arachidonic acid is first converted to hydroperoxyeicosatetraenoic acid (HpETE) by lipoxygenases such ALOX5, ALOX12, and ALOX15, and further to LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4.

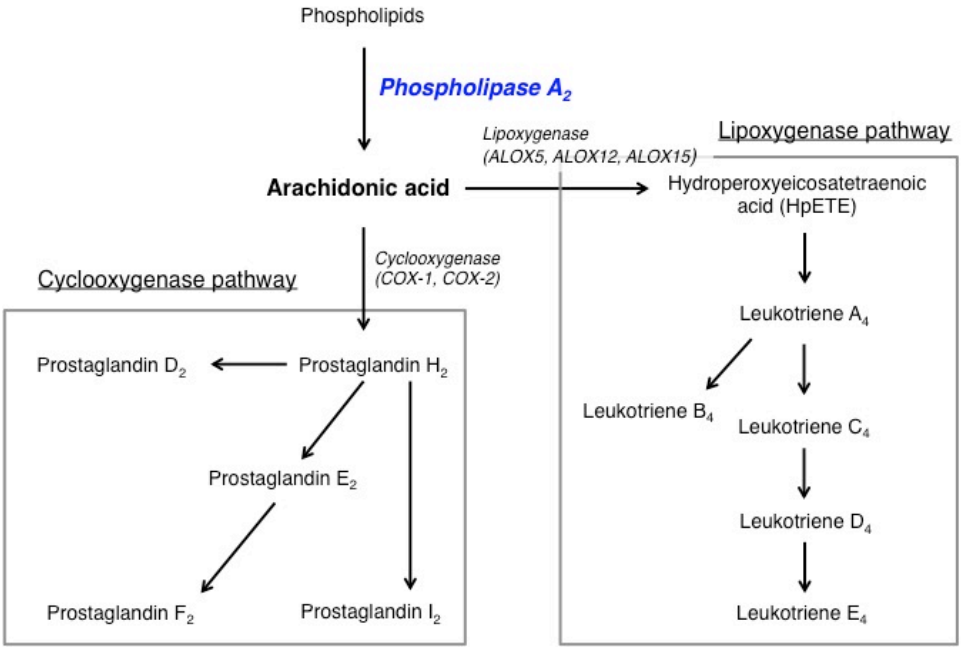


Figure 1.5 Arachidonic acid cascade

Expression of $cPLA_2$ can be found in various tissues and cell types. For example, PLA2G4A in alveolar macrophage and type II alveolar cells are responsible for the generation of lipid mediators in the lungs [291].

G4A is essential for the maturation of thymocytes [296]. Mast cells $cPLA_2$, which is under the regulation of mitogen-activated protein kinase (MAPK) [297] is important for both immediate and delayed production of lipid mediators [298].

Notably, PLA2G4D expression is elevated in the epidermis of psoriatic lesional skin compared to the skin of healthy and atopic dermatitis [299, 300]. The association of PLA_2 activity and psoriasis was first suggested when elevated level of PLA_2 products, including PGE_2, $PGF_{2\alpha}$, and 12-L-hydroxy-5Z,8Z,10E,13Z-eicosatetraenoic acid (12-HETE) were found in the epidermis of psoriatic lesions [301]. Several reports also revealed raised PLA_2 activity in the epidermal samples from psoriasis patients [302-305]. Expression of a novel PLA2G4D, was observed in psoriatic lesions yet was absent in healthy normal skin [299], which was further supported by a recent study showing psoriasis-specific gene expression of PLA_2 [300]. In addition, expression of non-pancreatic PLA_2 was also shown in psoriatic lesions [306]. Unlike $sPLA_2$, which immunological and pathological roles have been extensively studied, little is known about the pathological role of $cPLA_2$, notwithstanding its association with psoriasis, psychological disorders, and neurodegenerative diseases [307-309].

2. Aims and Objectives

While the association of elevated activity of phospholipase A_2 (PLA_2) and psoriasis has been long established, a definitive and reproducible identity of peptide antigens has yet remained elusive. Nonetheless, many studies have suggested the potential contribution of lipid antigens presented by CD1a in the T cell reactivity in skin diseases. However, little is known about whether PLA_2-dervied lipids could be CD1a ligands and contribute to the pathogenesis of psoriasis. In particular, it remains in question whether T cells of psoriasis patients are reactive to lipids presented by CD1a-expressing antigen-presenting cells.

In addition, while it was found that PLA2G4D, a cytosolic PLA_2, was preferentially expressed in psoriatic lesions, the source of it in the lesions has not been identified. Moreover, it is unclear that how it could affect the CD1a reactivity of T cells in the blood and skin of psoriasis patients.

Therefore, we hypothesised that the peripheral blood and skin of psoriasis patients contain enriched CD1a-autoreactive T cells and PLA_2-responsive CD1a-reactive T cells. The aims of the following chapters are listed below:

Chapter 4: To identify the CD1a-autoreactive T cells in the peripheral blood of healthy and psoriasis individuals

Chapter 5: To identify the source of PLA2G4D in psoriatic lesions, and examine the PLA_2-responsive CD1a reactivity of T cells in the peripheral blood of healthy and psoriasis individuals

Chapter 6: To identify the PLA_2-containing mast cell exosome-responsive CD1a reactivity of T cells in the peripheral blood and skin of healthy and psoriasis individuals

3. Materials and Methods

3.1 Reagents

3.1.1 Culture media

RPMI medium:

RPMI-1640 (R8758) (Sigma-Aldrich, USA)

R0 medium:

RPMI-1640 medium with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 *µ*g/ml streptomycin (Sigma-Aldrich, MO, USA)

R-HEPES medium:

RPMI-1640 medium with 2 mM L-glutamine, 100 IU/ml penicillin, 100 *µ*g/ml streptomycin (Sigma-Aldrich, MO, USA), and 10 mM HEPES (Life Technologies, CA, USA)

R10 medium:

RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 *µ*g/ml streptomycin (Sigma-Aldrich, MO, USA) and 10 mM HEPES (Life Technologies, CA, USA).

Complete medium:

RPMI-1640 medium supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, MO, USA), 2 mM L-glutamine, 1X non-essential amino acids (NEAA), 1 mM sodium pyruvate, 10 mM HEPES, and 500 μM 2-mercaptoethanol (Life Technologies, CA, USA).

T cell medium:

RPMI-1640 medium supplemented with 5% human serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Sigma-Aldrich, MO, USA), 1X NEAA, 1 mM sodium pyruvate, 10 mM HEPES, 500 μM 2-mercaptoethanol (Life Technologies, USA), and 2 nM IL-2 (PeproTech, London, UK).

T cell resting medium:

RPMI-1640 medium supplemented with 5% human serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Sigma-Aldrich, MO, USA).

K562 medium:

RPMI-1640 medium with 10% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Sigma-Aldrich, MO, USA), 1X NEAA, 1 mM sodium pyruvate, 10 mM HEPES, 500 μM 2-mercaptoethanol, and 50 μg/ml G418 antibiotic (Life Technologies, CA, USA).

Monocyte-derived dendritic cell (mDC) medium:

RPMI-1640 medium with 10% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Sigma-Aldrich, MO, USA), 1X NEAA, 1 mM sodium pyruvate, 10 mM HEPES, and 500 μM 2-mercaptoethanol (Life Technologies, CA, USA), and 50 ng/ml GM-CSF and 1000 U/ml IL-4 (PeproTech, London, UK).

LAD2 cell medium:

StemPro-34 SFM (Life Technologies, USA) supplemented with 100 ng/ml human recombinant stem cell factor (SCF) (PeproTech, London, UK).

Cord blood-derived mast cell (CBMC) medium:

IMDM containing 10% human serum, 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, MO, USA), 0.55 μM 2-mercaptoethanol (Life Technologies, CA, USA), human recombinant SCF (100 ng/ml) and human recombinant IL-6 (50 ng/ml) (PeproTech, London, UK).

3.1.2 Flow cytometry antibodies

CD1a-PE (1:50, BD Biosciences), CD3-Per-CP (1:50), CD4-FITC (1:25), CD8-APC (1:100) (BD Biosciences); LiveDead-Pacific Blue (1:50, Life Technologies, CA, USA).

3.1.3 Immunofluorescence antibodies

Primary antibodies: PLA2G4D (rabbit polyclonal, 1:50, ab111126), mast cell tryptase (mouse monoclonal, 1:100, ab81703) (Abcam Cambridge,

UK); CD63-PE (mouse anti-human, SAB4700218), Calcein-AM (17783) (Sigma-Aldrich, MO, USA)

Secondary antibodies: goat anti-rabbit IgG (H+L) Alexa Fluor 488 conjugate (1:500, A-11008), goat anti-mouse IgG (H+L) Alexa Fluor 568 conjugate (1:500, A-11031) (Life Technologies, CA, USA).

3.1.4 Western Blot antibodies

Primary antibodies: PLA2G4D (rabbit polyclonal, 1 μg/ml, ab173395), mast cell tryptase (mouse polyclonal, 1:1000, ab80703), GAPDH (rabbit polyclonal, 1:10000, ab128915), CD63 (mouse monoclonal, 1 μg/ml, ab59379) (Abcam, Cambridge, UK).

Secondary antibodies: Goat anti-rabbit IgG IRDye 800CW conjugate (1:10000, 925-32211), goat anti-mouse IgG IRDue 800CW conjugate (1:10000, 925-32210) (LI-COR Biosciences, NE, USA).

3.1.5 Antigen sources

Oleic acid C18:1 (O1008), palmitic acid C16:0 (P0500), lysophosphatidycholine (LPC) C16:0 (L5254) (Sigma-Aldrich, MO, USA); LPC C18:1 (845875) (Avanti polar lipids, AL, USA)

3.2 Isolation of PBMC from blood

Peripheral blood samples were collected from healthy adult donors and psoriasis patients with moderate-severe disease, but not on systemic therapy. Local ethical approval was given by Oxford Ethics Committee (09/H0606/71). Peripheral blood mononuclear cells (PBMC) were isolated from the whole by density gradient centrifugation. Heparinized blood was diluted 1:1 (v/v) with R-HEPES medium, followed by layering over 15 ml Lymphoprep (Nycomed, Roskilde, Denmark), and then centrifuged at 800 g for 20 minutes with the brake switched off. After the centrifugation, the buffy coat layer that contained the PBMC was collected, and washed twice with R-HEPES at 450 g. The PBMC was ready for subsequent experiments.

3.3 Isolation of $CD3^+$ T cells from donor PBMC

$CD3^+$ T cells were positively selected and purified from PBMC of healthy or psoriasis donors by CD3 MACS bead separation (130-050-101, Miltenyi Biotech, Germany). In brief, PBMC were resuspended in 2 ml of MACS buffer (PBS with 10% foetal bovine serum and 2 mM EDTA (Sigma-Aldrich, MO, USA)), and 20 μl of CD3 beads were added to per 10^7 cells, and mixture was incubated at 4ºC for 15 minutes with occasional shaking for the labelling of $CD3^+$ cells with the CD3 beads. After the incubation, 1 ml of MACS buffer was added to the mixture before centrifugation at 300 g

for 10 minutes. Supernatant was removed and the pellet was resuspended in 3 ml of MACS buffer for magnetic separation.

LS columns (130-042-401, Miltenyi Biotec, Germany) were installed on the separation magnet and pre-wetted with 3 ml of MACS buffer. PBMC were passed through pre-separation filters (70 μm) (130-095-823, Miltenyi Biotec, Germany) before applying to the LS columns. Cell mixtures were allowed to flow through by gravity. $CD3^+$ T cells remained to the LS columns, while other cells passed through the columns and collected as the negative fractions. The columns were then washed with 3 ml of MACS buffer. $CD3^+$ T cells were finally collected by passing through 3 ml of MACS buffer, followed by centrifugation and resuspending in T cell medium for further culture or experiments.

3.4 Monocyte-derived dendritic (mDC) cell culture

$CD14^+$ cells were positively selected and purified from PBMC from healthy or psoriasis donors by CD14 MACS bead separation (130-50-021, Miltenyi Biotec, Germany) in a similar method to $CD3^+$ T cell separation. After collecting $CD14^+$ cell pellet, cells were resuspended in mDC medium at 0.2-0.5 x 10^6 cells/ml cell density, and were cultured for 4 days, with hemidepletion of medium at day 3. mDCs were harvested at day 5 and

CD1a expression was verified by flow cytometry. The cells were then ready for further experiments.

3.5 K562 cell culture

Empty vector-transfected K562 (K562-EV) and CD1a-transfected K562 (K562-CD1a) cells were a gift from Branch Moody (Harvard) and were maintained in K562 medium in a T-75 flask, with hemidepletion of medium every 3 or 4 days. CD1a expression of K562-CD1a cells was checked routinely by flow cytometry.

3.6 LAD2 cell culture

LAD2 mast cell-like line was cultured in T-75 flasks with LAD2 medium, with SCF only added when in use. Hemidepletion of medium was performed every 3 to 4 days. Cell density was maintained below 0.5 x 10^6 cells/ml.

3.7 Human cord blood-derived mast cell (CBMC) culture

Human cord blood was obtained from National Blood Service, Oxford, UK. Mononuclear cells from human cord blood were isolated by

density gradient centrifugation using Lymphoprep (Nycomed, Roskilde, Denmark). Then $CD34^+$ progenitor cells were positively selected and purified from cord blood mononuclear cells using CD34 MACS bead separation (130-046-702, Miltenyi Biotec, Germany). Followed by $CD34^+$ cell separation, the cells were cultured and allowed for differentiation of cord blood-derived mast cells in CBMC medium, with SCF and IL-6 only added when in use, for 10 to 12 weeks at 37ºC, 5% CO_2.

3.8 Isolation of $CD3^+$ T cells from skin

Skin samples from abdominoplasty and face lift surgeries were obtained from patients with informed consent. After removing subcutaneous fat, skin sections were cut into 1mm pieces and incubated in R10 medium with the addition of 1 mg/ml of collagenase P (Roche, Sweden) overnight at 37ºC 5% CO_2 to digest the collagen within the tissues. On the following day, skin pieces were washed with cold PBS with 10 mM EDTA (PBS/EDTA) to quench the collagenase digestion, with repeated pipetting to break up the clumps of skin pieces. The samples were then passed through 70 μm nylon strainers and washed with 50 ml of PBS/EDTA, followed by 40 μm nylon strainers and collected as pellets after centrifugation at 300 g, 4ºC for 20 minutes. Then the pellets were resuspended with 30 ml of R0 medium and layered over 15 ml of Lymphoprep (Nycomed, Roskilde, Denmark) and centrifuged at 2000 rpm

for 20 minutes with the brake switched off. The buffy coat layer was collected and resuspended in R10 medium for temporary culture, or MACS buffer for a following MACS cell separation.

Polyclonal $CD3^+$ T cells from skin of healthy or psoriasis donors were positively selected and purified by CD3 MACS bead separation (Miltenyi Biotec, Germany) in a method similar to $CD3^+$ T cell isolation from blood samples. After separation, the cells were resuspended in T cell medium for culture or further experiments.

3.9 Flow cytometry

Flow cytometry was performed to determine the expression of CD3 of T cells isolated from the healthy and psoriasis donors, and the expression of CD1a of mDCs and K562-CD1a cells. In brief, the cells were fixed with Cytofix fixation buffer (BD Biosciences) and then stained with different antibodies at certain concentrations in R10 medium for 30 minutes at room temperature in the dark, followed by washing. Then the cells were resuspended in cold PBS and ready for flow cytometry analysis using CyAn^{TM} ADP Analyzer (Beckman Coulter), and data were analysed with WinMDI 2.8.

3.10 *Ex vivo* ELISpot assay

Peripheral blood- or skin-derived $CD3^+$ T cells from healthy or psoriasis donors were isolated by CD3 MACS bead separation and cells were cultured for 3-5 days in T cell medium prior to the assay for the regeneration of untouched CD3 molecules on the cell surface. On day 1, T cells were washed with twice with R-HEPES medium and allowed to rest in T cell resting medium overnight at 37ºC, 5% CO_2. On the other hand, K562-EV/K562-CD1a cells or mDCs were washed 3 times with R-HEPES medium, and pulsed with various antigen sources overnight at 37ºC, 5% CO_2. On the same day, ELISpot plates (Millipore Corp, USA) were pre-treated with 35% ethanol, washed 6 times with water, and then coated with anti-IFN-γ antibody (Mabtech AB, Sweden) overnight at 4ºC.

On day 2, ELISpot plates were washed 6 times with RPMI medium and blocked with R10 medium for 30-60 minutes, and then washed twice with RPMI medium. Each well was added with 50,000 T cells and 25,000 K562 cells/mDCs. Wells were set up in duplicates or triplicates. Phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (500 ng/ml) were used as positive control, whereas T cells alone served as the negative control. In some experiments, K562 cells were incubated with 10 μg/ml anti-CD1a antibody (eBioscience, CA, USA) or 10 μg/ml mouse control IgG antibody (BD Biosciences). For the mDC experiments, 10 μg/ml anti-HLA-ABC (W6/32) and anti-HLA-DR blocking antibodies (L243) were added for 2

hours prior to addition of T cells. In some experiments, inhibitors including chlorpromazine (10 μg/ml), methyl-β-cyclodextrin (2%), cytochalasin D (5 μg/ml), or arachidonoyl trifluoromethyl ketone (ATK) (10 μM) were added to mDC 30 minutes prior to mDC-T cell co-incubation for the inhibition of endocytosis. Cells were then incubated overnight at 37ºC, 5% CO_2.

On day 3, after collecting ELISpot supernatants for further experiments, ELISpot plates were firstly washed 5 times with PBS-Tween-20 (0.05%) and incubated with 1 μg/ml biotin-conjugated anti-IFN-γ monoclonal antibody (Mabtech AB, Sweden) for 2-3 hours at room temperature, followed by washing 6 times with PBS-Tween 20 (0.05%). Plates were then incubated with streptavidin-ALP solution (Mabtech AB, Sweden) for 1-2 hours, and washed 6 times with PBS-Tween-20. Spots were developed and visualized using AP conjugate kit (Bio-Rad, USA), and detected and analysed using ELISpot plate reader (ELISpot Reader Classic, Autimmun Diagnostika gmbh, Germany).

3.11 Cultured ELISpot assay

Peripheral blood-derived $CD3^+$ T cells from psoriasis donors were isolated by CD3 MACS bead separation, followed by T cell expansion using autologous mDCs pulsed with various lipid antigens at certain concentrations for 10-14 days prior to cultured ELISpot assays. On day 1,

T cells were washed with twice with R-HEPES medium and allowed to rest in T cell resting medium overnight at 37ºC, 5% CO_2. On the other hand, K562-EV/K562-CD1a cells were washed 3 times with R-HEPES medium, and pulsed with various antigen sources overnight at 37ºC, 5% CO_2. On the same day, ELISpot plates (Millipore Corp, USA) were pre-treated with 35% ethanol, washed 6 times with water, and then coated with anti-IFN-γ antibody (Mabtech AB, Sweden) overnight at 4ºC.

On day 2, ELISpot plates were washed 6 times with RPMI medium and blocked with R10 medium for 30-60 minutes, and then washed twice with RPMI medium. Each well was added with 50,000 T cells and 25,000 K562 cells. Wells were set up in duplicates or triplicates. Phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (500 ng/ml) were used as positive control, whereas T cells alone served as the negative control. In some experiments, K562 cells were incubated with 10 μg/ml anti-CD1a antibody (eBioscience, CA, USA) or 10 μg/ml mouse control IgG antibody (BD Biosciences). In some experiments, inhibitors including indomethacin (10 μM) and baicalein (10 μM) were added to K562 cells 30 minutes prior to K562-T cell co-incubation for the inhibition of cyclooxygenase and lipoxygenase pathways respectively. Cells were then incubated overnight at 37ºC, 5% CO_2.

On day 3, after removing ELISpot supernatants, ELISpot plates were firstly washed 5 times with PBS-Tween-20 (0.05%) and incubated with 1 μg/ml biotin-conjugated anti-IFN-γ monoclonal antibody (Mabtech AB, Sweden) for 2-3 hours at room temperature, followed by washing 6 times with PBS-Tween 20 (0.05%). Plates were then incubated with streptavidin-ALP solution (Mabtech AB, Sweden) for 1-2 hours, and washed 6 times with PBS-Tween-20. Spots were developed and visualized using AP conjugate kit (Bio-Rad, USA), and detected and analysed using ELISpot plate reader (ELISpot Reader Classic, Autimmun Diagnostika gmbh, Germany).

3.12 ELISA

Supernatants from ELISpot assays were collected for measurements of IL-17A and IL-22 secretion using ELISA kits (Human Ready-Set-Go!, eBioscience, CA, USA). In brief, 96-well plates (Nunc MaxiSorp, eBioscience, CA, USA) were pre-coated with 100 μl/ml of respective coating antibodies (eBioscience, CA, USA), which were diluted 1:250 in coating buffer (eBioscience, CA, USA), overnight at 4ºC. After coating, the plates were washed 5 times with PBS-Tween 20 (0.05%), and then blocked by 1% BSA/PBS (eBioscience, CA, USA) for 60 minutes at room temperature. Then the supernatants were added into the wells for an overnight incubation at 4ºC. On the next day, the ELISA plates were

washed with PBS-Tween 20 (0.05%, and incubated with streptavidin-horse radish peroxidase (HRP) (eBioscience, CA, USA) for 30 minutes at room temperature, followed by washing 4 times with PBS-Tween 20 0.05%. The plates were then developed using 3,3',5,5'-tetramethylbenzidine (TMB) (eBioscience, CA, USA) for 15 minutes at room temperature in the dark. Reaction was stopped using 1M sulphuric acid (Sigma-Aldrich, MO, USA). Cytokine levels were quantified by measuring the absorbance at 415nm with a microplate reader (Bio-rad iMark^{TM} Microplate Reader, Bio-Rad, USA).

3.13 Gene Expression Omnibus (GEO) study

A number of gene expression studies were accessible on the Gene Expression Omnibus (GEO), and various gene expressions were analysed using GEO2R. In brief, 6 gene expression studies were retrieved from GEO, and the results of 3 gene expression studies between non-lesional and lesional skin from psoriasis patients were collected. Their data were analysed using GEO2R, and expression levels of relevant genes were analysed and compared across the groups.

3.14 Immunofluorescence microscopy

Paraffin-embedded skin sections were first de-waxed for 10 minutes in Xylene Substitute solution (A5597, Sigma-Aldrich, MO, USA), and then rehydrated for 5 minutes in each of a series of ethanol-water solutions in descending gradients (95%, 90%, 75%, 50%, and 30% ethanol) and finally in water, and then boiled in 1X Target Retrieval Buffer (Dako) for 15 minutes for antigen retrieval, followed by cooling down in PBS for 10 minutes. Skin sections were then blocked with blocking solution (1% BSA/PBS) for 1 hour at room temperature. Skin sections were then ready for incubation with primary and secondary antibodies. In brief, primary antibodies were diluted, in according dilutions, in 1% BSA/PBS and added to the sections, and overnight incubation was performed at 4ºC in the dark. On the next day, the sections were washed 5 times with PBS-Tween 20 (0.05%), and incubated with secondary antibodies diluted in 1% BSA/PBS for 2 hours in the dark. Then they were washed 5 times with PBS-Tween 20 (0.05%), and mounted with Fluoroshield™ with DAPI (F6057, Sigma-Aldrich, MO, USA) and glass coverslip (VWR, PA, USA).

For imaging of LAD2 mast cell-like line, cells were seeded on culture slides (BD Biosciences) pre-treated with poly-D-lysine (Sigma-Aldrich, MO, USA) to improve cell adherence, and fixed and permeabilised with acetone for 10 minutes, then blocked with blocking solution for 1 hour

at room temperature, followed by staining with accordingly diluted primary and secondary antibodies.

Immunofluorescence images were acquired on an Axiovert S100 microscope (Carl Zeiss, Germany) coupled with an ORCA-ER C4742-80 digital camera (Hamamatsu, Japan). Laser intensity and amplifier gains were adjusted to avoid pixel saturation. Detection of fluorescence was performed independently and sequentially on each fluorophore. Images were processed by ZEN imaging software (Blue edition, Carl Zeiss, Germany).

3.15 Immmunoprecipitation

LAD2 mast cell-like line, or CBMC were lysed in RIPA buffer (Sigma-Aldrich, MO, USA) with protease inhibitor cocktail (04693116001, Roche, Sweden), and cell lysates were obtained in supernatants after centrifugation and removal of pellets. PLA2G4D protein was prepared using affinity column protein purification. In brief, the cell lysates were run through a resin column (Pierce ^{TM} Conjugate Kit, Thermo Fisher Scientific, USA) pre-coated covalently with anti-PLA2G4D antibody (Abcam, Cambridge, UK). After extensive washing of the column with PBS, PLA2G4D was eluted with elution buffer (Pierce ^{TM} Gentle Ag/Ab Binding and Elution Buffer Kit, Thermo Fisher Scientific, USA), and PLA2G4D

protein fraction was collected. Protein concentration was measured using bicinchoninic acid assay (BCA). In brief, the concentration of protein corresponds to the number of its peptide bond, which can convert the BCA reagent from green to purple, and absorbance at 562 nm was measured. The protein concentration was calculated according to a protein standard which is BSA.

3.16 Exosome preparation

Exosomes derived from LAD2 mast cell-like line were prepared from the culture supernatant using Total Exosome Extraction Reagent (Life Technologies, CA, USA), according to the manufacturers' instructions. In brief, 2 parts of culture supernatant were mixed with 1 part of exosome extraction reagent, and the mixture was vortexed for 30 seconds, followed by incubating overnight at 4ºC on a roller shaker. On the next day, the mixtures were centrifuged at 10,000 g and 4ºC for 1 hour. The reagent worked by tying up water molecules and forcing exosomes out of solution, and exosome fractions were collected as pellets. Pellets of exosomes were then dissolved in PBS or other desired buffer for further experiments.

3.17 Cytosolic PLA_2 biochemical activity assay

PLA_2 activity in culture medium, cell lysate, culture supernatant, and exosomes of LAD2 mast cell-like line was measured in colorimetric assay using cytosolic PLA_2 kit (Cayman Chemicals, MI, USA) according to the manufacturer's protocols. In brief, 10 μl of samples were added per well of a flat bottom 96-well plate, plus 5 μl of assay buffer, and then incubated for 1 hour at room temperature with arachionoyl thio-PC. Arachidonoyl thio-PC is a substrate for cytosolic PLA_2 by virtue of the presence of arachidonic acid at the *sn*-2 position of the glycerophospholipid. Hydrolysis of the arachidonoyl thioester bond at the *sn*-2 position by cytosolic PLA_2 releases a free thiol that can be detected by DTNB (5,5'-dithiobis(2-nitrobenzoic acid)).

3.18 SDS-PAGE and Western Blot

Protein expression of different samples was detected using either SDS-PAGE or Western Blot. First, various samples were diluted in loading buffer (NuPAGE® LDS Sample Buffer 4X, NP0007, Thermo Fisher Scientific, USA), reducing agent (NuPAGE® Sample Reducing Agent 10X, NP0009, Thermo Fisher, Scientific, USA) and water, and denatured at 90^{o}C for 10 minutes. During protein denaturation, 4-12% Bis-Tris protein gels (NuPAGE® Novex 4-12% Bis-Tris, 1.0mm, 12-well, NP0322BOX, Thermo Fisher Scientific, USA) were prepared with MES SDS Buffer

(NuPAGE® MES SDS Buffer Kit, NP0060, Thermo Fisher Scientific, USA). Gel electrophoresis was performed with 120V applied for 60 minutes, and protein gels were then ready for Coomassie Blue staining or Western Blot.

For Western Blot, the proteins on the gels were transferred to a PBDF membrane using iBlot® Gel Transfer Device (Thermo Fisher Scientific, USA) and iBlot Transfer Stack (IB401031, Thermo Fisher Scientific, USA), followed by washing twice with PBS-Tween 20 (0.05%). Membranes were blocked with Membrane Blocking Solution (000105, Thermo Fisher Scientific, USA) for 60 minutes at room temperature, followed by washing three times with PBS-Tween 20 (0.05%). Then the membranes were ready for staining with primary and secondary antibodies. In brief, primary antibodies diluted accordingly in PBS-Tween 20 (0.05%) were added to the membranes and overnight incubation was performed at 4ºC in the dark on a roller shaker. On the next day, they were washed 5 times with PBS-Tween 20 (0.05%), and then incubated with secondary antibodies diluted accordingly in PBS-Tween 20 (0.05%) at room temperature in the dark for 1 hour. After washing 5 times with PBS-Tween 20 (0.05%), the membranes were allowed to be dry in the air in the dark, followed by detection of protein expression using Odyssey® Imaging System (LI-COR Biosciences, NE, USA).

3.19 Statistics

Statistical analyses were performed using GraphPad Prism 6. Cohorts of healthy and psoriasis individuals for CD1a-autoreactive, PLA_2-specific and exosome-specific CD1a-restricted responses were analysed using one-tailed Wilcoxon matched-pairs signed rank test. Other T cell responses were analysed using two-way ANOVA.

4. CD1a autoreactivity in Psoriasis

4.1 Introduction and Aims

Psoriasis-associated peptide antigens

Considering the impaired function of physical barrier of the skin in psoriatic lesions, and together with the knowledge of Langerhans cells and dermal dendritic cells in psoriasis, it follows a rational line to question whether the antigens could be of foreign origin, which penetrate through cutaneous layers and come in contact with Langerhans cells in the epidermis or dermal dendritic cells in the dermis.

Bacterial flora on the skin became a major area of interest in search of the identity of the foreign antigens. Noble and Savin demonstrated that about 25% of psoriasis patients carried *Staphylococcus aureus* [205], while Marples *et al.* reported that half of psoriasis patients were carriers in their study [206]. A higher incidence of *Staphylococcus aureus* in psoriatic lesions than in uninvolved skin was reported [206, 207]. On the other hand, the association between the onset of acute guttate psoriasis,

especially in children and young adults, and streptococcal infection was commonly acknowledged [208, 210, 211]. Baker *et al.* demonstrated that streptococcal-specific T cells from psoriasis patients responded to group A streptococcal antigens and produced IFN-γ [209]. Intriguingly, Leung *et al.* proposed a "superantigen hypothesis", in which they put forward a putative mechanism describing the presentation of superantigens by MHC class II molecules to restricted T cells in an unconventional manner in psoriasis [310]. Different from usual peptide antigens, these superantigens do not bind to the antigen-binding groove, but interact with amino acid residues of the outer surface of the MHC molecules. Not requiring any intracellular processing to trim any excess parts in order to fit into the antigen-binding groove, they can be presented to restricted T cells, and hence initiate the production of cytokines by the T cells. Staphylococcal enterotoxins and streptococcal exotoxins are two groups of proposed superantigens. Brown *et al.* demonstrated that skin T cell lines reactive to group A streptococcal antigens were predominantly $CD4^+$, yet only some but not all lines produced high levels of IFN-γ and lower levels of IL-4 and IL-10 [212].

