CHARACTERIZATION OF PLP1+ CELLS AND NATURAL KILLER CELLS WITH HEIGHTENED ACTIVITY IN VITILIGO

by

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Bachelor of Medical Laboratory Science, The University of British Columbia, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

February 2012

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Abstract

Background: Vitiligo is a complex autoimmune skin condition characterized by the death of melanocytes, the principle pigment producing cells in the skin. Transcriptome analysis of vitiligo skin revealed significantly reduced levels of proteolipid 1 (PLP1) gene, which is known to be expressed by Schwann cells, as well as significantly up-regulation of genes that are associated with natural killer (NK) cell activity.

Hypothesis and Objectives: Schwann cells may be adversely affected in vitiligo and NK cells may potentially play a role in the overall disease pathogenesis. Therefore, the purpose of this study is to characterize the down-regulation of PLP1 and assess the presence of NK cell infiltration in vitiligo skin biopsies.

Materials and Methods: PLP1 expression analyses were performed on major types of skin cells as well as vitiligo and normal skin samples. Quantification of Schwann cells was performed on paired vitiligo samples using immunohistochemistry. Schwann cell conditioned medium was also tested for its ability to support the growth and survival of human melanocytes. To assess NK cell activity, explant skin cultures and immunofluorescence analyses were performed to localize activated NK cells in skin biopsies.

Results: Schwann cells were the primary source of PLP1 in human skin, although it is also expressed by melanocytes. Schwann cells were found to be decreased in vitiligo lesional skin as compared to peri-lesional and normal skin. In addition, conditional medium prepared from cultured Schwann cells significantly increased the survival of human melanocytes.

Furthermore, explant skin cultures and immunofluorescence studies revealed marked increase of NK cells with heightened activity in vitiligo lesional as well as peri-lesional vitiligo skin.

Conclusion: Results from our study suggest that the loss of melanocytes and reduction in Schwann cells may account for the down-regulation of PLP1 in vitiligo lesional skin. In addition, Schwann cells may play a role in the growth and survival of melanocytes and their decrease may have facilitated the development of vitiligo. Furthermore, this study lends support to the direct involvement of NK cells in the pathogenesis of vitiligo and suggests that they should be explored as cellular targets for development of better therapies in the future.

Preface

Part of Chapter 1 is based on a review article that has been accepted for publication. Yu R, Huang Y, Zhang X, Zhou Y. The potential role of neurogenic inflammatory factors in the pathogenesis of vitiligo. J Cutan Med Surg. Accepted. I was the one responsible for the entire original draft, including figures.

The project was approved by the Clinical Ethics Board of University of British Columbia (Approval number C0493), in accordance with the Declaration of Helsinki principles.

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List of Abbreviations

ABC	-	avidin-biotin complex
AD	-	atopic dermatitis
APC	-	antigen presenting cell
BDNF	-	brain-derived neurotrophic factor
cAMP	-	cyclic adenosine monophosphate
CANP	-	calpain
CD	-	cluster of differentiation
CGRP	-	calcitonin gene related peptide
CLEC2B	-	C-type lectin domain family 2, member B
CNS	-	central nervous system
COMT	-	catechol-O-methyltransferase
CTLA4	-	cytotoxic T-lymphocyte-associated protein 4
DA	-	dopamine
DAPI	-	4’,6-diamidino-2-phenylindole
DMEM	-	Dulbecco’s modified Eagle’s medium
ECM	-	extracellular matrix
EP	-	epinephrine
FB	-	fibroblast
FBS	-	fetal bovine serum
GAPDH	-	glyceraldehyde 3-phosphate dehydrogenase
GDNF	-	glial cell-derived neurotrophic factor
GWAS	-	genome-wide association study
HEKa	-	adult human epidermal keratinocyte
HEMC	-	adult human epidermal melanocyte
HKGS	-	human keratinocyte growth supplement
HLA	-	human leukocyte antigen
HMGS	-	human melanocyte growth supplement
HSP	-	heath shock protein
HVA	-	homovanillic acid
IF	-	immunofluorescence

IFN	-	interferon
Ig	-	immunoglobulin
IHC	-	immunohistochemistry
IL	-	interleukin
IMDM	-	Iscove's modified Dulbecco's medium
IR	-	immunoreactive
JRA	-	juvenile rheumatoid arthritis
KIR	-	killer immunoglobulin-like receptors
KLR	-	killer cell lectin-like receptor
KLRK1	-	killer cell lectin-like receptor subfamily K, member 1
LC	-	Langerhans cell
LS	-	lesional skin
Ly49	-	killer cell lectin-like receptor, subfamily A
MAO	-	monoamine oxidase
MBL2	-	mannose-binding lectin 2
MBP	-	myelin basic protein
Melan-A	-	melanoma antigen recognized by t-cells
MHC	-	major histocompatibility complex
MICA	-	MHC class I polypeptide-related sequence A
MICB	-	MHC class I polypeptide-related sequence B
MITF	-	microphthalmia-associated transcription factor
MLANA	-	see Melan-A
MTS	-	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NALP1	-	NLR family, pyrin domain containing 1
NB-UVB	-	narrow band-ultraviolet B
NCAM	-	neural cell adhesion molecule
NE	-	norepinephrine
NF	-	neurofilament
NGF	-	nerve growth factor
NK	-	natural killer
NKG2A	-	killer cell lectin-like receptor subfamily C, member 1
NKG2D	-	See KLRK1

NKp44	-	natural cytotoxicity triggering receptor 2
NKp46	-	natural cytotoxicity triggering receptor 1
NKp80	-	killer cell lectin-like receptor subfamily F, member 1
NPY	-	neuropeptide Y
NS	-	normal skin
NSV	-	non-segmental vitiligo
OCT	-	optimum cutting temperature
p75	-	low-affinity nerve growth factor receptor
PBMC	-	peripheral blood mononuclear cell
PBS	-	phosphate buffered saline
PGP 9.5	-	protein gene product 9.5
PLP1/DM20	-	proteolipid 1
PLS	-	peri-lesional skin
PMD	-	Pelizaeus–Merzbacher disease
PMT	-	Predictive Medicine and Therapeutics
PNS	-	peripheral nervous system
POSTN	-	periostin
PTPN22	-	protein tyrosine phosphatase, non-receptor type 22
PUVA	-	psoralen, ultraviolet A
RA	-	rheumatoid arthritis
ROS	-	reactive oxygen species
RPMI	-	Roswell Park Memorial Institute medium
RT-PCR	-	real-time polymerase chain reaction
S100	-	S100 calcium binding protein A1
SC	-	Schwann cell
SCP	-	Schwann cell precursor
SILV	-	premelanosome protein
SLE	-	systemic lupus erythematosus
SLEV1	-	systemic lupus erythematosus, vitiligo-related 1
SOX 10	-	SRY (sex determining region Y)-box 10
SPG2	-	spastic paraplegia 2
SV	-	segmental vitiligo

TBS	-	tris buffered saline
TCR	-	T cell receptor
TNF	-	tumor necrosis factor
TYR	-	tyrosinase
TYRP1	-	tyrosinase-related protein 1
ULBP	-	UL16 binding protein
VMA	-	vanilmandelic acid

Acknowledgements

I would like to offer my sincere and enduring gratitude to my supervisor, Dr. Youwen Zhou, for his strict supervision and guidance throughout my graduate study. I would also like to thank my supervisory committee members Dr. Kevin McElwee and Dr. Jan Dutz for their valuable advices and generous support. I own particular debt of gratitude for the support and assistance from Dr. Mingwan Su, Dr. Shencai Wei, Dr. Shengquan Zhang, Rachel Huang and other present and past members of the Molecular Medicine Laboratory. I would also like to extend my thanks to Dr. Raewyn Broady and Jie Yu in the Jack Bell Research Centre for their invaluable assistance in the explant culture of vitiligo skin biopsies, as well as Dr. Christopher Shaw's lab for granting me unrestricted access to their fluorescence microscope. The project would not be possible without any of your help. Finally, this work was supported by grants from National Natural Sciences Foundation of China (No. 30628021), Canadian Institutes of Health Research, Skin Research Training Centre at the University of British Columbia, Canadian Dermatology Foundation and Astellas Pharma Research Competition, Canada.

Dedication

This thesis is dedicated to my family, whose unconditional love and support, both morally and financially, have guided me throughout the course of my entire study.

Chapter 1: Introduction

The Laboratory of Predictive Medicine and Therapeutics (PMT) at the Chieng Genomic Center has been striving to better understand the pathogenic mechanisms of various skin diseases using genomic approaches. This thesis follows up an initial transcriptome analysis designed to screen for genes associated with vitiligo. The aim of this study is twofold: 1) to determine the significance of proteolipid 1 (PLP1) expression, which was identified to be down-regulated in the lesional skin (LS) biopsy of vitiligo patient as compared with paired unaffected peri-lesional skin (PLS) and skin from healthy individuals; 2) to evaluate and confirm the infiltration of activated natural killer (NK) cells in vitiligo skin. The significance of PLP1 down-regulation and especially its implications of Schwann cell involvement in vitiligo have never been reported. In addition, very few studies have demonstrated aberrant NK cell activities in vitiligo patients and none has ever suggested a role NK cell might play in the skin microenvironment of vitiligo patients. This is the first follow-up study to the only large-scale transcriptome analysis of full-thickness skin biopsies from generalized or non-segmental vitiligo (NSV) patients ever performed. Therefore, unveiling the significance of some aberrantly expressed genes in vitiligo will not only yield important information regarding the pathogenesis of the disease, but may also have novel therapeutic implications.

1.1 Vitiligo

Vitiligo, also called leukoderma, is either an acquired or familial, but highly complex pigmentation disorder in which melanocytes, the principle pigment-producing cells in humans, are destroyed. Affecting 0.5%-1% of population worldwide, vitiligo can be a highly

disfiguring disorder that is characterized by the development of smooth and porcelain-white patches of skin devoid of protective melanin pigmentation (Figure 1-1). The resulting depigmentation can affect anywhere from the retinal epithelium to the torso and extremities, and may even appear on the mucous membranes of the mouth and nose (Abu Tahir *et al.*, 2010; Grimes, 2005; Whitton *et al.*, 2008). Although vitiligo is traditionally viewed as a minor disease, it has been reported to have severe impact on the psychological well-being of the affected individuals, resulting in impaired social interactions and decreased quality of life. (Porter and Beuf, 1991; Thompson *et al.*, 2002). In addition to the cosmetic and psychosocial implications, there is increasing evidence of association between vitiligo and autoimmune diseases, such as systemic lupus erythematosus, hypothyroidism, diabetes and various disorders of the nervous system (Boelaert *et al.*, 2010; Deretzi *et al.*, 2010; Kocer *et al.*, 2009; Nikiforidis *et al.*, 1993; Ramagopalan *et al.*, 2007; Rashtak and Pittelkow, 2008; Sabate *et al.*, 1999; Varoglu *et al.*, 2010; Yacubian *et al.*, 2001). Therefore, the impact this disease has on the general well-being of patients should not be underestimated.

1.2 Classification of vitiligo

The simplest classification system divides vitiligo into two broad categories primarily based on the distribution of lesions: generalized or non-segmental vitiligo (NSV) and segmental vitiligo (SV) (Koga, 1977; Sehgal and Srivastava, 2007). NSV is defined as an acquired chronic depigmentation disorder where the white patches tend to have a symmetrical distribution throughout the body. In contrast, lesions in SV are distributed in an asymmetrical manner and often correspond with specific dermatomes, which are areas of skin supplied by individual spinal nerves. In addition, compared to NSV, the prevalence of

SV is rare and SV lesions are generally more refractive to therapy. Despite the differences in clinical manifestations, there is growing consensus that the two are not mutually exclusive and actually share common etiological mechanisms.

1.3 Vitiligo pathogenesis

To date, the exact pathomechanism of vitiligo has remained obscure, and the main reason lies in the multi-factorial nature of the disease, which progresses as a result of the interplay between multiple genes and environmental factors. In spite of this, several prevailing etiopathogenic theories have dominated the mainstream literature.

1.3.1 The autoimmune theory of vitiligo

Advances in technology and genomic studies have led researchers to map and sequence gene loci in large populations of vitiligo patients, which enabled the identification of specific genes involved in vitiligo susceptibility. Most of the candidate genes discovered using case-control genetic association studies are thought to be important in immune response regulation, such as CTLA4 (Alkhateeb *et al.*, 2005; Itirli *et al.*, 2005; Kemp *et al.*, 1999a), PTPN22 (Canton *et al.*, 2005), MBL2 (Onunu and Kubeyinje, 2003) and IL-10 (Abanmi *et al.*, 2008), most of which have been associated with various autoimmune diseases (Brand *et al.*, 2005; Gough *et al.*, 2005; LaBerge *et al.*, 2008; Le Poole *et al.*, 2001; Xia *et al.*, 2006). In addition, group and meta-analysis of multiple studies has found an association between the prevalence of vitiligo and certain major histocompatibility complex (MHC) alleles, such as HLA-DRB4 and HLA-A2 (Majumder *et al.*, 1988).

Recently, whole genome scans in genome-wide association studies (GWAS) revealed novel genetic markers associated with vitiligo susceptibility, notably SLEV1 (Onay *et al.*,

2007), which is associated with systemic lupus erythematosus, novel MHC loci (Chen *et al.*, 2005; Liu *et al.*, 2007; Quan *et al.*, 2010) and NRLP1 (Jin *et al.*, 2007), which encodes a critical regulator of innate immune system expressed mainly in cells of the myeloid lineage, such as monocytes, macrophages and dendritic cells.

The NRLP1 gene encodes a member of the NLR or Ced-4 family of apoptosis proteins. Ced-family members contain a caspase recruitment domain (CARD) and are known to be key mediators of programmed cell death. In addition, multiple members of the NLR family associate to form "inflammasomes", which are complexes that play a critical role in the inflammatory process mediated by immune cells of the myeloid origin. The CARD domain of NLRP1 recruits and activates caspase 1, which is responsible for the final maturation of several inflammatory cytokine precursors, most notably IL-1β and IL-18 (Lamkanfi *et al.*, 2011), which are predominantly produced by activated monocytes, macrophages and dendritic cells. The association of SNPs with vitiligo and concurrent development of other autoimmune diseases such as Addison's disease and type I diabetes suggest a model in which they may induce conformational changes in NLRP1 that reduce the threshold for assembly of the NRL1 inflammasome, thus triggering unwarranted activation of caspase-1 and excessive production of IL-1β and IL-18 in immune cells of patients.

Indeed, substantial evidence at the protein and cellular levels confirmed the genetic association studies and strongly attributed the progressive destruction of melanocytes to an autoimmune etiology (Garbelli *et al.*, 2005; Gopal *et al.*, 2007; Jin *et al.*, 2007; Kemp *et al.*, 2001b; Ongenae *et al.*, 2003; Ramagopalan *et al.*, 2007; Rezaei *et al.*, 2007; van den Boorn *et al.*, 2009). In addition to aberrant expression of genes encoding inflammatory mediators and their receptors (Birol *et al.*, 2006; Caixia *et al.*, 1999; Jin *et al.*, 2007; Moretti *et al.*,

2002), many pro-inflammatory cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, have been previously reported to be systemically elevated in the serum and lesional skin of vitiligo patients (De la Fuente *et al.*, 1993; Levite, 1998; Levite *et al.*, 1998; Song and Leonard, 1998). There is substantial evidence of specific cell-mediated and humoral immune responses to melanocytic antigens in vitiligo, especially those that are associated with melanogenesis, such as Pmel17, tyrosinase, tyrosinase hydroxylase and tyrosinase-related proteins (Jimbow *et al.*, 2001; Kemp *et al.*, 2011; Kemp *et al.*, 1997a; Kemp *et al.*, 1997b, 1998a; Kemp *et al.*, 1998b, 1999b, 2001a; Palermo *et al.*, 2001). In particular, a recent study by van den Boorn *et al.* (2009) has successfully isolated cytotoxic CD8+ T lymphocytes from the peri-lesional and lesional skin of vitiligo patients and demonstrated that those cells were specific for melanocytes in cytoxicity assays (van den Boorn *et al.*, 2009). Although a general consensus has been reached in the scientific community that specific adaptive immunity plays an important role in the death of melanocytes in vitiligo, evidence regarding the potential role of innate immune system is relatively limited. Infiltration of the peri-lesional and lesional skin of vitiligo patients by agents of the innate immune response, such as macrophages, Langerhans cells (LC), mast cells and eosinophils have been documented, although their role in vitiligo pathogenesis can only be speculated (Anbar *et al.*, 2009; Aroni *et al.*, 2010; Kroll *et al.*, 2005; Panuncio and Vignale, 2003; Prignano *et al.*, 2011; van den Boorn *et al.*, 2009; Wankowicz-Kalinska *et al.*, 2003). Recently, natural killer cells have been implicated to play a role in the destruction of melanocytes, although there has been no report of their presence in the skin of vitiligo patients (Basak *et al.*, 2008).

There is also evidence of an association between vitiligo and other autoimmune diseases, such as multiple sclerosis, hypothyroidism and diabetes (Boelaert *et al.*, 2010;

Deretzi *et al.*, 2010; Kocer *et al.*, 2009; Ramagopalan *et al.*, 2007; Rashtak and Pittelkow, 2008), all seen with increased incidence in vitiligo patients. Indeed, immunogenetic factors (i.e. MHC class II alleles) that predispose patients to autoimmune disorders in general have also been found to be associated with the onset of vitiligo, hence strengthening the theory that there are genetic and autoimmune components to the pathogenesis of vitiligo (Rezaei *et al.*, 2007).

1.3.2 The neural hypothesis of vitiligo pathogenesis

The speculation that the nervous system might be involved in vitiligo onset and progression first arose as a result of peculiar distribution of depigmented lesions along specific dermatomes, that is, following the pathway of major skin innervations, observed in SV patients. In addition, sympathectomy and the application of parasympathomimetic alkaloids, such as physostigmine, were able to halt the progression of SV in some patients (Koga, 1977), thus adding credence to the theory that sympathetic nervous system may play a role in the depigmentation process in segmental vitiligo.

Accumulating evidence in the past 20 years regarding the disease mechanism of vitiligo has demonstrated that involvement of the nervous system is not only limited to SV, but also in generalized vitiligo as well. Studies in the past decade have demonstrated evidence of neuroendocrine imbalance in vitiligo lesional skin in the form of altered numbers of different types of nerve fibers, specifically those that mediate stress responses. An increased number of neuropeptide-secreting nerve fibers were observed, such as neuropeptide Y (NPY) and calcitonin gene related peptide (CGRP), which could possibly lead to the destruction of melanocytes, either by direct mechanisms or indirectly through stimulation of the immune system (Al'Abadie *et al.*, 1994; Hristakieva *et al.*, 2000).

Significantly elevated levels of neuropeptide Y in the plasma and tissue fluids of vitiligo patients have also been observed (Tu *et al.*, 2001). In addition, it has been well documented that the catecholamine neurotransmitters, such as dopamine (DA), epinephrine (EP) and norepinephrine (NE), along with their metabolic products are found in significantly elevated levels in the serum and urine of vitiligo patients, which are suggested to facilitate the destruction of melanocytes through various mechanisms (Chu *et al.*, 2006; Cucchi *et al.*, 2000; Cucchi *et al.*, 2003; Morrone *et al.*, 1992; Schallreuter *et al.*, 1996). Furthermore, low affinity (p75) nerve growth factor receptor immunoreactive (NGFr-IR) nerve fibres, which is known for its ability to bind all neurotrophins of the nerve growth factor (NGF) family with equal levels of affinity and has recently been implicated in various autoimmune and inflammatory disorders, is increased in the lesional and peri-lesional skin of vitiligo patients (Liu *et al.*, 1996).

In addition, stress and psychological factors have been widely implicated in the initiation and exacerbation of autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and diabetes (Stojanovich, 2010). In fact, stress and emotional conditions have been implicated in vitiligo and other dermatoses, and their actions were suggested to be mediated through neurotransmitters and stress hormones (Chida *et al.*, 2008; Kodama *et al.*, 1999; Misery and Rousset, 2001; Misery *et al.*, 2008). A case control study on children afflicted with vitiligo and psoriasis has shown that the onset of vitiligo was mostly associated with psychological factors (Barisic-Drusko and Rucevic, 2004). In addition, another case control study done by Manolache and Benea has demonstrated that vitiligo patients are much more likely (odd ratio of 6.81) to encounter stressful events in their life (Manolache and Benea, 2007). Their result has also revealed that patients are much more

likely to experience one stressful event before the onset of vitiligo. Furthermore, it has also been suggested that patients with alexithymia (deficiency in the ability to express emotions) and those with poor social support are more susceptible to vitiligo due to their reduced ability to regulate emotions and cope with stress (Picardi *et al.*, 2003).

1.3.3 The oxidative and redox imbalance theory

There is considerable evidence of significant increases in the concentration of phenolic monoamines and their metabolites in the plasma and urine of vitiligo. Amongst these, catecholamines such as dopamine (DA), epinephrine (EP) and norepinephrine (NE) along with their metabolites, especially homovanillic acid (HVA) and vanilmandelic acid (VMA), seem to characterize the onset and progression of vitiligo in patients (Cucchi *et al.*, 2000; Morrone *et al.*, 1992; Salzer and Schallreuter, 1995; Schallreuter-Wood and Wood, 1996). Studies have consistently found that the urinary and plasma levels of catecholamines and their metabolites are higher in the early active phase of NSV than in normal controls (Cucchi *et al.*, 2000; Cucchi *et al.*, 2003; Morrone *et al.*, 1992). In addition to the adrenal gland, catecholamines are also produced by skin keratinocytes and melanocytes. Indeed, the accumulation of DA, EP and NE in vitiligo has been partially attributed to a defect in their biochemical synthesis pathway in keratinocytes (Schallreuter *et al.*, 1996).

Catecholamines are readily oxidized into phenolic metabolites, which can be further broken down to highly reactive oxygen species (ROS) such as peroxide (H_2O_2) and hydrogen oxide radical (·OH). These reactions can take place in the local microenvironment of melanocytes either via auto-oxidation or mediated by enzymes present in the skin, such as monoamine oxidases (MAO) and catechol-o-methyl-transferases (COMT), both of which have been found to exhibit significantly increased activities in the epidermis of patients with

vitiligo (Le Poole *et al.*, 1994; Morrone *et al.*, 1992; Schallreuter *et al.*, 1996). In addition, since NE is also a potent vasoconstrictor, chronic accumulation of catecholamines may induce tissue hypoxia, which in turn, directly stimulates the production of mitochondrial ROS (Chandel *et al.*, 2000). In any event, hydrogen peroxide was found to be significantly increased in the LS of vitiligo patients (Schallreuter *et al.*, 1999).

In addition to having direct melanocytotoxicity, ROS may modify the activity of various cellular enzymes, and even serve as important signaling molecules of the immune system (Griffiths, 2005; Schallreuter *et al.*, 1996). In addition, normal cellular proteins may undergo structural changes in chronic oxidative stress conditions to produce "neo-antigens", which exhibit structural homology to host antigens but can elicit auto-reactive immune response. This concept of free radical-generated "molecular mimicry" was originally described by Kannan (Kannan, 2006). Indeed, autoimmune responses against melanocytic antigens such as tyrosinase and tyrosinase-related proteins may be partially explained by this mechanism. Furthermore, tissue damage caused by chronic oxidative stress and "neo-antigens" may also result in the release of previously sequestered auto-antigens, thus leading to amplification of the initial immune response.

1.3.4 The melanocytorrhagy theory

Emerging evidence of the presence of residual melanocytes with abnormal morphological characteristics in vitiligo patients has led the development of the "melanocytorrhagy theory", which do not necessarily require the death of melanocytes for depigmentation to take place, but explains the loss of pigment either as a result of intrinsic defect in melanogenesis or the translocation of melanocytes due to defective adhesion mechanisms.

It has been discovered that in cases of chronic vitiligo, residual melanocytes are still present, however, that are abnormal in function and appearance. Some vitiligo patients demonstrated abnormal distribution of pre-melanosomes within keratinocytes in LS (Tobin *et al.*, 2000). This phenomenon has previously been explained by an intrinsic defect in the melanogenesis process whereby progressive decrease of the activity of critical enzymes such as tyrosinase would lead to the inhibition of melanization and even the death of melanocytes (Jarrett and Szabo, 1956; Schallreuter *et al.*, 1989).

There have been also observations that the onset of NSV is commonly precipitated by physical trauma such as wounds, pressure and repeated frictions. Therefore, this has led to the speculation that the melanocytes in certain subsets of patients exhibit defective adhesion mechanisms to the basement membrane and neighboring keratinocytes, and thus are easily detached (Morelli *et al.*, 1993). For example, tenascin, an anti-adhesive extracellular matrix molecule which may inhibit the attachment of melanocytes to fibronectin, has been found to be significantly increased in LS of NSV patients (Le Poole *et al.*, 1997). In addition, it has been reported that Ca^{+2}-mediated adhesion process in the keratinocytes and melanocytes of NSV patients might be defective, as demonstrated by impaired calcium uptake in cultured keratinocytes isolated from vitiligo LS (Schallreuter and Pittelkow, 1988). Morphologically, residual melanocytes isolated from NSV patients exhibit significant rounding and loss of dendrites, which are important for both attachment and transfer of melanosomes to surrounding keratinocytes (Jimbow *et al.*, 2001).

There has been a report of the presence of damaged melanocytes in the granular area of the skin in NSV patients (Morohashi *et al.*, 1977). It is speculated that translocated melanocytes may express abnormal stress markers, which in turn, may stimulate the resident

Langerhans cells in triggering an immune response cascade that would ultimately result in their destruction. Further, this chronic detachment and elimination process may result in the formation and release of auto-antigens that would further aggravate the condition.

1.3.5 Summary of vitiligo pathogenesis

The theories are not mutually exclusive and considerable overlap is present among them. For example, with emerging evidence of an intimate crosstalk between the nervous and immune systems (Downing and Miyan, 2000; Elenkov *et al.*, 2000; Straub *et al.*, 1998), it is likely that an over-expression of neuropeptides and catecholamines may result in melanocyte destruction not only by direct mechanisms and the generation of ROS, but also by stimulation of the immune system. Similarly, it has been proposed that weakening of the adhesion mechanisms of melanocytes that result in their detachment and clearance by the immune system may trigger the development of an immune cascade. Therefore, while every theory has its own proponents and advocates, from the currently available data it is most likely that the pathogenesis of vitiligo is not due to any single one of the proposed theories, but a combination of pathomechanisms working in unison.

1.4 Current interventions for vitiligo

Presently, treatment options for vitiligo patients are limited and their efficacy varies widely between individuals. Treatment typically involves the direct modulation of immune responses to melanocytes through local immunosuppression with topical corticosteroids, such as clobetasol propionate and betamethasone valerate, and calcineurin inhibitors, such as yclosporine, pimecrolimus and tacrolimus (Gawkrodger *et al.*, 2010; Hossani-Madani and Halder, 2010). Systemic treatment such as oral dexamethasone is also used, albeit with

common side effects including weight gain, acne and menstrual irregularities in women (Radakovic-Fijan *et al.*, 2001). Phototherapy such as psoralen in combination with ultraviolet A (PUVA) and narrow band ultraviolet B (NB-UVB) has also been the mainstream treatment option for vitiligo and various other dermatoses (Syed and Hamzavi, 2011). A number of dermatosurgery techniques have also been developed in attempt to repigment vitiligo lesions, among which cultured and non-cultured autologous melanocyte transplantation and split-thickness skin grafting have the highest success rates (Rusfianti and Wirohadidjodjo, 2006). However, permanent and complete repigmentation using surgical techniques are not guaranteed in most cases and the disease may continue to progress to even affect the treated areas.

The absence of a permanent and consistent cure for vitiligo highlights the complexity of the disease and therefore, a general overall lack of understanding of its pathogenesis. Therefore, additional studies at both the molecular and cellular levels are required to shed light on future therapeutic options.

1.5 Identification of genes with aberrant expression in vitiligo

In an attempt to further understand the pathogenesis of vitiligo, the Molecular Medicine Laboratory performed the first non-targeted and non-selective large-scale genomic expression analysis using oligonucleotide-based microarrays by comparing the well-established vitiligo LS and PLS and normal skin (NS) from healthy donors with similar anatomic properties. The study revealed a total of 30 genes that are either down-regulated or up-regulated in vitiligo skin as compared with normal skin. As expected, of the 17 down-regulated genes (Table A-1, Appendix A), most encode lineage markers or functional components of melanocytes, including TYRP1, TYR, Melan-A, and SILV (Kingo *et al.*,

2007; Kingo *et al.*, 2008; Kitamura *et al.*, 2004; Stromberg *et al.*, 2008). However, among these genes, the down-regulation of PLP1, which encodes a myelin sheath protein and also a critical component of the nervous system, is of particular interest. Among the up-regulated genes (Table A-2, Appendix A), the vast majority encode innate immunity regulators, such as β-defensin (Liu *et al.*, 1997) and CLEC2B, an activating ligand for natural killer cells that is primarily expressed by macrophages (Hamann *et al.*, 1997). Particularly striking is the up-regulation of genes in the killer cell lectin-like receptor (KLR) family, which include multiple activation and inhibitory markers for natural killer (NK) cells, another important player in innate immunity. Other notable up-regulated genes include CANP and POSTN, which code for markers of oxidative stress, tissue injury and repair (Andrianjafiniony *et al.*, 2010; Borg and Markwald, 2007; Dorn, 2007; Harbison *et al.*, 2010; Norris *et al.*, 2007; Yuan *et al.*, 2010) .

1.6 Proteolipid protein 1

1.6.1 General knowledge about PLP1

The PLP1 gene encodes a proteolipid protein that constitutes about 50% of all myelin proteins present in the central nervous system (CNS). While the PLP1 gene can produce multiple splice variants of the protein, the full-length PLP1 protein and its shorter variant DM20 are most abundantly produced in the nervous system (Edwards *et al.*, 1989; Sarret *et al.*, 2010; Wood *et al.*, 1984). In the CNS, PLP1 and DM20 have the highest expression levels in oligodendrocytes, which are the principle glial cells responsible for myelination. PLP1 plays an important role in the proper formation, and subsequent stabilization and maintenance of the myelin sheath membrane by interacting with myelin basic protein, while

DM20 is speculated to be involved in the differentiation and maturation of glial cells (Garbern *et al.*, 2002; Inoue, 2005).

Since myelin sheath is critical for nerve conduction in both the CNS and the peripheral nervous system (PNS), it is also produced by PNS glial cells, namely Schwann cells (Griffiths *et al.*, 1989). Although PLP1 and DM20 are expressed by Schwann cells, they are not major components of the peripheral myelin sheath. Therefore, the precise function of the PLP1 gene in the PNS is not known, although roles in the stabilization of myelin sheath (similar to its roles in the CNS) (Boison *et al.*, 1995) and function as an ion channel have been proposed (Helynck *et al.*, 1983).

The human skin is innervated by an intricate network of nerve fibers, which are often associated with Schwann cells (Tschachler *et al.*, 2004). Up until now, no cells other than Schwann cells in the PNS have been reported to express PLP1 at both the mRNA transcript and protein levels. On the other hand, a recent study employing novel DNA microarray techniques has hinted at the possible expression of PLP1 in skin melanocytes (Hoek *et al.*, 2008). Therefore, it remains possible that the PLP1 gene might be actively expressed in non-glial cells as well.

1.6.2 The association of PLP1 with diseases

Genetic mutations that lead to the under-expression and over-expression of the PLP1 gene or the production of defective protein products are associated with severe neurological conditions as a result of destabilization and improper formation of the myelin sheath. Missense mutations of the PLP1 gene is associated with the Pelizaeus–Merzbacher disease (PMD) or spastic paraplegia type 2 (SPG2—a milder version of PMD), which are a group of devastating neurological degenerative conditions characterized by massive inflammation of

the brain accompanied by widespread CNS dysmyelination that can ultimate result in impaired psychomotor development, behavior deficit, spasticity and even death (Cambi *et al.*, 1996; Inoue *et al.*, 1999; Kobayashi *et al.*, 1994; Osaka *et al.*, 1995; Saugier-Veber *et al.*, 1994). In addition, studies employing transgenic mice with over-expression of PLP1 produced a similar condition as PMD characterized by spasticity and loss of myelination (Edgar *et al.*, 2010; Espinosa-Jeffrey *et al.*, 2010; Karim *et al.*, 2010; Karim *et al.*, 2007; Tatar *et al.*, 2010). On the other hand, loss of PLP1 expression produced a disease similar to, but less severe than PMD/SPG2 characterized by demyelination and axonal degeneration and can involve both the CNS and PNS, while PMD/SPG2 is only found to affect CNS exclusively (Garbern *et al.*, 1997; Garbern *et al.*, 2002).

Although vitiligo has been found to be associated with occasional cases of nervous system disorders, such as encephalitis, auditory and visual sensory neuropathies (Flint and Scully, 1990; Legat *et al.*, 2002; Nikiforidis *et al.*, 1993; Nir *et al.*, 1995; Yacubian *et al.*, 2001), there has been no report of any association between vitiligo and PMD/SPG2 or peripheral neuropathies.

1.7 Natural killer cells

1.7.1 General overview of natural killer cells

NK cells are large and granular cells of the immune system derived from the common lymphoid progenitors that give rise to B and T lymphocytes (Colucci *et al.*, 2003; Yokoyama *et al.*, 2004). Therefore, NK cells are the third major subset of lymphocytes that play an important part in both innate and adaptive immune responses (Oldham, 1983; Sun *et al.*, 2009; Ugolini and Vivier, 2009). Contrary to both B and T lymphocytes, NK cells do not

have clonally recombinant receptors and thus do not express the T-cell antigen receptor (TCR), Pan T-cell marker CD3 or the surface immunoglobulin (Ig) B-cell receptors. The functional maturation of NK-cell development takes place in the bone marrow, from which they emerge into the circulatory system with two distinguishing surface markers—CD16 and CD56 (in humans), the expression levels of which vary between individual subsets (Yokoyama, 1998).

In humans, NK cells constitute about 5 to 15% of the total number of peripheral blood mononuclear cells (PBMC) in a healthy individual. The name "natural killer" cells was derived from the initial observation that they were able to successfully kill tumour cells without initial stimulation or sensitization, as normally required by other cells of the lymphocytic lineage. Advances in research revealed that as part of the innate immune system, NK cells play an indispensable role in the initial defense against transformed cells and exogenous pathogens, especially infections by viruses (Biron *et al.*, 1999; French and Yokoyama, 2003). The primary mechanism by which NK cells kill their targets is the release of cytotoxic granules that contain perforin and granzymes (Yokoyama *et al.*, 2004). Perforin is a cytolytic protein that inserts itself in the membrane of target cells and form pores (Rosado *et al.*, 2007), through which proteases, such as granzyme B can enter and cause cell apoptosis via the caspase pathway (Bots and Medema, 2006). In addition, NK cells can secrete a host of immunomodulatory and pro-inflammatory cytokines such as interferon-γ (IFN- γ) that can influence the development of an inflammatory cascade and an adaptive immune response (Fearon and Locksley, 1996; Horwitz *et al.*, 1997; Kos, 1998; Su *et al.*, 2001; Zitvogel, 2002). The spontaneity and the rapidity at which NK cells respond to

infections along with their strong cytolytic activity demand for tight regulation of their activity as a precautionary measure against their potential for auto-reactivity.

The activity of NK cells is regulated by cytokines and a repertoire of NK cell activating and inhibitory receptors. Although NK cells can respond directly to cytokines alone, such as interleukin (IL)-12 and IL-18, in most cases, the resulting action is dictated by a net response derived from the integration of signals from both cytokines and the stimulation of an appropriate combination of receptors (Figure 1-2). Some of the notable classes of receptors possessed by NK cells are: the C-type lectin-like receptors in the Ly49 family (in rodents but not humans); the C-type lectin-like receptors in the KLR family shared by both mouse and human, such as NKG2D and NKG2A; the receptors in the killer immunoglobulin-like receptors (KIR) family, as well as those in the natural cytotoxicity receptor family (for example, NKp44 and NKp46) (Campbell and Colonna, 2001; Lanier, 1998; Moretta *et al.*, 2002; Yokoyama, 1998; Yokoyama and Plougastel, 2003).

In order for NK cells to distinguish host normal tissues from harmful pathogens and prevent inappropriate responses directed at self-antigens all NK cells express at least one type of inhibitory receptor that recognizes the MHC class I molecules, which are present on every nucleated cell of the host body (Raulet, 1999; Raulet *et al.*, 2001). Therefore, as long as the host tissue maintains a normal expression level of MHC-1 molecules the cytolytic activity of NK cells would be restrained, although they still retain the ability to produce cytokines in response to other cytokines. Conversely, the "missing self" hypothesis states that abnormal cells in which MHC-1 molecules are significantly down-regulated or totally absent, such as in tumour and virus-infected cells, will exert correspondingly lower inhibitory effects on NK cells and thus making them potential targets for killing (Ljunggren

and Karre, 1990). In addition to the absence of inhibitory signals, the cytolytic activity of NK cells is strongly dictated by the presence of ligands that bind to one of the various activating receptors (Diefenbach and Raulet, 2001; Lanier, 2001; Moretta *et al.*, 2001; Smith *et al.*, 2001), such as NKG2D, whose ligands—MHC Class-1-related chain A (MICA) and chain B (MICB), are up-regulated in response to cellular stress or infection (Das *et al.*, 2001; Groh *et al.*, 2001; Tieng *et al.*, 2002).

1.7.2 Natural killer cells and autoimmune diseases

Defect in regulatory mechanism and impairment in the function of NK cells can predispose the host to a variety of autoimmune conditions, such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and diabetes (Baxter and Smyth, 2002; Flodstrom *et al.*, 2002; Grunebaum *et al.*, 1989).

Deficiencies in the number of circulating NK cells is associated with various autoimmune conditions, such as thyroid diseases (Bossowski *et al.*, 2003; Ciampolillo *et al.*, 2003) and psoriasis (Cameron *et al.*, 2003), a chronic immune-mediated condition with clinical manifestations of the skin. In addition, abnormalities in NK cell functions have been found in patients with MS, SLE and systemic-onset juvenile rheumatoid arthritis (JRA) (Erkeller-Yuksel *et al.*, 1997; Erkeller-Yusel *et al.*, 1993; Kastrukoff *et al.*, 1998; Munschauer *et al.*, 1995; Riccieri *et al.*, 2000; Takahashi *et al.*, 2001; Wouters *et al.*, 2002).

Alterations in the expression of NK cells activation and inhibitory receptors have also been associated with autoimmune diseases, such as diabetes, psoriasis and RA (Martin *et al.*, 2002; van der Slik *et al.*, 2003). Aberrant or skewed expression of various KIRs may potentially lower the activation threshold for NK cells and consequently results in the breakdown of immune tolerance. For example, in addition to the discovery that NK cells

constituted a significant fraction of auto-reactive lymphocytes in the synovial fluid of RA patients, the NK cells also exhibit abnormal up-regulation of NKG2D, one of the most potent activation receptor in the KLR family (Groh *et al.*, 2003). In addition, MICA, the corresponding ligand for NKG2D, was also found to be up-regulated in the synovium of RA patients, suggesting that stimulation of the activation receptor by its ligand results in heightened activity of NK cells. Furthermore, the aberrant expression of NK cell inhibitory receptors, especially CD94 and NKG2A, are associated with autoimmune conditions, such as psoriasis, SLE and RA (Dalbeth and Callan, 2002; Dalbeth *et al.*, 2004; Hervier *et al.*, 2011; Pridgeon *et al.*, 2003; Son *et al.*, 2009). Studies have shown that in some cases, inhibitory receptors such as CD94 and NKG2A were up-regulated while the opposite was observed in others. However, plausible explanations could be given to both phenomena since inhibitory receptors are mmune-modulatory in nature and their decrease could result in the initiation and/or the exacerbation of autoimmune conditions, while increased expression maybe a response to limit the progression of the underlying autoimmune and inflammatory conditions. Therefore, in different cases and/or at different stages of the disease, the expression of NK cell inhibitory receptors may vary.

As with many autoimmune diseases, previous studies have shed light on the possible involvement of NK cells in the pathogenesis of vitiligo. First, findings of association of SNPs located within the promoter region and the coding sequence of the NLRP1 gene with vitiligo and vitiligo-associated autoimmune diseases suggest heightened activity of the innate immune system, especially the dysregulated production of IL-1β and IL-18 by myeloid cells, both of which are critical for the proliferation, survival and function of NK cells (Cella *et al.*, 2010; Colonna, 2009; Gracie *et al.*, 2003)

In addition, there have also beendirect reports of increased number of circulating NK cells and other lymphocytes in the peripheral blood of vitiligo patients (D'Amelio *et al.*, 1990; Ghoneum *et al.*, 1987; Grimes *et al.*, 1986; Halder *et al.*, 1986; Mozzanica *et al.*, 1990; Mozzanica *et al.*, 1989; Mozzanica *et al.*, 1992; Ortonne and Alario, 1978). These and other studies have demonstrated that NK cells isolated from the peripheral blood of vitiligo patients also exhibited aberrant expression(Basak *et al.*, 2008) of surface receptors and were capable of killing melanocytes (Durham-Pierre *et al.*, 1995; Ghoneum *et al.*, 1987; Mozzanica *et al.*, 1989; Mozzanica *et al.*, 1992). However, as with reports of many autoimmune diseases, quantitative and qualitative assessment of NK cells were tempered by the fact that they have been based almost solely on those isolated from peripheral blood samples, which do not necessarily indicate that they actually play any significant role in target tissues. No study has ever investigated the possible infiltration and sequestration of NK cells with abnormal activities in the vicinity of melanocytes in the skin of vitiligo patients.

1.8 Hypothesis and objectives

Based on results from the transcriptome analysis of vitiligo skin biopsies, it is hypothesized that either melanocytes also express plp1 and its down-regulation in vitiligo lesions is simply a reflection of their absence or Schwann cells are also affected. In addition, given the significant up-regulation of various NK cell markers in vitiligo skin, NK cells with heightened activity may play a direct role in the immune-mediated pathogenesis of vitiligo. Therefore, the purpose of this study is twofold: (1) To identify the source of PLP1 deficiency

in vitiligo LS and whether Schwann cells are affected; (2) To evaluate the possible infiltration of NK cells with heightened activities in vitiligo skin.