Apart from foreign sources of antigens, several host-derived antigens, or autoantigens, were identified. A few studies reported that keratins were recognized by peripheral blood T cells from psoriasis patients, attributed to the sequence homology of streptococcal M protein with human keratins [214, 215]. LL-37, an antimicrobial peptide over-

expressed in psoriatic skin, has been identified as an autoantigen which can trigger T cell responses in psoriasis [213]. A recent finding revealed a melanocyte antigen was presented to HLA-C*06:02-restricted psoriatic T cells [216]. It was suggested that these autoantigens might play important roles in the predisposition towards psoriasis via autoimmune pathway. However, despite decades of effort of searching for peptide-based antigens, a definitive and reproducible identity of the antigens has remained elusive, hence raising a possibility of a role of non-peptide antigens.

Non-peptide antigenic responses in skin

Besides presenting peptide antigens to restricted T cells, Langerhans cells, as well as some dermal dendritic cells, are able to present non-peptide antigens, owing to their expression of CD1a molecules [220, 221]. In particular, human, but not mouse, Langerhans cells constitutively express high levels of CD1a molecules [246, 311]. Pena-Cruz *et al.* demonstrated that epidermal Langerhans cells presented microbial lipid antigens to T cells in a CD1a-depenedent manner [220], and Hunger *et al.* showed that Langerhans cells presented non-peptide antigens of *Mycobacterium leprae* to T cells of leprosy patients [221]. CD1a proteins can also present mycobacterial lipopeptide dideoxymycobactin to T cells [219]. Not only foreign non-peptide antigens,

CD1a can present self-lipids to T cells, such as sulfatide, fatty acids, wax esters, and squalene [217, 218].

Skin-homing markers, such as cutaneous lymphocyte antigen (CLA) and chemokine receptors CCR4 and CCR10, were found on the T cells that recognized lipid antigens presented by CD1a-expressing antigen-presenting cells [162, 222]. These skin-homing T cells were detected in the dermis and could be activated by Langerhans cells *in vitro* [218]. However, whether CD1a-expressing antigen-presenting cells, including Langerhans cells and dermal dendritic cells, could present lipid antigens and activate T cells *ex vivo* remained unclear. In particular, there is currently little understanding of whether these antigen-presenting cells could present self-lipids to CD1a-reactive T cells in psoriasis.

It is hypothesized that the CD1a-autoreactive response would be greater in psoriasis patients than healthy individuals, based on the knowledge of skin-homing CD1a-autoreactive T cells and the involvement of CD1a-expressing antigen-presenting cells in psoriasis.

4.2 Results

4.2.1 Enriched CD1a-autoreactive T cells in psoriasis patients

The CD1a autoreactivity of T cells in healthy and psoriasis cohorts was investigated. First, the CD1a-expressing K562 cells were used as the antigen-presenting cells for the T cell assays. K562 is a cell line derived from a 53-year-old female with chronic myelogenous leukaemia (CML) [312]. With little or no expression of HLA, they were susceptible to and readily killed by natural killer (NK) cells [313-315]. Therefore, K562 cells have been commonly used as a universal NK cell target. However CD1-transfected K562 cells have also been used as a lipid antigen-presenting population to compare cellular responses between unrelated individuals [217, 218, 260, 272, 273].

In the experiments, the $CD3^+$ polyclonal T cells were isolated from the peripheral blood of psoriasis patients or healthy controls using CD3 magnetic beads separation, and the expression of CD3 of the T cells was verified by flow cytometry (Figure 4.1). First, the lymphocyte population was selected based on size and scattering properties, and only singlet cells were included, while non-viable cells were screened out. The expression of CD3, CD4, and CD8 of the cells was determined. More than

90% of the cells expressed CD3, indicating a good yield following T cell isolation based on CD3 magnetic bead separation (Figure 4.1).

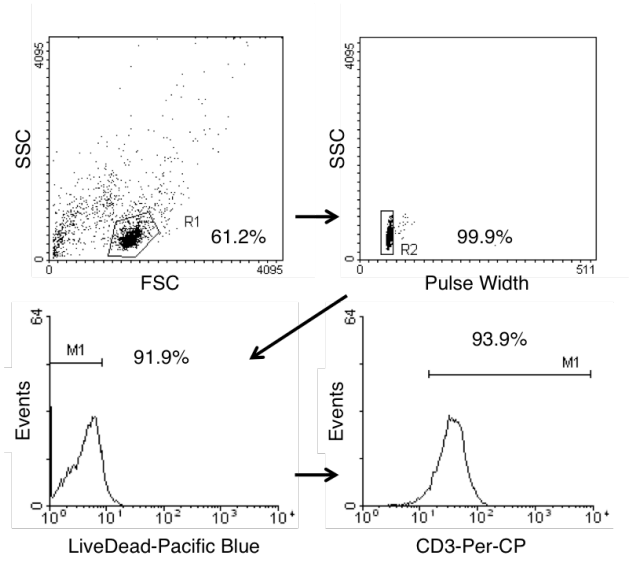


Figure 4.1 CD3 expression of T cells verified by flow cytometry

$CD3^+$ T cells were isolated from the peripheral blood of donors by MACS separation using CD3 magnetic beads, and the CD3 expression was verified by flow cytometry. Gating strategies are shown.

After the polyclonal $CD3^+$ T cells were isolated, they were incubated *ex vivo* with K562 cells that were transfected with CD1a (K562-CD1a), or were empty vector-transfected (K562-EV). Then T cell responses were measured on the basis of IFN-γ secretion using ELISpot assays (Figure 4.2). Before performing the *ex vivo* ELISpot experiments, the expression of CD1a of K562-CD1a was verified by flow cytometry (Figure 4.2A).

In the healthy cohort in Figure 4.2B, the amount of IFN-γ production by the T cells that were co-incubated with K562-EV (empty vector transfected K562) cells was indistinguishable from that by the T cells alone. The T cells isolated from healthy donors that were co-incubated with K562-CD1a cells produced a slightly increased amount of IFN-γ compared to those co-incubated with K562-EV cells, although the difference in response was not statistically different. The frequency of IFN-γ-producing CD1a-reactive T cells in healthy donors were determined to be about 0.01% of total blood T cells. Moreover, this small IFN-γ response could be inhibited by anti-CD1a blocking antibody, but not by control isotype IgG antibody, thus confirming that CD1a is crucial and necessary for the response (Figure 4.2B).

A similar set of experiments was performed on T cells from psoriasis patients. Again, both T cells alone and T cells that were co-

incubated with K562-EV cells demonstrated little or no IFN-γ production (Figure 4.2B). Surprisingly, T cells that were co-incubated with K562-CD1a cells demonstrated a greater IFN-γ response than those co-incubated with K562-EV cells ($p < 0.0001$) (Figure 4.2B). The frequency of CD1a-autoreactive T cells in the psoriasis cohort was much higher than that in the healthy cohort ($p < 0.0001$), being at about 0.03% of total blood T cells, which was three-fold of the frequency found in the healthy cohort (Figure 4.2B). This CD1a autoreactivity could be blocked by anti-CD1a blocking antibody ($p < 0.01$) but not control isotype IgG antibody, confirming the dependence of CD1a (Figure 4.2B).

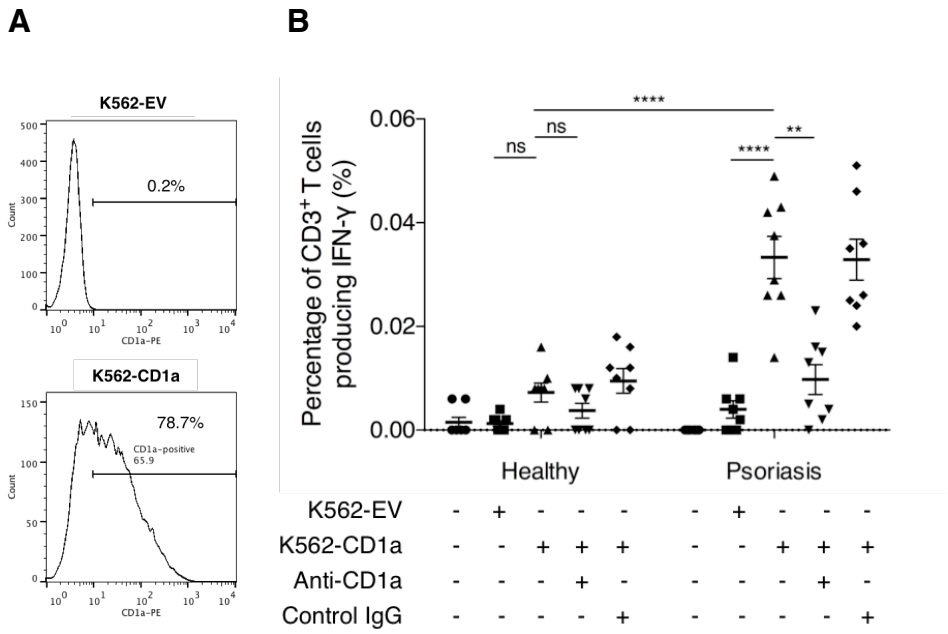


Figure 4.2 Circulating CD1a-autoreactive T cells in healthy and psoriasis individuals using K562 as antigen-presenting cells.

CD1a expression of K562-EV and K562-CD1a cells was determined by flow cytometry (**A**). T cells from healthy (n=8) or psoriatic donors (n=8) were isolated by CD3 MACS separation from peripheral blood, and incubated with CD1a-trasnfected (K562-CD1a) cells or mock-transfected (K562-EV) cells overnight, and IFN-γ production was measured by ELISpot in the presence or absence of anti-CD1a antibody or control IgG antibody. Data were means ± SEM (**B**). ** $p < 0.01$; **** $p < 0.0001$; ns no significance.

Altogether, the above findings demonstrated the enrichment of CD1a autoreactivity and elevated frequency of CD1a-autoreactive T cells in the peripheral blood of psoriasis patients, compared to healthy individuals. However, these experiments were performed using K562 cells as antigen-presenting cells. In addition to K562 cells, autologous monocyte-derived dendritic cells (mDCs) were used as another CD1a-expressing antigen-presenting cell type to confirm the CD1a-dependence. mDCs were derived from $CD14^+$ cells isolated from the peripheral blood of healthy or psoriasis donors using magnetic bead separation. Expression of CD1a was verified by flow cytometry (Figure 4.3A). The mDCs were fixed before performing flow cytometry, therefore the labelling antibody was not taken up non-specifically.

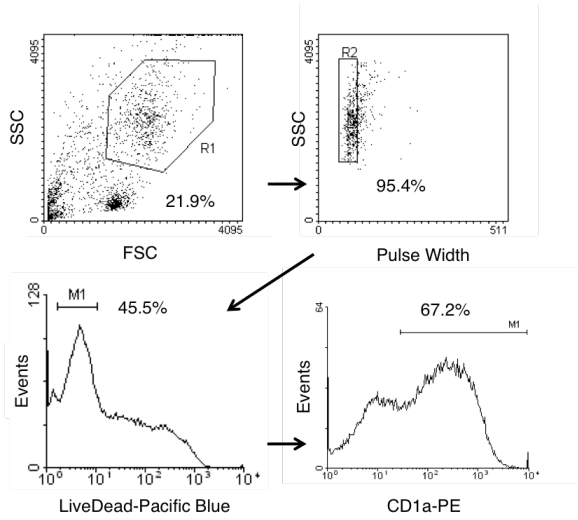
A similar set of experiments was performed on healthy and psoriasis cohorts using mDCs instead of K562 cells. Since mDCs express MHC I and II on their surface, anti-HLA-ABC (W6/32) and anti-HLA-DR blocking antibodies were added to prevent MHC-TCR interactions in the mDCs experiments. Using mDCs as antigen-presenting cells, the CD1a autoreactivity in both healthy and psoriasis cohorts was observed (Figure 4.3B). The frequency of IFN-γ producing CD1a-autoreactive T cells in the healthy cohort was determined to be approximately 0.01-0.04% of total blood T cells. Using K562-CD1a cells or autologous mDCs as CD1a-expressing antigen-presenting cells, here it was demonstrated that the

peripheral blood of normal healthy individuals contained a subpopulation of T cells that were reactive to CD1a-expressing antigen-presenting cells without the addition of CD1a ligands, thus CD1a-autoreactive. This is in agreement with the findings in our previous studies and others' [217, 218, 272, 274].

Furthermore, compared to the healthy cohort, there was a significantly higher frequency of CD1a-autoreactive T cells that produced IFN-γ in the psoriasis cohort, being 0.02-0.08%. The mean frequency was determined to be 0.04%, which was a two-fold increase compared to that of the healthy cohort ($p < 0.05$) (Figure 4.3B). Again, the CD1a-autoreactive T cell response could be inhibited by anti-CD1a blocking antibody but not control isotype IgG antibody ($p < 0.01$). This confirmed the CD1a-dependence (Figure 4.3B).

To summarize, it was demonstrated that the peripheral blood of psoriasis patients contained an enriched subpopulation of IFN-γ producing CD1a-autoreactive T cells compared to healthy individuals, using K562-CD1a and autologous mDCs as CD1a-expressing antigen-presenting cells.

A



B

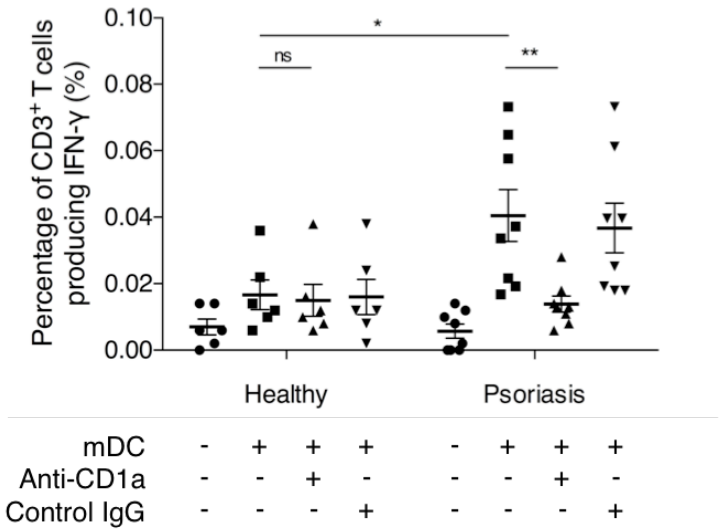


Figure 4.3 Circulating CD1a-autoreactive T cells in healthy and psoriasis individuals using mDCs as antigen-presenting cells

mDCs were derived from $CD14^+$ cells isolated from the peripheral blood of the donors by MACS separation using CD14 magnetic beads, and the expression of CD1a was determined by flow cytometry (**A**). T cells from psoriasis (n=8) or healthy (n=6) donors were isolated by CD3 MACS separation from peripheral blood, and incubated overnight with autologous

mDCs with anti-HLA-ABC (W6/32) and anti-HLA-DR (L243) blocking antibodies, and in the presence or absence of anti-CD1a antibody or control IgG antibody. IFN-γ production was measured by ELISpot. Data were mean ± SEM (**B**). ** $p < 0.01$; **** $p < 0.0001$; ns no significance.

4.2.2 CD1a-autoreactive T cells produce IL-17A and IL-22

Apart from IFN-γ, the production of IL-17A and IL-22 by the CD1a-autoreactive T cells was measured (Figure 4.4). The CD1a-autoreactive T cells from both healthy and psoriasis individuals produced IL-17A in response to K562-CD1a but not K562-EV cells ($p < 0.01$ and $p < 0.001$ respectively) (Figure 4.4A). Furthermore, while the CD1a-autoreactive T cells from healthy donors did not produce significant amount of IL-22, those from psoriasis patients produced higher level of IL-22 by comparison ($p < 0.01$) (Figure 4.4B).

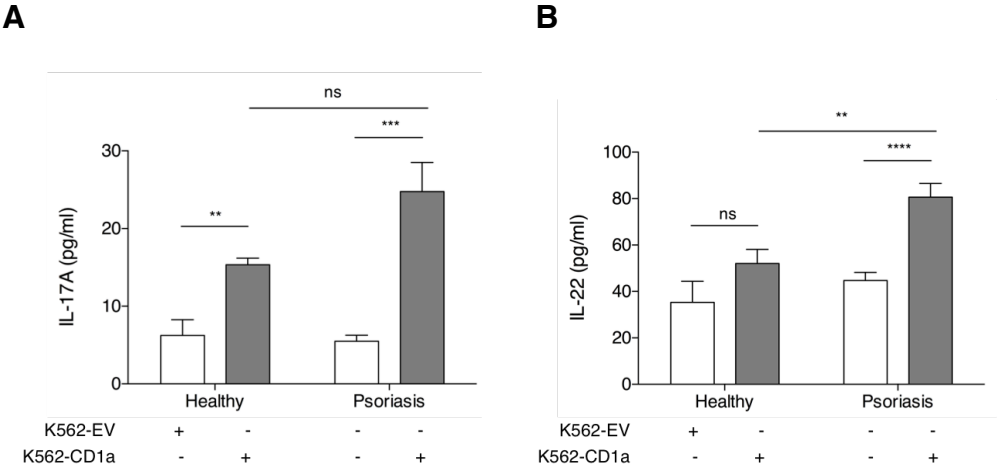


Figure 4.4 Circulating CD1a-autoreactive T cells produce IL-17A and IL-22

K562-EV/K562-CD1a cells were incubated with T cells from psoriasis patients (n=9) or healthy donors (n=6), and IL-17A (A) and IL-22 (B) production was measured using ELISA. Results were mean ± SEM. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns no significance.

4.2.3 CD1a presents host-derived lipids to T cells

Although a number of psoriasis-associated peptide antigens derived from exogenous or endogenous sources were suggested, recent studies have indicated the significance of non-peptide antigenic responses relevant to skin disorders [272-274]. Based on the findings in the previous section, there was an enriched population of CD1a-autoreactive T cells determined in the peripheral blood of psoriasis patients compared to healthy individuals. In addition, increased concentrations of a number of PLA_2-derived lipids, such as arachidonic acid, were described previously to be present in psoriatic lesions [301-304]. A series of cultured ELISpot experiments were performed to examine the antigenic potential of the lipids. In these ELISpot assays, T cells isolated from psoriasis patients were expanded using lipid-pulsed autologous mDCs for 10-14 days prior to the experiments.

First, the antigenic potential of arachidonic acid, a C20:4 polyunsaturated lipid, was investigated (Figure 4.5). CD1a-reactive T cells produced significant level of IFN-γ in response to arachidonic acid ($p < 0.05$). The concentration of arachidonic acid used in this experiment was based on previous similar experiments [272]. The reactivity was CD1a-specfic as it could be reduced in the presence of anti-CD1a blocking antibody but not isotype control (Figure 4.5). Interestingly, this response was potentiated in the presence of 10 μM of indomethacin, which is an

inhibitor of cyclooxygenase (COX), an enzyme upstream of prostaglandin synthesis pathways ($p < 0.05$) (Figure 4.5). On the contrary, the CD1a-reactive response was inhibited in the presence of 10 μM of baicalein, an inhibitor of lipoxygenase pathways ($p < 0.05$) (Figure 4.5).

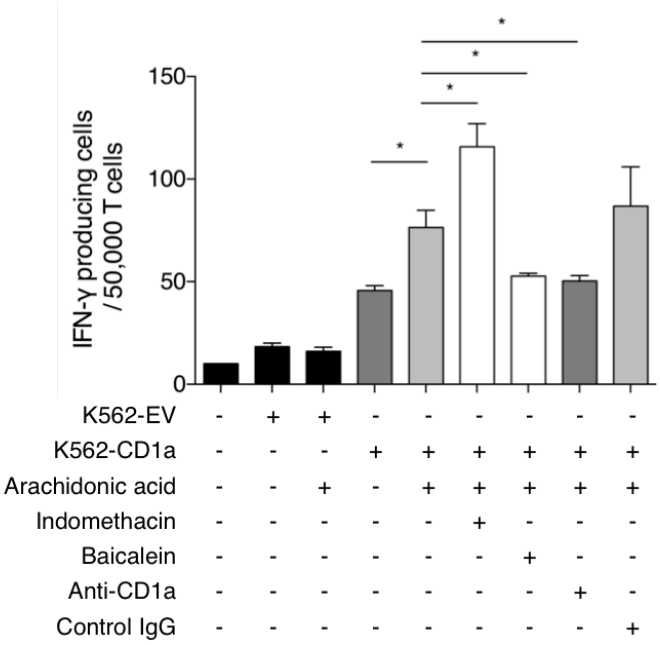


Figure 4.5 CD1a-reactivity of T cells in response to arachidonic acid

$CD3^+$ T cells from psoriasis donors were expanded using autologous mDCs that were pulsed with 10 μg/ml of arachidonic acid for 10-14 days prior to the ELISpot assay. For the ELISpot assay, K562-EV/K562-CD1a cells were incubated with 10 μg/ml of arachidonic acid, and then incubated with the T cells in the presence or absence of indomethacin (10 μM),

baicalein (10 μM), anti-CD1a blocking antibody or control IgG antibody, and IFN-γ production was measured by ELISpot assay. Results are typical representation of at least three individuals experiments. Data were mean ± SEM. * $p < 0.05$.

Based on the findings in Figure 4.5, the antigenic potential of a panel of C20 polyunsaturated lipids relevant to the syntheses of prostaglandins and leukotrienes was investigated. First, cultured ELISpot assays were performed using a number of lipids related to the lipoxygenase pathway. Strong CD1a reactivity of T cells were observed when the antigen source was 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraneonic acid (12-HETE) ($p < 0.0001$) (Figure 4.6A), of which elevated concentrations in the psoriatic lesions were reported previously [301]. Similar CD1a reactivity of T cells was also observed when using 15-hydroxyperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HpETE), and 13-hydroperoxy-9Z-11E-octadecadienoic acid (13-HpODE) ($p < 0.001$ and $p < 0.05$ respectively), but not 5-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HpETE) as the antigen source (Figure 4.6B-D). These findings supported the previous results that baicalein, an inhibitor of lipoxygenase pathways, could reduce the CD1a reactivity of T cells. The antigenic potential of two prostaglandins, PGE_2 and $PGF_{2\alpha}$, was also examined (Figure 4.7). Elevated levels of these two prostaglandins in psoriatic

lesions were also reported previously [301]. However, no significant CD1a reactivity of T cells was observed when using them as the antigen source (Figure 4.7). The concentrations of the lipids used in these experiments were based on the previous similar experiments [272].

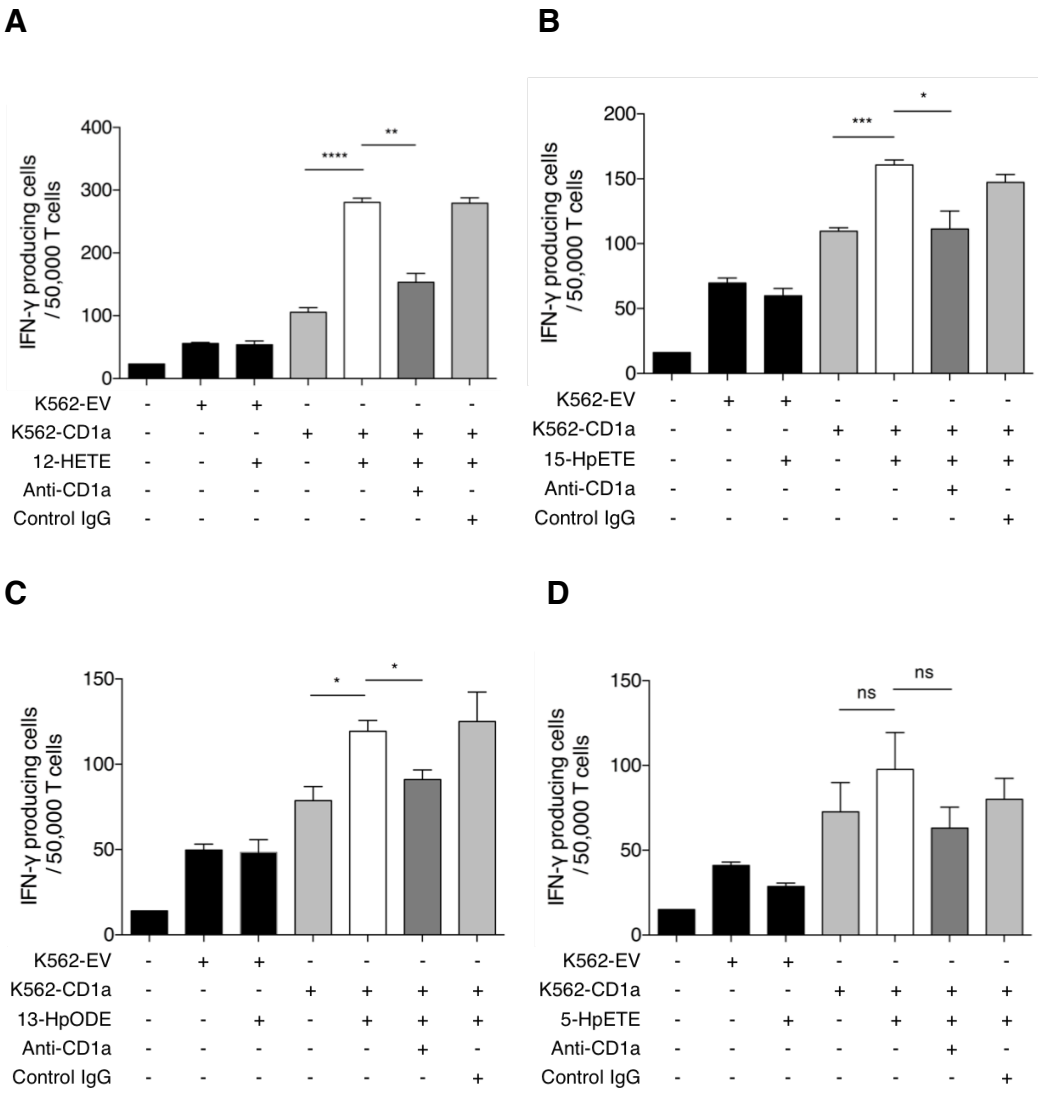


Figure 4.6 CD1a-reactivity of T cells in response to lipids relevant to lipoxygenase pathways

$CD3^+$ T cells from psoriasis donors were expanded using autologous mDCs that were pulsed with 10 μg/ml of different lipids: 12-HETE (**A**), 15-HpETE (**B**), 13-HpODE (**C**), and 5-HpETE (**D**) respectively for 10-14 days prior to the ELISpot assay. For the ELISpot assay, K562-EV/K562-CD1a cells were incubated with 10 μg/ml of the lipids, and then incubated with the T cells in the presence or absence of anti-CD1a blocking antibody or control IgG antibody, and IFN-γ production was measured. Results are

typical representation of at least three individual experiments. Data were mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns no significance.

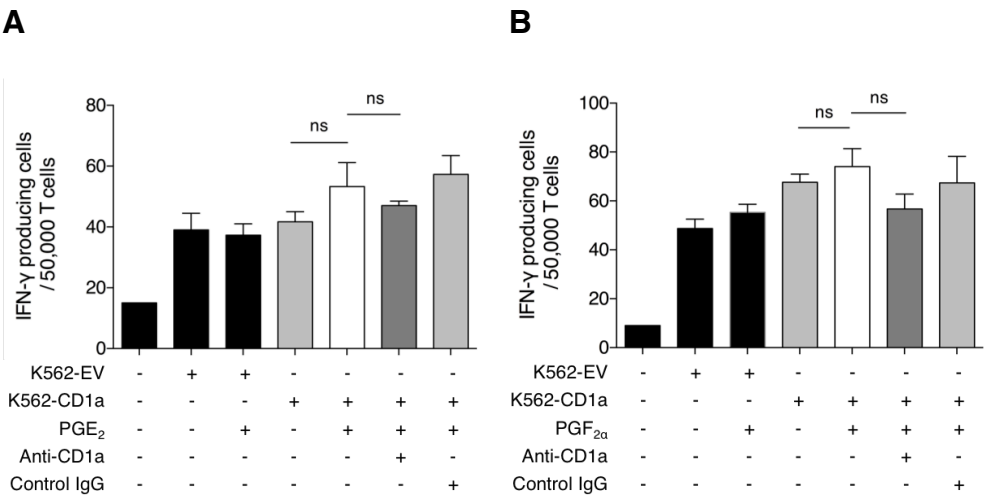


Figure 4.7 CD1a-reactivity of T cells in response to prostaglandins

$CD3^+$ T cells from psoriasis donors were expanded using autologous mDCs that were pulsed with 10 μg/ml of either PGE_2 or $PGF_{2\alpha}$ for 10-14 days prior to the ELISpot assay. For the ELISpot assay, K562-EV/K562-CD1a cells were incubated with 10 μg/ml of the prostaglandins, and then incubated with the T cells in the presence or absence of anti-CD1a blocking antibody or control IgG antibody, and IFN-γ production was measured. Results are typical representation of at least three individual experiments. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns no significance.

Next, a panel of lysophospholipids and fatty acids (FA), which are the two types of immediate products liberated from phospholipids by the action of PLA_2, was tested. Two lysophosphatidylcholine (LPC) with different chain length and degrees of saturation, namely LPC 18:1 and LPC 16:0, as well as two fatty acids, FA 18:1 (oleic acid) and FA 16:0 (palmitic acid), were used as the antigen source in this set of experiments, and significant CD1a reactivity was observed ($p < 0.05$, $p < 0.05$, $p < 0.05$, and $p < 0.05$ respectively) (Figure 4.8). The concentrations of the lipids used in these experiments were based on the previous similar experiments [272].