Chapter 2: Materials and Methods

2.1 Patient and sample collection

A total of 17 patients with non-segmental vitiligo (NSV) and16 healthy volunteers were included in this study (Table B-1, Appendix B). Paired skin biopsies from patients with other benign inflammatory skin conditions such as psoriasis and eczema were also taken for purpose of comparison. The diagnosis of vitiligo was based on characteristic acquired skin depigmentation with typical symmetrical distribution such as the torso, the extremities and the face. In fair skin colored individuals, Wood's lamp was used to help establish the diagnosis. Paired 5mm full-thickness punch biopsies were obtained from vitiligo lesional skin and the adjacent peri-lesional skin. The biopsies were taken in such a way as to ensure anatomic similarity between the paired LS/PLS and normal samples from healthy individuals. In addition, a Schwann cell-rich tumour skin biopsy was also taken for mRNA expression analyses from a patient diagnosed with Schwannoma. For mRNA expression analysis, the biopsies were placed in RNAlater solution (RNeasy protect mini kit; Qiagen, Valencia, CA, USA) immediately upon collection and stored at -20 ˚C for subsequent RNA extraction. A portion of the biopsies were placed in IMDM (Stemcell Technologies, Vancouver, BC, CA) and reserved for explant culture of natural killer cells. Rest of the samples were embedded in OCT medium (Tissue-Tek®; Sakura Finetek, Torrance, CA, USA) and immediately frozen at -80 ˚C for histological confirmation of vitiligo and immunohistochemistry (IHC) / immunofluorescence (IF) studies. All subjects signed the informed consent.

2.2 Cell culture

Adult human epidermal melanocytes (HEMC), adult primary human keratinocytes (HEKa), adult human dermal fibroblasts as well as peripheral blood mononuclear cells (PBMC) were cultured for mRNA expression analyses. All of the cell lines were kind gifts from Dr. Kevin McElwee of the Hair Research Laboratory (University of British Columbia). Melanocytes were cultured in Medium 254 with the addition of Human Melanocyte Growth Supplement (HMGS), keratinocytes in Medium 154 with Human Keratinocyte Growth Supplement (HKGS), fibroblasts in the standard Dulbecco's Modified Eagle's Medium (DMEM), and PBMCs in Gibco® RPMI Media 1640. All of the media and growth supplements were purchased from Invitrogen (Burlington, ON, CA). All of the cells were also supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Hyclone; Thermo Scientific, Ottawa, ON, CA) and cultured at 37 ºC with 5% CO_2. All cultures with exception of PBMCs were provided with fresh medium 3 times weekly and harvested at 80 to 90% confluence. PBMCs were harvested after culturing for 24 hours.

2.3 RNA extraction

For cellular samples, the cells were harvested in Trizol (Invitrogen, Burlington, ON, CA) and total mRNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Skin tissue samples were first trimmed of excess fat, chopped into fine pieces and homogenized in Trizol using a tissue homogenizer (Model 398; Biospec Products Inc, Bartlesville, OK, USA). Rneasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, U.S.A.) was used to extract total RNA from omogenized skin samples according to the

manufacturer's protocol. Total RNA quality was assessed by the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and RNA purity and concentration was determined by the Nanodrop 1000 spectrophotometer in the 260 to 280nm range (Thermo Scientific, Ottawa, ON, CA).

2.4 Quantitative real-time polymerase chain reaction analysis

Based on Molecular Medicine Laboratory's preliminary microarray results, the expression levels of the PLP1 gene as well as a melanocyte differential marker Melan-A was evaluated by quantitative real-time polymerase chain reaction (RT-PCR) analysis using the DNA Engine Opticon™ - Continuous Fluorescence Detection System (MJ Research, Inc. South San Francisco, CA, USA) in all 17 pairs of vitiligo skin tissues (LS+PLS) and 16 skin biopsies from healthy individuals. RNA was reverse transcribed to cDNA with Superscript II RT-enzyme and random hexamers according to the manufacturer's protocols (Invitrogen, Merelbeke, Belgium). Specific primers for each genes were designed using Primer Express 2.0 (Applied Biosystems, Lennik, Belgium). The primer pair sequence used for PLP1 gene detection is 5'-GGTTTCCCTGCTCACCT TCA-3' and 3'-TCAGAACTTGGTGCCTCGG-5', which can detect both PLP1 and DM20, the two major isoforms of the PLP1 gene (Trifilieff et al 1986 and Nave et al 1987). For the detection of Melan-A, the following pair of primers was used: 5'-GATCATCGGGACAGC AAAGTGTT-3' and 3'-AGTAAAGAGCCTGGGT TCTGGG-5'. Two step RT-PCR SYBR green assays were performed using a 25 µl mixture containing 12.5 µl of 2 times SYBR green PCR master mix (Applied Biosystems), 5µl of cDNA template, 300 nM of forward and reverse primer and 4.5µl of RNase free water. The cycling conditions are comprised of 2 min at 50 ^{o}C, 10 min

of polymerase activation at 95 ºC and 40 cycles at 95 ºC for 15 s and 60 ºC for 60 s.

A dissociation curve from 60 ºC to 95 ºC was performed after each run to exclude primer-dimer formation. The cycle threshold C(t) values were calculated by the Opticon Monitor™ software (MJ Research, Inc. South San Francisco, CA, USA), and relative gene expression levels were obtained after normalization to the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

2.5 Histological analyses of tissue samples

2.5.1 Immunofluorescence

Biopsies were embedded in OCT upon collection and transferred to -80 ˚C for storage. Sections of 7 to 14μm in thickness were cut using a cryostat and subjected to standard IF staining protocol. Briefly, the sections were fixed in 4% paraformaldehyde (Sigma-Aldrich, Oakville, ON, CA) at 4 ˚C for 20 min. For permeabilization, 0.15% Triton X (Sigma-Aldrich, Oakville, ON, CA) was applied to the sections for 10 min at 4 ˚C. Depending on the experiment, after blocking in 10% normal goat serum (NGS) (Sigma-Aldrich, Oakville, ON, CA) for 60 min at room temperature, the sections were incubated overnight at 4 ºC in the respective primary antibodies listed in Table 2-1. Mouse brain tissues (gifts from Dr. Kevin McElwee) were used as positive controls for neural markers while inflamed tonsil sections (gifts from Dr. Jan Dutz in the department of dermatology and skin science at the University of British Columbia) were used as positive controls for lymphocyte and NK cell markers. Then, the slides were treated with Alexa Fluor® goat 488/594 anti-mouse/anti-rabbit IgG (Invitrogen, Burlington, ON, CA). Finally, the sections were counter-stained for cell nuclei with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-

Aldrich, Oakville, ON, CA) and mounted in Gold anti-fade medium (Invitrogen, Burlington, ON, CA). The slides were visualized with a Zeiss Axiovert 200M inverted fluorescence microscope. Image processing and quantification were performed with AxioVision Rel. 4.6 software (Zeiss, Toronto, ON, CA).

2.5.2 Immunohistochemistry

Quantification of dermal Schwann cells were carried out on immunohistochemically stained tissue sections using the patented avidin-biotin complex (ABC) method originally established by Su-Ming Hsu and his associates (Hsu *et al.*, 1981). Avidin, a tetrameric protein isolated from egg whites, has extremely high binding affinity to biotin, a member of the water-soluble vitamin B family that plays an essential role in various cellular biochemical processes. The presence of four binding sites for biotin on every avidin molecule combined with the essentially irreversible binding between them allow the formation of macromolecular complexes (ABC) between avidin and biotinylated enzymes, such as peroxidase or alkaline phosphatase, common visualization reagents used in IHC. Therefore, when the ABC method is used with biotinylated secondary antibodies, immense signal amplification can be achieved, as each biotinylated antibody contains many binding sites for ABCs, each of which contains multiple biotinylated enzymes and is cross-linked with other ABCs.

As with IF, 14μm frozen sections in OCT fixed in 4% paraformaldehyde were used. IHC was carried out with the VECTASTAIN® Elite ABC system from Vector Laboratories (Burlington, ON, CA). Briefly, post-fixed tissues were subjected to a series of blocking steps in serum-free protein block (Dako, Burlington, ON, CA), hydrogen peroxide (Vector), avidin-biotin block (Vector), as well as 10% NGS prepared in PBS with 0.15% Triton X. The

sections were then incubated overnight at 4 ^{o}C in rabbit polyclonal anti-human PLP1 (Table 2-1). After extensive washing in tris-buffered saline (TBS), the samples were incubated in biotinylated goat anti-rabbit (Vector) for 1 hr at room temperature. After incubation with the secondary antibody, ABC solution (avidin-biotinylated peroxidase complex) prepared according to manufacturer's protocol was applied to the tissues. After incubation in ABC solution for 30 minutes, visualization was carried out with the substrate solution under a Nikon light microscope, followed by dehydration/clearing and mounting in DPX (VWR, Mississauga, ON, CA).

2.5.3 Quantification

Quantitative analyses of Schwann cells were performed on immunohistochemically stained tissues under 200x magnification. Quantification was limited to the non-myelinating Schwann cells in the superficial dermis (<400 μm from the epidermis), as myelinating Schwann cells in the deep dermis were much less evenly distributed and there was no consistently reliable way of quantifying them using current tissue sections. Both the numerical number and total length of Schwann cell bodies were quantified across entire tissues sections in consecutive fields of view. The length of Schwann cell bodies was measured with the AxioVision Rel. 4.6 software using the interactive measurement module.

Quantification of CD3-/NKG2D+ NK cells and PLP1-positive melanocytes in tissues stained with the IF method were performed at 400x magnification across 5 fields of view.

2.6 Assessing the effect of Schwann cell conditioned medium on melanocytes

2.6.1 Preparation of Schwann cell conditioned medium

Schwann cell conditioned medium was prepared by growing either human Schwann cells isolated from neurofibromatosis type 1 (NF1) patients (Cat. No. CRL-2885, ATCC, Manassas, VA, USA) or rat Schwann cells (Cat. No. CRL-2765, ATCC) in DMEM supplemented with 10% FBS in T-75 culture flasks to approximately 60% confluency. When the appropriate cell density has been achieved, they were washed with Medium 254 (melanocyte medium without HMGS). The Schwann cells were then cultured in fresh Medium 254 under serum-free condition for an additional 24 hours. Conditioned medium was obtained by collecting the supernatant from the Schwann cell cultures, followed by centrifugation at 5000 g for 10 min under 4 ^{o}C to remove any detached cells. Collected conditioned medium was stored in 50 ml BD Falcon™ tubes at 4 ^{o}C.

2.6.2 Melanocyte culture in Schwann cell conditioned medium

HEMCs were seeded into a 96-well microtiter plate at 1000 cells per well and initially cultured for 24 hours in Medium 254 with the addition of HMGS and 10% FBS. The wells were then washed with 1x PBS and divided into 4 groups, each cultured in one of the following media: human Schwann cell conditioned medium, rat Schwann cell conditioned medium, Medium 254 with supplement or Medium 254 without supplement. All of the media were also supplemented with 0.5% FBS. The number of viable cells was monitored over a 120-hour period with the MTS assay. Figure 2-1 presents the detailed layout of the experimental groups in the 96-well plate.

2.6.3 MTS assay

The number of viable melanocytes in the 96-well plate was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96® A_{Queous} One Cell Proliferation Assay, Promega, Madison, WI, USA). MTS was prepared and added to the wells according to the manufacturer's protocol, and incubated for 2 hours. MTS can be readily reduced to formazan by active mitochondrial enzymes from living cells. The coloured formazan product can then be measured at 490 nm by a spectrophotometer, with 650 nm as background control. Conversely, non-viable cells do not have active mitochondrial enzymes and thus are not able to catalyze the reaction of the conversion of MTS to formazan. Therefore, the amount of reduction is positively correlated with the number of viable cells in the sample, which can be obtained on a standard curve.

2.7 Explant culture of natural killer cells

2.7.1 Isolation of lymphocytes from skin biopsies

Traditionally, skin-resident T cells were isolated via mechanical disaggregation of tissue biopsies in conjunction with treatment with chelating agents and digestion with collagenase. However, in addition to producing very little yield of T cells, the cells isolated with these techniques tend to lose their original surface marker expression patterns (Armerding and Kupper, 1999). Clark *et al.* (2006) has recently discovered that by placing skin explants on artificial three-dimensional matrices, a wound-healing microenvironment can be simulated, which will result in the migration of fibroblasts out of the tissue and into the matrix, followed by T cells, which migrate out of the tissue in response to the various chemokines secreted by fibroblasts (Clark *et al.*, 2006). They have also found that not only is

this much more efficient than the traditional method in isolating lymphocytes, the cells isolated with this method also tend to retain their surface expression phenotypes, such as cutaneous homing receptors.

This novel method was used to isolate immune cells from the skin biopsies of 6 vitiligo patients, 5 healthy individuals, as well as 6 patients with psoriasis (another benign inflammatory skin condition) for purpose of comparison (Broady *et al.*, 2010; Clark *et al.*, 2006). Briefly, Cellfoam matrices (Cellsciences Pte Ltd, Singapore) were treated with rat tail collagen I (BD Biosciences, Bedford, MA, USA) and served as three-dimensional scaffolds that separate dermal fibroblasts and skin-resident lymphocytes. The skin explants were minced and placed on the surface of the matrices and cultured in 12-well 0.4mm pore size polyester transwell culture plates (Corning, Corning, NY, USA) in the presence of 25 ng/ml IL-2 and 20 ng/ml IL-15 (R&D systems, Minneapolis, MN, USA) in IMDM (Stemcell Technologies, Vancouver, BC, CA). The cultures were supplemented with 10% heat-inactivated FBS, penicillin and streptomycin (Sigma-Aldrich, Oakville, ON, CA). The cells that have migrated out of the explants were analyzed after 3 weeks.

2.7.2 Flow cytometric analysis of natural killer cells

Flow cytometry of the migrated lymphocytes was performed using fluorophore-conjugated antibodies against T cell receptors and natural killer cell markers. Cells were stained with antibodies against CD3, CD56, granzyme B and Ki67 (BD Biosciences, Bedford, MA, USA) and natural killers were distinguished from the rest of the lymphocytes by positive expression of CD56 and negative expression of CD3. The samples were analyzed on a BD FACSCanto flow cytometer (BD Biosciences, Bedford, MA, USA) and data was analyzed with the software FCS Express Pro 3 (De Novo Software, Los Angeles, CA, USA).

The proportions of $CD56^{bright}$ NK cells cultured from vitligo and normal skin explants were tabulated, plotted and compared statistically.

2.8 Statistical analysis

Statistical analyses were performed with the GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA, USA). The non-parametric Mann-Whitney U test was used to for comparisons between vitiligo and normal skin samples, as well as between melanocytes cultured in different conditioned media while the paired Student's t-test was used to compare between paired vitiligo LS and PLS.

Chapter 3: Results

3.1 Comparison of PLP1 gene expression in vitiligo and normal skin

In order to confirm the differential expression of PLP1 gene observed in Molecular Medicine Laboratory's preliminary transcriptome analysis of paired vitiligo and normal skin further comparison of the PLP1 gene expression levels on 17 paired vitiligo LS and PLS along with 16 NS biopsies was performed using RT-PCR. Comparisons were made between paired vitiligo LS and PLS as well as between paired vitiligo and NS samples. The expression levels were standardized against either vitiligo PLS for paired analysis or NS for comparison between vitiligo and normal skin samples. A bar plot was constructed with the average of the calculated ratios of all the paired samples (Figure 3-1A). For comparison between paired vitiligo and normal skin samples, a separate experiment was done side-by-side with 9 samples from either groups and a bar plot was constructed with the average ratio of vitiligo samples (LS and PLS) against NS (Figure 3-1B).

As shown in Figure 3-1, there was a statistically significant difference of approximately 1.6 fold (LS/PLS = 0.61, $p = 0.0004$) between paired vitiligo LS and PLS and twofold between vitiligo LS and NS (LS/NS = 0.46, $p < 0.0001$) (Figure 3-1b), which was consistent with the data from the transcriptome analysis (Table A-1). In addition, there appeared to be no statistically significant difference (PLS/NS = 0.84, $p = 0.15$) in PLP1 expression levels between vitiligo PLS and NS (Figure 3-1b).

3.2 Characterization of the cellular origins of PLP1 expression in normal skin

Two steps were taken to establish the cellular origins of the PLP1 gene: quantification of messenger RNA in representative cell types of the human skin using RT-

PCR, and IF staining of normal skin sections using antibodies specific for various neural and melanocytic markers.

3.2.1 Comparative PLP1 expression analysis in cells of the human skin

First, quantitative polymerase chain reaction was used to determine the mRNA abundance of the PLP1 gene in melanocytes, fibroblasts, keratinocytes, peripheral blood mononuclear cells (PBMC) and Schwann cells derived from Schwannoma tissues. The gene expression levels were standardized with the housekeeping gene GAPDH.

The PLP1 gene was expressed at the highest levels in Schwann cells among all the cell types screened, although modest levels were present in melanocytes and detetable levels were also observed in keratinocytes (Figure 3-2A). On the other hand, fibroblasts and PMBCs displayed negligible levels of PLP1, which was to be expected. In addition, the pattern of expression for PLP1 was distinctly different from a melanocyte differential marker, such as Melan-A (MLANA) (Figure 3-2B), which was expressed the highest in melanocytes, whereas the level was undetectable in Schwann cells. It is intriguing that melanocytes expressed a significant amount of PLP1, although its expression level was lower than in Schwann cells. This suggested that in human skin, both Schwann cells and melanocytes are the major cellular sources of PLP1 expression at the mRNA level.

3.2.2 PLP1 localization at the protein level in normal human skin

To further confirm the cellular sources of PLP1 and evaluate its expression at the protein level, immunofluorescence analysis was performed on normal skin using specific antibodies against the PLP1 protein, neural cell adhesion molecule (CD56/NCAM), which also stains Schwann cells, as well as peripheral nerve fibers, such as neurofilament 200 (NF 200) and the pan-neural marker protein gene product 9.5 (PGP 9.5).

In general, PLP1-positive Schwann cells were relatively abundant in the upper epidermis of human skin (Figure 3-3), some of them in close proximity to the basal epidermal layer and seemed to have established connections with the basement membrane (Figure 3-3A). Double-stain analysis using the Schwann cell marker NCAM and PLP1 revealed that the two markers almost perfectly co-localize with each other (Figure 3-3A, arrows), which confirmed the fact that the PLP1-positive structures observed in the dermis of human skin were indeed Schwann cells. This was further confirmed by the absence of NCAM stain in the epidermal area.

Further, co-localization of intermediate neurofilament (200 kDa) (Figure 3-3B) and PGP 9.5 (Figure 3-3C) with PLP1 or NCAM revealed an intricate spatial relationship between Schwann cells and peripheral nerve fibers in the skin. Larger nerve fibers enveloped by Schwann cell bodies were confined entirely in the dermis, while fine cutaneous sensory nerve endings free from Schwann cell processes extended all the way into the epidermis.

3.2.3 PLP1 expression in melanocytes

Given the modest levels of PLP1 mRNA present in cultured melanocytes as revealed by RT-PCR analysis, it remains important to assess whether PLP1 was also expressed at the protein level in human skin. Therefore, IF analysis was performed on normal and vitiligo human skin biopsies with antibodies against PLP1 and Melan-A. By viewing the samples at 400x magnification under a fluorescence microscope and focusing on the basal epidermal layer it can be observed that most, if not all melanocytes were strongly positive for both PLP1 and Melan-A (Figure 3-4, arrows) in both the normal and vitiligo peri-lesional skin, where residual melanocytes can still be detected. In contrast, neither PLP1 nor Melan-A can be dectected in the basal layer of the epidermis in vitiligo lesional skin where there was a

complete absence of melanocytes. Therefore, the down-regulation of PLP1 gene observed at the mRNA level in vitiligo lesional skin can be partially attributed to the death of melanocytes.

3.2.4 Localization of myelinating and non-myelinating Schwann cells

In order to distinguish between the two major types of Schwann cells, the myelinating and non-myleinating Schwann cells, myelin basic protein (MBP), the principle constituent of myelin sheath, was used as a marker along with PLP1 in the IF analysis of normal human skin biopsies. The resulting staining pattern demonstrated that the PLP1-positive Schwann cells found in the papillary dermis and the dermo-epidermal junction (Figure 3-5A) were negative for MBP, while the majority of the Schwann cells found in the reticular dermis were MBP-positive (Figure 3-5B), which was indicative of myelin sheath presence. This suggested that the Schwann cells in the superficial dermis of human skin are non-myelinating while those in the deep dermis are predominantly myelinating in nature, which are typically associated with large nerve bundles.

3.2.5 Summary of PLP1 expression in normal skin

Overall, PLP1 was found to be expressed mainly by Schwann cells in the skin and these cells are closely associated with nerve fibers and some of them, melanocytes. Furthermore, these cells do not express the melanocyte differential marker Melan-A. These results established Schwann cells as the main cellular source of PLP1 gene expression in the skin, although it is also expressed at both the mRNA and protein levels in a minor subset of melanocytes.

3.3 Assessment of Schwann cells in vitiligo skin

3.3.1 Assessment of Schwann cells and nerve fibers in vitiligo and normal skin with immunofluorescence

In order to get an initial qualitative perspective on the state of Schwann cells and nerve fibers in vitiligo LS as compared with PLS and NS, IF analysis was performed on 13 paired vitiligo and 6 normal skin biopsies using antibodies specific to PLP1, MBP, PGP 9.5 and Melan-A. Observations across all of the vitiligo and normal skin samples did not reveal any significant qualitative difference in the morphology of non-myelinating Schwann cells in most samples, although in some vitiligo lesions the Schwann cells exhibited a more fragmented appearance with shorter cell body processes (Figure 3-6A). Furthermore, there appeared to be a net numerical decrease in vitiligo LS as compared to PLS and NS. Myelinating Schwann cells in the deep dermis were generally much more sparsely distributed than their non-myelinating counterpart and no consistent qualitative and quantitative difference could be observed between vitiligo and normal skin (Figure 3-6B). In addition, there appeared to be no difference in the morphology and distribution of PGP 9.5-immunoreactive sensory and autonomic nerve fibers between vitiligo LS, PLS and normal skin (Figure 3-6C), which has also been previously reported (Liu *et al.*, 1996).

3.3.2 Quantification of Schwann cells in paired vitiligo skin

To further assess the cellular defect associated with PLP1 down-regulation in vitiligo LS, a systematic quantification of Schwann cells was performed in 13 paired vitiligo skin samples (LS+PLS). Due to the sparseness and uneven distribution of deep dermal myelinating Schwann cells, quantification was limited to the superficial non-myelinating Schwann cells (depth < 200 μm), which constitute the vast majority of Schwann cells in

human skin (Griffin and Thompson, 2008; Tschachler *et al.*, 2004). IHC of PLP1 was performed on the samples and quantification of both the number and total length of PLP1-immunoreactive structures were performed across the span of the entire tissue sections.

On average, there appeared to be a decrease in both the number and length of Schwann cell bodies in vitiligo LS as compared with the corresponding PLS (Figure 3-7A). While the difference in the average number of Schwann cells per 200x field of view of all the samples was not statistically significant (LS/PLS = 0.89, p = 0.19) (Figure 3-7B), the total length of the Schwann cell bodies per field of view was significantly shorter (about 22%) in LS as compared to PLS (LS/PLS = 0.82, p = 0.032) (Figure 3-7C). Quantification data for individual patient are also given in Figure C-1 and 2 (Appendix C). In general, this suggested that Schwann cells are indeed affected in vitiligo, which could partially explain the observed down-regulation of PLP1 at the mRNA level.

3.4 The influence of Schwann cells on melanocyte growth and survival

Decreases in the number and length of Schwann cell bodies and the down-regulation of PLP1 in vitiligo LS raised an important question of whether defect in Schwann cells may have had an effect on melanocyte death during the depigmentation process. In an attempt to shed light on this question, melanocytes were cultured using conditioned medium from cultured rat primary Schwann cells and human Schwann cells isolated from neurofibromatosis patients.

As shown in Figure 3-8, there were significantly greater numbers of melanocytes present after 5 days in cultures enriched with either HMGS or rat and human Schwann cell-conditioned medium as compared to the group with Medium 254 alone. Growth curves

constructed based on standardized MTS absorbance values (over the initial value on the day the cells were seeded) for the different experimental groups over a period of 5 days (Figure 3-8E, F) demonstrated that melanocytes cultured in Schwann cell-conditioned medium exhibited increased survivability (around the same level as those cultured in Medium 254 enriched with HMGS) as compared with the control cells, which were cultured in Medium 254 alone. The pro-survival effect of Schwann cell-conditioned medium was evident after 24 hours. The difference between Schwann cell-conditioned media (both human and rat) and the control consistently increased throughout the entire span of the experiment, with the greatest being observed after 120 hours, where the Schwann cell conditioned groups had approximately 8 times (rat) (Figure 3-8E) and 7 times (human) (Figure 3-8F) the starting number of cells, respectively, whereas the control group had grown approximately 3.5 times since the beginning of the experiment. Overall, this suggested that Schwann cells secrete various factors that may aid in the growth and survival of melanocytes in human skin.

3.5 Assessment of NK cell activity in the LS and PLS of vitiligo patients

Since the gene expression analysis revealed marked increase of multiple markers of activated NK cells in the lesional and peri-lesional vitiligo skin, it is speculated that skin in vitiligo subjects contains abnormal infiltration of activated NK cells. To test this speculation, skin resident NK cells were isolated from cultured vitiligo skin explants (Broady *et al.*, 2010; Clark *et al.*, 2006) and analysed for CD3 (a pan T cell marker), CD56 (a natural killer cell marker) and granzyme B (a marker for cytotoxicity) expression. Skin of healthy volunteers from anatomically similar locations served as controls. In addition, paired PLS and LS skin biopsies from psoriasis patients were also used for comparison purpose between benign

inflammatory skin conditions. The explant culture method has previously been shown to accurately reflect the *in situ* immune cell compositions in the tissues by retaining the expression ratio of surface markers (Clark *et al.*, 2006).

As shown in Figure 3-9, normal skin contained only a small number of NK cells. In contrast, in the skin obtained from vitiligo patients, there was a significant increase in NK cells not only in the vitiligo lesion ($p = 0.0043$), but also in the normal-appearing pigmented skin in the vicinity of the lesion ($p = 0.0043$) as compared to normal skin (Figure 3-9A). There is a noticeable increase in the number of NK cells in the LS of psoriasis patients as compared to NS, however, on average, the number is significantly less than that found in vitiligo LS. Further, a small percentage of $CD3^{bright}$ $CD56^{bright}$ cells were isolated from all tissue biopsies, indicating the presence of cytotoxic T cells and/or natural killer T cells (NKT) in the skin (Terabe and Berzofsky, 2008), although no quantitative difference were observed among the samples.

In addition, the NK cells isolated from vitiligo LS and PLS exhibited high expression of the serine protease, granzyme B (Figure 3-9B), indicating that these cells are activated and cytotoxic (Afonina *et al.*, 2010). Further, these cells do not express Ki67, a marker of proliferation and its expression has been shown to be inversely correlated with the stage of cellular differentiation (Scholzen and Gerdes, 2000). The Ki67-negative status indicated that the NK cells are terminally differentiated and not actively proliferating, which is consistent with their activation status.

3.6 Infiltration of NKG2D-positive NK cells in the skin of vitiligo patients

The role of activated natural killer cells in vitiligo is less well-characterized than that of other lymphocytes. The transcriptome analysis of vitiligo skin from Molecular Medicine Laboratory revealed the significant up-regulation of NKG2D, one of the most potent activating NK cell receptors in the KLR family. Therefore, to identify and confirm the presence of activated natural killer cells with high NKG2D expression, IF analysis using specific antibodies against NKG2D (KLRK1) was performed on skin biopsies taken from vitiligo patients, normal individuals as well as soriasis and eczema patients for comparison.

In vitiligo skin (both LS and PLS), three general populations of lymphocytes were observed using NKG2D and the CD3 pan-T cell markers—CD3+/NKG2D-, CD3+/NKG2D+ and CD3-/NKG2D+ cells (Figure 3-10A, C-3(Appendix C)). In normal skin, only the sparsely distributed CD3+/NKG2D- lymphocytes could be clearly seen, with little or no NKG2D+ cells. In addition to the increase in cytotoxic T cell infiltrates characterized by the expression of both CD3 and NKG2D (Figure C-3, arrows), which is in agreement with a previous study (van den Boorn *et al.*, 2009), a significant portion of cells in both the PLS and LS of vitiligo patients were either CD3+/NKG2D- or CD3-/NKG2D+ (Figure 3-10A, arrows), which can be identified to be activated NK cells due to the lack of CD3 expression. The majority of cells were found to reside in the upper dermis of the skin, especially activated NK cells, with some observed in close proximity with the basement membrane of the dermal papillae. Quantification of CD3-/NKG2D+ cells has demonstrated the presence of NKG2D+ natural killer cells in vitiligo skin that were not commonly found in anatomically similar normal skin.

Finally, immunofluorescence co-staining of Melan-A and NKG2D in the transitional area of vitiligo skin (borderline region between PLS and LS) revealed the infiltration of activated NK cells in the epidermis, within close proximity of the residual melanocytes (Figure 3-10B), with some of them even in direct physical contact with NKG2D+ cells (arrow). Even though NK cells have also been implicated to play a role in the disease pathogenesis of other benign inflammatory skin conditions such as psoriasis and eczema (Buentke *et al.*, 2002; Son *et al.*, 2009), our result demonstrated that even if they are present, NKG2D+ NK cells are not found in the epidermal and upper dermal area (Figure 3-10C), which strengthened the speculation that activated NK cells may be directly involved in the destruction of melanocytes in vitiligo.

Chapter 4: Discussion

In this study, PLP1 was found to be down-regulated at the mRNA level in vitiligo lesional skin as compared to peri-lesional skin and normal skin. The major cellular source for PLP1 expression is the Schwann cells, although it is also expressed at a respectable level in melanocytes. Therefore, the down-regulation of PLP1 in vitiligo LS can be partially explained by the death of melanocytes as well as the observed decrease in both the number and total length of Schwann cell bodies. In addition, the infiltration of NK cells with heightened cytotoxicity and activity in vitiligo skin is an important indication that they may play both a direct and indirect role in the depigmentation process. The following sections will attempt to discuss in detail the implications of PLP1 down-regulation and NK cell activation in vitiligo and shed light on potential new pathomechanisms suggested by the results of this study.

4.1 Down-regulation of PLP1 in vitiligo lesions

4.1.1 Expression of PLP1 in both Schwann cells and melanocytes

Results of this study have demonstrated that PLP1 is expressed in both Schwann cells and melanocytes at the mRNA and protein levels in the skin. Being one of the very important myelin sheath proteins, PLP1 has been known to be expressed in oligodendrocytes and Schwann cells, the glial cells of the CNS and PNS, respectively (Fulton *et al.*, 2010; Garbern *et al.*, 1997; Sarret *et al.*, 2010; Wood *et al.*, 1984). Therefore, it would seem surprising at first that melanocytes, which are not known to participate in myelin formation and are only peripherally involved with the nervous system, also express one of the most important myelin proteins.

On the other hand, this was not totally unexpected, since melanocytes share the same lineage as Schwann cells in that they are both first originated from multipotent progrenitor cells of the neural crest, which migrate throughout the body at the embryonic stage to give rise to a wide variety of tissue types that include the neurons and glials cells of the PNS (Chimge and Bayarsaihan, 2010). In addition, a recent landmark study by Adameyko *et al*. (2009) has demonstrated that Schwann cell precursors from skin innervations can differentiate into both melanocytes and Schwann cells and thus may serve as a source for both (Adameyko *et al.*, 2009). Therefore, it is reasonable to expect the expression of common molecular markers by both melanocytes and Schwann cells.

In fact, in addition to PLP1, both S100 (a commonly used marker to differentiate cells of melanocytic lineage from others) and SOX10 (a HMG box transcription factor expressed in neural crest stem cells) have been shown to be expressed in both melanocytes and Schwann cells (Bishop *et al.*, 1993; Levy *et al.*, 2004; Nonaka *et al.*, 2008). In particular, the mutation of SOX10 can result in Waardenburg syndrome, an autosomal dominant disorder characterized by both neurological and pigmentation abnormalities (Bondurand *et al.*, 2000; Cheng *et al.*, 2000; Jiao *et al.*, 2004; Lee *et al.*, 2000; Potterf *et al.*, 2000).

Prior to the current study, another group has employed a novel two-step DNA microarray analysis on melanocytes and has found that PLP1 could potentially be one of the down stream targets of microphthalmia-associated transcription factor (MITF), the master gene regulator of melanocytes (Hoek *et al.*, 2008). The presence of PLP1 in both melanocytes and Schwann cells of the superficial dermis, both of which are non-myelinating cells, highly suggests its involvement in functions unrelated to the formation of myelin.

Evidence in the literature has linked PLP1 with cellular functions such as ion exchange, cell signaling and migration, as well as survival and programmed cell death.

One of the earliest evidence linked PLP1 to the formation of transmembrane ion exchangers, in particular the voltage-dependent Ca^{2+} channels (Helynck *et al.*, 1983; Ting-Beall *et al.*, 1979). Diaz *et al.* (1990) has demonstrated using artificial proteoliposomes that Ca^{2+} movement across the plasma membrane is highly dependent on its interaction with PLP1 and other proteins (Diaz *et al.*, 1990). The maintenance of proper intracellular Ca^{2+} levels is critical for various cellular functions. In melanocytes, calcium is an important regulator of melanogenesis, melanosome aggregation, trafficking and distribution to the surrounding keratinocytes (Bush and Simon, 2007; Martina *et al.*, 2009; Yamada and Fujii, 2002). In addition, calcium channels are an indispensable part of the signal transduction system in neurons. There is also evidence that melanocytes are intimately connected with nerve fibers in the skin (Hara *et al.*, 1996), which makes it reasonable to assume that PLP1 may also play a role in signal transduction and cellular communication between neurons and melanocytes.

As a transmembrane protein, PLP1 is well situated in the plasma membrane to participate in the signal transduction process between the cell and the extracellular environment. A couple of studies done by Gudz *et al.* (2002, 2006) have shown that PLP1 can interact with integrins (Gudz *et al.*, 2006; Gudz *et al.*, 2002), which are cell surface receptors for extracellular matrix molecules (ECM) that can trigger various cellular responses, such as migration, proliferation and differentiation, and even survival (Harburger and Calderwood, 2009). Specifically, the studies demonstrated that binding of PLP1 to integrins in oligodendrocytes reduced interaction of integrins with ECM and subsequently

increased their motility. Studies have shown that human hair follicles and peripheral nerves can serve as cellular reservoirs for both Schwann cells and melanocytes (which explains their recovery in vitiligo repigmentation process) (Adameyko *et al.*, 2009; Biernaskie, 2010; Ernfors, 2010; Sieber-Blum *et al.*, 2004). Neural crest stem cells in hair follicles and Schwann cell precursors in peripheral nerve sheaths can migrate through the ECM and differentiate into either melanocytes or Schwann cells to replenish those as needed. In this regard, PLP1 may very well play a role in guiding the differentiation and migration of Schwann cells and melanocytes in the skin.

Further studies have demonstrated that PLP1 may regulate mitochondrial function and general survival in cells of neural origin (as well as some non-neural cell types). Aberrant expression in the PLP1 gene can result in mitochondrial dysfunction, depolarization of mitochondrial membrane potential, deficiency in ATP levels, drastic increase in intracellular acidity and subsequent trigger of apoptosis (Skoff *et al.*, 2004a; Skoff *et al.*, 2004b). In general, the function of PLP1 in cells other than glial cells is not well-established and further investigations into this matter are warranted.

4.1.2 Schwann cell defect in vitiligo lesions

Back in the mid-1960s and 1990s, two independent groups performed ultra-structural studies of peripheral nerve fibres in vitiligo skin using electron microscopy. In their studies, Breathnach *et al.* (1966) and Al'abadie et al. (1995) found signs of degenerative changes in Schwann cells and their associated nerve axons in vitiligo lesions as compared to normal skin (Al'Abadie *et al.*, 1995; Breathnach *et al.*, 1966). Both groups noticed duplication and fragmentation of Schwann cell membranes, followed by swelling and extrusion of associated nerve axons. Schwann cells in vitiligo lesions also appeared to have significantly heightened

activity levels in the form of increased numbers of rough endoplasmic reticulum and mitochondria, which are signs of periods of regeneration interspersed with degeneration. Both groups agreed that the degenerative changes were observed more frequently in superficial non-myelinating Schwann cells and their associated axons, while Breathnach *et al.* had observed them in some myelinating Schwann cells as well. However, both also acknowledged the fact that electron microscopy was severely limited in its ability to perform a systematic quantification of the changes.

Therefore, although the current study is not the first to point out at the possibility of Schwann cell defect in vitiligo, our work is the first systematic quantification of Schwann cells in vitiligo skin biopsies using IHC techniques . Our work revealed an overall decrease in both the number and length of non-myelinating Schwann cell bodies in vitiligo LS.

While myelinating Schwann cells are primarily associated with larger nerve bundles, and their primary functions are the formation and maintenance of myelin sheath, non-myelinating Schwann cells are often associated with smaller and finer nerve fibres, also called Remak fibres, typically those that are present in the superficial dermis of the skin (Griffin and Thompson, 2008). Schwann cells have been shown to undergo programmed cell death when their associated nerve axons are severely injured or when they are totally denervated, which have been extensively documented in animal studies (Grinspan *et al.*, 1996). Although it is possible that the defect observed in Schwann cells in our studies as well as those from the electron microscopy studies may be secondary to peripheral nerve injuries, several lines of evidence point against this explanation. As opposed to myelinating Schwann cells, non-myelinating Schwanns associated with Remak fibres have shown to be “fast responders” to nerve injury and denervation. They respond to denervation by increasing their

proliferation rate and secreting various growth factors to aid in the regeneration of nerve axons (Murinson *et al.*, 2005; Murinson and Griffin, 2004). In addition, an overall increase in certain types of superficial dermal nerve fibres has been observed in vitiligo skin, such as those that are immunoreactive for neuropeptide Y (NPY) and calcitonin gene related peptide (CGRP) (Al'Abadie *et al.*, 1994; Hristakieva *et al.*, 2000; Lazarova *et al.*, 2000; Liu *et al.*, 1996). These observations suggest that Schwann cells should, in fact, be increased in numberin LS of vitiligo patients.

A more plausible explanation of the decrease in Schwann cell number seen in vitiligo LS can be proposed on the basis of the pathogenic nature of the disease and the shared origin between melanocytes and Schwann cells. There is widespread evidence of autoreactivity against melanocytes in vitiligo, such as the presence of autoantibodies against melanocytic markers (Jimbow *et al.*, 2001; Kemp *et al.*, 2011; Kemp *et al.*, 1997a; Kemp *et al.*, 1997b, 1998a; Kemp *et al.*, 1998b, 1999b, 2001a; Palermo *et al.*, 2001) and infiltration of cytotoxic lymphocytes specific to melanocytes in vitiligo lesions (van den Boorn *et al.*, 2009). Since melanocytes and Schwann cells are derived from the same precursor cells and share many characteristics in common, including surface markers, it is reasonable to speculate a certain degree of cross-reactivity between these two cell types. In fact, Hedstrand *et al.* (2001) has established SOX 10 as a novel autoantigen for vitiligo, in that a significant number of patients in their study exhibited strong immunoreactivity against the pan-Schwann cell-melanocyte marker (Hedstrand *et al.*, 2001). In addition, non-specific tissue damage in the microenvironment of melanocytes caused by chronically up-regulated pro-inflammatory cytokines (Birol *et al.*, 2006; Tu *et al.*, 2003) and oxygen radicals (Schallreuter *et al.*, 1999) may also contribute to Schwann cell destruction as well as explain the degeneration-

regeneration cycle observed by Breathnach *et al.* (1966) and Al'abadie et al. (1995) (Al'Abadie *et al.*, 1995; Breathnach *et al.*, 1966).

The landmark study done by Adameyko *et al*. (2009) has not only established a new source of both Schwann cells and melanocytes in the form of Schwann cell precursors (SCP) in peripheral nerve sheaths, but has also demonstrated that mature Schwann cells still retain the ability to de-differentiate and re-differentiate into melanocytes (Adameyko *et al.*, 2009). Therefore, this raises the possibility that some Schwann cells may spontaneously transform into melanocytes to replenish those lost as a result of the depigmentation process. In this regard, the decrease in the length of Schwann cell bodies and their fragmented appearance in some cases of vitiligo lesions may reflect the state of transformation that some of these cells were in.

4.1.3 Implications of Schwann cell defect on melanocytes

Several studies have attempted to elucidate the close physical relationship shared by melanocytes and nerve fibres in the dermo-epidermal junction (Breathnach *et al.*, 1966; Eriksson *et al.*, 1968; Hara *et al.*, 1996; Mihara *et al.*, 1982). Amongst these, the study by Hara *et al.* (1996), which utilized both confocal immunofluorescence microscopy and electron microscopy to detect innervations of melanocytes by Schwann-cell enveloped nerve fibres, was considered the most significant and widely accepted (Hara *et al.*, 1996). In their study, Hara *et al.* discovered that structures characteristic of non-myelinating Schwann cell axonal complexes either end in what appeared to be synaptic contact with melanocyte cell bodies or penetrate into melanocyte cytoplasm. In addition, studies have provided support for the neuroendocrine control of melanocyte activity (ex. Melanogenesis) by the cutaneous nervous system (Legat *et al.*, 2002). In fact, Hara *et al.* in this same study has also revealed

that neuropeptides associated with these cutaneous nerve fibres have a profound effect on both the survival and activity of cultured melanocytes, in the form of increased proliferation rate and intracelluar cAMP levels, which is known to regulate melanogenesis (Grando *et al.*, 2006). Since Schwann cells are critical for the development and maintenance of nerve fibres, their defect would adversely affect the cutaneous nervous system and potentially disrupt the neuroendocrine imbalance in the microenvironment of melanocytes, which in turn may potentially result in pigmentation abnormalities as seen in vitiligo and other skin diseases.