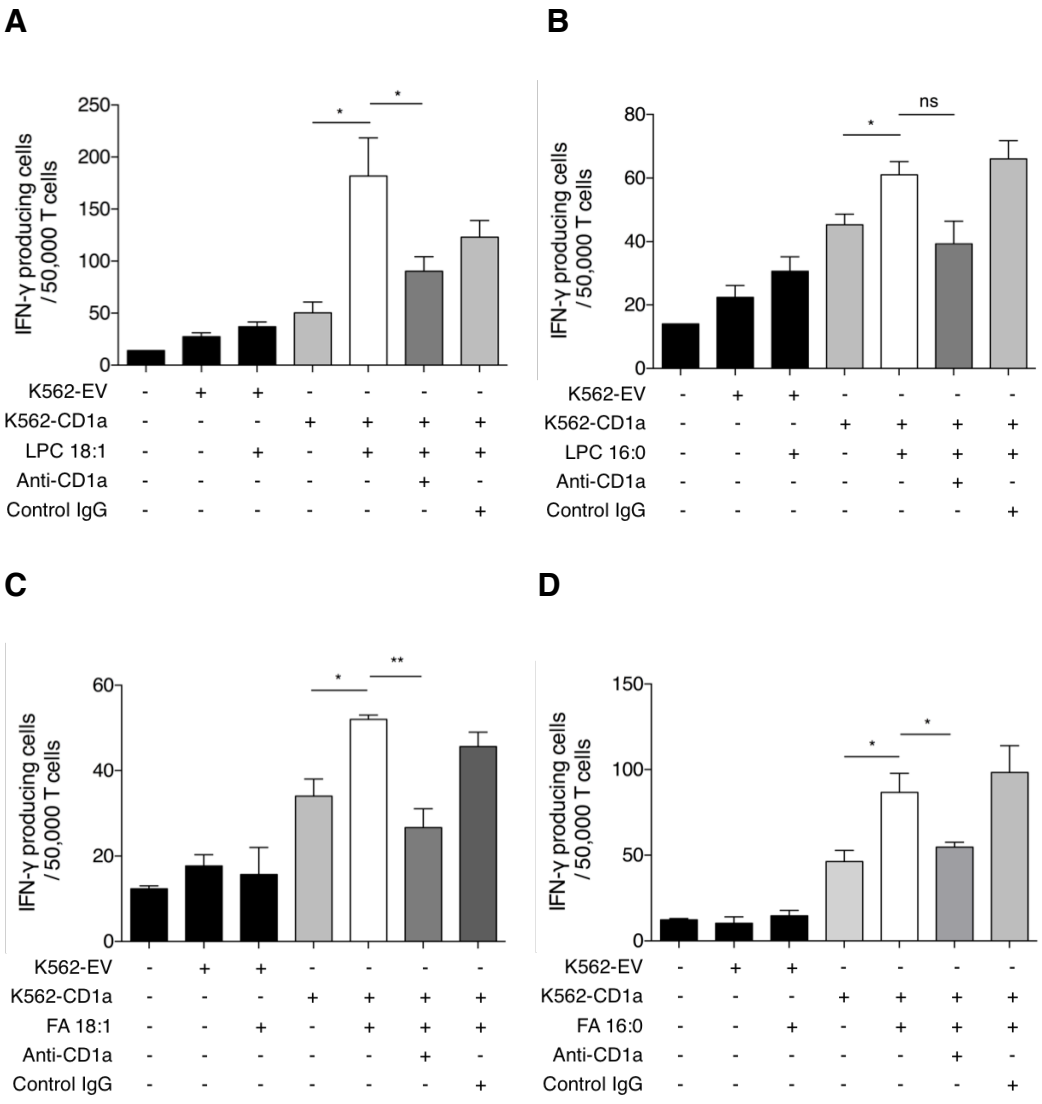


Figure 4.8 CD1a-reactivity of T cells in response to lysophospholipids and fatty acids

$CD3^+$ T cells from psoriasis donors were expanded using autologous mDCs that were pulsed with 10 μg/ml of lysophosphatidylcholine (LPC) (LPC 18:1 (**A**) and LPC 16:0 (**B**)) or fatty acids (C18:1 (**C**) and C16:0 (**D**)) for 10-14 days prior to the ELISpot assay. For the ELISpot assay, K562-EV/K562-CD1a cells were incubated with 10 μg/ml of the lipids, and then incubated with the T cells in the presence or absence of anti-CD1a blocking antibody or control IgG antibody, and IFN-γ production was

measured. Results are typical representation of at least three individuals experiments. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; ns no significance.

4.3 Discussion

Psoriasis is a complex inflammatory skin disease with a complicated pathogenesis due to a number of cell types involved in the process. The disease is commonly accepted as a lymphoid cell-mediated disorder due to the increased infiltration of leukocytes including $CD4^+$ and $CD8^+$ T cells and ILC3s. Besides T cells, antigen-presenting cells, such as Langerhans cells and dendritic cells play a number of important roles in the disease as well. Numerous efforts have been invested in the search for peptide antigens for the T cell response in psoriasis. Several foreign antigens, such as streptococcal and staphylococcal antigens, as well as self-antigens including keratins, melanocyte antigens, and LL-37 were described [213-216].

Nonetheless, some evidence has suggested a potential role of non-peptide antigens for T cell responses. Firstly, given the association of abnormal migration of Langerhans cells and psoriasis [200], it remains possible there is a potential role of CD1a, a non-peptide antigen-presenting molecule highly expressed on Langerhans cells. Secondly, de Jong *et al.* demonstrated that the CD1a-autoreactive $CD45RO^+$ memory T cells in peripheral blood express CLA, a skin-homing marker [218]. Thirdly, the same group has recently shown that CD1a could present “headless” skin oils such as squalene, fatty acids, and wax esters [217], and

proposed that the $CD1a^+$ Langerhans cells can migrate out of epidermis and present these lipids upon physical injury of the skin or infection [316]. Although it has been known that CD1a-autoreactive T cells are a normal part of T cell repertoire in normal healthy individuals [218], little is known about the role of CD1a-expressing antigen-presenting cells and CD1a-autoreactive T cells in psoriasis.

In this study, the role of CD1a and CD1a-autoreactive T cells in psoriasis was investigated. Polyclonal $CD3^+$ T cells were isolated from the peripheral blood of healthy and psoriasis individuals using CD3 magnetic bead separation, and a high yield of $CD3^+$ T cells was confirmed by more than 90% of total cells expressing CD3 (Figure 4.1).

The CD1a autoreactivity in peripheral blood T cells from both healthy individuals and psoriatic patients was determined by measuring IFN-γ production. The concentration of IFN-γ, a major cytokine in the Th1 response, is usually elevated in the lesional skin of psoriasis, and the association of Th1 response and psoriasis has been long established [317, 318]. Also, based on the prior knowledge that CD1a-autoreactive T cells could secrete a number of cytokines including IFN-γ, TNF-α, and IL-22 [218], the IFN-γ production of the CD1a-autoreactive T cells was determined in the current study using *ex vivo* ELISpot assays. Using K562-CD1a cells, as well as autologous mDCs as the CD1a-expressing

antigen-presenting cells, the IFN-γ producing CD1a-autoreactive T cells were identified in both healthy and psoriasis individuals. In particular, the frequency of CD1a-autoreactive T cells in psoriasis patients, being around 0.04%, was higher than that in healthy individuals, which was about 0.01-0.02% (Figure 4.2 and 4.3). According to the findings by de Jong *et al.* in 2010, the frequency of CD1a-autoreactive T cells in peripheral memory T cells in healthy individuals was about 0.02-0.4% [218]. It is of note that they determined the frequency of CD1a-autoreactive T cells out of $CD45RO^+$ memory T cells rather than total peripheral blood T cells as in our study. Therefore such frequency in the current study was expected to be lower. Nonetheless, our findings, consistent with past studies, indicated that peripheral blood of healthy individuals contains a subpopulation of T cells that are autoreactive to CD1a.

Apart from IFN-γ, these T cells could also produce IL-17A and IL-22 (Figure 4.4), suggesting that these cells might be responsible for the increased concentrations of IL-17A and IL-22 in the lesional skin [156, 158], and hence indicating their potential role in the Th17 and Th22 responses in the lesions of psoriatic patients.

The data show the enrichment of circulatory CD1a-autoreactive T cells in psoriasis patients, compared to healthy individuals. The two-fold increase in frequency of CD1a-autoreative T cells in the blood implied the

significance of CD1a autoreactivity in psoriasis. While several conventional peptide antigens that are relevant to psoriasis have been described, recent studies have suggested that self-antigens, including LL-37 and melanocytes antigens, are relevant to psoriasis [213, 216]. However, de Jong and colleagues revealed that CD1a-autoreactive T cells, that are part of normal T cell repertoire in healthy individuals, could recognize self-lipids derived from the skin [217, 218]. The current findings have highlighted the relevance of CD1a autoreactivity to psoriasis, and suggested that CD1a autoreactivity might potentially contribute to the pathogenesis of the disease. One possible explanation for the enrichment of CD1a-autoreactive T cells in the peripheral blood of psoriasis patients is that it could be triggered by the initial antigen presentation by $CD1a^+$ antigen-presenting cells. In the early phase of psoriasis, physical injury to the skin or infection initiates the early response of psoriasis, and $CD1a^+$ antigen-presenting cells, such as Langerhans cells and dermal DCs, may be potentially exposed to non-peptide antigens, and present such antigens to CD1a-autoreactive T cells, leading to the expansion of the T cells. As a result, there is a higher frequency of CD1a-autoreactive T cells in the circulation during the chronic phase of psoriasis. However, despite the frequency of circulating CD1a-autoreactive T cells of the psoriasis patients being higher than that of the healthy individuals, the significance of 0.04% of total (1 in 2500) of these T cells in the blood of psoriasis patients in contributing to the disease might be questionable. By comparison, CD1d-

restricted iNKT cells represent 2% (1 in 50) in the peripheral blood of healthy individuals. The relatively low frequency of CD1a-autoreactive T cells in the circulation might be attributed to the tight regulation of potential T cell autoreactivity. Nonetheless, the frequency of these T cells was determined based on ELISpot assays without the addition of lipid antigens. Therefore, the CD1a reactivity in the presence of non-peptide antigens was then investigated.

Some evidence presented here supported that non-peptide antigen presentation by CD1a is relevant to psoriasis. The data have suggested that the endogenous lipids expressed by K562 and mDCs represent CD1a ligands, raising the possibility that pathways which generate fatty acids may be relevant to disease. Such ligands can include headless antigens including fatty acids. It has been known that the phospholipase A_2 (PLA_2) activity is elevated in skin of psoriasis patients [301]. Furthermore, the levels of metabolic products of PLA_2 activity were higher in the lesional skin than in the uninvolved skin of psoriasis [302-304]. Based on these studies, cultured ELISpot assays were performed to examine the antigenic potential of a panel of PLA_2-derived lipids. Arachidonic acid, a C20:4 fatty acid liberated from phospholipids by the action of PLA_2, was firstly tested, and it was shown that CD1a-reactive T cells could be activated in response (Figure 4.5). Furthermore, the arachidonic acid-responsive CD1a reactivity could be potentiated by indomethacin, which is an inhibitor of

cyclooxygenase (COX)-1 and COX-2. The inhibition of COX pathways could lead to a diversion of PLA_2-derived lipid synthesis towards lipoxygenase pathways. As a result, in the presence of indomethacin, the lipid syntheses downstream of COX would be reduced, while those downstream of lipoxygenase would be increased due to a diversion to this pathway. This was supported by reduction of arachidonic acid-responsive CD1a reactivity in the presence of baicalein, an inhibitor of lipoxygenase pathways (Figure 4.5).

Therefore, four different C20 lipids relevant to the lipoxygenase pathways were used as the antigen source in the following ELISpot assays, and found that 12-HETE, 15-HpETE, and 13-HpODE, but not 5-HpETE, were able to activate CD1a-reactive T cells to produce IFN-γ (Figure 4.6). It is notable that elevated level of 12-HETE in psoriatic lesions was described previously [301], hence supporting the current findings that lipids, especially those relevant to lipoxygenase pathways, might contribute to the lipid-responsive CD1a reactivity of T cells in psoriasis. This was supported by the findings that no significant CD1a reactivity of T cells was detected when using prostaglandins as the antigen source (Figure 4.7). Next, the antigenic potential of lipids in different chain lengths and degrees of saturation was then examined. Two LPC, namely LPC 18:1 and LPC 16:0, and two fatty acids, oleic acid (FA 18:1) and palmitic acid (FA 16:0) were tested. These lipids were included

because they are immediate enzymatic products liberated from phospholipids by the action of PLA_2. Significant LPC- and FA-responsive CD1a reactivity of T cells were detected (Figure 4.8), consistent with a previous study [272]. These results showing that CD1a-expressing antigen-presenting cells could present PLA_2-derived CD1a ligands to CD1a-reactive T cells were consistent with the studies showing that bee venom- and house dust mite-derived PLA_2 can generate neolipid antigens for antigen presentation by CD1a [272, 274]. Altogether, the findings have suggested the association of PLA_2-derived lipid-responsive CD1a reactivity of T cells and psoriasis.

Recently Birkinshaw *et al.* have proposed that broad groups of antigens can be “permissive” for CD1a interaction with T cells, and the above data using a range of PLA_2-derived lipids would fit with that hypothesis [260]. They showed that permissive antigens facilitated TCR interaction through modulating the position of the alpha1/2 helices of CD1a, which in turn allowed TCR binding. In contrast, non-permissive antigens disrupted the CD1a:TCR contact zone and abrogated binding. Interestingly, many of the permissive antigens (including C18:1 tested above) did not make direct contact with the TCR which would also explain recognition of antigens with small head groups or no head groups (“headless”). The data support the previously described possibility that PLA_2-derived lipids represent CD1a ligands but show that enhanced

responses may be relevant to psoriasis. Furthermore the data show that modulation of the PLA_2 pathway can affect the generation of CD1a binding ligands, with potential therapeutic implications, such as the use of inhibitors of lipoxygenase pathway for treatment of psoriasis.

To summarise, the peripheral blood of psoriasis patients, compared to that of healthy individuals, contained an enriched subpopulation of CD1a-autoreactive T cells that are able to produce IFN-γ, IL-17 and IL-22. The CD1a reactivity of these T cells could be potentiated by a number of host-derived PLA_2-derived lipids. The data have led to a question of the source of PLA_2 in psoriatic lesions, and the role of PLA_2 in the CD1a-lipid presentation in psoriasis, and detailed discussion is included in the next chapter.

5. Phospholipase A_2 activates CD1a-reactive T cells in Psoriasis

5.1 Introduction and Aims

Potential relevance of phospholipase A_2 activity in psoriasis

The potential role of phospholipase A_2 (PLA_2) activity in psoriasis was described in the 1970s and 1980s. Hammaström *et al.* demonstrated the increased concentrations of the immediate and downstream products of PLA_2, including arachidonic acid, 12-HETE, PGE_2, and $PGF_{2\alpha}$ in the involved epidermis, compared to uninvolved epidermis, of psoriasis [301]. On the other hand, Forster and colleagues reported that raised PLA_2 activity was present in the epidermis of uninvolved, rather than involved, psoriatic skin [302-304]. Nonetheless, Verhagen *et al.* showed that both uninvolved and involved epidermis of psoriasis contained elevated PLA_2 activity [305]. Despite the relative differences between these conclusions, all suggested that PLA_2 activity might be relevant to psoriasis.

Proposing a unique PLA_2 group potentially associated to psoriasis, Bergers *et al.* however did not further describe the identity and properties of such PLA_2 [319]. Andersen *et al.* reported an elevated expression of

non-pancreatic PLA_2, as well as cytosolic PLA_2, in psoriatic tissues [306]. Although some phenomena of psoriasis could be explained by the action of eicosanoids generated through the arachidonic acid cascade downstream of PLA_2, the true identity of a psoriasis-associated PLA2 had not yet been understood, until Chiba *et al.* revealed a novel PLA_2, namely $cPLA_2\delta$, or PLA2G4D, of which expression was higher in psoriatic lesions than in normal healthy skin [299]. RNA expression of PLA2G4D was detected in the upper epidermis of psoriatic lesions, while its protein expression was detected in both the upper epidermis and dermis of lesions. On the contrary, no expression of PLA2G4A ($cPLA_2\alpha$) was found in psoriatic lesions, implying that the elevation of PLA_2 activity was attributed to PLA2G4D but not PLA_2 in general. Therefore, this was the first evidence of the potential relevance of PLA2G4D in psoriasis. Moreover, a recent study by Quaranta *et al.* supported this idea. In their intraindividual genome expression analysis using psoriasis and eczema cohorts, they revealed the up-regulation of PLA2G4D in psoriatic lesions [300]. However it came into question how the products of PLA2G4D could contribute to the pathogenesis of psoriasis. Our group has previously reported that PLA_2 derived from exogenous sources could generate neolipid antigens for presentation by CD1a-expressing antigen-presenting cells to CD1a-restricted T cells in atopic dermatitis [272-274]. Therefore we sought to investigate the link between PLA2G4D and CD1a-antigen presentation. Details are discussed below.

Phospholipase A_2 generates neolipid antigens for presentation by CD1a

Bourgeois and Subramaniam *et al.* revealed that bee and wasp venom-derived PLA_2 activated T cells by generating neolipid antigens [272]. Some but not all PLA_2-derived neolipids could activate T cells via CD1a-antigen presentation. T cells produced IFN-γ in response to lysophosphatidylcholine 18:1 (LPC 18:1) but not palmitic acid, a C_{16} fatty acid, suggesting the importance of carbon chain length of the lipids. Significant reduction of CD1a-restricted T cell response by manoalide, a $sPLA_2$ inhibitor, indicated that the CD1a reactivity was mainly contributed by the neolipids generated by PLA_2 itself, rather than the existing lipids in the venom extract. Inhibition of CD1a reactivity by anti-CD1a antibody confirmed the CD1a dependence in the T cell response.

Similarly, bee and wasp venom-responsive CD1a reactivity was observed in the venom-allergic individuals [273]. In brief, Subramaniam *et al*. demonstrated that circulatory T cells from bee/wasp venom allergic individuals, but not healthy controls, responded to neolipid antigens presented by CD1a-transfected K562 cells and released IFN-γ, GM-CSF, and IL-13. Moreover, cross-venom T cell responses were observed, implying that PLA_2 from both venoms might generate similar products

upon acting on host phospholipids, therefore eliciting similar CD1a-reactive responses.

On the other hand, the generation of CD1a neolipid antigens by house dust mite-derived PLA_2 was described by Jarrett *et al.* [274]. In the study, it was found that CD1a-reactive T cells that responded to house dust mite (HDM) extract were enriched in patients with atopic dermatitis (AD), and they produced IFN-γ, GM-CSF, and IL-13. Both peripheral blood and skin CD1a-reactive T cells of AD patients showed HDM-specific CD1a reactivity towards HDM challenge. In addition, filaggrin could inhibit the CD1a-reactive response of T cells from peripheral blood and skin of atopic dermatitis patients, suggesting the potential role of filaggrin for providing both barrier function and direct inhibition of cutaneous lipid-specific immune responses.

Combining these studies, there is an obvious role of PLA_2 and PLA_2-derived neolipids for antigen presentation by CD1a-expressing cells in the skin. It is noted that the sources of PLA_2 of above studies were all exogenous. However, according to the current understanding of pathogenesis of psoriasis, there is little evidence about the involvement of exogenous PLA_2 in the initiation of psoriasis. Although several psoriasis-associated exogenous antigens, such as from streptococcus and staphylococcus, were identified, recent findings have suggested that

endogenous antigens could be more pivotal to psoriasis [213, 216]. Hence it is key to look for the endogenous source of CD1a antigens, especially given that PLA_2 activity is elevated in the skin of psoriasis.

Therefore, we hypothesised that the PLA2G4D, a cytosolic PLA_2, is the endogenous source of CD1a neolipid antigens for CD1a-reactive T cell response in psoriasis.

5.2 Results

5.2.1 PLA2G4D expression in psoriatic lesions

Based on the prior knowledge that PLA_2 activity was elevated in psoriasis skin [301-304], and in particular the association of the expression of PLA2G4D and psoriasis [299, 300], the potential role for PLA_2 in the generation of CD1a ligands was investigated. The published gene expression studies accessible in the Gene Expression Omnibus (GEO) were analysed. GEO is a public functional genomic database storing experimentally validated array- and sequence-based data. Up to date there are over 3800 “DataSets” (GDS), which contains above 67000 data series (GSE) in the GEO system. All data in GDS and GSE can be analysed by GEO2R, an interactive web tool that allows the comparison across two or more groups of samples, and results are presented as a table of the top 250 genes ranked by P-value. In other words, top 250 genes with most significant P-value are listed as a result.

The results of six human gene expression studies that are relevant to psoriasis from the Gene Expression Omnibus (GEO) were collected (Table 1). Gene expression in normal healthy skin, and non-lesional and lesion skin from psoriasis patients was analysed and compared across the

groups using GEO2R. In some studies, comparisons of gene expression were made between healthy, psoriasis non-lesional and psoriasis lesional skin groups, while in others comparisons were only available for the latter two skin groups.

Entry	Summary	Reference
GSE6710 (GDS2518)	Uninvolved and involved skin biopsies of 13 patients with mild to severe plaque psoriasis	[320]
GSE13355	Skin biopsies of 64 normal healthy donor; uninvolved and involved skin biopsies of 58 psoriasis patients	[321]
GSE14905 (GDS3539)	Skin biopsies of 21 normal healthy donor; uninvolved and involved skin biopsies of 28 psoriasis patients	[322]
GSE30999	Uninvolved and involved skin biopsies of 85 patients with moderate to severe psoriasis	[323]
GSE34248	Uninvolved and involved skin biopsies of 14 psoriasis patients	[324]
GSE41662	Uninvolved and involved skin biopsies of 24 psoriasis patients	[324]

Table 1 Gene Expression Omnibus entries relevant to psoriasis studies in humans

We analysed a number of genes that are relevant to PLA_2 and psoriasis, and summarized them in Figure 5.1 to 5.5. Notably, in three GEO Series, namely GSE30999, GSE34248 and GSE41662, we found the elevation of *PLA2G4D* gene expression in psoriasis in lesional skin compared to non-lesional skin, and it was among the top 250 genes with

most significant P-values (Figure 5.1). Elevation of *PLA2G4D* gene expression was in line with the findings by Quaranta *et al.*, which revealed the increased *PLA2G4D* gene expression level in the total skin biopsies from lesional skin of psoriasis patients [300]. This was also consistent with the findings by Chiba *et al.*, that PLA2G4D protein was preferentially expressed in psoriasis lesional skin [299].

Apart from *PLA2G4D* gene, other PLA_2 genes, and genes that are related to PLA_2 or psoriasis were also analysed. In Figure 5.2, expression results of GEO DataSet GDS2518, containing genes of *IL-17A*, various $sPLA_2$ and $cPLA_2$, secretory PLA_2 receptor 1 (*PLA2R1*), and PLA_2 activating protein (*PLAA*), were listed. PLA2R1 is a transmembrane receptor protein for $sPLA_2$. PLAA can increase eicosanoid production through PLA_2 pathway [325], and is thought to be related to inflammatory bowel disease [326]. As expected, *IL-17A* expression level was higher in lesional than in non-lesional skin of psoriasis. It was notable that there was a slight elevation in expression levels of two $sPLA_2$, *PLA2G2F* and *PLA2G3*, and one other $cPLA_2$, *PLA2G4B*, while other PLA_2 gene levels showed no significant difference across two skin groups. Expression levels of two PLA_2-related genes, *PLA2R1* and *PLAA*, remained relatively constant between non-lesional and lesional psoriasis skin groups (Figure 5.2). Interestingly, in GEO Series GSE13355, *PLA2G4B* gene level was also found elevated in psoriasis lesional skin group compared to psoriasis

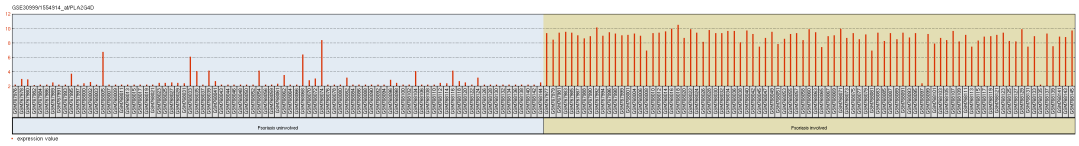
non-lesional and normal healthy skin (Figure 5.3). PLA2G4B, similar to the other group members in the $cPLA_2$ family, can cleave phospholipids and generate free fatty acids and lysophospholipids. In GEO DataSet GDS3539, again *PLA2G2F*, *PLA2G3* and *PLA2G4B* showed slightly elevated gene expression levels in psoriasis lesional skin group, which were not found in other PLA_2 (Figure 5.4). Also similar to results of GDS2518, *IL-17A* gene expression level was higher in lesional skin group. Intriguingly, the analysis of GSE30999 demonstrated the elevation of expression of *PLA2G4B*, which was similar to the findings from the analysis of GDS2518, GSE13355 and GDS3539 (Figure 5.5).

In particular, *PLA2G4B* appeared among the top 250 genes in two out of the all four aforementioned analyses. Although the association of PLA2G4D and psoriasis has been investigated by several studies, whether PLA2G4B has any role in psoriasis was not yet known. Nonetheless, considering that both PLA2G4B and PLA2G4D are $cPLA_2$, it might be possible that they might share a common role in the pathogenesis of psoriasis. However, the definition of such role requires further investigation.

Altogether, the analyses of six gene expression results retrieved from GEO concluded that PLA2G4D is highly relevant to psoriasis, as the results indicated that *PLA2G4D* gene expression was significantly

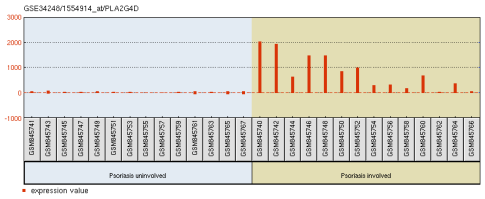
elevated in psoriasis lesional skin. Based on these, the protein expression of PLA2G4D in psoriatic lesional skin was examined as shown in the next section.

PLA2G4D (GSE30999)



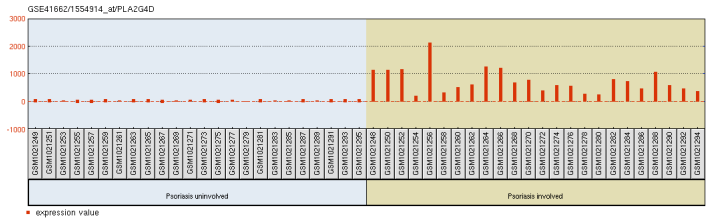
Raw P-value = 1.17 x 10^{-77}; adjusted P-value = 9.14 x 10^{-74}

PLA2G4D (GSE34248)



Raw P-value = 5.98 x 10^{-8}; adjusted P-value 1.53 x 10^{-5}

PLA2G4D (GSE41662)

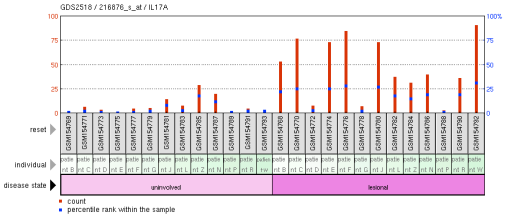


Raw P-value = 8.91 x 10^{-20}; adjusted P-value = 2.00 x 10^{-16}

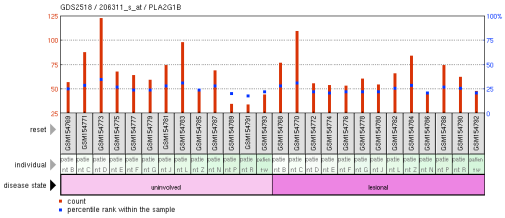
Figure 5.1 Gene Expression Omnibus shows elevated *PLA2G4D* gene expression in psoriatic skin

Data sets from three gene expression studies of skin biopsies from psoriasis patients were retrieved from Gene Expression Omnibus (GEO) series GSE30999, GSE34248 and GSE41662. Gene expression levels were compared across psoriasis uninvolved (non-lesional) and psoriasis involved (lesional) skin groups. Y-axis represents arbitrary gene expression value. Raw and adjusted P-values are listed below each series.

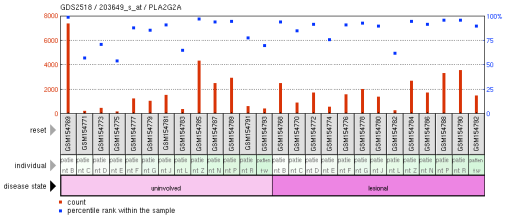
IL-17A



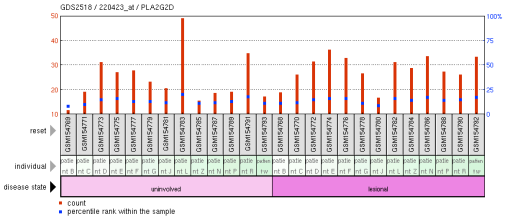
PLA2G1B



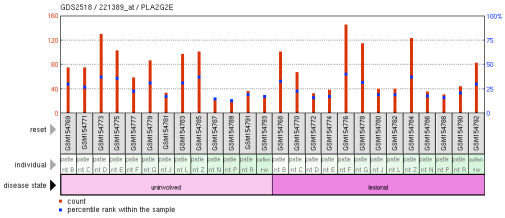
PLA2G2A



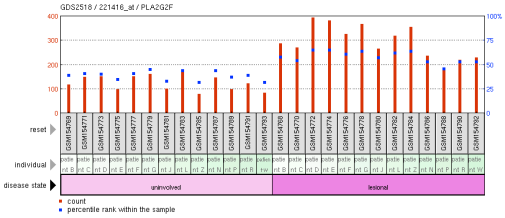
PLA2G4D



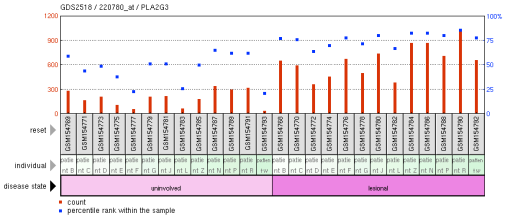
PLA2G2E



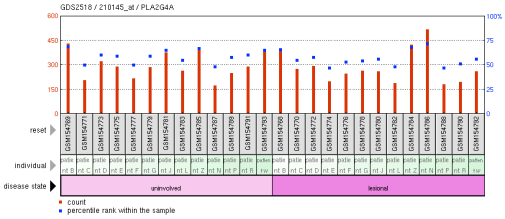
PLA2G2F



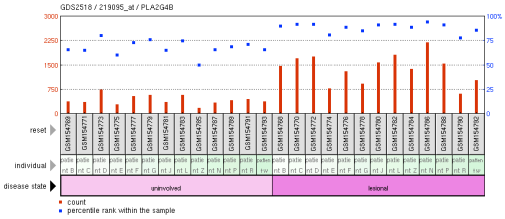
PLA2G3



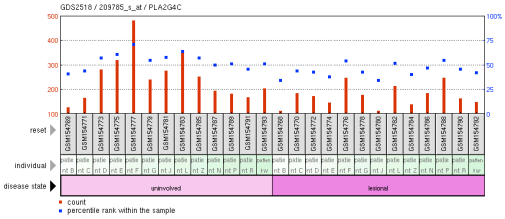
PLA2G4A



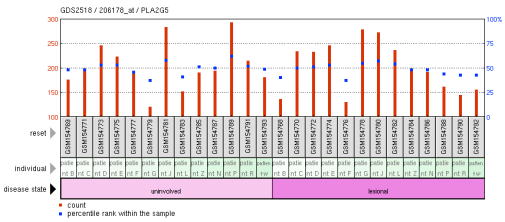
PLA2G4B



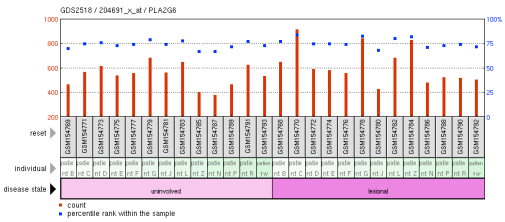
PLA2G4C



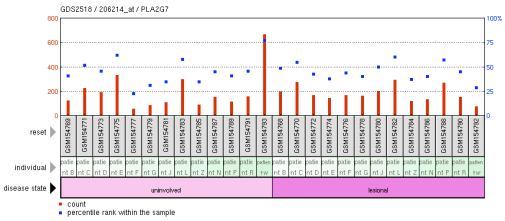
PLA2G5



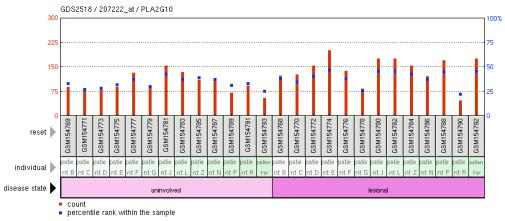
PLA2G6



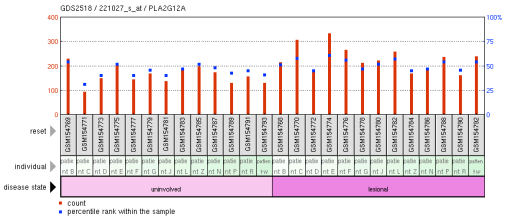
PLA2G7



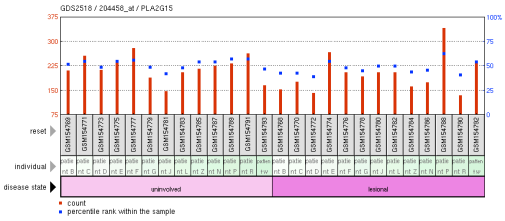
PLA2G10



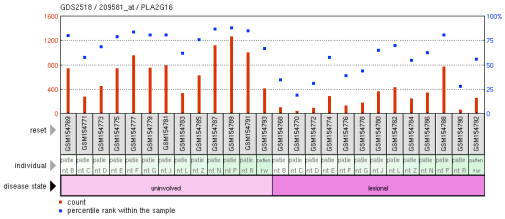
PLA2G12A



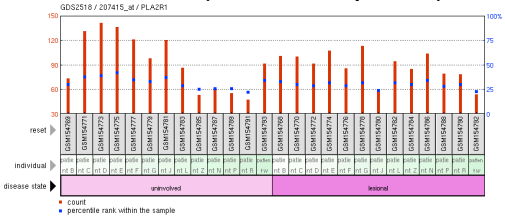
PLA2G15



PLA2G16



PLA2R1 (PLA_2 receptor 1)



PLAA (PLA_2 activating protein)

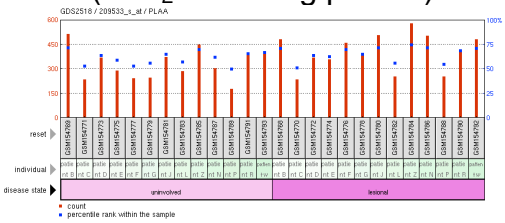


Figure 5.2 PLA_2- and psoriasis-relevant gene expression results in GEO DataSet GDS2518

Gene expression results of *IL-17A*, various PLA_2 groups, PLA_2 receptor 1, and PLA_2 activating protein were retrieved from GEO DataSet GDS2518. All the genes listed did not appear among the top 250 genes using GEO2R analysis.

PLA2G4B

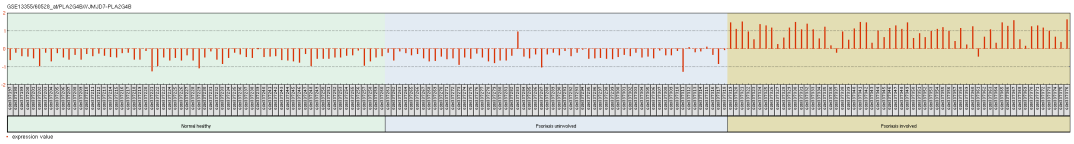
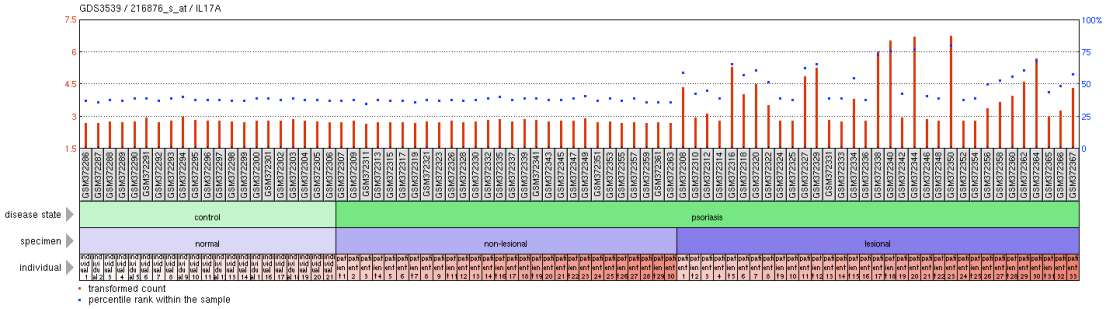


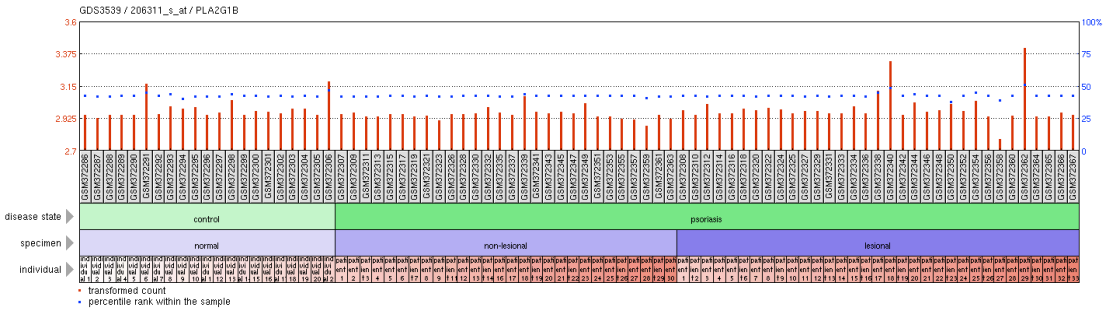
Figure 5.3 *PLA2G4B* gene expression results in GEO Series GSE13355

Gene expression results of *PLA2G4B* were retrieved from GEO Series GSE13355. *PLA2G4B* gene in this study appeared among the top 250 genes using GEO2R analysis.

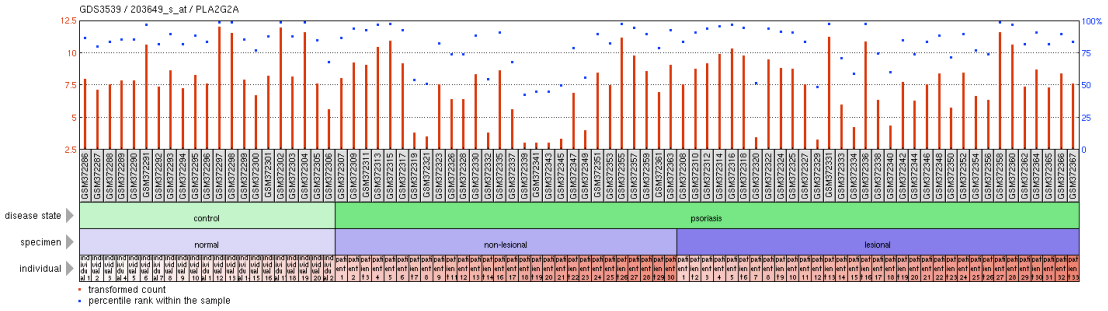
IL-17A



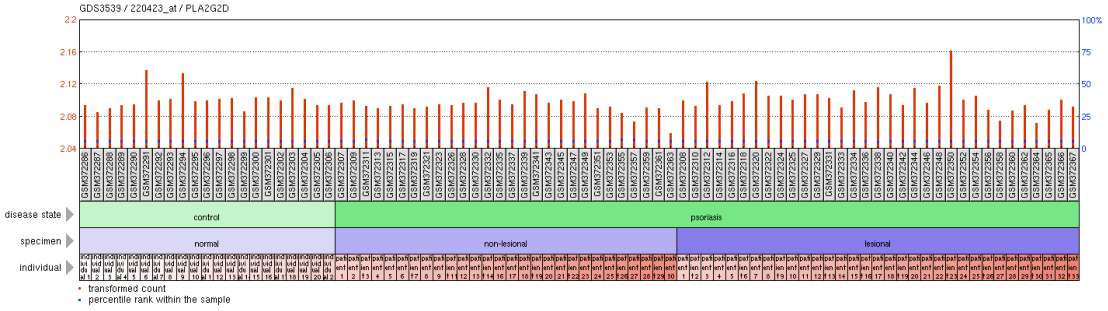
PLA2G1B



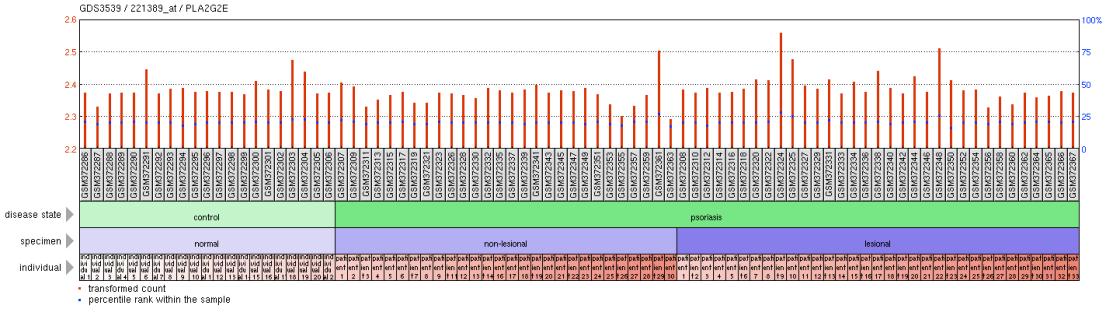
PLA2G2A



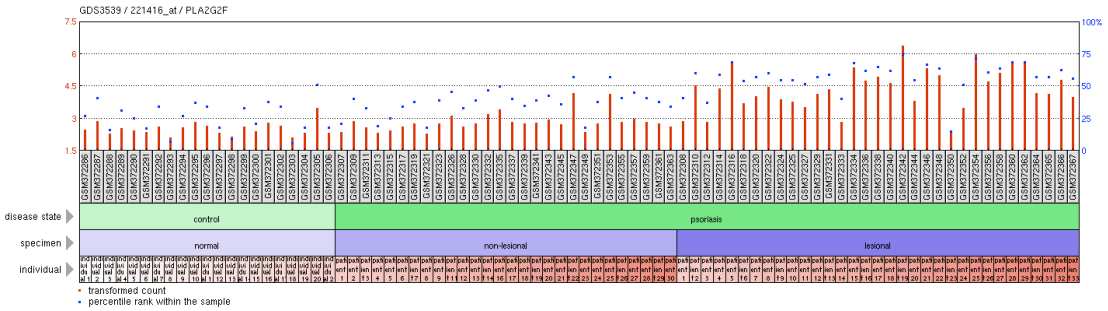
PLA2G2D



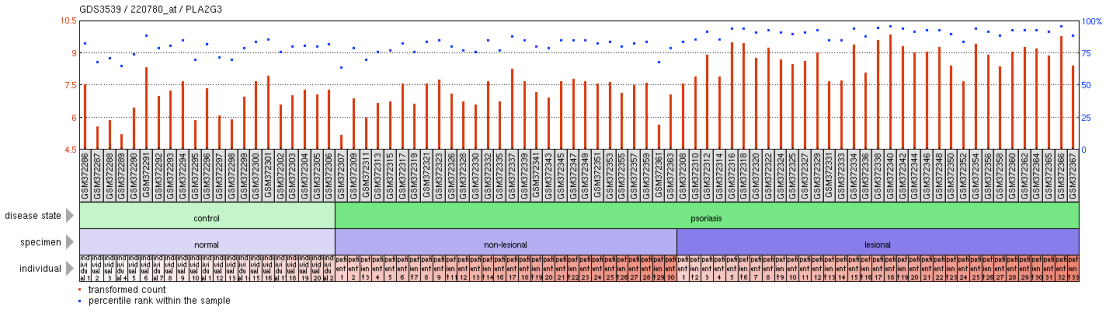
PLA2G2E



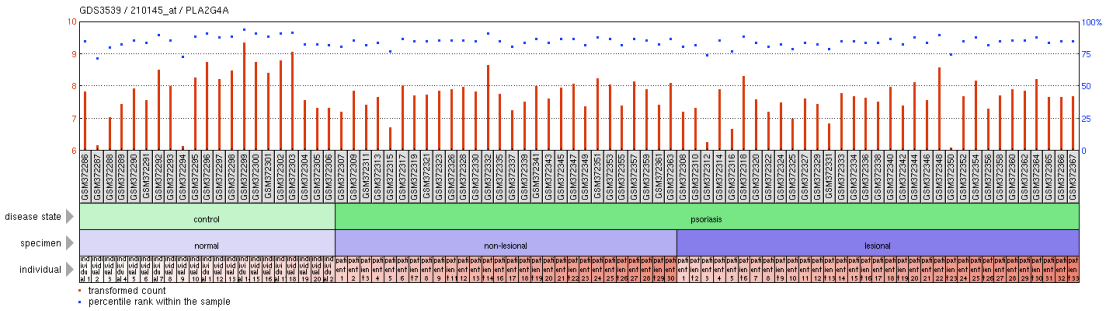
PLA2G2F



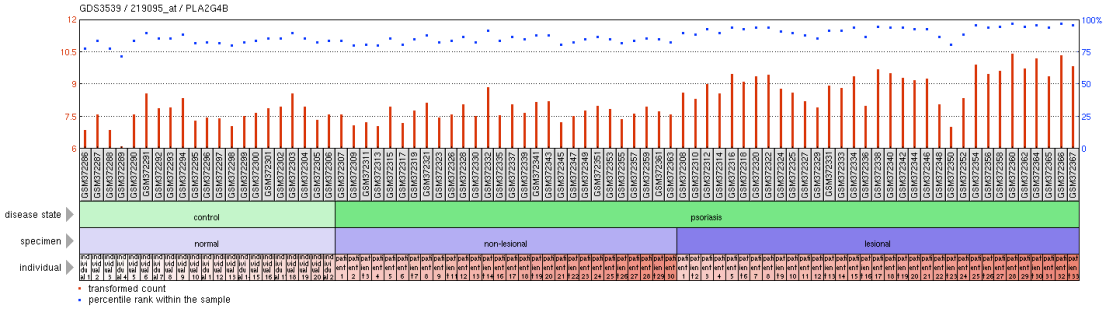
PLA2G3



PLA2G4A



PLA2G4B



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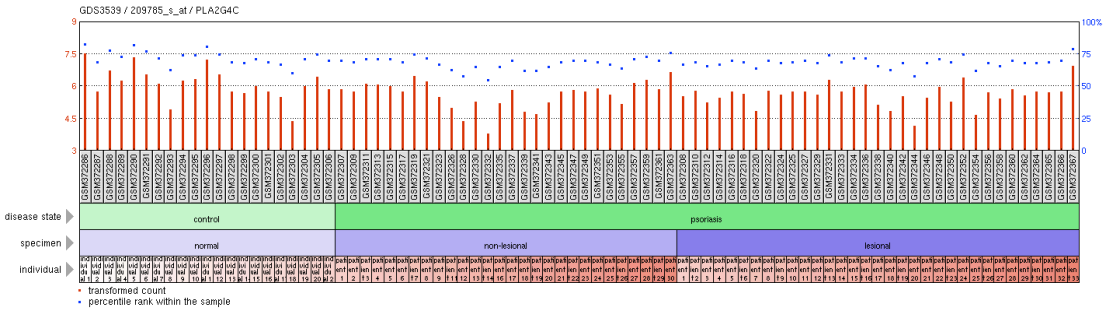
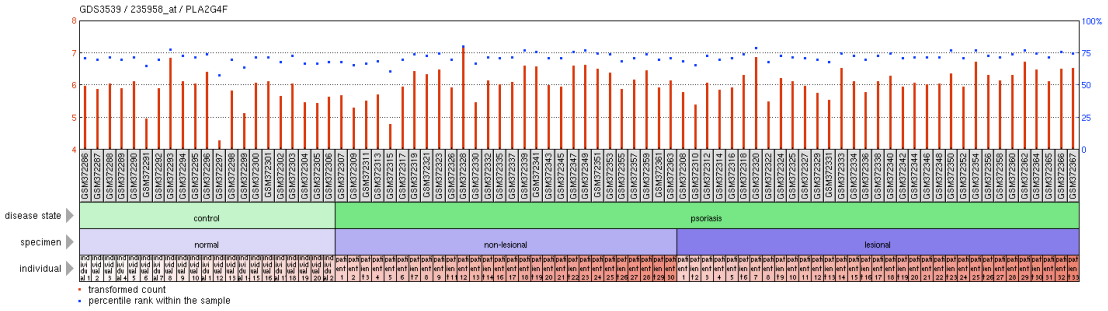
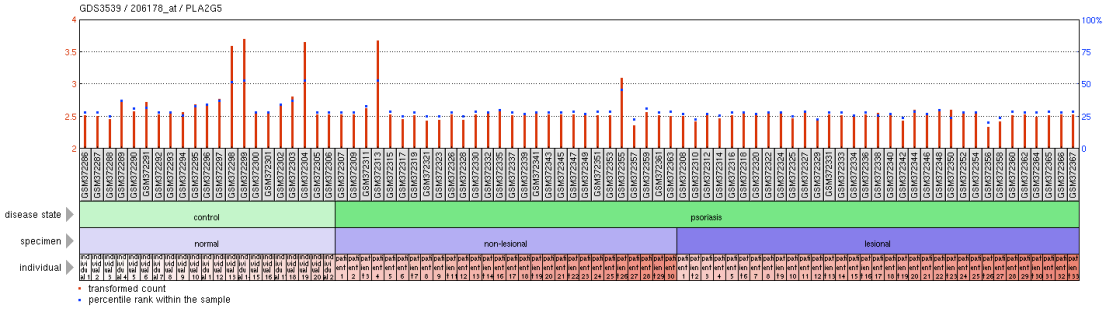


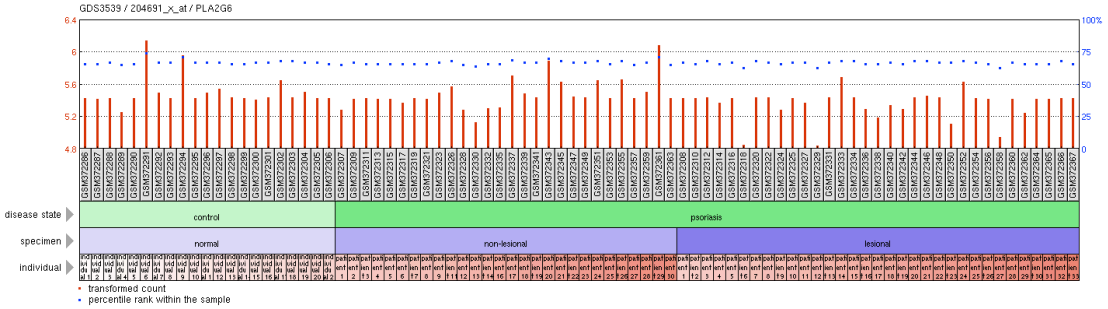
PLA2G4F



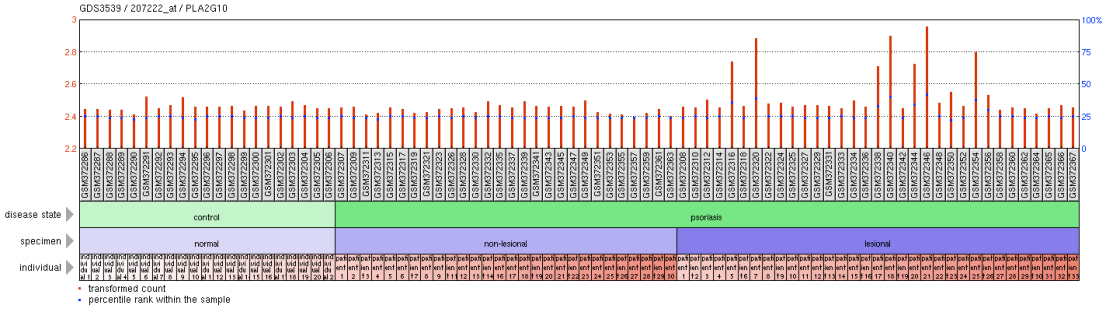
PLA2G5



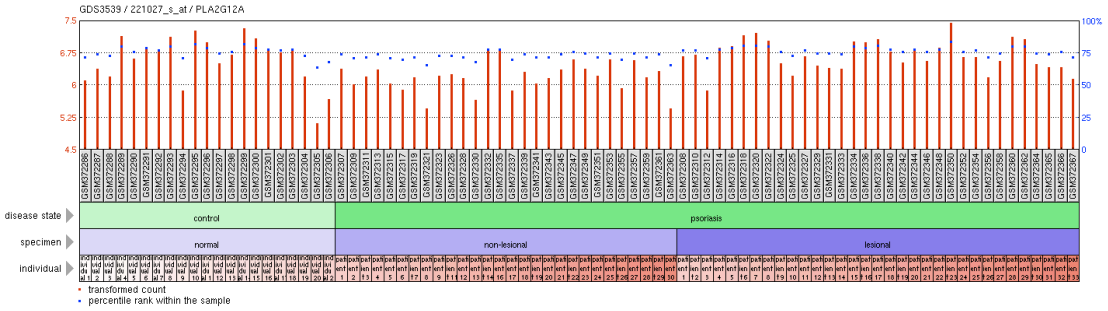
PLA2G6



PLA2G10



PLA2G12A



PLA2G12B

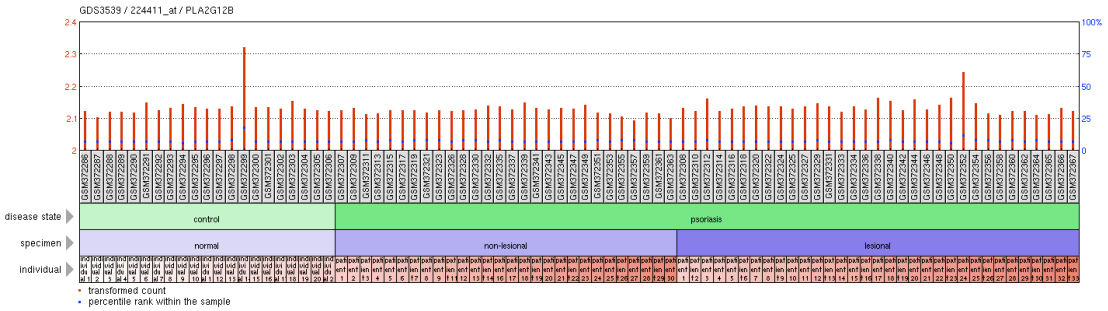


Figure 5.4 PLA$_2$- and psoriasis-relevant gene expression results in GEO DataSet GDS3539

Gene expression results of *IL-17A* and various PLA_2 groups were retrieved from GEO DataSet GDS3539. All the genes listed did not appear among the top 250 genes using GEO2R analysis.

PLA2G4B

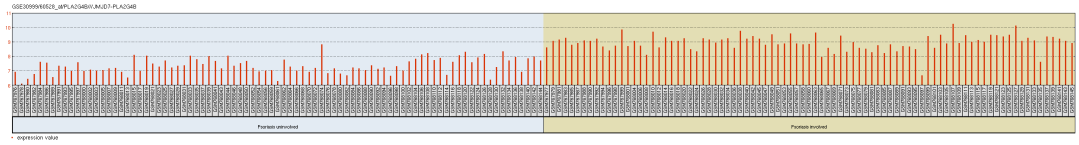


Figure 5.5 *PLA2G4B* gene expression results in GEO Data Series GSE30999

Gene expression results of *PLA2G4B* were retrieved from GEO Series GSE30999. *PLA2G4B* gene in this study appeared among the top 250 genes using GEO2R analysis.

Next, the presence of PLA2G4D in psoriatic lesional skin was verified. Here, the protein expression of PLA2G4D between normal healthy skin and lesional skin of psoriasis patients was compared using immunofluorescence (Figure 5.6A). The protein expression of PLA2G4D (red) was observed in the dermis, but interestingly not in the epidermis, of psoriatic lesions, while no detectable PLA2G4D expression was seen in normal skin (Figure 5.6A). This was consistent with the findings in Figure 5.1 and others' [299, 300] that PLA2G4D expression was detected in psoriasis skin but not in normal healthy skin. We then investigated the origin of PLA2G4D in the dermis of psoriatic lesions, and found that the PLA2G4D expression co-localized with the expression of mast cell tryptase (green), which is a protease specifically found in granules of mast cells, and is hence mast cell-specific (Figure 5.6B). This implied that mast cells are a source of PLA2G4D in psoriatic lesions.

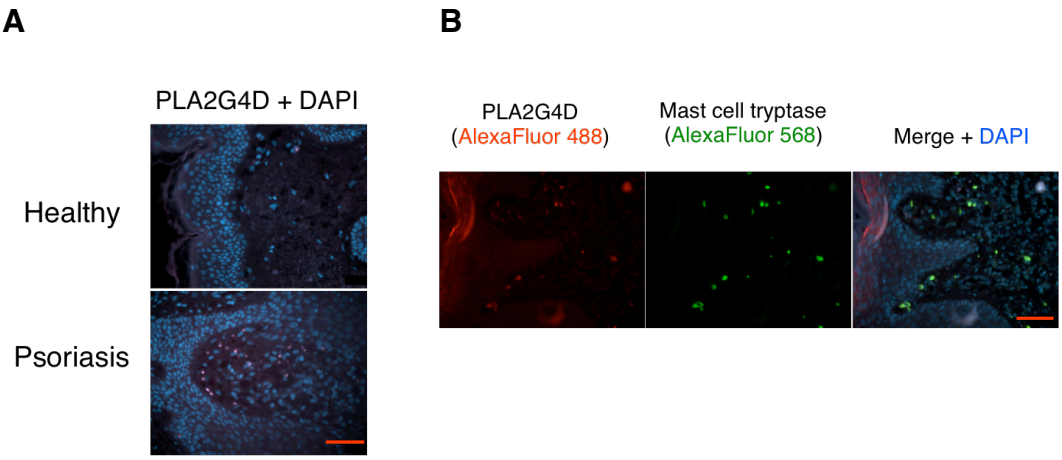


Figure 5.6 Mast cells are a source of PLA2G4D in psoriatic lesions

PLA2G4D (red) protein expression in healthy control and psoriatic lesional skin was determined by immunofluorescence (**A**). Co-localisation of PLA2G4D (red) and mast cell tryptase (green) protein expression were observed in the dermis of psoriatic lesional skin (**B**). Results were typical representation of 12 psoriasis donors and 10 healthy donors, and at least three individual experiments. Scale bar represents 60 μm.

To confirm the presence of PLA2G4D protein expression in mast cells, the PLA2G4D expression in the LAD2 cell line, which is a mast cell-like line similar to tissue mast cells [327], was examined. The protein expression of PLA2G4D in LAD2 cells was confirmed using immunofluorescence (Figure 5.7A). A strong expression of PLA2G4D (red) was detected in the cytoplasm of the cells. Then, the presence of PLA2G4D protein was also confirmed in the lysate of LAD2 cells using Western Blot and mast cell tryptase and GAPDH as the positive controls (Figure 5.7B). However, the expression of other isoforms of $cPLA_2$, including PLA2G4A and PLA2G4B, were not observed. To confirm that mast cells express PLA2G4D, the PLA2G4D expression in cord blood-derived mast cells (CBMCs) was then examined. Mature CBMCs were differentiated from $CD34^+$ progenitor cells isolated from human cord blood of healthy donors in the presence of stem cell factor (SCF) and IL-6, according to the protocol described previously [328]. Similarly, the protein expression of PLA2G4D was also determined in the lysate of CBMCs using Western Blot (Figure 5.7C), but not immunofluorescence due to limited cell number. Altogether the results supported the findings in Figure 5.6 that mast cells are a source of PLA2G4D.

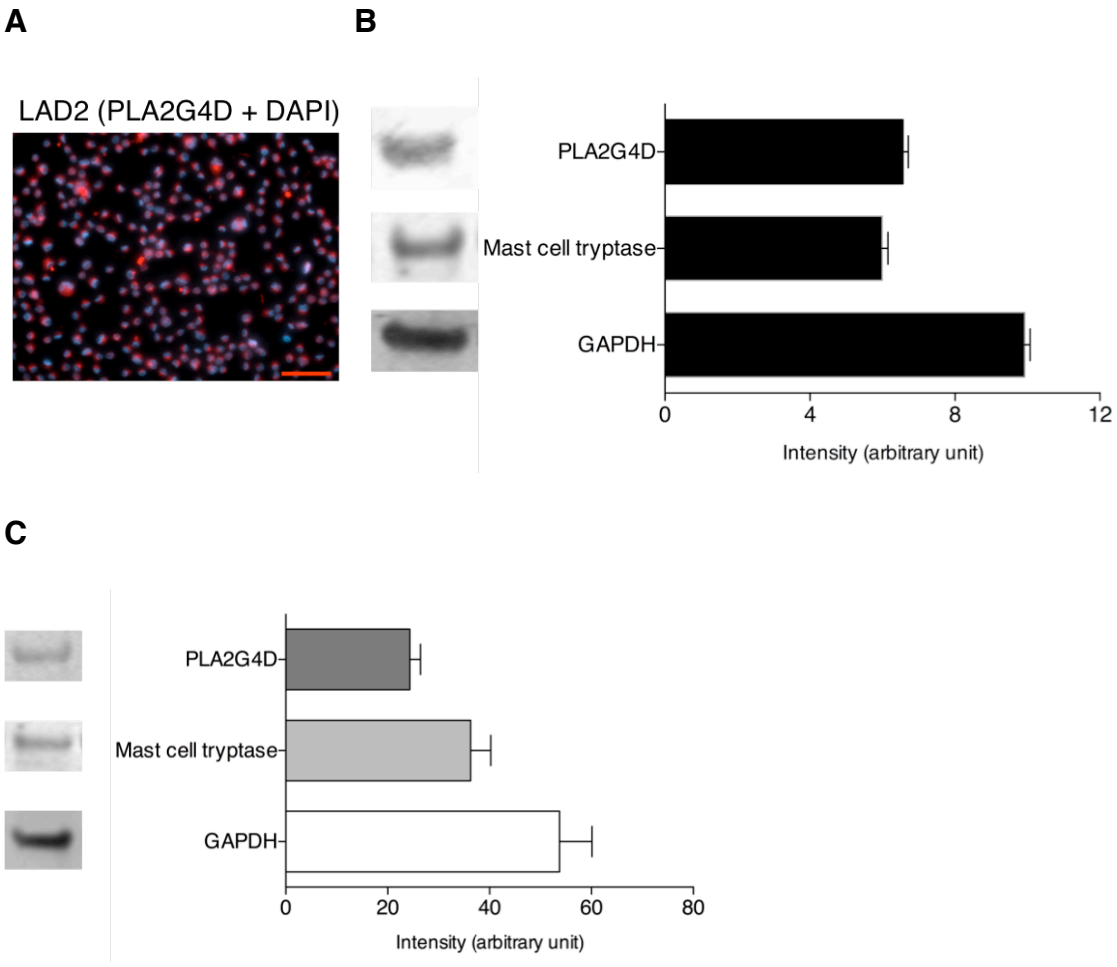


Figure 5.7 PLA2G4D protein expression in LAD2 mast cell-like line and cord blood-derived mast cells

PLA2G4D (red) protein expression was detected in LAD2 mast cell-like line by immunofluorescence. Scale bar presents 100 μm (**A**). PLA2G4D expression in LAD2 cells (**B**) and cord blood-derived mast cells (CBMC) (**C**) was also detected by Western Blot, using mast cell tryptase and GAPDH as positive control proteins. Western Blot staining intensity was evaluated by Image J software. Results were typical representation of at least three individual experiments. Data were mean ± SEM.