The current study has also demonstrated that Schwann cells produce various factors that aid in the growth and survival of melanocytes *in vitro*. In fact, melanocytes cultured in Schwann cell-conditioned medium exhibited similar level of growth as those cultured in fully supplemented melanocyte growth medium. Studies have shown that Schwann cells produce various neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and various nerve growth factors (NGF), which are essential growth and differentiation factors for cells of both neural and non-neural origin (Murinson *et al.*, 2005). These neurotrophic factors have shown to play an important role in the survival, differentiation and migration, dendrite development and maintenance, and even melanin synthesis of melanocytes (Bothwell, 1997; Marconi *et al.*, 2006; Sieber-Blum, 1998; Yaar *et al.*, 1994). However, some of these factors are also produced in keratinocytes and fibroblasts, which, in the absence of more detailed studies on additional factors that may be produced by Schwann cells, makes it difficult to assess the true impact that reductions in Schwann cells may have on melanocytes.

4.2 Heightened NK cell activity in vitiligo skin

4.2.1 Evidence of NK cells in vitiligo and other immune-mediated skin diseases

Natural killer cells are an important component of the innate immune system and considered to be the first line of defense against tumours as well as bacterial, viral and parasitic infections. On the other hand, considerable evidence has demonstrated an important role NK cells might play in the initiation and/or perpetuation of various autoimmune diseases (French and Yokoyama, 2004; Perricone *et al.*, 2008). In particular, NK cells have previously been associated with various autoimmune skin conditions. In particular, significantly elevated numbers of $CD56^{bright}$ NK cells were found in the blood and skin lesions of patients with systemic lupus erythematosus and both the peripheral blood and synovial fluid of patients with psoriatic arthritis (Spadaro *et al.*, 2004; Viljaranta *et al.*, 1987). Natural killer cells have also been implicated in the pathogenesis of atopic dermatitis (AD), as evidence has revealed the infiltration of $CD56^{bright}$ NK cells in the dermal infiltrate of AD lesions (Buentke *et al.*, 2002; Kawakami *et al.*, 2009). In addition, NK cells isolated from the lesions of AD patients exhibited the ability to spontaneously release various pro-inflammatory cytokines, indicative of its heightened activation status (Aktas *et al.*, 2005). Findings of NK cells have been reported in other skin conditions, such as alopecia areata (Baadsgaard and Lindskov, 1986; Chiarini *et al.*, 2008; Ito *et al.*, 2008) and pemphigus vulgaris (Stern *et al.*, 2008; Takahashi *et al.*, 2007).

As expected, NK cells have also previously been associated with vitiligo. Previous studies have shown an increase in the number of circulating NK cells in the blood of vitiligo patients (Basak *et al.*, 2008; Durham-Pierre *et al.*, 1995; Ghoneum *et al.*, 1987; Mozzanica *et al.*, 1989; Mozzanica *et al.*, 1992). In particular, Basak *et al.* (2008) have shown that the

number of $CD16^{bright}$ $CD56^{bright}$ NK cells was significantly elevated in the blood of vitiligo patients. NK cells characterized by high expression of CD56 have recently been demonstrated to be one of the most versatile subpopulations, in that they exhibit both high degrees of cytotoxicity (Tsuda *et al.*, 2011) and are able to produce various pro-inflammatory cytokines, such as IFN-γ and TNF-α (Dalbeth and Callan, 2002; Pridgeon *et al.*, 2003; Tsuda *et al.*, 2011), both of which have been previously reported in the serum and LS of vitiligo patients (Birol *et al.*, 2006; Tu *et al.*, 2003). Therefore, the $CD56^{bright}$ subpopulation of NK cells have been proposed to play an important role in the initiation and propagation of autoimmune diseases, in the form of heightened cytotoxicity and dysregulated production of pro-inflammatory cytokines, which facilitate the ultimate breakdown of immune tolerance.

The present study has further provided support for the pathogenic role that NK cells may potentially play in vitiligo and other immune-mediated conditions by demonstrating for the first time the infiltration of $CD56^{bright}$ and NKG2D+ natural killer cells in the disease end tissues, the skin microenvironment of melanocytes in both the LS and PLS of vitiligo patients. In addition, further flow cytometric analysis of these cells revealed heightened cytotoxicity characterized by the expression of high levels of granzyme B.

4.2.2 Potential role of NK cells in the pathogenesis of vitiligo

The presence of activated natural killer cells within the infiltrate of vitiligo skin raises questions regarding their role in the pathogenesis of vitiligo—of particular interest is whether they are involved in the direct destruction of melanocytes. One of the findings from the current study that supports this notion is that the cells isolated from the skin of vitiligo patients all expressed high levels of granzyme B, which has long been a characteristic of elevated activities of cytotoxic T lymphocytes and NK cells in inflammatory conditions

(Afonina *et al.*, 2010; Goldbach-Mansky *et al.*, 2005; Ronday *et al.*, 2001; Tak *et al.*, 1994). In addition to the commonly attributed cytotoxic role via activation of the caspase pathway, granzyme B has been implicated in the generation and presentation of self-antigens (Darrah and Rosen, 2010). For example, a recent study has shown that cleavage of transaldolase by granzyme B can result in highly antigenic autoimmune epitopes in patients with multiple sclerosis (Niland *et al.*, 2010). Since granzyme B cleavage sites are contained within many autoantigens, it is possible that melanocyte death in vitiligo may be initiated by activated NK cells via direct cytotoxic action of granzyme B, which may result in the structural modification of melanocytic antigens, thereby inducing the formation of "neo-antigens" (Kannan, 2006). The uptake of neo-antigens by professional antigen presenting cells such as resident Langerhans cells in the skin may then trigger specific adaptive immune responses against melanocytes.

Of potential importance is the up-regulation of the killer cell activating receptor NKG2D characterized by the presence of NKG2D+/CD3- NK cell infiltration in vitiligo skin, as demonstrated by quantitative IF analysis. NKG2D is an immunoreceptor expressed by NK cells, NKT cells, and a subset of cytotoxic T lymphocytes. Engagement of NKG2D by its ligands, which are MHC class I-like proteins in the ULBP and MIC families, result in the killing of target either directly or via cytokine production (Stern-Ginossar and Mandelboim, 2009). One of the well-known ligands in the ULBP family—ULBP2, has been associated with various cancers and autoimmune diseases (Gao, 2010; Lopez-Soto *et al.*, 2009; Nuckel *et al.*, 2010; Serrano *et al.*, 2010; von Lilienfeld-Toal *et al.*, 2010), and incidentally, has been up-regulated in vitiligo skin in Molecular Medicine Laboratory's transcriptome analysis as well, although the data did not meet the strict cut-off criteria

(increased by 1.5 folds as opposed to 2 folds in lesional skin as compared to that of normal individuals). The increased expressions of NKG2D and ULBP2 in vitiligo skin suggest engagement of NK cells and their ligands in the local milieu.

It has been shown previously that the overexpression of NKG2D ligands—ULBP and their homologues, can be readily induced in tissues in the event of local and systemic stress, as well as via the inflammatory cascade, and can result in marked alteration in the immune microenvironment (Champsaur and Lanier, 2010; Strid *et al.*, 2008). Interestingly, periostin, a protein that plays an important role in collagen modeling and organization, tissue injury/repair especially following physical strain (Rani *et al.*, 2010; Rani *et al.*, 2009), has been revealed by our transcriptome analysis to be significantly up-regulated in vitiligo LS and PLS (Table A-2). Previous evidence has demonstrated that melanocytes could be mechanically detached through repeated light physical friction and speculated that physical trauma and non-specific tissue injury could result in melanocytorrhagy and subsequently, vitiligo (Gauthier *et al.*, 2003a; Gauthier *et al.*, 2003b). Given these lines of evidence, we conjecture that in genetically susceptible individuals, one of the possible mechanisms for the initiation of vitiligo follows the activation of NK cells as a result of melanocyte mechanical detachment, which induces the aberrant expression of stress markers on melanocytes, among which are NK cell receptor ligands, such as those in the ULBP family. In this case, periostin may serve as a marker for physical stress.

Oxidative stress has been one of the proposed pathomechanisms of vitiligo that can result in the destruction of melanocytes. One of the mechanisms by which oxidative stress may activate NK cells is through the induction of heat shock protein 70 (HSP 70) in melanocytes (Kroll *et al.*, 2005). Recently, Denman *et al.* has demonstrated via a gene gun

vaccination approach that HSP 70 significantly accelerated the depigmentation process in a mouse model of autoimmune vitiligo (Denman *et al.*, 2008). Since heath shock proteins, in particular the HSP 70 family, are a potent inflammatory mediators and ligands known to significantly enhance the proliferation and cytotoxic capabilities of NK cells (Multhoff *et al.*, 1999), it is conceivable that they may be partially responsible for the observed heightened NK cell activity in vitiligo skin. This is further strengthened by the up-regulation of CANP, an oxidative stress marker, in vitiligo skin, as demonstrated in Molecular Medicine Laboratory's transcriptome analysis (Table A-2).

Furthermore, NK cells could be selectively recruited and activated by other cells of the immune system, especially dendritic cells, macrophages and monocytes. The CLEC2B gene, which encodes an activating ligand of the NK cells, has also been up-regulated in vitiligo skin, as shown in Molecular Medicine Laboratory's transcriptome analysis (Table A-2). It is mainly expressed by monocytes and macrophages, and can activate NK cells by binding to the NK cell activating receptor NKp80 (Kuttruff *et al.*, 2009; Renedo *et al.*, 2000; Welte *et al.*, 2006). Since CLEC2B can be up-regulated by toll-like receptor stimulation, and topical imiquimod (an activator of the toll-like receptors) has been shown to induce vitiligo (Jacob and Blyumin, 2008; Senel and Seckin, 2007; Serrao *et al.*, 2008), it can be speculated that the increased expression of CLEC2B is a reflection of the activated innate immune system in vitiligo skin that also involves monocytes and macrophages.

4.3 Proposed pathomechanisms for vitiligo

Based on findings and concepts in this and other studies, an attempt has been made to propose new mechanisms of melanocyte destruction mediated by NK cells and possibly

further facilitated by the reduction in Schwann cells (Figure 4-1). Needless to say, not everyone who experience injury and redox imbalance within their tissues will develop an immune response that induces vitiligo. A general consensus has been reached that genetic disposition and immune backgrounds likely play important roles in the initiation and development of the disease.

4.4 Impact of the study

This study specifically highlights the quantitative defect in non-myelinating Schwann cells of the dermo-epidermal junction in vitiligo LS. This finding, when combined with the observation that Schwann cells secrete factors which have protective and possibly other beneficial effects on melanocytes, suggests that these factors could be explored for the development of better therapies in the future. In addition, the current study has also revealed the presence of activated NK cells in the local skin microenvironment and their possible role in the destruction of melanocytes, and suggests that they should be explored as cellular targets for development of better therapies in the future. Further, given the fact that NK cells constitute a major part of the body's natural defense against cancerous cells and that increased NK cells in vitiligo are associated with a decrease in numbers of melanocytes, it remains interesting to see whether or not NK cell activation may represent valuable therapeutic strategy against metastatic melanoma.

4.5 Pitfalls

There are a couple of potential pitfalls in this study. First of all, questions may be raised regarding the reliability of our quantification methods, especially since the histological

analyses performed in this study employed traditional vertically-cut tissue sections to visualize the cutaneous nervous system, which is mainly horizontally oriented. An attempt has been made to circumvent or at least minimize the statistical bias inherent in this kind of approach by performing quantification of Schwann cells across a large number of consecutive fields of views. Secondly, due to the degree of difficulty involved in the isolation of Schwann cells, especially from the cutaneous neurosensory system, commercially available Schwann cells isolated from neurofibromatosis patients were used for preparation of Schwann cell conditioned medium. That being said, this cell line has been tested by ATCC to be immunopositive for the pan-Schwann cell markers p75 and S100. In addition, the cell line exhibited a normal karyotype and was able to produce the normal full-length neurofibromin protein.

4.6 Future directions

The expression of PLP1 at both the mRNA and protein level in melanocytes warrant further investigations into its functional significance. A propose study might be to silence the PLP1 gene in cultured melanocytes and to evaluate its effect on melanogenesis, adhesion to extracellular matrix as well as general survival and growth.

This study provided strong support for quantitative reduction of non-myelinating Schwann cells in vitiligo. In order to reliably assess the state of myelinating Schwann cells, which are much more scarcely distributed in the skin, it is proposed, in the future, to adopt the horizontal dermal sheet preparation method originally established by Tschachler *et al.* (2004) in conjunction with confocal microscopy, which will provide a much larger window

and more accurate three-dmensional topography of the cutaneous nervous system (Tschachler *et al.*, 2004).

In addition, since the present study demonstrated that Schwann cells are capable of secreting factors that are beneficial to the growth and survivability of melanocytes, the identity and function of these factors warrant further investigations. As a start, neutralization assays can be performed whereby antibodies directed against known growth factors (such as BDNF, GDNF and various NGFs) can be used in Schwann-cell conditioned melanocyte cultures to identify the agents in question, which can be confirmed by further assays such as western blot.

Finally, the present study lends support for the direct involvement of activated NK cells in the pathogenesis of vitiligo. Further functional assays should be performed with activated NK cells isolated from vitiligo skin to assess their role in the destruction of melanocytes (for example, NK cell cytotoxicity assays against melanocytes).

Chapter 5: Conclusion

Vitiligo is a complex immune-mediated skin condition that has been difficult to treat due to a lack of total understanding of its pathogenesis. Results from this study suggest that the loss of melanocytes and reduction in Schwann cells may account for the down-regulation of PLP1 in vitiligo lesional skin, a finding based on previous transcriptome analysis. In addition, Schwann cells appeared to play a role in the growth and survival of melanocytes and their decrease may have facilitated the development of vitiligo. Finally, natural killer cells with heightened activity and cytotoxicity have been demonstrated for the first time within the infiltrate of vitiligo skin and their presence in the microenvironment of melanocytes indicate that they may be directly involved in the pathogenic process of vitiligo.

Tables and Figures:

Tables:

Table 2-1. List of antibodies used in histological studies

Name of Antibody	Host	Dilution factor	Source	Structures Stained	References
Anti-PLP1	Rabbit Polyclonal	1:100	Sigma-Aldrich, Oakville, ON, CA	Schwann cells	(Garbern *et al.*, 1997; Griffiths *et al.*, 1989)
Anti-CD56/NCAM	Mouse Monoclonal	1:50	Dako, Burlington, ON, CA	Neural structures, Schwann cells, NK cells	(Tschachler *et al.*, 2004; Whiteside and Herberman, 1990)
Anti-NF 200	Mouse Monoclonal	1:200	Sigma-Aldrich, Oakville, ON, CA	Heavy neurofilament (200 kDa)	(Leermakers and Zhulina, 2010)
Anti-PGP 9.5	Rabbit Polyclonal	1:2000	Cedarlane, Burlington, ON, CA	Nerve fibers (all)	(Teunissen *et al.*, 2002)
Anti-MBP	Mouse Monoclonal	1:25	Millipore, Billerica, MA, USA	Myelin sheath, myelinating Schwann cells	(Griffiths *et al.*, 1989)
Anti-Melan A	Mouse Monoclonal	1:50	Dako, Burlington, ON, CA	Melanocytes, melanoma cells	(Nicholl *et al.*, 2011)
Anti-NKG2D	Mouse Monoclonal	1:2000	Abcam, Cambridge, MA, USA	Activated NK cells, cytotoxic CD8+ T cells	(Groh *et al.*, 2001)
Anti-CD 3	Rabbit Polyclonal	1: 100	Dako, Burlington, ON, CA	T cells (all)	(Kim *et al.*, 2010)

Figures:

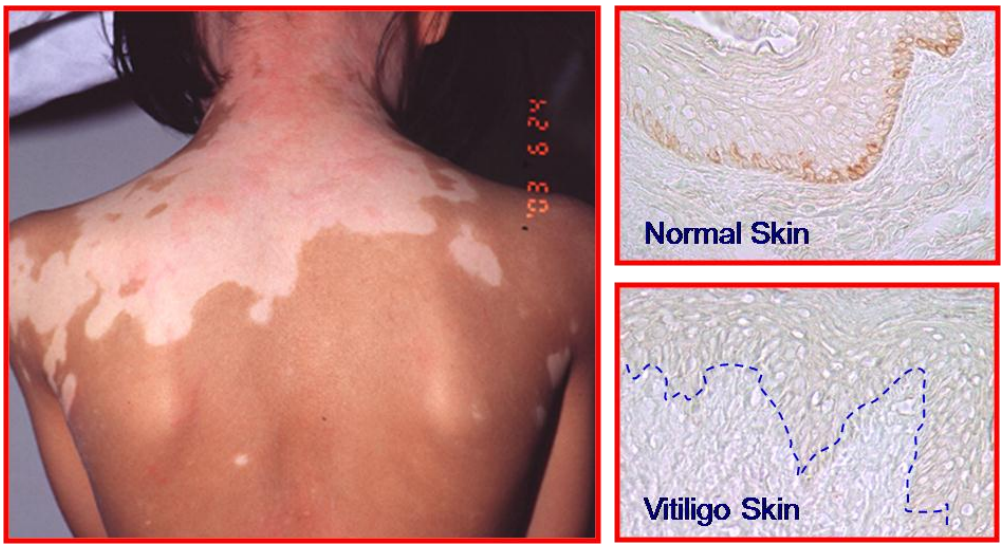
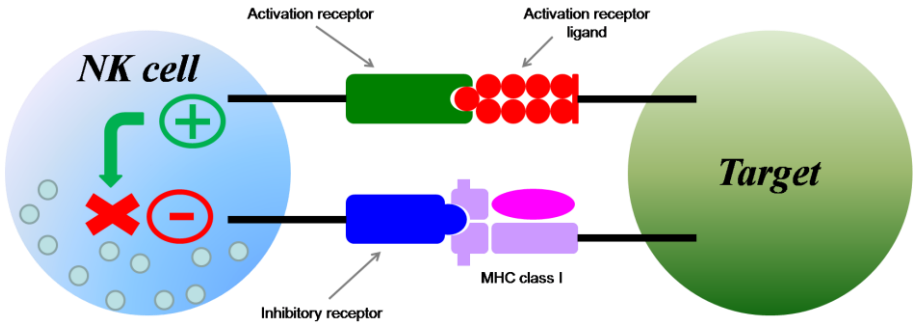
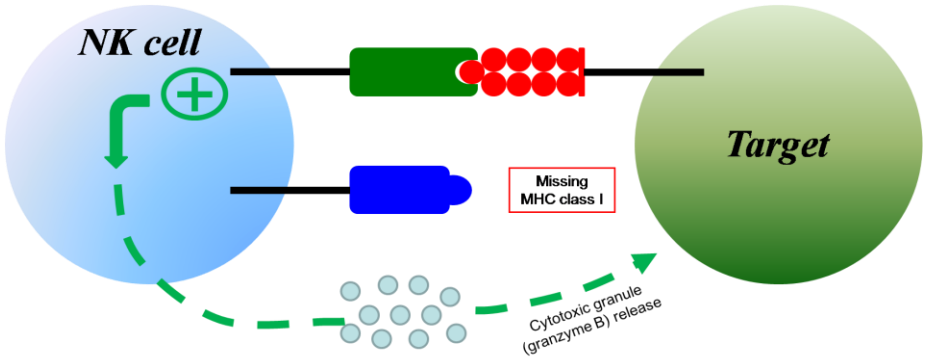


Figure 1-1. Characteristics of vitiligo. Vitiligo is characterized by the presence of depigmented skin patches on the body of patients. Histological analysis reveals the absence of melanin along the basal layer of the epidermis in vitiligo skin as compared to normal skin.

A


Activation receptor
Activation receptor ligand
NK cell
Target
MHC class I
Inhibitory receptor

B


NK cell
Target
Missing MHC class I
Cytotoxic granule (granzyme B) release

C

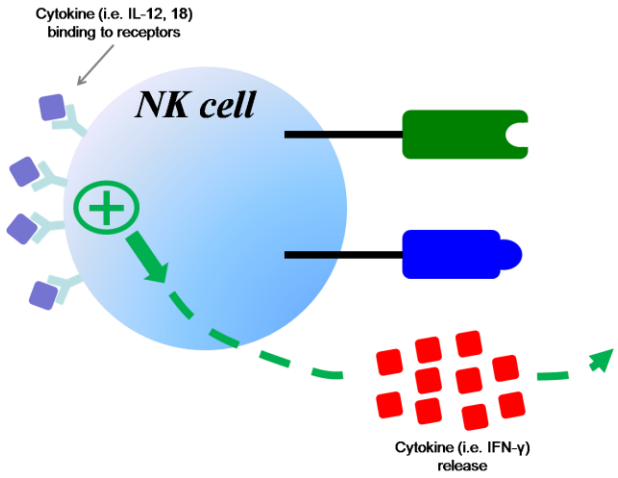

Cytokine (i.e. IL-12, 18) binding to receptors
NK cell
Cytokine (i.e. IFN-γ) release

Figure 1-2. Mechanism of natural killer (NK) cell activation. The activity of NK cells is controlled by signal input from the stimulation of both the activation and inhibitory receptors. (A) Activation receptors (such as NKG2D) bind to appropriate ligands on target cells that increase the activity levels of NK cells, while inhibitory receptors bind to MHC I molecules and suppress NK cell activation. (B) When NK cells encounter target cells with little or no MHC I expression, NK cell activity will no longer be restrained, although cytokine production and the release of cytotoxic granules are still regulated by the interaction of activation receptors with their corresponding ligands. In summary, the activity of NK cells is dependent on the net balance of signals from the expression and stimulation of the opposing activation and inhibitory receptors. (C) In addition, NK cells can be stimulated by cytokines such as IL-12 and IL-18 to produce and release their own cytokines, one of which is IFN-γ, in a manner independent of MHC-I expression levels.