Given that mast cells express PLA2G4D protein, the PLA2G4D protein was purified from the cell lysate of LAD2 cells for further functional experiments using column purification. Figure 5.8A shows the result of an SDS-PAGE with Coomassie Blue staining. First, a resin column was coated with PLA2G4D-capturing antibody by covalent conjugation. PLA2G4D-capturing antibody was absent in the eluent (second lane) after chemical conjugation, indicating a successful conjugation of antibody to the column (Figure 5.8A). LAD2 cell lysate (third lane) contained a mixture of proteins (Figure 5.8A). Flow through (fourth lane) was collected directly after LAD2 cell lysate was allowed to pass through the PLA2G4D-capturing column, and PLA2G4D protein was immobilised onto the column. After several rounds of washing, PLA2G4D protein was collected eluted as the eluent (fifth lane) (Figure 5.8A). The presence of PLA2G4D protein in the concentrated eluent, but not in the concentrated flow through, was confirmed with Western Blot (Figure 5.8B). To confirm that the purified PLA2G4D protein was enzymatically active, the $cPLA_2$ activity in both LAD2 cell lysate and the PLA2G4D-containing fraction was examined, and both showed $cPLA_2$ activity (Figure 5.8C). While the $cPLA_2$ activity of LAD2 cell lysate was determined to be about 0.001 μmol/min/ml, fraction-derived PLA2G4D showed an almost four-fold increase of activity, being at around 0.0038 μmol/min/ml ($p < 0.0001$) (Figure 5.8C). This confirmed that the purified PLA2G4D was enzymatically active and functional.

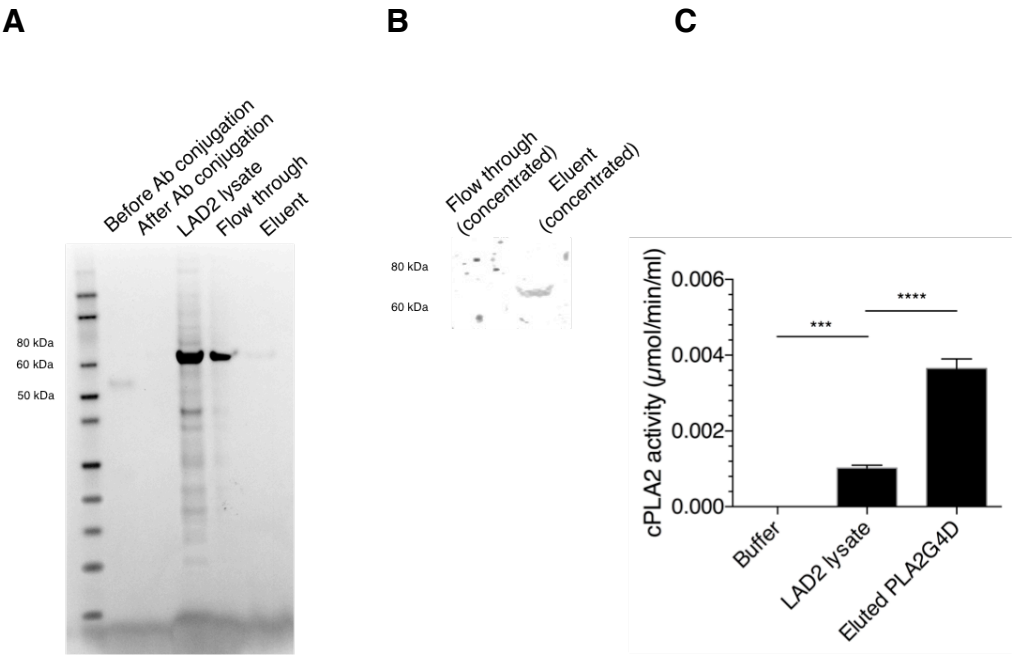


Figure 5.8 Column purification of PLA2G4D protein from LAD2 cell lysate

PLA2G4D protein was isolated from LAD2 cell lysate by column purification using PLA2G4D-capturing antibody. In the SDS-PAGE with Coomassie Blue staining, the capturing antibody was present before conjugation of the antibody to the column (first lane), but absent after the conjugation (second lane). PLA2G4D protein was prepared from LAD2 cell lysate using immunoprecipitation and collected in the eluent (fifth lane) (**A**). The presence of PLA2G4D protein in the eluent (concentrated), but not in the flow through (concentrated), was confirmed by Western blot (**B**). Cytosolic PLA_2 activity of LAD2 cell lysate and binding fraction-derived PLA2G4D was determined. The enzyme activity was measured using detection of free thiol release from arachidonoyl thio-PC, where eluent refers to protein-binding material (**C**). Results were typical representation

of at least three individual experiments. Data were mean ± SEM. *** p< 0.001; **** p< 0.0001.

5.2.2 LAD2-derived PLA2G4D activates CD1a-restricted T cells

In the previous chapter, the enrichment of circulating CD1a-autoreactive T cells in the peripheral blood of psoriasis patients was demonstrated. These T cells responded to CD1a-expressing antigen-presenting cells, including K562-CD1a cells and autologous mDCs, without the addition of antigen source and released IFN-γ. Based on the prior knowledge that PLA2G4D was thought to be associated with psoriasis [299, 300], and that PLA_2 derived from bee and wasp venom and house dust mite extract could activate CD1a-restricted T cells in allergic individuals [273, 274], the CD1a reactivity of T cells from psoriasis patients in response to LAD2-derived PLA2G4D was investigated. We hypothesized that PLA2G4D can generate CD1a ligands by cleaving phospholipids in the self cellular plasma membrane and producing neolipid antigens fro presentation to CD1a-reactive T cells.

First, *ex vivo* ELISpot assays were performed to test whether PLA2G4D could generate CD1a ligands for antigen presentation by CD1a-expressing antigen presenting cells to T cells. Polyclonal circulating $CD3^+$ T cells from healthy and psoriasis donors isolated from peripheral blood *ex vivo* were used in the experiments (Figure 5.9). In the experiments, both CD1a-transfected K562 (K562-CD1a) and mock-transfected K562 (K562-EV) cells were pulsed with purified PLA2G4D protein overnight for the cleavage of membrane phospholipids extracellularly. The concentration of

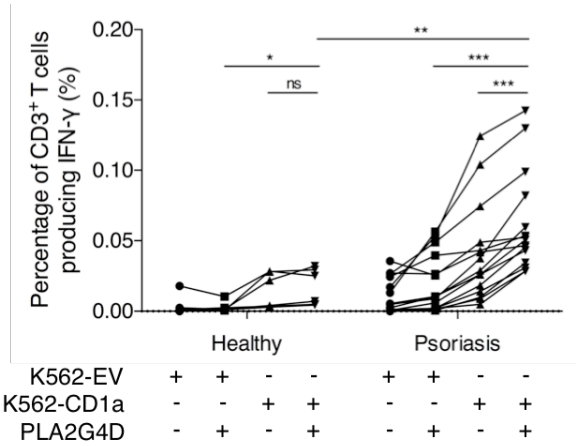
PLA2G4D protein used was based on the result in Figure 5.10. Then the T cells were co-incubated with K562-EV/K562-CD1a cells overnight and IFN-γ secretion was determined on the next day.

In the healthy cohort, while T cells co-incubated with either unpulsed or PLA2G4D-pulsed K562-EV did not produce high level of IFN-γ, T cells demonstrated a small but detectable CD1a autoreactivity when co-incubated with unpulsed K562-CD1a cells as expected (Figure 5.9A). However, the PLA2G4D-responsive CD1a-reactive T cell response was not significantly different from CD1a autoreactivity in the healthy cohort (Figure 5.9A). The frequency of these T cells remained relatively low at 0.01-0.03% of total T cells (Figure 5.9A).

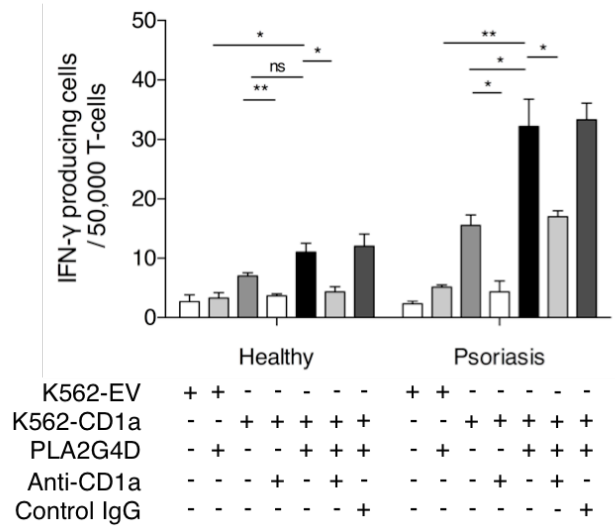
In the psoriasis cohort, while CD1a autoreactivity was observed when T cells were co-incubated with unpulsed K562-CD1a cells, there was a greater IFN-γ production from T cells that were co-incubated with PLA2G4D-pulsed K562-CD1a cells ($p < 0.001$) (Figure 5.9A). The frequency of PLA2G4D-responsive CD1a-reactive T cells in the psoriasis cohort ranged from 0.03% to as high as 0.14% of total peripheral blood T cells (Figure 5.9A). This reactivity is specific to CD1a as the response from T cells co-incubated with PLA2G4D-pulsed K562-EV cells was significantly lower ($p < 0.001$) (Figure 5.9A). Moreover, by comparing both cohorts, the PLA2G4D-responsive CD1a reactivity was much greater in

the psoriasis cohort ($p < 0.01$) (Figure 5.9A). Therefore, the IFN-γ responsiveness to PLA2G4D was CD1a-dependent and limited to psoriasis patient T cells only. The T cell response in both healthy and psoriasis individuals could be reduced by anti-CD1a antibody ($p < 0.05$), but not control IgG antibody, confirming the CD1a dependence (Figure 5.9B). Moreover, the antigenic potential of PLA2G4D protein was also confirmed by the IFN-γ production by the expanded PLA2G4D-respsonsive CD1a-reactive T cells determined by cultured ELISpot assay (Figure 5.9C).

A


Percentage of CD3+ T cells producing IFN-γ (%)
0.20
0.15
0.10
0.05
0.00
*
ns
**
***
***
Healthy
Psoriasis
K562-EV + + - - + + - -
K562-CD1a - - + + - - + +
PLA2G4D - + - + - + - +

B


IFN-γ producing cells / 50,000 T-cells
50
40
30
20
10
0
*
ns
**
*
**
*
*
*
Healthy
Psoriasis
K562-EV + + - - - - - + + - - - - -
K562-CD1a - - + + + + + - - + + + + +
PLA2G4D - + - - + + + - + - - + + +
Anti-CD1a - - - + - + - - - - + - + -
Control IgG - - - - - - + - - - - - - +

C

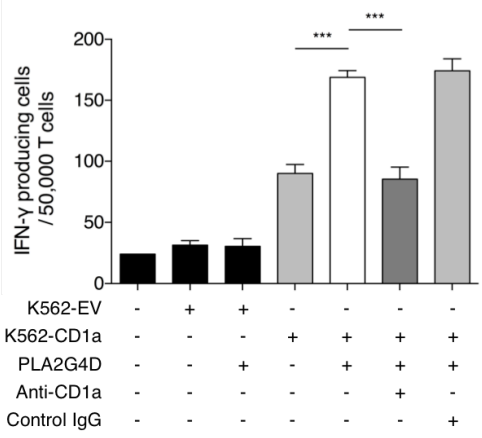

IFN-γ producing cells / 50,000 T cells
200
150
100
50
0
***
***
K562-EV - + + - - - -
K562-CD1a - - - + + + +
PLA2G4D - - + - + + +
Anti-CD1a - - - - - + -
Control IgG - - - - - - +

Figure 5.9 LAD2-derived PLA2G4D protein activates CD1a-restricted T cells in blood of psoriasis patients

K562-EV/K562-CD1a cells were incubated with 1.0 μg/ml PLA2G4D, and then incubated with T cells from psoriasis patients (n=15) or healthy donors (n=6). IFN-γ production was measured by *ex vivo* ELISpot (**A**). K562-EV/K562-CD1a cells were incubated with 1.0 μg/ml PLA2G4D, and then incubated with T cells from one psoriasis patient and one healthy donor in the presence or absence of anti-CD1a antibody or control IgG antibody. The data are representative of 15 psoriasis patients and 6 healthy donors (**B**). T cells from psoriasis donors were expanded using autologous mDCs that were pulsed with 1.0 μg/ml of PLA2G4D protein for 10-14 days prior to the cultured ELISpot assay. For the assay, K562-EV/K562-CD1a cells were incubated with 1.0 μg/ml of PLA2G4D, and then incubated with the T cells in the presence or absence of anti-CD1a blocking antibody or control IgG antibody, and IFN-γ production was measured. Results are typical representation of at least three individual experiments. Data are mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns no significance.

Next, the effects of different concentrations of PLA2G4D protein for K562-CD1a pulsing on the PLA2G4D-responsive CD1a reactivity of T cells from healthy and psoriasis individuals were investigated. Both healthy and psoriasis T cell responses varied with various amounts of PLA2G4D, and peaked at 1.0 μg/ml of the protein (Figure 5.10). However they declined when up to 5 μg/ml of PLA2G4D was used for K562-CD1a pulsing (Figure 5.10), potentially due to PLA_2 toxicity to cell membranes.

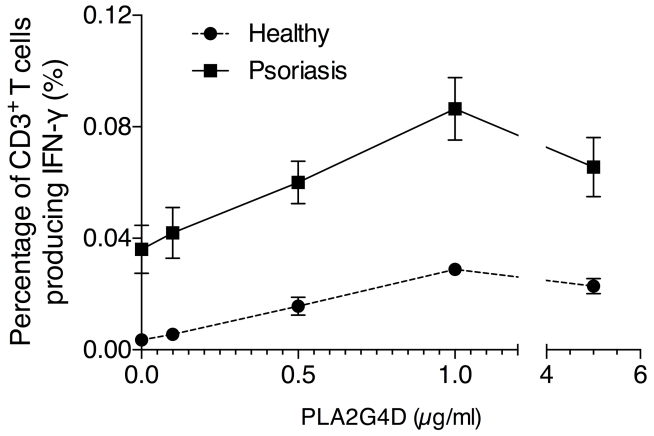


Figure 5.10 Dose-dependent PLA2G4D-responsive CD1a reactivity of T cells from healthy and psoriasis individuals

K562-EV/K562-CD1a cells were incubated with increasing concentrations of PLA2G4D, and then incubated with T cells from psoriasis patients (n=5) or healthy donors (n=3). IFN-γ production was measured by ELISpot assay. Data are mean ± SEM.

5.2.3 Cytosolic PLA_2 inhibitor reduces PLA2G4D-dependent CD1a reactivity

In order to confirm that the T cell CD1a reactivity was specific to the $cPLA_2$ activity of PLA2G4D but not other non-specific effects, the T cell responses of both healthy and psoriasis T cells in the presence of arachidonoyl trifluoromethyl ketone (ATK), which is a specific $cPLA_2$ inhibitor that readily diffuses across plasma membrane [329], were examined. A series of concentrations of ATK was used and an inhibitory effect on IFN-γ production by the T cells was observed (Figure 5.11A). The PLA2G4D-responsive CD1a reactivity of T cells from psoriasis patients declined with the increasing concentration of ATK, while the reactivity of T cells from healthy individuals remained low throughout (Figure 5.11A). Also, no inhibitory effect by ATK on the CD1a autoreactivity of T cells from both healthy and psoriasis individuals was observed (Figure 5.11B), therefore ruling out the toxicity and non-specific response of ATK on K562-CD1a cells (Figure 5.11B).

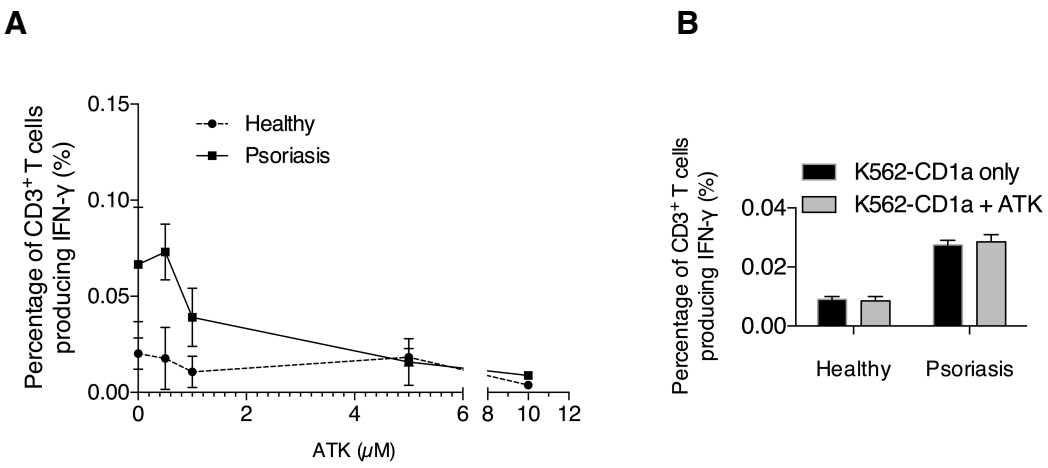


Figure 5.11 Reduction of PLA2G4D-responsive CD1a reactivity by a cPLA$_2$ inhibitor

K562-EV/K562-CD1a cells were incubated with 1.0 μg/ml PLA2G4D, and then incubated with T cells from psoriasis patients (n=3) or healthy donors (n=3) in the presence or absence of arachidonoyl trifluoromethyl ketone (ATK), which is a specific cytosolic PLA$_2$ inhibitor, and IFN-γ production was measured by ELISpot assay. Data were mean ± SEM.

5.2.4 Autologous mDCs activate PLA2G4D-responsive CD1a-reactive T cells

The PLA2G4D-responsive CD1a-reactive T cell responses were further tested using autologous mDCs as the CD1a-expressing antigen-presenting cells. In the healthy cohort, the T cell responses remained low regardless of the presence of PLA2G4D for pulsing (Figure 5.12). In the psoriasis cohort, while CD1a reactivity was detected in T cells co-incubated with unpulsed mDCs, there was a significantly higher frequency of IFN-γ producing T cells that were co-incubated with PLA2G4D-pulsed mDCs ($p < 0.01$), ranging from 0.05% to 0.18% of total peripheral blood T cells (Figure 5.12). This PLA2G4D-responsive CD1a-reactive T cell frequency was consistent with the frequency we determined earlier using K562 cells in Figure 5.9. Moreover, in the presence of ATK, the CD1a reactivity drastically reduced ($p < 0.001$) (Figure 5.12). As expected, the reactivity could be inhibited by anti-CD1a blocking antibody ($p < 0.001$) but not control IgG antibody, thus confirming the dependence of CD1a (Figure 5.12).

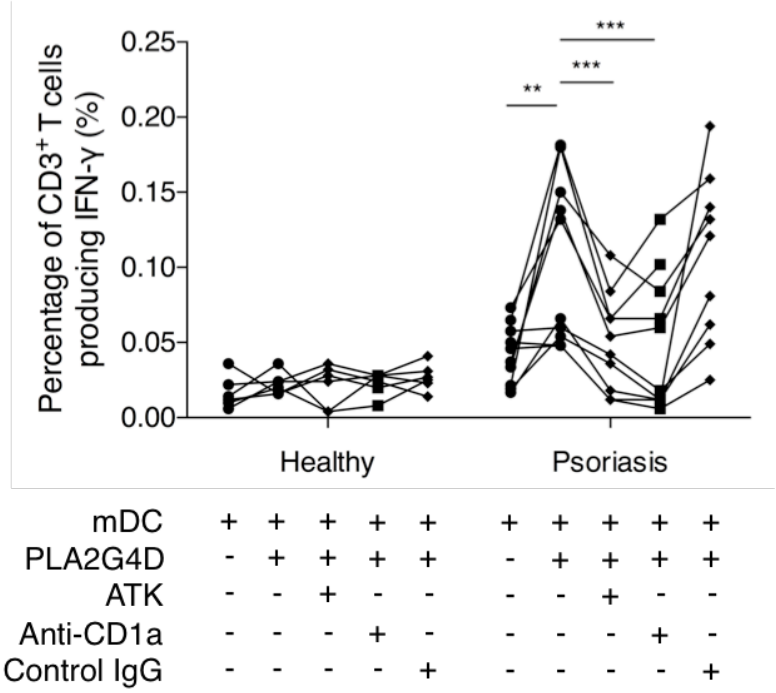


Figure 5.12 PLA2G4D-responsive CD1a reactivity using autologous mDCs as antigen-presenting cells

Autologous mDCs were incubated with 1.0 μg/ml PLA2G4D, and then T cells from healthy donors (n=6) (**A**) or psoriasis patients (n=10) (**B**) in the presence of anti-HLA-ABC (W6/32) and anti-HLA-DR blocking antibodies (L243), and in the presence or absence of anti-CD1a antibody, control IgG antibody, or ATK. IFN-γ production was measured by ELISpot assay. ** $p < 0.01$; *** $p < 0.001$; ns no significance.

5.2.5 PLA2G4D-responsive CD1a-reactive T cells produce IL-17A and IL-22

Next, whether the CD1a-reactive T cells could produce cytokines other than IFN-γ was investigated. Based on the prior knowledge that Th17 response plays a pivotal role in the pathogenesis of psoriasis, we went on to test whether these T cells could produce IL-17A and IL-22, which are the hallmark cytokines commonly found in psoriatic lesions. We performed ELISA to measure the production of IL-17A and IL-22 from the T cells of healthy and psoriasis individuals (Figure 5.13). Figure 5.13A demonstrated the profile of IL-17A production by T cells from healthy and psoriasis individuals. In the healthy cohort, T cells co-incubated with unpulsed K562-EV cells produced low levels of IL-17A, while the CD1a-autoreactive T cells that were co-incubated with unpulsed K562-CD1a cells, produced more IL-17A ($p < 0.01$) (Figure 5.13A). This indicated that CD1a-autoreactive cells in healthy individuals produced IL-17A in response to CD1a-expressing antigen-presenting cells. Furthermore, comparing with T cells that were co-incubated with PLA2G4D-pulsed K562-EV cells, T cells co-incubated with PLA2G4D-pulsed K562-CD1a cells produced a higher level of IL-17A ($p < 0.001$) (Figure 5.13A). There was a slight, albeit not statistically significant, difference in IL-17A production by PLA2G4D-responsive CD1a-reactive T cells compared to CD1a-autoreactive T cells in the healthy cohort (Figure 5.13A).

In the psoriasis cohort, a substantial production of IL-17A in CD1a-autoreactive T cells was observed, compared to T cells that were co-incubated with unpulsed K562-EV cells ($p < 0.001$) (Figure 5.13A). Moreover, CD1a-autoreative T cells from psoriasis patients produced a higher level of IL-17A than those from healthy individuals ($p < 0.05$) (Figure 5.13A). In addition, PLA2G4D-responsive CD1a-reactive T cells produced greater amount of IL-17A comparing with T cells co-incubated with PLA2G4D-pulsed K562-EV cells ($p < 0.001$), indicating that CD1a was necessary for the IL-17A responsiveness. Comparing PLA2G4D-responsive CD1a reactivity between both cohorts, there was a higher IL-17A production in the psoriasis cohort (Figure 5.13A). Altogether, these findings suggested that both CD1a-autoreactive T cells and PLA2G4D-responsive CD1a-reactive T cells might contribute to Th17 response through the production of IL-17A.

Figure 5.13B demonstrated the IL-22 production profile of the healthy and psoriasis cohorts, which was similar to the IL-17A profile as shown in Figure 5.13A. In brief, in the psoriasis cohort, CD1a-autoreactive T cells produced higher level of IL-22 compared to T cells co-incubated with unpulsed K562-EV cells ($p < 0.0001$), again highlighting the importance of CD1a (Figure 5.13B). The IL-22 production by CD1a-autoreactive T cells in the psoriasis cohort was significantly greater than in the healthy cohort ($p < 0.01$) (Figure 5.13B). The PLA2G4D-responsive

CD1a-reactive T cells from psoriasis patients produced a large amount of IL-22 compared to the psoriatic T cells co-incubated with PLA2G4D-pulsed K562-EV cells ($p < 0.0001$), as well as the counterpart in the healthy cohort ($p < 0.0001$) (Figure 5.13B). Altogether, these findings showed that IL-22, together with IL-17A and IFN-γ, are the major cytokines produced by both CD1a-autoreactive T cells and PLA2G4D-responsive CD1a-reactive T cells in psoriasis patients. This was consistent with their contribution towards elevated levels of IL-17A and IL-22 found in psoriatic lesions.

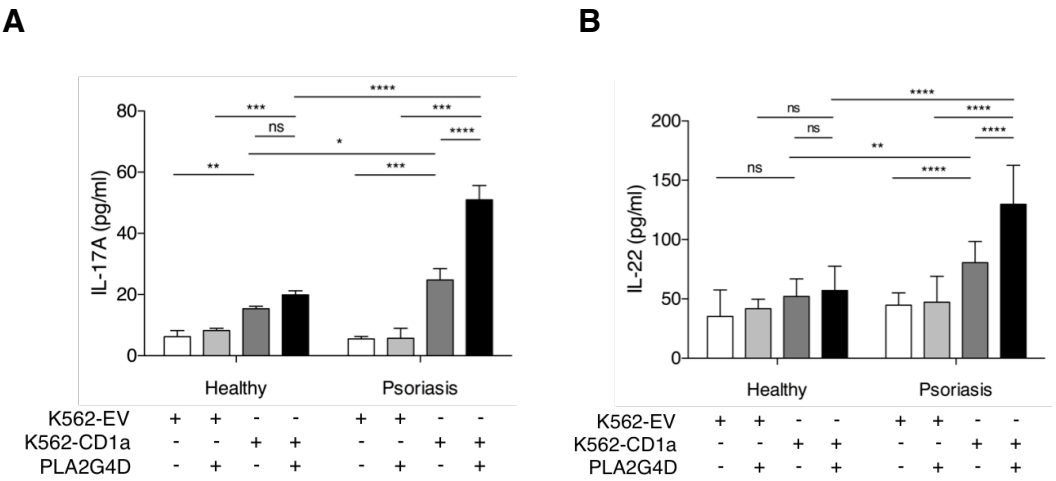


Figure 5.13 IL-17A and IL-22 production by PLA2G4D-responsive CD1a-reactive T cells

K562-EV/K562-CD1a cells were incubated with 1.0 μg/ml PLA2G4D, and then incubated with T cells from psoriasis patients (n=9) or healthy donors (n=6), and IL-17A (**A**) and IL-22 (**B**) production was measured using ELISA. Data are mean ± SEM. *** $p < 0.001$; **** $p < 0.0001$; ns no significance.

5.2.6 Cord blood mast cells-derived PLA2G4D activates CD1a-reactive T cells

Based on the previous findings that CMBCs express PLA2G4D protein, the CD1a reactivity of T cells in response to CMBC-derived PLA2G4D (CBMC-PLA2G4D) was examined. While the IFN-γ production by the CD1a reactive T cells from healthy individuals, which responded to PLA2G4D derived from CBMC-PLA2G4D, was low, a much greater IFN-γ production was observed in the T cells from psoriasis patients, and this could be inhibited by the blockade of CD1a ($p < 0.0001$) (Figure 5.14).

Summarizing all the findings discussed above, there was an enrichment of PLA2G4D-responsive CD1a-reactive T cell population in the peripheral blood of psoriasis patients, compared to healthy individuals. We determined the frequency of such T cells in the circulation of psoriasis patients to be 0.03-0.18% of total blood T cells. This CD1a reactivity is dependent on not only the amount of PLA2G4D for pulsing of antigen-presenting cells, but also on the enzymatic activity of PLA2G4D. Apart from IFN-γ, these T cells also produced significant amounts of IL-17A and IL-22, implying their potential contribution to Th17 and Th22 responses in psoriasis.

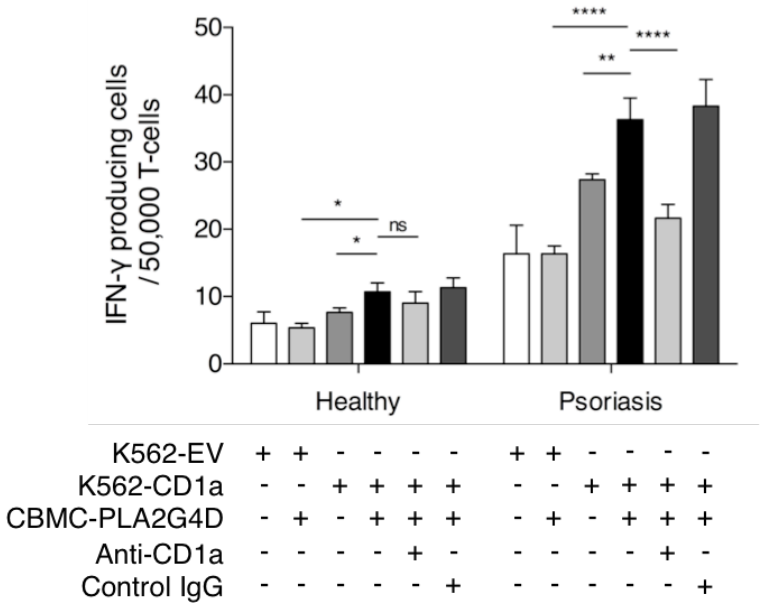


Figure 5.14 PLA2G4D derived from cord blood-derived mast cells activates CD1a-reactive T cells

K562-EV/K562-CD1a cells were incubated with 1.0 μg/ml PLA2G4D isolated from cord blood-derived mast cells (CBMC), which are differentiated from $CD34^+$ progenitor cells from healthy human cord blood, and then incubated with T cells from a healthy donor (**A**) and a psoriasis patient (**B**) in the presence or absence of anti-CD1a antibody or control IgG antibody. Data were typical representation of 5 healthy donors and 4 psoriasis patients. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$; ns no significance.

5.3 Discussion

A potential role of phospholipase A_2 (PLA_2) in the pathogenesis of psoriasis was initially suggested three decades ago when the increased activity of PLA_2 was found in the skin of psoriasis [301-304]. However, a defined identity of a psoriasis-associated PLA_2 had not been revealed until the discovery of PLA2G4D, a cytosolic PLA_2, by Chiba *et al.*, in which they reported the elevated mRNA and protein expression of PLA2G4D in psoriatic lesional skin [299]. A recent finding of Quaranta *et al.* about the elevated gene expression of PLA2G4D in psoriasis skin was in line with this [300]. Apart from endogenous phospholipase, potential microbial triggers of psoriasis were proposed, such as infection with Streptococcus and Malassezia spp, which contain secretory PLA_2 ($sPLA_2$) activity [330-332]. However, many recent studies have reported endogenous antigens, including melanocyte antigen and LL-37 antimicrobial peptide, that are relevant to psoriasis [213, 216], implying the importance of endogenous triggers of psoriasis.