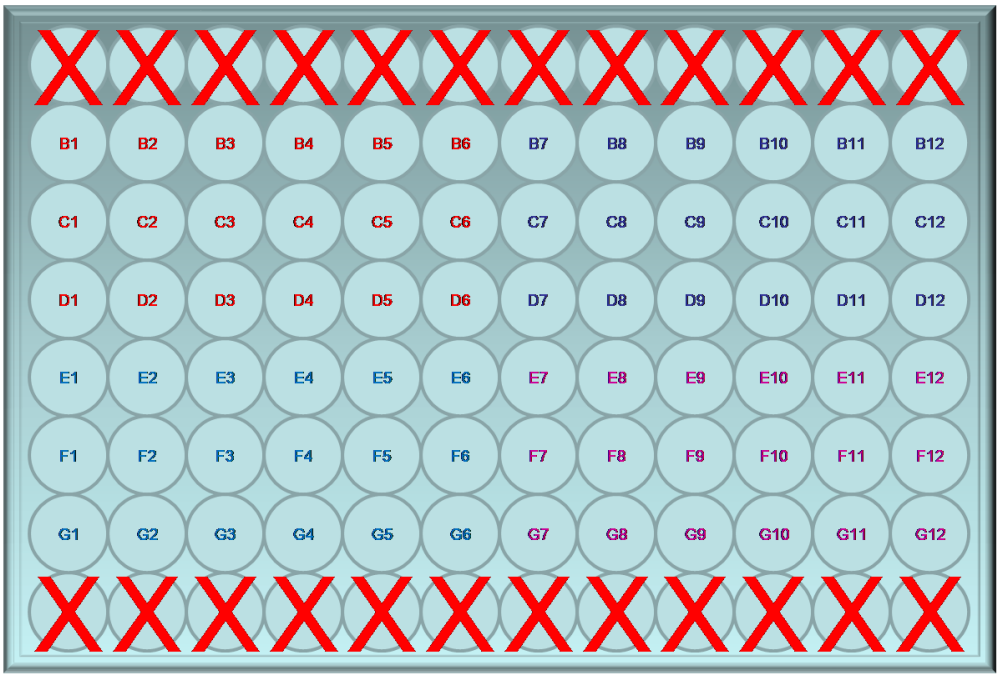


Figure 2-1. Experimental layout for investigating the effects of Schwann cell-conditioned media on the growth and survival of melanocytes. Melanocytes were cultured in four types of media in a 96-well plate and monitored with the MTS assay every day (24 hours) for 5 days (120 hours), with each of the time points performed in triplicates. Letters represent replicate samples, while numbers refer to time points from day 0 to day 5. B,C,D (1-6): human Schwann cell conditioned medium; B,C,D (7-12): rat Schwann cell conditioned medium; E,F,G (1-6): Medium 254 with supplement; E,F,G (7-12): Medium 254 without supplement.

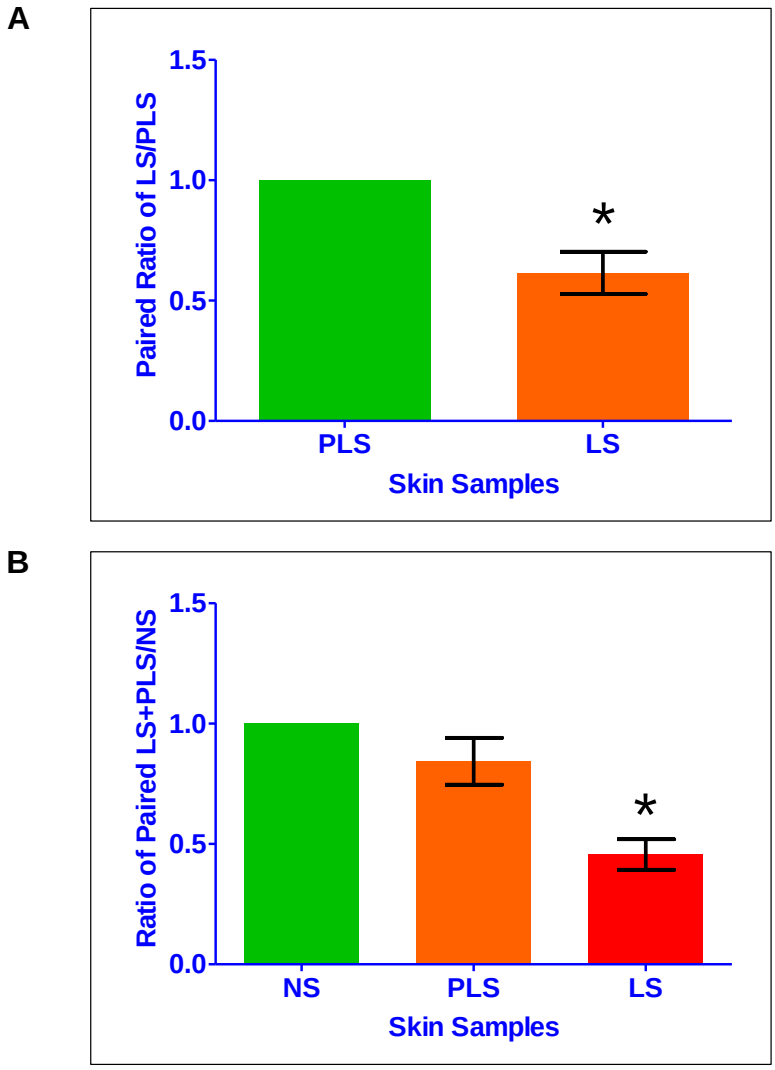


Figure 3-1. Comparative RT-PCR analysis of PLP1 gene expression in vitiligo and normal skin. (A) The average mRNA expression levels of PLP1 in 17 paired vitiligo lesional skin and peri-lesional skin are displayed in a bar plot. The data is expressed as ratios of expression levels in LS over that in the corresponding PLS. There appeared to be a statistically significant down-regulation of PLP1 in LS as compared with PLS ($p = 0.0004$; mean ± SEM). (B) In addition, PLP1 expression in vitiligo LS ($n = 9$) was also significantly lower ($p < 0.0001$; mean ± SEM) than that detected in normal skin (NS) ($n = 9$), while no statistically significant ($p = 0.15$; mean ± SEM) difference was detected between vitiligo PLS ($n = 9$) and NS. The data is expressed as ratios of expression levels in LS or PLS over that in NS. Abbreviations: NS: normal skin; PLS: peri-lesional skin; LS: lesional-skin

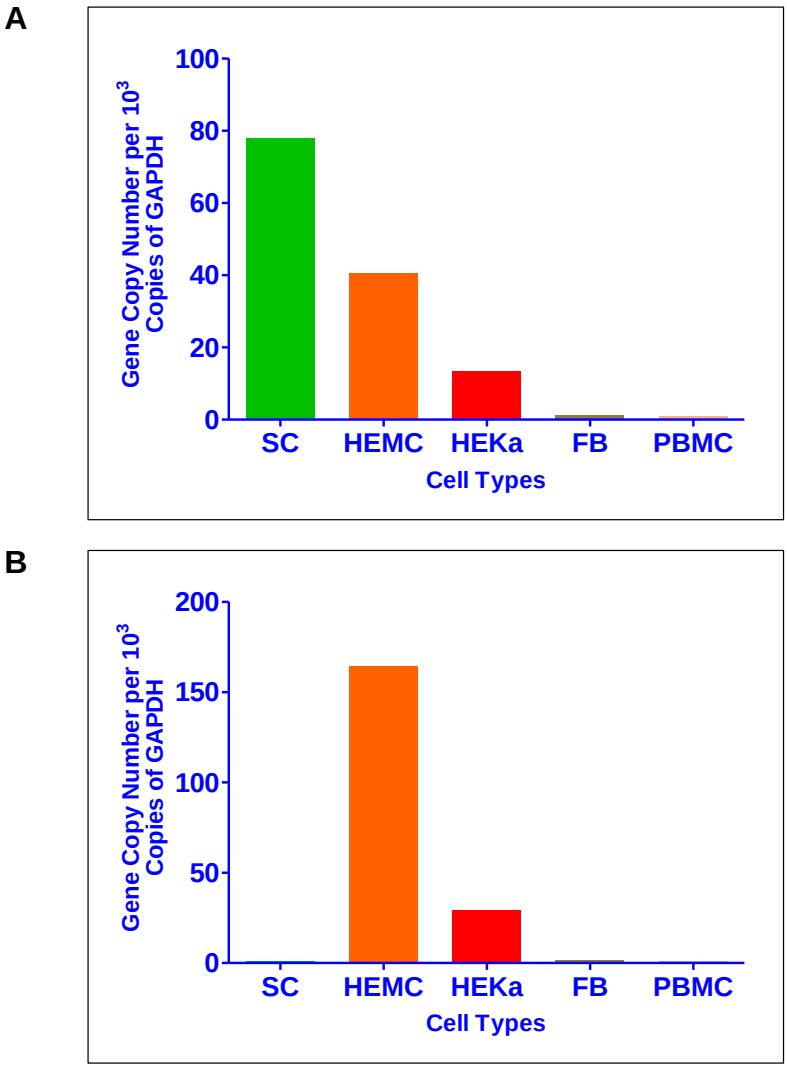


Figure 3-2. Differential expression of PLP1 in cells of normal human skin. RT-PCR assessment of PLP1 expression was performed on cultured human melanocytes, keratinocytes, fibroblasts, peripheral blood mononuclear cells, as well as Schwann cell-rich tumor biopsies obtained from Schwannoma patients. (A) Schwann cells were found to have the highest expression of PLP1, followed by melanocytes. (B) In contrast, the melanocyte differential marker Melan-A (MLANA) was not found to be expressed in appreciable levels in all cell types but melanocytes. Abbreviations: FB: fibroblast; HEKa: adult human epidermal keratinocyte; HEMC: adult human epidermal melanocyte; PBMC: peripheral blood mononuclear cell; SC: Schwann cell

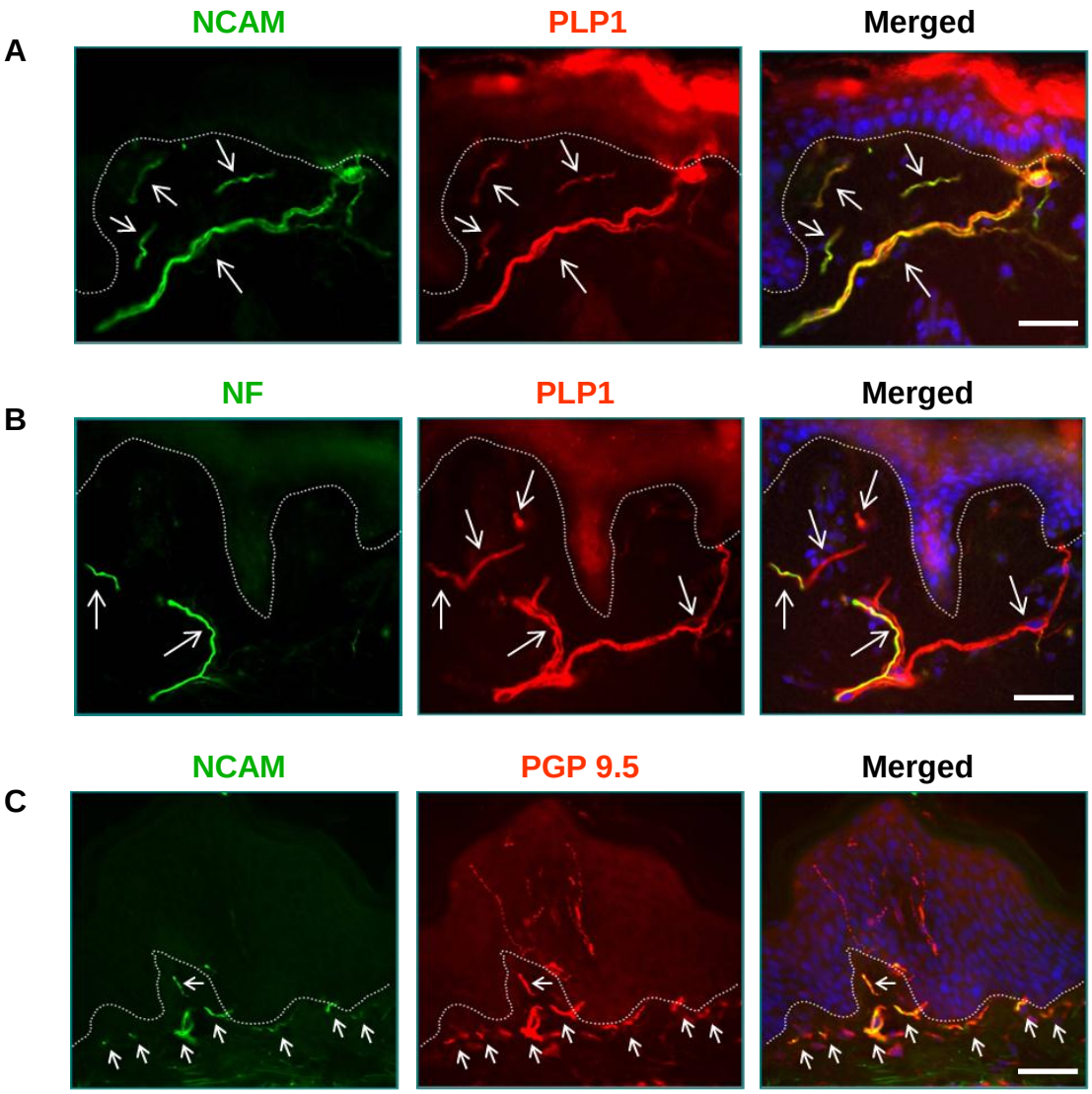


Figure 3-3. Localization of PLP1-expressing Schwann cells in normal human skin. Immunofluorescence was performed on normal skin tissues to investigate the location and distribution of PLP1-expressing Schwann cell bodies in human skin. (A) NCAM almost completely co-localizes with PLP1. (B) Schwann cell processes envelope NF-immunoreactive nerve fibers in the upper dermis. (C) Nerves associated with Schwann cells are entirely confined in the dermal region while free nerve endings (PGP 9.5+) extend all the way up into the epidermis. (green: NCAM/CD56 (A, C), NF (B); red: PLP1 (A, B), PGP 9.5 (C); blue: DAPI; magnification: 400x; scale bar: 50μm)

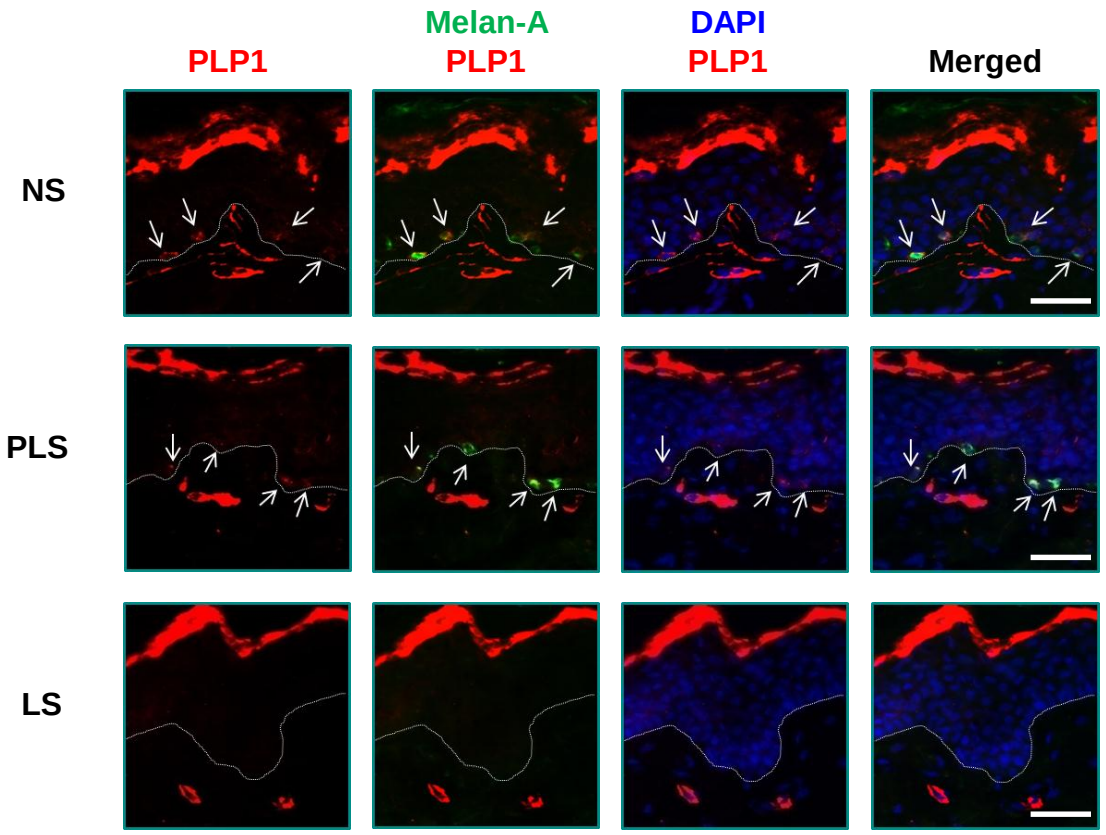


Figure 3-4. PLP1 expression in melanocytes in normal and vitiligo skin. Melanocytes appeared to express PLP1 at the protein level in normal human skin, as demonstrated by the co-localization of Melan-A and PLP1 in the basal epidermal layer in both normal skin and vitiligo peri-lesional skin. In contrast, vitiligo lesional skin characterized by the loss of melanocytes exhibited no expression of both markers. (green/yellow: Melan-A; red: PLP1; magnification: 400x; scale bar: 50μm)

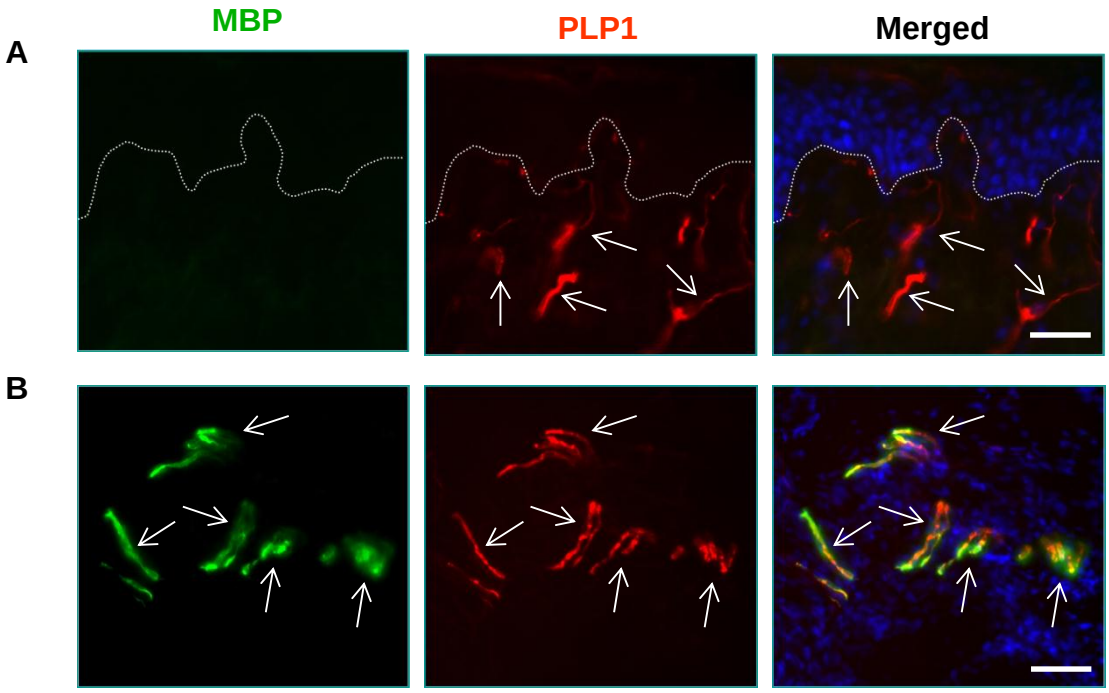


Figure 3-5. Identification of myelinating and non-myelinating Schwann cells in normal human skin. (A) Schwann cells in the upper dermis (< 500 µm from the skin surface) are exclusively non-myelinating in nature as demonstrated by the absence of MBP expression. (B) In contrast, myelinating Schwann cells are located in the reticular dermis, indicated by the co-expression of MBP and PLP1. (green: MBP; red: PLP1; blue: DAPI; magnification: 400x (A) and 200x (B) ; scale bar: 50µm)

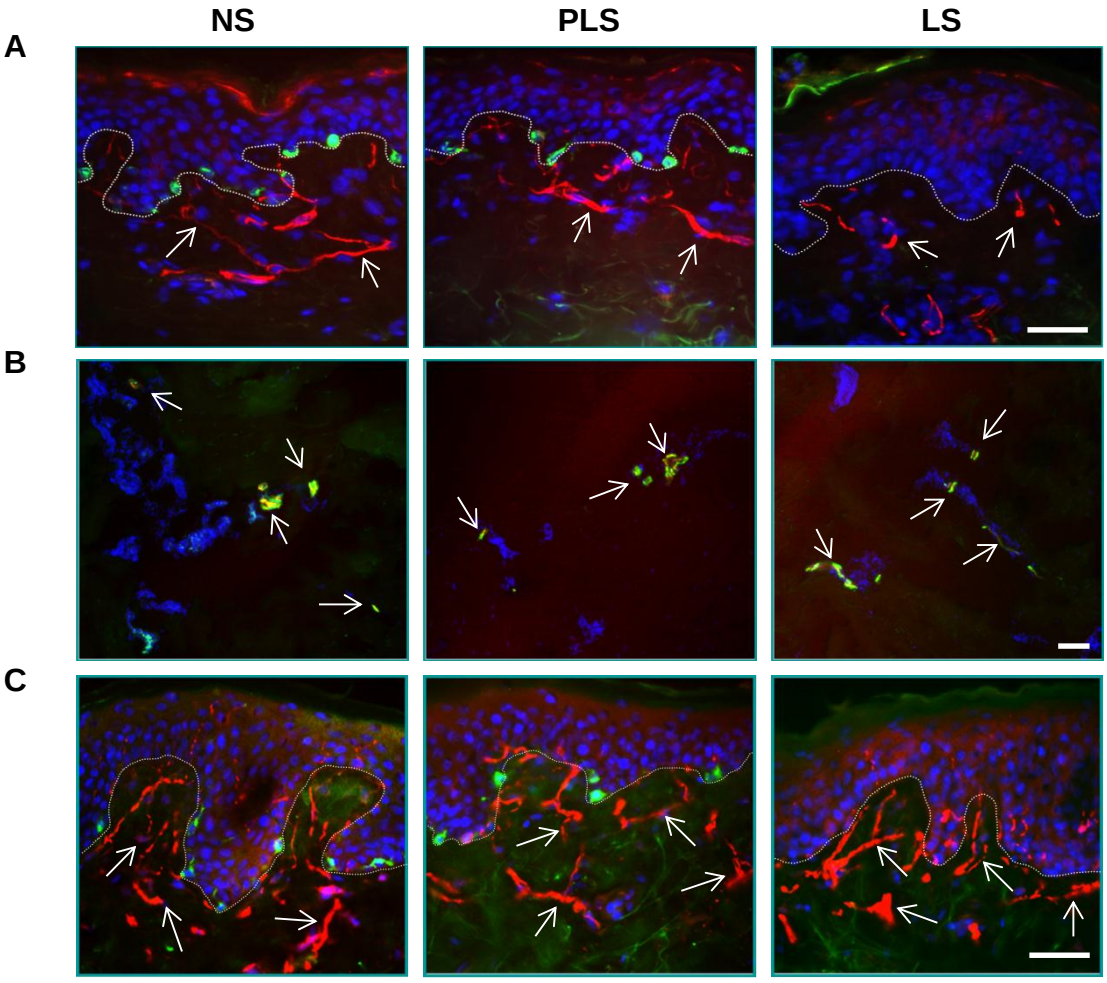


Figure 3-6. Immunofluorescence of Schwann cells and nerve fibers in normal and vitiligo skin. (A) In general, PLP1-immunoreactive non-myelinating Schwann cells in vitiligo lesional skin appeared to have shorter cell bodies and processes as compared to the paired peri-lesional skin and normal skin. (B) There did not appear to be a significant difference in the morphology or the number of deep dermal myelinating Schwann cells between vitiligo and normal skin. That being said, our current method of analysis might not prove to be adequate in assessing the much more sparsely distributed myelinating Schwann cells. (C) No significant qualitative difference was found in PGP 9.5-immunoreactive nerve fibers between vitiligo and normal skin. Abbreviations: NS: normal skin; PLS: peri-lesional skin; LS: lesional-skin. (green: Melan-A (A, C), MBP (B); red: PLP1 (A, B), PGP 9.5 (C); blue: DAPI; magnification: 400x (A, C) and 100x (B) ; scale bar: 50μm)

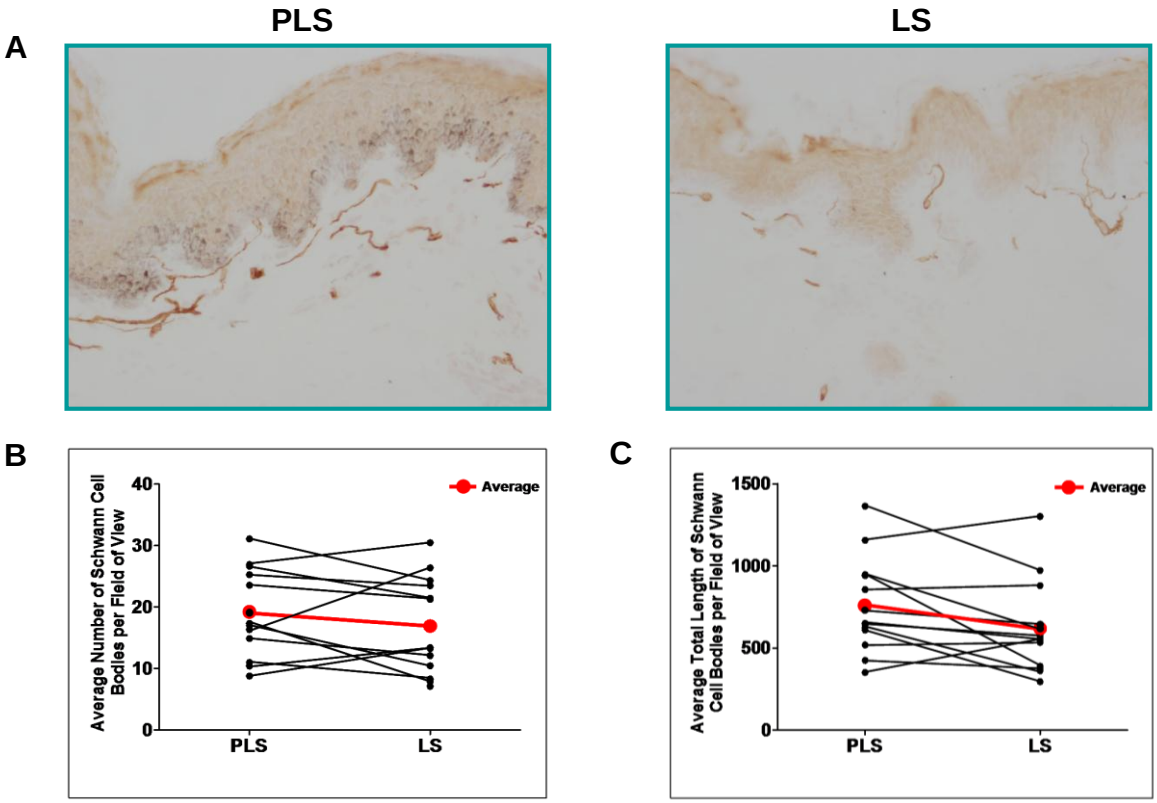


Figure 3-7. Quantification of non-myelinating Schwann cells in paired vitiligo skin samples. Immunohistochemistry of PLP1 was performed on paired vitiligo samples (n = 13) and quantification of both the number and total length of non-myelinating Schwann cell bodies were performed on consecutive 200x fields of view across the entire span of tissues. The average number and total length of Schwann cell bodies per field of view in all 13 paired samples were plotted and paired student t-test was performed to determine statistical significance. (A) Micrographs of representative areas in vitiligo peri-lesional and lesional skin (demonstrated by the lack of pigmentation in the basal epidermal layer) were taken at 200x magnification. (B) On average, there appeared to be a decrease in the number of Schwann cell bodies in vitiligo LS as compared to PLS, although the difference was not statistically significant ($p = 0.19$). (C) On the other hand, there was a statistically significant decrease in the total length of Schwann cell bodies in LS (about 22% reduction) as compared to PLS ($p = 0.032$). Abbreviations: PLS: peri-lesional skin; LS: lesional-skin. (magnification: 200x)

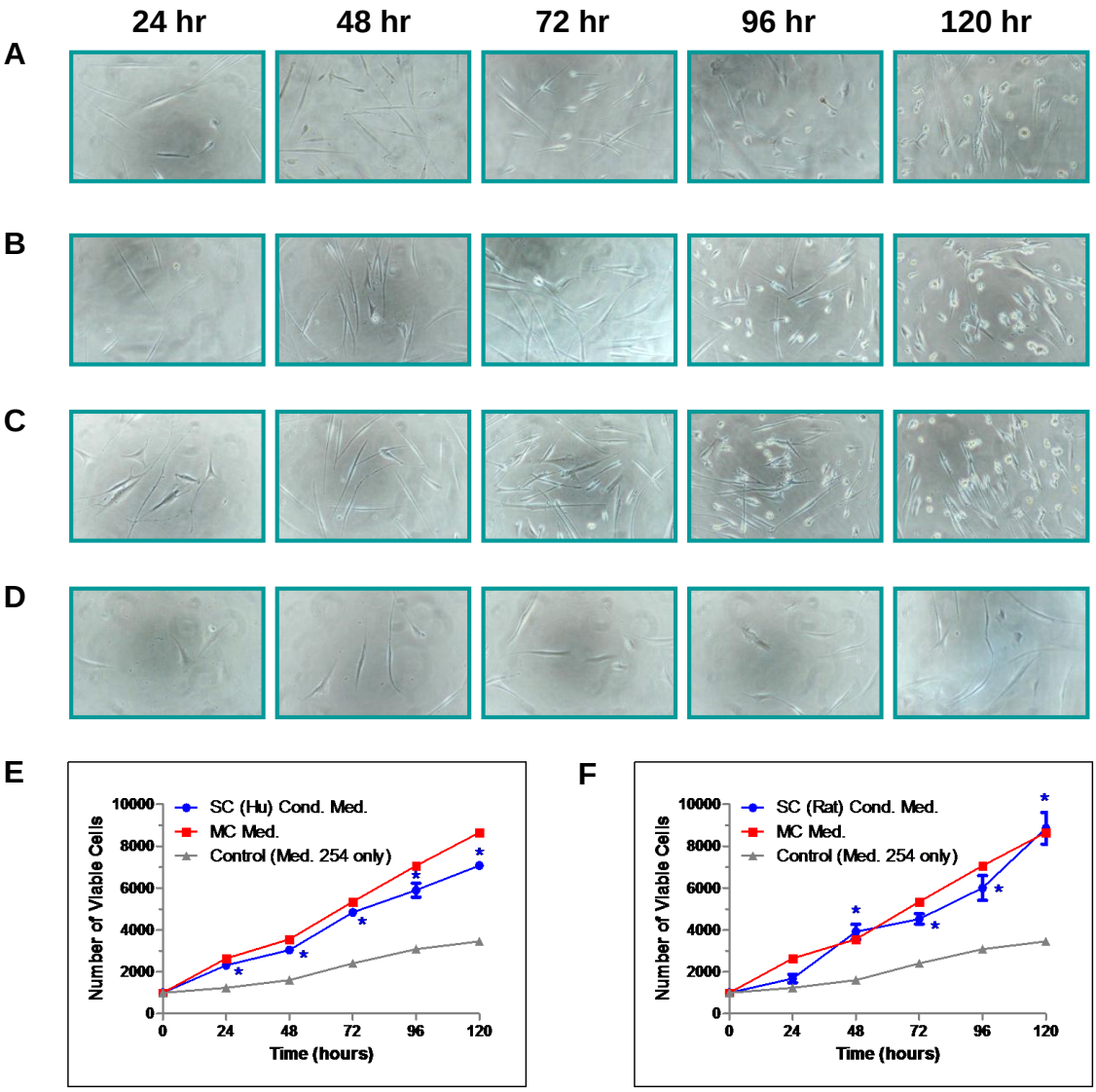


Figure 3-8. The effect of Schwann cell-conditioned medium on the growth and survival of melanocytes. Melanocytes were cultured in conditioned media prepared from human and rat Schwann cells and MTS assay was used to monitor their growth over a period of 120 hours. Series of photographs were taken every 24 hours to qualitatively assess melanocyte growth in the following media: (A) human Schwann cell conditioned medium; (B) rat Schwann cell conditioned medium; (C) Medium 254 with supplement; (D) Medium 254 without supplement. (E) Melanocytes in human Schwann cell-conditioned medium exhibited significantly increased ($n = 3$, $p = 0.0034$, mean ± SEM) growth rate just after 24 hours of culture as compared with those in Medium 254 alone (control). The difference maintained throughout the rest of the experiment. (F) Similarly, melanocytes in rat Schwann cell-conditioned medium also grow significantly faster than those in the control group after 48 hours ($n = 3$, $p = 0.012$, mean ± SEM) and maintained a comparable level of growth to those cultured in standard melanocyte medium (Medium 254 with supplement). (magnification: 400x). Abbreviations: MC: melanocytes; SC: Schwann cells.

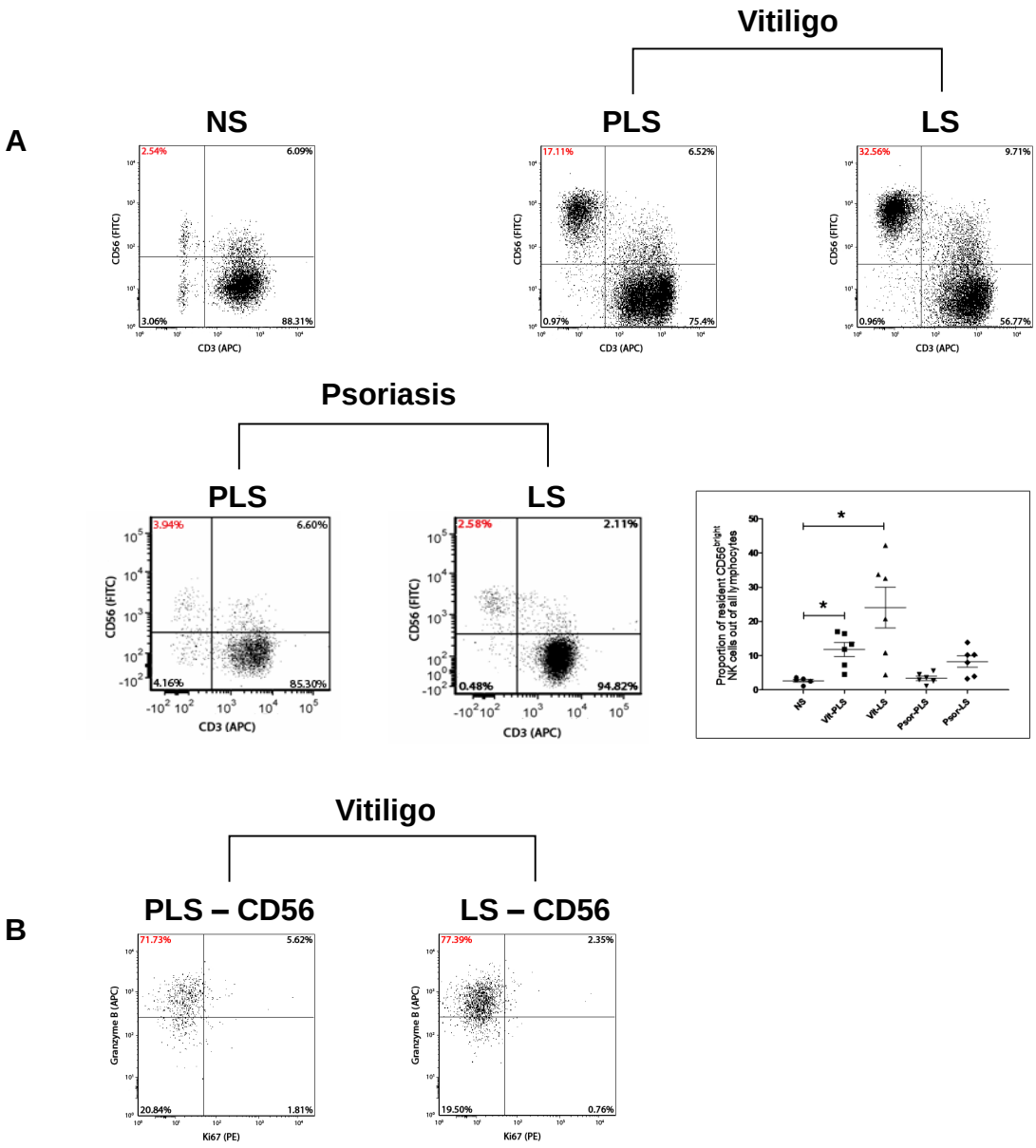


Figure 3-9. Explant culture of natural killer cells from vitiligo skin. Natural killer cells from 6 pairs of vitiligo skin, along with 5 normal and 6 psoriasis skin explants were cultured with Cellfoam matrices and analyzed using flow cytometry. (A) Flow cytometry plots demonstrated a significantly increased proportion of skin-resident $CD56^{bright}$ natural killer cells in vitiligo skin, and to a lesser extent, in psoriasis skin, as compared with normal skin. The numbers of resident natural killer cells in vitiligo PLS ($p = 0.0043$; mean ± SEM) and LS ($p = 0.0043$; mean ± SEM) were both significantly increased as compared to normal skin. (B) Majority of the $CD56^{bright}$ natural killer cells in vitiligo skin are granzyme B-positive. Abbreviations: NS: normal skin; PLS: peri-lesional skin; LS: lesional-skin.

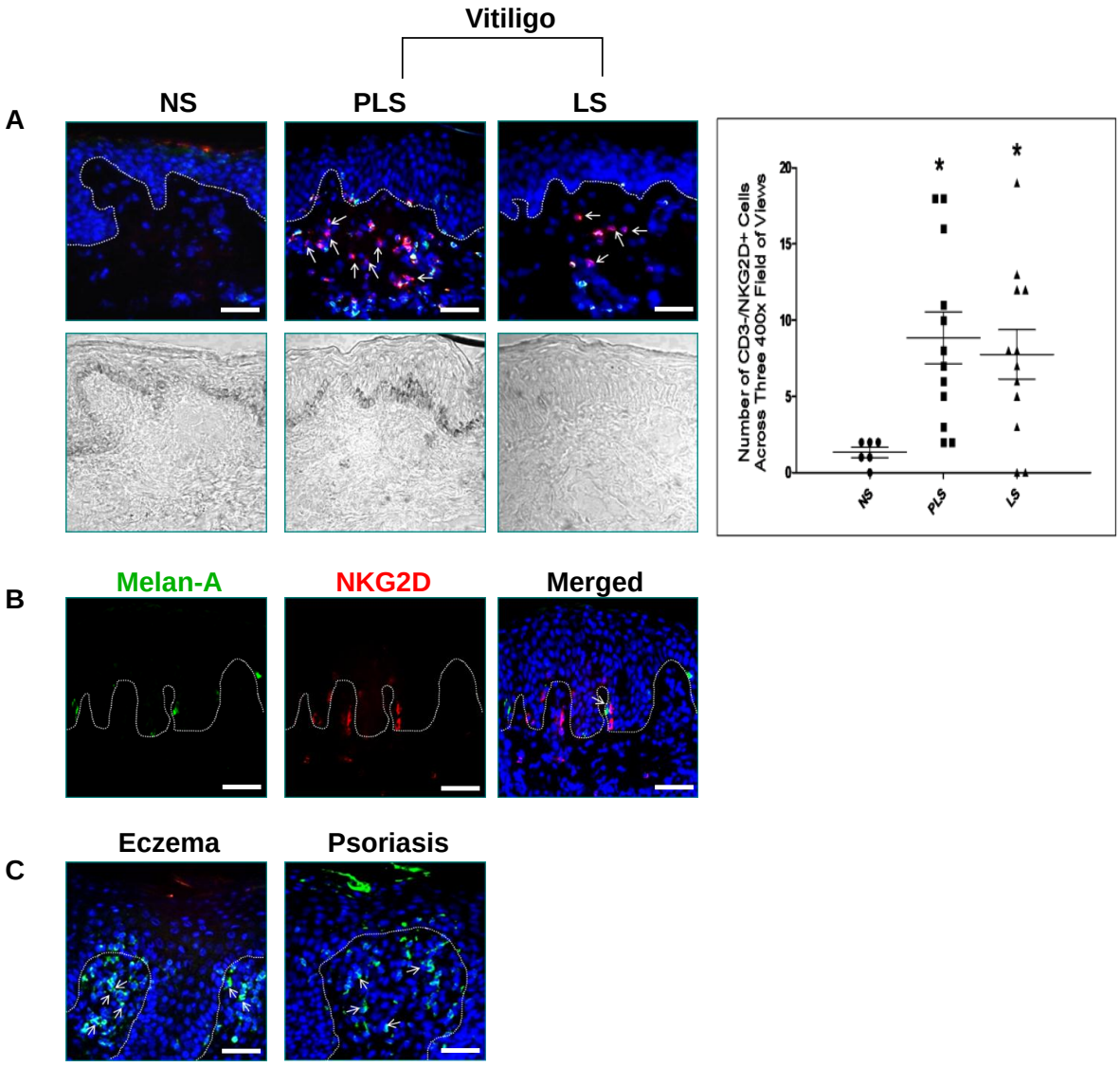


Figure 3-10. Characterization of NKG2D+ natural killer cells in vitiligo skin. Skin biopsies taken from 12 vitiligo patients and 6 normal individuals were subjected to immunofluorescence analysis of activated natural killer cells. (A) CD3-/NKG2D+ natural killer cell infiltrate were found in the skin of vitiligo patients but not in normal skin. Quantification of CD3-/NKG2D+ cells demonstrates a statistically significant increase of activated natural killer cells in vitiligo PLS ($p < 0.0021$; mean ± SEM) and LS ($p = 0.021$; mean ± SEM) as compared with normal skin. (B) NKG2D+ cells are often found in close vicinity of remnant melanocytes in the peri-lesional skin of vitiligo patients. (C) There is no evidence of NKG2D+ NK cells in the epidermis and upper dermal area of LS from eczema and psoriasis patients. (green: CD3 (A, C), Melan-A (B); red: NKG2D; blue: DAPI; magnification: 400x; scale bar: 50μm). Abbreviations: NS: normal skin; PLS: peri-lesional skin; LS: lesional-skin.