Although the association of PLA_2-associated CD1a reactivity and psoriasis has not been elucidated before this study, the relevance of PLA_2 to the skin has been recently investigated. Bourgeois and Subramaniam *et al.* revealed that bee and wasp venom contained PLA_2 activity, which can

generate neolipid antigens by cleaving host phospholipids for antigen presentation by CD1a in healthy individuals [272]. PLA_2-responsive CD1a-restricted T cell responses were also reported in individuals allergic to bee and wasp venoms or house dust mite respectively [273, 274]. Interestingly, in the study by Bourgeois and Subramaniam *et al.*, they found that CD1a reactivity was higher when CD1a antigens that contained C18 fatty acid chains were used, while the reactivity was significantly lower when added with C16-containing lipids [272]. This suggested that C18-lipids might be permissive ligands of CD1a and play important role in CD1a reactivity in the skin. Interestingly, Chiba *et al.* also reported that PLA2G4D demonstrated strong activity, compared to PLA2G4A, with linoleic acid (C18:2)-containing and oleic acid (C18:1)-containing phospholipids as substrates; while both PLA2G4D and PLA2G4A had similar activity with arachidonic acid (C20:4)-containing phospholipid. This reinforced the potential role of PLA2G4D in psoriasis, specifically that PLA2G4D might produce C18 lipids which act as CD1a ligands for antigen presentation by CD1a-expressing antigen-presenting cells in the skin of psoriasis.

Elevation of *PLA2G4D* gene expression, but not other PLA_2 or PLA_2-related genes, was common in the lesional skin group in the gene expression studies (Figure 5.1 to 5.5). Although the expressions of other PLA_2 genes, such as *PLA2G3*, and *PLA2G4B*, were slightly higher in the

lesional skin group in some studies, their P values were not statistically significant and hence were not included in the top 250 gene using GEO2R analysis. Considering the past evidence suggesting the PLA2G4D expression is strongly associated with psoriatic lesions, the relevance of PLA2G4D in psoriasis was examined, and the current findings revealed the elevated expression of PLA2G4D in the lesional skin of psoriasis patients compared to healthy skin (Figure 5.6A). Interestingly this expression co-localized with the expression of mast cell tryptase, indicating that mast cells could be a source of PLA2G4D in psoriatic lesions (Figure 5.6B). This was verified by showing that LAD2 mast cell-like line and cord blood-derived mast cells (CBMCs) also expressed PLA2G4D protein by immunofluorescence and Western Blot (Figure 5.7). These findings were hence in line with previous relevant studies [299, 300]. However, little or no detectable protein expression of PLA2G4D was observed in the keratinocytes of the epidermis of psoriatic lesions (Figure 5.6), hence suggesting that mast cells in the psoriatic lesions are a main source of PLA2G4D in the lesions.

After purifying the PLA2G4D protein and showing that it was enzymatically active (Figure 5.8), the antigenic potential of the protein was tested, and an elevated frequency of CD1a-reactive T cells that were responsive to PLA2G4D was observed in the peripheral blood of psoriasis patients, but not healthy individuals (Figure 5.9). The frequency of

PLA2G4D-responsive CD1a-reactive T cells in psoriasis patients was determined to be 0.03-0.14%, while such frequency in healthy individuals was around 0.02% of total blood T cells (Figure 5.9A). This CD1a reactivity was dependent on the amount of PLA2G4D (Figure 5.10), and could be reduced by ATK, an inhibitor of $cPLA_2$ activity (Figure 5.11). Therefore, the results showed that $cPLA_2$ activity of PLA2G4D is critical to the PLA2G4D-responsive CD1a reactivity due to the generation of neolipid antigens by cleaving membrane phospholipids extracellularly. These lipophilic antigens can readily diffuse across plasma membrane and can be loaded onto CD1a molecules for lipid antigen presentation to restricted T cells. Moreover, this CD1a reactivity was confirmed with the use of autologous mDCs (Figure 5.12). Notably, these CD1a-reactive T cells did not only produce IFN-γ, but also produce IL-17A and IL-22 (Figure 5.13), which are two hallmark cytokines commonly found in psoriatic lesions. The increase of IL-17A and IL-22 production could be due to the increase in production of these cytokines, or the increased number of T cells producing them.

Lastly, the antigenic potential of PLA2G4D that was derived from CBMCs, which are more physiologically relevant, was confirmed (Figure 5.14). This further supported the association of mast cells and psoriasis which was described in some studies [140, 333]. Altogether, the current findings confirmed the presence of PLA2G4D in psoriatic mast cells, which could activate CD1a-reactive T cells of psoriasis patients to produce IFN-

γ, IL-17A, and IL-22, and hence potentially contributing to the Th17 and Th22 responses in psoriasis.

However, there are three major questions regarding to the current proposed model. First, the mechanism of how PLA2G4D, as a cytosolic PLA_2, can generate neolipid antigens and activate CD1a-restricted T cells remains unclear. Unlike secretory PLA_2 that can be secreted out of the cells or venom-derived PLA_2 that already exist extracellularly, cytosolic PLA2G4D remains in cytosol of the mast cells. Although it might be possible that PLA2G4D can generate lipid products inside mast cells, where these lipids can diffuse out of the mast cells through plasma membrane, and then come into contact with CD1a-expressing antigen-presenting cells (e.g. Langerhans cells) for lipid loading either on the cell surface or intracellularly, this process seems highly inefficient and ineffective. Hence, in the next chapter, the mechanisms underlying the role of PLA2G4D and/or its lipids was investigated. Another question is that the role of IFN-α has not yet been linked to the current model of psoriasis pathogenesis. Based on the prior knowledge that IFN-α can be produced by pDCs in psoriatic skin and trigger the initial phase of psoriasis [56], it remains unknown that whether IFN-α can activate mast cells for the initiation of CD1a-restricted T cell responses. Also, since the experiments so far were based on the T cells from the peripheral blood of healthy or

psoriasis individuals, the CD1a reactivity of cutaneous T cells was still in question. These questions will be addressed in the next chapter.

6. Mast Cell Exosome-responsive CD1a Reactivity in Psoriasis

6.1 Introduction and Aims

A link between PLA_2, mast cells, and psoriasis

The association of mast cells and psoriasis has been long known. In brief, a potential role of mast cell degranulation for the evolution of acute guttate psoriasis was suggested [334]. Also, increased density of dermal mast cell was found in psoriatic lesional, as well as non-lesional skin compared to normal healthy skin [134]. It was also reported that mast cells are a source of IL-17 in the psoriatic lesional skin [140]. IL-33 produced by keratinocytes in psoriatic skin, in response to TNF-α, can lead to mast cell activation [333]. A recent study confirmed that dermal mast cells in experimentally induced psoriatic skin expressed IL-33 receptor [335]. On the other hand, the level of mast cell chymase was found elevated in skin of atopic dermatitis but not in psoriatic skin [336], suggesting that degranulation of mast cell might be differentially regulated in psoriasis. Although many studies have attempted to investigate the role of mast cells in psoriasis, a defined mechanism of mast cell response in the disease has not yet been established.

According to past studies and our findings described in the previous chapters, it has been known that PLA_2 is important for CD1a-restricted T cell responses in psoriasis, for it generates CD1a ligands by cleaving host phospholipids. While an increased concentration of lysophosphocholine (LPC) was reported in psoriatic lesions [337], interestingly it can activate mast cells and induce their secretion of β-hexosaminidase, a lysosomal enzyme cleaving molecules containing terminal N-acetyl hexosamines [338]. The association between PLA_2 and mast cells has been studied previously. For example, lung mast cells can produce release multiple $sPLA_2$ [339]. Also, it was recently revealed that group III PLA_2 was critical for the maturation of mast cells [286]. Altogether, these studies have suggested that PLA_2 might be an important link between psoriasis and mast cells.

However, it is still unclear that how PLA2G4D, a $cPLA_2$ which normally remains in the cytoplasm, exerts a distant effect to antigen-presenting cells as suggested by findings in the previous chapters. Therefore an intercellular transport mechanism should be involved. It has been widely known that cells can secrete proteins to the extracellular environment via either the release of storage granules, for instance mast cell degranulation, or vesicular transport through exocytosis. Hence there

is in need to examine whether such vesicles might explain the distant effect of PLA2G4D derived from mast cells on antigen-presenting cells.

Exosomes for intercellular communication

Many cells can actively secrete proteins, as well as lipids and nucleic acids, through the production of extracellular vesicles. These vesicles can serve as “messengers” for intercellular communication by transferring materials from one cell to another. Different types of extracellular vesicles are categorized according to their sizes and origins. They include apoptotic bodies, exosomes, microparticles and microvesicles.

Exosomes are extracellular vesicles with the diameter of 40-100 nm derived from multivesicular body (MVB) fusion with the plasma membrane, which is a Rab GTPase-dependent process [340-342]. Exosomes can by produced a diversity of cell types, including B lymphocytes, DCs, Tc cells, neurons, and mast cells [343, 344]. Exosomes are also found in various body fluids including blood, urine, saliva, breast milk, and cerebrospinal fluid [345-349].

The composition of exosomes can be complex, and is usually unique according to the cells that produce them. Since exosomes are

derived from MVB, they contain a number of endosome-associated proteins, such as SNAREs and Rab GTPase, and MVB-related proteins, such as Alix and Tsg101 [350]. Depending on the cell of origin, the membrane of exosomes can contain a number of surface proteins, including MHC II and the tetraspanins CD63, CD81, and CD82 [344] [351]. In particular, exosomal content is usually lipid-rich. For example, exosomes are commonly enriched in cholesterol, sphingomyelin, and hexosylceramides, as well as phosphatidylcholine (PC) and phosphatidylethanolamine in relatively smaller amounts [352-355]. Saturated and monounsaturated fatty acids are also found in the exosomes [344]. Recent studies have shown that exosomes also carry RNA. For instance, mRNA could be delivered to the target cells where the mRNA could be translated into proteins [356, 357]. Moreover, exosomes can also deliver other RNA, such as miRNA, small interfering RNA, structural RNA, Y RNA, and tRNA fragments [358, 359]. ExoCarta is an online database containing the information of lipids, proteins, and nucleic acids that are associated with exosomes. Vesiclepedia is a similar database for vesicles in general. All the entries on ExoCarta and Vesiclepedia were experimentally verified.

The interaction between exosomes and their target cells begins with the binding of exosomes to the plasma membrane of the target cells. At this stage exosomes can remain stable on the membrane or dissociate

from it. To facilitate the uptake, exosomes can either directly fuse with the plasma membrane or be internalized through endocytosis by the target cells. Direct fusion with the plasma membrane results in the immediate loading of exosomal content into the cytosol of the target cells. Once the exosomes being endocytosed, they enter the endosomal pathway of the target cells, in which the exosomes may fuse with the endosomal delimiting membrane. Eventually exosomes may be targeted to lysosomes for degradation. One remarkable physiological function of exosomes is the intercellular transport of antigens. A notable example is that exosomes derived from intestinal epithelial cells could deliver MHC II/peptide to dendritic cells [360, 361]. In addition, transfer of MHC I/peptide in between dendritic cells via exosomes was also described [362]. These evidences supported the immunogenic role of exosomes.

Evidence for intercellular transport of PLA_2 and PLA_2-associated lipids

There are some clear examples of the intercellular transport of PLA_2 and PLA_2-associated lipids in vesicles. Table 2 summarises the five studies retrieved from Vesiclepedia, which identified PLA_2-associated lipids in different types of extracellular vesicles.

Two studies demonstrated that extracellular vesicles found in body fluids contained PLA_2-associated lipids. Fourcade *et al*. (Vesiclepedia_233) identified a number of lipids in the microvesicles found in inflammatory fluids in humans. These lipids included LPC, PC, phosphatidylethanolamine, and lysophosphatidic acid (LPA), as well as phosphatidylserine (PS), and sphingosine (SPH) [363]. A study by Losito *et al*. (Vesiclepedia_529) provided another example that blood microparticles contained a number of LPC composed of different chain lengths, ranging from C16 to C22, and varying degrees of saturation [364].

Entry	Species	Sample	Vesicle type	Reference
Vesiclepedia_89	*Rattus norvegicus*	Rat basophilic leukaemia cell line	Exosomes	[365]
Vesiclepedia_91	*Homo sapiens*	T cells	Exosomes	[366]
Vesiclepedia_129	*Rattus norvegicus*	Rat tumoural mast cells	Exosomes	[354]
Vesiclepedia_233	*Homo sapiens*	Inflammatory fluids	Microvesicles	[363]
Vesiclepedia_529	*Homo sapiens*	Blood	Microparticles	[364]

Table 2 Five studies reporting the presence of PLA_2 lipids in extracellular vesicles from immune cells and fluids

Another three studies reported the lipid contents of exosomes produced by different immune cells. In the study of exosomes derived from RBL-2H3, a rat basophilic leukaemia cell line (Vesiclepedia_89), Subra *et al.* showed that these exosomes contained fatty acids that were saturated, monosaturated, and polyunsaturated; and they also contained PC, LPC, phosphatidylethanolamine, phsophatidic acid (PA), as well as prostaglandins including PGD_2, PGE_2, and $PGF_{2\alpha}$ [365]. On the other hand, a lipodomic study by Laulagnier *et al*. (Vesiclepedia_129) showed that rat tumoural mast cells produced exosomes that contained LPC, PC, and phosphatidylethanolamine [354]. Alonso *et al*. (Vesiclepedia_91) also identified the presence of LPA in the exosomes from T cells [366]. Altogether these studies showed that extracellular vesicles could carry lipids that are relevant to PLA_2 activity.

Some other studies even demonstrated the ability of exosomes/microvesicles to carry the mRNA and protein of PLA_2, especially the $cPLA_2$. Table 3 summarized the four studies that reported the presence of $cPLA_2$ protein and mRNA in the exosomes/microvesicles in humans. Skog *et al*. (Vesiclepedia_203, 204, and 205) showed that microvesicles derived from glioblastoma cells contained mRNA for two $cPLA_2$, namely PLA2G4B and PLA2G4D [367], while Hong *et al*. reported the presence of mRNA for PLA2G4C in the microvesicles produced by colorectal cancer cells [368]. Yet, whether the mRNA could be translated

into $cPLA_2$ proteins in the target cells upon uptake of the microvesicles was not described. Intriguingly, two studies reported the presence of $cPLA_2$ proteins, and particularly PLA2G4D, in exosomes [369, 370]. In one of the studies, the PLA2G4D-containing exosomes were derived from dendritic cells [369]. However, the authors did not study whether these $cPLA_2$ were enzymatically active, or they had any physiological effects on the target cells. Nonetheless, these are the direct evidences confirming that cytosolic enzymes could be transported intercellularly via exosomes.

Entry	Sample	Target	mRNA / Protein	Vesicle type	Reference
Vesiclepedia_203 / 204 / 205	Glioblastoma cells	PLA2G4B, PLA2G4D	mRNA	Microvesicles	[367]
Vesiclepedia_303	Colorectal cancer cells	PLA2G4C	mRNA	Microvesicles	[368]
Vesiclepedia_437	Urine	PLA2G4B, PLA2G4D	Protein	Exosomes	[370]
Vesiclepedia_562 / 563 / 565	Dendritic cells	PLA2G4A, PLA2G4D	Protein	Exosomes	[369]

Table 3 Four studies reporting the presence of mRNA and protein of cytosolic PLA_2 in extracellular vesicles

Based on this evidence, it is then logical to ask whether human mast cells could also “send out” cytosolic PLA2G4D via exosomes to the target cells, especially CD1a-expressing antigen-presenting cells, for the activation of CD1a reactivity of T cells of psoriasis patients.

6.2 Results

6.2.1 Cytosolic PLA_2 activity in LAD2 exosomes

Based on the findings in the previous chapter, that PLA2G4D protein was expressed in dermal mast cells within psoriatic lesional skin and in the LAD2 mast cell-like line as well as cord blood-derived mast cells, the generation of neolipid antigens by PLA2G4D, as a $cPLA_2$, for CD1a-lipid presentation to T cells by CD1a-expressing antigen-presenting cells was investigated. To understand the exact localisation of PLA2G4D inside the LAD2 cells, firstly a panel of antigen sources, including purified PLA2G4D protein, LAD2 cell lysate, LAD2 culture medium and LAD2 supernatant, was screened by ELISpot assay using K562-CD1a as presenting cells (Figure 6.1). As expected, T cells from both healthy and psoriasis patients produced a substantial level of IFN-γ (Figure 6.1). IFN-γ production was detected in T cells from healthy or psoriasis individuals when using LAD2 cell lysate as the antigen source (Figure 6.1). This is in agreement with our previous findings in Figure 5.3 and 5.4 that the cell lysate contained PLA2G4D protein, which was enzymatically active.

Surprisingly, when using LAD2 supernatant as the antigen source, a strong IFN-γ production by the T cells from both healthy and psoriasis

individuals was detected (Figure 6.1). Hence the possibility that the T cells responded to non-specific content in the LAD2 culture medium was ruled out, as little or no IFN-γ production was detected when using the culture medium as antigen source (Figure 6.1). Therefore, it is possible that the supernatant might contain substances that were derived from LAD2 cells, which could activate the T cell CD1a reactivity. It was suspected the substances were related to $cPLA_2$, thus examined the $cPLA_2$ activity in the supernatant, and confirmed activity compared to the baseline activity in LAD2 culture medium ($p < 0.05$) (Figure 6.2). $cPLA_2$ normally remains in the cytosol of mast cells and is not transported to extracellular environment via mast cell degranulation. Therefore, the alternative way that $cPLA_2$ could be released by mast cells is the vesicular pathway. It was proposed that PLA2G4D, which was conventionally located in the cytosol of LAD2 cells, could be exported via exosomal pathway of the cells.

To confirm this, exosomes derived from LAD2 cells were prepared from the LAD2 supernatant, and the CD1a reactivity of T cells was tested using LAD2 exosomes as the antigen source. As a result, a significantly greater IFN-γ production by the CD1a-reactive T cells from psoriasis patients was observed, compared to using antigen sources such as LAD2 supernatant, culture medium, LAD2 cell lysate and even purified PLA2G4D protein ($p < 0.0001$, $p < 0.001$, $p < 0.001$, and $p < 0.001$ respectively) (Figure 6.1). This exosome-responsive CD1a reactivity was

greater in T cells from psoriasis patients than those from healthy individuals ($p < 0.01$) (Figure 6.1). The concentration of exosome used in this experiment was based on the result in Figure 6.6. Also, the enriched $cPLA_2$ activity was confirmed in the LAD2 exosomes, compared to LAD2 supernatant ($p < 0.001$) (Figure 6.2).

The protein expression of PLA2G4D in the exosomes was then examined. Using Western Blot, we confirmed that exosomes contained PLA2G4D protein, with CD63, a tetraspanin and a typical marker for exosome, as the positive control for exosome proteins (Figure 6.3). To confirm the purity of exosome preparation, the expression of mast cell tryptase was also determined. Mast cell tryptase is constitutively expressed by mast cells, and is normally released during mast cell activation but not exocytosed through exosomal pathway. The purity of the exosome samples was ensured as low expression of mast cell tryptase was observed in the exosome samples (Figure 6.3). Therefore, here it was demonstrated the exosome-responsive CD1a reactivity of T cells, and that the LAD2 exosomes contained PLA2G4D which is potentially responsible for the CD1a reactivity.

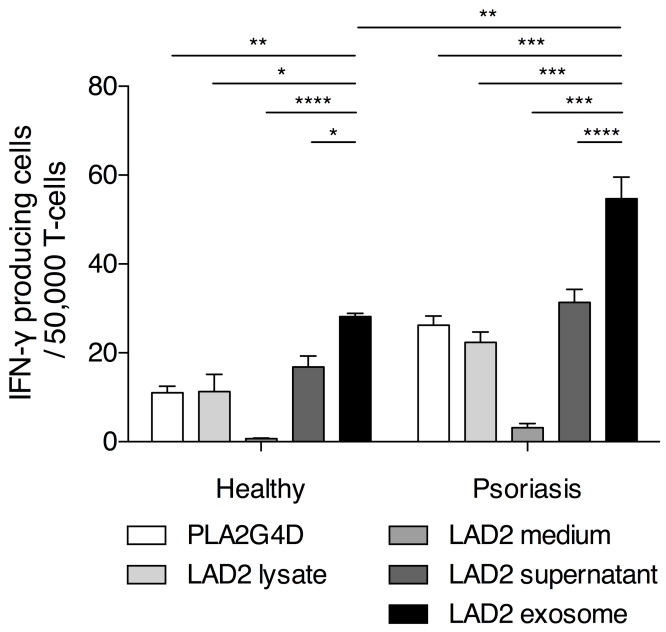


Figure 6.1 LAD2-derived exosomes activate T cells from psoriasis individuals

K562-CD1a cells were pulsed with either PLA2G4D (1 μg/ml), LAD2 lysate (10 μg/ml protein content), LAD2 culture medium (1 ml per 1 x 10^6 cells), LAD2 supernatant (1 ml per 1 x 10^6 cells), or LAD2 exosomes (10 μg/ml protein content). The cells were then incubated with T cells from patients with psoriasis (n=3) or controls (n=3). IFN-γ production was measured by ELISpot assay. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

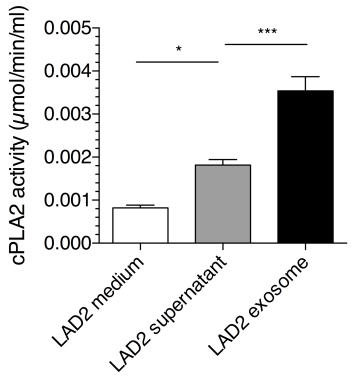


Figure 6.2 Cytosolic PLA_2 activity in LAD2 supernatant and exosomes.

Cytosolic PLA_2 was measured in LAD2-derived exosomes, LAD2 supernatant, and LAD2 culture medium using biochemical activity assay. Results were typical representation of at least three individual experiments. Data were mean ± SEM. * $p < 0.05$; *** $p < 0.001$.

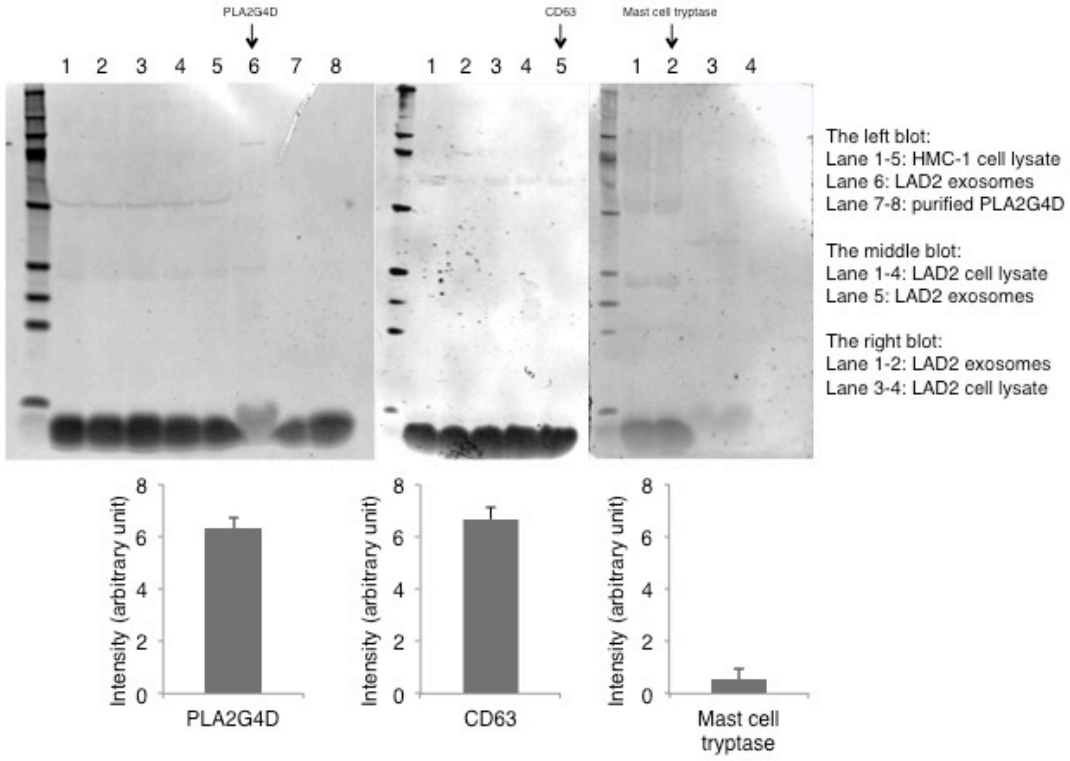


Figure 6.3 PLA2G4D protein expression in LAD2 exosomes

Protein expression of PLA2G4D in LAD2-derived exosomes (lane 6, left blot) was detected using Western Blot and intensity was evaluated by Image J software, and compared to the expressions of CD63 (lane 5, middle blot), a tetraspanin and a known exosome marker, and mast cell tryptase (lane 2, right blot) in the exosomes. Results were representation of at least three individual experiments. Data were mean ± SEM.

6.2.2 Uptake of LAD2 exosomes by K562 cells

After confirming the expression of PLA2G4D protein in exosomes derived from the LAD2 mast cell-like line, the interaction between the exosomes and the antigen-presenting cells was then examined. It remained unclear how cytosolic PLA2G4D derived from LAD2 cells could exert a distant effect on K562 cells with the aid of an exosomal transport system. We hypothesised that the LAD2 exosomes could deliver PLA2G4D from LAD2 cells to K562 cells. Here two fluorochromes were used to represent cytosol and membrane of LAD2 exosomes and investigate the process of exosome docking and loading to the K562 cells under immunofluorescence microscopy (Figure 6.4). In brief, PE-conjugated anti-CD63 antibody (red) could bind to the surface CD63 on the LAD2 cells, thus representing the membrane of LAD2 cells as well as the membrane of LAD2 exosomes. On the other hand, calcein-AM (green) could enter the LAD2 cells and remain in the cytosol, thus could also enter the LAD2 exosomes, hence representing the exosomal content. As a result, LAD2 cells produced exosomes which were double-labelled. After obtaining the exosomes from the supernatant, they were co-incubated with K562 cells for 4 hours, followed by washing and the examination of fluorescence in the K562 cells (Figure 6.4). Detectable fluorescence of both anti-CD63-PE and calcein-AM was observed in the K562 cells, indicating the successful uptake of double-labelled LAD2 exosomes by these cells and the loading of exosomal content (Figure 6.4). This

confirmed that PLA2G4D derived from LAD2 cells could be delivered to antigen-presenting cells via exosomal transport.

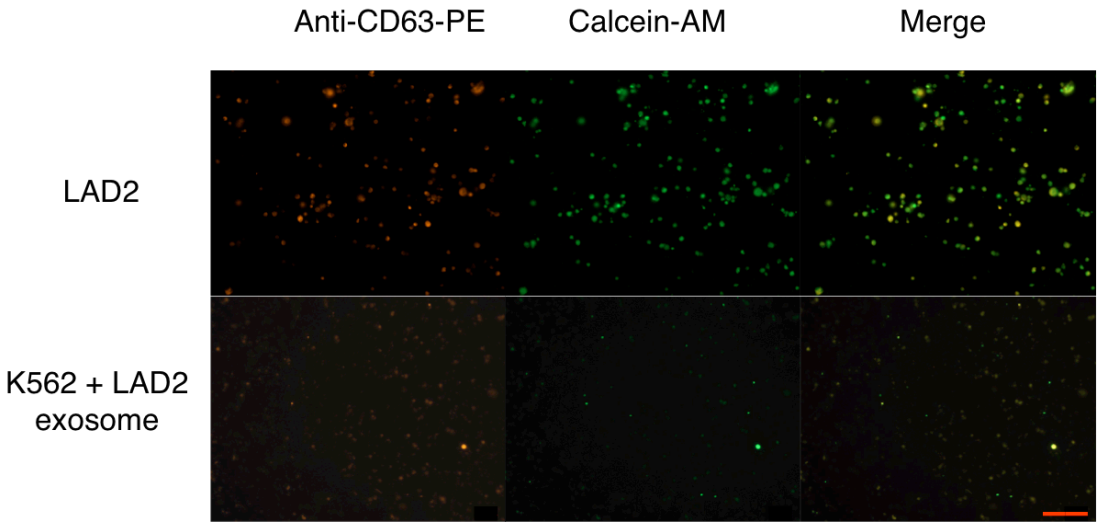


Figure 6.4 Uptake of LAD2 exosomes by K562 cells

LAD2 mast cell-like line was stained with anti-CD63-PE (representing membrane) and calcein-AM (cytosolic) for 30 minutes, then washed and cultured overnight to allow the production of exosomes which were stained with both fluorochromes. Followed by 24-hour incubation, culture supernatants were collected and the exosome fraction was collected using total exosome extraction reagent. K562 cells were incubated with the double-stained exosomes for 4 hours. After three times of washing, fluorescence from both fluorochromes was detected using fluorescence microscopy. Results were typical representation of at least three individual experiments. Scale bar represents 100 μm.

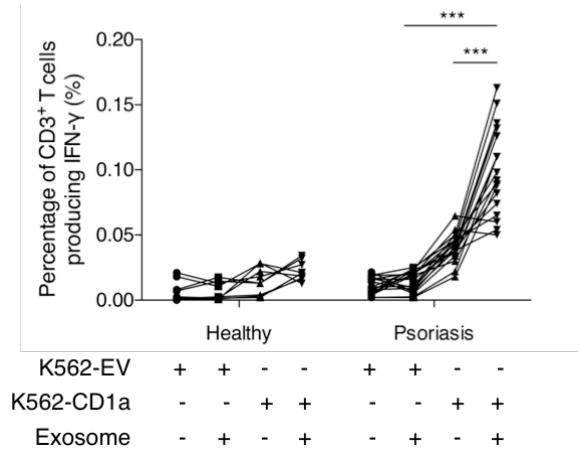
6.2.3 LAD2 exosomes activate CD1a-reactive T cells

The previous findings have confirmed the following: exosomes derived from LAD2 cells contained enzymatically active PLA2G4D; LAD2 exosomes could activate CD1a reactivity in T cells from psoriasis patients; and PLA2G4D protein derived from LAD2 cells could be delivered to K562 cells via exosomal transport. Therefore, a series of functional assays were performed to investigate the antigenic potential of mast cell-derived exosomes.

First, the nature of the exosome-responsive CD1a reactivity of T cells in the healthy and psoriasis cohorts was further examined (Figure 6.5A). In the psoriasis cohort, a high frequency of exosome-responsive CD1a-reactive T cells in the peripheral blood of psoriasis patients was detected, ranging from 0.05% to 0.16% of total blood T cells (Figure 6.5A). This reactivity was significantly higher that the CD1a autoreactivity and the response of T cells that were co-incubated with PLA2G4D-pulsed K562-EV cells (both $p < 0.001$) (Figure 6.5A). By contrast, the T cell response in the healthy cohort remained low throughout (Figure 6.5A). As expected, this CD1a reactivity could be inhibited by anti-CD1a blocking antibody ($p < 0.05$) but not control IgG antibody (Figure 6.5B). Moreover, the antigenic potential was further confirmed by the IFN-γ production of the expanded exosome-responsive CD1a-reactive T cells determined by cultured ELISpot assay (Figure 6.5C). Altogether, it has been demonstrated that

peripheral blood of psoriasis patients contained an enriched subpopulation of CD1a-reactive T cells that were responsive to LAD2 exosomes.