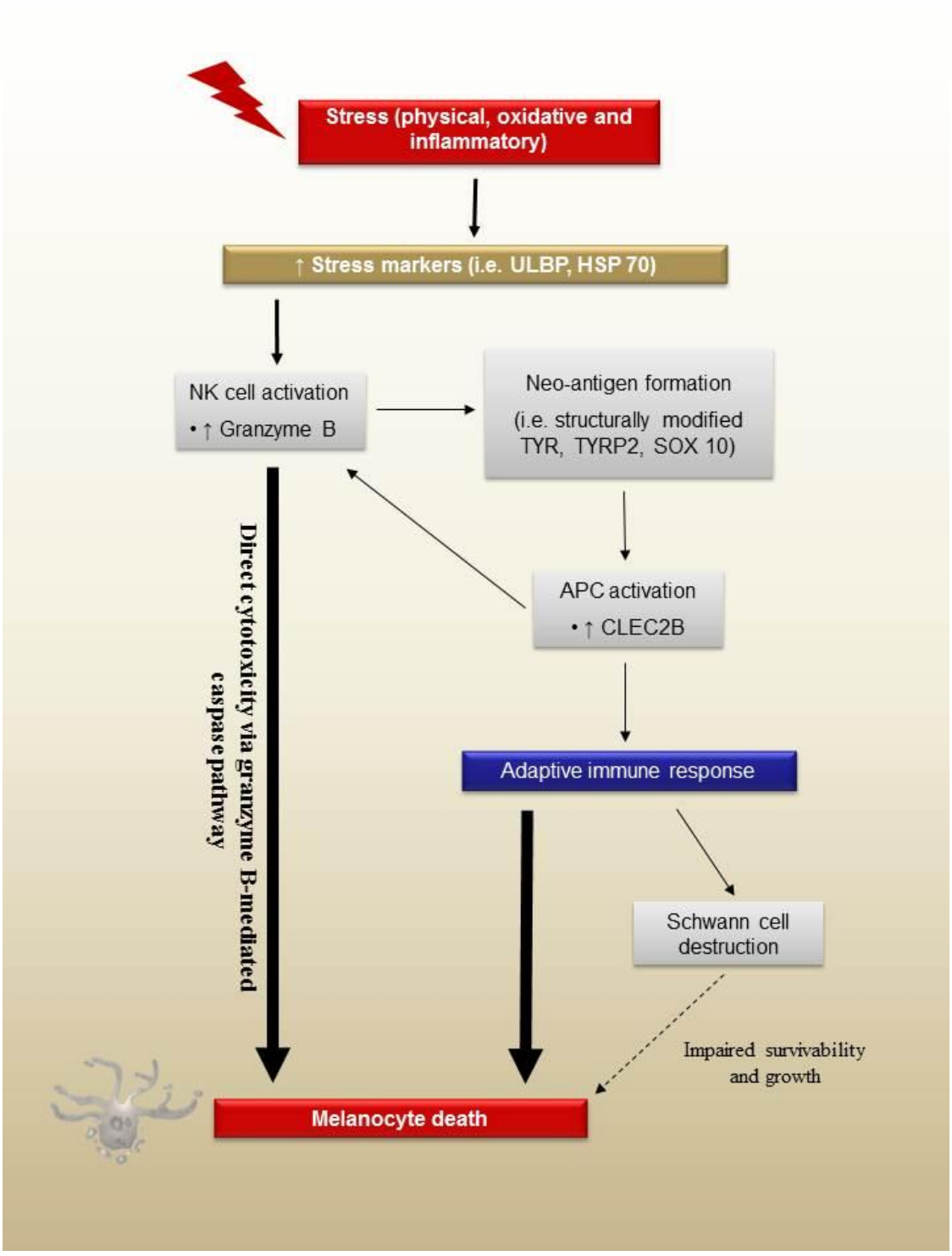

Stress (physical, oxidative and inflammatory)
↑ Stress markers (i.e. ULBP, HSP 70)
NK cell activation
• ↑ Granzyme B
Neo-antigen formation
(i.e. structurally modified TYR, TYRP2, SOX 10)
APC activation
• ↑ CLEC2B
Adaptive immune response
Schwann cell destruction
Direct cytotoxicity via granzyme B-mediated caspase pathway
Impaired survivability and growth
Melanocyte death

Figure 4-1. Proposed mechanism of natural killer cell-mediated melanocyte destruction. Factors such as physical injury, redox imbalance and inflammation in genetically predisposed individuals may trigger up-regulated expression of local and systemic stress markers such as ULBP and HSP 70, which also serve as potent ligands for the activation of natural killer cells. Aberrant expression of such ligands in melanocytes will recruit and activate NK cells, which in turn may cause their destruction via granzyme-B-mediated apoptosis. The consequence of chronic activation of NK cells is the formation of structurally modified melanocytic antigens, or neo-antigens, such as TYR, TYRP2 and SOX 10, which is also a Schwann cell marker. The subsequent uptake of neo-antigens by professional antigen presenting cells such as macrophages may further activate or enhance the activity of NK cells, as well as trigger specific adaptive immune responses against melanocytes and Schwann cells, the death of which may also render melanocytes vulnerable to destruction. Abbreviations: APC: antigen-presenting cell; NK: natural killer.

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Appendices

Appendix A

Table A-1. Down-regulated genes in vitiligo skin

Down-regulated Genes in Vitiligo

Gene	Chromosome	[1] Ratio LS/NS	[2] Ratio PLS/NS	Full name	Function
TYRP1	9	0.06	0.97	Tyrosinase-related protein	Melanogenesis
TYR	11	0.08	1.09	Tyrosinase	Melanogenesis
MLANA	9	0.13	1.08	Melan-A	Melanogenesis
TRPM1	15	0.11	0.89	Transient receptor potential cation channel M1	Melanogenesis and signal transduction
DCT	13	0.13	0.90	Dopachrome tautomerase	Melanogenesis
PMEL	12	0.21	1.02	Premelanosome protein	Melanogenesis
CA14	1	0.21	0.89	Carbonic anhydrase XIV	Physiological processes
SFTPC	8	0.35	1.21	Pulmonary-associated protein C	Surfactant
SOX10	22	0.31	0.80	SRY (sex determining region Y)-box 10	Stem cell development
OCA2	15	0.58	1.44	Oculocutaneous albinism II	Melanogenesis
PCSK2	20	0.36	0.89	Proprotein convertase subtilisin/kexin type 2	Hormone synthesis
PLP1	X	0.50	1.14	Proteolipid protein 1	Myelin sheath protein
GMPR	6	0.40	0.84	Guanosine monophosphate reductase	Nucleoside reductase
BCAN	1	0.37	0.78	Brevican	Cell motility
LZTS1	8	0.31	0.63	Leucine zipper putative tumor suppressor 1	Tumor suppressor
GPR143	X	0.10	0.51	G protein-coupled receptor 143	Melanogenesis and signal transduction
C19orf28	19	0.22	0.44	Clone PP3501	Unknown

[1] Ratio LS/NS = Mean expression level on 17 vitiligo lesional skin divided by Mean expression level on 16 normal skin

[2] Ratio PLS/NS = Mean expression level on 17 vitiligo peri-lesional skin divided by Mean expression level on 16 normal skin

Table A-2. Up-regulated genes in vitiligo skin

Up-regulated Genes in Vitiligo

Gene	Chromosome	[1] Ratio LS/NS	[2] Ratio PLS/NS	Full name	Function
KLRC1	12	6.27	1.71	Killer cell lectin-like receptor subfamily C, member 1	Natural killer cell receptor
KLRC2	12	5.88	1.44	Killer cell lectin-like receptor subfamily C, member 2	Natural killer cell activating receptor
KLRK1	12	2.48	2.02	Killer cell lectin-like receptor subfamily K, member 1	Natural killer cell activating receptor
KLRG1	12	2.46	2.05	Killer cell lectin-like receptor subfamily G, member 1	Natural killer cell receptor
KLRC4	12	3.91	2.27	Killer cell lectin-like receptor subfamily C, member 4	Natural killer cell receptor
LPAL2	6	6.37	3.28	Lipoprotein, Lp(a)-like 2	Pseudogene
CANP	11	3.56	1.92	Calpain	Oxidative stress
DEFB103A	8	2.27	1.38	Defensin, beta 103A	Innate immunity
CLEC2B	12	3.18	2.01	C-type lectin domain 2B	Ligand for natural killer cell receptor
SP8	7	6.48	4.15	Sp8 transcription factor	Transcription factor
POSTN	13	5.70	3.68	Periostin	Tissue injury and repair
RGS20	8	3.10	2.02	Regulator of G-protein signaling 20	Signal transduction
EREG	4	7.46	5.37	Epiregulin	Epidermal growth factor

[1] Ratio LS/NS = Mean expression level on 17 vitiligo lesional skin divided by Mean expression level on 16 normal skin

[2] Ratio PLS/NS = Mean expression level on 17 vitiligo peri-lesional skin divided by Mean expression level on 16 normal skin

Appendix B

Table B-1. Demographics and clinical features of patients with non-segmental vitiligo

Patient No.	Gender	Ethnic Origin	Age (yrs)	Autoimmune Disease	Family History of Autoimmune Disease	Disease Extent-BSA (%)	Biopsy Site
1	M	Chinese	28	None	None	30	Elbow
2	M	Caucasian	70	Myathenis gravis	None	2	Hands
3	M	S. Asian	54	None	None	5	Abd
4	M	Chinese	10	None	Vitiligo	11	Legs
5	F	S. Asian	35	None	None	10	Abd.
6	M	S. Asian	75	None	None	3	Neck
7	F	Chinese	21	None	None	5	Torso
8	M	S. Asian	8	None	None	22	Abd.
9	F	Chinese	71	None	None	2	Abd.
10	M	Chinese	36	None	None	30	Abd.
11	F	Chinese	33	Atopic dermatitis	None	80	Flank
12	F	Korean	28	None	None	20	Flank
13	F	S. Asian	57	None	None	6	Neck
14	M	Chinese	51	None	Diabetes	2	Neck
15	F	Caucasian	36	None	Vitiligo	3	Face
16	F	S. Asian	47	Hypothyroidism	None	3	Neck
17	M	Chinese	26	None	None	1	Buttocks

Abbreviations: F = Female; M = Male; BSA = body surface area; Abd. = abdomen

Appendix C

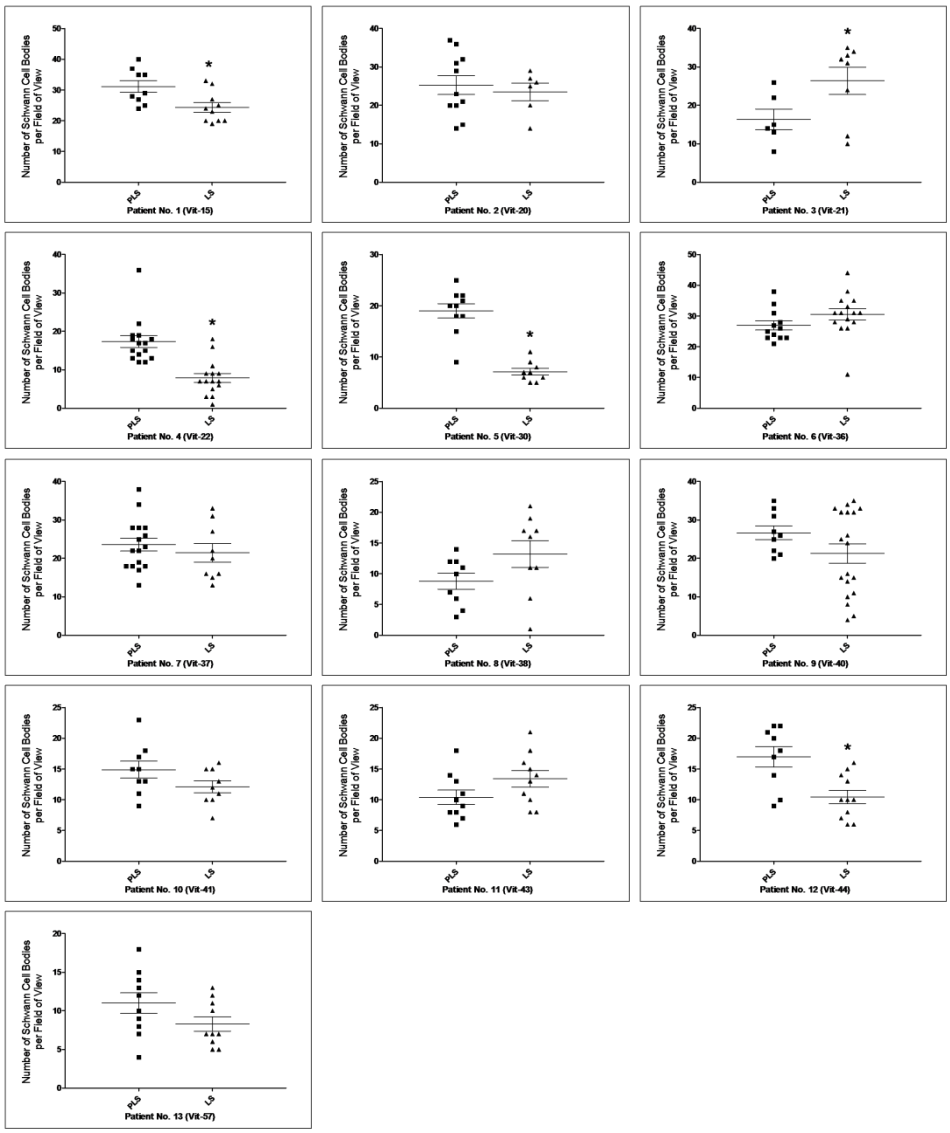

Figure C-1. Quantification of Schwann cell number in paired vitiligo skin samples for individual patient. Immunohistochemistry of PLP1 was performed on paired vitiligo samples (n = 13) and quantification of the number of non-myelinating Schwann cell bodies was performed on consecutive 200x fields of view across the entire span of tissues. Each data point represents the quantification result for a single field of view. There were a reduction in the number of Schwann cells in LS as compared to PLS in patients 1, 2, 4, 5, 7, 9, 10, 12, 13, among which, the differences seen in patients 1, 4, 5 and 12 were statistically significant ($p = 0.047$, 0.0004, < 0.0001, 0.013, respectively). On the other hand, the number of Schwann cells in LS was increased as compared to PLS in patients 3, 6, 8 and 11, with the difference been statistically significant ($p = 0.044$) in patient 3. Abbreviations: PLS: peri-lesional skin; LS: lesional-skin.

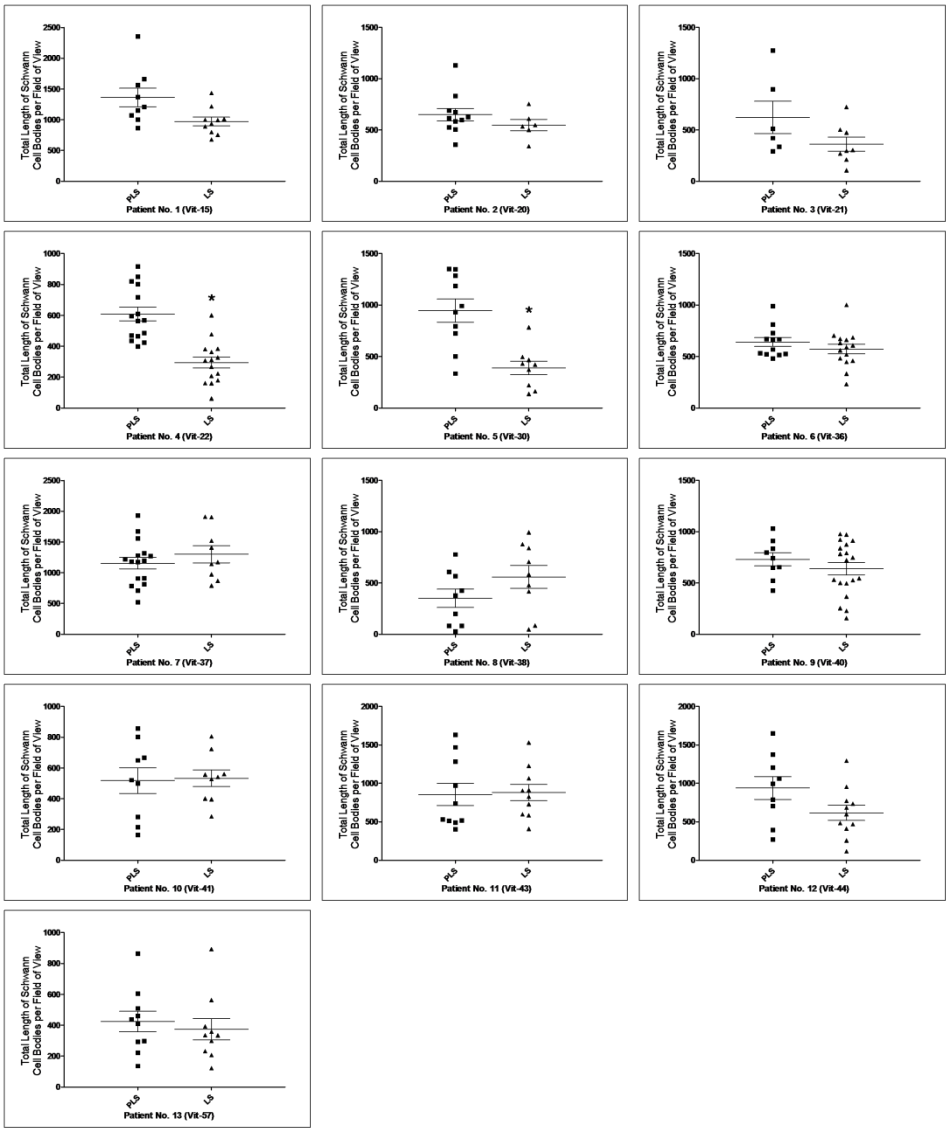

Figure C-2. Quantification of Schwann cell body length in paired vitiligo skin samples for individual patient. Immunohistochemistry of PLP1 was performed on paired vitiligo samples (n = 13) and quantification of the total length of non-myelinating Schwann cell bodies was performed on consecutive 200x fields of view across the entire span of tissues. Each data point represents the quantification result for a single field of view. There were a reduction in the total length of Schwann cell bodies in LS as compared to PLS in patients 1, 2, 3, 4, 5, 6, 9, 12, 13, among which, the differences seen in patients 4 and 5 were statistically significant ($p \leq 0.0001$). On the other hand, the total length of Schwann cell bodies in LS was either increased or around the same level as compared to PLS in patients 7, 8, 10 and 11. Abbreviations: PLS: peri-lesional skin; LS: lesional-skin.

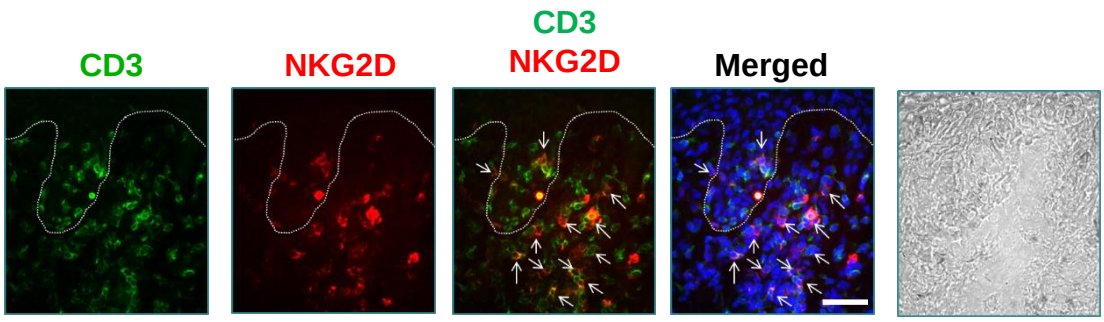

Figure C-3. Infiltration of CD3+/NKG2D+ cells in vitiligo lesional skin. The presence of numerous CD3+/NKG2D+ cytotoxic T cells as well as CD3+ singly positive T cells (presumably CD4+ T cells) can be seen within the upper dermal lymphocytic infiltrations in vitiligo lesional skin. (green: CD3; red: NKG2D; blue: DAPI; magnification: 400x; scale bar: 50μm)