A



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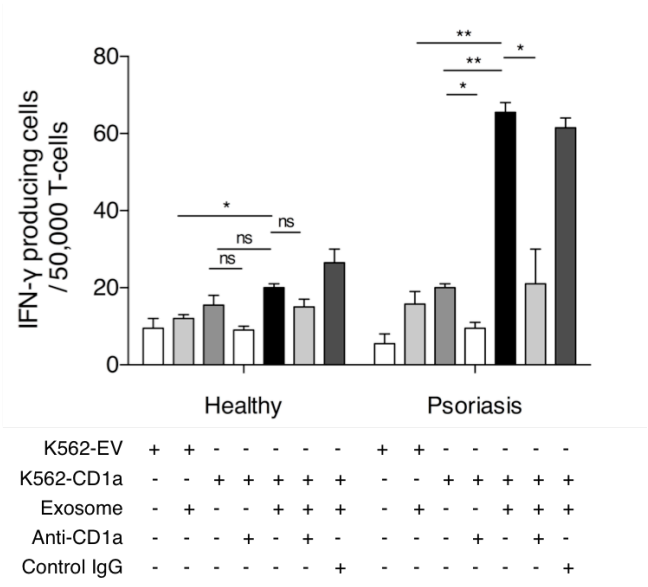

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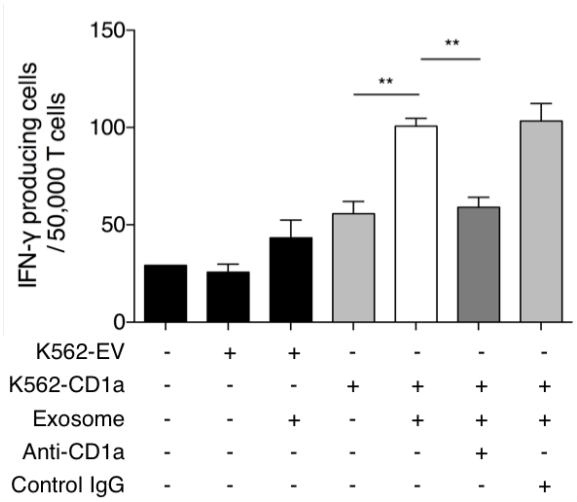

Figure 6.5 Exosome-responsive CD1a reactivity of T cells from psoriasis patients

K562-EV/K562-CD1a cells were incubated with LAD2-derived exosomes (10 *μ*g/ml protein content), and then incubated with T cells from psoriasis patients (n=18) and healthy donors (n=12). IFN-γ production was measured by ELISpot assay (**A**). K562-EV/K562-CD1a cells were incubated with LAD2-derived exosomes (10 *μ*g/ml protein content), and then incubated with T cells from one psoriasis patient and one healthy donor in the presence or absence of anti-CD1a antibody or control IgG antibody. Results are typical representation of at 18 psoriasis donors and 12 health donors, and of at least three individual experiments (**B**). T cells from psoriasis donors were expanded using autologous mDCs that were pulsed with LAD2-derived exosomes (10 *μ*g/ml of protein content) for 10-14 days prior to the cultured ELISpot assay. For the assay, K562-EV/K562-CD1a cells were incubated with 10 *μ*g/ml of the exosomes, and

then incubated with the T cells in the presence or absence of anti-CD1a blocking antibody or control IgG antibody, and IFN-γ production was measured by ELISpot assay. Results are typical representation of at least three individual experiments (**C**). Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns no significance.

Also whether the amount of LAD2 exosomes used for K562-pulsing could affect the T cell CD1a reactivity was examined. The K562-CD1a cells were pulsed with varying amounts of exosomes, and the CD1a reactivity of T cells from healthy or psoriasis individuals was tested (Figure 6.6). While the reactivity of T cells from healthy individuals remained low, the T cell reactivity in psoriasis patients varied with different concentrations of exosomes. Such reactivity increased with the amount of exosomes until it reached the peak at approximately 12 μg/ml of exosomes used, and then slightly dropped as the exosome amount further increased (Figure 6.6). Thus, it demonstrated that the exosome-responsive CD1a reactivity is dependent on the amount of exosomes used for pulsing of antigen-presenting cells.

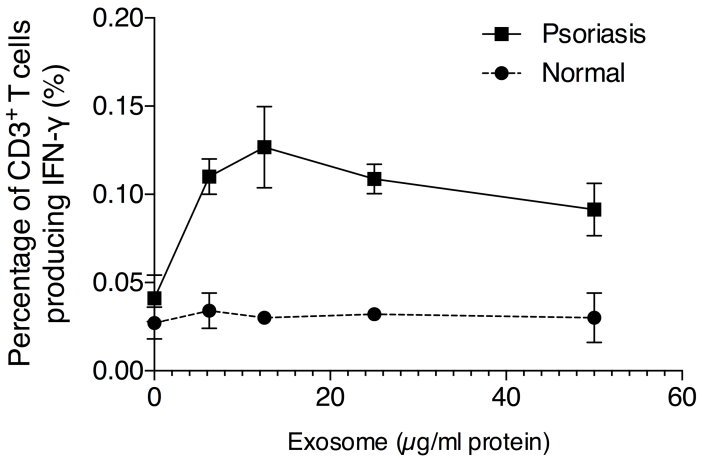

Figure 6.6 Dose-dependent exosome-responsive CD1a reactivity of T cells from psoriasis patients

K562-CD1a cells were incubated with varying concentrations of LAD2 exosomes, and then incubated with T cells from psoriasis patients (n=3) or controls (n=3). IFN-γ production was measured by ELISpot assay. Data were mean ± SEM.

6.2.4 Exosome-responsive CD1a-reactive T cells produce IL-17A and IL-22

Besides IFN-γ production, the production of IL-17A and IL-22 by the activated exosome-responsive CD1a-reactive T cells from healthy and psoriasis individuals was also examined (Figure 6.7). Similar to PLA2G4D-responsive CD1a-reactive T cells, these T cells also produced IL-17A and IL-22. Again, T cells from psoriasis patients produced higher levels of IL-17A and IL-22 than those from healthy individuals ($p < 0.05$ and $p < 0.001$ respectively) (Figure 6.7). This is consistent with the previous findings that the CD1a-reactive T cells could produce IL-17A and IL-22, which might contribute to the Th17 and Th22 responses in psoriasis.

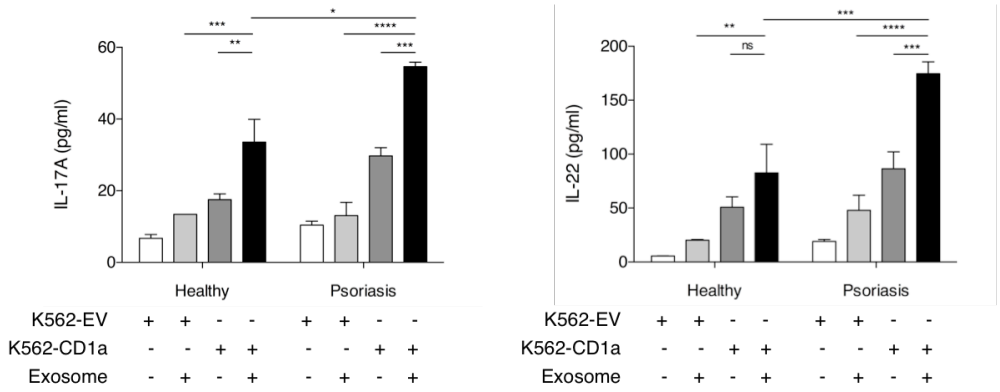

Figure 6.7 IL-17A and IL-22 production by exosome-responsive CD1a-reactive T cells

K562-EV/K562-CD1a cells were incubated with LAD2 exosomes (10 μg/ml protein content), and then incubated with T cells from one psoriasis patient and one healthy donor. IL-17A and IL-22 production were measured by ELISA. Results are typical representation of at least three individual experiments. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns no significance.

6.2.5 Autologous mDCs activate exosome-responsive CD1a-reactive T cells

A similar set of ELISpot experiments was performed using autologous mDCs as antigen-presenting cells. Again, there was a strong exosome-responsive T cell CD1a reactivity in the psoriasis cohort (Figure 6.8). The frequency of such T cells was significantly higher than the frequency of autoreactive T cells ($p < 0.01$), and it could reach as high as 0.2% of total blood T cells (Figure 6.8). Dependence of CD1a was confirmed by the successful reduction of T cell reactivity by anti-CD1a blocking antibody ($p < 0.05$) but not control IgG antibody (Figure 6.8). Importantly, the exosome-responsive CD1a reactivity could be inhibited by ATK, a $cPLA_2$ inhibitor ($p < 0.05$) (Figure 6.8). This suggested that $cPLA_2$ activity was important for the activation of exosome-responsive T cell response. By contrast, the exosome-responsive CD1a reactivity remained low throughout in the healthy cohort (Figure 6.8).

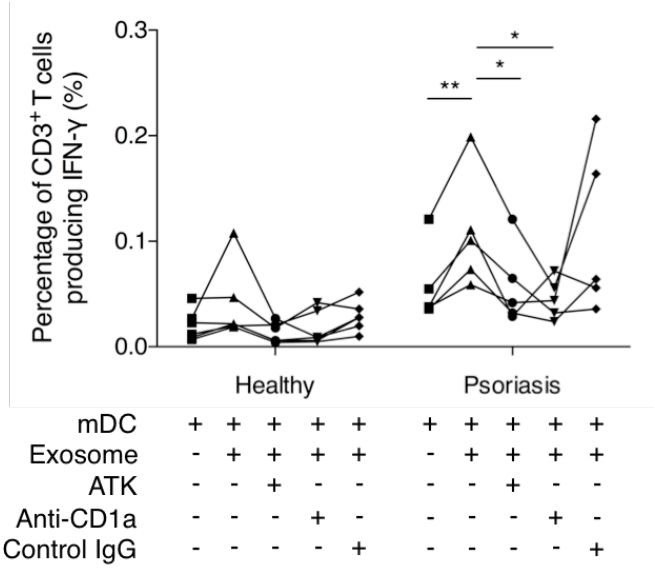

Figure 6.8 Exosome-responsive CD1a reactivity using autologous mDCs as presenting cells

mDCs were incubated with LAD2 exosomes (10 μg/ml protein content), and then incubated with T cells from psoriasis patients (n=5) and healthy donors (n=6) with anti-HLA-ABC (W6/32) and anti-HLA-DR blocking antibodies (L243), and in the presence or absence of anti-CD1a antibody or control IgG antibody, or specific cytosolic PLA_2 inhibitor ATK. IFN-γ production was measured by ELISpot assay. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$.

6.2.6 Inhibition of endocytosis reduces exosome-responsive CD1a reactivity

It has been previously shown in Figure 6.4 that exosomes derived from LAD2 mast cell-like line can be taken up and internalized by K562 cells. To study the mechanism of exosomal uptake, the endocytic pathway of K562 cells with the use of a panel of inhibitions of endocytosis was investigated (Figure 6.9). In this series of experiments, autologous mDCs were used as the antigen-presenting cells. mDCs were pre-incubated with the endocytosis inhibitors for 30 minutes before the addition of LAD2 exosomes for mDC-pulsing.

In the presence of cytochalasin D, which inhibits actin polymerization, a significant reduction in CD1a reactivity was observed in the psoriasis cohort ($p < 0.05$), but not in the healthy cohort (Figure 6.9A). As the process of endocytosis requires actin polymerization, an inhibition to it could halt the endocytic uptake of exosomes. Therefore, a reduction of CD1a reactivity in the presence of cytochalasin D indicated that endocytic uptake of exosomes by mDCs was essential for CD1a reactivity of the T cells. This was supported by the significant reduction in the CD1a reactivity of T cells in both healthy and psoriasis cohorts due to the presence of methyl-β-cyclodextrin (both $p < 0.01$) (Figure 6.9B). Methyl-β-cyclodextrin alters the plasma membrane fluidity by depleting the cholesterol, hence inhibits endocytosis. Altogether, any inhibition to the

uptake of exosomes by the endocytosis of mDCs could result in a significant reduction in the exosome-responsive CD1a reactivity of T cells.

It has been known that exosomal content can enter the cells via either clathrin-dependent or clathrin-independent pathways. We sought to study whether the endocytic uptake of exosomes by mDCs was a clathrin-mediated process. In the presence of chlorpromazine, which is an inhibitor of clathrin-mediated endocytosis, the exosome-responsive CD1a reactivity in both healthy and psoriasis cohorts drastically reduced (both $p < 0.01$) (Figure 6.9C). This implied that the uptake of LAD2 exosomes by mDCs was through clathrin-mediated endocytic pathway. In addition, we ruled out the possibility of toxicity and non-specific reactions caused by the above three inhibitors, as they showed no reduction of CD1a autoreactivity of T cells from both healthy and psoriasis individuals (Figure 6.9D).

Altogether, it has been demonstrated that the endocytic uptake of exosomes by antigen-presenting cells is essential for the activation of CD1a-reactive T cells, and this endocytosis is a clathrin-mediated process.

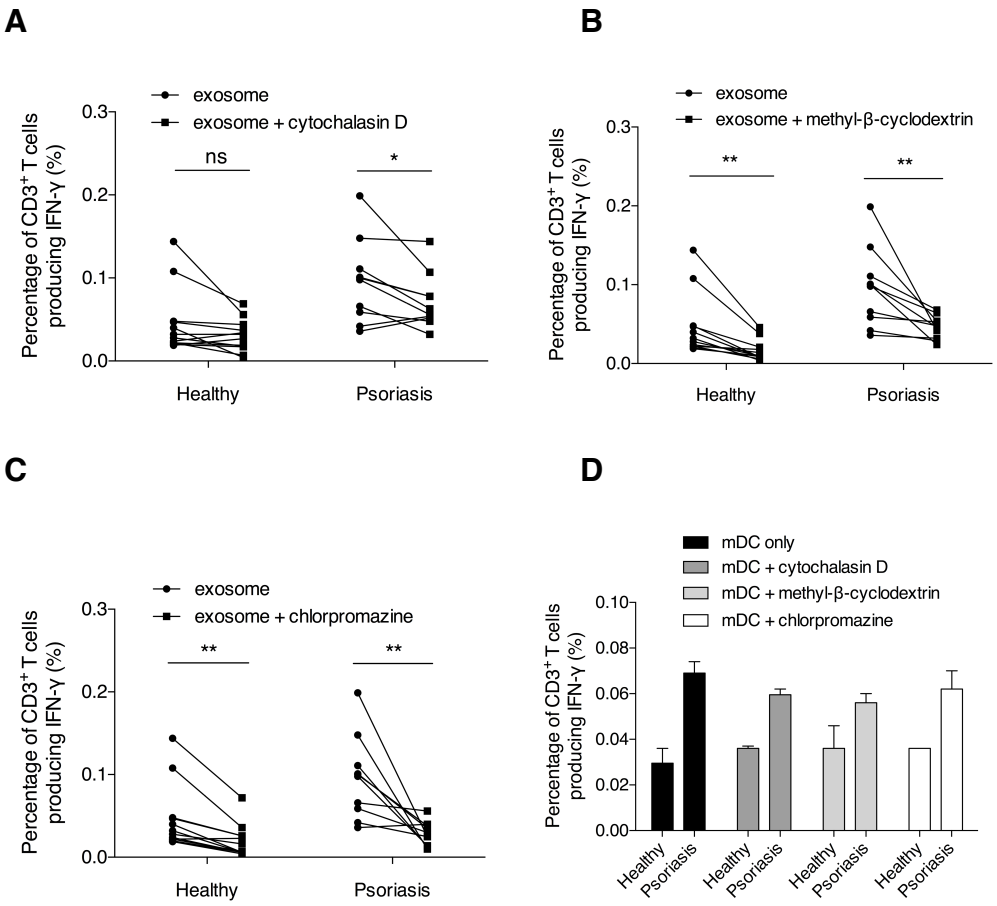

Figure 6.9 Clathrin-mediated endocytosis is critical for exosome-responsive CD1a reactivity of T cells

Autologous mDCs were incubated with LAD2 exosomes (10 *μ*g/ml protein content) in the presence of anti-HLA-ABC (W6/32) and anti-HLA-DR (L243) blocking antibodies, and with or without 30-minute pre-incubation of inhibitors of the endocytosis pathway (**A**: 5 *μ*g/ml cytochalasin D; **B**: 2% methyl-β-cyclodextrin; **C**: 10 *μ*g/ml chlorpromazine), and were then incubated with T cells from psoriasis patients (n=10) or healthy donors (n=12) (**A**, **B**, **C**) Autologous mDCs were pre-incubated with or without inhibitors of endocytosis pathway (5 *μ*g/ml cytochalasin D, methyl-β-cyclodextrin, or 10 *μ*g/ml chlorpromazine) for 30 minutes, and were then

incubated with T cells from psoriasis patients or healthy donors. IFN-γ production was measured by ELISpot assay. Results were representative of 10 psoriasis patients and 12 controls. Data were mean ± SEM (**D**). * $p < 0.05$; ** $p < 0.01$; ns no significance.

6.2.7 IFN-α potentiates exosome-responsive CD1a reactivity

It has been demonstrated that the CD1a-reactive T cells could produce IFN-γ, IL-17A, and IL-22 in response to CD1a-expressing antigen-presenting cells using either PLA2G4D protein or PLA2G4D-containing exosomes derived from LAD2 mast cell-like line. On the other hand, it has been known that IFN-α might play an important role in the initiation of psoriasis. For instance, plasmacytoid dendritic cells (pDCs) could initiate psoriasis through the production of IFN-α, contributing to elevated level of IFN-α in the psoriatic lesions [56]. However, little is known about how IFN-α could affect the CD1a pathway. Here, the LAD2 cells were pre-treated with IFN-α overnight, and on the next day exosomes were collected from the supernatant and used as an antigen source. In the psoriasis group, compared to exosomes from untreated LAD2 cells, exosomes produced by IFN-α-treated LAD2 cells could activate T cells and generate a two-fold increased T cell response ($p < 0.05$), which could be inhibited by anti-CD1a blocking antibody ($p < 0.05$) but not control IgG antibody (Figure 6.10). In the healthy group, T cell response remained relatively low regardless of the antigen sources (Figure 6.10). Hence these findings indicated that IFN-α could potentiate that exosome-responsive CD1a reactivity of T cells from psoriasis patients.

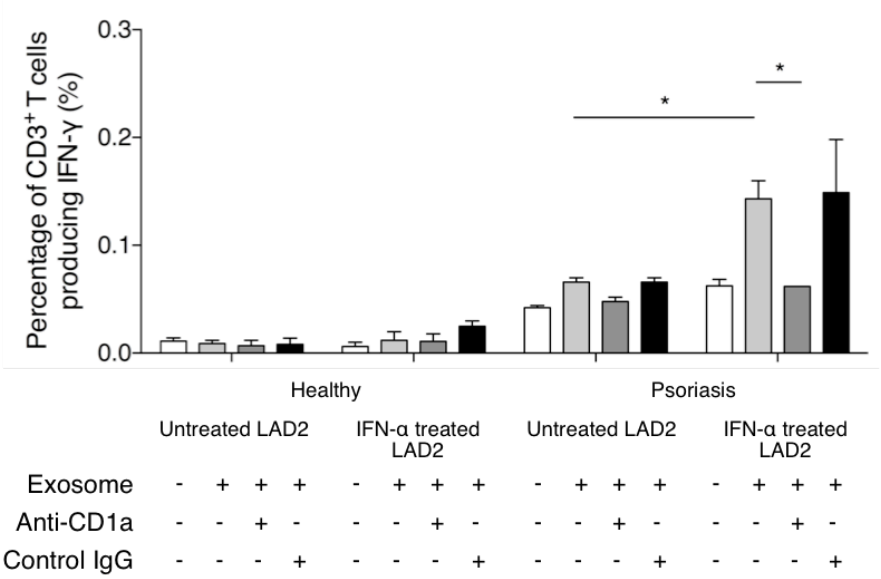

Figure 6.10 IFN-α potentiates exosome-responsive CD1a reactivity

K562-CD1a cells were either incubated with exosomes from untreated LAD2 cells or exosomes from IFN-α (4U/ml) treated LAD2 cells (both 10 μg/ml protein content), and then incubated with T cells from one psoriasis patients or one healthy donors in the presence or absence of anti-CD1a antibody or control IgG antibody. IFN-γ production was measured by ELISpot assay. Results were representative of four patients with psoriasis and five controls. Data were mean ± SEM. * $p < 0.05$.

6.2.8 Enriched exosome-responsive CD1a-reactive T cells in psoriatic lesions

The previous findings have demonstrated the antigenic potential of exosomes derived from LAD2 mast cell-like line, and that the PLA2G4D-containing exosomes could enter the CD1a-expressing antigen presenting cells via clathrin-mediated endocytosis, and lead to the activation of exosome-responsive CD1a-reactive T cell response, resulting in the production of IFN-γ, IL-17A and IL-22. However, all these experiments were based on the T cells isolated from the peripheral blood of the donors. Whether T cells derived from the skin could also elicit a similar response was still in question. Given that in the psoriatic lesions there is usually an infiltration of leukocytes, and many of them are T cells, we hypothesized that skin-resident T cells could respond to the exosomes-derived antigens which are presented by CD1a-expressing antigen-presenting cells, and hence could contribute to the immune response in the psoriatic lesions.

Cutaneous T cells were isolated from lesional and non-lesional skin of psoriasis patients for the study of LAD2 exosome-responsive CD1a reactivity *ex vivo*. After the isolation of skin-resident T cells, they were divided into three experimental conditions due to the limited number of cells obtained from each biopsy; they were either co-incubated with unpulsed K562-CD1a cells, exosome-pulsed K562-CD1a cells in the presence of anti-CD1a blocking antibody, or exosome-pulsed K562-CD1a

cells in the presence of control IgG antibody (Figure 6.11A). In the healthy skin, the frequency of CD1a-autoreactive T cells was about 0.1% of total skin T cells, while the frequency of exosome-responsive CD1a-reactive T cells was about 0.1-0.3% (Figure 6.11A). Comparing with the frequencies of CD1a-autoreactive T cells and exosome-responsive CD1a-reactive T cells in the peripheral blood determined in previously (~0.01% and ~0.02% respectively), there was an about 10-fold enrichment of responding T cells in the healthy skin.

In the non-lesional psoriatic skin, while the frequency of CD1a-autoreactive T cells remained as low as 0.1%, the frequency of exosome-responsive CD1a-reactive T cell subpopulation was about 0.3% of total skin T cells (Figure 6.11A). However, such frequency was not statistically different from that in the healthy skin (Figure 6.11A).

In the lesional psoriatic skin, the frequency of CD1a-autoreactive T cells was only about 0.2%, yet the frequency of exosome-responsive CD1a-reactive T cells was 0.5-0.6%, which was almost double of that in the non-lesional psoriatic skin ($p < 0.05$), and triple of that in the healthy skin ($p < 0.01$) (Figure 6.11A). Inhibition of the CD1a reactivity by anti-CD1a blocking antibody ($p < 0.01$) confirmed the CD1a dependence (Figure 6.11A). Comparing with the frequencies of CD1a-autoreactive T cells and exosome-responsive CD1a-reactive T cells in the peripheral

blood of psoriasis patients (0.02-0.08% and 0.03-0.14% respectively), we observed a 2 to 10-fold enrichment of CD1a-autoreactive T cells, and 4 to 20-fold enrichment of exosome-responsive CD1a-reactive T cells in the psoriatic lesions. Yet the enrichment of the both types of T cells was much lower in the non-lesional psoriatic skin.

For each donor of psoriatic skin, we compared the exosome-responsive CD1a reactivity between the T cells from peripheral blood, non-lesional and lesional psoriatic skin (Figure 6.11B). Within the same donors, the frequency of exosome-responsive CD1a-reactive T cells was generally the highest in the lesional skin, followed by the non-lesional skin, compared to the peripheral blood of psoriasis patients (Figure 6.11B).

Furthermore, we have examined the production of IL-17A and IL-22 from the cutaneous T cells of healthy donors and psoriasis patients. While exosome-responsive CD1a-reactive T cells from healthy skin produced relatively low levels of IL-17A, the T cells from both non-lesional and lesional psoriatic skin produced significantly greater amounts of IL-17A ($p < 0.01$ and $p < 0.001$ respectively) (Figure 6.11C). Also, the IL-17A production by the T cells in the psoriatic lesional skin was greater than that in the non-lesional skin ($p < 0.05$), and such production could be inhibited by anti-CD1a blocking antibody ($p < 0.05$) (Figure 6.11C). A similar trend was observed in the IL-22 profile (Figure 6.11D). While T cells from the

healthy skin produced low levels of IL-22 in all experimental conditions, exosome-responsive CD1a-reactive T cells from psoriatic lesional skin produced significantly greater amount of IL-22 ($p < 0.001$) (Figure 6.11D). Similarly, such IL-22 production could also be inhibited by anti-CD1a blocking antibody ($p < 0.05$) (Figure 6.11D). The IL-22 production by the exosome-responsive T cells from the non-lesional psoriatic skin was lower compared to the production by T cells from the lesional skin ($p < 0.05$), yet had no significant different from that by T cells from the healthy skin (Figure 6.11D). Altogether, these findings have showed that exosome-responsive CD1a-reactive T cells, as well as CD1a-autoreactive T cells, were enriched in the lesional skin of psoriasis, and these T cells produced not only IFN-γ, but also IL-17A and IL-22.

To summarize all the findings in this chapter, the current findings have demonstrated that LAD2 mast cell-like line could release cytosolic PLA2G4D, which was enzymatically active, to the extracellular environment via exosomes. Through exosomal transport, enclosed PLA2G4D could be delivered from LAD2 cells to antigen-presenting cells by the uptake of exosomes through a clathrin-mediated endocytic process. We also demonstrated the antigenic potential of LAD2 exosomes, as they could activate the CD1a-reactive T cells from psoriasis patients and lead to the production of IFN-γ, IL-17A, and IL-22. This exosome-responsive CD1a reactivity is dependent on the presence of CD1a and the amount of

exosomes. Moreover, this CD1a reactivity could be potentiated by the action of IFN-α on the LAD2 cells prior to the production of exosomes. At last we revealed a significant enrichment of exosome-responsive CD1a-reactive T cell subpopulations in the total skin-resident T cell population in the psoriatic lesional skin, and these T cells could also produce substantial levels of IFN-γ, IL-17A, and IL-22, which might contribute to the Th17 and Th22 responses in the lesions.

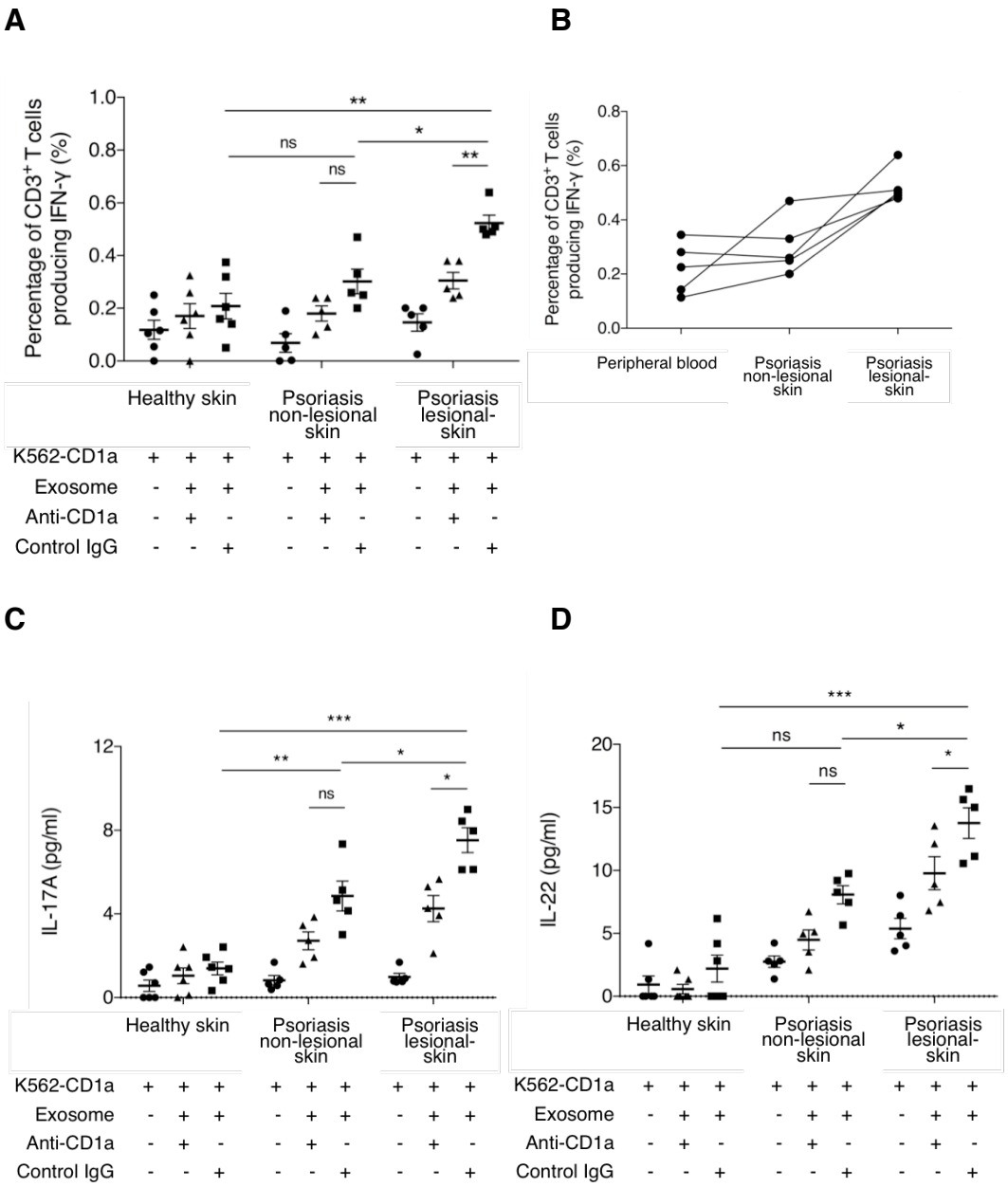

Figure 6.11 Skin-resident CD1a-autoreactive and exosome-responsive CD1a-reactive T cells in psoriatic lesions

K562-EV/K562-CD1a cells were incubated with LAD2 exosomes (10 *μ*g/ml protein content), and then incubated with T cells from skin biopsies from psoriasis patients (n=5) or healthy donors (n=6) in the presence or absence of anti-CD1a or control IgG antibodies. IFN-γ production was measured by ELISpot assay (**A**). K562-EV/K562-CD1a cells were

incubated with LAD2 exosomes (10 μg/ml protein content), and then incubated with T cells from blood, non-lesional skin and lesional skin biopsies from psoriasis patients (n=5). IFN-γ production was measured by ELISpot assay (**B**). K562-EV/K562-CD1a cells were incubated with LAD2 exosomes (10 μg/ml protein content), and then incubated with T cells from skin biopsies from psoriasis patients (n=5) or healthy donors (n=6) in the presence or absence of anti-CD1a or control IgG antibodies. IL-17A (**C**) and IL-22 (**D**) production in the supernatant was measured by ELISA. Data were mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns no significance.

6.3 Discussion

In the previous chapter, it has been demonstrated that the peripheral blood of psoriasis patients, compared to healthy individuals, contained an enriched subpopulation of PLA2G4D-responsive CD1a-reactive T cells. The results also showed that mast cells in psoriatic lesions are a source of the PLA2G4D. Although PLA2G4D protein alone can activate the CD1a reactivity in T cells, it remained unclear how a $cPLA_2$ from mast cells can act on another cells. Therefore, it was reasoned that there must be an intercellular transport mechanism that allows the delivery of PLA2G4D from the mast cells to the antigen-presenting cells.

Mast cells can release preformed enzymes and other proteins in their granules in a rapid process namely degranulation. Apart from this, mast cells can also synthesize a number of lipid mediators upon activation and release them to extracellular environment. PLA2G4D and other $cPLA_2$ that are expressed by mast cells normally remained in the cytosol, and not secreted via mast cell degranulation. Nonetheless, past studies about the lipid and protein content of extracellular vesicles have revealed that $cPLA_2$ and relevant lipids could be delivered to target cells via extracellular vesicular pathways. For instance, exosomes produced by rat tumoural mast cells and rat basophilic leukaemia cell line contained PC, LPC, and

other related lipids [354, 365]. Moreover, another four studies revealed that exosomes and microvesicles contained the mRNA and proteins of $cPLA_2$ [367-370]. In particular, one of the above studies has recently shown that exosomes derived from dendritic cells contained PLA2G4D protein [369]. This further supported that PLA2G4D could be carried by intercellular transport mechanism via extracellular vesicles. Hence our findings about the PLA2G4D-containing exosomes derived from LAD2 mast cell-like line (Figure 6.1 and 6.3) were in agreement with the aforementioned studies.

Another issue is the activity of the $cPLA_2$ in exosomes. As enzymes require optimal pH for maximal enzymatic functions, whether the LAD2 exosomes could provide a suitable pH for the activity of PLA2G4D remains a question. Although little is known about the pH inside the exosomes and other extracellular vesicles, it is logical to assume that it is mildly acidic, based on the fact that multivesicular body (MVB), which the exosomes originate from, has a pH value of about 5 [371]. Generally, the optimal pH for PLA_2 is about 5.5-6.5 [372], therefore it is probable that the PLA2G4D could function normally inside the LAD2 exosomes. This was confirmed by detecting $cPLA_2$ activity in the exosomes (Figure 6.2).

Besides the activity of PLA2G4D in the exosomes, successful loading of the enzyme to the target cells, i.e. antigen-presenting cells, is

also important. We have demonstrated the successful docking and internalization of LAD2 exosomes by K562 cells (Figure 6.4) through clathrin-mediated endocytic pathway (Figure 6.9). It was suggested that an acidic pH in the extracellular environment could facilitate the uptake of exosome by target cells [373]. However we did not test whether the pH in the culture medium could affect the rate of exosome uptake by the K562 cells. Although it has been shown that the skin surface pH of psoriasis lesional skin is slightly higher than that of psoriasis non-lesional skin [374], little is known about the pH within the epidermis or dermis of psoriatic lesions. Therefore it remains unclear the effect of pH on the uptake of exosomes by antigen-presenting cells.

After understanding the mechanism of exosome uptake by antigen-presenting cells, we have demonstrated the exosome-responsive CD1a reactivity of T cells from psoriasis individuals with the use of K562 cells as CD1a-expressing antigen-presenting cells (Figure 6.5), and also autologous mDCs as antigen-presenting cells, which are more physiologically relevant (Figure 6.8). This CD1a reactivity is dependent on the amount of exosomes (Figure 6.6). We determined the frequency of exosome-responsive CD1a-reactive T cells in psoriasis patients ranging from 0.05% to 0.16% of total blood T cells. Such frequency was consistent with the frequency of PLA2G4D-responsive CD1a-reactive T cells in the psoriasis patients in the previous chapter (0.03-0.14%). Also, in healthy

individuals the frequencies of PLA2G4D- and exosome-responsive T cells in blood were similarly low, at around 0.02%. This indicated that the CD1a-reactive T cells in the peripheral blood of psoriasis patients could elicit a similar level of responses when using PLA2G4D-containing exosomes or PLA2G4D protein itself. Similar to PLA2G4D-responsive T cells, exosome-responsive T cells also produced IL-17A and IL-22 (Figure 6.7), implying the potential contribution to Th17 and Th22 responses.

We also investigated the effect of IFN-α on the LAD2 cells. In psoriasis lesions, it is commonly accepted that pDCs are associated with early lesions of psoriasis. For instance, LL-37/DNA complexes, as a result of antimicrobial production by keratinocytes and self-DNA released by damaged tissues, can activate plasmacytoid dendritic cells, which can initiate psoriasis by producing IFN-α [56, 69]. IFN-α can stimulate or activate myeloid DCs, Th17 cells, and keratinocytes [45, 148]. Here we have demonstrated that IFN-α could potentiate the exosome-responsive CD1a reactivity of T cells by acting on the LAD2 cells (Figure 6.10), hence revealing a link between IFN-α, mast cells, CD1a-expressing antigen-presenting cells, and CD1a-reactive T cells. However, the detail molecular action of IFN-α on the LAD2 cells/mast cells had not been investigated.

Apart from blood T cell responses, we also examined the responses of T cells in the skin (Figure 6.11). We found that there was an

up to 10-fold enrichment of exosome-responsive CD1a-reactive T cells in the lesional skin compared to the blood of psoriasis patients. By contrast, there was no significant difference in the frequency of the CD1a-reactive T cells between healthy skin and non-lesional psoriasis skin. Notably, we determined the frequency of CD1a-autoreactive T cells in the lesional skin to be approximately 0.2% of T cells, which was at least half the frequency of exosome-responsive T cells (0.5-0.6%). This implied that the exosome-responsive CD1a reactivity might play a more prominent role than the CD1a autoreactivity to account for the immune response in the lesions. It is noted that the CD1a-autoreactivity was not completely reduced by the presence of anti-CD1a antibody (Figure 6.11), and this might be attributed to non-specific stimulation to T cells, which is not dependent on CD1a, by the addition of exosomes.

Here a model is proposed in attempt to explaining the exosome-responsive CD1a reactivity of T cells. After being internalised, the LAD2 exosomes, as well as the PLA2G4D inside, are directed to the sorting/early endosomes of the antigen-presenting cells. Notably, due to the mildly acidic (~ pH 6.2) environment of these endosomes [371], PLA2G4D might also generate lipids inside them. Coincidentally, PLA2G4D and its derived lipids might meet CD1a that is being recycled to the sorting/early endosomes, therefore the exchange of CD1a ligands could occur locally. The lipid autoantigens, which are initially on the CD1a,

are replaced by the neolipid antigens generated by the activity of PLA2G4D. As a result, when CD1a returns to the surface of the antigen-presenting cells, the neolipid antigens could be presented to CD1a-reactive T cells, and hence leading to the activation of T cells and the production of IFN-γ, IL-17A, and IL-22.

This proposed model is based on the assumption that the exchange of CD1a ligands mainly occurs inside the cells. Indeed the ligand exchange could also occur at the cell surface of CD1a-expressing cells [375]. As we have previously shown that PLA2G4D was active inside the exosomes, it is then in question whether PLA2G4D could generate neolipid antigens by cleaving the membrane of exosomes, and that these lipids could diffuse out of exosomes and replace the autoantigens on the CD1a at the cell surface. Nonetheless, in some experiments we have shown that this is probably not the case, or at least this is not a major pathway. We have learned that the CD1a reactivity was attributed to successful uptake of exosome by the antigen-presenting cells via endocytosis, whereas the blockade of this process greatly reduced the CD1a reactivity (Figure 6.9). Therefore, it is more probable that the sorting/early endosomes is the main location of exchange of CD1a ligands.

It might also be argued that the CD1a autoantigens could be replaced by the lipids that already exist in the exosomes. One possible source of these lipids could be those that are generated by PLA2G4D during exosomal transport or even before the biogenesis of exosomes. However, our results demonstrated that the T cell CD1a reactivity could be greatly reduced by the inhibition of $cPLA_2$ activity (Figure 6.8), and hence it was more probable that the lipids were generated after the internalisation of exosomes. Another possible source of the lipids that already exist in the exosomes could be directly derived from the LAD2 cells. Again, the results regarding to the reduction of CD1a reactivity by $cPLA_2$ inhibitor (Figure 6.8) and inhibitors of endocytosis (Figure 6.9) serve as the grounds for the counter-argument. It is more probable that the generation of neolipids antigens for CD1a ligand exchange inside the sorting/early endosomes is the necessary factor for the CD1a-lipid presentation to CD1a-reactive T cells.

7. General Discussion

7.1 General discussion

It is widely accepted that psoriasis is a mainly lymphoid cell-mediated immune disease of the skin, supported by the infiltration of leukocytes, especially $CD4^+$ and $CD8^+$ T cells, and the elevation of Th1 and Th17 cytokines in the lesions [94, 155, 156]. A number of peptide antigens have been suggested. Past studies reported the association of psoriasis and bacterial flora on the skin [206-208, 210, 211], and T cells from some psoriasis patients responding to protein antigens derived from staphylococcus [209]. Apart from foreign sources, many recent studies have identified a number of self-antigens, such as keratins, antimicrobial peptide LL-37, and melanocyte antigens [213-216]. This indicates that self-antigens might play a more significant role in the pathogenesis of psoriasis. However despite considerable effort to identify peptide antigens that are relevant to psoriasis, the studies have rarely been replicated, suggesting that non-peptide antigens may represent an additional antigen class.

The association of phospholipase A_2 (PLA_2) and psoriasis has been long established. Elevation of PLA_2 activity and increased concentrations of a number of PLA_2-derived lipid products were identified in the skin of

psoriatic lesions [301-305]. Elevated expression of non-pancreatic PLA_2 and cytosolic PLA_2 ($cPLA_2$) proteins were also reported [306]. In particular, a defined $cPLA_2$ protein, namely PLA2G4D, was discovered in the psoriatic lesions, where its mRNA and protein expression were increased [299]. This was further supported by the findings of an intra-individual genomic analysis of psoriasis patients [300]. However, the above studies did not establish a direct role of PLA_2 and particularly PLA2G4D in the pathogenesis of psoriasis. Nonetheless, the recent findings about the non-peptide antigen presentation by CD1a in the skin shed some light on the role of PLA_2 in psoriasis. CD1a, highly expressed on Langerhans cells and some myeloid dendritic cells, can present a range of lipid-based antigens to CD1a-restricted T cells, where these antigens can be of exogenous or endogenous sources. de Jong *et al.* showed that CD1a-autoreactive T cells (i.e. T cells recognising self-antigens presented by CD1a) are a normal part of the T cell repertoire in normal healthy individuals [218]. CD1a-associated self-lipids include fatty acids, wax esters, and squalene, which are commonly found in sebum [217]. CD1a lipids can be derived from exogenous sources such as bee and wasp venom [272] and house dust mite [274]. In particular, our previous recent findings described that allergic individuals contained elevated frequencies of CD1a-reactive T cells in the blood and skin [273, 274]. Based on these findings, it is suggested that CD1a might be the link between elevated PLA_2 activity and the T cell response in the lesions of psoriasis; and Langerhans cells and

myeloid dendritic cells in the skin, which are $CD1a^+$, might be responsible for the lipid antigen presentation to CD1a-restricted T cells.

Notably, the altered mobilisation of Langerhans cells is associated with psoriasis. In the lesional skin, the migration rate of Langerhans cells was reduced in response to stimuli [35, 200, 201], and it might be due to an epidermal microenvironment rather than the abnormality of the cells themselves [202]. On the other hand, studies showed that dermal dendritic cells in psoriatic lesions could interact and activate T cells to produce IL-17 and IFN-γ [203, 204]. Therefore, these studies further supported the potential link between PLA_2 activity and T cell response in psoriasis.

In Chapter 4 the relationship between CD1a and psoriasis was investigated. The relevance of CD1a autoreactivity in psoriasis was first reported by showing the greater IFN-γ response of CD1a-autoreactive T cells in the peripheral blood of psoriasis patients compared to healthy individuals. We determined the subpopulation of IFN-γ producing CD1a-autoreactive T cells in the blood of psoriasis patients to be 0.02-0.08% of total blood T cells, whereas that of healthy individuals to be 0.01-0.04%. Our determined frequency of CD1a-autoreactive T cells in healthy individuals was consistent with our previous findings [273, 274]. The frequencies were determined without the addition of external source of antigens. It has remained in question how such small population of CD1a-

autoreactive T cells in the circulation could contribute to the CD1a-mediated responses in psoriasis. To explain the relevance of CD1a autoreactivity in the lesional skin of psoriasis, it is hypothesised that the immune response might be initiated when self-lipids, which are originally located in the upper layer of epidermis, are brought to deeper layers of skin due to barrier dysfunction of psoriatic lesions. In the steady state, such self-lipids remain in their original place and do not meet the $CD1a^+$ Langerhans cells and dermal dendritic cells, therefore do not contribute to CD1a-autoreactive responses, and hence comparatively lower frequency of CD1a-autoreactive T cells are found in the blood of healthy individuals. However during psoriasis, the $CD1a^+$ cells encounter the “invasion” of self-lipids to the deeper skin, and present these lipids to CD1a-restricted T cells, resulting in CD1a-autoreative response in the psoriatic lesions. Upon activation, CD1a-autoreactive T cell expansion occurs, thus contributing to the elevated frequency of CD1a-autoreactive T cells in the peripheral blood of psoriasis patients compared to healthy individuals. To test this hypothesis, the lipid-responsive CD1a reactivity of the T cells were expanded with autologous mDCs, which were pulsed with a panel of host-derived lipids. Results showed that the expanded T cells could recognise host PLA_2-derived lipids, such as arachidonic acid, lysophosphatidylcholine (LPC), and fatty acids. These findings were consistent with the elevated PLA_2 activity in the lesional skin of psoriasis [301-304].

After establishing the link between CD1a and psoriasis, the contribution of PLA_2 to the CD1a-mediated T cell response in psoriasis patients was the investigated in Chapter 5. According to the literature, and based on the analysis of the past studies, expression of PLA2G4D is highly relevant to psoriatic lesions. Therefore, we examined the source of PLA2G4D in psoriatic lesions, and found that mast cells in the lesions are a source of the $cPLA_2$ by confirming the protein expression of PLA2G4D in the LAD2 mast cell-like line and cord blood-derived mast cells. The PLA2G4D protein was then purified from the LAD2 cells for subsequent functional experiments. Conventionally, mast cells are well known for their contribution to IgE-mediated immunity and Th2 response. However, apart from these roles, mast cells indeed contribute to diverse immune responses due to the ability of producing a wide range of cytokines, enzymes, lipids, and other biologic mediators [80]. Indeed, the association of mast cells and psoriasis has long been suggested. For example, increased number of mast cells in the lesions of psoriasis was reported previously [134]. Furthermore, mast cells can be activated by a number of mediators, such as IL-33 produced by keratinocytes in psoriatic lesions, as well as LPC, an immediate product of PLA_2 of which activity was elevated in psoriatic lesions [333, 338]. Moreover, the secretion of IL-17 from mast cells could also contribute to Th17 responses in the lesions [140]. Furthermore, PLA_2 is also an important component of normal mast cell

functions. For example, lung mast cells express multiple types of secretory PLA_2 ($sPLA_2$) [339], and indeed the group III PLA_2 is critical for mast cell maturation [286]. Therefore, based on the evidence above, it is suggested that PLA2G4D expressed by mast cells in the psoriatic lesions might generate lipids, which could be presented by CD1a-expressing antigen-presenting cells to CD1a-restricted T cells. Also, the $cPLA_2$ activity found in the mast cells might also account for the elevated PLA_2-associated lipids concentration in the psoriatic lesions.

Based on our previous findings in Chapter 4 that CD1a-autoreactive T cells can be activated by CD1a-expressing antigen-presenting cells that present self-lipids by CD1a, in Chapter 5 it was investigated whether these T cells could respond to the PLA2G4D-derived lipids presented by CD1a. It was found that PLA2G4D derived from LAD2 cells could activate the response of CD1a-reactive T cells in psoriasis patients. It is noted that apart from IFN-γ, the activation could also lead to the production of IL-17A and IL-22 from the T cells. Therefore we provided the evidence for 1) the antigenic potential of a $cPLA_2$ related to CD1a-reactive T cell response, and 2) the contribution of $cPLA_2$-expressing mast cells in T cell response in psoriasis.

However, there seems a paradox that $cPLA_2$, normally remaining in the cytosol of the cells, can nevertheless exert a distant effect on the

CD1a-expresing cells. In order to explain this paradox, we examined the extracellular transport system of mast cells. While there was little knowledge about whether $cPLA_2$ could be secreted through mast cell degranulation, there were a number of studies confirming the presence of PLA_2-associated lipids in the extracellular vesicles derived from T cells, mast cells, blood and inflammatory fluids [354, 363-366]; as well as two types of $cPLA_2$, including PLA2G4D, in the exosomes derived from dendritic cells [369]. In Chapter 6.2.1, we presented direct evidence of the presence of PLA2G4D in the exosomes produced by LAD2 cells, by showing that the exosome contained $cPLA_2$ activity and PLA2G4D protein expression. We also demonstrated that K562 cells could be the target cells of the exosomes (Chapter 6.2.2). Based on these findings, it is probable that PLA2G4D, although normally remaining in the cytosol, could be delivered to the antigen-presenting cells through an extracellular transport system, namely by exosomes, of the mast cells.

Chapter 6.2.3 shows the antigenic potential of PLA2G4D-containing exosomes. We observed an elevated frequency of exosome-responsive CD1a reactivity of T cells in the peripheral blood of psoriasis patients, but not healthy individuals. These exosome-responsive T cells, like the PLA2G4D-responsive cells, also produced IFN-γ, IL-17A, and IL-22 (Chapter 6.2.4). Inhibitor experiments confirmed that this CD1a reactivity was dependent on $cPLA_2$ activity, implying the response was not due to

the existing lipids inside the exosomes, but the *de novo* synthesis of neolipids. Similarly, the exosome-responsive CD1a reactivity was confirmed using autologous mDCs as antigen-presenting cells (Chapter 6.2.5).

Although the exchange of CD1a ligands is thought to mainly occur in the sorting/early endosomes during the recycling of CD1a, one possibility is that such an event might alternative occur on the cell surface as described before [375]. However, we showed that successful uptake of exosomes by antigen-presenting cells via clathrin-mediate endocytosis is critical for the CD1a reactivity (Chapter 6.2.6). Therefore the ligand exchange is not likely to occur when endocytosis of exosomes is blocked. Therefore it is probable that the sorting/early endosomes is a site for the generation of neolipid antigens by PLA2G4D, and the exchange of CD1a ligands. PLA2G4D-derived lipids are then presented to CD1a-reactive T cells after CD1a returns to the cell surface of the antigen-presenting cells.

In addition, it was shown that IFN-α could potentiate the CD1a reactivity by acting on the LAD2 cells (Chapter 6.2.7). The production of IFN-α by pDCs is an important trigger of the early phase of psoriasis [56, 69]. Here a link between IFN-α and mast cells in psoriasis has been established. It is likely that IFN-α can stimulate the expression of PLA2G4D of mast cells, and/or increase their exosome production. Detail

mechanisms underlying this observation are important for further investigation.

Apart from the peripheral blood, an enriched exosome-responsive CD1a-reactive T cell population was also detected in the lesional skin of psoriasis patients, compared to non-lesional skin and skin of healthy individuals (Chapter 6.2.8). In addition, CD1a-autoreactive T cells were also detected in the psoriatic lesions, albeit not significantly enriched compared to non-lesional psoriasis skin and healthy skin. These findings confirmed the relevance of CD1a reactivity to the skin of psoriatic lesions. The CD1a-reactive T cells in the psoriatic lesions could recognise neolipids, that are generated by mast cell exosomes-derived PLA2G4D, presented by CD1a and produce IFN-γ, IL-17A, and IL-22. The frequency of these T cells in the lesions was determined to be 0.5-0.6%, which is ten times more than the frequency in the blood. This could be due to the recruitment of T cells, especially those expressing markers relevant to skin homing, from the circulation to the lesions in response to chemokines. Although these T cells only represent 1 in every 200 skin-resident T cells, the production of IFN-γ, IL-17A and IL-22 by these T cells could lead to a number of inflammatory responses in psoriasis. For example, IL-17 and IL-22 together stimulate the production of antimicrobial peptides from keratinocytes [148]. IL-17 and IFN-γ, on the other hand, together induce the production of proinflammatory cytokines from keratinocytes [150]. IL-

22 promotes the proliferation, yet inhibits the differentiation, of keratinocytes [149]. Therefore, by producing these cytokines, the exosome-responsive CD1a-reactive T cells might contribute to the Th1, Th17, and Th22 responses in the lesions. However, the expression of CD4 and CD8 of these CD1a-reactive T cells was not determined in this study. While both $CD4^+$ and $CD8^+$ T cells were identified in psoriatic lesions [376-378], some evidence showed that $CD8^+$ T cells in the lesional skin could produce IL-17A and IL-22, therefore representing the effector Tc17 and Tc22 cells [156]. Therefore further investigation is required to reveal the identity of the CD1a-reactive T cells in the psoriatic lesions, and the cytotoxic properties of these cells, by releasing cytotoxins (such as granzyme and perforin) or via Fas-FasL interaction, should they belong to the Tc subsets.

To summarise, connecting all the relevant findings discussed above, we have established a novel pathogenic model to explain the pathogenesis of psoriasis, depicted in Figure 7. In the normal skin, the skin barrier is intact and there is no abnormal proliferation and differentiation of keratinocytes. In the steady state, $CD1a^+$ Langerhans cells reside in the epidermis, while dermal dendritic cells are the CD1a-expressing antigen-presenting cells in the dermis and antigenic self-lipids are excluded from direct contact.

During early phases of psoriasis, a breach in skin barrier occurs upon physical injury, resulting in the increased exposure to environmental pathogens. In response to microbial invasion, keratinocytes secrete antimicrobial peptides, including β-defensins and LL-37, to support the clearance of foreign microbes. Damage to cells due to physical injury or infections release RNA and DNA. Notably, LL-37 can bind to self-DNA, which is derived from damaged cells due to physical injury or infections. LL-37/DNA complexes in turn bind to the Toll-like receptor (TLR) 9 on the surface of pDCs in the dermis, and induce their production of IFN-α. LL-37 can also bind to self-RNA, and LL-37/RNA complexes can activate pDCs via TLR7 to produce IFN-α. LL-37/RNA and IFN-α can also activate myeloid DCs through TLR8 and lead to the production of IL-12 and IL-23 by the dendritic cells. While IL-12 promotes a Th1 response, IL-23 promotes the activation of Th17 and Th22 cells, resulting in the production of IL-17 and IL-22 respectively. Collectively, IL-17, IL-22, and IFN-γ are the hallmark cytokines found in high levels in the psoriasis lesions, where they cause keratinocyte proliferation and the production of antimicrobial peptides, cytokines and chemokines. Chemokine CCL20 can recruit DCs and IL-17 producing $CD4^+$ and $CD8^+$ T cells, while CXCL1, CXCL2, and CXCL8 can recruit neutrophils to the lesional skin. This forms an amplification loop leading to the exacerbation of psoriasis.

In our proposed model, skin-resident mast cells release the PLA2G4D (a $cPLA_2$)- containing exosomes in response to IFN-α produced by pDCs. These exosomes are taken up through clathrin-mediated endocytosis by the dendritic cells or migratory Langerhans cells in the dermis. Inside the sorting/early endosomes of these antigen-presenting cells, PLA2G4D generates neolipid antigens, which can replace the initial ligands on CD1a molecules. CD1a loaded with PLA2G4D-derived lipids are eventually recycled back to the surface of the antigen-presenting cells, and present the lipids to CD1a-restricted T cells, resulting in the production of IFN-γ, IL-17, and IL-22. Furthermore, during breaches of the skin barrier, more self-lipids that are originally in the epidermis can reach the dermis, where the antigen-presenting cells (DCs and migratory Langerhans cells) can present them to and activate the CD1a-autoreactive T cells to produce IFN-γ, IL-17, and IL-22.

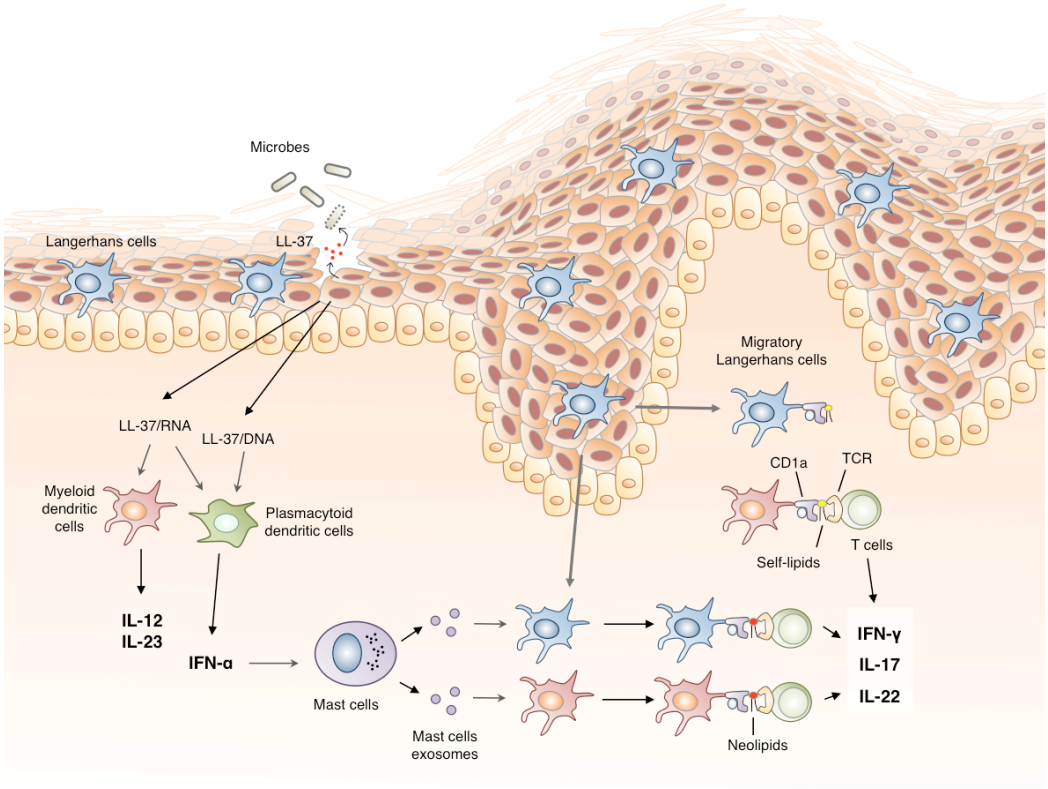

Figure 7 A proposed model for CD1a-mediated pathogenesis of psoriasis

Considering the pivotal role of PLA_2 in the CD1a-mediated T cell responses in the psoriatic lesions, this raises the possibility that PLA_2 might be a potential therapeutic target for the treatments of psoriasis. It is noted that topical corticosteroids, which are effective in psoriasis, contain inhibitory effect on PLA_2 activity. However, there is no evidence showing that the inhibition of PLA_2 by corticosteroids directly contributes to the reduction of CD1a-mediated T cell reactivity in the lesions. Indeed, the broad PLA_2 inhibition by the corticosteroids might affect the diverse roles of PLA_2 in homeostasis and inflammatory responses. Therefore specific

inhibitors targeting certain PLA_2, cytosolic PLA_2 in particular, would represent promising and effective treatments.

The current findings showed that the CD1a-reactive T cells of psoriasis patients could produce significant levels of IL-17A, and IL-22, which are hallmark cytokines found in the lesions of psoriasis. Current immunotherapeutic approaches include the targeting of IL-17 and IL-12/23 pathways. For instance, Secukinumab and Ixekizumab target IL-17A, and Brodalumab targets IL-17RA, and hence they can inhibit the action of IL-17 on target cells. On the other hand, Ustekinumab, by targeting IL-12/23 p40 subunit, can inhibit IL-23-driven Th17 differentiation. IL-22 is also a major cytokine contributing to the pathogenesis of psoriasis, particularly by activating keratinocytes and increasing their proliferation rate in the psoriatic lesions. However no biologics targeting IL-22 are currently available. Therefore IL-22-targeting strategies may represent novel immunotherapeutic approaches in the future.

Furthermore, understanding that exosomes play an important carrier role to transfer cytosolic PLA_2 from mast cells to neighbouring CD1a-expressing antigen presenting cells, blockade of exosomal transport can be a novel therapeutic intervention. While a number of chemicals are available for intervening with the exosome synthesis, secretion, and internalisation by target cells, a specific inhibitor that preferentially targets

mast cell exosomes is required, for exosomal transport is crucial for intercellular communication in general. However, one limitation was that the current experiments involving exosome work did not examine the effect of non-PLA_2 and non-CD1a-mediated pathways. For instance, it remains unclear whether co-stimulatory molecules were involved in the CD1a reactivity. Future experiments can include the blocking of the co-stimulatory molecules to determine their involvement in the exosome-mediated responses.

This study has shed light on the involvement of non-peptide antigen presentation by CD1a in the pathogenesis of psoriasis, however it did not determine the relationship between non-peptide- and peptide-specific responses in the skin of psoriasis. It is noted that the association between *HLA-Cw6* and psoriasis has been long described and extensively examined [379-381]. It is proposed that the non-peptide-specific responses represent an early phase of T cell responses in psoriasis, which results in recruitment of T cells and other immune cells into the site of inflammation, and leads to a shift to peptide-specific responses for a late phase of T cell responses in psoriasis.

Similar elevated CD1a reactivity has been recently reported in atopic dermatitis, another inflammatory skin disorder [274]. Further experiments can examine the role and significance of CD1a reactivity in

other inflammatory skin disorders, and determine whether CD1a reactivity represents a hallmark response for inflammatory skin disorders in general.

7.2 Future works

A number of future works can lead to a deeper understanding of various aspects of the CD1a-mediated pathogenic model of psoriasis. For example, it remains in question whether the skin-resident CD1a-reactive T cells can recognise skin host-derived lipids, such as squalene, that were described previously [217]. Based on the findings in this study, it is speculated that there might be a higher frequency of CD1a-reactive T cells in the lesional skin of psoriasis that can recognise these lipids. Furthermore, the antigenic potential of a panel of different PLA_2-derived lipids with different chain lengths and varying degrees of saturation could be examined. Altogether, these experiments could attempt to identify the common properties from a number of CD1a ligand candidates that can activate CD1a-restricted T cells.

Also, the regulation of PLA_2 activity in the exosomes of mast cells can also be investigated, since whether PLA_2 is regulated through phosphorylation or by some regulatory proteins, such as PLA_2 activating protein (PLAA), is unclear. Moreover, based on the prior knowledge that mast cells could be activated by a number of cytokines as well as PLA_2,

whether the cytokines produced by the exosome-responsive CD1a-reactive T cells, or PLA_2 generated by mast cells, could potentiate the mast cell responses and hence create certain positive feedback loops. This could contribute to the amplification of T cell-mediated responses in the psoriatic lesions.

Previous published data suggest that CD1a-restricted T cells express a diverse TCR repertoire and can be $CD4^+$ or $CD8^+$ but it will be important to examine whether this is the case in the setting of disease. For example, within psoriatic lesions, it may be that distinct clonotypes of CD1a-restricted T cells emerge as being important in disease pathogenesis. This could be approached through T cell cloning experiments, or potentially through next generation single cell TCR sequencing [382].

Overall, there are many future experiments to be undertaken, but it is hoped that by identifying lipids as a novel class of antigen relevant to psoriasis that the thesis study will make a contribution to our understanding of psoriasis and the identification of novel therapeutics.

8. References

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